Science, society and biosafety of a field trial with transgenic biofuel poplars

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IUFRO Tree Biotechnology Conference 2011: From Genomes to Integration and Delivery

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INTRODUCTION

A1

IUFRO Tree Biotechnology 2011: "From genomes to integration and delivery"

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Forest trees have unquestionably entered the genomic era. The updated version of the *Populus* genome, the recently released *Eucalyptus grandis* genome and the concerted efforts towards the generation of genome sequences for spruces (*Picea sp.*) and pines (*Pinus sp.*) by several groups worldwide, are fueling a multitude of inter-disciplinary studies and applications in sustainable forest production and conservation. Time now calls for the integration of scientific fields with an increased sense of urgency for delivery of effective biotechnologies.

The IUFRO (International Union of Forestry Research Organizations) Tree Biotechnology biannual conference has established a solid tradition for over 20 years as the official meeting of the IUFRO working group 2.04.06 – Molecular biology of forest trees. This conference has convened scientists and foresters interested in the genetics, genomics, molecular biology and physiology of forest trees, and the application of this knowledge to tree improvement and conservation. The Tree Biotechnology Conference has undoubtedly been the premiere international forum where the most cutting edge research in tree biotechnology developed both in academia and industry is presented. "From genomes to integration and delivery", this was the theme chosen for the 2011 edition of the IUFRO Tree Biotechnology Conference, first time to be held in South America. Our intention was to promote a more integrated and applied dialogue on tree biotechnology and genomics, beyond the mainstream discussion of the fundamental advances on the genetic mechanisms that underlie tree phenotypes.

In nine scientific sessions some of the current advances of genomics applied to forest conservation, tree physiology, stress response, molecular breeding, in vitro and propagation technologies, wood development and genetically modified (GM) trees were highlighted. With 340 registered participants, the Conference brought to Brazil most of the world's brain power in forest tree genomics and biotechnology. An outstanding team of international scientists shared their results and visions on the present and future of this fast moving area of forest science, while a brilliant group of young scientist and students delivered a very energetic and diverse collection of high-quality scientific presentations. Forty two countries were represented at the Conference with almost 100 different laboratories from tens of Universities, research institutions and private companies.

During the seven days of the Conference 26 invited lectures, 63 oral and 185 poster presentations were delivered, totaling 274 papers made available as extended abstracts into this BMC Proceedings supplement. The special workshop on the hot topic of "Genomic Selection in tree breeding" and the several reports on whole-genome studies, made this conference edition inaugurate a deliberate effort towards a better integration between the quantitative genomics, the "single-gene" and the system biology approaches to more efficiently unravel the complex relationships between genotypes and phenotypes in forest trees. A field trip to the forest plantations, nurseries and mill of VERACEL Cellulose was a definite highlight and a welcome break from the scientific sessions, providing an overview of some of the advances and challenges facing the translation of research into plantation forestry.

In closing this introductory statement, acknowledgements are due to the outstanding financial support provided by the competitive grants of the Brazilian Ministry of Science and Technology through the National Research Council (CNPq) and the Ministry of Education through its agency for graduate studies (CAPES). Major support was also provided by EMBRAPA (Brazilian Corporation of Agricultural Research), and VERACEL Cellulose, the host organizations, together with an exceptional suite of private sponsors. Besides the organizations that backed this conference and an active Scientific Committee involved in abstract review a number of people were involved in the organization and logistics. The conference would not have been possible without the valuable contributions of all these players.

Given the rewarding feedback received after the Conference, the original goal of providing an exceptional mix of science, social activities and field exploration in a relaxed atmosphere was truly accomplished. The IUFRO Tree Biotechnology Conference 2011 made a significant contribution to advance the forest biotechnology research community one step ahead on the challenging task of moving from gene and genome discoveries to the delivery of valuable technologies into sustainable forestry.

S1. POPULATION GENOMICS, CONSERVATION AND ADAPTATION

I1

Missing heritability and missing Fst of candidate genes: why does gene variation differ from trait variation in trees?

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BMC Proceedings 2011, **5(Suppl 7):**I1

Molecular footprints of phenotypic variation are usually explored by two different approaches in trees. On the one hand, association genetics is

seeking for statistical correlation between SNPs and traits owing to the significant heritability that is usually observed in progeny tests. On the other hand, detection of outlier Fst values of single SNPs has also been implemented to account for the very large differentiation of traits that are observed in provenance tests. The rationale of both approaches is based on the extensive within or between population genetic variability that has been widely recorded in fields tests. While the cataloguing of candidate genes has steadily increased in trees, so has the inventory of their diversity in natural or breeding populations. There is now a rapidly growing body of experimental results showing a very large discrepancy between the expectations of both approaches and the SNP variation that is monitored in candidate genes. Most association studies show that single SNPs explain usually less than 5% of the phenotypic variation of the trait, while Fst values are at best of the same value than neutral markers. In my presentation I will explore the reasons of the decoupling between trait and gene variation, by focusing on the multilocus structure of traits, as compared to the monolocus SNP information. Indeed, association studies and Fst outlier detection are essentially based on single locus approaches, while traits are multidimensional structures. There are at least three properties of multilocus structures that will be investigated: cumulativity, interactions and covariation of gene effects. I will show that cumulativity and interactions may explain the discrepancy in the case of association studies, while covariation of gene effects (at the between population level) explain the missing Fst of genes underlying adaptive traits. These conclusions are supported by experimental results, theoretical background and simulation predictions.

I2

Population and conservation genomics of forest trees: seeing the forest for the trees

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BMC Proceedings 2011, **5(Suppl 7):**I2

Background: Forest trees exhibit striking adaptations to the environments in which they grow. A long history of quantitative genetic experimentation has established the genetic basis for many traits, which are likely adaptive since many of them are also correlated with environmental heterogeneity. The genes underlying these traits, however, have largely remained elusive. Recent applications of high-throughput sequencing and genotyping technologies to natural populations of forest

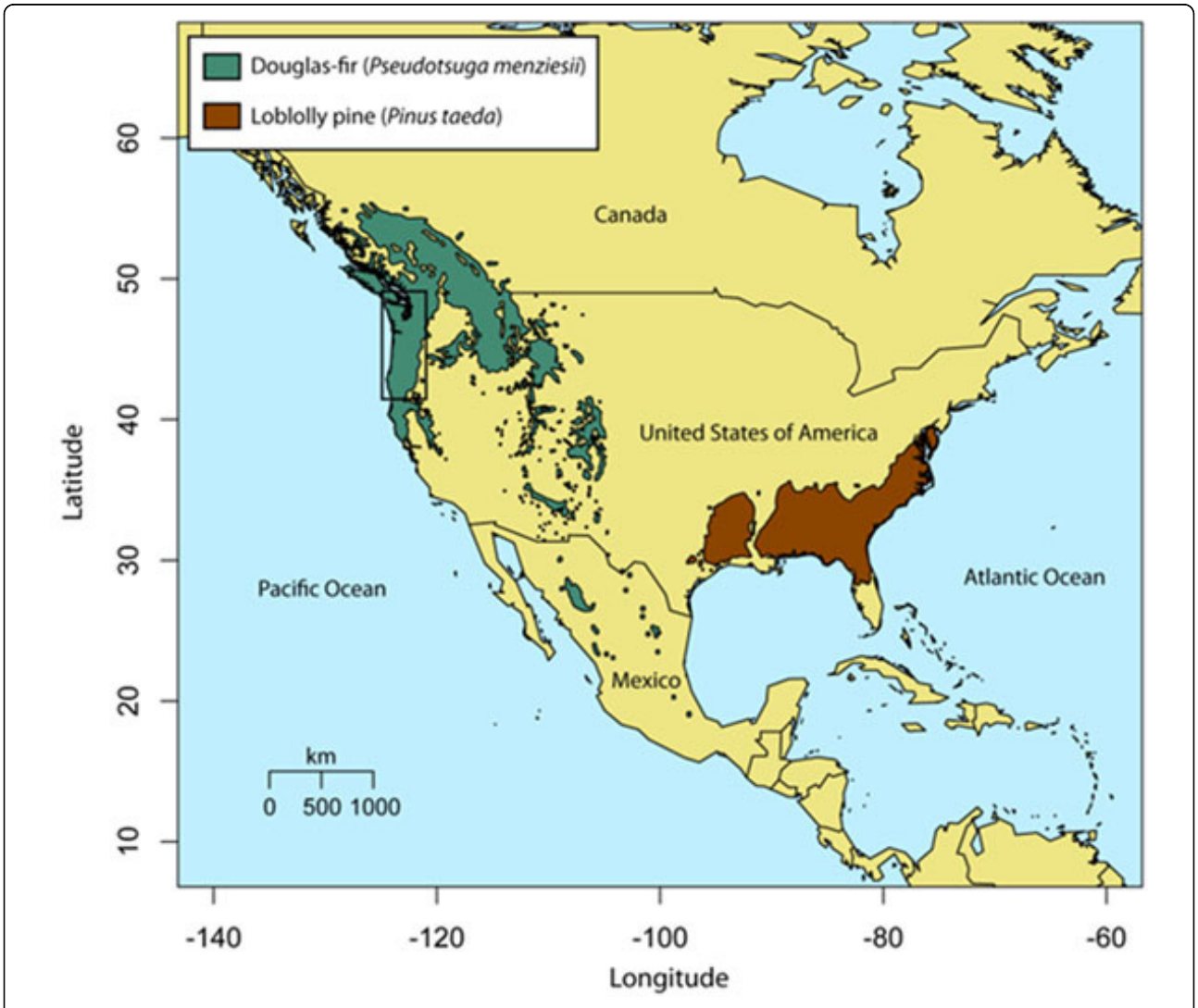

Figure 1(abstract I2) The distribution of Douglas-fir and loblolly pine in North America. The black box surrounds the sampling area for Douglas-fir, while the entire range for loblolly pine was sampled.

trees have identified several promising candidates for genes underlying complex and adaptive traits (reviewed by [1]). The diversity of analytical approaches employed in those studies, however, begs the question of the generality of reported results. Here, I exploited the diversity of analytical approaches used previously to identify genes underlying adaptive traits for two conifer species to assess the logical consistency among results generated from different conceptual frameworks.

Materials and methods: Data sets comprised of genotyped single nucleotide polymorphisms (SNPs) were gathered for two North American conifers (Fig. 1): loblolly pine (*Pinus taeda* L.) and coastal Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco var. *menziesii*). These data sets were generated through resequencing of diversity panels (n = 18-24 megagametophytes) for a sample of expressed sequence tag (EST) unigenes (n = 7,535) and cold-hardiness related candidate genes (n = 121), respectively. Genotyping was performed using Illumina's Infinium (loblolly pine) or GoldenGate (coastal Douglas-fir) array technologies. For each data set, associations to phenotypes and environmental variables were gathered from the literature or performed as described elsewhere

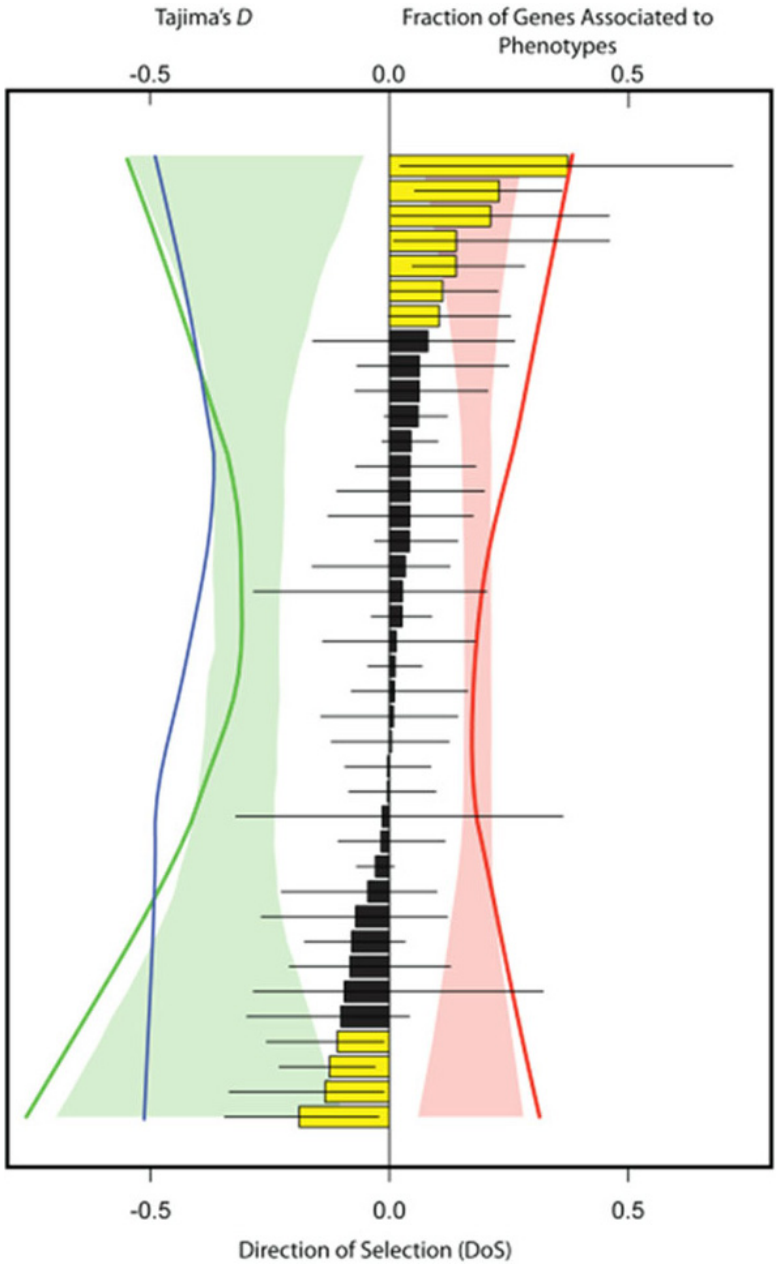

Figure 2(abstract I2) An example of logical consistency for loblolly pine between genes associated to phenotypes or environmental variables and gene categories that have average deviations from neutrality consistent with positive or negative selection. Error bars give 99% bootstrap confidence intervals for the direction of selection statistic (DoS), with yellow bars having confidence intervals excluding zero. Lines give average Tajima's D for nonsynonymous (blue) and synonymous sites (green), as well as the proportion of genes associated to at least one phenotype (red). Shaded areas give null distributions (99% quantiles) generated via permutations of genes among categories (n = 10,000 permutations). All lines, including those forming the null distributions, were smoothed using lowess smoothing.

(see references in [1]). For each associated gene, I asked two questions: (1) Are genes associated to phenotypes more often also associated to environmental variables than randomly chosen genes? (2) Are associated genes also outliers for nucleotide diversity and site-frequency spectrum based statistics, nucleotide divergence or F_{ST} more often than randomly chosen genes? Permutation tests were used to assess whether or not observed patterns were different than those produced by chance.

Results: A total of ~30 genes were associated with cold-hardiness phenotypes or with climate data for coastal Douglas-fir, while ~850 associated genes were identified for loblolly pine. Genes associated with phenotypes for coastal Douglas-fir were more often associated to environmental variables than randomly chosen genes ($P = 0.005$), which was only moderately apparent for loblolly pine ($P = 0.067$). Genes associated to phenotypes or environmental variables were not more likely to be outliers for nucleotide diversity, site-frequency spectrum based statistics, nucleotide divergence or F_{ST} for Douglas-fir ($P > 0.05$). There was some evidence, however, for non-neutral evolution for associated genes using a statistic based on the McDonald-Kreitman table [2]. In this case, associated genes had too many extreme values for the direction of selection (DoS) statistic than expected from randomly resampling the available genes ($P < 0.05$). Associated genes on average had skewed site-frequency spectra for loblolly pine, especially for synonymous sites, as well as too many extreme values for the DoS statistic. Further classification of genes for loblolly pine into functional categories revealed striking trends indicative of non-neutral processes underlying some of the associations (Fig. 2). A total of 11 gene categories were consistent with positive ($n = 7$) or negative ($n = 4$) selection. In both cases, the frequency of associated loci increased for these categories and the synonymous site frequency spectra became more skewed. Many associations for loblolly pine may thus reflect linked selection, with molecular phenotypes (e.g. gene expression) accounting for all the associations in gene categories indicative of negative selection and environmental associations (e.g. aridity) being largely located in gene categories consistent with positive selection.

Conclusions: Many of the genes associated to phenotypes for both species were also correlated to environmental variables and exhibited patterns of non-neutral evolution. Thus, associated genes are prime targets for conservation efforts. The questions posed here, however, make the strong assumption that genes associated to phenotypes or environmental variables should also show non-neutral patterns of evolution. This is not always expected to be the case [3,4], yet the lack of consistency is often interpreted as such and is one explanation for those genes or sets of genes reported here as lacking non-neutral signals. The search for logical consistency among analytical approaches, however, often focuses on uninformative patterns. To illustrate this point, I employed a novel environmental association approach that correlates genetic divergence to environmental change and show that most of the site-frequency spectrum based outliers for coastal Douglas-fir [5] are correlated to change in climate variables but not to extant climate patterns. Taken together these results illustrate that non-neutral genes are often identified during association analyses, that departures from neutrality for genes driving associations are not only those due to recent directional selection, and that further work is needed to understand the population genetic processes underlying associations between genotypes, phenotypes and the environment.

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I3

Tracing the history of South-American neotropical savannas and seasonally dry forests evidences from comparative phylogeography

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Background: The Neotropical Seasonally Dry Forests (SDF) are tree-dominated ecosystems that occur in disjunct areas of fertile soils throughout the Neotropics. The hypothesis that the vicariance of a formerly continuous seasonal woodland formation, which may have reached its maximum extension during a dry-cool period 18,000–12,000 bp (the LGM), the Pleistocenic Arc Hypothesis, has been raised to explain the disjunct distribution [1]. Alternatively, based on the distribution of contemporary SDF species in Amazonia Forest and palynological data, Pennington [2] also proposed that SDF may have expanded into Amazonia Basin during the Pleistoce, with rain forest and montane taxa largely confined to gallery forest. In addition, a number of studies based on the fossil pollen record now available show that during the early Holocene period (until ca. 6000-5000 14C B.P.), the climate was drier in most of the South American savannas and distribution of savanna-like vegetation in Central and Southeast Brazil was more extensive in early compared with the late Holocene [3-5]. In southeastern Brazil, the current vegetation exist in the region only in the latest Holocene period (since 970 or 600 B.P. for some regions) under the current wet climatic conditions, with an annual dry season of about 4 months. Hence, the fossil record shows that savanna expansion in the Quaternary, especially in southeastern Brazil was characterized mainly by herbaceous and grass savanna which were favored by the drier and highly seasonal climate. It is possible that arboreal savanna taxa became restricted to sites with moist climatic conditions, which served as refugias. We are interested in test these hypotheses using the genus *Tabebuia* as model group. We have chosen five species based on the pattern of geographical distribution: *T. aurea* and *T. ochracea*, from savanna vegetation (cerrado sensu stricto), *T. impetiginosa*, T. roseo-alba from SDF, *T. serratifolia*, widely distributed in Mata Atlantica, SDF, riparian forests and Amazonia. Here, we present the results based on the phylogeography of *T. impetiginosa*.

Methods: We first generated a phylogenetic hypothesis based on sequences from three non-coding regions of cpDNA and ITS from nuclear rDNA. At least 16 individuals from 14 populations were sampled and sequenced for three chloroplast intergenic spacers (1635bp). We also sequenced individuals of *T. ochracea*, *T. chrysanta*, *T. chrysotricha* and *Cybstax antisyphilitica*, as outgroups to estimate coalescence time and better understand the biogeographical history of *T. impetiginosa*. Time to most recent common ancestor was estimated based on coalescent analysis implemented in BEAST 1.4.7 software [6]. For distribution modeling, we used 77 records of *T. impetiginosa* and four climatic variables (mean annual rainfall and variability, average temperature of the warmest and coldest months) derived from four different coupled Atmosperic-Oceanic Global Circulation Models (AOGCM): CCSM3, CSIRO, HADCM3 and ECHAM. Five different niche models were used: the BIOCLIM , Euclidean Distances (EUCL) Mahalanabis Distances (MAHAL), Genetic Algorithm for Rule Set Production (GARP), and Maximum Entropy (MAXENT). For each of the NM, a total of 750 different models were generated, using distinct combinations of dataset partition and variable selection The paleoclimate scenarios were based on the GCM Genesis 2 [7].

Results and discussion: We have no evidences of a recent connection of the SDF in the LGM. Coalescent analyses showed that *T. impetiginosa* populations probably originated at ~7 Myr BP, but populations started to diverge only ~2 Myr BP, with the divergence of two major clades, one that comprises the populations from the East and West boundaries of Cerrado Biome, and the other that corresponds to the forests from Central, West and Northeast Brazil. Major divergences in this last clade coincide with the Quaternary glaciations. Our results strongly support that the disjunct distribution of *T. impetiginosa* may be derived from vicariance and that

long distance gene flow is unlikely, since we found a deep geneanalogy with alopatric lineages and a remarkable differentiation among populations (AMOVA, Arlequin v. 3.11) [8]. Paleodistribution modeling together with coalescent simulation showed no connection among SDFs in the LGM. Thus, glaciations may have most likely caused population divergence and differentiation due to retreat and range shift of an ancient forest widely distributed throughout the Central, Southwest and Northeast Brazil. Hence, we hypothesize that the isolation of an ancient forest due to a global cooling and drying during the glaciations of Pliocene/Pleistocene is responsible for the disjunct distribution of the SDF and *T. impetiginosa*. Our results also indicated that the last glaciation in Pleistocene were most likely responsible for local differentiation because lineage diversification within localities coincides with ~ 100 kya BP but without important demographic effects because populations showed a constant effective population size and no exponential growth or shrink. In conclusion, the range shift of *T. impetiginosa* toward the Northwest and the lack of a connection among SDFs in the LGM support the hypothesis of a kind of SDF in Amazonian Basin during Pleistocene glaciations but did not support the Pleistocenic Arc Hypothesis.

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S2. LINKAGE AND ASSOCIATION MAPPING

I4

Genomics-based breeding in forest trees: are we there yet?

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BMC Proceedings 2011, **5(Suppl 7):**I4

Efforts to develop genetic marker based approaches to breeding forest trees began in the late 1980s. Approaches based on first generation of markers, allozymes, were not feasible due to the very limited number of markers (<50). The first DNA-based markers, RFLPs, brought more hope as moderately dense genetic maps could be constructed to scan the genome and map quantitative trait loci (QTLs). This approach was quite effective toward mapping QTLs in many forest tree species but the approach could not be brought to application in tree breeding due to low levels of linkage disequilibrium (LD) in forest tree breeding populations and recombination between flanking markers and QTLs with each generation. The next generation of DNA markers based of the polymerase chain reaction (PCR), RAPD, AFLP and SSR, did not solve the LD and recombination problem, even though more markers were available and throughput increased.

The situation began to change in the early 2000s with the availability of automated DNA sequencing technology and single nucleotide polymorphism (SNP) genetic markers. Now association studies could be performed where SNPs within candidate genes controlling complex traits could be identified and thus "solving" or minimizing the LD and recombination limitation. This approach to complex trait dissection has been widely applied in forest trees and the early approach of QTL mapping in segregating populations has been mostly abandoned. The association genetic approach has been used to find candidate gene SNPs associated to a broad array of quantitative traits of interest (wood properties, growth, abiotic stresses and disease resistance). However, like QTL mapping before, individual SNP x trait associations only account for a small proportion of the variation (generally less than 2-3% of the total phenotypic variance) and the total variation explained by all markers is generally less than 50%. In human genetics, this situation is called the "missing heritability" and there has been great debate over whether this problem can ever be solved in complex trait dissection in humans. In a few agricultural systems however, notably dairy cattle, researchers are now accounting for nearly all the heritable variation.

Efforts to realize genomics-based breeding in conifer tree improvement in the US have culminated under a collaborative research project funded by the USDA. The Conifer Translational Genomics Network Coordinated Agricultural Project (CTGN CAP) has successfully brought genomics-based breeding to application in the four major cooperative forest tree breeding programs in the US. The project has completed the basic research on allele discovery of economic traits in conifers, conducted the translational research, and provided education and training to tree breeders to realize this goal during the four-year project period (2007 to 2011). Genomics-based breeding can now be applied to the production of over 1.3 billion loblolly pine, slash pine and Douglas-fir seedlings planted annually in the US.

The CTGN CAP obtained high-density single nucleotide polymorphic (SNP) genotypes (over 7000 SNPs) on nearly 10,000 loblolly pine, slash pine, and Douglas-fir trees from the breeding cooperatives. These data were used to estimate molecular breeding values that can now be used to make selections as an alternative to breeding values based on mature traits that can take many years to the time of evaluation and can be expensive to measure in field tests. The CTGN CAP was the first to apply the advanced Illumina Infininium SNP-genotyping technology that is now being used in the breeding of most major crops such as corn, wheat, tomato, potato, and many more.

The CTGN CAP culminates a long-term investment by the USDA in this team of researchers and their work on the technological advancement of tree breeding in the US. USDA's efforts began more than 20 years ago with funding of a series of grants under the National Research Initiative Plant Genome Program and continued under the CSREES IFAFS and AFRI programs. Recently, NIFA has awarded a grant to members of this team to conduct full genome sequencing in loblolly pine, sugar pine, and Douglas-fir. The full genome sequence will soon allow tree breeders to practice genomics-based breeding using genetic variation from the entire genome (genomic selection), and thus minimizing or even eliminating the missing heritability problem in forest trees. The partnership between USDA research programs and this team of researchers has resulted in a modernization of tree breeding technology in the US that will secure US competitiveness in the production of forest products.

I5

Identification of genes and alleles influencing wood development in Eucalyptus

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BMC Proceedings 2011, **5(Suppl 7):**I5

The goal of many forest tree breeding programs is to increase the quantity and quality of wood products from plantations. Due to their outcrossing breeding systems, long generation times and relatively short history of domestication, breeding populations of most forest trees

closely resemble the wild state. Consequently, vast stores of genetic variation are available for selection. Because wood traits are under polygenetic control (quantitative), genetic improvement will rely on selection of multiple alleles, each of relatively small individual effect.
Marker-assisted selection may enhance tree breeding programs by enabling informed selection of parents for crossing; fixing desirable alleles in the homozygous state; increasing selection intensity through screening large numbers of individuals; enabling early selection in seedlings and by reducing phenotyping costs. The low linkage disequilibrium found in most forest trees makes them ideally suited to candidate gene-based association mapping approaches for marker discovery. This approach seeks to find alleles which affect phenotype and that remain linked to the trait across populations and over many generations. This methodology is well suited to tree breeding programs which aim to maintain a broad genetic base i.e. programs with a large number of families.
We are using association studies to identify genes and allelic variation that influences wood fibre properties in *Eucalyptus nitens*. Candidate genes are being selected on the basis of their known involvement in cell wall synthesis pathways expected to impact wood traits. Single nucleotide polymorphisms (SNPs) are identified in candidate genes by sequencing in a number of unrelated individuals. Selected SNPs are being genotyped across large unrelated *E. nitens* populations that have been extensively phenotyped for wood properties including cellulose and lignin content, pulp yield, MFA, and density. Several SNPs significantly associated with wood properties have been identified and subsequently validated in other provenance or mapping populations growing in different environments. Selected SNPs are being investigated further to determine whether or not the SNP is the causative polymorphism and how the polymorphism influences the trait. DNA markers identified in this research may be used to complement existing index selection strategies in *E. nitens* breeding programs. Strategies for exploiting SNPs for marker-assisted selection in seedling-based breeding programs will be discussed.

I6

Gene mapping in white spruce (*P. glauca*): QTL and association studies integrating population and expression data

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Background: Connecting phenotype with genotype is the basis for developing forest genetic applications such as marker assisted selection (MAS). Quantitative Trait Locus (QTL) mapping and genetic association mapping (or linkage disequilibrium (LD) are two major approaches to find genes that control phenotypes of interest in forest trees. Quantitative trait loci (QTL) and association mapping experiments in white spruce (*Picea glauca* [Moench] Voss) aimed to identify genes linked to or associated with growth, adaptation, and wood property traits.
Gene mapping in conifer trees presents us with specific challenges, including very large genome sizes, low level of linkage disequilibrium, and large effective size in breeding populations. Therefore, association mapping experiments have relied on testing candidate gene targets rather than genome-wide association scans. We have explored different approaches to utilize gene expression and SNP outlier data to identify candidate genes, to help to explain the findings of gene mapping experiments and provide a broader understanding of observed phenotypic variations.
Results: Growth and phenology: The genomic architecture of bud phenology and height growth was investigated by assessing QTLs across pedigrees, years, and environments (1).A total of 11 distinct QTLs for bud flush, 13 for bud set, and 10 for height growth were localized on a linkage map highly-enriched in gene markers. Nearly 50% of the QTLs were stable across environments and/or years and 20% were replicated between populations. The proportion of phenotypic variance explained by QTLs ranged from 3% to 22.2%, and QTLs accounted for up to 70% of trait variance. These outcomes were integrated with findings from studies aimed identifying local adaption genes and gene expression associated with bud formation.
A genome-wide scan of 534 SNPs localized in 345 expressed genes was used to detect genes putative linked to local adaptation (2). We identified 5.5% of genes as outliers with FST at the 95% confidence level, and 14% of genes as candidates for local adaptation with a Bayesian method. The list of candidate genes and outliers includes sequences which co-localized with the QTLs for bud phenology.
A bud set roadmap was constructed by comprehensive microarray and qRT-PCR analysis of dormancy transition in bud, stem, needle, and root tissues over a time course, under short and long days (3). Tissue expression profiles were used to identify genes expressed only or preferentially in developing buds, which we hypothesize to play a more prominent role in bud formation. A core group of genes likely involved in the initiation of bud formation included about 100 of the bud-prominent genes and several sequences encoding potential regulatory proteins. Several of the bud set roadmap genes including bud-prominent genes co-localized with QTLs for the time of bud set.
Wood properties: Wood physical traits were assessed using SilviScan technology in a population of 1700 trees comprising 215 open-pollinated families. In a pilot study, we tested for associations between single nucleotide polymorphisms (SNP) in 550 candidate genes and wood traits (4). We found 13 SNPs significantly associated with wood traits. The phenotypic variance explained reached up to 11% with approaches combining several SNPs.
Most association studies of wood properties have tested candidate genes that are highly expressed in secondary xylem, hypothesizing that genes that are preferentially or strongly expressed during wood formation are more likely to control wood properties. However, this hypothesis had not been tested. The genotyped sequences included genes with diverse expression profiles. Their transcript accumulation profiles were determined in trees grown under controlled conditions with a large-scale custom oligonucleotide microarray representing 25,094 different spruce genes. Of the 550 genes tested for association, 29% accumulated preferentially in secondary xylem compared to both secondary phloem and needles, but as many genes (29%) were phloem preferential. Xylem-preferential RNA accumulation was found for 10 of the 13 genes harbouring SNPs significantly associated. Our findings confirm that expression data were relevant for selecting candidate genes but not all of the genes containing significant SNPs were xylem preferential.
Transcript accumulation was also studied in secondary xylem of trees from the provenance-progeny trial, to further characterize the genes containing SNPs significantly associated with wood traits. In some cases, significantly different transcript levels were found among the different SNP genotypes. Xylem-preferential RNA accumulation was shown for the majority of these genes, which indicates that. Our results suggest that differential expression may be associated with SNP genotypes.
Large-scale genotyping: A meta-analysis was used to integrate data from multiple experiments in order to identify and assign priorities to approximately 5000 candidate genes for association mapping experiments. The candidate gene selection considered the above gene expression data, findings from transcriptomic investigations of gene regulation, studies investigating transcriptional variation within mapping populations trees, and outlier data related to local adaptation. A large-scale genotyping chip was developed and data were obtained for 7000 SNPs from nearly 2500 genes.
Conclusions: This report describes QTL mapping and genetic association mapping results. We have illustrated ways in which gene expression and population data may be of value in these approaches, whether they are used to select candidate genes or to characterize the physiological processes underlying marker-trait associations.

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S3. GENOMICS ASSISTED BREEDING

I7

Capturing and genotyping the genome-wide genetic diversity of trees for association mapping and genomic selection

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Background: Growing demand for food and fiber, and a rapidly changing climate will require that plant breeders accelerate the improvement of germplasm adapted to new sources of biotic and abiotic stress. In trees, the threat from climate change is more evident and the solutions more challenging than in any other plant species, due to the complexity and cost of breeding programs, and the long breeding cycles. Therefore, the discovery of genetic polymorphism that can be exploited for early selection of better adapted and productive individuals is essential. Quantitative trait loci (QTL) analysis provided an initial glimpse at the architecture of complex traits, but limited transferability across populations and resolution hampered the adoption of markers in tree breeding programs. Recently, association studies have become the method of choice for detection of markers implicated in trait variation, because of higher resolution, population transferability and allelic diversity captured relative to the QTL approach. However, in tree species, association studies have been largely constrained to sampling the genetic diversity in a limited fraction of the genome, and in small populations. Evidence from genome-wide association studies (GWAS) in humans and advanced crops clearly show that larger populations, and the sampling of regulatory variants and rare alleles is critical to dissect the genetic control of complex traits for marker-assisted breeding (MAB). As the limitations of QTL and GWAS approaches become evident, "hybrid" intermediate strategies that combine the advantages of both methods have emerged. Notably, genomic selection has become an alternative to MAB. Genomic selection (GS), which relies on developing genome-wide marker-based models that predict the genetic value of progeny, will be particularly valuable for early selection in tree breeding programs. However, the implications of GS may also be highly valuable to identify mating designs that generate progeny with optimal allelic combinations for superior growth and wood properties, and adaptive capabilities.
Methods: To address limitation of recent association studies in forest tree species we have adopted an approach that combines targeted sequence-capture followed by high-throughput sequencing, to genotype eastern cottonwood (*Populus deltoides*) and loblolly pine (*Pinus taeda*) populations. To identify genetic polymorphisms that regulate biomass productivity, wood quality, and disease resistance, we have optimized methods of sequence-capture targeted regions of the genome for unbiased, high-throughput and low cost recovery of coding and regulatory sequences. This establishes the foundation for GWAS in both species, addressing a critical limitation of these studies in tree genomes – i.e. sampling of regulatory variants and rare alleles.
While the work described above resolves part of the challenges of association studies, the use of GWAS will be most useful for discovery of genes to be targeted for genetic modification, rather than MAB. This is the case because the fraction of the total genetic variation explained by association studies is likely to remain small, considering the limitations of the existing populations. In an effort to implement marker-based breeding, we recently completed the first assessment of the utility of GS in an experimental population of loblolly pine, where we explored the contribution of factors such as age of model estimation and site location on the accuracy of prediction models. Furthermore, the incorporation of non-additive effects to prediction models has been evaluated for improvement of their accuracy.
Results and conclusions: Two association studies are currently underway, in loblolly pine and *Populus deltoides*, where a large fraction of the coding and regulatory sequences of their genomes are being re-sequenced for SNP detection. Existing results from sequence capture indicate that the majority of targeted regions can be effectively captured, even in genomes of very high complexity, such as that of loblolly pine. In parallel we have now verified the suitability of applying genomic selection to a breeding population of loblolly pine. Estimates generated for prediction models developed for this population indicates that accuracies that are comparable to traditional phenotypic selection can be obtained. Therefore, considering the significant reduction in the breeding cycle length due to early identification of elite genotypes, the increase in efficiency per unit of time in the selection response of genomic selection is almost twice as high, compared to traditional breeding. By combining GS with advanced methods of vegetative propagation, breeding and seedling production, a breeding cycle can be reduced from decades to less than 5 years. While early selection of superior genotypes is an obvious application of GS to tree improvement programs, prediction models can be utilized to guide the mating design of future breeding cycles to favor stacking of favorable alleles over multiple generations. Towards this end, we have initiated crosses aimed at generating families that are predicted to have exceptional adaption, as well as biomass growth and quality properties for bioenergy, pulp and paper and timber production.
Acknowledgements: This work was supported by the University of Florida Genetics and Genomics Graduate Program, the Plant Molecular and Cellular Biology Graduate Program, the Forest Biology Research Cooperative (FBRC), the US Department of Agriculture National Institute of Food and Agriculture Plant Breeding and Education Program (award no. 2010-85117-20569) and the National Science Foundation (award no.DBI-0501763).

I8

Genomic selection in loblolly pine - from lab to field

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Background: Tree breeding is logistically complex and expensive, and breeders have long sought to use molecular markers to accelerate breeding. A candidate gene approach based on testing for association between the presence of DNA sequence variation in or near candidate genes, and phenotypic variation in a population has long been explored [1,2]. However, using candidate gene approach (QTLs) has not been successful in breeding [3,4]. QTL-trait associations detected in one genetic background are often not observed in other families, because of recombination of genes during the segregation and low levels of linkage disequilibrium in the population. A new technology called genomic selection (GS) is revolutionizing dairy cattle breeding. In GS, marker effects are first estimated in a large training population (>500) with both phenotypic and genotypic data. Subsequently, estimated marker effects are used to predict breeding values in validation populations for which marker genotypes but not phenotypes are available [4]. Several dairy cattle breeding companies now routinely use GS to select and market bulls. The success of GS in cattle breeding is largely based on bovine genome sequencing and discovery of thousands of SNP markers. GS application, if successful, will have a great impact on forest tree breeding because of their complex and logistically difficult breeding programs. Although, there have been several simulation studies examining the effective population size, linkage disequilibrium, and heritability on the predicted accuracy of

GS in tree breeding [5], GS has not yet been demonstrated for forest trees using empirical markers data, mainly due to lack of sufficient dense markers.

Methods: Biallelic SNP markers provided by the CTGN project (http://dendrome.ucdavis.edu/ctgn/) were used for genotyping. A population of 149 cloned full-sib offspring of loblolly pine (*Pinus taeda* L.) was phenotyped. Fitting 3406 informative SNP markers simultaneously, we estimated genome-wide breeding values and compared them with breeding values based on pedigree model. Variances explained by the marker additive and dominant effects were obtained.

Results: The accuracy of the genomic estimated breeding values ranged from 0.30 to 0.83 for growth and wood quality traits. Lignin and cellulose content had great accuracy values from GS compared to growth traits. The accuracies were comparable with breeding values that were calculated based on the traditional pedigree model. If we take into account time needed to complete progeny testing, GS would be more efficient than classical progeny testing for some traits. The marker additive effects explained 18% and 23% for lignin and cellulose, respectively. Variances could not be determined for height and volume, because the Gibbs sampler failed to converge, even after five million iterations. We speculate that observed accuracies in this study trace familial linkage rather than historical LD with trait loci, because of small population size and relatively deep pedigrees. The markers are sampling the haplotypes and thus constructing the pedigrees rather than explaining phenotypic variance. Nevertheless, the results are promising, and we expect that with decreases in genotyping cost, GS has a potential to fundamentally change tree breeding in the near future.

Challenges of GS applications in tree breeding: Despite promising results from some early work based on empirical data, there some challenges to overcome to routinely use GS in tree breeding. Conifers have genome size with a range between 18,000 and 40,000 Mbp [6]. Their populations have low levels of LD which decays rapidly. LD in loblolly pine decays to less than r^2=0.25 within 2000 bp [7]. Low LD is due to genetic recombination over the evolutionary history of the species and causes inconsistency of QTL-marker association. Large genome size and historically low LD require large numbers of dense markers to explain a considerable amount of phenotypic variation in complex traits.

Another challenge is the lack of genetic maps in forest trees. With a few exceptions, the genomes of forest trees have not been sequenced, and thus precise locations of SNP markers are lacking, which hinders the use of haplotypes. Using haplotypes reduces the dimensions of the data and thus requires much smaller computing resources to analyze. More importantly, with haplotypes, larger variation between trees can be obtained using allelic combinations, although larger training populations are required to adequately sample the effects of all the haplotypes.

High marker genotyping cost is the major obstacle in applications of GS in forest trees. More cost efficient genotyping technologies, such as genotyping-by-sequencing and restriction digestion are being explored to reduce cost of markers. On the other hand, advances in computer power have made it possible to analyze large amount of complex data, but bioinformatics challenges still remain to analyze sequence data and SNP marker calling.

Further research is needed in development of training models and calibration of prediction model. The number of generations that statistical models can be used before losing accuracy remains to be determined in forest trees. Another question is the validity of models across different populations. In cattle breeding, lower accuracies of GS for dairy versus beef cattle remains a challenge. For some tree species, GxE interaction could be an issue to be addressed; observed marker-trait association observed in one population may not hold in another environment.

Conclusions: We are currently working on construction of realized genomic relationship matrix based on SNP markers to use in predictions of breeding values. This method provides flexibility in terms of fitting common environmental effects in mixed models. We expect that decreases in marker genotyping costs will make GS in pine breeding feasible in the near future. Our group will work on pilot projects with forestry companies in the southern US, and plans are underway to revise breeding strategies to incorporate genomic selection.

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S4. REPRODUCTION, GROWTH AND DEVELOPMENT

I9

Salicylate metabolism in Populus

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Phenolic metabolites that contain salicylic acid (SA)-like moieties are major non-structural constituents in *Populus* leaves, shoots and roots. These so-called phenolic glycosides (PGs) are taxonomically limited to the Salicaceae, where they are known to mitigate insect and animal herbivory. SA itself is an important signaling molecule in plant defense and abiotic stress responses, and is derived from the isochorismate or phenylpropanoid pathways, depending on the species [1,2]. Hyper-accumulation of SA reduces growth in *Arabidopsis*[3], while high levels of PG have been associated with reduced growth in *Populus*[4]. Although the common PGs, salicin, salicortin, and their derivatives contain a salicyl moiety, a direct metabolic relationship between PG and SA in *Populus* has not been shown. We have therefore been using stable isotope tracers, cell culture feeding, functional genomics and transgenic perturbation to probe the relationship between SA, PG and growth in *Populus*.

Stable isotope incorporation confirmed a hydroxycinnamate-benzoate origin of the salicyl moiety of PGs, and provided the first empirical evidence that the bioactive hydroxycyclohexenone moiety of salicortin is also a phenylpropanoid derivative [5]. Therefore it is unlikely that PG biosynthesis involves the salicylic acid pathway. To further address any possible involvement of the isochorismate pathway in PG biosynthesis, isochorismate synthase (ICS) was characterized. *ICS* is present as a single-gene in most sequenced plant genomes, but is duplicated in *Arabidopsis*. In *Arabidopsis*, chorismate-derived SA is the obligatory route for defense signaling, mediated by *AtICS1* in response to various biotic and abiotic cues. In contrast, the single-copy *Populus ICS* is not stress-inducible, and is involved in biosynthesis of phylloquinone for photosynthetic electron transport [2]. We found no evidence of *ICS* involvement in SA or PG biosynthesis in transgenic *Populus*, pointing to lineage-specific evolution of the ICS-derived SA pathway in *Arabidopsis*.

We transformed *Populus* with the bacterial genes for the biosynthesis and degradation of SA for a more in-depth investigation of the possibility of an SA interaction with PG regulation. SA-hyperaccumulating and SA-deficient lines were generated by expressing a salicylate synthase and a salicylate hydroxylase, respectively. SA was converted to SA-glycoside (SAG) and gentisic acid-glucoside (GAG) in the hyperaccumulating lines. SAG and GAG were very low in wild-type and were not detected in the SA-deficient lines. Despite these clear differences, no changes in PG levels were detected. These results argue against SA as potential precursor of PGs. Survival and establishment of rooted cuttings were negatively affected by SA-hyperaccumulation. Once established, however, growth rates were similar among plant lines, in sharp contrast to SA-over-producing *Arabidopsis*. SA-hyperaccumulating lines exhibited altered thermal tolerance, based on electrolyte leakage assays and metabolite profiling.

Together, our results provide support for a phenylpropanoid origin of PGs, and argue against the involvement of SA in PG biosynthesis. The data is consistent with complementary roles for SA and PG in *Populus* fitness.

A number of other experiments were conducted to investigate the regulation of PG homeostasis in *Populus*. Administration of a putative PG precursor salicyl alcohol to cell cultures yielded the glucoside, salicin. Salicyl alcohol-feeding also altered the partitioning of carbon into condensed tannin (CT) biosynthesis, another quantitatively important phenylpropanoid product [6]. Salicyl alcohol-feeding induced expression of several glycosyltransferases (GT1), as well as genes associated with sucrose catabolism, glycolysis and the Krebs cycle, presumably to accommodate the increased glycosylation. Conversely, transcript levels of most of the flavonoid pathway genes were reduced, consistent with reduced CT synthesis. Given that glycosylation was the only PG biosynthetic step occurring in the fed-cells, the results suggest that glycosylation of phenolic products can play a role in regulating the tradeoff between competing chemical defenses, *e.g.*, PGs and CTs.

Correlation analysis was used to identify PG-coregulated genes from a number of inductive treatments. One such candidate, *SUT4* encoding a tonoplast sucrose transporter, was of particular interest because of the reported salicin transport activity of its *Arabidopsis* ortholog. RNAi-silencing of *SUT4* altered sucrose distribution between source and sink organs, and reduced PG accrual by 20-30%, accompanied by altered biomass partitioning [7]. Chemical and molecular analyses revealed a complex network linking PG accrual and carbohydrate homeostasis.

PG-coregulated GT1 members were also targeted for characterization, due to the prominent role of glycosylation in PG biosynthesis. We identified a novel salicyl alcohol glycosyltransferase (UGT71L1). Over-expression of UGT71L1 in transgenic *Populus* led to a ~2-fold increase in concentrations of salicin and salicortin in roots. The quantitative effect on secondary metabolism is significant, as PGs are the second most abundant non-structural phenolics, after CTs, in the roots of this poplar clone. UGT71L1 is phylogenetically distinct from the well-characterized SAGTs. It belongs to a *Populus*-specific clade with no close homologs in *Arabidopsis*, *Vitis*, *Medicago* and *Oryza*, consistent with the limited taxonomic range of PGs. Overall, the experiments described highlight the important contributions of sugar transport and phenolic glycosylation to PG homeostasis, while also showing that SA and PGs make independent but complementary contributions toward the maintenance of *Populus* fitness.

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I10

Division of labor: multiple and specialized controls of vegetative growth and development in a poplar tree

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Plants size, shape and adaptability are determined in large by their capacity to sustain, spur, redirect or arrest localized and whole-plant growth. We show that GA signaling and metabolism determines the level and extent of shoot and root growth. Increase of GA concentrations or signaling leads to increased shoot growth but suppresses root development. These GA-related responses are underpinned by sets of highly specialized in their functions enzymes and signaling factors and cross-talk with other hormonal pathways. The differential effects of GA on root and shoot growth and development are likely associated with a regulatory mechanism responding to optimum and stress conditions. Ongoing work in the laboratory employs genomics and genetics approaches to more thoroughly understand poplar growth and development under stress conditions including nitrogen and water limitations. We use genetic networks analysis and forward genetics approaches to identify key regulators of poplar roots response to stress. Besides overall growth, trees show incredible repertoire of spatiotemporal regulation of growth in relation to control of organ size, growth periodicity and tropic responses. We have identified and characterized novel regulators of these processes in poplar.

I11

Regulation of shoot-system development in *Populus*

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Shoot phenology interacts with crown morphology to determine leaf production and duration over the growing season and throughout a tree's life span; and thus, this interaction has a major role in determining whole-tree photosynthesis and biomass yield. For several years, the pattern of shoot meristem activity determines *Populus* crown architecture, but after the first onset of flowering the specification of meristem identity (inflorescence vs. vegetative) also has a major influence on crown form. To study how shoot meristem activity and identity are regulated in *Populus*, we are focusing on two pathways that have been extensively characterized in annual plants: 1) the *MORE AXILLARY BRANCHING* (*MAX*) genes that are involved in the synthesis and perception of upwardly mobile strigolactones that inhibit axillary bud outgrowth, and 2) members of the *TERMINAL FLOWER1* (*TFL1*)/*FLOWERING LOCUS T* (*FT*) and *APETALA1*(*AP1*)/*FRUITFULL*(*FUL*) gene families that regulate flowering. We are taking a multi-pronged approach that includes field and controlled-environment study of poplar transgenics, transcriptome analyses, cell-specific expression studies, and QTL and association mapping.

QTL mapping in a hybrid poplar pedigree localized three of seven poplar *MAX* genes in QTL for bud set and two *MAX* genes in sylleptic branching QTL. All *MAX* transgenics were established in a field trial and phenotyping of phenology, crown morphology, and growth is in progress. Greenhouse studies of poplar transgenics with the seven different poplar *MAX* gene promoters driving the reporter gene GUS show that most of the *MAX* genes are expressed in vascular tissues. However, vascular expression patterns are different, especially in stems undergoing secondary growth. Surprisingly, none of the RNAi transgenics showed differences in branching in the greenhouse under standard growing conditions. High nitrogen levels can induce sylleptic branching in poplar and greenhouse experiments with *MAX* transgenics indicate a possible role for one *MAX* gene in nitrogen-induced sylleptic branching. Ongoing studies of the effects of decapitation of the shoot apex with and without subsequent auxin application to the cut stem indicate that, as predicted based on studies in annual plants, the poplar *MAX* RNAi transgenics show a reduced response to apically-applied auxin.

Recent work has revealed that poplar *FT1* and *FT2* have diverged in regulation and function with *FT1* promoting the transition to flowering in response to winter temperatures, whereas warm temperatures and long days promote *FT2* expression and shoot growth [1]. In Arabidopsis, *FT* activates *AP1*, a key regulator of flower initiation. Expression and transgenic studies in poplar indicate that similar to the two poplar *FTs*, poplar *AP1* subfamily members have roles in both flowering and bud set in response to short-days and abiotic stress. Ongoing work is aimed at defining the roles of *FT* and *AP1* modules in these processes and to

delineate the roles of the five different poplar *AP1/FUL* genes. Finally, *TFL1* acts opposite to *FT* to promote vegetative meristem identity in Arabidopsis, and *AP1* and *TFL1* repress each other's expression. The poplar *TFL1* homologs, *CEN1* and/or *CEN2* have roles in maintaining vegetative identity and in the dormancy-growth transition [2] and we are currently working to define the individual roles of the poplar *CENs* and their relationships to *FT* and *AP1* activities.

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S5. BIOTIC AND ABIOTIC INTERACTIONS

I12

The genomics of poplar-rust interactions to improve tree resistance against fungal disease

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With their long life cycle, trees must have accurate mechanisms to perceive microbial invasion and elaborate signalling networks in order to activate the appropriate defense response through transcriptional reprogramming. Transcriptional activators and repressors participate in the tight regulation of the stress response, which is key to minimise the fitness costs associated with an activated response. With the availability of its whole genome sequence, ease of growth and clonal propagation, and routine transformation, poplar (*Populus* spp.) is considered a model tree species for genomics research and also a good system in forest pathology [1]. Moreover, genomes of a cortege of associated microorganisms are being sequenced including a tree pathogen, *Melampsora* poplar rust. We pursued various approaches to identify poplar genes involved in the interaction with the biotrophic *Melampsora* rust pathogen.

Recent transcriptome analyses from our lab have shown that the expression of genes encoding several transcription factors are up-regulated during infection by *Melampsora* rust. Similarly we have also shown that mitogen activated protein kinases (MAPKs) are associated with poplar disease resistance against *Melampsora* rust [2]. New data obtained from various experimental approaches have directed our focus on two important families of transcription factors; the jasmonate ZIM-domain (JAZ) and Cys2/His2 zinc-finger protein (ZFP) families. We have identified a novel MAPK-interacting partner, *Pti*ZFP1, which belongs to the C2H2 ZFP family of transcriptional EAR repressors. The JAZ family of transcriptional repressors were recently identified as key negative regulators of jasmonate (JA) responses.Transcript analyses show that some ZFP and JAZ members exhibit hormone-related expression profiles and up-regulation by rust infection. This up-regulation of JAZ and ZFP transcripts after rust infection strongly suggests that a hormonal response including JA is a key component of the poplar defence response against *Melampsora*. Late and sustained kinetics of *PtiZFP1* and specific*JAZ* up-regulation suggest that the corresponding proteins may be required for late regulation of defense mechanisms.

Several recent data have shownthat proteolytic cleavage of transcriptional repressors is a general mechanism used by plants to activate gene induction. This mechanism is now well documented for jasmonate signaling, which depends on proteasome-mediated degradation of the JAZ repressors [3,4]. In the present work, we also obtain clear evidence that MAPKs promote degradation of *Pti*ZFP1 through the 26S proteasome. Our work suggests that *Pti*ZFP1 and JAZs are part of *Melampsora* specific hormone-related responses, and by correlation as putative transcriptional repressors, participate in the regulation of the transcriptional responses downstream of these stress hormones.We hypothesize that the observed gene induction play a negative feedback loop needed to replace *Pti*ZFP1 and JAZ proteins that were degraded following through the 26S proteasome. Newly synthesized repressor proteins could contribute to defense attenuation and therefore prevent a runaway response.

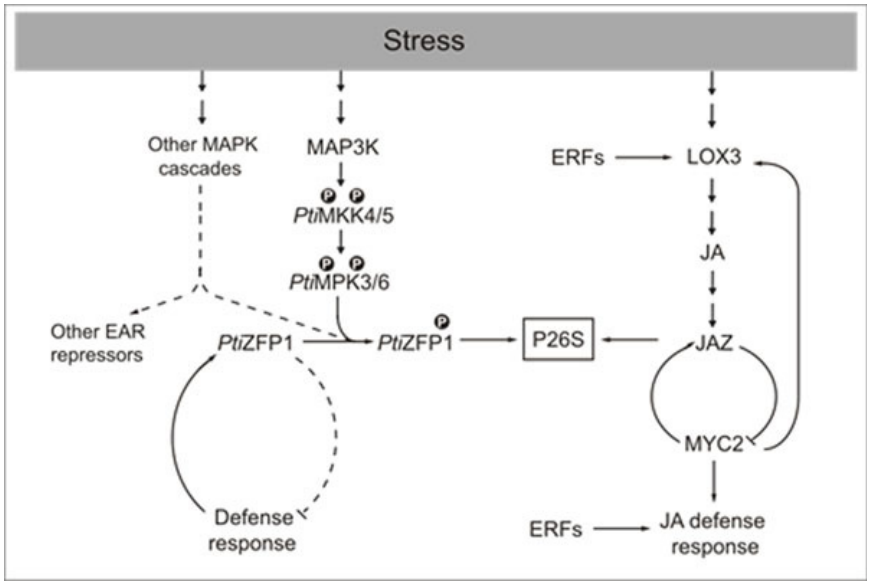

Figure 1(abstract I12) Proposed model of *Pti*ZFP1 function in the plant defense response.

Based on our data, we propose a model (Figure 1) where two important families of transcription factors play a predominant role in poplar defense response to the biotrophic fungus *Melampsora*. The activation of poplar MAPKs would lead to*Pti*ZFP1 degradation and as a result to transcriptional activation of defense-related genes. In parallel we also observed that several genes involved in the JA response (including JAZ) are induced following *Melampsora* infection. This model highlights the importance of the 26S proteasome in regulating protein pools of transcriptional repressor. For future studies we intend to uncover which *cis* elements and target genes are recognized by *Pti*ZFP1 and perform functional approaches to uncover JAZ interacting proteins in poplar.

During normal conditions, direct transcriptional repressors such as *Pti*ZFP1 inhibit the expression of stress-related genes. Following stress perception, a MAPK signaling cascade is activated leading to phosphorylation of *Pti*ZFP1. Phosphorylation targets *Pti*ZFP1 for degradation via the 26S proteasome (P26S), thus relieving repression of defense genes. Defense signaling in turn activates *PtiZFP1* gene transcription in order to replenish normal *Pti*ZFP1 protein levels and complete a regulatory cycle necessary to attenuate the defense response. A similar regulatory cycle exists for jasmonic acid (JA) signaling. JA biosynthesis is induced by stress which then promotes proteasome-mediated degradation of JAZ proteins. These indirect transcriptional repressors sequester the bHLH MYC2, thus inhibiting expression of defense genes under non-stressed conditions. Upon release, MYC2 ensures both positive and negative feedback loops by activating *LOX3* and *JAZ* genes respectively. Confirmed and hypothetical pathways are presented in bold and dashed lines respectively.

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I13

Adaptation of forest trees to climate change

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There is an urgent need to determine the adaptive potential of forest trees given their importance in ecosystem functioning and the associated ecological and economic services they provide. Indeed, underestimating rapid adaptation could lead to unnecessary recommendations such as the use of non-native (and perhaps non-adapted) genetic material for reforestation. Overestimating adaptive potential could have detrimental consequences if population decline massively and prove enable to regenerate.

A critical step to study individual's and specie's responses to variation in climate is to examine the basis of adaptation (at the phenotypic and molecular levels) under natural conditions and across multiple years. In this context, these sessile and long lived species have emerged as a model system for studying adaptation into the wild. Whether forest trees will be able to adapt in situ fast enough to outstrip the rate of such modifications relative to their longevity is still largely debated and remains an open question.

The objectives of this presentation are three fold:

i/ provide an overview of the concept of ADAPTATION: a process whereby a genotype or a population becomes better suited to its habitat. Adaptation to climate change can occur through phenotypic plasticity (a mechanism by which individuals can withstand large environmental fluctuation without genetic change) and evolution (i.e. response to natural selection pressure when the level of standing genetic diversity or diversity introduced by gene flow permits it).

ii/ provide some clues (from historical and contemporary data) on how closely can forest tree adaptation be expected to track climate change as rapid as envisioned for the future. They have high phenotypic plasticity which allows them to tolerate wide environmental variations during their lifetime. Besides, there are some evidence against the idea that rapid environmental changes overwhelms evolutionary processes preventing adaptation to local environments.

iii/ review how genes that matter for adaptation can be detected using 'omics technology in combination with population and quantitative genetics approaches.

S6. WOOD FORMATION

I14

A systems biology approach to understanding the regulation of lignin biosynthesis

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BMC Proceedings 2011, **5(Suppl 7):**I14

Lignin is a complex phenolic structural component of the secondary cell walls of all vascular plants. It is an irreversible end point of a major metabolic pathway in plant secondary metabolism. Lignin is fundamental to the adaptation of plants to land, the evolution of vascular transport and the resistance of plants to pests and pathogens. Lignin is a major barrier to the utilization of biomass for energy, for papermaking, and for forage digestibility due to the interaction of lignin with cellulosics in the plant cell secondary wall.

The past research on lignin biosynthesis is substantial, creating one of a few opportunities in higher plants to integrate genomics, proteomics, biochemistry, chemistry and modeling to develop a comprehensive understanding of biosynthesis and structure of a major component of morphology and adaptation. We are conducting a systems biology study on regulation of lignin biosynthesis in wood formation. We seek to build models to quantitatively illustrate how the entire pathway is organized and regulated and to reveal regulatory and metabolic flux control mechanisms, leading to lignin quantity and structures. We use the model woody plant, *Populus trichocarpa* (Nisqually-1), and the systems approach including advanced quantitative methods of genomics, proteomics, metabolomics, biochemistry and structural chemistry, to provide a comprehensive analysis of the regulation of lignin biosynthesis. A perturbation strategy is used to systematically knock down the expression of all pathway and regulatory genes known to be involved in lignin biosynthesis during wood formation, and the effects on lignin biosynthesis (gene transcripts, proteins, metabolites, quantity and structures) analyzed using advanced genomic methods available. This information forms the foundation of statistics-based mechanistic modeling and lignin quantity/structural predictions for a quantitative model of lignin biosynthesis. Details of systems data generation, data analyses and model development will be presented. Substantial data have been generated for gene-specific (amiRNA & RNAi) transgenic *P. trichocarpa*, enzyme kinetics, protein regulation and numerical modeling, and stable-isotope-dilution based absolute quantitation of proteins and of metabolites. These results will be discussed. Our long term goal is a predictive model of lignin biosynthesis and quantity/structure for greater understanding of the plant response to environmental stress and for more precise strategies to improve plant productivity and the production of energy, biomaterials and food.

I15

Ethylene signaling via Ethylene Response Factors (ERFs) modifies wood development in hybrid aspen

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Background: The phytohormone ethylene (ET) has the potential to regulate secondary growth of plants and wood formation in trees.

Application of exogenous ethylene or its *in planta* precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), to wood forming tissues of hybrid aspen (*Populus tremula x Populus tremuloides*) enhances xylem growth [1]. In the same study it was demonstrated that stimulation of enhanced xylem formation (tension wood, TW) at the upper side of leaning stems is mediated by endogenous ET. The production of endogenous ET in TW forming tissues is further supported by the increase of ACC oxidase gene transcript and enzyme activity on the TW side [2].

The ET perception and signal transmission cascade in Arabidopsis has been linked to the transcriptional activation of Ethylene Response Factors (ERFs) [3,4]. As transcription factors, ERFs regulate the expression of various specific downstream target genes by binding to *cis*-elements in their promoters [5]. We hypothesize that ERFs participate in xylem development through ethylene signaling and that they are involved in ET responses during TW formation.

Results and conclusions: We identified 169 *ERF* genes in the *Populus trichocarpa* genome versions 2.0 and 2.2 using regular expression method and pfam search for the ERF domain. These ERFs grouped into 11 distinct groups, similar to ERFs identified in Arabidopsis and rice [6]. Using qPCR we showed that a majority of the *ERF* transcripts were detectable in stem tissues of in vitro or greenhouse grown hybrid aspen. The responsiveness of all *ERF*s to short term ACC treatments (10h, in vitro plants) and to short or long term ethylene treatments (24 or 2 weeks, greenhouse grown plants), was assessed by qPCR. Most of the *ERF*s responded to at least one of the treatments, mostly by increased transcript accumulation. We identified *ERF*s that were specifically induced within the early ACC/ET- or the late ET-response. In addition, some *ERF*s showed prolonged induction up to 2 weeks of ET treatment. These different transcript patterns indicate that different *ERF*s may be involved in distinct, temporally distinguished processes during the ACC/ET-induced secondary xylem growth response.

According to their expression and capacity to be induced by ET or ACC, we selected 26 *ERF* candidates and investigated whether those were responsive to endogenous ET-signals in leaned stems during TW formation. We compared the induction of those 26 candidate *ERF*s in TW with *ERF* accumulation during long-term (2 weeks) ET treatment. Interestingly, a significant overlap of *ERF* induction in both conditions was found. From the 20 *ERF*s that were induced after long-term ET treatment, 16 had an increased transcript abundance during tension wood formation. This indicates that on an *ERF* transcript basis, tension wood formation is largely comparable to a long-term ET treatment.

Based on the transcript data, twenty ERFs were selected for overexpression in hybrid aspen cambium/xylem under the *pLMX5* promoter [1]. Successful overexpression of the selected *ERF*s in transgenic plants was confirmed and two to seven lines of each overexpressed ERF were phenotyped in a greenhouse trial. In general, *ERF* overexpression caused only mild alterations of overall plant stature (height and radial growth). Only overexpression of one *ERF* candidate led to a severe dwarf phenotype with thin stems, reduced fiber and vessel size, reduced height growth and smaller leaves. The absence of any striking phenotypes in all other plants suggests that other regulators in addition to the overexpressed *ERF*s may be necessary to mimic the enhanced growth response observed during ET/ACC/TW mediated stimulation of secondary xylem growth.

A Fourier-Transformed Infrared spectroscopic and Pyrolysis GC-MS based screening of five lines for each of the 20 overexpressed ERFs revealed that five *ERF*s led to changes in cell wall composition in xylem tissues when overexpressed. This suggests that these ERFs have the ability to modify cell wall composition in wood forming tissues and may regulate the expression of cell-wall biosynthesis genes. This hypothesis is now under further investigation.

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I16

How sweet it is – making the most of carbohydrate metabolism

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Like all plants, trees constantly monitor endogenous and environmental cues, and use this information to make adjustments in resource allocation, both to maximize resource acquisition and to minimize exposure to deleterious phenomena. To accomplish this, trees possess mechanisms that integrate and interpret the information provided by internal and environmental signals. Ultimately, these phenomena are controlled at the level of gene expression, which consequently translates into the accumulation of a variety of soluble metabolites, including carbohydrates. Trees are relatively "plastic" in their ability to allocate resources, and such plasticity has an adaptive significance in that it allows trees to (i) match resource allocation with resource acquisition, (ii) acquire new resources more effectively, and in some instances (iii) avoid adverse conditions. The inherent plasticity in resource acquisition can have a profound effect not only on the development and physiology of trees, but also on the industrial harvesting and utilization of the terminal lignocellulosic resource.

Photosynthetic carbon capture by trees represents a major sink for atmospheric CO_2, ultimately terminating in the synthesis of a secondary plant cell wall – a complex matrix of polysaccharides intricately linked to lignin. Therefore, engineering plant where resource allocation is directed to vegetative biomass and fibre properties should have an effect on the plant cell wall traits. As such we have targeted the biosynthetic processes governing sucrose metabolism by mis-regulation of key enzymes associated with the catabolism and anabolism of sucrose in attempts cellulose production – both quantity and quality. This paper will discuss the results of these efforts, and illustrate the altered performance of such plants for industrial use.

S7. PROPAGATION AND IN VITRO TECHNOLOGIES

I17

From genes towards products and the significance of gene delivery

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SweTree Technologies is a plant and forest biotechnology company providing products and technologies to improve the productivity and performance properties of plants, wood and fiber for forestry and fiber related industries.

In the area of Biotech Trees, we have been testing and developing genes and biotech trees. Since 2002 we have successfully screened well over one thousand genes in our gene testing program. The program has been mainly directed towards increased growth, improved wood properties and stress tolerance. The current outcome of this program will be presented. The primary gene developing program is performed in hybrid aspen for which we have a very efficient transformation protocol.

However for practical tree biotechnology the genes and DNA constructs have to be transformed into the best commercial clones. Therefore we have been setting up efficient transformation protocols for both *Eucalyptus* and *Populus* elite clones. Another crucial part of a practical tree biotechnology program is to test the GM trees with commercially interesting genes under field conditions. We have initiated field trials and plan to have over 35 different types of transgenic trees in field trials by the end of this year. These field experiments are performed by SweTree or in collaboration with academic partners. The importance and requirements for elite clone transformation and field trials will be discussed in the presentation.

I18

Hybrids and mini-cutting: a powerful combination that has revolutionized the *Eucalyptus* clonal forestry

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Background: Eucalypt canker disease (*Chrysoporthe cubensis*) was the main driver of the concept of eucalypt clonal forestry in Brazil. Initially clonal forestry was based on outstanding spontaneous hybrids resistant to canker disease. Since the recognition that cloning superior inter-specific hybrids could be an important general strategy, several crosses were made for many different purposes, especially pulp & paper, charcoal and more recently veneer and solid wood. This strategy has been responsible for a significant advance in the forest productivity of *Eucalyptus*. The underlying foundation for this strategy has been the exploitation of heterosis observed in most inter-specific crosses.

Due to its importance, hybridization and cloning currently constitute a key component of almost all forest-based industrial plantations in Brazil. Significant progress has taken place in the last few years with the different techniques used to carry out controlled crosses and cloning.

Controlled crosses: The fact that *Eucalyptus* flowers are hermaphrodite and protandreous makes the traditional method of controlled crosses difficult to perform. This method is based on the exploitation of protandry, which involves emasculation and isolation, prior to pollination. It needs several visits resulting in a technique of low operational yield.

After discovering that different treatments in stile and stigma during anthesis could immediately induce flower receptivity [3], it was developed the OSP (One Stop Pollination) technique [4]. Later it was found that this receptiveness could be achieved just before anthesis [5]. Combining these two discoveries a new technique was developed [2], called AIP (Artificially Induced Protogyny). This technique consists of the artificial "transformation" of protandry into protogyny, obtained by cutting the top of the floral bud operculum together with the upper third of the stile. This is done during the pre-anthesis stage, i.e., when the flower is still closed. Pollen can be applied immediately after induction. Additionally the concept of indoor breeding orchards was developed, precluding the need to isolate individual flowers, umbels or branches, enabling the isolation of whole plants in a collective way.

The combined use of these technologies (AIP and indoor breeding orchards) currently allows large scale controlled crosses, and enables the production of highly superior full-sib families, a task formerly considered technically and economically difficult in *Eucalyptus*. Operational productivity improved from 35 to 400 pollinated flowers/person/hour.

Cloning techniques: Cloning techniques have been significantly enhanced in the last years. The first major leap occurred in 1992, when micro-cutting was invented [1] and later mini-cutting. The micro-cutting is a rooting method, where the propagules are obtained exclusively from shoot apices, originated from micro-propagated plants. Mini-cuttings, on the other hand, come from axillary sprouts of rooted stem-cuttings. After the establishment of the mini-clonal hedges, the two techniques are identical, varying only in the origin of the initial source of propagules. Micro-cutting and mini-cutting are the most modern concepts for large scale cloning of *Eucalyptus* species. They are implemented using hydroponic systems such as sand bed with drip irrigation or intermittent flooding with very specific nutritional parameters that allow commercial scale production of sprouts. Although significant progress has been made in these robust and functional systems, new approaches to clone *Eucalyptus* species are opening important opportunities to improve operational results and also contemplating recalcitrant species in the general hybridization & cloning strategy. One of these new approaches is cloning selected trees from lignotuber tissue. Tested originally at Arcelor Mittal Bioflorestas, cloning from lignotubers constitute a new advance to be soon incorporated in the operational procedures. Another promising approach is to cover mini-stumps with mini-tunnels. Preliminary results of this method at Aperan Bioenergia have shown that productivity, rooting rate and root quality are improved, reducing the formation of callus.

After more than thirty years of experience on deriving clones from inter-specific hybrids, this general strategy, together with the evolution of silvicultural practices, have been the major drivers of the progress experienced in forest productivity in Brazil. The evolution of pulp productivity obtained by cloning superior hybrids has been impressive. While in 1970 the average MAI-Cellulose was 6 ton/ha/year it now reaches 12 ton/ha/year. New projections now work with the possibility of achieving a potential productivity of about 16 ton/ha/year by 2015. One of the key elements for this further jump in productivity is the rapidly increasing use *E. globulus* in hybrid breeding programs. *E. globulus* has superior wood qualities such as low lignin content and high S/G ratio (one of the highest in woody plants) that result in easier cooking, low specific consumption (high wood density and pulp yield) and good bleaching ability. The use of adapted species and clones in crosses with *E.globulus* is yielding a new breed of elite hybrid clones for subtropical and tropical areas.

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I19

Opening the gateway to enhanced *Eucalyptus*

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Eucalyptus is the second most planted tree on earth. Despite this ranking, its mature breeding programs, clonal propagation and deployment and short rotation time make it a leading target for biotechnology improvement. The recently completed eucalyptus genome project provides an enormous opportunity for understanding and developing new traits for eucalyptus forestry. The main obstacle for widespread trait enhancement remains the recalcitrance of commercial eucalyptus clones to transformation and thus the lack of a suitable prototyping system for this species. We believe that the future success of trait development in eucalyptus will be enhanced by close collaboration between academic centers and industrial partners. Some of FuturaGene's models for academic collaboration will be described in this presentation.

An example of such potential collaborations may involve BRASUZ1, which constituted the germplasm base for the eucalyptus genome project. This key clone was donated by Suzano Pulp and Paper, FuturaGene's parent, to the genome project. We will present our progress with the transformation protocol for BRASUZ1, which could provide a platform for collaborations. We will also present our perspective on the development of improved transgenic eucalyptus, and an outline for such development programs.

S8. GENOMIC, PROTEOMIC AND METABOLOMIC TECHNOLOGIES

I20

The *Eucalyptus grandis* Genome Project: Genome and transcriptome resources for comparative analysis of woody plant biology

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Background: The International Year of Forests - 2011 [http://www.un.org/en/events/iyof2011/] will be a milestone for forest tree genomics. The draft genome sequence of *Eucalyptus grandis* was released in January 2011 in the USA (Phytozome [http://www.phytozome.net]) and in Belgium (BOGAS, [http://bioinformatics.psb.ugent.be/webtools/bogas/]). The genome sequencing was funded by the US Department of Energy (DOE) and performed at the DOE Joint Genome Institute (JGI) in collaboration with members of the *Eucalyptus* Genome Network (EUCAGEN, [http://www.eucagen.org]) who contributed genetic materials, linkage maps, EST resources and bioinformatics support. The *E. grandis* genome together with that of *Populus trichocarpa* [1]and other woody plant genomes recently completed (e.g. *Vitis*, *Cacao*, *Prunus*, *Citrus and Malus*)will provide excellent opportunities for comparative studies of the unique biology of woody plants. Eucalypts are currently the most widely grown hardwood fibre crop in the world and eucalypt breeding programs will benefit greatly from the new genomic resources. The reference genome sequence of *Eucalyptus*, a foundation tree genus in Australia comprising more than 70% of the native forest estate, will also offer important benefits for ecological and evolutionary biology studies. We report the sequencing, assembly and annotation of the *E. grandis* genome.
Genome sequencing and assembly: Whole-genome (8X) shotgun sequencing was performed for a partially inbred (S1), 17-year-old tree of *E. grandis* (est. genome size 640 Mbp, n = 11), BRASUZ1 (Suzano, Brazil). A total of 7.7 million Sanger reads (5.4 Gbp) were produced from plasmid, fosmid and BAC libraries. An inbred genotype was selected to circumvent perceived problems with the assembly of a highly heterozygous eucalypt genome. However, microsatellite genotyping showed that BRASUZ1 was much less homozygous than expected, with large parts of the genome remaining heterozygous presumably due to viability selection. This finding was confirmed during the assembly of the S1 genome - approximately 25% of the assembly occurred in two haplotypes of 3-4X coverage, while the remainder of the genome assembled into a single haplotype of 6-7X coverage. Linkage maps with over 2400 DArT and microsatellite markers were subsequently used as a framework for the assembly of 11 large chromosome scaffolds. The chromosome scaffolds contained 88% (605 Mbp) of the draft assembly, with the remainder of the assembly sequence (85 Mbp) in 4941 smaller scaffolds. Based on similarity searches with 1.6 million ESTs from BRASUZ1, it was estimated that 96% of expressed gene loci were included in the 11 chromosome assemblies.
Genome annotation: Genome annotation was performed in parallel at the JGI and at the University of Ghent. Both annotation teams used *ab initio* and homology-based annotation approaches supported by over 4 million 454-FLX-Titanium ESTs produced by the JGI, as well as Sanger, 454 and Illumina EST data provided by collaborators. The two annotations revealed that the 11 chromosome scaffolds contain more than 90% of the predicted protein-coding loci (total 44,974 - JGI, 47,974 - UGent). More than 70% of the predicted genes had EST support and 9,961 (18%) alternatively spliced transcripts were detected. The two annotations are being compared and a joint annotation may be released for the main *E. grandis* genome paper.
Genome duplication: The *Vitis* genome [2], representing an early diverging Rosid lineage (Vitales), was found to contain the ancient hexaploidization event shared by Rosids and Asterids, but none of the more recent genome duplications found in the Rosid lineages represented by *Arabidopsis* and *Populus*. A preliminary analysis performed at UGent of genome duplication in *E. grandis* (representing the Rosid order Myrtales) suggested that the *Eucalyptus* genome most likely contains one more recent duplication event, in addition to the paleohexaploidy event.
Genome resequencing: *E. globulus* is a temperate eucalypt with superior wood properties compared to *E. grandis* and is viewed as the premier eucalypt species for pulping. The two species occur in different sections (*Maidenaria* and *Latoangulatae*) of the subgenus Symphyomyrtus and their genome sizes differ substantially (*E. globulus* - 530 Mbp, *E. grandis* - 640 Mbp, [3]). The JGI has performed genome-wide resequencing (>30X Illumina PE) of an *E. globulus* clone (X46, Forestal Mininco, Chile). Approximately 75% of the *E. globulus* Illumina reads mapped to the *E. grandis* reference genome and sequence analysis in these regions revealed an average sequence divergence of 1.5% between the two genomes. Other eucalypt genomes currently being resequenced by collaborators will generate a valuable resource for studies of eucalypt genome evolution.
Transcriptome resources: The large amount of transcriptome sequence data was produced the project includes 1.9 million xylem and leaf ESTs (454 reads) from BRASUZ1 and 2.1 million 454 reads from *E. globulus* (X46) xylem and leaf tissues. Together with other large 454 datasets (e.g. [4]) and Illumina mRNA-Seq data [5]produced by collaborators, the *Eucalyptus* research community now have access to excellent transcriptome resources some of which are already available in integrated genome and transcriptome browsers (Eucspresso [http://eucspresso.bi.up.ac.za/]).
Conclusions: The *E. grandis* genome sequence will be the first reference for the Rosid order Myrtales and will be informative for comparative genomic studies within the Eudicots. It will also deliver powerful tools for the application of genomics in eucalypt breeding programs.

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I21

Populus resequencing: towards genome-wide association studies

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Genome-wide association studies (GWAS) have been used to identify regions of the genome related to various phenotypes in humans, corn, rice and cattle. Successful application of this approach to bioenergy crops such as *Populus* requires 1) an appropriate mapping population, 2) high-quality

phenotypic data and 3) informative genotypic data. With the goal of reducing the recalcitrance of lignocellulosic biomass for economic production of biofuels and understanding basic mechanisms of cell wall formation in *Populus* we established 4 clonally replicated common gardens experiments each with 1100 native *P. trichocarpa* genotypes collected from along the northwest coast of the U.S. and Canada. A high-throughput phenotyping pipeline was developed to measure cell wall chemistry, pretreatment response and enzymatic sugar release. Initially 18 genotypes were resequenced to an average 30X depth in order to design a SNP array to test for statistical association using MMAX and PCA methods of testing among ca. 2500 candidate genes. Genetic structure and linkage disequilibrium (LD) was assessed using SSR and SNP markers. Outlying genotypes were excluded from the analyses and estimates of LD were used to design the bead array. Candidate genes were selected based on QTL intervals, expression profiling within developing xylem and expert opinion. MMAX and PCA results revealed similar significant associations for all measured phenotypes and several SNPs within the candidate gene set explain a relatively high degree of the phenotypic variance. As a result, resequencing has continued in order to conduct GWAS in *Populus*; the complete set of 1100 genotypes will be complete in 2012.

I22

Eucalyptus research in the post-genome era

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With the efforts of the Joint Genome Institute (JGI) through the EUCAGEN Eucalyptus Genome Network reaching the final release of a *Eucalyptus grandis* reference genome (BRASUZ1), it is anticipated that this accomplishment will profoundly shape the future research of this global tree genus [1]. One of the first steps toward this end has been the refinement of the genome annotation. Robust models have been generated for protein-coding genes. Here we report the annotation of other pivotal genome constituents, transposable elements (TE) and micro RNA genes. TEs are not only the most dominant elements but are also major drivers of genome plasticity. Several bioinformatic strategies were employed to perform "de novo" and homology based prediction of repetitive elements [2]in the current genome release (version 1.0). A total of 53 distinct TE families could be identified, with the retrotransposon super-family being widely over-represented, as observed for the majority of plant genomes sequenced.

Micro RNAs (miRNA), key players in post-transcriptional gene regulation, were annotated using a combination of massively parallel sequencing of small RNA libraries and a genome-wide computational screening to ascertain a compatible secondary structure of the precursor. Both experimental and "in silico" evidences enabled the annotation of 206 distinct miRNA loci comprising 36 different mir gene families including several miRNA isoforms. The blueprint provided by a high-quality reference genome, both in terms of sequence completeness and annotation, will leverage efforts to better characterize intra- and inter-specific sequence variation underlying the marked phenotypic differences among the hundreds of species comprising this genus.

Recent advances of DNA sequencing technologies permit a comprehensive interrogation of several other individuals at a fraction of the cost. In this context, we have used Illumina (2x75bp) short read sequencing data of *E. globulus* clone X46 generated by JGI and made available through the EUCAGEN network to carry out two comparative genomics experiments. From 40X raw sequence data provided it was possible to use an equivalent of 20X coverage (~12Gbp). We first attempted to perform a "de novo" assembly using VELVET [3]. A total of 161,000 contigs were obtained the largest one sizing at ~3,5kb. In spite of the easy access and low cost of next generation sequencing technologies, these results suggest that even for relatively small forest tree genomes, current technical and computational limitations preclude comprehensive assembly, likely due to the ubiquitous occurrence of repetitive elements in such genomes. Nevertheless when we mapped the *E. globulus* sequencing data against the BRASUZ1 reference genome, 55% of the reads could be mapped with high confidence. From these, approximately 800,000 high quality single nucleotide polymorphisms (SNPs) could be identified clearly showing the key role that the reference genome will have for future genomic undertakings. The sheer number of molecular markers discovered in this experiment not only fosters more powerful studies on the evolutionary history and population genomics of eucalypts, but also inaugurates a new era in molecular breeding of species of this genus, providing genome-wide coverage for genomic selection and association studies.

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S9. BIOSAFETY, CERTIFICATION AND ECONOMICS OF TREE BIOTECHNOLOGY

I23

Science, society and biosafety of a field trial with transgenic biofuel poplars

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Background: Global warming, environmental disasters, and increasing oil prices have catalyzed a worldwide trend to use plant biomass as a renewable source for liquid biofuels and bio-based materials. Plant biomass can be processed into fermentable sugars by enzymatic depolymerization of the cell wall polysaccharides, followed by fermentation. However, the presence of lignin in the cell wall constitutes a major recalcitrance factor because it limits the accessibility of polysaccharidases to the cellulose microfibrils. To overcome this hurdle, plant biomass is pretreated in a costly and energy-requiring process. One approach to overcome the recalcitrance problem is to engineer lignin amount or alter its composition to make lignin more susceptible to chemical degradation [1]. Cinnamoyl-CoA reductase (CCR), the enzyme that converts feruloyl-CoA into coniferaldehyde, is considered the first enzyme in the monolignol-specific branch of the phenylpropanoid pathway. Poplar trees down-regulated in CCR have been produced in the early nineties and planted in a field trial in France to produce sufficient wood for small scale chemical pulping tests [2]. These trees had 20% lower lignin levels and relatively more cellulose per gram of wood [2]. Given that lignin is one of the main limiting factors limiting the conversion of plant biomass into fermentable sugars, and that poplar is considered as a promising second generation biofuel crop, we have re-grown these trees in the greenhouse and in the field, and evaluated wood produced from these trees by saccharification experiments.

Methods: Cinnamoyl-CoA reductase (CCR) expression was down-regulated in poplar by sense and antisense strategies [2]. Transgenic trees were evaluated for lignin amount and composition [2] and for sugar release by saccharification assays [3]. After obtaining permission from the regulatory authorities, two transgenic lines were planted in a field trial in Belgium and two in a field trial in France, both under short rotation coppice culture to maximize biomass production. Wood was saccharified with and without acid pretreatment.

Results: Wood from CCR down-regulated trees that were grown in the greenhouse was saccharified with and without acid pretreatment. Interestingly, wood from the transgenic lines released more glucose in saccharification assays performed without pretreatment than wood from

wild type trees when saccharified with a pretreatment, demonstrating the improved processing of the cell wall polysaccharides by the enzyme cocktail. These promising results prompted us to ask permission for a field trial to verify whether wood from these trees, when grown in an open field, would still be more easily saccharified. After all, when trees are grown in the greenhouse, they do not experience wind, rain and seasons, and they need to be stacked to grow upward. After a one year long calvary to obtain regulatory permission for the Belgian field trial [4], the trees were grown under short rotation coppice culture in replicated clonal blocks in a small plot of a total of ~ 500 trees. The plot has a fence and is surveilled by cameras. In Spring, the trees are regularly monitored for flowering as they are not allowed to flower. Flowering is not expected because the coppice cycles are as short as 3 years. The possible effects of the low lignin trait on rust infection and insects is being monitored, but it is not expected that these effects will be larger than those associated with conventionally bred poplar hybrids or will be specific to the transgenic trees taking the large genetic diversity of the wild poplar germplasm into account. Activist groups that are concerned that these trees pose different risks than conventionally bred hybrids or pose a risk for human health are invited for a discussion with scientists and the biosafety manager at VIB.

After one year of growth in the field, trees were coppiced to allow the development of multiple shoots in the next spring. The basal 20 cm part of the stem was debarked. Wood harvested from wild type trees was white in appearance, but wood from the transgenic trees was red-brown, most likely due to reactions with ferulic acid, derived from the substrate of CCR, in the cell wall [5]. Importantly, the red coloration was not uniform, but varied significantly in a single trunk, and among trunks from clonally propagated trees. Saccharification potential increased to ~50% per gram dry weight.

Our data show that reducing lignin amount in trees improves saccharification potential and should reduce the chemical or energy cost of biomass pretreatments. The trees will be harvested again in winter 2012-2013, i.e. three years after the first coppice. This should provide sufficient biomass for evaluation at semi -industrial scale at the pilot biorefinery in the Port of Ghent [6].

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I24

Biotech *Eucalyptus* can sustainably address society's need for wood: the example of freeze tolerant *Eucalyptus* in the southeastern U.S

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Background: *Eucalyptus* species are among the fastest growing woody plants in the world and represent about 8% of all planted forests (~18 million hectares) grown in 90 countries [1]. Only a limited number of species are grown commercially, and these have been the focus of extensive breeding to improve desirable wood properties such as basic density, cellulose content, fiber length and improved growth. Improved *Eucalyptus* varieties grown in managed plantations as a source of timber and pulpwood have provided socio-economic benefits for both small and large land owners. *Eucalyptus* also provides a cost-effective source of lignocelluloses for the production of energy and advanced biofuels. However, the large scale plantings of the most productive *Eucalyptus* species in the Southeast U.S. are currently limited to regions of central and southern Florida where no hard freezes occur in winter. Plantings of hardwood species to support the traditional forest industry in the Southeastern U.S. are infrequent due to slow growth rates and high plantation establishment and management costs. The biotech Freeze Tolerant *Eucalyptus* would provide an economically viable plantation hardwood for the Southeastern U.S.

Methods: Genetically engineered Freeze Tolerant Eucalyptus (AGEH427) was developed by the introduction of plasmid pABCTE01 into the EH1 genotype of *E. grandis x E. urophylla* using the *Agrobacterium* transformation system described by Cheah (2001) [2]. pABCTE01 contains a CBF2 expression cassette comprised of Arabidopsis cold-inducible promoter rd29A [3] driving the Arabidopsis *CBF2* (C-Repeat Binding Factor) cDNA [4,5]. It also contains a pollen control cassette consisting of a modified *barnase* gene from *Bacillus amyloliquefaciens* under the control of a *Pinus radiata* anther-specific promoter (PrMC2) [6]. Single insert, backbone free transgenic lines were vegetatively propagated to enable 21 replicated field trials established at eight different locations representing USDA Hardiness Zones 8a (potential kill zone), 8b (target freeze-stress zone), and 9a (freeze stress-free zone) across the Southeast US. A simple comparison of pre-winter and post-winter live height measurements was used to calculate a percent dieback of the main stem, and provided appropriate assessment of freeze tolerance and growth performance under field conditions. Data were collected over five winter/growing seasons. Pollen ablation was studied by collecting multiple flowers from field grown transgenic and non-transgenic trees prior to anthesis and microscopic assessment of the frequency of normal pollen.

Results: The transgenic line AGEH427, had significant freeze hardiness that enabled it to grow in USDA Hardiness Zone 8b. The growth of AGEH427 was comparable to EH1 non-transgenic control trees when grown under low- or no-freeze challenge conditions (Zone 9a). In the target freeze-challenged environment (Zone 8b), AGEH427 grew better than the non-transgenic control. After the severe winter of 2009/2010 at a site with a minimum temperature of 16.8 °F, the average live height of control trees was approximately 0.3 feet as compared to 52.4 feet for AGEH427. AGEH427 had approximately 10% dieback from the top. In the zone 8a (winter temperatures as low as 8.4 °F), complete dieback and defoliation were observed, with post-winter tree heights for both AGEH427 and the non-transgenic control being only 0.1 ft. AGEH427 also did not produce pollen, whether or not it was grown in freeze challenged locations. No pollen was produced over multiple years, different flowering seasons, different sites, and different physiological ages of trees while the control trees did produce pollen. Additional phenotypic evaluations of AGEH427 in comparison to the control were performed in field studies across a broad range of environmental conditions in the Southeast U.S., and no differences, other than the freeze tolerance and pollen ablation traits were observed. Neither the transgenic nor the control trees demonstrated any tendency to spread beyond a managed plantation, with low seed set and lack of any seeded volunteers observed in the field trials.

Conclusions: The cumulative multi-season data obtained from these trials demonstrated conclusively that the freeze tolerant trait in AGEH427 enabled this tropical tree to grow in the subtropical, freeze-prone USDA Hardiness Zone 8b. The productivity of AGEH427 makes it ideal as a short-rotation plantation hardwood in the Southeast U. S. The yield achievable with freeze tolerant *Eucalyptus* is predicted to meet or exceed the required productivity for an economically feasible hardwood plantation to meet traditional forest industry and bioenergy needs [7]. There is no evidence from the literature or from our field trials which indicates that EH1-427 would be invasive or negatively impact endangered species. EH1-427 is currently the subject of a petition for deregulation in the U.S.

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I25

Transgenic biotechnology in forestry: what a long strange trip it's been

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In the 1980s, when the production of transgenic plants was first demonstrated and was soon followed by production of healthy transgenic poplars in the US and then in Belgium, many of us, including some of the most conservation minded in forestry science, embraced genetic engineering/genetic modification as an important new technology for forestry. We had this sense of following the yellow brick road to Oz. It seemed that we could finally get beyond the narrow limitations of the slow outbreeding, quantitative breeding system, and begin an era of qualitative genetics to complement the dominant quantitative genetic paradigm. Unfortunately, when we got closer to the Wizard of Oz things looked a bit different than on the famed Yellow Brick Road (Figure 1).

What happened? Most important, the technology, and how it was structured, ran into a social and political buzz-saw. The world was becoming richer and more sensitive, both in terms of environment and respect for life. A diversity of social movements were taking place, powered by the new internet, that was uncomfortable with the notion of transgenics and who controlled them, and a number of organizations with resources and power opposed transgenics at every turn. A patent system was put in place that only very large companies or a few wealthy foundations could afford to negotiate. Rich consumers and vulnerable consumers sensed risk and little or no direct benefit to them, and thus also opposed the technology in large numbers. Regulations were pushed through that made the costs of field research, marketing, permits, international trade, liability insurance, and labeling huge barriers to investment and commercial adoption. Nearly the only crops to survive produced huge economic benefits and had to be pushed through the regulatory system by the multinational companies that consumers feel the least trust in.

Unfortunately, national and international regulations, once in place, are very hard to change—so this legacy will be with us for many years. Moreover, because the regulations treat all transgenes as hazards until

Figure 1(abstract I25) On the road to transgenic Oz in the early days of plant and forest biotechnology.

"proven safe," they pose severe problems for conducting high quality field trials of most types of transgenic forest trees. This is a result of their wild and feral relatives, low level of domestication, and ability to produce pollen and seed that can move over large distances. Thus, it's very hard to integrate transgenic trees into conventional breeding and field testing programs—which is essential for their development and application. Meanwhile, social perspectives and the structure of research funding has led to extraordinary scrutiny and amplification of every possible risk of transgenic trees, usually far out of proportion to its significance compared to conventional agriculture and forestry breeding. Thus, the trip has felt much more like a certain Electric Kool-Aid Test (Figure 2), than a normal technological progression, and not at all like the start of the trip to Oz.

On the science side, despite our early successes, when we finally got to meet the Wizard, there was also a number of revelations that gave us pause about the robustness of transgenic tree biotechnology, and most remain significant to this day. 1) It was much harder to transform/

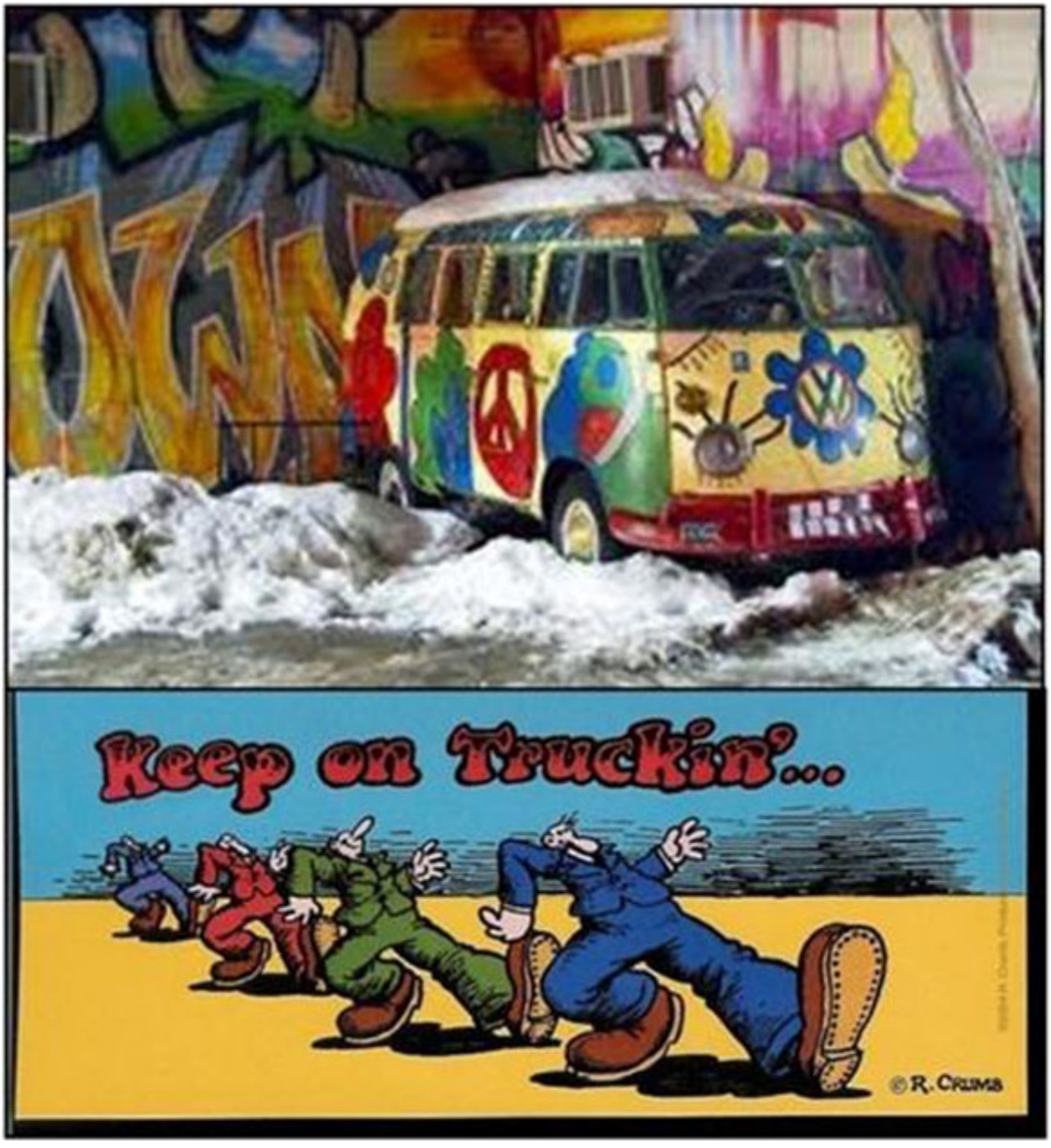

Figure 2(abstract l25) The trip to transgenic Oz was far risker, but also far richer and more informative about ourselves and our society, than we dard to imagine. We will keep on working and innovating with transgenic tools, both on biological and sociopolitical fronts, because our thirst, imagination, and world demands it.

regenerate most commercially important forest tree species/genotypes than we had imagined, and very little in the way of public research funding has been directed to overcome this in a meaningful way. 2) There was a great deal of hype about some of the early successes, such as in the area of growth rate improvement and lignin modification, with insufficient field trials to judge their true merit. Many have not proven themselves, but the results are often hidden by business confidentiality, academic self-interest, and a paucity of high quality field research 3) Beyond 35S-type overexpression for traits like herbicide and Bt-insect resistance, the tools in place for control of gene expression were found to be coarse, unpredictable, and imprecise, likely due to lack of control of insertion location, chromatin state, and poor understanding of RNAi/siRNA mechanisms. 4) While genetic containment would solve a lot of social and regulatory problems, due to high social concerns, short term funding, strict regulations over field trials, and immature technology, it's unclear if we can attain, in the near to middle term, the nearly perfect level of predictable, field-validated containment that appears to be required. 5) The extraordinary advances in genomics and phenomics in conventional breeding, combined with the high costs of regulations, are pushing transgenic applications to focus on the most new, novel, and valuable applications that cannot be attained by conventional or genome-assisted breeding—which are also those for which attaining regulatory approval will be most difficult. 6) Meaningful, field-based ecological studies of fitness and non-target effects, especially of pest-resistant, stress-resistant, bioindustrial, and bioremediation types of transgenic trees, have not been conducted—leaving a wide swath for models and speculations of severe ecological impact that are likely to be vastly overstated—but push regulations toward ever greater stringency. In sum, a great deal of fundamental science, from the genic to the ecological, and technology development to efficiently identify and transfer genes and pathways, remains to be done by the next generation of scientists and practitioners.

Propelled by the ongoing successes of some pioneering companies, I sense that transgenic forest biotechnology, though facing great difficult challenges at present, will "keep on trucking" in the parlance of "The Trip" (Figure 2). This will be driven by science and technology successes that continue at an impressive rate; urgency in finding solutions in the face of severe global economic and environmental problems; and the human thirst for novelty, innovation, and truth.

KEYNOTE LECTURE PRESENTATION

K1

Exponential advances in Forest Genetics: the past 25 years and the next 25 weeks

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A history of Forest Biotechnology will be briefly reviewed, from the first cloned tree gene, transgenic trees, DNA markers, genetic maps, QTLs, marker assisted breeding to genome sequencing. Perspectives of newer methods and their applications will be discussed, including integration of genetics and RNA-seq, metabolomics, and proteomics. New applications may be anticipated or expected for breeding, vegetative propagation, phenotypic plasticity, epigenetics, symbiosis, adaptation to biotic and abiotic stress and the directed evolution of some forest tree species.

Forest Biotechnology began over 25 years ago with the advent of new technology of molecular genetics able to advance the fundamental and applied science of forest trees. Methods of gene cloning and gene transfer held the promise of bypassing the long generation times that made genetic analysis and breeding so difficult. This vision has come to pass and the presentations of this meeting show how far we have come. Today, we are deeply involved in the next generation of genetic technology, a second revolution, using genomic sciences, based on the technology and concepts derived from whole genome sequencing, with the same objective, advancing the fundamental and applied science of forest trees.

The path ahead appears to have a third phase, based on an extension of the genomic paradigm, to systems biology and predictive modeling, directed to the same basic goals. Each new phase has been highly integrative, the first bringing molecular genetics into many traditional disciplines. The second phase, integrated areas of molecular and quantitative genetics with high throughput processing and bioinformatics. What appears to be the third phase will extend this paradigm to incorporate the biochemistry of proteomics, the chemistry of metabolomics and the organization, structure and dynamics of cell biology and whole plant physiology. This integrated paradigm further incorporates an engineering perspective and describes the integrated data as predictive mathematical models, subject to experimental test and manipulation. Much of this new biology will be at the interface of biology and systems engineering. Biologists will learn to think like engineers, and engineers will learn a lot of biology. In time, the distinctions between these disciplines may be lost.

What makes a tree a tree? We now know that a relatively small number of genes regulate the woody phenotype, because a small combination of gene mutations can produce a woody form of Arabidopsis. Similarly, small numbers of genes regulate key aspects of tree dormancy. Much remains to be determined regarding the control of tree growth and development, and little is known about the genetic basis of the wide diversity in morphology, metabolism and adaptation. Beyond the descriptive understanding of tree phenotypes and their diversity, lies the evolutionary question, how did the diversity of trees get to be that way.

Comparative genomics has been a powerful tool to derive gene function by comparisons of woody and herbaceous plants, because a large number of functions are conserved. A major barrier to a broad understanding of trees and all other plants still lies in the lack of information on the function of the majority of plant genes. The functions of most genes are known only by superficial annotation based on the time and place of transcript expression, or response to an external stress. Even for many genes of presumed known function, we do not know if that is the only function or the primary function. Even as more genes are subjected to gain of function /loss of function tests, interpretation suffers from effects of redundancy and lethality.

In the near future, genomic scale integration of processes at the levels of molecules, cells, tissues and organisms, as part of a systems biology approach, will bring about progress toward this goal. This goal appears daunting. One challenge comes from the knowledge of epistasis, or genetic interactions, that indicate substantial complexity even for simple traits. QTL analysis provides a general picture of this complexity, with a frequent genetic architecture of a small number of genes of large effects with a larger number of genes with small effects. Thanks to genomics and computers, the quantitative effects of a large number of genes may now be studied at the molecular level as is being done for genomic selection.

Traditionally, new concepts and technology developed in model systems see earlier applications in medicine and agriculture than in forestry. The lag time may be decreasing. The paradigm for genomics was established by the human genome project, where the draft sequence was initiated in 1989 and a first draft released in 2000. The sequence of Arabidopsis was completed in 2000, rice in 2002, and the first tree genome, black cottonwood, completed in 2006. We see application of new technology to traditional problems, in some cases with great success, and in others with great promise.

Only a few years after gene transfer was demonstrated in model plants, an herbicide resistance gene was inserted into poplar and a tree regenerated. Deployment of genetically modified crops has met with resistance, however, today worldwide; there are about 120 million hectares of GM crops, with half in the USA. GM trees are still highly regulated in the US. In China, GM trees have been widely deployed in the last decade. GM trees still face substantial resistance in the US and more so in Europe.

Chestnut serves as an important model for a Genetically Modified Tree. The prospects of development of a blight resistant American chestnut appear quite good based on a combination of backcross breeding; map based cloning and genetic transformation. A genome sequence for Chinese chestnut is in progress (Carlson et al.). The use of gene transfer for chestnut restoration is compelling because of the high value of the species, and because GM may be required to restore the species. The restoration of the American chestnut would have great value to Appalachian forest ecosystems, as a resource of high value wood, and potentially for bioenergy. The restoration would serve as an example for the application of biotechnology and genomics to many valuable species currently threatened by pests and pathogens or climate change.

S1. POPULATION GENOMICS, CONSERVATION AND ADAPTATION

O1

Comparative genomics of resistance of spruce to the white pine weevil in British Columbia

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We present results of two large scale comparative studies of the genomic basis of resistance of Interior spruce to the white pine weevil. Both volume growth, the main objective of the spruce breeding program in British Columbia, and white pine weevil resistance, are examined. "Interior spruce" is a species complex involving mainly *Picea glauca* (White spruce) but introgressed with *P. engelmannii* (Englemann spruce), depending upon locality. In the first study, we compared constitutive expression of 17825 genes between 20 resistant and 20 susceptible trees to the weevil; 54 upregulated and 137 downregulated genes were found in resistant phenotypes, with implications discussed in regard to volume growth. In particular, we will be surveying these genes for SNPs that differ between these two classes of trees in the next year. In the second study, we developed a 1536 Illumina SNP chip based upon candidate genes for weevil resistance. In a novel experimental design, we assayed 945 open-pollinated progeny of the Prince George breeding population (176 parents), and 654 open-pollinated progeny of the Prince Rupert breeding population (134 parents); parents were also genotyped. Within each family of 100 progeny, we identified the highest ranked 3 progeny and the lowest ranked 3 progeny, based upon BC Ministry of Forests scores for volume growth and resistance. These were genotyped and used in a novel test analogous to the transmission disequilibrium test to detect both SNP associations and QTLs linked to SNP markers. Discoveries about associations and QTL are discussed, with the added caution about genotyping error. Both studies illustrate how operational tree breeding populations can provide valuable inferences about tree genomics.

O2

Genomics of adaptation and wood properties in *Populus trichocarpa*

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BMC Proceedings 2011, **5(Suppl 7):**O2

Populus trichocarpa (black cottonwood) and *P. balsamifera* (balsam poplar) are native to North America where large natural populations exist. *P. trichocarpa* has an extensive north-south range along the west coast, while *P. balsamifera* has a northerly range across the continent from Alaska to the North American east coast. Both species are well suited for highly productive bioenergy biomass plantations in north-temperate climates. We are investigating the large reservoirs of natural genotypic and phenotypic variation in wild populations of these species, with an initial focus on *P. trichocarpa*, to identify allelic variation underlying optimal biofuel and biomass traits that could be used for accelerated domestication. We sampled genetic variation in *P. trichocarpa* by Illumina transcriptome resequencing of 20 individuals from provenances along a latitudinal gradient from 60?N to 44?N. This analysis revealed extensive nucleotide, gene expression and alternative splicing polymorphism in 10,000 xylem-expressed genes. Alignment of transcript sequences to the *P. trichocarpa* Nisqually-1 reference genome was used to identify over 500,000 SNPs. These SNPs were combined with a larger SNP set generated by whole genome resequencing of multiple *P. trichocarpa* individuals carried out by the US DOE Bioenergy Sciences Center (BESC) to populate a *P. trichocarpa* SNP database. Using the Illumina iSelect process, we generated an Illumina Infinium bead array for genotyping of 34,000 SNPs that tag 3,700 candidate genes selected for their potential involvement in biomass, adaptation, and wood traits. Using this bead array in collaboration with BESC, we obtained candidate gene SNP genotypes for over 700 *P. trichocarpa* individuals grown in common gardens. This analysis of these data revealed extensive genotypic polymorphism in the targeted gene set. In parallel, we carried out phenotyping of the trees from the wild populations grown in common gardens, quantifying variation in over 85 morphological, growth, physiological, wood chemistry, and wood quality traits. Population genetic analysis of the SNP data, its use for genotype-phenotype association studies to identify candidate gene alleles underlying variation in traits of interest, and the prospects of using the results for marker assisted breeding will be discussed.

O3

Lodgepole pine, jack pine, and their hybrids: molecular markers reveal mountain pine beetle host-range expansion into jack pine of the boreal forest

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Background: Lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia*) is found in western North America, extending from the Yukon into British Columbia and Washington, and along the Rocky Mountains and eastern slopes to Colorado [1]. Jack pine (*Pinus banksiana* Lamb.) is a closely related species found east of the Rockies, mainly in Canada's boreal forest from the Northwest Territories to Quebec [1]. Lodgepole and jack pine ranges overlap in northern Alberta and the Northwest Territories, where these species hybridize (Fig. 1). In the absence of reliable molecular markers, morphological characteristics are commonly used to distinguish lodgepole pine, jack pine and hybrids. However, hybrids and pure species can be difficult to visually distinguish, particularly at the presumed periphery of the hybrid zone, which is poorly described.
Mountain pine beetle (MPB; *Dendroctonus ponderosae* Hopkins) is indigenous to western North America. In the current MPB outbreak, more than 14 million hectares of mainly lodgepole pine forests have sustained MPB-caused mortality in British Columbia alone [2]. Following MPB long range dispersal into northwestern Alberta in 2006, MPB has continued its apparently unprecedented eastward spread into the lodgepole x jack pine hybrid zone of Alberta (Fig. 1).
To better define this hybrid zone, we developed microsatellite and SNP markers that distinguish the pure species from hybrids. We then used the microsatellite markers to test the hypothesis that the MPB epidemic has spread into jack pine.
Materials and methods: Foliage was collected from 678 trees representing 25 localities in British Columbia, Alberta, Saskatchewan, Ontario, and Minnesota in 2007, 2008 and 2010, including 154 MPB-attacked trees from British Columbia and Alberta.
Eleven microsatellite markers amplifying both lodgepole and jack pine were developed from published loci [3] and loci isolated from a microsatellite enriched (GT_n/CT_n) lodgepole pine library. Genotyping and scoring was carried out as described [4]. There were less than 2% missing data, with a 0.8% genotyping error rate. Loci met Hardy-Weinburg equilibrium criteria; pairs of loci in linkage disequilibrium were negligible. Genetic diversity measures were calculated in GENEPOP [5], GenAlEx [6] and HP-RARE [7]. Assignment tests were conducted in NEWHYBRIDS [8] and STRUCTURE [9].
In silico species-discriminating SNP detection was carried out using CLC Genomics with lodgepole and jack pine cDNA 454 sequence data, and validated with PCR.
Results and discussion: Genetic diversity was high among localities, and was higher in lodgepole than jack pine. Differentiation among localities (F_{ST} = 0.125) and between species (F_{ST} = 0.133) was high. Within species, differentiation among localities was generally low ($F_{STlodgepole}$ = 0.033, F_{STjack} = 0.016).
The efficacy of the microsatellite loci to distinguish pure species and hybrids was tested by assignment of individuals to their correct class with NEWHYBRIDS and STRUCTURE, using five simulated datasets (ten iterations each) containing multiple levels of admixture generated with known pure lodgepole and jack pine as benchmarks. Using Q_T greater than or equal to 0.9 to assign pure species and Q_T less than 0.9 to designate hybrids, we achieved accurate resolution of first and second generation hybrids in simulated datasets using both NEWHYBRIDS and STRUCTURE, but

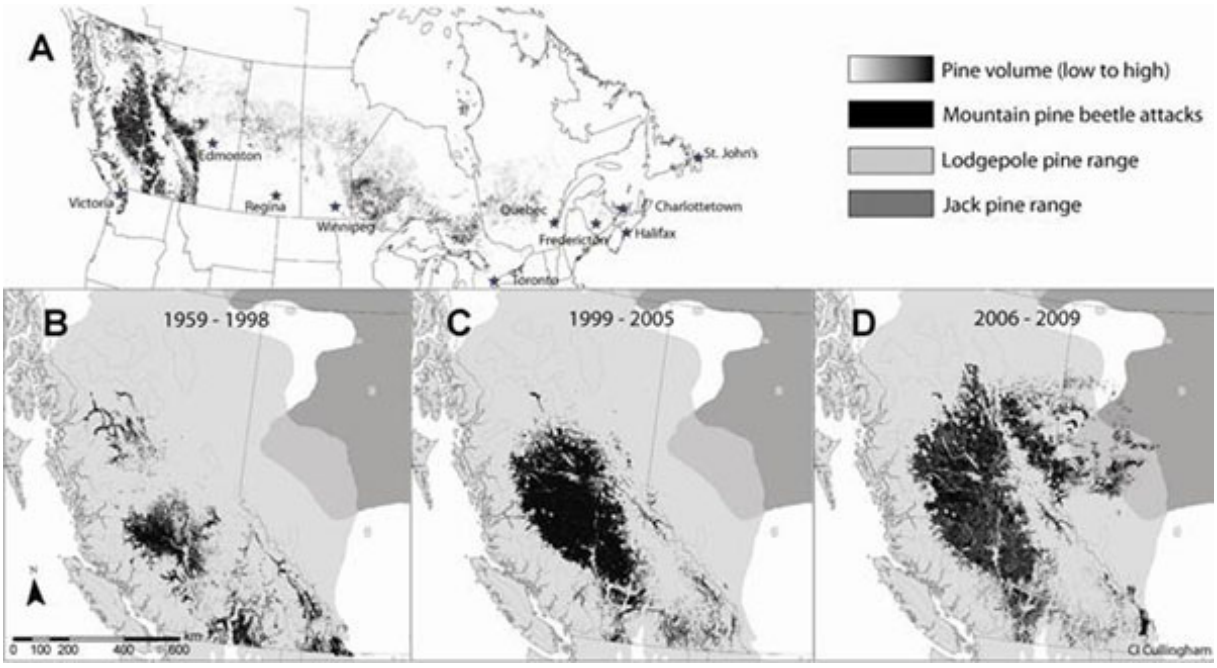

Figure 1(abstract O3) Canadian data for A. pine distribution, B. historical MPB range, C. extent of the current MPB outbreak prior to 2006, D. extent of the current MPB outbreak following dispersal into northern Alberta. MPB data are overlaid on classic lodgepole and jack pine ranges [1]. Pine volume and MPB attack data courtesy of the Canadian Forest Service.

encountered diminishing power with advanced generations. There was very high agreement between NEWHYBRIDS and STRUCTURE; a decision tree was developed to resolve the small number of disagreements. Assignment of the 678 genotyped individuals suggests that the intersection of the ranges classically described for lodgepole and jack pine [1] underestimates the distribution of hybrids. Pure lodgepole and jack pine were identified within the lodgepole x jack pine hybrid zone, with lodgepole pine often occupying higher elevations.

Eight of 154 MPB-attacked trees genotyped with the microsatellite loci were identified as jack pine. This first evidence of MPB mass-attack and reproduction on jack pine in natural stands was presented to key forest managers and decision makers via the National Forest Pest Strategy – a Canadian Forest Service initiative coordinating federal and provincial forest management and policy development – and led to accelerated development of a consensus inter-jurisdictional strategic plan.

We have developed 9 nuclear, 12 chloroplast, and 3 mitochondrial SNP markers that distinguish lodgepole pine, jack pine, and hybrids. These markers are being used to further delineate the hybrid zone, investigate gene flow and introgression, and continue monitoring MPB invasion of the boreal forest.

Conclusion: Microsatellite markers reliably distinguishing lodgepole pine, jack pine and their hybrids were used to better delineate Alberta's lodgepole pine x jack pine hybrid zone and to conclusively demonstrate that MPB has undergone host range expansion into jack pine of the boreal forest, a new habitat for this devastating forest pest.

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O4

Geographic shifts in climatically suitable areas and loss of genetic variability under climate change in a neotropical tree

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Background: Many species are expected to suffer a strong shift in geographic ranges due to climate changes in the next fifty years, depending on their ecological tolerance and current demographical parameters, which were in turn shaped by their evolutionary history. These shifts may also cause a change in genetic population structure and variability, because local extinctions or reduction in fitness are not expected to be random in geographical space. Here we used an ensemble forecast approach of Species Distribution Modeling (SDM hereafter, also known as niche modeling) to derive current and future geographic distribution of the Neotropical tree *Dipteryx alata* ("Baru" tree, Fabaceae). We then obtained a series of genetic parameters for the

species after generating extinctions in areas of low future habitat suitability.

Methods: We obtained a total of 448 occurrences of *D. alata* throughout the Brazilian Cerrado, which were recorded in a grid with 6240 cells of 0.5° of latitude/longitude covering South America. These occurrences were modeled as a function of eight climatic variables (WORLDCLIM), for the current time and projected into2050,for three different Global Circulation Models (AOGCMs - CCCma, Csiro, HadCm3) [1,2].

Occurrences were modeled using six different SDM techniques [3]. Methods used were BIOCLIM, Euclidian, Gower and Mahalanobis distances, GARP and MAXENT. For each of these methods, models were built using 255 combinations of the climatic variables, each one tested using 50 cross-validations using True-Skill statistics (models with TSS < 0.7 were excluded). A Principal Component Analysis (PCA) of the estimated frequencies of occurrence, obtained by SDMs and AOGCMs, was used to compare the maps. Variance components of these sources were obtained and mapped [2].

Genetic data for *D. alata* consisted in microsatellites markers analyzed for 25 widely distributed local populations, encompassing species' geographical range. A total of 644 individual trees were genotyped for eight microsatellite loci. Genetic parameters were used to estimate the total amount of polymorphism and genetic diversity currently found in *D. alata*. We estimated the number of alleles per locus, genetic diversity (expected heterozygosity under Hardy–Weinberg equilibrium), and the expected

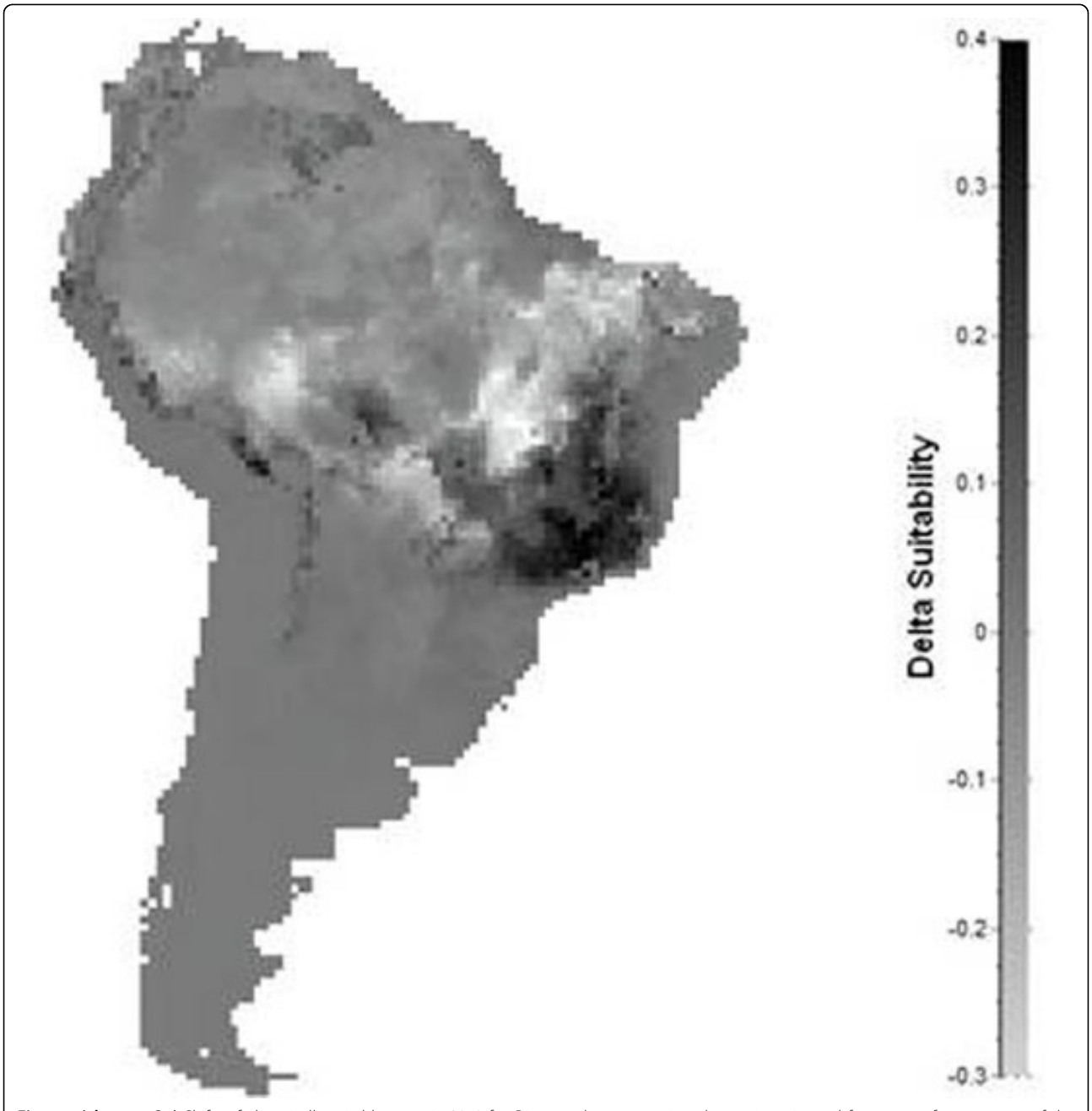

Figure 1(abstract O4) Shifts of climatically suitable areas in 2050 for *Dipteryx alata*, expressing change in estimated frequency of occurrences of the species based on six species distribution models projected into three GCMs.

heterozygosity under mutation-drift equilibrium. Moreover, we recalculated these parameters by assuming that, under climate change, there will be a displacement of climatically suitable areas for the species and, consequently, that only populations found in regions above certain levels of suitability in the future will persist.

Results and discussion: The ensemble forecasting approach reveals that *D. alata* will shift its geographic range and climatically suitable areas from Central towards Southeastern Brazil (Fig. 1). The first principal component explains 74.3% of the variation among maps and, on average, 95% of the variation among them is due to SDMs used.

The changes in the climatically suitable areas in *D. alata* imply a reduction in the genetic parameters. The parameters remain approximately constant up to a 50% threshold, which is the minimum by assuming a majority consensus of frequency of occurrences. However, after this critical threshold there is an abrupt reduction in all parameters, although the magnitude of shift is only about 10% of the current values, on average (Fig. 2). There is a wide variation of shifts direction and magnitude among loci for each parameter, and actually these trends are usually driven by two or three loci.

The wide current range and ecological tolerance of *D. alata* explains why low levels of loss in genetic diversity were observed here, contrasting with previous results for other species (i.e., the "pequi" tree *Caryocar Braziliense*). Because of the wide range, currently and in the future, some of the local populations with highest genetic diversity will potentially remain in highly suitable areas in the future, even using very conservative thresholds of 80%. However, it is important to highlight that coalescence analyses and the expected heterozygosity under mutation-drift equilibrium suggest strong population bottlenecks for the species in the recent past, which were corroborated by hind cast projections of the SDMs using paleoclimatic data. This explains the low level of genetic variability *in D. alata*, despite its wide geographic range.

Thus, despite the shift in geographic range size and climatically suitable areas towards Southeastern Brazil and the expected downward shift in the genetic parameters, the analyses performed here do not show a strong loss of genetic diversity in *D. alata*. Even so, it is important to realize that these results are mainly due to the relatively low current genetic variability of the species, probably associated with recent population bottlenecks. In this case, climatic shifts can have more serious adaptive consequences and further investigations are necessary to avoid a false indicative of high probability of persistence of this species based on a low relative level of loss of genetic diversity.

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O5

Genomic signatures of selection in response to sulfate air pollution in natural populations of red spruce

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Rapid environmental changes, such as anthropogenic air pollution, can create significant evolutionary pressures on populations, species and ecosystems. Evolutionary processes occurring in natural populations at very short time scales, especially in response to human-induced environmental changes, are not well understood. Confounding effects of geographic variation and demography cannot be easily separated from signatures of recent selection in natural populations. We investigated the genetic response of declining red spruce (*Picea rubens*) populations at high elevation sites in Southern Appalachians to anthropogenic sulfate depositions. Red spruce seedlings are more sensitive to drought and cold stresses elicited by exposure to anthropogenic sulphate air pollution, than old trees. Genetic variation in seedlings and young trees was significantly reduced in heavily polluted stands. Several candidate genes involved in cold acclimation and calcium metabolism demonstrated signatures of selection corresponding with sulfate pollution levels. SNP allele frequencies at one gene involved in calcium metabolism demonstrated directional selection in response to

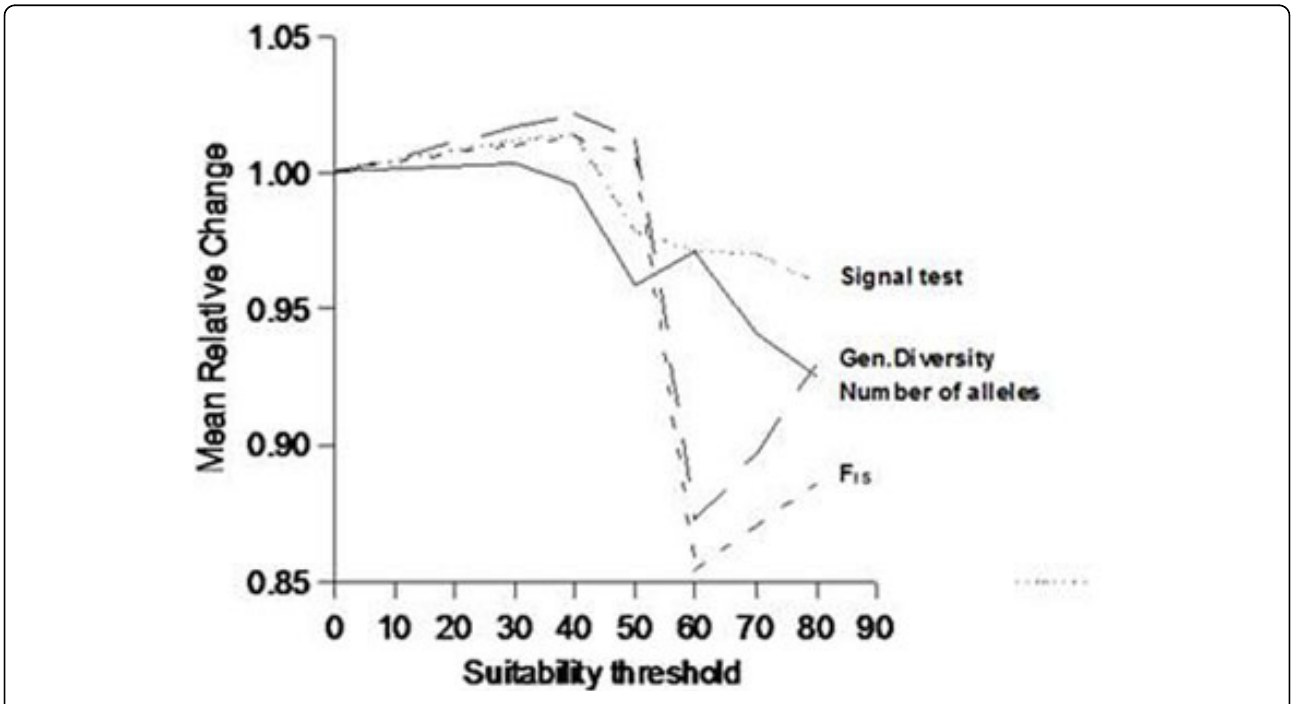

Figure 2(abstract O4) Reduction in genetic parameters of *D. alata* estimated at increasingly levels of environmental suitability estimated for 2050.

anthropogenic sulfate deposition in red spruce growing at severely polluted high elevation sites, which corresponds well with the putative role of this gene in adaptation to acidification stress. Unlike range-wide experimental designs (e.g. the popular F_{ST} outlier test) and nucleotide diversity-based association studies, our within-population testing approach disentangled the confounding effects of geographic variation and demography from the genetic effects of recent selection.

O6

Effects of forest fragmentation on the effective and realized gene flow of Neotropical tree species: implications for genetic conservation

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Tree species are key organisms of forest ecosystems, due their size and extended life, providing the environmental setting for many other living organisms. The levels of genetic diversity and the effective population sizes are essential issues for the maintenance and survival of tree species populations, because they direct adaptation to the current environment and future environmental changes. Forest fragmentation around the world is a fact, affecting many animal and plant populations in all continents. In Brazil, forest fragmentation and selective logging are the two main problems affecting populations of tropical tree species. For example, the Atlantic Forest was intensively destroyed and fragmented during the last century. Today, only between 11 and 17% of the original areas remain, generally split into small forest fragments or isolated trees in pastures and agricultural lands. In the Atlantic Forest, only 3% of the original area of the Araucaria Forest biome in southern Brazil, remains. Forest fragmentation is especially drastic in tropical forest biomes, due to the very high species diversity, associated to low population density (<1 tree/ha). Thus, after forest fragmentation takes place, the remaining fragmented forest may contain the same species diversity, but the populations are strongly reduced, and, in some cases, less than a dozen reproductive individuals remain. The bottleneck (size reduction of the reproductive population) caused by forest fragmentation associated to spatial isolation of the remaining stands may cause the loss of genetic diversity, an increase in inbreeding and relatedness, reducing the effective population size and blocking seeds and pollen migration inside the stands. These hypotheses have been tested in some tropical tree species of the Araucaria Forest and semicidual Atlantic Forest, including *Araucaria angustifolia*, *Copaifera langsdorffii*, *Myracrodruon urundeuva* and *Hymenaea stigonocarpa*, using microsatellite markers and parentage analysis. More specifically, in these studies we tried to answer the following questions: Can spatial isolation of tree populations following forest fragmentation block seed and pollen migration (gene flow)? What is the distance and patterns of seed and pollen dispersal in populations of tree species living within fragmented stands? Is there intra-population spatial genetic structure (SGS) in the adults and regeneration, and where is it higher, in adults or regeneration after forest fragmentation? Can forest fragmentation really reduce the genetic diversity and effective population size and increase the inbreeding and relatedness within populations? What is the necessary number of seed trees to collect seeds aiming at *ex situ* conservation and environmental reforestations plans with a reference effective population size that provides a minimum evolutionary potential?
Knowledge of these issues is crucial to delineate strategies for *in situ* and *ex situ* conservations, tree breeding and environmental restoration plans. As many tropical trees are long lived individuals, established before forest fragmentation had occurred (<100 years), seeds, seedlings and juveniles, established after forest fragmentation have been included in the samples. Thus, these studies have been based on the sampling of all reproductive trees regenerates (realized seed and pollen dispersal), as well as open-pollinated seed (effective pollen dispersal). All adult trees and regenerates were also mapped (x and y coordinates), the diameter at breast height (dbh) and/or total height measured and in the case of dioecious species, all reproductive trees were also sexed. We used a set of microsatellite loci to arrive to a high exclusion power, to guarantee that each candidate parent displays a unique multilocus genotype for parentage analysis. Our results showed an essentially perfect isolation of seed gene flow, with total absence of seed immigration, probably due to the large distance separating the remaining forest fragments, associated to an intensive use of surrounding land (agriculture, highways, urban areas, etc). Within populations, although long distance seed dispersal events were detected, seeds generally disperse intensively near the mother trees following a classic isolation by distance model. These hypotheses have also been supported by studies of SGS in both adults and juveniles and mating system, revealing significant rates of mating among relatives. In contrast, substantial realized and effective pollen immigrations have been detected in the studied species (ranging from 4 to 10%), evidencing longer-distance pollen dispersal than seed dispersal. Within populations, although the estimates are downward biased due to a substantial rate of pollen immigration in the stands, pollen generally disperses with high intensity at short distances, in a near neighbour pollen dispersal pattern, determined by the individual flowering patterns and behaviour of pollination vectors. Matings are not random, due to selfing, mating among relatives and correlated mating, resulting in higher inbreeding relatedness and lower effective population size in open-pollinated seeds than expected under a panmitic model. High inbreeding and relatedness and lower effective population size were also seen in the regeneration. Lower genetic diversity has been found in seeds, seedlings and juveniles when compared to the adult population, suggesting the occurrence of genetic drift. Taken together our results in the targeted species has indicated a requirement to maintain the genetic connectivity among the forest fragments using strategies of reforestation in riparian areas and forest corridors. Our results also suggest the need to collect seeds from a large number of trees for ex situ conservation, tree breeding and environmental reforestation, due to a typically limited effective population size contained in open-pollinated individuals. This small number of species studied so far does not represent all the genetic, demographic, reproductive and ecological characteristics of the tree diversity in the Atlantic Forest. To provide a stronger support to our hypotheses and results to date, new studies of gene flow, mating system, SGS, genetic diversity and effective population size are underway with other species. Finally, it is never enough to reiterate the importance of conserving tropical tree diversity for future generations so that they can benefit from them as we currently do. Although, *in situ* conservation is the indicated method for this purpose, conserving not only the target species, but also other organism in the same biome using *ex situ* strategies is also necessary due the continued fragmentation and degradation of the Atlantic Forest.

S2. LINKAGE AND ASSOCIATION MAPPING

O7

Genetic dissection of transcript, metabolite, growth and wood property traits in an F2 pseudo-backcross pedigree of *Eucalyptus grandis x E. urophylla*

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Background: *E. grandis* is used extensively for the production of pulp and paper due to its rapid growth, good form and ease of vegetative propagation. *E. urophylla* exhibits tolerance to fungal diseases that limit the growth of *E. grandis* in tropical and subtropical regions. Interspecific hybrids of these two species are, therefore, commonly used to produce fast-growing, disease tolerant hybrids for clonal eucalypt plantations in tropical and subtropical regions (e.g., South Africa, Congo and Brazil). These hybrids often exhibit superior growth and wood quality compared to the pure species, but the underlying genetic basis of the observed hybrid superiority remains unclear. Phenotypic variation observed in interspecific mapping populations has been used to identify QTLs in several genetic linkage studies in *Eucalyptus*. However, QTL intervals have generally been wide (20 to 30 cM) and may include several hundred

genes. To bridge the gap between fine mapping and QTL validation studies, the expression levels of genes and metabolites in individuals from the segregating population can be treated as quantitative traits and used for eQTL and mQTL mapping, respectively. Co-localization of wood property, expression and metabolite QTLs will facilitate the identification of positional candidate genes and other components of regulatory networks underlying phenotypic variation.

Methods: To identify genetic factors controlling growth and wood property traits in *Eucalyptus*, an F_2 pseudo-backcross mapping family derived from a cross between an F_1 hybrid (GUSAP1, *E. grandisxE. urophylla*, Sappi Forest Research) and an *E. urophylla* parent (USAP1), consisting of 555 individuals, was used for genetic linkage map construction using microsatellite and DArT markers. Phenotypic trait assessment included physical measurements of tree diameter and wood density performed on 319 three-year-old individuals. Klason (acid-soluble & -insoluble) lignin and cell wall sugar content were determined for a selection of 100 backcross progeny and used for near-infrared analysis (NIRA) calibration. NIRA predictions for glucose, xylose, arabinose, cellulose and total lignin content, as well as pulp yield were made for all 315 individuals. Total lignin and S:G ratios were also separately measured for the 315 individuals. Immature xylem tissues, collected from 192 backcross progeny, were used for metabolite profiling (ORNL, Oak Ridge, TN) and Illumina mRNA-Seq (PE50, BGI Americas) quantification of transcript levels.

Results and discussion: Parental genetic linkage maps were constructed for the F_1 hybrid (GUSAP1, *E. grandis* x *E. urophylla*) and the *E. urophylla* backcross parent (USAP1, Kullan et al. submitted).An integrated parental linkage map, constructed using the cross pollinator (CP) population type in JoinMap®4, was used for QTL mapping as this allows for the detection of the effect of alleles inherited from a single parent and the interaction between the alleles inherited from both parents. In addition to more than 50 growth and wood property trait QTLs (physical measurements, NIRA predictions and wood chemistry traits) and more than 80 metabolite QTLs (mQTLs), representing variation in 22 known metabolites, we will present progress made towards the mapping of gene expression QTLs (eQTLs) based on mRNA-Seq profiling of the first 96 backcross individuals.

Conclusions: High-density genetic linkage maps, as constructed during this investigation, enable the accurate identification of genomic regions harbouring QTLs, mQTLs and eQTLs. This is an on-going investigation in which we will integrate different genomics data with the aim of dissecting regulatory networks underlying growth and development in *Eucalyptus*.

O8

QTL analysis for growth and wood properties across multiple pedigrees and sites in Eucalyptus globulus

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BMC Proceedings 2011, **5(Suppl 7):**O8

Background: *Eucalyptus globulus* is the most widely planted species for pulpwood production in temperate regions of the world and there are breeding programs in numerous countries. There is interest in molecular approaches to breeding, particularly marker assisted selection of wood properties. QTL analysis has an important role in identifying positional candidate genes responsible for variation in wood properties. This is one approach to targeting genes which may harbour functional allelic variants (SNPs). The objective of this study was to detect and validate QTL across multiple sites and pedigrees, in order to identify genomic regions and genes affecting growth and wood properties with wide applicability in the species. We also aimed to determine the proportion of QTL which were stable in their expression across sites of contrasting productivity. Such information will be important to exploit the full potential of the impending *Eucalyptus* genome sequences.

Methods: Linkage mapping and QTL analysis were conducted in 650 individuals from four separate pedigrees; a clonally replicated outbred F_2 family grown on a single site (112 genotypes), and three F_1 families (180 individuals each) grown at two additional widely separated sites. Trees were assessed for growth, wood density, cellulose content, pulp yield, klason lignin content and the syringyl: guaiacyl (S:G) ratio of lignin at 7 years of age. Chemical traits were estimated using NIR spectroscopy [1]. Saturated linkage maps were constructed in each family, predominantly from Diversity Array Technology (DArT) markers [2] as well as some SSRs, candidate genes and AFLP markers using Joinmap. Common markers were used to construct a consensus map of all families which was used for the QTL analyses. QTL analyses were conducted at various levels (across all families and sites combined, in each family and in each family by site combination) in order to investigate the stability of QTL expression across families and sites.

Results and discussion: Ninety eight QTL were detected across all analyses, of which 87 affected wood properties and 11 affected growth. QTL for several different chemical wood properties were co-located, consistent with their high phenotypic correlations. Several of the QTL detected co-located with previously reported QTL in *E. nitens*; *E. globulus*; and *E.grandis*[3-5] and candidate genes including COBL4 [5,6], and CCR [4]. Major QTL were also identified in regions of the genome where no candidate genes or QTL had been previously mapped. Nineteen of the QTL were significant in more than one family, and therefore validated. These validated QTL would be the priority for searches for positional candidate genes in the genome sequence of *Eucalyptus grandis*.

Significant QTL by environment interaction was found for 17 out of the 98 (17%) wood property QTL and for 5 out of 11 (45%) growth QTL. This was consistent with significant family by site interactions exhibited across these sites, particularly for growth. The higher level of QTL by environment interaction for growth compared to wood properties was consistent with quantitative genetic studies in the genus [7]. Nevertheless, there was a surprising proportion of wood property QTL (including validated QTL) which were expressed on one site, but not the other within the same family. While there are few multi-site QTL or association mapping studies in forest trees, in most such studies high levels of QTL (or SNP) by environment interactions have been found [8,9].

Conclusions: Comparative mapping demonstrates that the genomes of commercially important eucalypt species (within Symphyomyrtus) are highly syntenic and co-linear (e.g. Hudson in prep), suggesting QTL results may be applicable across eucalypt species. It is therefore important to use transferable markers such as DArTs and SSRs to facilitate comparison of QTL results between studies. Considering the findings of this and previous QTL studies in the genus, growth and wood properties appear to be highly polygenic. Several of the QTL detected co-located with previously reported QTL in *E. nitens*; *E. globulus*; and *E.grandis* and also with some well characterised wood property candidate genes. However, the specificity of many QTL to particular families and sites in this study reinforces the importance of considering the effect of site and genetic setting may complicate the application of marker assisted selection (MAS) or genomic selection.

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O9

SNP discovery and association mapping in Eucalyptus pilularis (blackbutt)

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Background: This research explores the universality of genetic variation in genes controlling wood formation across the genus *Eucalyptus*.
Breeding and deploying Eucalypts for improved wood quality is constrained by the delay before wood traits can be measured reliably. Marker assisted selection (MAS) offers a way to make earlier selection of wood properties, by selecting Single Nucleotide Polymorphisms (SNPs) in the DNA which can predict specific phenotypic traits (genotype-phenotype links). SNPs shared between species (trans-specific SNPs) may have broad application to multiple species. When the species are more distantly related, ancient SNPs shared between subgenera (trans-subgeneric SNPs) are likely to be of adaptive importance and persist in separate lineages due to balancing selection [1].
Sample and methods: The focal species of this investigation was *Eucalyptus pilularis* of the subgenus *Monocalyptus*, one of the most important species for solid wood production in Australia. As part of the development of suitable SNP markers for *E. pilularis*, two other important species were included for comparative purposes. The first comparison was made with the closely related *Eucalyptus pyrocarpa*, which grows in parapatry with *E. pilularis* and is distinguished by minor morphological characteristics including a larger leaf and capsule size [2]. More distant comparisons were made between both *Monocalyptus* species and the alternate *Symphyomyrtus* subgenus, represented by *Eucalyptus globulus* subspecies *globulus*.
DNA was pooled from 30 individuals representing the natural geographical range of each of three species. A total of 34 genes were amplified from each pool and sequenced on an Illumina GAIIx. SNPs were identified in individual species pools and classified as trans-specific if they shared the same reference position in two or more species.
The association study focused on *E. pilularis* and utilized 561 destructively sampled trees from a nine-year-old progeny trial established by Forests NSW at Hannam Vale, near Port Macquarie in New South Wales, Australia (Latitude 31°40', Longitude 152°33). This sample represented the genetic diversity of 284 open-pollinated families, collected across 37 provenances that encompassed a large part of the natural distribution of this species. We tested 40 traits covering growth, wood chemistry (lignin, cellulose, hemi-cellulose complexes), and wood dimensional stability, strength and stiffness [3]. Eight SNPs were genotyped in pectin methylesterase 6 and 7 (*PME6* and *7*) using Sequenom iPLEX gold chemistry. Association testing was performed using a General Linear Model (GLM) in TASSEL where 1,000 permutations were applied. Kinship between family members was a problem and was addressed by the inclusion of a kinship matrix as covariate data within the model. The large population size used also allowed for validation of results in smaller subsets of the population containing only single representatives from each family. Previous studies have reported no geographical population structure for the provenances included in the breeding program [4].
Results and discussion: Among the three species 6,852 SNPs were identified in total. Pairwise comparisons between species representative of divergent subgenera revealed that 20-23% of SNPs were shared. These trans-subgeneric SNPs are likely to have persisted in separate lineages for tens of millions of years following the split of the *Monocalyptus* and *Symphyomyrtus* subgenera and may be indicative of adaptive variation maintained by "balancing selection". The primary signatures of balancing selection were significantly higher proportions of trans-subgeneric SNPs in exons and promoters, compared to introns and 3' untranslated regions. Further analysis revealed that ten of the 34 genes investigated were likely to be influenced by balancing selection with a significantly higher proportion of trans-subgeneric SNPs and high nucleotide diversities.
PME6 and *PME7* were two of the genes considered likely to be influenced by balancing selection. Association testing revealed several significant correlations between SNPs in these genes and solid wood properties of *E. pilularis*. *PME6* was primarily associated with shrinkage of drying timber. This is consistent with a role for pectin as a hydrophilic polysaccharide, and patterns of methyl-esterification are known to affect the water holding capacity of pectin gels. *PME7* was primarily associated with cellulose and pulp yield, and inversely with lignin content. Selection of specific alleles in these genes may enable identification of trees with superior wood quality for breeding and deployment. For some SNPs, the heterozygote was superior, indicating that selection to fix one allele in the breeding population would not be appropriate. In combination with haplotype blocks, a heterozygote advantage at one or more *PME7* loci may explain the absence of one homozygous class at all SNPs investigated in this gene. Alternatively the absent homozygous genotypes may be disadvantaged during early life stages.
Conclusions: Using comparative genomics we provided direct DNA-based evidence that many candidate genes for wood formation are under balancing selection and therefore of adaptive importance. Such knowledge of genetic variation maintained by balancing selection could be critical to ensure that selection for alleles linked to desirable phenotypes does not compromise the maintenance of adaptability in the breeding population. This concept needs to be tested, and our experiments provide a number of suitable markers which could provide the basis for further investigation.
Each of these informative SNPs in both *PME* genes could be used for MAS, subject to cost-benefit analysis. Given the large number of trans-subgeneric SNPs identified in these genes, many informative SNPs are likely to transfer to other species, facilitating the development of SNP markers in other commercial species within the diverse *Eucalyptus* genus.

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O10

Comparative genomics of resistance of spruce to the white pine weevil in British Columbia

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We present results of two large scale comparative studies of the genomic basis of resistance of Interior spruce to the white pine weevil.

Both volume growth, the main objective of the spruce breeding program in British Columbia, and white pine weevil resistance, are examined. "Interior spruce" is a species complex involving mainly *Picea glauca* (White spruce) but introgressed with *P. engelmannii* (Englemann spruce), depending upon locality. In the first study, we compared constitutive expression of 17825 genes between 20 resistant and 20 susceptible trees to the weevil; 54 upregulated and 137 downregulated genes were found in resistant phenotypes, with implications discussed in regard to volume growth. In particular, we will be surveying these genes for SNPs that differ between these two classes of trees in the next year. In the second study, we developed a 1536 Illumina SNP chip based upon candidate genes for weevil resistance. In a novel experimental design, we assayed 945 open-pollinated progeny of the Prince George breeding population (176 parents), and 654 open-pollinated progeny of the Prince Rupert breeding population (134 parents); parents were also genotyped. Within each family of 100 progeny, we identified the highest ranked 3 progeny and the lowest ranked 3 progeny, based upon BC Ministry of Forests scores for volume growth and resistance. These were genotyped and used in a novel test analogous to the transmission disequilibrium test to detect both SNP associations and QTLs linked to SNP markers. Discoveries about associations and QTL are discussed, with the added caution about genotyping error. Both studies illustrate how operational tree breeding populations can provide valuable inferences about tree genomics.

O11

Using the Eucalyptus genome to understand the evolution of plant secondary metabolites in the Myrtaceae

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Background: *Eucalyptus* trees (family Myrtaceae) are well-known for their high foliar content of several classes of secondary metabolites and these have a strong effect on the feeding patterns of several species of marsupials and at least some insects. Best known are the essential oils, which is mostly a mixture of terpenoids, but there are also significant concentrations of flavonoid and formylated phloroglucinol compounds. There is extensive quantitative and qualitative variation within and between species of Myrtaceae in these chemical groups and all appear to be under strong genetic control with heritabilities (H^2) between 0.3 and 0.9. As well as being important ecologically, the terpenes in particular are valued as industrial and medicinal products and Australia supports a strong essential oil industry focused on *Eucalyptus* and *Melaleuca* foliar oils.
Results and discussion: The *Eucalyptus grandis* genome provides the opportunity to discover the genetic makeup of the biosynthetic pathways for secondary metabolites. We present data from pathways leading into the biosynthesis of terpenes, flavonoids and lignins. The homology of genes and gene families were investigated and compared to a variety of other species including poplar (*Populus trichocarpa*), grape (*Vitis vinifera*) and apple (*Malus* x *domesticus*). For example, terpene synthases (the gene family responsible for the final step in the terpene biosynthesis) has 120 members in the genome of *Eucalyptus grandis*, compared to 44 and 99 in poplar and grape, respectively (Table 1). Genes of the biosynthetic pathways for secondary metabolites were mapped to the *Eucalyptus grandis* genome and their location was compared to a number of quantitative trait loci (QTL) studies that investigated variability in secondary metabolites and wood properties in eucalypts. This approach allowed the discovery of candidate genes for a large number of QTL.
Understanding the genetic basis of variations in quantitative traits provides insights into ecosystem function and at the same time may help breeders in the essential oil industry. We have characterized trait associations with polymorphisms from *Eucalyptus globulus*, investigating 200 SNPs and roughly 40 traits ranging from terpenoids to terpene-adducts to flavonoids and to tannin-related traits. We discovered several significant trait associations between allelic variants in the chloroplastic MEP pathway and monoterpenes and between the cytosolic MVA pathway and sesquiterpenes, as well as one allelic variant in a prenyl pyrophosphate synthase that associates with the ratio of monoterpenes to sesquiterpenes. Loci with significant associations were mapped to the *Eucalyptus grandis* genome and compared to published QTL datasets that investigated similar traits. These results represent the first species wide analysis of the molecular basis of quantitative variation in secondary metabolites in any tree.
Conclusions: The publicly available genome sequence of Eucalyptus grandis is a great resource that can be applied to a variety of questions including the genetic make-up of gene families for biosynthesis of plant secondary metabolites, genome organization of these genes and evolution of traits such as resistance to herbivores or the ability to re-sprout after fire. Combining studies of association genetics, QTL studies together with the genome sequence helps to shed light on the underlying control mechanisms of phenotypic variation.

O12

Functional variability of two lignification genes in *Eucalyptus urophylla*

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Lignin quantity and composition are major components of wood quality in eucalypts breeding programs. Optimizing these traits can have a huge economical impact for charcoal and pulp production. However, little is known about the genetic determinism of these traits. Our objective is to establish efficient early selection criteria to identify ideotypes for these traits using gene based markers. To reach this goal, we first estimated genetic parameters for lignin quantity and composition using a factorial design comprising 16 founders and 328 progenies of *E. urophylla*. We found high heritability for both Klason Lignin ($h\hat{A}^2$=0.85) and Syringyl to Guaiacyl ratio (S/G: $h\hat{A}^2$=0.62). Then, nucleotide and haplotype diversities were described for two linked genes involved in the lignification process (*CCR* and *ROP1*). High levels of nucleotide diversity and rapid decay of linkage disequilibrium were observed in a sample of 16 trees. A SNP by SNP association mapping was carried out in the 328 progenies using a mixed linear model (available in Tassel). A total of 4 SNP (3 in *CCR* and one in *ROP1*) were significantly associated with S/G ratio explaining between 1 and 1.8% of the trait variation. Finally, the additive effects of haplotypes (defined by all SNPs) were estimated using a Bayesian approach of the mixed model including the pedigree (MCMCglmm package available in R software). This analysis confirmed the existence of two distinct additive effects for *CCR* and *ROP1* on S/G ratio, suggesting the existence of two S/G QTLs for these two linked genes in *E. urophylla*.

Table 1(abstract O11) Number of TPS loci in annotated genomes and putative loci in *E. grandis*

	A. thaliana	*P. trichocarpa*	*O. sativa*	*V. vinifera*	*E. grandis*
TPS-a (sesqui)	23	21	31	42	53
TPS-b (mono)	6	13	0	30	38
TPS-b (hemi)	0	3	0	3	11
TPS-c, -e, -f	3	5	13	8	2
TPS-g (mono)	1	2	2	16	16
Sum of TPS genes	33	44	46	99	120

S3. GENOMICS ASSISTED BREEDING

O13

Genomic selection in tree breeding: testing accuracy of prediction models including dominance effect

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Background: The concept of Marker Assisted Selection (MAS) is rapidly evolving in animal and plant breeding. With the advent of high throughput molecular technology, numerous molecular markers distributed throughout the whole genome can be produced to characterize many genetic entries involving new perspectives in methodology of selection. An important research activity has begun in the animal world given the first theoretical framework for a methodology called genomic selection (GS) [1]. Several statistical approaches have been proposed for the prediction of genomic breeding values and numerous results are available that validates the interest of this method in animal breeding. In plants the GS is still limited to very advanced model species involved in genetic improvement and especially from scenario-based simulation [2,3].
In tree breeding the GS could significantly reduce the cost of genetic improvement schemes by limiting the size and number of field experiments; and facilitating the early selection at the nursery stage [4]. If most of the studies on GS have addressed the prediction of breeding value, taking into account the gene additive effects, there is still a lack of analyses dealing with the total genetic value (genotypic value) including both additive and dominance effects. This aspect is important in plant and especially in tree breeding where the goal of some programs is the production of clones or elite families. The aim of this study is to investigate the performance of GS in the context of tree breeding when the selection is based on genotypic value. The proposed approach allows taking into account both additive and dominance effect [5] for each marker in the statistical model. Six scenarios are simulated to test the reliability of the GS in the frame of recurrent selection scheme.
Methods: Simulation: The data used to evaluate the accuracy of the model have been simulated using HaploSim package in R software [6].
Firstly, populations were simulated for 1000 generations at an effective size of 100 to reach a mutation-drift balance. Fifty parent trees were then selected to start a breeding scheme that was conducted during two generations. At each generation, a progeny test was implemented using a factorial mating design. The fifty percent parents were selected and crossed using circular design to constitute the following generation. At each generation, 670 individuals issued from the mating of 16 females and 34 males were evaluated for clonal selection. The 670 individuals were genotyped for 400 SNP markers equally-spaced across one chromosome of one Morgan corresponding to an efficient marker density.
The broad sense heritability HÂ² was equal to 0.3. A gamma distribution was used to sample the 44 QTL effects.
The additive (breeding), dominance and genotypic values were simulated for each individual. The ratio of dominance to additive variance was equal to 0.1, 0.5 and 1. Six scenarios were evaluated for predicting the genotypic value: three different ratios and two different QTL distributions (high proportion with small or medium effects).
Analysis model: Genomic selection consists in following steps: (i) estimation of the effects of all markers in a 'training data set', where the individuals are phenotyped and genotyped; (ii) prediction of the genetic values of other 'evaluation' individuals by combining their marker genotypes with the estimates obtained in step (i) .
A Bayesian implementation of the Lasso method with BLR package in R [7,8] was used to estimate the substitution and dominance effects for each of the 400 SNP. This method allows predicting the genotypic value using all markers simultaneously with different variances for each marker effect. We evaluated the performance of the statistical model with and without dominance effects. The training and validation set corresponded, respectively, to the first and second generation containing each 670 individuals. The criterion to compare the different scenarios was the accuracy calculated as the correlation between true and predicted genotypic value. Each simulated data set and analysis was replicated 30 times.
Results: The accuracy of two models decreases when the ratio of dominance to additive variance increases whatever the QTL distribution (table 1). In all scenarios GS is superior to basic phenotypic selection. In addition, the model with dominance effects shows a higher accuracy, especially when the variance ratio increases. For the second generation the same trend is observed but the model with dominance effects is more accurate when the ratio of variances is greater than 0.5. A lower accuracy is observed in the second generation; it can be attributed to the low linkage disequilibrium of this breeding population two generations after selection in simulated wild population.
Conclusions: The model including dominance effects is more accurate to predict the genotypic value especially when the dominance-additive variance ratio increases. These results are particularly interesting for tree improvement in hybrid populations where dominance effects are marked and clonal varieties are produced (eucalyptus, poplar, for example).

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Table 1(abstract O13) Accuracy (se) of GS in the first and second generation without and with dominance effects for six different scenarios and accuracy of phenotypic selection (30 replicates)

ratio	QTL distribution: small effects			QTL distribution: medium effect		
Dominance variance/ additivevariance	Phenotypic selection	Model without dominance	Model with dominance	Phenotypic selection	Model without dominance	Model with dominance
First generation						
0.1	0.52(0.03)	0.80(0.03)	0.81 (0.03)	0.53(0.03)	0.79 (0.03)	0.80(0.03)
0.5	0.51(0.03)	0.70 (0.05)	0.73 (0.04)	0.53(0.04)	0.68 (0.04)	0.73(0.04)
1	0.51(0.03)	0.61(0.06)	0.69(0.05)	0.53(0.03)	0.64(0.04)	0.71(0.04)
Second generation						
0.1	NO	0.63(0.13)	0.63(0.13)	NO	0.62(0.08)	0.62(0.08)
0.5	NO	0.53(0.13)	0.55(0.12)	NO	0.53(0.11)	0.54(0.10)
1	NO	0.42(0.13)	0.48(0.13)	NO	0.42(0.10)	0.47(0.9)

NO: not observed.

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O14

Stability of Genomic Selection prediction models across ages and environments

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Background: A tree breeding program is characterized by long generation intervals which, over time, result in a much smaller number of breeding cycles when compared to annual crops. Moreover, most economically important traits in a tree-breeding program are quantitatively inherited, display low heritability and are expressed late in the life cycle. Genomic Selection (GS) is expected to be particularly valuable for tree species, leading to shorter generation intervals and improved genetic gain over time.

The main factors that affect the accuracy of GS prediction models are the level of linkage disequilibrium (LD) in the training population, the training population size, the heritability of the trait and the number of QTL regulating its variation. However, it is yet largely unknown how stable prediction models are across environments and different ages. This knowledge is critical for tree breeders that wish to use genomic selection in their genetic improvement program.

Here, we report the first assessment of the utility of genomic selection in a conifer species. We developed prediction models for growth traits measured at multiple sites, to evaluate the impact of genotype by environment interactions in their accuracy. Training populations were also measured over multiple ages and models were developed to assess their value in predicting breeding values later in the lifecycle.

Material and methods: Here we analyzed a population of 790 to 840 individuals of loblolly pine, clonally replicated in four sites in the southeastern US: Palatka and Nassau (Florida, USA), Cuthbert and B.F. Grant (Georgia, USA). The population is derived from 61 full-sib families, established by crossing 32 parents in a circular mating design. The traits analyzed in this study were diameter at breast height (DBH) measured when trees were three, four and six years old; and total height (HT) measured when trees were one, two, three, four and six years old. All the individuals were genotyped with a total of 3,938 SNPs [1]. Single marker regression association analyses were initially performed treating the markers as fixed effects. The markers that were selected in this association analysis had their effects estimated adjusting all the allelic effects simultaneously using a genomic BLUP procedure [2]. These analyses were performed across all sites, traits and ages and the estimated effects of the markers were validated using a 10-fold cross validation approach. The selection gain of genomic selection was compared to classical phenotypic selection considering a reduced breeding cycle due to early selection.

Results and discussion: The accuracies of the prediction models for GS developed using phenotypic data measured in each site at year 6 ranged from 0.65–0.75 for DBH, and 0.64–0.77 for HT. To evaluate the performance of GS relative to traditional breeding methods, we estimated the accuracies of BLUP-based selection [3] and used it as a benchmark for the comparison of the accuracies obtained by GS. The increase in efficiency per unit of time in the selection response of GS was 53–95% higher for DBH, and 58–118% higher for HT, assuming a conservative reduction of 50% in the length of the breeding cycle.

To evaluate if models generated at early ages would predict well the phenotype at mid-rotation, we assessed the accuracy of models developed for HT based on data collected at ages 1 to 4, but validated with measurements from the same populations at age 6. Accelerating model estimation is beneficial because the sooner models that accurately predict phenotypes at rotation age can be developed, the faster genomic selection can be adopted. However, the models developed for HT early in the rotation (age 1 to 3) had limited accuracy in predicting phenotypes at age 6.

Next, we tested the suitability of models estimated in each individual site, in predicting phenotypes across different sites. The accuracies reduced up to 86% (Table 1) and the decrease parallels the increase in geographic distance between the site for which models were estimated, and the site where they were validated. Therefore, environment × genotype interactions appear to severely affect the transferability of models across breeding zones.

Out of the total number of markers, 177 and 83 were significantly associated with traits across all sites for DBH and HT, respectively. Those were selected for model estimation since they may reflect marker-trait associations that are independent, or at least less affected, by genotype × environment interactions. Initially, models were re-estimated based on data from each site, using only the subset of markers, and re-validated in the same site. Accuracies were only slightly lower (3–9% for DBH, 6–13% for HT) than those obtained when models included the full set of significant makers (Figure 2). Therefore, a significant component of the genetic variance appears to be captured by relatively few markers (~100).

Next, prediction models were estimated based on a combine-site model and validated individually in each of the four sites (Figure 1). While accuracies remained relatively high across sites (0.43–0.51 for DBH, 0.46–0.54 for HT), there is a decrease of up to 30% relative to the accuracy obtained when the full set of markers and site-specific specific data was used for model estimation (Figure 1). Therefore, while the same reduced set of markers is relevant for explaining the genetic variation in all sites, the magnitude of their effect changes significantly from site to site.

In conclusion, the results in efficiency demonstrated that incorporating genomic selection would dramatically increase the genetic gains per unit of time of a conifer's breeding program. Moreover, even at relatively low marker density, the accuracy of prediction models could significantly impact the genetic gain efficiency. However, the use of a prediction model should be constrained within a breeding zone once genotype x environment can affect the prediction and reduce the accuracy of those models.

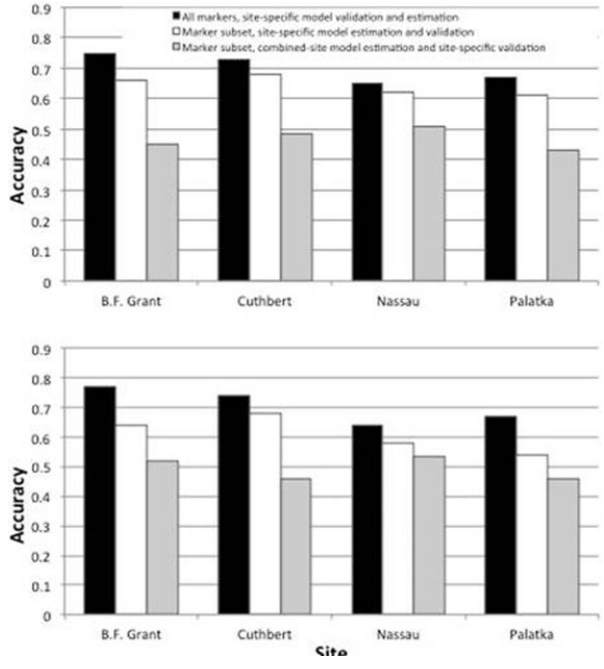

Figure 1(abstract O14) Accuracy of genomic selection models estimated using the complete (black bar) or subset of markers significant across all sites (white and grey bars) for DBH (upper panel) and HY (lower panel). Prediction models were estimated and validated in each individual site (black and white bars), or estimated based on a combined-site model and validated in idiviudal sites (grey bar).

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O15

Candidate gene-based association mapping of growth and wood quality traits in *Eucalyptus globulus* Labill

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Background: The identification of polymorphisms that underlie complex phenotypic traits presents new and exciting challenges to molecular genetics. Association mapping, which uses linkage disequilibrium (LD) to map trait variation with nucleotide polymorphisms, has proved suited for this purpose in outcrossed tree species. Association mapping can be undertaken either at a candidate gene level or at the whole-genome level however; until sufficiently dense marker assays are developed the candidate gene approach will remain the most effective way of dissecting complex traits in tree species. To date, this approach has led to the identification of several quantitative trait nucleotides (QTN) that associate with a variety of breeding traits including, early wood specific gravity, percentage latewood [1], microfibril angle [2], cellulose [3]and carbon isotope discrimination [4].
Aim: Our research aims to identify single nucleotide polymorphism (SNP) markers using candidate gene-based association mapping that can predict growth and wood quality in *Eucalyptus globulus*.
Materials and methods: A *Eucalyptus globulus* provenance-progeny trial, planted in 1989 near Latrobe in north-central Tasmania, Australia, by Gunns Ltd, was used as the association discovery population. An eight-year-old Southern Tree Breeding Association (STBA) breeding trial growing near Frankland, Western Australia, is being used for validating marker-trait associations. Twenty functional candidate genes for wood and fiber formation, were selected for this study. SNPs were discovered by direct sequencing of PCR products from 11 to 28 trees.
The iPLEX Gold assay (Sequenom Inc.)was used to genotype 98 selected polymorphisms in up to 385 individuals from the discovery population. This subset includes individuals from eight races of *E. globulus*. Linkage disequilibrium between SNPs was estimated using GEVALT [5]and Hardy-Weinberg equilibrium was estimated using FSTAT ver 2.9.3.2 [6]respectively.
To account for genetic structure, 18 SSR markers were genotyped in all the discovery samples. Ancestry (Q) co-efficients were estimated using the model-based clustering method as implemented in STRUCTURE [7]. A matrix of pairwise kinship coefficients (K) was calculated as described in Ritland [8], using the software SpaGeDi [9]. Marker-trait associations were tested using a mixed linear model (MLM) [10], which accounted for both population structure and familial relatedness using TASSEL version 2.0.1 [11].
Results: At a 5% significance level, only 4 SNPs deviated from Hardy Weinberg expectations in more than one race. With all races pooled, LD between SNPs was very low with only 1.8% of the pairwise comparisons having r^2 values greater than 0.33. Only where r^2 is greater than 0.33 is there sufficiently strong LD to be useful for association mapping [12]. Only one pair of SNPs, *EgCSA3*_4186 and *EgMYB2*_1380 between genes was found to be in LD ($r^2 > 0.5$). When LD was computed within races 1.3 to 2.7% of the pairwise comparison had r^2 values greater than 0.33.
Of the 98 polymorphisms tested against 12 traits, 33 associated significantly ($P < 0.05$) with one or more traits giving a total of 62 associations. Individual polymorphisms explained between 0.9 and 3.8% of the phenotypic variation observed. Marker-trait associations found in the discovery population are currently being validated by testing their consistency using the validation population.
Conclusions: Candidate gene based association mapping studies are a useful means of dissecting complex quantitative traits in species like *E. globulus* with low LD and high nucleotide diversity. This study, like other tree association mapping studies, shows that the percentage of phenotypic variation explained by a single polymorphism will often be small. This is not unexpected because of the complex nature of most wood quality traits. The small proportion of the phenotypic variation explained so far using association mapping should not detract from its future use in tree breeding, since genotyping costs are expected to fall while throughput increases, thereby facilitating larger scale association mapping efforts in the near future.

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O16

Genomic Selection for growth traits in *Eucalyptus*: accuracy within and across breeding populations

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Background: Genomic selection (GS) involves selection decisions based on genomic breeding values estimated as the sum of the effects of genome-wide markers capturing most QTLs for the target trait(s). GS is revolutionizing breeding practice for complex trait in domestic animals. The same approach and concepts can be readily applied to forest tree breeding. Trees also have long generation times and late expressing traits. Differently from association genetics that aims at dissecting complex traits in their discrete components, GS precludes the discovery of individual marker-trait associations and focuses on prediction of performance. By capturing the "missing heritability" of complex quantitative traits beyond the few effect variants that association genetics has so far typically identified, GS might soon cause a paradigm shift in forest tree breeding. In a prior deterministic study we assessed the impact of linkage disequilibrium (modeled by Ne and inter-marker distance), the size of the training set, trait heritability and the number of QTL on the predicted accuracy of GS [1]. Results indicate that GS has the potential to radically improve the efficiency of tree breeding. The benchmark accuracy of conventional BLUP-based phenotypic selection (0.68) was reached by GS even at a marker density ~2 markers/cM when Ne ≤30, while up to 10 markers/cM are necessary for larger Ne. Shortening the breeding cycle by 50% with GS provides an expected increase ≥100% in selection efficiency. To validate these simulation results we carried out a large multi-population proof-of-concept study of GS in tropical Eucalyptus. In this report we present results of this on-going study for two populations and three different quantitative traits.

Methods: This study was carried out in two structured populations of Eucalyptus represented by two progeny trials of independent breeding programs. The first was a progeny trial developed by CENIBRA (CEN) with 43 full-sib families generated by an incomplete diallel mating design carried out by intercrossing 11 highly selected *E. grandis x E. urophylla* hybrids clones therefore having an effective population size of Ne= 11. The second population was a progeny trial installed by FIBRIA (FIB) with 232 full-sib families involving 120 elite parents, the vast majority F1 hybrids of *E. grandis*, *E. urophylla*, *E. globulus* and *E. maidenii*. For the GS study however only the most phenotypically relevant 75 families were used derived from intercrossing 55 parents therefore limiting the effective population size to Ne= 55. A sample of trees was taken in a stratified way from all families to provide a balanced representation of all families with similar numbers of individuals per family. Bark or leaf samples were collected, DNA extracted and genotypes obtained for 783 and 920 trees from CEN and FIB respectively. Genotyping was carried out using a high-throughput genotyping platform developed earlier [2]that provided 3,120 and 3,564 robustly scored dominant DArT markers. De-regressed phenotypes for Height (H), Diameter at Breast Height (DBH), wood density (WD), pulp yield, lignin content and *Puccinia* rust resistance were obtained. Single marker regression association analyses were initially performed with all the traits, treating the markers as fixed effects. Markers selected in the previous association analysis had their effects estimated adjusting all the allelic effects simultaneously using Random Regression Best Linear Unbiased Predictor. Each population was then divided in an training set comprising 90% of the total number of individuals and a validation set with the other 10%. The estimated effects of the markers were validated using a cross validation approach using random sub-sampling with ten replications so that all individuals had their phenotypes predicted by GS. Details of the overall analytical approach have been described [3,4].

Results and conclusions: To date results have been obtained for height, DBH and wood density while the remaining traits are under analysis. Realized accuracies by Genomic Estimated Breeding Values (GEBV) for H and DBH were 0.67 and 0.69 for CEN and 0.62 and 0.54 for FIB while for WD in FIB it was 0.53. These accuracies match or surpass those obtained by conventional BLUP (Best Linear Unbiased Prediction) based phenotypic selection. Not surprising GEBV accuracies were low (~0.18) across populations implying variable genotype-phenotype associations across backgrounds so that population-specific GS models will be necessary. GS-based reduction in breeding time by 50%, i.e. by reducing a breeding generation time from 12 to 6 years should provide gains ≥100% in selection efficiencies. If a reduction from 12 to 3 years using flower induction can be achieved, gains in selection efficiency theoretically could surpass 300%. These are among the first experimental results of GS in forest trees and in plants in general. With the technological advances and declining costs of genotyping methods we anticipate that GS will soon be implemented operationally and revolutionize *Eucalyptus* tree breeding practice.

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O17

High-throughput targeted SNP discovery using Next Generation Sequencing (NGS) in few selected candidate genes in *Eucalyptus camaldulensis*

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BMC Proceedings 2011, **5(Suppl 7):**O17

Background: The present era of high throughput technologies offer immense promise and innovative applications for SNP discovery and high quality parallel genotyping [1,2]. Using advancements in the next generation sequencing (NGS) technologies, the *en masse* SNP discovery for targeted genomic regions is possible for eucalypts. The river red gum or *Eucalyptus camaldulensis* (*Ec*) is a fast growing, hardy and highly adaptable eucalypt species acclimatized to Indian climatic conditions and these new advancements would aid in developing new tools and techniques for its improvement. In our knowledge, limited efforts have been undertaken to identify SNP markers in eucalypts either by employing RNA sequencing [3] or by using few genes available in the literature [4]. Despite these miniscule efforts, useful SNP markers were discovered in *Cinnamoyl CoA Reductase* (*CCR*) gene with potential application [5]. Using the recently released whole genome sequence of *E. grandis* (*Eg*), herein we describe targeted SNP discovery in 41 candidate genes by employing Illumina's 72-bases paired end sequencing technology.

Materials and methods: The DNA was isolated from a SNP discovery panel consisting 96 individuals from a naturally mating *Ec* population from Australia following standard procedures (modified CTAB method). Twelve primary DNA pools were constituted by mixing equimolar concentrations of eight DNAs @ 10 ng/mL. Forty one genes selected for SNP discovery were identified from *Eg* genome (http://eucalyptusdb.bi.up.ac.za/gbrowse8x) by employing Arabidopsis TAIR 9 gene IDs. Further the primer pairs were designed to amplify the gene fragments. The individual primary DNA pool was amplified (Veriti-ABI) using *Paq* DNA polymerase (Agilent Technologies), all amplicons pooled (figure 1), eluted if necessary (*Ec*CRE-AHK4, *Ec*OBP1), precipitated using ethanol and dissolved in TE (0.1).

A paired end library suitable for 72-bases read length was prepared and sequenced on an Illumina GAIIx sequencer and analyzed using bwa and samtools with appropriate parameters (outsourced to Genotypic Technologies Ltd, Bangalore). The SNP data was adjusted for read depth ($1/10^{th}$ SD) and rare allele frequency (<5%). Further approximate equal frequency (EF) blocks were manually estimated by nearest neighborhood (NN) analysis in MS Excel (MS Office 2007), wherein, a block of NN SNPs having frequency difference of less than 0.02-0.03 was considered as single EF block. Web-based gene prediction tool FGENESH (http://linux1.softberry.com) was used for identifying genic regions such as UTRs, exons and introns with *Arabidopsis thaliana* gene model.

Results and discussion: Forty one growth and adaptive genes were selected based on literature search [6, TAIR database]. A total of 100.5 kb genomic sequence from *Ec* genome spread over ~1055 Mbp reads was generated (~94% high quality reads with average read depth 6124). A total of 11,329 SNPs were polymorphic within *Ec* and 378 SNPs exhibited

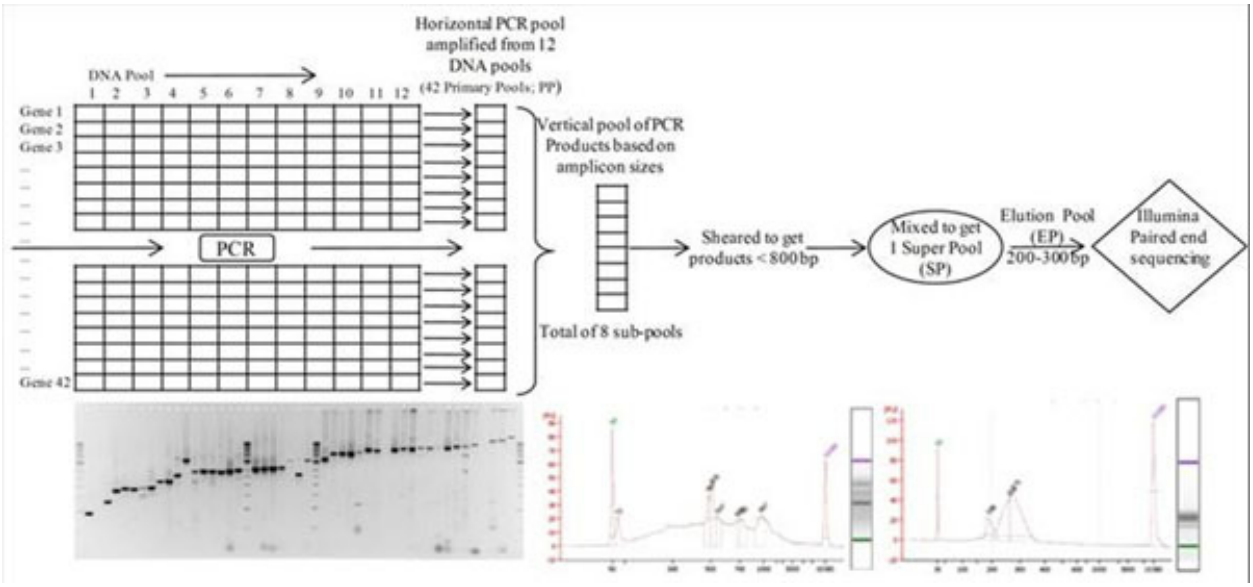

Figure 1(abstract O17) Stategy for hierarchical pooling of 96 DNAs and PCR products for SNP discovery using Illumina NGS platform.

Table 1(abstract O17) Results from SNP discovery in 41 candidate genes

Predicted gene region	SNP frequency parameters			SNP classification			
	SNP count (range)	*SNP Frequency in bases/SNP (range)*	*Total length in bp (range)*	*Ts*	*Tv*	*Total*	*Ratio(Ts/Tv)*
5'UTR(n=1)	4	40.8	163	1	3	4	0.33
Exons(n=176)	427 (1-52)	105.8 (0-1339)	45,177 (200-3487)	310	117	427	2.65
Introns(n=136)	536 (0-64)	71.3 (0-472)	38,210 (68-5079)	332	204	536	1.63
3'UTR(n=27)	54 (0-11)	81.6 (0-358)	4,405 (7-674)	33	21	54	1.57
Unclassified(n=13)	69 (0-25)	62.7 (0-425)	4,329 (4-425)	40	29	69	1.38
Nongenic(n=17)	101 (0-25)	82.6 (0-974)	8,340 (6-1481)	63	38	101	1.66
Total(n=370)	1,191 (1-115)	84.5 (38.2-974)	100,624 (634-9864)	779	412	1191	1.89

Note: n: number of units detected from 41 genes; Ts: transitions, Tv: transversions.

inter-species polymorphism between *Ec* and *Eg*. In addition, 75 insertions and 90 deletions within *Ec* and eight intra-specific deletions in comparison to *Eg* were detected. After appropriate corrections as described, the 'useful' SNP number reduced to 1,191 which was ~10.5% of the original SNP count (~frequency of 1 per 84.5 bp). Table 1 describes findings from the present analysis of SNPs. A total of 198 putative EF blocks containing 541 SNPs, grossly comparable to LD blocks, with 55, 65 and 34 in exons, introns, exon-intron junctions respectively were detected (rest all were small in numbers) with an average length of ~105 bp (SD: ± 182; range: 1-1234 bp, distribution shown in figure 2; ~3 SNPs/block) and would aid in selection of SNPs. The comparable mean lengths adjusted for the respective amplicon lengths were around 0.014 to 0.016 (SD: ±0.013 to ±0.015) for exons, introns and nongenic regions whereas for intron-exon junctions it was 0.028±0.023, significantly longer than the rest (p=0.03).

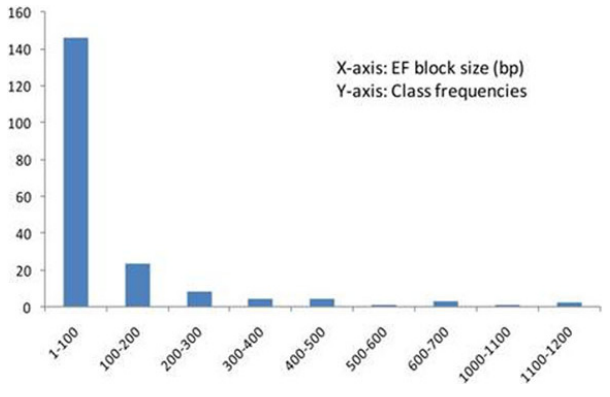

Figure 2(abstract O17) Bar graph showing distribution of 198 frequency (EF) blocks according to their length (bp).

Conclusions: Herein, NGS (Illumina) platform was successfully used for identifying ~1,200 SNPs in 41 targeted genes in *Ec* which has shed important light on quantitative and qualitative distribution of SNPs. In addition, the analysis of EF blocks also provided important guidelines for selection of SNPs for genotyping.

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O18

Mapping and transcriptomic approches implemented for understanding disease resistance to *Phytophthora cinammomi* in *Castanea sp*

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BMC Proceedings 2011, **5(Suppl 7):**O18

The European chestnut, *Castanea sativa* Mill, covers a total area of 2.53 million hectares two million of which are chestnut forests, i.e. forests where chestnut is the dominant tree species, being the remaining 0.53 million hectares devoted to fruit production (20.9% of the total chestnut-growing area). Chestnut fruit production in Europe declined considerably during the XX century to the current 200,000 t (almost 300 million euros). This decline arose mainly due to serious diseases and changes in the structure of society. Towards the end of the century, there was a sharp increase in chestnut demand which triggered new planting and the restoration of old orchards throughout Europe, although ink disease (*Phytophthora* spp.), canker blight (*Cryphonectria parasitica* (Murrill) M.E. Barr.) and more recently *Dryocosmus kuriphilus* Yasumatsu, still represent major threats to the species. In Europe, chestnut breeding has been focused on the improvement of cultivars and rootstocks through selection and hybridisation with Asian species resistant to ink disease. Considerable success was obtained using this approach, however little information was acquired until now on the genetic basis of resistance in chestnut.
A set of interspecific crosses were established between European chestnut and Asian species and two separate full-sib pedigrees have been produced, SC (*C. sativa* x *C. crenata*) and SM (*C.sativa* x *C. mollissima*). The goal is to perform DNA marker:trait association analysis to identify QTLs related to ink disease metrics and also to identify putative resistance genes to *P cinnamomi* using a transcriptomic approach. An overview of the study will be presented including the genotyping of the mapping population (parents and progenies) with nuclear SSR markers designed for *Castanea mollissima* and provided by the American team of Fagaceae Genomics Project, for the construction of the first genetic linkage map. The methodologies implemented for the determination of the metrics of resistance of mapping population, for further identification of QTLs, will also be presented.

O19

Sequence-based breeding; application to unravel traits of the 6F crops: food, feed, fiber, flowers, fuel and fun

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BMC Proceedings 2011, **5(Suppl 7):**O19

The genomes of the world's most important crop species differ enormously in size, ploidy levels, repeat composition and germplasm diversity, posing a tremendous challenge to utilize them for breeding and trait improvement. Keygene's Crop Genome Center addresses this challenge by developing and applying sequence-based methodologies to advance building genome assemblies and elucidating genetic diversity of 6F crops. For example, novel strategies to build very high quality genome sequence assemblies make use of our Whole Genome Profiling (WGP) technology and high parallel local sequence assemblies. For sequence-based genotyping we developed random Sequence-Based Genotyping (rSBG), a technology which incorporates the high-throughput sequencing capacity of the Illumina Genome Analyzer (GA)-*II* and the genome complexity reduction capabilities of AFLP®. rSBG allows for simultaneous sequence-based marker discovery and detection and is therefore a highly suitable technology for cost efficient, highly flexible, sequence-based genotyping, not requiring prior sequence information and custom reagents. These technologies, together with KeyGene's lead discovery and molecular mutagenesis platforms, significantly increase the rate with which the genetic basis for (complex) traits can be unraveled. In this presentation, I will present recent applications of these technologies in our breeding research programs.
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O20

The regulation of quantitative variation of foliar terpenes in medicinal tea tree *Melaleuca alternifolia*

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Background: Terpenes are important mediators of plant-environment interactions as well as often being economically important. Terpenes are biosynthesized via two spatially separate pathways: the MEP pathway in the plastid, the site of monoterpene biosynthesis and the MVA pathway in the cytosol, the site of sesquiterpene biosynthesis (McGarvey and Croteau 1995; Eisenreich *et al*. 1998; Rohmer 1999). Studies using transgenic plants have demonstrated that when the MEP genes *dxs* and *dxr* and MVA pathway gene *hmgr* are up-regulated plants exhibit an increase in foliar terpene concentrations (Chappell *et al*. 1995; Wildung and Croteau 2005; Carretero-Paulet *et al*. 2006). However, as yet, the importance of the expression of these genes has not been demonstrated for naturally occurring quantitative variation in foliar terpene concentrations.
Aims and methods: *Melaleuca alternifolia*(medicinal tea tree) is a small tree in the family Myrtaceae that is grown in plantations for its valuable terpene-rich leaf oil. We studied the expression of MVA and MEP pathway genes to identify those that are correlated with variation in foliar terpene concentration. Diagnostic primers were designed for seven MEP pathway genes, three MVA pathway genes and three genes downstream of both pathways. Transcript abundance for all 13 genes was then quantified from 48 individuals on the Fluidigm biomark platform.Foliar terpene concentrations were determined by GC of ethanol extracts containing an internal standard.
Results: Foliar terpene concentration varied from 5 to 12.2% of dry matter (DM), terpinen-4-ol concentration from 2.1 to 6.6% of DM and bicyclogermacrene concentration from 0.1 to 0.5% of DM. The transcript abundance of five genes within the MEP pathway were correlated significantly with variation in foliar terpinen-4-ol concentrations. The transcript abundance of three MEP pathway genes and one MVA pathway gene were significantly correlated with foliar bicyclogermacrene (the most abundant sesquiterpene) concentrations. However, the expression of the genes within both pathways were co-dependent and showed strong pair-wise correlations within pathways. Therefore, multiple regression models were formulated to account for interactions amongst the genes. A multiple regression model using only the genes in the MEP pathway explained 87% of variation in foliar terpinen-4-ol concentrations (Figure 2). A multiple regression model of the genes within both the MEP and MVA pathways explained 60% of variation in foliar bicyclogermacrene concentrations (Figure 2). Two sub-models using only the genes from either pathway explained significantly less variation in bicylclogermacrene concentrations than the full model (MEP only, r^2= 0.5; MVA only, r^2= 0.15) and indicated that both pathways contribute to the

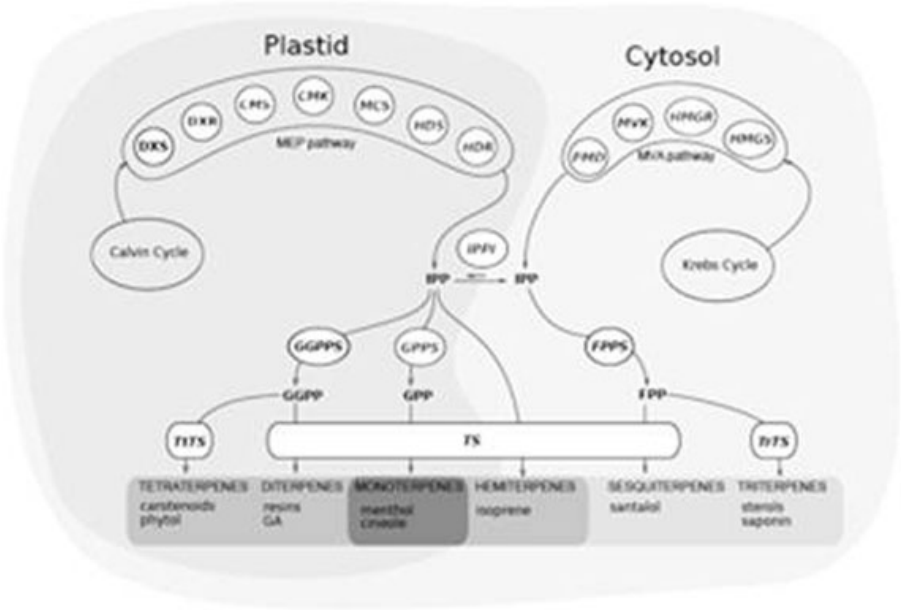

Figure 1(abstract O20) Simplified schematic of the terpene biosynthetic pathway in plants, showing the catalyzing enzymes for each step.

pool of isopenthyl pyrophosphate (IPP) used to biosynthesize bicyclogermacrene.

Discussion: Several studies have shown that the expression of *dxr* and *dxs* has a strong influence on foliar terpene yield; however, this has only ever been demonstrated in transgenic plants and never on naturally occurring, quantitative variation in terpene concentrations. Our results show that a large proportion of the quantitative variation of foliar terpene concentration in *M. alternifolia* can be explained by quantitative variation of transcript abundance of genes in the early stages of terpenoid biosynthesis. A number of MEP pathway genes are correlated

Terpinen-4-ol

Predictor	Slope	P value
dxs2	70.883	<0.001
dxr	37.682	0.018
mct	4.129	<0.001
mcs	53.857	0.18
hds	9.499	0.585
gpps	8.3	0.019
I(hds^2)	92.068	<0.001
I(mcs^2)	41.043	0.125
mct:gpps	3.603	<0.001
dxr:mcs	77.482	0.018
dxr:hds	54.508	0.01
hds:I(mcs^2)	60.705	0.014
dxs2:I(mcs^2)	52.135	0.017
dxs2:mcs	145.007	0.001
Overall R^2	0.871	
Model p value	<0.001	

Bicyclogermacrene

Predictor	Slope	P value
dxs2	1.708	0.206
dxs3	1.198	0.159
dxr	6.472	0.004
cmk	12.013	<0.001
mcs	7.398	0.002
hds	12.472	0.005
pmd1	2.503	0.08
mvk	2.205	0.111
hmgs	1.228	0.208
mcs:hds	9.575	0.009
dxs2:cmk	12.352	<0.001
dxs2:hds	6.972	0.001
Overall R^2	0.603	
Model p value	0.001	

Figure 2(abstract O20) Multiple linear regression models of gene expression against terpinen-4-ol and biocyclogermacrene concentrations.

with variation in both the monoterpene terpinen-4-ol as well as the sesquiterpene bicyclogermacrene, which is also correlated with one MVA pathway gene. The multiple regression models for both terpinen-4-ol and bicyclogermacrene, each explained a significant amount of variation in the concentrations of each terpene.

The correlation between monoterpenes and transcripts from the MEP pathway was expected as they both occur in the plastid, however we also found a significant correlation between MEP pathway genes and bicyclogermacrene, a sesquiterpene synthesized in the cytosol. These correlations, as well as the results of the multiple regression analysis, strongly suggests that in *M. alternifolia*,the IPP from the plastid contributes significantly to the pool of IPP used for sesquiterpene biosynthesis.

Conclusions: This is the first study that shows the importance of variation in transcript abundance of terpene biosynthesis genes in determining naturally occurring variation in foliar terpene concentrations. The results also suggest that IPP originating in the plastid contributes significantly to the pool of precursors used for sesquiterpene biosynthesis in the cytosol in *M. alternifolia*. These results open the way for future studies to identify markers that can be used to improve early selection of plants with an enhanced yield and thus greater profitability.

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S4. REPRODUCTION, GROWTH AND DEVELOPMENT

O21

A roadmap of apical bud formation in white spruce identifies potential regulators of time to bud set

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Background: Bud development is an adaptation that temperate forest trees have acquired to survive inclement winter conditions and resume growth the following spring. Bud development is a complex physiological and developmental process comprising bud formation, cold and desiccation tolerance development, and dormancy acquisition [1]. Because bud formation is accompanied by growth cessation, the timing of bud formation represents a trade-off between acquisition of winter hardiness and duration of active growth [2]. This is particularly true for determinate species such as white spruce (*Picea glauca* [Moench] Voss), where preformed stem units contained within the apical bud constitute most of next season's growth. Timing of bud set in white spruce shows genetic variation [3]. Our goal is to identify genes that exert genetic control over time to bud set in white spruce. As first steps towards that goal, we have conducted microarray gene expression profiling of shoot tips during the transition from active growth to dormancy in white spruce, and identified candidate regulators of bud formation by comparing these differentially expressed (DE) genes to results obtained from QTL analyses of time to bud set conducted using the same genes as molecular markers.

Materials and methods: Two year old white spruce nearing completion of active growth were placed in either short day (SD; 8 h days / 16 h nights, 20°C) or continued long day (LD; 16 h days / 8 h nights, 20°C) photoperiods, and samples harvested at 0, 1, 3, 7, 14, 28 and 70 d. After an additional 8-15 weeks in SD, plants were transferred to low temperatures (LT) under continued SD for 3-4 weeks prior to final harvest. Light microscopy, microarrays, SYBR Green qRT-PCR, and hormone analyses were conducted as described [4].

Results and discussion: Unlike indeterminate species such as *Populus* spp., white spruce does not require environmental cues such as reduced photoperiod or cooler temperatures to initiate bud formation, although these cues may be required to complete bud development and acquire endodormancy. However, SD hastens and synchronizes bud formation. To reduce biological variation, we conducted most analyses with seedlings under SD. Microarray analyses revealed 4460 DE genes in shoot tips during SD-induced bud formation. These analyses revealed the timing of molecular processes that occur during bud formation, identifying DE groups of genes associated with meristem transitions, identity and maintenance; organogenesis; protein synthesis and turnover; chromatin assembly and remodeling; cell wall biosynthesis and expansion; carbohydrate and lipid metabolism; secondary metabolism; and general stress responses. Changes in gene expression correlated with anatomical changes occurring at the shoot apex during the transition from active growth to endodormancy. Comparison of DE genes in developing buds under SD and LD uncovered ca. 2000 genes that are DE under both photoperiods at comparable phases of bud formation. This common set of genes is likely important for bud formation, particularly during early stages. Many genes DE shortly after transfer of plants to SD are likely photoperiod-responsive but not essential for bud formation. Genes DE only under SD at later stages of bud formation could be involved in processes associated with completion of bud formation and transition to endodormancy, events which do not appear to occur fully under LD. Further comparison of shoot tip, needle and stem transcript abundance profiles during this time course revealed approximately 100 genes that are DE only in developing buds and show greater transcript abundance in developing buds than other tissues. Several genes DE during bud formation – including some genes both preferentially expressed and only DE in buds – are putatively associated with hormone signalling. Quantification of ABA, cytokinins, auxin and their metabolites during SD-induced bud formation revealed distinct profiles for these hormones during bud formation, with shifts in hormone levels coinciding with morphological events such as bud scale development. A small number of genes DE in developing buds fall within QTL regions identified for time of bud set [5]. Some of these genes also show signatures of selection in natural populations of white spruce via identification of Fst outliers [6]. These candidate genes may exert genetic control over time to bud formation, and are currently being studied in more detail An eQTL experiment and association study are underway to identify additional candidate regulators of time to bud set.

Conclusions: This comprehensive study of gene expression changes that occur at the shoot apex during the transition from active growth to dormancy in white spruce has revealed timing of processes important for overwintering preparation, and groups of genes implicated in these processes. Use of these same genes as molecular markers in QTL analyses has enabled us to tentatively identify candidate genes that may exert genetic control over the time of bud formation in white spruce. These results provide new insight into an important adaptive trait, and lay the groundwork for more detailed studies into regulators of time of bud set in conifers.

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O22

Greenhouse and field evaluation of transgenic poplars with modified gibberellin metabolism and signaling genes

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Summary: Although there have been many observations of transgenes leading to improved plant growth in greenhouse experiments, there is a paucity of data that have been validated by multiple greenhouse experiments or field studies. Genes that encode regulators of gibberellin metabolic pathways were among the first reported to improve biomass growth in trees, and thus were the logical focus of this study. Poplars were transformed with eight different transgenic constructs designed to modify gibberellin biosynthesis and/or signaling. Dramatic improvements in growth rate were observed for several constructs under greenhouse conditions. However, these were often not seen in repeated greenhouse studies, and were statistically unconfirmed for most constructs in the field. Our results underline the importance of multiple greenhouse and field trials during studies with transgenes that modify hormonal homeostasis.

Genes studied: The genes under investigation in this study were *GA20-oxidase*, *PHOR1-1*, and *SPINDLY*. GA20-oxidase catalyzes the conversion of C_{20}-gibberellins to C_{19}-gibberellins through a series of sequential oxidation steps using 2-oxoglutarate as a co- substrate [1]. This enzyme was first isolated from pumpkin cotyledons [2], and is involved in the penultimate step in the production of bioactive gibberellins. The *PHOR1-1* gene is a positive regulator that was first isolated from leaves of potato plants subjected to a short day photoperiod (SD) [3]. Altered feedback regulation was observed in transgenic plants produced by antisense inhibition of this gene, indicating the role of the PHOR1-1 protein as an intermediate in the gibberellin signaling pathway. *SPINDLY* (*SPY*) is a gene that codes for an O-linked N-acetylglucosamine transferase [4]. In *Arabidopsis*, it has been shown to inhibit GA signaling, and thus is considered a negative regulator of GA signaling [5].

Methods: Combinations of the three different genes and seven different promoters were used (Table 1).

In addition to constitutive promoters, we used native promoters of poplar genes which were identified by screening whole-genome microarray expression data. Plants transformed with all eight constructs were grown in the greenhouse, while those transformed with six of these constructs were also grown in the field. For four constructs, the greenhouse trials were repeated at least once. In all, 2,066 trees were studied in the greenhouse (286 independent transformations) and 556 trees (94 independent transformations) were studied in the field. Transgenic and non-transgenic controls were included in both the greenhouse and field studies. Height and diameter measurements were recorded in both the greenhouse and the field, and shoot and root biomass were measured on the greenhouse-grown plants. Levels of bioactive gibberellins were also measured on a subset of plants transformed with the two cisgenes (a gene having driven by its own promoter and ending with its own terminator), as well as in plants that had the RGL and the GA2-oxidase promoters driving the *PtGA20-oxidase* gene.

Results and discussion: When considering the best growth rate improvements for constructs that had repeated greenhouse trials, the maximum growth rate improvement seen (the mean of the best two events relative to the control) varied from 50 to 220% i.e., a fractional growth rate improvement ranging from 0.5 to 2.2 (Figure 1).

However, results were not consistent between the different greenhouse trials. The best events showed more than 100% in maximum growth rate improvement (fractional growth rate improvement > 1) in six different greenhouse experiments (Fig. 1); however, these events did not have a similar extent of improvement when the experiments were repeated. Five out of eight constructs produced a statistically significant improvement in growth rate (Figure 1).

Only one out of the six constructs tested in the field produced a statistically significant improvement in volume index, and this was for the RGL promoter driving the *PtGA20-oxidase* gene. The GA20-oxidase and the PHOR1 cisgenic plants, when grown in the greenhouse, had substantial increases (73-133%) in the levels of bioactive gibberellins in developing shoots compared to control plants, however, there was no significant correlation between gibberellin levels and volume index of each event. The levels of the gibberellin biosynthetic precursors were also measured and found to be greater than the bioactive gibberellin level, suggesting feedback inhibition—a possible mechanism for the lack of association with growth. Our results suggest that physiology-modifying transgenes strongly interact with growth environments. This implies that, as in conventional plant breeding, greenhouse results are of limited value and field trials are essential for evaluation of varieties with improved productivity.

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Table 1(abstract O22) Summary of constructs used in the transformation experiments

Promoter	Gene	Terminator	Number of events studied (GH/Field)	Total number of trees (GH/Field)
PtGA20ox7	*PtGA20ox7*	PtGA20ox7	38/35	345/278
PtPHOR1	*PtPHOR1*	PtPHOR1	26/3	327/24
RGL1-1	*PtGA20ox2-2*	NOS	34/17	444/83
GA2ox1	*PtGA20ox2-2*	NOS	30/15	425/75
B-expansin	*PtGA20ox2-2*	NOS	24/0	120/0
PtCESA1	*PtGA20ox2-2*	NOS	16/0	80/0
35S	*AtSPY*	OCS	33/12	163/48
35S	*HvSPY*	OCS	33/12	162/48

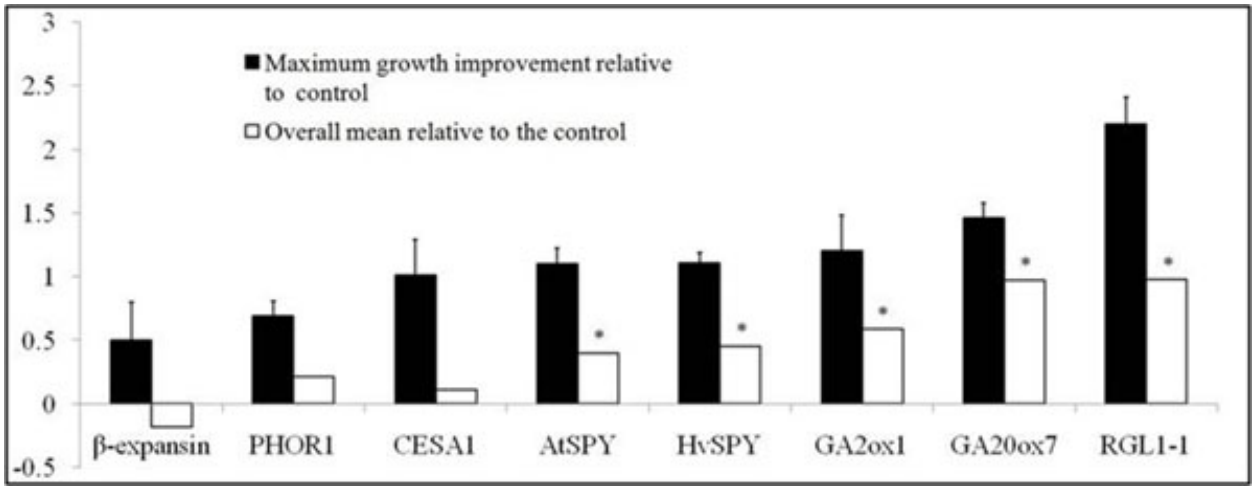

Figure 1(abstract O22) Results from greenhouse trials of transgenic plants. The constructs are denoted by the promoter used, except in the case of *SPY* genes. Dark bars show the maximum fractional rate improvement (best two transgenic events from best greenhouse experiment) compared to controls. Open bars show the mean fractional growth rate improvement for all transgenic events in that greenhose experiment. Brackets denote one standard error. A "*" above an open bar indicates statistically significant difference between the transgenic and control groups.

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O23

Enhanced cytokinin signaling stimulates cell proliferation in cambium of Populus

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Introduction: Understanding the regulation of radial growth that underlies wood development is of great importance for future use of tree products as a renewable resource. For this purpose, we have performed a detailed analysis of cytokinin function in the regulation of cambial development in a tree stem. Furthermore, we have identified potential cytokinin signaling response genes active during the secondary development in Arabidopsis and currently studying the best candidate genes also in Populus.
Results and discussion: In the cambium, expression of cytokinin receptor genes peaks in the actively dividing cells. This indicates that cytokinin signaling pathway is a major regulator of cambial cell proliferation. We have previously shown in transgenic Populus trees that we can slow down the cambial activity by reducing the level of cytokinin signaling in it [1]. To study if cytokinin signaling is truly a rate-limiting process for cambial activity, we have now engineered transgenic Populus trees with an enhanced cambial cytokinin signaling level. In these trees, we were able to see that elevated cytokinin signaling stimulated the cambial cell division activity and led to increased biomass production. Furthermore, based on microarray profiling in Arabidopsis, we have been able to identify several transcription factors functioning downstream of cytokinin signaling during the secondary development. These transcriptional regulators have stimulatory effect on radial growth in Arabidopsis root. We are currently studying if the Populus orthologs of these genes have similar function in the cambium of the tree stem.
Conclusions: We have shown that the cambial cell division activity, and through it the production of stem biomass, can be enhanced or decreased through elevated or decreased cytokinin signaling. Together, our results show that cytokinins are major rate-limiting hormonal regulators of cambial development. Our study provides potential for applied research towards future breeding of faster growing tree cultivars.
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O24

WAT1 (WALLS ARE THIN1) defines a novel auxin transporter in plants and integrates auxin signaling in secondary wall formation in Arabidopsis fibers

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Background: Our knowledge of signaling mechanisms involved in secondary cell wall (SCW) formation is quite limited. To discover novel markers of SCW, a genomics approach using *Zinnia elegans* xylogenic cultures was undertaken that identified hundreds of gene candidates expressed at the onset of secondary wall formation [1]. *Arabidopsis* homologs and the corresponding T-DNA mutants for each *Zinnia* gene were identified and the panel of *Arabidopsis* cell wall mutants was subjected to developmental and wall-related phenotyping.
Results and conclusion: Among the most interesting mutants was *wat1* (*walls are thin1*). The most conspicuous phenotypic feature of *wat1* was the severe reduction (sometimes to the extent of being inexistent) of SCW in xylary and interfascular stem fibers. Interestingly, xylem vessel wall thickness and morphology were not modified by the mutation.

In addition to the SCW phenotype, *wat1* was characterized by 5-Me-tryptophan seedling toxicity, severely decreased auxin transport and content in stems, and massive down-regulation of auxin-related genes. These data led us to the conclusion that WAT1 acts as an upstream regulator of SCW deposition in fibers, presumably through an auxin-mediated mechanism [2].

Bioinformatic analysis of WAT1, annotated as 'homolog to a *Medicago truncatula* nodulin gene, *MtNOD21*, suggested that WAT1 encoded a putative transporter belonging to the Plant Metabolite Exporter family [3]. WAT1:GFP fusion protein experiments localized WAT1 on the tonoplast, confirming the prediction that WAT1 is a membrane protein. Although WAT1 is plant-specific, it shares structural similarities with bacterial amino acid transporters in that it consists of ten transmembrane domains encompassed within a tandem Domain of Unknown Function 6 (DUF6).

To characterize WAT1 function, we recently tested its capacity to transport tryptophan and/or auxin in both yeast and *Xenopus*oocytes. Neither WAT1-expressing yeast cells nor *Xenopus* oocytes were able to facilitate radiolabeled Trp import or export. However, we have been able to demonstrate that WAT1 facilitates auxin import in both expression systems. These results clearly place WAT1 among the ranks, along with PINs, AUX/LAXs andABCB/MDR/PGPs, as a novel, bona fide auxin transporter in plants.

This study constitutes the first functional characterization of any of the 46 members of the WAT1 gene family in *Arabidopsis* and our hope is that this discovery will help pave the way in identifying the functions of other family members. Moreover, the *wat1* mutant will be an ideal tool to address the question as to how auxin subcellular homeostasis plays a role in fiber SCW formation in Arabidopsis. Our current efforts to understand poplar WAT1-mediated auxin signaling in wood formation in trees will also be discussed.

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O25

Molecular dissection of an adaptive epigenetic memory mechanism in norway spruce

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In Norway spruce, environmental conditions during the reproduction can greatly influence progeny performance. We found that the temperature during post meiotic megagametogenesis (zygotic embryogenesis) and seed maturation shift the growth cycle program of the embryos in the seeds, resulting in significant and long lasting phenotypic changes in the progeny. Traits that are affected include the timing of dehardening and bud burst in the spring; leader shoot growth cessation in the summer, and bud set and cold acclimation in the autumn. All processes are advanced or delayed in correspondence with the temperature during female reproduction. Colder reproductive environment advance bud set and cold acclimation during autumn and dehardening and bud burst during spring in their progenies. Temperature dependent difference in timing of terminal bud formation in identical clones was equivalent to a 4–6° latitudinal ecotypic difference. The progeny "remember" the temperatures and photoperiod prevailing during zygotic embryogenesis and seed maturation and this memory, affecting the climatic adaptation in this species, is an epigenetic phenomenon.

This phenomenon is not only of evolutionary significance but has clear practical implications. This memory can help the conifer to cope with the anticipated rapid change in climatic conditions. It will have importance for the deployment of seedlings produced in seed orchards containing clones that are translocated to warmer sites, and it may be used to produce seedlings that have specific adaptive properties. So, it is possible to produce distinct phenotypes (epitypes) in Norway spruce, however this type of long lasting effects is not well documented in other organisms so far.

The molecular mechanism behind this striking epigenetic memory phenomenon is not yet unraveled but transcriptional changes are clearly involved. In epigenetically different progenies, transcriptional analysis revealed that seedlings from full-sib families produced at different embryogenesis temperature under long and short day conditions differed. Suppressive subtracted cDNA libraries revealed significant differences in their transcriptomes. Using qRT-PCR, microRNA pathways genes *PaDCL1* and *2* and *PaSGS3* as well as transposons related genes are differential expressed in the epigenetically different progenies with phenotypic differences in bud burst and bud set.

MicroRNAs (miRNAs) are endogenous small RNAs that can exert epigenetic gene regulatory impacts. We have examined the possible role of miRNA in the epigenetic phenomena, and found that Norway spruce contains a set of conserved miRNAs as well as a large proportion of novel non-conserved miRNAs. From concatemerized small RNA libraries from seedlings from the same parents, originated from seeds developed in a cold and warm environment from a family with distinct epigenetic effects, contrasted to one from a family with little response, miRNAs potentially involved in this epigenetic memory was identified. Most of the miRNAs target unknown genes or genes with no known function. The expression of seven conserved and nine novel miRNAs showed significant differences in transcript levels in progenies with distinct epigenetic difference in bud set, but not in the progenies from a non-responding family, making them excellent candidate miRNAs. The differentially expression of specific miRNAs in genetically identical but epigenetically different progeny indicate their putative participation in the epigenetic regulation.

Epigenetic mechanisms influence phenotype through altered regulation of gene expression that is mitotically (and sometimes meiotically) propagated. Understanding the mechanisms involved in the initiation, maintenance, and heritability of epigenetic states is an exiting aspect of research in current biology. Epigenetic regulation may be realized through several interconnected molecular pathways including DNA methylation, histone modification and chromatin remodeling, small non-coding RNAs and transposable element regulation.

Among spruce ESTs we found 64 homologs of genes described as involved in DNA methylation, histone modification and chromatin remodeling and small RNA biogenesis in other plant species. In general, known epigenetic mechanism related genes are very well represented in the spruce genome. We analyzed the transcription patterns of these genes using RT-PCR in epigenetically different zygotic embryogenic samples on different stages of development and in seedlings, originated from full-sib families clearly differed in epigenetic response. The largest difference in gene expression of selected genes was found at the earlier stages of embryogenesis while in seedlings a low number of these genes were differentially expressed. Most of the known epigenetic mechanism related genes in seedlings were steadily expressed in all studied samples independently of their epitype.

To get a deeper analysis of epigenetic related transcriptome we used high-throughput sequencing (RNA-seq and miRNA-seq) in cooperation with GenXPro GmbH. Using MACE (massive cDNA 3'end sequencing) deep mRNA sequencing on the Illumina GSII platform, we analyzed the genes differentially expressed in *P. abies* during early stages of embryonic development. We selected genes which could be involved into epigenetic response by comparison warm and cold originated "embryonic epitypes" from the same full-sibs family somatic embryos developed in cold (18°C) and warm (30°C) environmental conditions. Additionally, for more distinct analysing of the large amount of "no database hit" reads we sequenced one normalised library using 454 Titanium GS FLX sequencing to get reference transcript set of expressed genes. The sequencing data is currently under processing and we are going to discuss main results here.

To proceed with our initial study of miRNAs in spruce, we used Illumina/SOLEXA sequencing to identify small RNAs expressed at the same epigenetic responsive family developed in warm and cold environment

progenies following short-day treatment. The identification of novel miRNA candidates are in progress and the confirmation of conserved and novel miRNA by qRT-PCR analysis will be presented.

O26

Dual function of auxin during leaf abscission in poplar

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Background: Leaf abscission is an important trait for biomass production and seasonal acclimation in deciduous trees of the temperate region. Various plant hormones are involved in the timing of abscission. For example, ethylene signaling is required to induce hydrolysis of cell walls, while an auxin gradient [1] was suggested to act upstream of ethylene on the onset of leaf abscission. Besides pharmacological application of auxins on cut surfaces of explants, experimental evidence for such a gradient is however lacking. In addition to its function in temporal control, auxin has also been suggested to be a positional signal specifying the cells of the abscission zone [2].
Methods: We established an experimental system on intact Populus trees, which allows us to induce abscission synchronously under controlled conditions. Leaf blades were bagged in aluminum foil and abscission was recorded daily. Cumulative abscission followed a sigmoidal curve for dark-induced leaves, whereas control leaves in transparent bags of the same weight as the aluminum foil bags were not separated from the stem. Abscission was preceded by senescence in the petiole but not in the leaf.
Results and conclusions: Local auxin applications directly onto the abscission zone, as well as onto the distal end of the petiole, delayed dark induced abscission indicating that auxin could range not only as a short but also as a long distance signal. Similarly, an inhibitor of polar auxin transport retarded separation from the plant body. By contrast, auxin applied onto mature abscission zones only delayed abscission slightly in comparison to auxin applications before the development of an abscission zone. Taken together this points to a distinct function of auxin in early stages of abscission. Interestingly, we found shortly after dark-induction a new auxin response maximum on the abaxial side of the petiole, highlighting the incipient abscission zone. This auxin response maximum progressively moved from the abaxial to the adaxial side of the petiole, preceding the maturation of the abscission zone, presumably providing positional information for the formation of the abscission zone. Microarray data identified the auxin efflux carriers *PIN1* and *PIN5*, as well as a novel auxin transporter, to be down-regulated after dark induction. Immunolocalizations of those carriers will reveal if their subcellular localization and expression can explain the novel auxin response maximum.

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S5. BIOTIC AND ABIOTIC INTERACTIONS

O27

Biomarkers and gene copy number variation for terpenoid traits associated with insect resistance in Sitka spruce: An integrated genomic, proteomic, and biochemical analysis of (+)-3-carene biosynthesis

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Conifers have evolved complex chemical defenses in the form of oleoresin terpenoids to resist attack from pathogens and herbivores. The large diversity of terpenoid metabolites is determined by the size and composition of the terpene synthase (TPS) gene family, and the single- and multi-product profiles of these enzymes. The monoterpene (+)-3-carene is associated with resistance of Sitka spruce (*Picea sitchensis*) to white pine weevil (*Pissodes strobi*). We used a combined genomic, proteomic and biochemical approach to analyze the (+)-3-carene phenotype in two contrasting Sitka spruce genotypes. Resistant trees produced significantly higher levels of (+)-3-carene than susceptible trees, in which only trace amounts were detected. Biosynthesis of (+)-3-carene is controlled, at the genome level, by a small family of closely related (82-95% amino acid sequence identity) (+)-3-carene synthase (*PsTPS-3car*) genes. Transcript profiling identified one *PsTPS-3car* gene (*PsTPS-3car1*) which is expressed in both genotypes, one gene (*PsTPS-3car2*) expressed only in resistant trees, and one gene (*PsTPS-3car3*) expressed only in susceptible trees. The *PsTPS-3car2* gene was not detected in genomic DNA of susceptible trees. Target-specific selected reaction monitoring substantiated this pattern of differential expression of members of the PsTPS-3car family on the proteome level. Kinetic characterization of the recombinant PsTPS-3car enzymes identified differences in the activities of PsTPS-3car2 and PsTPS-3car3as a factor for the different (+)-3-carene profiles of resistant and susceptible trees. In conclusion, variation of the (+)-3-carene phenotype is controlled by *PsTPS-3car* gene copy number variation, variation of gene and protein expression, and variation of catalytic efficiencies.

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O28

Integrative approach involving RNA-Seq, foliar traits and growth measurements revealed genotype-specific plasticity on *Eucalyptus* subjected to seasonal water shortage

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Background: In the context of climate change, water availability will become an important limiting factor threatening biomass production, particularly in intensively managed forest tree plantations. The complexity of drought stress response calls for integrative approaches combining physiological, anatomical and molecular investigations at different scales ranging from particular cells to the whole plant. Such a strategy was developed for two eucalyptus clones used in industrial plantations in the Republic of Congo and known for their differences in term of productivity and water-use efficiency.
Methods: The two genetic units used in this study consisted in one more productive hybrid of *E. urophylla* * *E. grandis* (18-50), and one less productive hybrid obtained from open-pollination of E. *alba* (1-41). These genotypes were subjected to two watering regimes (irrigated during two dry seasons, versus non-irrigated) in open field conditions. Growth parameters such height, diameter at breast height, stem, leaf and root biomasses as well as physiological parameters, such carbon discriminationand specific leaf area, were regularly measured over two growing seasons. At the end of the second dry season (i.e. 14 months-old trees), polyphenols accumulation and anatomical measurements were performed on mature leaves. RNA of shoot apices from trees representing the four conditions (i.e. 2 genotypes subjected to 2 watering treatments) were extracted and used to create non-normalized libraries. Using 454/Roche sequencing, we performed digital expression analysis to highlight differentially expressed transcripts showing genotype, treatments and/or genotype by treatment interaction effects. Expression of candidates genes revealed by this first analysis were assessed by RT-qPCR on shoot apices and on mature leaves.
Results and discussion: Growth parameters and stem biomass analysis revealed that the two studied genotypes presented similar degrees of phenotypic plasticity in response to watering treatment during the

dry season. Nevertheless, comparison of growth increments during wet seasons suggested that the more productive genotype had higher ability to recover from water shortage during the dry seasons and to use favorable conditions to increase its above-ground biomass. We found that the more productive genotype displayed greater plasticity of foliar traits in the non-irrigated treatment during the dry season, suggesting that it could adjust more quickly its leaf parameters when rainfalls resume.

On mature leaves from trees subjected to water deficit, we identified mechanisms established to limit water losses by cuticle reinforcement and stomatal density decrease, to preserve leaf turgor by increase of collenchyma layer, to enhance hydraulic conductance with xylem vessel diameter decrease, and to preserve cells from reactive oxygen species through accumulation of hydroxycinnamates. Those adjustments were more intense for the more productive genotype, suggesting that this genotype may entailed higher cost strategies to preserve its leaf functions.

On shoot apices, 454-sequencing provided a catalogue of 129,993 unigenes (49,748 contigs and 80,245 singletons) from the initial sequencing of 398Mb corresponding to 1.14Mreads. Digital expression analysis identified 1,280 contigs displaying differential expression between the two genotypes, 155 contigs between treatments, and 274 contigs displaying genotype x treatment interaction effects.The more productive genotype mainly under-expressed genes related to photosynthetic activity during the dry season, activating genes to reallocate resources through major changes in primary metabolism, whereas the less productive genotype displayed lower levels of variation in gene abundance between treatments. This analysis allowed to reveal a set of candidate genes that could be related to phenotypic variation observed in mature leaves.

Conclusions: The productivity of the two genotypes used in this study differed essentially in terms of ability to recover from water shortage. These differences could be explained by different strategies of investment in preservation of leaves. In the environmental gradient tested, it seemed that higher investments enabled genotype 18-50 to increase its leaf resistance, and subsequently to recover quickly high growth performance once environmental conditions become favorable. Genes that potentially underlie such physiological plasticity were revealed through integrative approaches. More investigations are now needed to valid their putative roles in phenotypic plasticity in response to water deficit. Forward genetic approaches could be used to screen the variability of gene expression and other more-integrated phenotypic traits, the nucleotide diversity of these candidate genes and their covariations on a larger genetic background.

O29

Influence of water deficit on the induced and constitutive responses of pines to infection by mountain pine beetle fungal associates

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Background: The ongoing outbreak of mountain pine beetle (MPB; *Dendroctonus ponderasae* Hopkins) and its associated pathogenic fungi (e.g. *Grosmannia clavigera* [Robinson-Jeffrey and Davidson] Zipfel, de Beer and Wingfield) in western North America has resulted in the loss of more than 13 million hectares of pines since 1999 in British Columbia alone [1]. MPB has principally attacked lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia*) in British Columbia. However, since 2006 MPB has spread into northern Alberta, where lodgepole pine hybridizes with jack pine (*Pinus banksiana* Lamb.) [2]. Few studies have compared lodgepole and jack pine defense responses, but given that lodgepole pine and MPB share a co-evolutionary history [1] whereas jack pine is a new host for MPB [2], it is reasonable to expect that differences might exist. Some regions affected by the current outbreak have experienced drought conditions during the last decade. Water deficit can limit carbon assimilation, potentially increasing tree susceptibility to MPB and their symbiotic fungi [3]. We are testing the hypotheses that lodgepole and jack pine defenses against MPB and *G. clavigera* differ, and that water deficit affects these responses.

Materials and methods: The relationship between water availability and tree defense was evaluated in (1) two year old lodgepole and jack pine seedlings in growth rooms, and (2) ca. sixty year old lodgepole x jack pine hybrids in naturally regenerated, thinned stands. Soil relative water content was monitored using time-domain reflectometry. Seedlings were subjected to watering or water deficit for one week prior to wounding or wounding plus *G. clavigera* inoculation. Mature trees were either watered or water limited via tarps for six weeks before wounding plus *G. clavigera* inoculation. In both experiments, water deficit conditions were maintained throughout the time course.

Tree physiological status was evaluated by measuring gas exchange and stomatal conductance using a LiCor 6400, stem hydraulic conductivity using a low-pressure flow meter and safranin dye xylem perfusion, and HPLC. Defense responses were assessed by lesion measurements histochemistry, and qRT-PCR.

Results and discussion: Stomatal conductance and photosynthesis significantly decreased under water deficit for both lodgepole and jack pine seedlings, but seedling hydraulic conductivity was not affected. The mild water deficit applied to the mature trees reduced stomatal conductance and photosynthesis, but not significantly.

Stem lesions are a means of killing and compartmentalizing invading organisms [4]. *G. clavigera*-induced lesions developed more slowly in jack pine than lodgepole pine seedlings. Stem hydraulic conductivity decreased in inoculated lodgepole but not jack pine seedlings, likely because of greater tracheid occlusion caused by increased fungal growth and/or resin production in lodgepole pine [5]. Water deficit reduced lesion development rates at early time points in inoculated lodgepole and jack pine seedlings, as well as in mature trees at 5 weeks post-inoculation. Lesion length has been considered an indicator of tree defense capacity [6], with longer lesions reported to reflect increased release of toxic and/or inhibitory substances [7]. Accordingly, we interpret slower lesion development to indicate a slower defense response. Our results suggest (1) more rapid defense responses to *G. clavigera* in the co-evolved lodgepole pine host than in the new jack pine host, and (2) defense responses are slowed by water limitation.

We then examined the effect of water deficit on transcript abundance corresponding to genes classically associated with drought and defense responses. We conducted qRT-PCR transcript abundance profiling of secondary phloem from mature lodgepole x jack pine hybrids. We first profiled four *aquaporin* and five *DREB* genes, families associated with water stress responses. Although the mild water deficit did not significantly alter expression of these genes, expression of one *aquaporin* and one *DREB* decreased in response to *G. clavigera* inoculation. We then profiled five *chitinase* and four *terpene synthase* defense-associated genes. Expression of two *chitinases* was significantly induced by water deficit but not *G. clavigera*. Expression of other *chitinases* significantly increased in response to fungalinoculation, but the response was attenuated by water deficit. Expression of one *terpene synthase* significantly increased with fungal inoculation, but this response was also attenuated under water deficit. In contrast, water deficit increased constitutive expression of another *terpene synthase*. Higher constitutive expression of the *monoterpene synthase* under mild water stress suggests a pre-emptive defense via higher biosynthesis of volatile monoterpenes. Microarray and qRT-PCR analyses of the lodgepole and jack pine seedling experiment are underway.

Conclusion: Our analyses suggest that defense responses of jack pine differ from those of lodgepole pine. Molecular analyses are underway to further characterize these differences. Both constitutive and induced defense responses are modulated in pines by water deficit, and this response appears to be gene-specific. This study shows evidence of cross talk between the water stress and defense responses of pine trees.

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O30

Heterobasidion spp, triggers non-specific defence responses in bark of Norway spruce

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Norway spruce [*Picea abies* (L.) Karst.] is one of the economically most important conifer species in Europe. The major pathogen on Norway spruce is *Heterobasidion parviporum* (Fr.) Niemelä & Korhonen. The completed genome sequence of *H. irregulare* opens up new possibilities to understand the interactions between *Heterobasidion* spp. and spruce. However to date there are no completed genome sequencing projects of conifer genomes available for complementing studies. To achieve a better understanding of induced transcriptional defence responses in Norway spruce upon *Heterobasidion* spp. attack, we compared transcriptional responses in bark to *H. parviporum* infection to the response to wounding using cDNA-AFLP and transcriptome sequencing.

In an initial study bark samples were harvested at 3, 7 and 14 days post inoculation (dpi) and untreated bark was used as negative control. About 2500 transcribed derived fragments (TDFs) generated by cDNA-AFLP were screened. 199 TDFs were investigated further based on band intensity in the inoculated bark in relation to either untreated bark or wounded bark. Out of these, 119 TDFs had a putative homology and a consistent band intensity pattern between replications. A majority of these TDFs showed homology to genes known to associate with defence e.g. *3-deoxy-d-arabino-heptulosonate 7-phosphate synthetase* (*DAHP*), *Pathogenesis-related protein 1* (*PR1*), *Lipoxygenase* (*LOX*), *ACC-synthase* (*ACS*), *ACC-oxidase* (*ACO*) and *Jasmonate ZIM-domain 1* (*JAZ1*). Many of these are found in Salicylic acid- or Jasmonic acid/ethylene-signalling pathways. The majority of the TDFs showed a similar expression pattern for all treatments but samples inoculated with *H. parviporum* generally showed an enhanced reaction (induction/repression) compared to wounding alone. Expression patterns were confirmed by qPCR in material treated with wounding and inoculation with *H. parviporum* or *Phlebiopsis gigantea*. Our data suggest that infection with *H. parviporum* in Norway spruce induces a broad defence, with many similarities to non-specific defence responses in angiosperms. Additionally signs of reallocation of carbon from primary to secondary metabolism were evident.

With this information at hand we analysed four Norway spruce genotypes with either high or low susceptibility to *Heterobasidion* spp. [1] sampled 0, 5, 15 and 28 dpi with *H. annosum*. The bark phenol-composition was profiled in each sample. The 500,000 454-reads were assembled into 17,228 contigs that assembled in 14,364 putative transcript units (PTU) using the sequence assembler software Newbler™ (http://www.454.com). The assembled reference file was annotated with the software Blast2Go and the PTUs were submitted to differential expression analysis. Data on associations between gene expression levels and phenol composition in bark upon *H. annosum* inoculation and level of susceptibility will be presented.

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O31

Transcriptome sequencing of *Eucalyptus camaldulensis* seedlings subjected to water stress

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Water stress limits plant survival and production in many parts of the world. Identification of genes responding to water stress conditions will underpin efforts to breed plants better adapted to drought. We studied the effect of water stress on *Eucalyptus camaldulensis* seedlings derived from three natural populations. Physiological and growth traits were measured and gene and allelic expression in leaves was examined by RNA sequencing (RNA-seq). Water stress had a significant impact on all the physiological and growth traits, while differences between the populations were not significant. Genes differentially expressed in leaves were identified by *de novo* assembly and by *ab initio* transcriptome mapping using the *Eucalyptus grandis* reference genome sequence. Gene ontology (GO) enrichment tests with 2,500 significantly differentiated genes revealed 128 stress-related gene categories were up-regulated while 28 gene categories belonging to photosynthesis and other metabolic processes were down-regulated under stress treatment. More than 190,000 single nucleotide polymorphisms (SNPs) and small indels were detected and 4,053 of these revealed differential allelic expression between control and drought stressed seedlings. Allelic expression of 70% of these variants was correlated with total gene expression. These variants may be *cis*-acting variants or in high linkage disequilibrium with such variants. The SNPs and indels identified in this study form a useful resource for further testing in association studies.

O32

The relationship between intra-specific variation in the *Populus* transcriptome, stomatal development, and the metabolome in response to drought

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Drought is one of the most significant factors limiting tree growth. Trees in the genus *Populus* are particularly noted for their drought sensitivity; therefore, understanding the mechanisms by which these economically and ecologically important forest trees respond to drought is of paramount importance. The ability of *Populus* trees to contend with drought is dependent on the responsiveness of the genome, and in turn, the ability of the transcriptome to appropriately remodel growth, metabolism and development. Amassing evidence indicates that different species of *Populus* have divergent mechanisms and adaptations to contend with drought stress; however, individuals within a given species also display divergent drought responses. In order to investigate the intra-specific variation underpinning the drought response, we examined six genotypes of *P. balsamifera*. Using Affymetrix Poplar GeneChips, we found a positive correlation between the magnitude of drought-induced changes in the transcriptome and the capacity of the genotype to maintain growth. Surprisingly identifiable differences at the transcriptome were observed, and similar responses were observed within the metabolome. Although common drought responses could be identified within the species, the complexities of these responses must be taken into consideration when defining species- or genus-level drought responses.

O33

New insights in the molecular events underlying actinorhizal nodulation in the tropical tree *Casuarina glauca*

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Background: Actinorhizal root nodules result from the interaction between a nitrogen-fixing actinomycete called *Frankia* and roots of dicotyledonous trees and shrubs belonging to 8 plant families and 25 genera. Most actinorhizal plants are capable of high rates of nitrogen fixation comparable to those found in legumes. As a consequence, these trees are able to grow in poor and disturbed soils and are important elements in plant communities worldwide. This facility for adaptation has drawn great interest to actinorhizal plants, particularly to several species of *Casuarinaceae* such as *Casuarina glauca*, which can be used for fuelwood production, agroforestry, and land reclamation in the tropics and subtropics.

The basic knowledge of the symbiotic association between *Frankia* and actinorhizal plants is still poorly understood, although it offers striking differences with the *Rhizobium*-legume symbiosis [1]. Recently, the development of genomics in some actinorhizal plants such as *C. glauca* [2], together with the possibility to obtain transgenic actinorhizal plants following *Agrobacterium* gene transfer [3], offer new approaches to understand the molecular basis of the actinorhizal process. We will highlight recent progress in the molecular knowledge of the early stages of the actinorhizal symbiosis. A comparative analysis of the symbiotic pathway in actinorhizal trees and legumes will be presented.

Methods: *Plant material. Casuarina glauca*Sieb. Ex Spreng seeds were obtained from B&T World Seeds (Aigues-Vives, France) and germinated in sterile conditions as described [4].

Genetic transformation of Casuarina glauca. Composite plants of *C. glauca*were obtained following the genetic transformation by *Agrobacterium rhizogenes* A4RS containing the appropriate binary vector [5]. For promoter analyses, binary vectors were derived from pBIN19 and for RNAi experiments, hairpin constructs were cloned into the pHKN29 vector using standard molecular methods.

Nodulation by Frankia. Composite and control plants were nodulated with a suspension of *Frankia*CcI3 [4].

Results and discussion: CgSymRK is involved in the actinorhizal signal transduction pathway: The development of genetic and genomic tools for the model legumes *M. truncatula*a nd *Lotus japonicus* has greatly facilitated the cloning of genes required for root symbiosis [6]. Some of these genes were found to be involved in the establishment of both rhizobia and mycorrhiza symbioses, and designated as common SYM genes constituting the common SYM pathway. They include genes encoding a leucine-rich-repeat (LRR) receptor kinase (SymRK), cation channels, nuclear pore complex proteins, a calcium and calmodulin-dependent protein kinase (CCaMK) and a nuclear coiled protein. The question was raised whether some of these symbiotic genes were shared in the signal transduction pathway in response to *Frankia* factors and rhizobial Nod factors.

To answer this question, a functional study of *CgSymRK*, a gene isolated from *C. glauca*, homologous to the receptor-like kinase gene *SymRK* required for nodulation and mycorrhization in legumes, was undertaken. Downregulation of *CgSymRK* resulting from a RNA interference approach revealed that the frequency of nodulated RNAi-*CgSymRK* plants was reduced 2-fold compared to control *C. glauca* plants [7]. In addition, a range of morphological alterations was observed in the down-regulated CgSymRK-nodules. Additional experiments revealed that CgSymRK was also necessary for the establishment of the symbiosis with the arbuscular mycorrhiza *Glomus intraradices*. Therefore, the function of SymRK is conserved between legumes and actinorhizal plants.

Search for other candidates of the signalling pathway: Our group has developed the first genomic platform to identify plant genes involved in the symbiotic process between *Frankia* and *C. glauca* [2]. Based on the comparison with legumes sequences, ESTs sharing significant homologies with genes from the Nod signalling pathway were identified. Functional analyses of the candidate orthologues to the *CCaMK* and *NIN* genes are in progress. CCaMK is a calcium and calmodulin-dependent protein kinase that is presumed to decode and transduce Nod-factor specific calcium spiking response in legumes. Preliminary experiments suggest that CgCCaMK is necessary for both nodulation and endomycorrhization in *C. glauca*, thereby suggesting a conservation of the whole common SYM pathway in the actinorhizal plants.

Conclusion: Although several actinorhizal genera contain species that are economically important in forestry and land regeneration, progress has been slower in our knowledge of the actinorhizal symbiosis than that of the legume rhizobium interaction. The availability of a method for the genetic transformation of Casuarinas has resulted in a major breakthrough since it opened the way to functional gene analysis in the actinorhizal host plant. Deep sequencing analyses are now in progress in *C. glauca* and mining these data will undoubtedly to contribute to dissecting the molecular dialogue between *Frankia* and the host plant in the different stages of development of the actinorhizal nodule.

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O34

Potential use of the *Eucalyptus globulus* vacuolar pyrophosphatase 1 (EgEVP1) to generate drought and salt resistant plants

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Desertification is a phenomenon that affects vast territories worldwide usually caused by high soil salt concentrations, being salt and water stress one of the main problems that affect plant growth. Part of the strategy developed by the plant to face these stresses consists on the compartmentalization of cytoplasmic Na^+ within the vacuole, by a coordinated expression of a H^+ pump that uses pyrophosphate as a source of energy (known as vacuolar pyrophosphatase) and a Na^+/H^+ exchanger, both located on the vacuole membrane. The vacuolar pyrophosphatase has been described in various plant species, much of which are of commercial importance, such as rice, wheat and corn, along some woody species such as poplar and apple. In our laboratory we isolated the cDNA of the *Eucalyptus globulus* vacuolar pyrophosphatase (EVP1), establishing by real-time PCR that its transcript expression increases when *Eucalyptus globulus* plants are subjected to water and salt stress. An analysis of its genomic sequence allowed us to identify four introns and a promoter region of approximately 2Kb. The fusion of

the GUS reporter gene to the promoter region as well as to the first EVP1 intron allowed us to establish for the latter a possible regulatory function, in addition to identifying the promoter tissue-specific expression in *A.thaliana* plants. Finally, *A. thaliana* transgenic plants generated by overexpressing EVP1 produced seeds that exhibited a higher germination rate in high salt conditions compared to the wild-type plants. Furthermore, adult transgenic plants had more leaves in the rosette and a higher tolerance to salt and water stress.

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S6. WOOD FORMATION

O35

Overexpression of walldof transcription factor increases secondary wall deposition and alters carbon partitioning in poplar

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Background: Of the total worldwide biomass produced by land plants, 70% is represented by cell walls [1]. In angiosperm trees, the fixed carbon is accumulated mainly in the secondary walls of xylem cells, which turns xylem cell wall formation a major carbon sink in trees[2]. The understanding of mechanisms that regulate carbon flux to the synthesis of cell wall components is a fundamental goal to maximize the amount of lignocellulosic biomass.

Several families of transcription factors have been shown to be important regulators of secondary wall biosynthesis, but how plants control and direct carbon partitioning among wall components still remains unclear [3,4].

In an attempt to identify transcription factors that participate in the regulation of carbon flux into secondary wall formation, a systematic search for poplar transcription factors expressed in a wood-preferred manner was performed. We have used the collection of over 400,000 ESTs from *Populus sp*. available at GenBank to determine the tissue-specific pattern of genes encoding transcription factors. Among these, a DOF (DNA-binding with one finger) domain transcription factor family member (which we named *WALLDOF*) that presents a cambium-preferred expression profiling was selected for further characterization.

DOF proteins are plant-specific transcription factors suggested to participate in the regulation of biological processes such as light-regulated gene expression, photosynthetic carbon assimilation, accumulation of seed-storage proteins, germination, response to phytohormones, guard cell-specific gene expression, flavonoid metabolism, and lipid biosynthesis [5,6].

In this work, we report that overexpression of *WALLDOF* in transgenic poplar is capable of increasing secondary wall deposition and altering carbon partitioning in poplar stems.

Methods: The collection of over 400,000 ESTs from *Populus sp*. available in GenBank was searched for the tissue-specific pattern of expressed genes. For this purpose, ESTs from 100 cDNA libraries were grouped into 15 representative tissues/organs including apical shoot, bark, cambial zone, catkins, cultured cells, flower buds, leaves, mixed tissues, petioles, roots, seeds, shoot meristem, shoots, vegetative buds and xylem (including xylem and wood libraries). A set of clusters generated by CAP3 was searched for those composed of at least 60% of EST reads from libraries representing poplar cambial zone and stem tissues.

One cluster representing a DOF transcription factor family member with a cambium-preferred expression profiling was identified. Using a cDNA synthesized from total RNA from stems of *Populus deltoides* plants, the full-size ORF of the *WALLDOF* gene was cloned under the control of a 1.0 kb fragment of a *Populus deltoides* cinnamate 4-hydroxylase (C4H) promoter. Wild-type aspen hybrid (*Populus tremula x Populus alba*) was transformed with *Agrobacterium tumefaciens* carrying the construct.

Results and conclusions: We obtained 12 transgenic poplar lines that were transferred to soil and grown in the greenhouse. After two months, they were harvested and screened for changes in lignin deposition by phloroglucinol staining of transverse stem sections. The initial screen showed that the intensity of the staining was higher in seven of twelve lines, suggesting a stronger lignification in those plants. Based on the initial lignin screen, four of the seven transgenic lines were randomly chosen for in-depth characterization. Overexpression of *WALLDOF* in transgenic poplar leads to a dramatic thickening of secondary wall and a reduction in the lumen area, which resulted in an increase of as much as 78% of the area occupied by wall in fiber cell cross-sections. Wall thickening at the expense of lumen area also resulted in higher wood density in transgenic lines. Moreover, *WALLDOF* overexpressing plants presented increased cellulose content, a general reduction in hemicellulose carbohydrates, mainly mannose and arabinose, and alteration in lignin composition. Starch content in stems was also reduced in transgenic plants.

Aiming to figure out which genes could be related to the altered phenotype, we proceed with a xylem transcriptome analysis of *WALLDOF* events using Affymetrix Genechip Poplar Genome Array. We have identified 825 genes that are differentially expressed, including a set of genes that participate in carbon and nitrogen metabolism.

The results indicate that *WALLDOF* is involved in carbon flux regulation and carbon partitioning through cell wall formation, and can contribute for increasing biomass production.

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O36

Boundary issues in regulation and evolution of wood formation

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Wood has played an indispensable part in the rise of human civilization, evolution of modern plants and has cornerstone importance for the sustained long term health and stability of managed and unmanaged ecosystems. Despite its importance, the molecular mechanisms involved in wood formation are still poorly understood.

Wood formation initiates in a lateral meristem known as vascular cambium which consists of meristem cells (cambial initials) organized in single-cell radial file that form continuous cylinder around the stem. Cambium initials divide to produce xylem, phloem, and ray mother cells that in turn undergo several rounds of divisions followed by differentiation into respective cell types. Because of their importance to regulation the quantity and quality of wood production these processes are of substantial theoretical and applied interest but are poorly understood and thus very difficult to manipulate. The progression from meristem to fully differentiated cells is a complex process requiring highly organized transition from different developmental states, most prominently, loosing the pluripotency of meristem cell and committing to a specific cell/tissue fate (e.g., xylem, phloem or rays). Because of the importance of this transition plants establish developmental boundaries that insulate cells in different stages of this progression. We have recently shown that plant-specific transcription factors of the LATERAL ORGAN BOUNDARIES DOMAIN (LBD) family play key role in the establishment and function of these boundaries [The Plant Cell, 2010, 22: 3662–3677].

The discovery leading to the realization of the importance of LBD proteins in woody development was the identification and subsequent characterization of an activation tagged mutant that shows enhanced woody growth. We have found that the activation of a LBD encoding gene, *PtaLBD1* (*Populus tremula x P. alba LBD1*) with uncharacterized previously function in poplar and *Arabidopsis*, is primarily involved in differentiation of secondary phloem and ray cells. Transgenic gain and loss-of-function experiments demonstrated that PtLBD1 is a positive regulator of secondary phloem growth and development. Expression and localization experiments showed predominant expression in the phloem and localization of the transcript on the boundary between the differentiating phloem and the cambium zone. Genes encoding regulators of meristem identity genes like *ARBORKNOX1* (*ARK1*) and *ARBORKNOX2* (*ARK2*) were downregulated while key regulators of phloem initiation like *ALTERED PHLOEM DEVELOPMENT* (*APL*) gene was upregulated in the activation tagged mutant line. The phenotype, localization and misexpression of *ARK*1, *ARK2* and *APL* genes indicate that LBD1 is a critical component in the establishment and function of the developmental boundary between the vascular cambium and the differentiating phloem. It shows that the boundary genes like LBD have dual function to repress meristem identity but promote proliferation and differentiation. We have also found that LBD1 is downregulated by auxin and is co-expressed with strong repressors of auxin response. Because auxin concentrations peak in the cambium and LBD1 is downregulated by auxin we hypothesize that part of the molecular mechanism that establishes the boundary is through auxin-mediated repression of LBD1 in the cambium zone.

Vascular cambium of most extant plants is bifacial – producing xylem to the inside and phloem to the outside of the stem trunk. We then asked if the boundary on the xylem side of vascular cambium is organized and regulated in a similar manner. A broad preliminary survey of the LBD family in *Populus* identified 57 members; microarray results display that four LBD genes (*PtaLBD1*, *PtaLBD*4, *PtaLBD15*, *PtaLBD18*) show predominant expression in stems undergoing wood production with two genes expressed in the phloem (*PtaLBD1* and *PtaLBD*4) and two in the xylem (*PtaLBD15*, *PtaLBD18*). This suggests that a similar regulatory mechanism is in operation during xylem growth and differentiation.

Our studies also shed light on possible mechanisms in the evolution of woodiness and wood anatomy. The LBD proteins are present in most genomes of the sequenced species from the plant kingdom with exceptions of algae. This suggests that the LBD gene family has evolved after the colonization of land plants. Comparative sequence analysis of putative LBD1 orthologs in several sequenced plant genomes, indicates a large and highly significant expansion of the LBD1 gene in *Vitis vinifera*. There were 12 putative LBD1 orthologs. *Vitis* is vine and has a unique wood anatomy. Most notably, the rays are highly multiseriate and secondary phloem is well-developed with multiple growth rings. The intercalation of less-lignified tissues in the xylem of vines and lianas and proportionally more parenchyma tissues is an adaptive feature allowing more stem flexibility. The multiseriate rays and increased secondary phloem production in *Vitis* resembles the poplar transgenics with increased *PtaLBD1* expression. Thus the putative increased LBD1 gene dosage in *Vitis*, well-corresponds to its wood anatomical features and suggests that LBD genes like LBD1 may have played important role in evolution of wood anatomy as an adaptive mechanism reflecting species biology and life habit.

Our findings have broad importance with respect to regulation and evolution of wood formation. They provide tools for manipulation of woody growth and development and provide clues for the mechanisms involved in evolution of wood anatomy with relation to plants' biology and ecology.

O37

Vascular-related NAC-domain 7 directly regulates a broad range of genes for xylem vessel differentiation

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Background: Xylem functions in conduction of water and minerals throughout the plants, and supports the plant body. One of the features of xylem cells is development of secondary wall structure between plasma membrane and (primary) cell wall. Recently, it is expected that knowledge on xylem development can be utilized for application of improvement of the plant biomass, since most portion of wood, which represents one of important sources of woody biomass, is mainly composed of two types of xylem cells, xylem vessels and fiber cells.

Previously we established the *in vitro* transdifferentiation system, in which Arabidopsis suspension cells could synchronously transdifferentiate into xylem vessel elements. A number of genes whose expression is elevated during the transdifferentiation processes have been isolated by using microarray analysis [1]. We revealed that one of the identified genes, which encoded a NAC domain protein, *VND7* (*Vascular-Related NAC Domain Protein7*), plays a pivotal role in promoting the xylem vessel differentiation [1,2].

Recently, to efficiently obtain xylem vessel elements, we used a glucocorticoid-mediated post-translational induction system [3]. The transgenic Arabidopsis plants exhibited transdifferentiation of most of cells into xylem vessel elements, and the plants died. This induction system worked in poplar trees and in suspension cultures of cells from Arabidopsis and tobacco. These data demonstrate that the induction systems controlling VND7 activity can be used as powerful tools for understanding xylem cell differentiation.

Objectives of the research: Several studies report that VND7 regulates expression of downstream of some transcription factors, suggesting that existence of transcriptional network regulating xylem vessel differentiation. Here, in order to identify direct target genes of VND7, we performed global transcriptome analysis using Arabidopsis transgenic lines in which VND7 activity could be induced posttranslationally.

Methods: We generated a transgenic Arabidopsis plant expressing *VND7-VP16-GR*[3] driven by *CaMV35S* promoter. *VND7-VP16-GR* and *VP16-GR* seedlings were soaked with water containing 10 μM cycloheximide (CHX), a protein synthesis inhibitor, for 2 hours. After removal of the solution, the seedlings were re-soaked with water containing 10 μM CHX, with or without 10 μM dexamethazone (DEX), for 4 hours. Microarray analysis was performed using GeneChip ATH1 Arabidopsis genome arrays. The effector, reporter, and reference plasmids [4], were delivered into the rosette leaves of Arabidopsis by particle bombardment. After overnight incubation, luciferase activity was assayed. For electrophoretic mobility shift assay (EMSA), promoter fragments were labeled with biotin. The poly-His-tagged N-terminal region of VND7 (His-VND7$^{1-161}$) protein was purified from *Escherichia coli*.

Results: As shown previously, overexpression of *VND7* induces expression of many genes related to the differentiation of vascular vessels [4]. Microarray analysis revealed that 300 genes are upregulated by more than two-fold in the transgenic *VND7* plant. To identify direct target genes of VND7 among the upregulated genes, we used a glucocorticoid-mediated posttranslational induction system. We generated a transgenic Arabidopsis plant expressing chimera *VND7-VP16-GR* gene under the control of the *CaMV35S* promoter [3]. We subjected *VND7-VP16-GR* seedlings to pre-treatment with CHX, followed by treatment with or without DEX. Microarray analysis using these samples revealed that, among the 300 genes upregulated in the *VND7-YFP* plants, 63 were also upregulated (fold change > 2; FDR < 0.1 [$P < 0.026$]) in the *VND7-VP16-GR* plants in response to DEX treatment. These genes encode a broad range of proteins such as transcription factors, IRREGULAR XYLEM proteins, and proteolytic enzymes, known to be closely associated with xylem vessel formation [5].

To define the promoter region responsible for the upregulation of gene expression by VND7, we carried out transient reporter assays using the *XCP1* promoter sequence. We constructed reporter plasmids by linking various lengths of *XCP1* promoter sequences to a minimal *CaMV 35S* promoter driving the firefly luciferase gene and the *VND7* gene driven by *CaMV 35S* promoter was used as an effector plasmid. We concluded that the region of *XCP1* promoter between residues -211 and -96 is necessary and sufficient for gene expression induced by VND7 [5].

In order to investigate the direct DNA/protein interaction between the *XCP1* promoter sequence and the VND7 protein, we carried out EMSA. Two distinct regions of the *XCP1* promoter were demonstrated to be responsible for VND7 binding. Furthermore, we also showed that VND7 protein binds to several promoter sequences present in candidate direct target genes [5].

Conclusions: These findings indicated that VND7 upregulates, directly and/or indirectly, many genes involved in a wide range of processes in xylem vessel differentiation. Interestingly, VND7 directly regulates lots of genes involved in programmed cell death, while most of genes controlling secondary cell wall biosynthesis could be regulated through the MYB transcription factors. Further investigation of consensus DNA binding sequences of target genes will help elucidate the regulation of gene expression in xylem vessel formation. To obtain a view of xylem vessel differentiation, we also need to reveal how the direct target genes function during xylem vessel formation.

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O38

Populus biomass protein-protein interactions and their functions

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Proteins are molecular machines that play roles in almost all biological activities through interactions with other molecules such as carbohydrates, lipids, nucleic acids and other proteins. We are mapping protein-protein interactions relevant to woody biomass production by focusing on proteins co-expressed in poplar secondary xylem. In addition to revealing novel regulatory mechanisms important to woody biomass production, mapping the poplar protein-protein interactome will provide fundamental information relevant to xylem differentiation and secondary growth. We have cloned a portion of the poplar biomass ORFeome, specifically 374 ORFs that are upregulated in xylem versus phloem, for use in protein-protein interaction research. Here we summarize the techniques we are using to discover and study protein-protein interactions and results to date.

1. Yeast two-hybrid (Y2H) binary assays: 108,205 Y2H binary assays involving over 300 biomass ORFeome members have been performed and 11 interaction pairs identified. The proportional yield from our binary screen is similar to that represented by the current preliminary binary screen data from the Arabidopsis interactome project.

2. Y2H cDNA library screening: We have used 40 bait proteins to screen our poplar xylem cDNA prey library, and a total of 60 biomass ORFeome members comprising putative regulators of lignocellulose synthesis will be screened in 2011. For the 26 ORFs that are completely through the library screen, we have identified 44 unique interacting sequences. Thus far, the proportional yield of interactors for proteins catalyzing metabolic reactions (such as cellulose synthase, PB138) is much lower than that for regulatory proteins (such as NIMA kinase, PB223). Results from these Y2H screens are available at our project website (http://xylome.vbi.vt.edu/index.html). Additional website information includes a project overview, project objectives, progress to date, sequences for all ORFs and primers, clone availability, and Y2H protocols.

3. Confirmation of selected protein-protein interactions identified by Y2H screens: Selected Y2H interactions are being confirmed by independent methods including bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation/affinity purification using plant transient or stable expression systems. We have developed improved vectors and protocols for reducing background fluorescence during BiFC experiments.

4. Functional analysis: We are characterizing the functions of selected interacting pairs in both poplar and Arabidopsis by ectopically expressing or suppressing genes singly and in combination. For ectopic and/or over-expression experiments we are using *35S*, *SUC2* (phloem specific) and *XCP2* (xylem specific) promoters. Co-expression of *35S::PB15* and *35S::PB129* in Arabidopsis resulted in expanded interfascicular regions containing enlarged fibers compared to fibers in wild-type plant interfascicular regions of the inflorescence stem. Importantly, this phenotype was not observed in transgenics overexpressing just one of these genes, showing the potential of novel interactome data to be translated into alteration of wood phenotypes.Arabidopsis mutants carrying T-DNA insertions within Arabidopsis orthologs of *PB15* and *PB129* are also being evaluated, and *Populus* RNA interference (RNAi) lines have been generated to study the effects of silencing *PB15* and/or *PB129* and their paralogs.

5. Transgenic poplar field trial: We prepared transgenic poplar overexpressing 11 biomass genes as tandem affinity purification-(TAPa)-tagged fusions and established these in a replicated field trial, which will be evaluated for overexpression phenotypes and serve as a source of xylem for identification of co-purified proteins.

O39

Identification of laccases involved in lignin polymerization and strategies to deregulate their expression in order to modify lignin content in Arabidopsis and poplar

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BMC Proceedings 2011, **5(Suppl 7):**O39

Lignins have a major impact on the agro-industrial uses of plants. Until now, most of the strategies considered for lignin reduction have targeted the monolignol pathway since the genes involved in these metabolic steps have been identified in many plants. Less is known about the other steps and in particular on lignin polymerization in the cell wall. While it is established that peroxidases are involved in the polymerization of lignin precursors, it is not yet clear whether laccases (EC 1.10.3.2) participate in constitutive lignification.

In order to address this issue, laccase genes (*AtLAC4* and *AtLAC17*) that are highly expressed in Arabidopsis stems were studied. *AtLAC17* was specifically expressed in the interfascicular fibers while *AtLAC4* was expressed in vascular bundles and interfascicular fibers. *Arabidopsis* T-DNA insertion mutants were selected and characterized. Two double mutants were obtained by crossing the *AtLAC17* (*lac17*) mutant with two *AtLAC4* mutants (*lac4-1* and *lac4-2*). The single and double mutants displayed normal growth, except the *lac4-2 lac17* mutant that sometimes had a semi-dwarf phenotype and collapsed vessels. While the single mutants had moderately reduced lignin levels, the stems of *lac4-1 lac17* and *lac4-2 lac17* had lignin content reduced by 20% and 40%, respectively. This lower lignin level improved their saccharification yield. Thioacidolysis revealed that disrupting *AtLAC17* mainly affected the deposition of G lignin units in the interfascicular fibers and that complementation of *lac17* with *AtLAC17* restored the normal lignin profile. This study provides evidence that both *AtLAC4* and *AtLAC17* contribute to the constitutive lignification of *Arabidopsis* stems and that *AtLAC17* is involved in the deposition of G lignin units in fibers, suggesting a role in early lignification (Berthet et al, in press).

The double mutants cannot be obtained for species that are propagated vegetatively such as poplar. In order to produce plants with lower laccase activity and reduced lignin content, we therefore used a miRNA strategy. The overexpression of two miRNA (miR397 and miR408) targeting several laccase genes was tested in different plants including Arabidopsis and poplar. These miRNAs were expressed constitutively under the control of

the CaMV 35S promoter or of lignin-specific promoters such as *CAD* and *C4L* in transgenic Arabidopsis and poplar.
Results obtained using of this miRNA strategy in *Arabidopsis* and preliminary results for poplar will be presented.

O40

An AC-type element mediates transactivation of secondary cell wall carbohydrate-active enzymes by PttMYB021, the *Populus* MYB46 orthologue

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Background: The transcription factor MYB46, together with its redundant paralog MYB83, regulates expression of secondary cell wall biosynthesis genes in Arabidopsis [reviewed in 1].
We isolated the Populus MYB46 ortholog, PtxtMYB021 from hybrid aspen (Populus tremula x tremuloides) [2]. Transiently expressed MYB021 transactivated gene promoters of Populus xylan-active enzymes GT43A, GT43B and Xyn10A. Analysis of conserved motifs within these promoters identified the sequence CCACCAAC, which is similar to the AC elements mediating transactivation by MYB transcription factors during lignin biosynthesis, and we showed that this motif is enriched in xylem-specific carbohydrate active enzyme (CAZyme) promoters.

Methods: To establish whether the AC-type motif in CAZyme promoters is important for their function, we analyzed loss-of-function and gain-of-function GUS reporter constructs of the GT43A promoter in a transient transcription assay, by co-infiltration with a 35S:MYB021 effector in Nicotiana benthamiana leaves.
Results and discussion: We show that mutation of the AC-type element in the GT43A promoter abolishes transactivation by MYB021, whereas an AC multimer confers MYB021 transactivation to a minimal 35S promoter. Others have shown that the recombinant Eucalyptus grandii MYB46/MYB83 ortholog, EgMYB2, directly binds the AC-motif variant CCACCTACC found in the EgCCR gene promoter [3], suggesting that MYB021 directly binds the AC-type motif. We propose that, in angiosperms, the AC-type regulatory element mediates MYB46's transactivation of its target genes. Further, MYB46 activates lignocellulose synthesis in a feed-forward loop with downstream MYBs, involving both direct activation of downstream MYBs and joint regulation of downstream secondary cell wall synthesis targets (Fig. 1). Recent results show direct activation of downstream lignocellulose synthesis targets by higher hierarchy NAC domain regulators [4,5].
Interestingly, the downstream MYB regulators of lignin biosynthesis MYB58 and MYB63 bind the AC element but reportedly do not regulate CAZyme-mediated secondary cell wall polysaccharide biosynthesis [6]. Other downstream MYBs that bind variants of the AC element are the EAR domain lignin repressors, such as EgMYB1 and ZmMYB31 (Table 1). Then, binding of the distinct lignocellulose synthesis MYB transcription factors to particular AC-element variants may be mediated by motif sequence, motif context and/or interactions with cofactors. Also, some AC element variants overlap extensively with each other and with the AC-type element defined

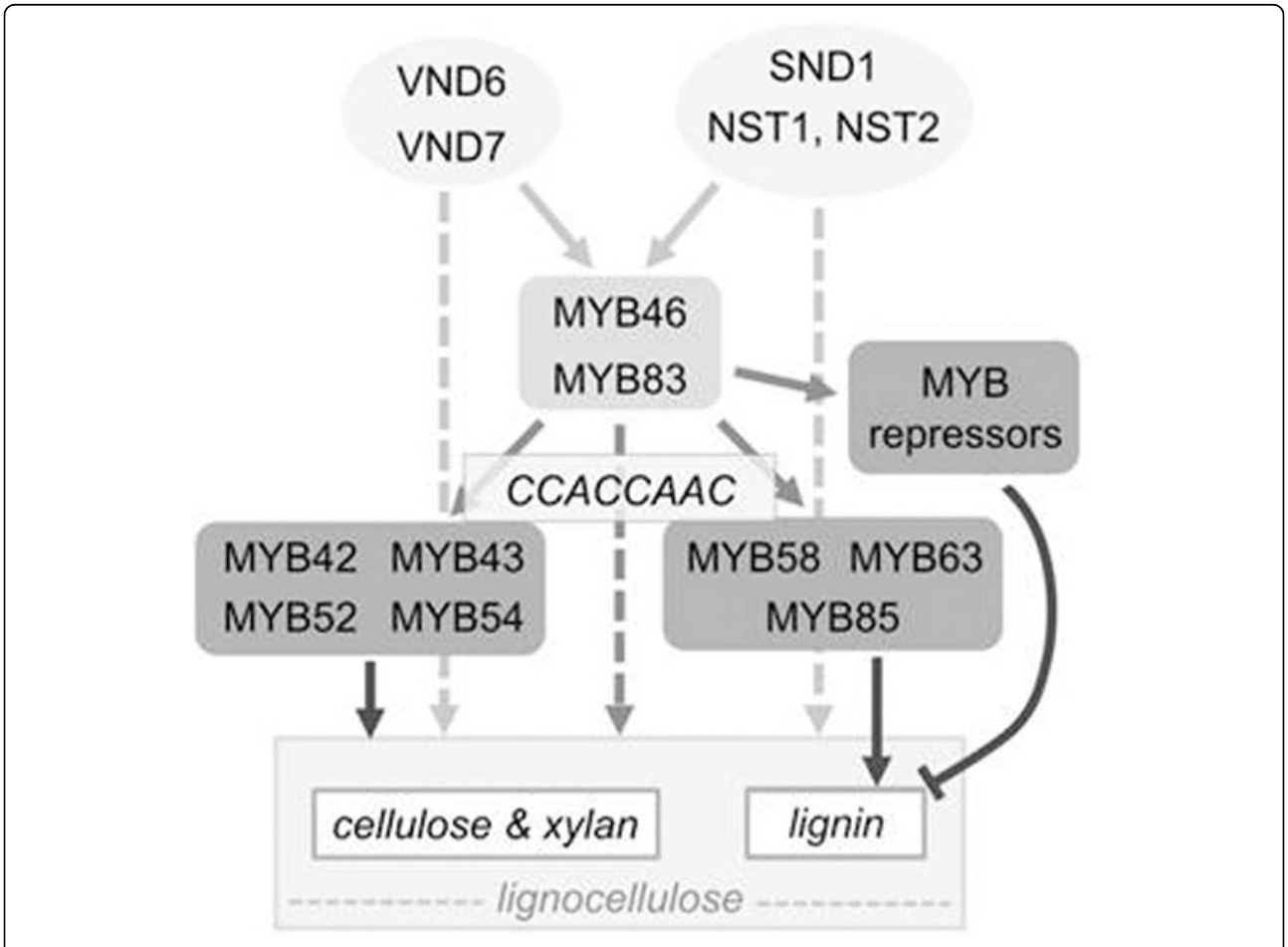

Figure 1(abstract O40) Regulatory networks in lignocellulose synthesis in angiosperms, shown using Arabidopsis gene nomenclature, involve feed-forward loop circuits. Dashed arrows highlight direct target gene activation by higher hierarchy regulators. White boxes at the bottom indicate target gene programs. For simplicity, some regulators, including ASL19, MYB103 and KNAT7, are omitted. Adapted from [1] and references therein.

Table 1(abstract O40) Sequence overlap in AC motifs

Species	Genbe	Motif name	Motif sequence	MYB protein[†]	Reference
Phaseolus	**PAL2**	**AC-I**	CCCACC**T**ACC	**n.d.**	[9]
Phaseolus	**PAL2**	**AC-II**	CCACCAACCC...	**n.d.**	[9]
Petroselinum	**4CL1**	**AC-II**	C**T**CACCAACCC	**n.d.**	[10]
Populus	**CCoAOMT**	**AC-II**	C**T**CACCAACCC...	**n.d.**	[7]
Eucalyptus	**EgCCR**	**MBSIIG**	**c**CACC**T**ACC	**EgMYB2[§]**	[3]
Eucalyptus	**EgCCR**	**MBSIIG**	**c**CACC**T**ACC	**EgMYB1**	[11]
Arabidopsis	**4CL1**	**AC-II**	**t**CACCAAC	**MYB58/63**	[6]
Zea	**ZmCOMT**	**AC-II**	**tc**ACCAAC	**ZmMYB31**	[12]
Populus	**GT43A**	**AC-type**	CCACCAAC **** **	**PttMYB021[§]**	[2]

In motif sequences, lowercase indictates ases outside the defined motif, and asterisks highlight invariant bases. Similarity to the GT43A AC-type elements is underlined. [†]Indicates which MYB protein binds the motif. [§]Ortholog of MYB46.

by us (Table 1), so it remains to be established whether they are all representatives of one identical core sequence motif, as previously proposed [7]. Establishing the exact sequence requirements for AC-element recognition by distinct MYBs will facilitate elucidation of lignocellulose-related regulatory networks in different species, through bioinformatic analysis [8].

Conclusions: The AC regulatory element, here preliminarily redefined as the sequence CCACCAAC, is a true "lignocellulose synthesis response element", mediating MYB46-dependent transactivation of the whole secondary cell wall gene program. This knowledge will enable inference of MYB46-centered regulatory networks in different species, through bioinformatic analysis.

Acknowledgements: We thank David Caparrós-Ruiz for helpful comments and discussions.

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S7. PROPAGATION AND IN VITRO TECHNOLOGIES

O41

Analysis of cellulose synthase (CesA) promoter function in trees using Induced Somatic Sector Analysis (ISSA)

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Detailed knowledge of the tissue specificity of gene expression is of central importance not only for our understanding of developmental processes during wood formation, but also is a prerequisite for the deliberate manipulation of xylogenesis candidate genes. Today, much of our knowledge about specific gene expression is based on annual model plants in part because perennial tree systems are often seen as too cumbersome for detailed promoter studies. Here we address this issue in a novel way with the investigation of a number of well characterised cellulose synthase (CesA) genes in the stems of perennial tree species using the *in vivo* Induced Somatic Sector Analysis (ISSA) system. Three primary and four secondary cell wall associated CesA gene promoters from *Eucalyptus grandis* and *Arabidopsis thaliana* were previously isolated and cloned in front of the GUS reporter gene then, using *Agrobacterium*-mediated transformation, transformed into cambial initials in stems of eucalypt and poplar plants. A CAMV35S promoter::GUS vector was also used as a control. After a period of growth, stems were harvested and GUS-staining patterns were analysed and interpreted. Results from this work show that in eucalypts the staining patterns are consistent with observations in other species whereas in poplar conflicting results were observed. These findings and the general utility of ISSA for these studies are discussed.

O42

Variation of telomeric repeats in Scots pine (*Pinus sylvestris*) – is there a connection to ageing and loss of regeneration ability?

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The shortening of telomeres, the specific structures of repeated DNA sequence at the end of the eukaryotic chromosomes, has been connected with ageing and loss of cell replication or regeneration capacity. There is, however, only limited information available on telomeres of long-living trees. Physiological ageing, on the other hand, represents a serious

problem for vegetative propagation of conifers, while in deciduous tree species ageing has no such a strict influence on regeneration ability. The aim of this study was, for the first time, to determine the variation in telomeric repeats in an economically important Nordic conifer, Scots pine (*Pinus sylvestris* L.), and to study potential connections to tree ageing.
Scots pine individuals ranging from immature embryos to 200-year-old trees were examined. In 1- and 5 year-old seedlings and 50-, 100- and 200-year old trees, cambium, bud and needled samples were collected from different positions within a tree. The length of telomeric repeats in the extracted total genomic DNA was then determined by Southern hybridisation. In addition, telomerase enzyme activity was measured from the protein extracts prepared from the same tissues using TRAP (Telomeric Repeat Amplification Protocol) .
In Scots pine samples, the size of telomeric repeats detected was variable, ranging from 0.9 kb up to 26 kb. Based on exonuclease treatment, high molecular weight repeats seem to be genuine telomeres at the ends of the chromosomes, while the low molecular weight signals probably originate at interstitial or centromeric positions. The Southern hybridisation was thus adjusted to show only high-molecular weight signals, and analyses were performed based on them.
A decline in the average length of the telomeric repeats was observed with increasing level of tissue differentiation: immature and germinating embryos had the longest repeats, followed by cambium, elongating buds with tiny meristems and, finally, full-sized needles without meristems having the shortest repeats. When embryo samples were excluded from the data, the age of the tree had no significant effect on the length of telomeric repeats, although there was a tendency for telomere shortening with increasing age. In cambium samples, also the position of the tissue within tree (stem base versus top) affected the length of telomeric repeats, the telomeres shortening towards top of tree. The telomeric repeats also varied remarkably between tree individuals. Potential connections to regeneration ability are discussed.

O43

Agrobacterium-mediated co-transformation of American Chestnut (*Castanea dentata*) somatic embryos with a wheat oxalate oxidase gene

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BMC Proceedings 2011, **5(Suppl 7):**O43

Background: American chestnut is a tree of great historical, ecological, and economical importance. It once dominated forests in eastern United States until the introduction of chestnut blight fungus (*Cryphonectria parasitica*) in the late 19th century. Within 50 years, *C. parasitica* killed almost all of the 4 billion American chestnut trees in the eastern United States. The fungus first infects wounded stem, secretes oxalic acid to decrease the pH of the infected tissue to toxic levels for the tree, but optimum for fungal enzymes, and then mycelia fans spread forming a canker which when it girdles a branch prevents water and nutrient transport, eventually killing the tree above the canker. The fungus does not infect the roots, thus allowing the growth of adventitious shoots to keep the tree alive. However, this survival is only temporary because these spouts will again get infected by the fungus and die back to the ground. It is this continuing circle that made American chestnut, once a great canopy tree, to no more than an early-succession-stage shrub today. Efforts to restore American chestnut back to its native range are currently being made. In our labs, we use an *Agrobacterium*-mediated co-transformation system to deliver potential resistant genes into somatic embryos of American chestnut together with a selectable marker gene. The gene of interest we used is an oxalate oxidase gene from wheat (*Triticum aestivum*). Oxalate oxidase (OxO) can degrade the fungus secreted oxalic acid to carbon dioxide and hydrogen peroxide. This action has a duel-effect: bringing up the pH of the infected sites thus neutralizing the virulent effect of oxalic acid, and increasing the expression of defense related genes induced by hydrogen peroxide byproduct. The selectable marker we used is Green Florescent Protein (GFP) from *Aequorea victoria*,which allows us to have a direct visual selection of successful transformants.

Methods: *Agrobacterium tumefaciens* strain EHA105 was used for the co-transformation. Vectors containing OxO and GFP were introduced to EHA105 separately via electroporation so each *Agrobacterium* had only one vector. Transformed EHA105 were grown separately under the selection of kanamycin and rifampicin until an O.D. of 0.8-1.2 was reached. *Agrobacterium* with optimum O.D. were harvested and re-suspended in *vir* induction medium containing acetosyringone. To approximately equalize the concentration of bacteria samples with different vectors, the amount of *vir* induction medium added was calculated by multiplying the O.D. value with 50ml. Bacteria samples were incubated in *vir* induction medium for three to four hours under room temperature with slow agitation. After *vir* induction, bacteria samples containing different vectors were mixed together in two different volume ratios: 2 (OxO):1 (GFP) and 4 (OxO):1 (GFP). The mixture was then added to embryo clusters to bring a total volume of 6ml followed by co-cultivation on a turning machine for an hour at room temperature. After the co-cultivation, embryo clusters were taken out to put in desiccation plates (plates with 200ul ddH_2O saturated filter paper) and kept in dark for two days under room temperature. After desiccation, embryos were transferred to *Agro*-kill medium containing carbenicillin and cefotaxime and kept in dark under room temperature. After a week, embryos were transferred to selection medium containing carbenicillin, cefotaxime, and appropriate selection agents (herbicide PPT and paromomycin in our case). Embryos were transferred to fresh selection medium every two weeks and were examined for GFP expression under a UV microscope (Figure 1).
GFP positive embryos were separated and multiplied in selection medium followed by genomic DNA extraction and PCR. Positive transformants with both GFP and OxO together with six GFP-only events were selected for regeneration as previously developed in our labs. The expression and copy number of OxO were tested by RT-PCR (Real Time-PCR).
Results and discussion: Thirteen experiments with a total of 208 embryo clusters were done using the 2 (OxO):1 (GFP) ratio resulting in 50 GFP positive events. 10 out of the 50 events were also OxO positive, giving a co-transformation efficiency of 20%, or 4.8% per embryo cluster. Three experiments with a total of 150 embryo clusters were done using the 4 (OxO): 1 (GFP) ratio resulting in 45 GFP positive events. 18 out of the 45 events were also OxO positive, giving a co-transformation efficiency of 40%, or 12% per embryo cluster. Doubling the ratio of vector containing gene of interest to vector containing the selectable marker from 2:1 to 4:1 gave a 2.5-fold increase in co-transformation efficiency. All of the 28 events went through the regeneration process together with 6 GFP-only events. 4 out of the 10 events produced by 2:1 ratio regenerated into whole plants. 17 out of the 18 events produced by 4:1 ratio regenerated into whole plants. All 6 GFP-only events regenerated into whole plants. These regenerated American chestnut plants seem to exhibit normal morphology. A gradient of OxO expression levels were found with the highest being more than 200 fold higher than the lowest expressed event.

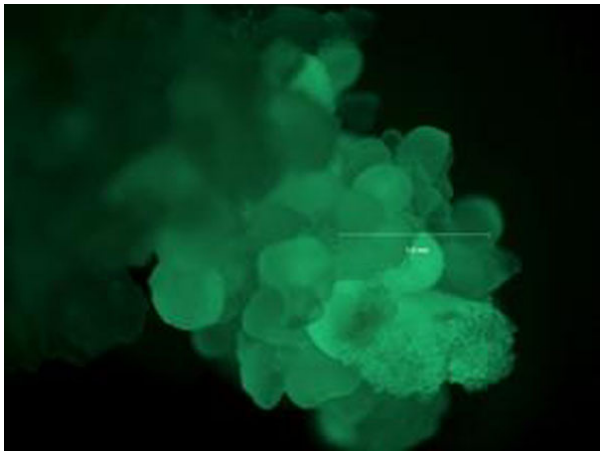

Figure 1(abstract O43) GFP expression in transformed American chestnut embryo cluster under UV light.

Copy numbers of the transgene *oxo* were also tested via RT-PCR showing most events with 2 copies, few with single copy, and a couple with higher copy numbers. The difference in regeneration ability and expression levels among these events was likely a result of position effect, which the expression of foreign gene is affected by the insertion site and nearby regulatory factors in the genome.

Conclusions: The efficiency of *Agrobacterium*-mediated co-transformation of American chestnut somatic embryos improved significantly when increasing the ratio of vector containing the gene of interest to vector containing the selectable marker in the co-cultivation mixture. Other factors of co-transformation including *Agrobacterium* strain, co-cultivation time, and desiccation time, etc. can also be investigated to improve the overall efficiency of this process. Co-transformation proved to be an effective way to introduce foreign genes into American chestnut to test their effectiveness on chestnut blight resistance. Having GFP on a separate plasmid provided a useful tool for removing of the marker gene in future. However, further tests need to be done to determine whether the GFP gene is linked to *oxo* in the genome.

O44

Evaluation of reference genes for quantitative PCR analysis during somatic embryogenesis in conifers

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BMC Proceedings 2011, **5(Suppl 7):**O44

Clonal propagation by somatic embryogenesis (SE) has a great application potential in conifers, but its efficiency still needs further improvement. Quantitative PCR (qPCR) is widely used for gene expression analysis during SE as part of studies to characterize gene function. In qPCR, data is normalized against a reference gene with a uniform expression during the tested conditions. Studies have shown that no single housekeeping gene is universal for all experiments, so a systematic characterization of several endogenous genes for concrete conditions is fundamental for biological accuracy.

We evaluated 10 genes present in *Pinus pinaster* selected among those commonly used as endogenous reference in conifers (*actin*, *α-tubulin*, *elongation factor 1 α*, *adenosine kinase* and *ubiquitin*), previously tested in model plants (*Histone 3*, *SAND* protein and *clathrim adaptor complex* -CAC- subunit), and with a lower variation of expression in five RNA samples at different embryogenesis stages according to microarray hybridization results (homologous to a heat shock protein -HEATSK- and to an ether reductase -REDUC-) (Simões *et al*., unpublished results). Stability of the expression in three developmental stages (embryogenic tissue in proliferation, mature embryos and germinated embryos) was evaluated with two different algorithms (implemented by programs geNorm and Normfinder), both in *Pinus pinaster* and *Picea abies*, so results could be expanded to other conifers. Previously, efficiency was calculated with programs Linreg and qPCR miner and the distribution of Cts by statistical boxplot analysis, for each amplicon.

Results evidenced that the geometric averaging of two-to-four of these genes is accurate enough. Using genes *elongation factor 1 α*, *SAND* and *α-tubulin* as reference is the better generic combination, and so probably the preferable option to consider in other conifers. Among them, *SAND* and *α-tubulin* had a reduced range of Cts, while it was higher in *elongation factor 1 α*, especially in *Pinus pinaster*. Some discrepancies were detected in the ranking base on stability for the other genes, so this generic combination can be improved in certain stages or species, like *adenosine kinase* in *Pinus pinaster* or CAC when analysing embryogenic tissue in proliferation. *Adenosine kinase* and *ubiquitin* are also acceptable options, as they showed small ranges of Cts and medium stability values in the three stages in both species. By contrast, several genes showed a higher variation in our experiments, as both selected from the microarray analysis (REDUC and HEATSK), and one commonly used reference gene (*actin*), which evidence the importance of these kind of studies.

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O45

Optimisation of methods for Agrobacterium rhizogenes mediated generation of composite plants in *Eucalyptus camaldulensis*

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Background: The wide adaptability, fast growth rate, good form, excellent wood and fiber properties make *Eucalyptus* the most widely planted genus of plantation forest trees. With the availability of Eucalyptus genome sequence information, assignment of function to genes contributing to desired traits require functional validation via overexpression or silencing in the context of the plant environment. However, plant regeneration from transformed tissues has been the most time consuming and rate-limiting step for rapid gene function analysis in tree species. The composite plants with transgenic hairy roots derived via *Agrobacterium rhizogenes* mediated transformation are therefore increasingly being used in species with such limitations to study root development and root-biotic interactions [1]. In this paper we describe a protocol developed for generation of composite plants using *A. rhizogenes* mediated transformation of *Eucalyptus camaldulensis*.

Methods: Transformation experiments were carried out using an agropine mannopine-type strain A4RS, harbouring a green fluorescent protein (sGFP) based binary vector pHKN29, constructed from pCAMBIA 1300 by replacing hygromycin phosphotransferase (hpt) with the sGFP(S65T) [2]. Infection was carried out using a sterile needle swabbed with *A. rhizogenes* grown for 48 hours in YEB plates containing 100 μM Acetosyringone. The infected plants of *E. camaldulensis* were then placed under dim light on modified MS [3] medium comprising half strength MS macro elements, full strength MS minor elements and organic supplements. The petridishes were placed slantingly with the plants in an upside-down position. Following cocultivation, the cultures were transferred to fresh medium with plants in an upright position. Hairy roots were made bacteria-free by making transfers every 15 days to fresh medium containing 500mg/l cefotaxime. The hairy roots generated were examined under a stereo fluorescence microscope (Nikon, Japan) for visualization of GFP following excitation by blue light (Excitation filter 495nm, Barrier filter 520nm). The nontransformed roots were removed using sterile blades. The composite plants were placed in test tubes, and different media including full MS, modified MS, Knop's, B5 and Hoagland's solutions were evaluated for growth under hydroponic conditions. The composite plants were shifted to the transgenic greenhouse, where in the cotton plugs were loosened gradually and finally removed after a period of 2 weeks. The plants were then placed so that roots were immersed in the liquid media and shoots were above the test tube rim covered with aluminium foil. Few of these plants were subjected to stepwise increasing concentration of 50mM NaCl to determine their tolerance to salt stress.

Results and discussion: Inoculation with A4RS resulted in roots, arising mainly from the injured surface of the explants. Root initiation occurred within first 7 days after inoculation of explants with *A. rhizogenes*. The roots reached a length of 8-10cm in 45 days. The results were similar to the report of MacRae and Van Staden (1993), where in genetic transformation using the *A. rhizogenes* strain LBA 9402 was assessed by the detection of opines in the root generated from *in vitro* propagated seedling explants of *E. grandis*, *E. dunni* and *E. nitens*. In our study, the bright green fluorescence due to co-transformation of the pHKN29 vector harbouring the sGFP(S65T) was used for screening of transgenic roots (Figure 1). No fluorescence was observed in untransformed control roots of Eucalyptus.

Effect of explant type: Seedlings showed root induction in 68% of the explants at an average of 1.08 roots per plant of which 36% were cotransformed with the pHKN29 as confirmed by the GFP expression. The *in vitro* propagated clonal plantlets showed root induction in 37.5% of the explants at an average of 0.45 roots per plant of which 4.1% showed GFP expression.

Effect of cocultivation conditions: In a separate experiment, out of the four different basal media used for cocultivation, modified MS media gave the highest average number of hairy roots at 1.3 per plant, when

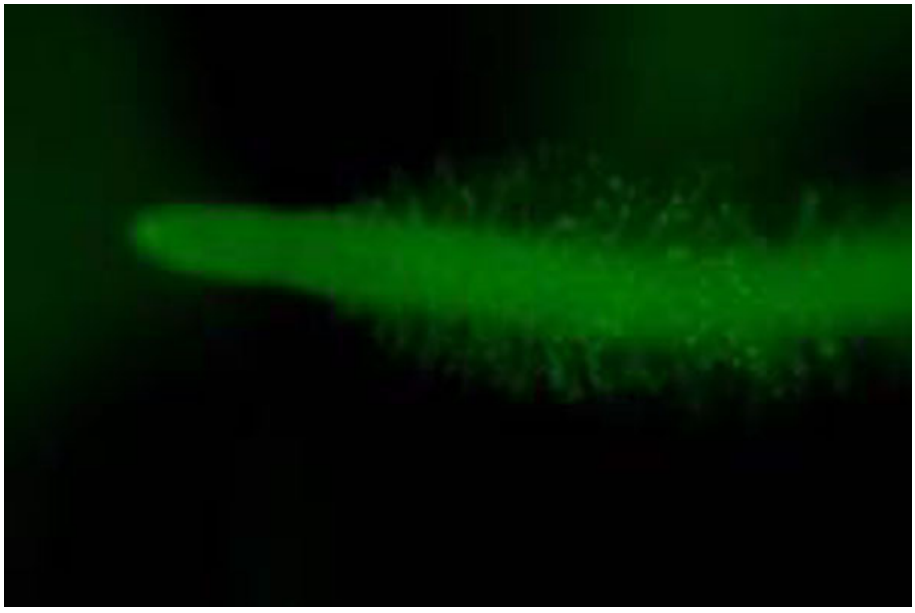

Figure 1(abstract O45) GFP expressing hairy root of *E. camaldulensis*.

compared to 0.8, 0.7 and 0.6 in case of 0.1 MS, Knop's and Hoagland's media (Figure 2).

Different supplements like phosphate buffer and calcium chloride were included in the cocultivation media to evaluate their influence on hairy root induction. Addition of calcium chloride to the cocultivation media reduced the average number of hairy roots induced per plant by 30%. Similarly, phosphate buffer also did not significantly influence the hairy root generation.

However, the inclusion of 100 μM acetosyringone in the cocultivation medium increased the average number of hairy roots to 1.6 per plant as compared to 1.3 for media without acetosyringone (Figure 2). Acetosyringone has been known to enhance transformation efficiency through activation of *vir* genes in *Agrobacterium*.

Preliminary experiments indicated that, cotransformation efficiency was higher when cocultivation was carried out at 16.5°C for 14 days when compared to 22°C for 7 days.

Effect of different liquid media on hardening of composite plants: Different liquid media were evaluated for hardening of composite plantlets. Hoagland's solution showed the highest survival (73.3%) when compared to MS (20%), modified MS (16%), B5 (0%) and Knop's (3.3%).

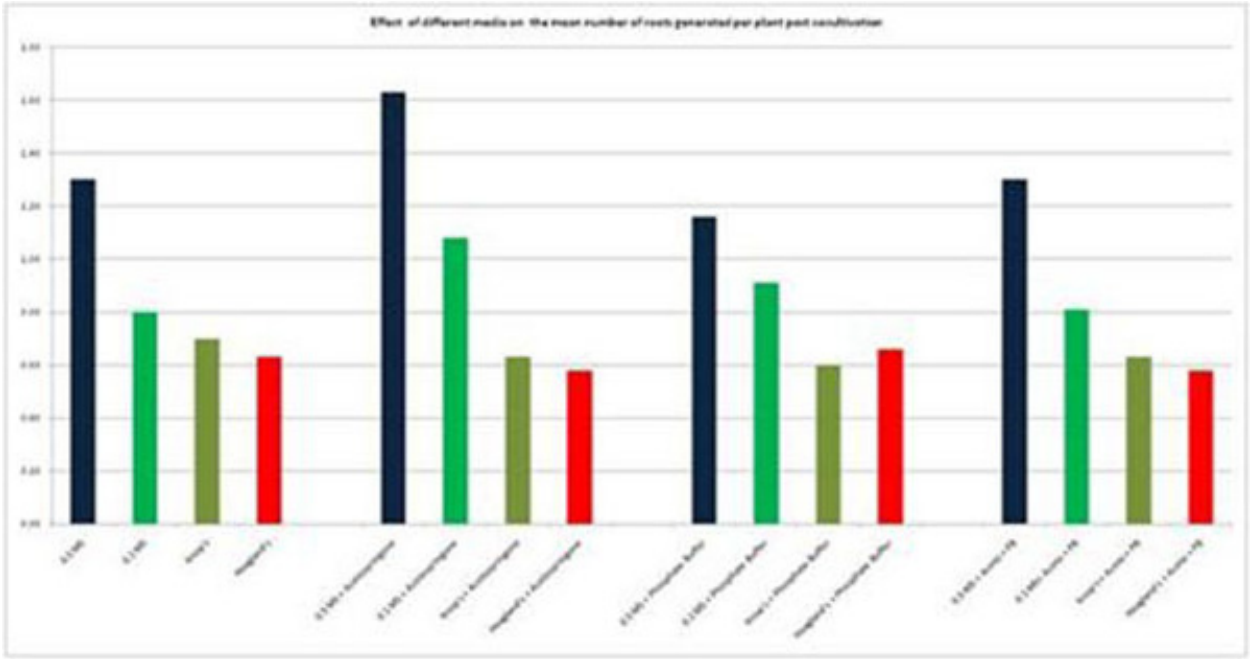

Figure 2(abstract O45) Effect of different media / supplements on the average number of roots generated per plant post cocultivation.

To determine the tolerance of these composite plants under salt stress, they were subjected to weekly increase in the concentration of NaCl by 50mM. Fifty percent of the Eucalyptus composite plants were able to tolerate up to 300 mM NaCl stress.

Conclusion: A composite plant strategy was developed utilising GFP based screening of transgenic hairy roots. Different parameters were evaluated to increase transformation efficiency using *A. rhizogenes*. Our studies showed that, modified MS media containing 100 μm Acetosyringone was optimum for enhancing the cotransformation efficiency. Hoagland's solution was identified as an appropriate media for hardening of Eucalyptus composite plants under transgenic green house conditions. Roots being the major portal of entry of ions to plants, the composite plant strategy will provide a rapid tool for the functional analysis of the genes vital for ion transport and root development. The composite plant strategy will be used to validate the function of *EcHKT1/EtHKT1*, the homologue of which have been reported to contribute to Na^+ uptake and xylem unloading of Na^+ in model plant and crop species.

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O46

New cytokinin derivatives – their discovery, development and use for micropropagation of endangered tree species

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Elms (*Ulmus* sp.) are highly valuable trees for their great strength, tightly twisted grain, and durability, as well as for their cold and salt tolerance [1]. Wych elm (*Ulmus glabra*, Huds.) is a native species in Europe. In the Czech Republic, it is spread especially in mountain regions. During the years 1970–80, the second epidemic of Dutch elm disease destroyed most elm populations in Europe. The elm trees, which have survived the disease, are capable to maintain a high degree of resistance against the repeated infection. Micropropagation represents promising alternative of their vegetative reproduction.

The wild service tree (*Sorbus torminalis* (L.) Crantz) is a slow-growing tree reaching maximum height of 20–25 m at around 80–100 years. According to the latest data, the species is also disappearing from forests of the Czech Republic and it is considered to be endangered. On the other hand, the wild service tree is rated as one of the most valuable hardwoods [2] with a great potential for wider use in forestry and forest ecology, and also for its importance in the timber industry.

The *in vitro* induction of organogenesis, rooting and acclimatization of above mentioned elm species as well as wild service tree were optimalized and standartized. Widely used BAP is an important and affordable cytokinin routinely utilized for its effective stimulatory properties in micropropagation practice. On the other hand, BAP may negatively influence the growth, rooting and acclimatization processes in some crops [3]. It is known that hydroxylated aromatic cytokinins (topolins) are more resistant to cytokinin oxidase (CKX), more stable and in some biological systems active at lower concetrations than the isoprenoid CKs. In addition, *meta* - topolin does not inhibit root formation which is a typical inconvenient effect of high concentrations of BAP. These properties might help to enhance the future productivity of plant tissue culture industry crops [3,4]. The development of other new CK derivatives exhibiting high morphogenetic activity might consequently be of a great practical importance in plant biotechnology. During our recent search for naturally occurring aromatic cytokinins (ARCKs) in plants, another new group of endogenous BAP derivatives – methoxytopolins – exhibiting high biological, especially anti-senescence (and surprisingly also anti-cancer) activity, was discovered. Based on these results, we synthesized several groups of their synthetic analogues that exhibited high activity in three different CK bioassays and showed the ability to activate cytokinin receptors and/or to inhibit CKX. Best compounds so far (6-(3-hydroxybenzylamino)purine (mT) and the 6-(3-methoxybenzylamino) purine-9-ribofuranoside (MeomTR)) were used for retardation of senescence during multiplication stage of micropropagation of above mentioned tree species as well as for rooting support. Subsequently, wide range of endogenous plant hormones (isoprenoid cytokinins, IAA, ethylene) were quantified (using UPLC-MS/MS and/or GC/FID, respectively) and compared in relation to cytokinin exogenously used.

The results about optimal endogenous plant hormone concentrations and their dependence on different exogenous cytokinin used in the cultivation media, may improve *in-vitro* micropropagation efficiency as well as quality of *ex vitro* acclimatized plants of above mentioned elm species, wild service tree and possibly also of other tree species.

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S8. GENOMIC, PROTEOMIC AND METABOLOMIC TECHNOLOGIES

O47

Haploid transcriptome analysis reveals allelelic gene expression variants, co-expressed gene groups, and linkages between expression and copy number variation

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Background: Genetic variation can cause changes in gene expression (mRNA abundance) among individuals. This so-called heritable variation in gene expression is affected by genetic variants that are co-segregating with the gene locus (local/*cis* effects) and/or segregating independently from it (distant/*trans* effects). Genetic variation in gene expression can be measured to estimate the extant of variation in gene expression within a population, and to determine to what degree expression alleles of different genes are connected within regulatory networks. Furthermore, determining whether variation in the expression of a gene is linked to local or distant effects allows us to make inferences about how heritable variation may change depending on gene function, the number of interacting partners, genetic architecture and evolutionary history [1,2].

Detecting heritable variation in gene expression can be a challenging task in diploid organisms, mainly because of tissue-specificity and dominance effects of allelic expression. For example, up to 70% of gene expression alleles in *Drosophila* may be masked by dominance [3]. We developed an experimental system to overcome these obstacles by utilizing the conifer

seed's maternally derived haploid tissue, the megagametophyte. Analyzing a set of sibling megagametophytes allows us to first, measure separately the expression each of the two alleles in the maternal genome in the absence of dominance and second, identify genes whose expression levels are co-segregating. In addition, the megagametophyte allows us to categorize the underlying genetic variants into local or distant with a simple co-segregation assay.

Methods: We set out to characterize segregating variation in gene expression in white spruce (*Picea glauca* [Moench] Voss). We analyzed the transcriptomes of germinating sibling megagametophytes from two controlled-crossed families (C9412516: male 2388 x female 77111 & C9612856: male 80109 x female 80112) with a custom microarray comprised of 32,000 spotted oligonucleotides, which represent over 25,000 unique white spruce genes. Each megagametophyte was split into two halves to provide technical replicates that were analyzed separately. A separate comparison of microarray results and RNA-Seq data has been carried out to validate the quality of the microarray.

The single-color microarray data was background-corrected and quantile-normalized with the R package Limma [4]. We used the R package Mclust [5] to test for unimodal vs. bimodal expression distributions of each gene across sibling megagametophytes. Genes that exhibited at least two expression alleles, which segregated within the 95% IC of their expected proportions, were selected. The segregation had to be repeatable with a replicate sample set to be considered valid.

Results and discussion: Analysis of two families of sibling megagametophytes (n=18) identified close to a thousand genes with segregating gene expression patterns in both of the two families. Approximately 10% of these genes were shared. Zero replicates are expected to have the same clustering pattern by chance alone (binomial $p \approx 3.810\text{-}6$| n=18). The number of variable genes is comparable to that found between 50 nearly isogenic *Drosophila* lines [6].

We have discovered a large number of genes with gene expression patterns segregating in a Mendelian way in white spruce. We are presently analyzing the relationships between gene function and paralog number with the variation in its expression in order to determine whether heritable variation in gene expression is associated with same genetic attributes in white spruce as what has been reported in model organisms. We have also begun investigating the contribution of local vs. distant effects on the expression alleles identified in the megagametophytes, and their nature, by studying the co-segregation of gene expression, SNPs and copy number variations (CNVs). Preliminary comparative genomic hybridization data suggests a significant portion of genes which show segregating gene expression alleles also exhibit CNVs. In follow-up experiments, we will address the amount of dominance between the gene expression alleles by comparing gene expression in megagametophytes versus self-fertilized embryos.

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O48

Targeted sequencing in the loblolly pine (*Pinus taeda*) megagenome by exome capture

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Background: An essential use of genomics is in the discovery of genes controlling complex, quantitative traits. In forestry, attempts to identify genes that regulate quantitative variation are still limited to a few Association Studies (AS) focused largely on candidate genes [1]. In most studies, few markers have been identified in association with quantitative traits.

Recent advances in DNA sequencing create the potential for high-throughput SNP genotyping at low cost, by re-sequencing genomes of interest [2]. For the particular case of conifers, two obstacles remain: (i) the lack of a reference genome to align the DNA sequences and identify SNPs, and (ii) the size and complexity of the genome that hinders the *de novo* assembly of reads. Whereas whole-genome sequencing of a large number of conifer genotypes is still unfeasible, concentrating the sequencing on gene-rich regions is an alternative to generate markers that are more likely to capture variation associated to complex traits. Here we report our approaches to develop methods of genotyping based on whole-exome capture using in-solution target enrichment (Agilent's SureSelect) followed by high-throughput sequencing (Illumina's GAIIx).

Methods: Probes for target enrichment: To capture a defined genic portion of the *Pinus taeda* genome we designed 54,773, unique, 120-mer RNA probes that tilled across a collection of 14,729 EST assemblies (unigenes). For 3,615 genes we could predict gene models and avoid probes on known exon-intron junctions.

Genetic material, library preparation and sequencing: A total of 72 seeds collected from a female tree (10-5 genitor) were used for library preparation. Haploid megagametophyte tissue was dissected and DNA was extracted using standard protocols. Libraries were prepared as recommended by Illumina, with some custom modification of concentration, adapter sequence and fragment size selection. Custom barcoded adapters were used to pool up to eight genotypes in a single hybridization, which followed Agilent's SureSelect protocol. Captured-libraries were sequenced using GAIIx with 115nt single-end run.

Results and discussion: Preliminary validation: To test the potential of target enrichment in pine we prepared sequencing libraries from three different genetic complexities from single genotypes/haplotypes: haploid cDNA from megagametophyte (n=12), haploid DNA from megagametophyte (n=12) and diploid DNA extracted from needles (2n=24). After hybridization of these libraries to SureSelect probes, we cloned each captured library into vectors and sequenced 96 fragments using Sanger sequencing. After reads were aligned to the reference sequences used for probe design (BLAST e-value=$1x10^{-10}$), 70, 84 and 80% of the quality-filtered reads from haploid cDNA, haploid DNA and diploid DNA aligned uniquely to a unigene, respectively. Therefore, sequence capture is highly specific, even when the complexity of the material increases.

Pipeline development for mapping population: We modified the sequence capture protocol to increase throughput by constructing libraries from haploid DNA of 72 megagametophytes in 96-well plates. An average of 25.9 million single-end reads were obtained from flow-cell channel containing 8 barcoded megagametophytes, for a total of 56 megagametophytes sampled across 7 channels lanes. The remaining two pools (16 individuals) have not been sequenced. Despite multiplexing a large number of megagametophytes, we detected low variation in read numbers between haplotypes within a multiplex reaction (Figure 1). Due to the lack of a reference genome, bioinformatics experiments were designed to test whether the best approach to identify polymorphisms would result from aligning the sequences directly to the unigene sequence using Mosaik (http://bioinformatics.bc.edu/marthlab/Mosaik), or by alignment of reads to a new reference sequence defined by *de novo* assembly of the captured reads with the ABySS [3]short read sequence assembler.

The analysis using Mosaik compared different levels of polymorphism between reads and the unigene sequences resulting from alignments over a range of mismatch tolerances (from 1% to 50%). Figure 2A shows that the number of reads that aligned to a single region of the reference (i.e. uniquely aligned) increases linearly until 10% of mismatches in the alignment are accepted, and stabilizes after that. Interestingly, the

percentage of reads non-uniquely aligned remains lower than 2%, regardless of the mismatch rate. Next we tested if accepting higher mismatch rates adds more reads to the same genes, or if genes previously not captured are now represented. Again, at a 10% mismatch rate (Figure 2B) the total number of genes with at least one read aligned reaches its maximum, with 22% of the reads aligning to more than 10,000 genes.

Analyzing the data using a *de novo* assembly approach does not appear to be more suitable. ABySS assemblies were performed utilizing a range of k-mer values (50 to 95, increments=5) and the contigs formed were analyzed. As expected, the number of contigs drops considerably as a function of the k-mer size (Figure 2C). To evaluate the quality of contigs generated at each k-mer, we compared them to the unigene sequences (BLAST identity≥85%) and counted the number of genes represented by at least one contig. This number decreased as a function of the k-mer size (Figure 2D); and, within the same genotype the sequence assemblies generated using a high k-mer were already presented in the assembly using a lower k-mer. However, when comparing two genotypes assembled with the same k-mer value about 30% of the genes are unique to each genotype. This suggests that additional sequencing is necessary for an adequate analysis of the efficiency of sequence capture.

We are currently sequencing the same libraries from both ends (i.e. paired-end sequencing) to increase coverage and depth. This sequencing data is expect to identify additional SNP markers for segregation analysis, and to help define the sequencing requirements to confidentially perform target-enrichment resequencing and genotyping in complex genomes.

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O49

The *Eucalyptus* genome integrative explorer (EucGenIE): a resource for *Eucalyptus* genomics and transcriptomics

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Background: The fast growth and good wood properties of *Eucalyptus* tree species and hybrids make them excellent renewable sources of fiber for pulp and paper production, and woody biomass for bioenergy production. Our research is aimed at understanding the genetic regulation of wood formation in eucalypts, with a focus on transcriptomes, regulatory sequences and gene families involved in secondary cell wall biosynthesis.

Methods: We have performed deep mRNA sequencing (using Illumina RNA-Seq technology) of several primary and secondary tissues of three *Eucalyptus grandis* trees with the aim to investigate the transcriptional control of cellulose biosynthesis and wood formation. The transcriptome datasets range from nearly mature xylogenic tissues to immature shoot tips, and consists on average 35 million paired-end 80bp short reads. The Illumina short reads were mapped to the latest *Eucalyptus grandis* genome sequence (DOE-JGI v. 1.0, http://www.phytozome.net), and the set of predicted gene models provided by the Joint Genome Institute (JGI). We calculated tissue specific gene expression (FPKM) profiles for each of the ~44 000 predicted genes across the sequenced transcriptome datasets. The results were stored in a relational database for further analysis and analysis of co-expression patterns in the expression profiles. We ultimately aim to identify genes that are differentially expressed and co-regulated during different stages of wood formation.

Results: We describe the initial development of an Integrative *Eucalyptus* Genome Explorer (EucGenIE), modeled after the poplar resource, PopGenIE (http://www.popgenie.org, Sjödin *et al*, 2009). EucGenIE relies on a relational database system that allows for the efficient storage and retrieval of gene models and expression values from the database, which is then presented to the user in novel and intuitive ways. The web-based front-end makes use of tools available in the Generic Model Organism Database (GMOD, http://gmod.org) toolkit to enrich the query interface as well as result visualization. Links to the Phytozome GBrowse instance provides genomic context to the expressed gene sets. Custom queries allows the user to find genes with similar gene expression profiles across the various datasets, as well as perform bulk searches on sequence features annotated on the gene set. EucGenIE also provides access to common analyses tools, such as homology searching and online clustering tools. The first version of the EucGenIE database and online portal is available at http://eucgenie.bi.up.ac.za with restricted access.

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O50

Investigation of genetic variation in *Jatropha curcas* by Ecotilling and ISSR

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Background: The ability of species to adapt to different environments resides in their genetic diversity. This diversity, most commonly manifested as Single Nucleotide Polymorphisms (SNPs), can provide clues to the adaptive processes and population histories that have played a role in the species' evolution. A number of different techniques for identifying SNPs have been developed, all having their limitations.

Reverse genetics approaches rely on the detection of sequence alterations in target genes to identify allelic variations in natural or mutant populations. Ecotilling, a variant of TILLING (Targeting Induced Local Lesions IN Genomes) technique, allows high-throughput analyses of natural genetic diversity in plants [1], particularly in species with limited genetic diversity.

Jatropha curcas L. is a perennial, monoecious shrub of the *Euphorbiaceae* family, native to America but distributed widely in the tropical and subtropical areas [2]. Wild or semi-cultivated types of *J. curcas* can grow well under unfavourable climatic and soil conditions [3]. *J. curcas* has attracted a great deal of attention worldwide, regarding its potential as a new energy plant. The seeds of *J. curcas* contain 30-45% oil [4] with a high percentage of monounsaturated oleic and polyunsaturated linoleic acid [5]. For genomic analyses, *J. curcas* is an interesting model species, since it has a relatively small genome (2C DNA content of 0.850 ± 0.006 pg or C DNA content of 0.416 × 109 bp) [6].

However, to achieve specific breeding goals in *Jatropha* for wider ecological adaptation, disease resistance and novel seed quality, the use of germplasm from different group and regions is necessary. Understanding the population structure of the alternative bioenergy plant *Jatropha curcas* is challenging due to limited genetic variability and information on phylogenetic relationships between accessions and related species. The development of cultivars of *Jatropha curcas* by conventional breeding will profit largely from biotechnological support (pathogen-free accessions with specific traits, non-toxic, high yielding varieties).

The knowledge about *J. curcas* remains limited and little genomic research has been done so far [7]. In fact, the genetic map of *J. curcas* is not well-developed and only few molecular markers exist that could be used to clearly distinguish world wide accessions. Therefore, a resource database of SNPs in *J. curcas* would provide researchers with a tool for answering questions concerning population structure or adaptation and allow comparison of this species with related species.

Methods: The identification of novel SNPs that account for natural variation was used to study genetic diversity and the relationships between and within *Jatropha* species. ISSRs (*Inter* Simple *Sequence*

Repeats) also were considered as a tool in selecting germplasm for breeding purposes.

An *in vitro* germplasm collection of 1300 accessions from 12 countries was established. This collection will serve different purposes: a) conserve valuable genetic resources, b) survey genetic variation, and c) serve as starting material for genetic improvement with different breeding goals.

Ecotilling was applied to 12 different genes of interest related to stress tolerance, toxin and oil metabolism. 50 ISSR primers were used to assess the genetic diversity of *Jatropha curcas* and related species. Four different pooling strategies were used to identify homozygous and heterozygous SNP variations. In fact, variation was analyzed both within a single tree (heterozygous) as well as between individual trees and a reference samples. Due to the reported low variations between *Jatropha* accessions [8,9] and large size of our collection, the 8 x 8 pooling strategy was chosen to estimate the level of variations among 12 selected genes.

Results and conclusions: To elucidate genetic relationship among *Jatropha* accessions from different regions and related species, a dendrogram was produced using NJ analysis of NeiÂ´s genetic distance for 5 ISSR markers. The dendrogram is divided into two groups, one containing all *Jatropha* accessions and the other containing the related species. The main *Jatropha curcas* cluster is divided into two subclusters, one containing samples from Kenya and the other containing the remaining *Jatropha* accessions. The data showed clear variations not only among individuals but also between different regions.

Ecotilling was found to be more efficient for large-scale studies of genetic variation in *Jatropha*, compared to RAPD, SSR and AFLP. Ecotilling is a low cost, high-throughput reverse genetic method for haplotyping and SNPs discovery.The level of differentiation observed was based on the geographic distribution pattern, i.e. it was higher in the centre of origin. ISSR analysis yielded highly reproducible patterns with 5/50 primers.

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O51

Activation tagging in poplar by using an inducible Ac/Ds transposon system

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Background: The sequence of the whole genome of black cottonwood (*Populus trichocarpa*) was made available in public domain (http://www.phytozome.net/poplar) few years ago. Sequencing and annotation of the genome of an organism yields a tremendous amount of data and information on putative genes, however, often without any idea on their functions. To understand how a cell works one needs to know the function of almost every gene in its genome. Once the whole-genome information is available for an organism, the challenge turns from identifying the parts to understanding their function as well as to improving genome structure. In the short term, the first goal is to assign some element of function to each of the genes in an organism also referred to as 'functional genomics', and to do this with high-throughput, systematic approaches.

Material and methods: Gene technology is a very powerful tool in forest tree breeding programs as well as for functional genomics in order to unraveling gene functions. Various reverse and forward genetics strategies are ongoing to determine the functions of genes and regulatory sequences. A significant progress has been made by using T-DNA as a vehicle to induce either "Knock-out" or "Knock-in" ("gain-of-function" or Activation tagging") in different plant species including poplar [1].

The application of a transposon-based activation tagging system for poplar has been proposed early by [2]. It could be shown that the maize transposable element *Ac* is functional in the *Populus* genome [3], and re-integrations occur in high frequencies in or near coding regions [4]. Further, the majority of the re-integrations were found scattered over many unlinked sites on other scaffolds than the one carrying the original integration locus, confirming that *Ac* does in fact cross chromosome boundaries in poplar [5].

Results: In this paper, we describe for the first time the development of an efficient activation tagging system for poplar based on a non-autonomous "Activation Tagging Ds" (ATDs) system [6] in combination with a heat-inducible *Ac*-transposase. First, seven independent transgenic lines were obtained transformed with HSP::*transposase* construct. From these, two transgenic lines were selected for super-transformation with the ATDs-*rolC* construct based on the construct by [6]. The ATDs original integration could be determined from 18 double transgenic lines.

Induction of the transposase gene and mobility of the ATDs element could be confirmed. Four activation-tagged populations comprising in total 12,083 individuals from 22 different ATDs transgenic lines have been produced and phenotyped. So far, from 18 different putatively tagged variants the new ATDs genomic position was successfully determined. Sequences obtained were blasted against the publicly available genome sequence of *P. trichocarpa* v2.0 (Phytozome v5.0; http://www.phytozome.net/poplar). E-values of hits ranged from -35 down to zero. Annotation of the sequences obtained against *P. trichocarpa* revealed for 13 variants possible transcripts. For five putative proteins either no functional annotation or unknown function was found.

In a second approach, 300 randomly selected individuals revealing no obvious phenotype from the forth activation-tagged population were screened for ATDs excision. In approximately one third of the investigated individuals transposition of ATDs was confirmed, and analyses of the new genomic positions of ATDs reveal a very high percentage of tagged genes.

Conclusions: Tagging approaches based on T-DNA insertion are effective only for plant species (like Arabidopsis and poplar), whose are easily transformable in combination with high frequencies of tagged lines obtained. An advantage of T-DNA based activation tagging could be that even T-DNA insertion sites are not randomly distributed in the genome but do show some insertion site preferences to the 5'UTR of a gene coding region. For transposable elements, however, new insertion sites were found scattered throughout the genome at many unlinked sites. But similar to [5] results for poplar, also other reports describe preferential transposon insertion around transposon donor sites [7].

The fact that a transposon is able to jump to other chromosomes, thus passing chromosomal boundaries, leads to the convenient situation that only a few primary transposon transgenic lines are sufficient for the establishment of large transposon tagging populations in order to tag at least theoretically every gene in a tree genome. This is obviously difficult for T-DNA tagging as plant transformation is time consuming and, therefore, the genome can't easily be saturated with T-DNA tags.

Taken together, so far the strategy for both T-DNA and transposon activation tagging is first to phenotype an existing tagging population and then to determine the new genomic insertion locus of the tag. A novel strategy of activation tagging can be suggested based on the described power of the ATDs transposon approach and the simplicity to induce ATDs transposition *in vitro*. The ATDs-based strategy allows first the production of a very high number of independent ATDs-transposed plants whose can be screened for new ATDs flanking genomic loci.

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O52

Building up resources and knowledge to unravel transcriptomics dynamics underlying *Eucalyptus globulus* xylogenesis

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The economic importance of some *Eucalyptus* species, including interspecific hybrids, has been extended from the traditional interest of pulp and paper production to the emergent areas of bio-fuels and bio-materials. New genomic resources and high throughput technologies have provided the *Eucalyptus* research international community with the opportunities to identify genomic regions of interest in order to comprehensively dissect, catalogue and characterize genes involved in the determination of wood formation and quality. Similar strategies can be now applied to identify key regulator genes and better understand the cellular mechanisms by which they modulate the complex molecular events occurring in xylogenesis.

The *Geneglob*^{wq} project (2006-2010) produced and used a set of genomic tools which, associated with Next Generation Sequencing (NGS) technologies, allowed us to expand the knowledge of the *Eucalyptus* genome by focusing on regions potentially involved in the determination of wood properties, namely pulp yield and lignin content. We first start to characterize two *E. grandis* BAC libraries [1] constructed by the Arizona Genome Institute (with DNA from the clone Brasuz S1 whose genome was sequenced recently by the DOE [http://www.jgi.doe.gov/sequencing/why/99176.html], and two *E. globulus* BAC libraries made available by RAIZ [http://www.raiz-iifp.pt/]. We than used 3D-pools of BAC libraries and BAC macroarrays to characterize genomic environment of several lignin and lignin-regulator genes (e.g. *EguCCR*, *EguCAD2* and *EguRAC1*) both in *E. grandis* and *E. globulus*. The shotgun sequencing of selected BAC clones containing those genes generated a high amount of sequencing data that made it possible to map the *E. globulus* BAC sequenced clones against the *E. grandis* genome (8X coverage). These comparative analyses showed extended microlinearity between both genomes, at least in the studied regions. Additionally, we have sequenced and annotated the chloroplast genome of *E. grandis* (GeneBank Accession NC_014570) [1].

A global approach to unravel *E. globulus* transcriptome dynamics has also been included and structured in the *Geneglob*^{wq} project, aiming at the identification of genomic hotspots of transcription activity. Various *E. globulus* xylogenesis "models" have been considered comprising several paired, contrasting wood forming tissues (provided by RAIZ): i) xylem samples collected along the year (season variation); ii) juvenile and adult individuals of a single genotype; iii) contrasting genotypes for pulp yield. Samples from these tissues were used for transcriptome sequencing using *IlluminaHi-Seq* technology (*mRNA-SEQ*). The same *E. globulus* genotype used for both *E. globulus* BAC libraries (a parent tree used in controlled crosses by RAIZ) has been re-sequenced (pair-end 100bp), and provided the first draft of an *E. globulus* genome. This resequencing data was mapped against the *E. grandis* Brasuz S1 reference genome. Transcriptomic data were also blatted against the gene models annotated in *E. grandis* genome, to evaluate *in silico* the expression of each gene.

More recently, the *microEGo* project (2010-2012) started the identification and characterization of *Eucalyptus globulus* microRNAs and their target genes, involved in the regulation of wood formation. The *E. globulus* season variation xylogenesis "model" was used considered for this project as well as an *E. globulus* reaction wood "model". The latter comprises reaction wood tissues (tension / opposite wood) formed in bent trees at different kinetic times of gravitropic stimulation and control wood (non-bent trees). Small RNA libraries have been generated from those tissues and sequenced using *IlluminaHi-Seq* technology (*Small RNAs-SEQ*). The sequencing data from both *microEGo* and *Geneglob*^{wq} projects together with a genome wide bioinformatics analysis of *E. grandis* reference genome and *E. globulus* genome are being used for identification of miRNA gene and putative their putative target-genes.

These projects will hopefully constitute an important piece in the assemblage of whole new categories of knowledge and genomic resources for the *Eucalyptus* community, providing insights into the nature of the molecular machinery involved in wood formation and most importantly in the identification of key players determining the variability of wood characteristics and its end-uses.

Acknowledgments: This work has been partially supported by Fundação para a Ciência e Tecnologia (Portugal): http://PTDC/AGR-GPL/098179/2008, "microEGO - Did you ask for something small? The microRNAs power in a *Eucalyptus* tension world! (https://sites.google.com/site/microegopublic/); PTDC/AGR-GPL/66564/2006, "GenEglob^{wq} - Scanning for candidate genes underlying a pulp yield QTL in *Eucalyptus globulus*" (http://geneglob.inesc-id.pt/). We would like to aknowledge ALTRI FLORESTAL SA (Portugal) and RAIZ (Portugal) for providing the plant material used in these studies.

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S9. BIOSAFETY, CERTIFICATION AND ECONOMICS OF TREE BIOTECHNOLOGY

O53

Highly regulated but can't be certified as sustainable. Responsible use principles are bridging the gap for biotech trees

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Biotech trees are some of the most heavily regulated plants in the world, yet no forest management system certifies the use of them as sustainable. The Responsible Use: Biotech Tree Principles are the first, and

only, set of practices designed specifically for the long-term stewardship of biotech trees. These Principles may serve as a framework that certification systems can use when considering whether biotech trees meet their certification standards.

Responsible use principles were developed to guide the long-term stewardship of biotech trees. As forest biotechnology has taken root and genetically modified trees are being field-tested for biofuels, species restoration, and disease resistance, the need for a set of global guiding principles for responsible use of these trees became apparent. Since this need was not being met by the international forestry certification schemes, the Institute of Forest Biotechnology (IFB) developed the Responsible Use: Biotech Tree Principles in a transparent, stakeholder driven process over nearly three years.

A broad set of stakeholders have set aside the issues of whether biotech trees should be used and created these stewardship principles. The Principles are in recognition that responsibly used biotech trees have the potential to benefit society, economies, and the environment in ways that other trees cannot. Central to these Principles are core beliefs that:

• Biotech trees should benefit people, the environment, or both
• Risks and benefits of biotech trees must be assessed
• Transparency is vital and stakeholders must be engaged
• Social equity and indigenous rights are important and must be respected
• Biotech tree use must follow regulations of the appropriate country

Regulations for biotech trees are stringent in most parts of the world. From product inception in the lab, to seedlings grown by tree farmers, it can take years and cost millions of dollars – and the biotech tree may never get deregulated. Long growth cycles make gathering scientific information necessary for deregulation difficult in many countries. However, regulations vary widely in practice from one country to another. We will discuss the regulatory frameworks of biotech trees in Brazil, Chile, U.S., Canada, South Africa, China, and New Zealand.

Certification systems have yet to address biotech tree use in a holistic, scientific manner. We will discuss how the top certification systems in Brazil, Chile, U.S., Canada, South Africa, China, and New Zealand address biotech trees. Along with the regulatory review of these countries, a framework will be developed that shows where regulations and certification systems overlap, diverge, or have significant gaps. This framework will be a tool to begin a dialogue with certification systems on how to best bridge these gaps using the Responsible Use: Biotech Tree Principles.

People have highly disparate opinions about using biotech trees. The reality is that biotech trees are already in use today, and they will be put to use even more in the future. It is important that we engage society, assess environmental risks and benefits, and follow best practices for the stewardship of biotech trees and their products. The IFB, through the Responsible Use Initiative, will work with relevant forest certification systems to provide science-based, stakeholder driven assessments that will help meet the requirements of their own sustainable forestry mechanisms.

The IFB is the only organization to address the sustainability of forest biotechnology on a global scale. The IFB has been in operation for 10 years as a non-profit organization. In December 2010 the IFB published the Responsible Use: Biotech Tree Principles. The Principles along with a comprehensive Biotech Tree Primer are available at http://www.responsibleuse.org. Learn more at IFB's main website, http://www.forestbiotech.org.

O54

Assessing ectomycorrhizal associations and transgene expression in transgenic *Castanea dentata*

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Background: American chestnut (*Castanea dentata*) once dominated the forests of the eastern United States until the introduction of a Chinese fungal pathogen *Cryphonectria parasitica*(causal agent of the chestnut blight) decimated the species. This tree was important not only ecologically, but also played a significant role economically. Restoration programs are implementing a variety of techniques, including genetic transformation, to develop a blight resistant tree that can be reintroduced into the native chestnut range (S.A. Merkle, 2007, *Tree Genetics and Genomics*, 3, 111-18).

Specific genes involved in plant defense responses are being introduced into American chestnut via *Agrobacterium*-mediated transformation, with the hope that one gene or a combination of genes will aid in chestnut defense against blight (L.D. Polin, 2006, *Plant Cell, Tissue, and Organ Culture*, 84, 69-79). One gene of particular interest to the transgenic project is oxalate oxidase, a defense related gene from wheat that has also been shown to enhance the defense response of other plants when introduced through genetic transformation (H. Liang, 2001, *Plant Molecular Biology*, 45, 619-29). American chestnut transformation with a binary vector containing oxalate oxidase has produced a number of transgenic events, many of which have already been regenerated plants (unpublished data).

Before releasing any transgenic plant it is necessary to assess any non-target impacts the introduced genes may have on associated microbial communities. *C. parasitica*is a fungal pathogen, so we are particularly interested in studying the effects of any transgene on the symbiotic relationship between the host plant and mycorrhizae-forming fungi. Prior studies have examined the potential impact of transgenic plants on ectomycorrhizal fungi (K. L. Oliver, 2008, *Applied and Environmental Microbiology*, 74, 5340-48).

Objectives: 1. Analyze and compare transgene expression in root, stem, and leaf tissue in American chestnut trees transformed with a gene for oxalate oxidase.

2. Assess potential non-target impacts on mycorrhizal fungi by comparing ectomycorrhizal associations with wildtype American chestnut and a transgenic American chestnut expressing a gene for oxalate oxidase.

Methods: Wildtype American chestnut seeds and transgenic American chestnut ('Darling4') trees were grown in a potting mix containing field soil in a soil bioassay to bait for mycorrhizal fungi. All trees were grown in a greenhouse for at least one year before mycorrhizal root tips were harvested. The root system of each tree was rinsed over a fine sieve, and segments of root were collected. Mycorrhizal root tips were quantified and grouped based on morphotype. Fungi were identified using RFLP and sequence analysis of the fungal ITS region.

RNA was extracted for gene expression studies from all tissues using a CTAB extraction method. RNA samples were then DNase treated and used to synthesize cDNA, which was used as a template in all subsequent quantitative RT-PCR experiments. qRT-PCR studies were conducted to determine the relative level of transgene expression in leaf, stem, and root tissue in 'Darling4' trees.

Results: Gene expression study results based on qRT-PCR indicate that the oxalate oxidase gene is expressed in root tissue of 'Darling4' American chestnut and not in the wildtype control (Fig. 1). Transgene expression results for both stem and leaf tissue are comparable to that found in root tissues.

Based on mycorrhizal tip quantification for transgenic and wildtype American chestnut, it appears that there is not a significant difference in total percent fungal colonization between these tree types (Fig. 2).

Discussion and conclusions: The results of the gene expression portion of this study show oxalate oxidase is expressed in the root tissue of 'Darling4' transgenic American chestnut trees. As expected, there is no oxalate oxidase expression in root tissue of wildtype American chestnut trees. Oxalate oxidase expression in root tissue has the potential to influence associated microbial communities, specifically mycorrhizal fungi that are in direct contact with chestnut roots. It is therefore necessary to assess mycorrhizal associations in transgenic chestnut to determine if there are any non-target impacts as a result of this expression. Mycorrhizal root tips were quantified for both the Darling 4 transgenic chestnut and the wildtype. The results of the total percent ectomycorrhizal fungal colonization portion of the study show that there is no significant difference in overall levels of mycorrhizal abundance between the two tree types. It can be inferred from this that American chestnut trees expressing oxalate oxidase are able to form mycorrhizal associations in a similar manner to the wildtype.

As with any other transgenic plant, 'Darling4' and other transgenic American chestnut trees are required to go through extensive deregulation procedures before they can be released back to the native chestnut range. The results from this study will be used in conjunction with other ecological impact studies to inform the deregulation process in

an effort to restore the American chestnut. This study will also contribute to a body of existing work examining the potential environmental impacts of transgenic forest trees that can inform future policy and procedures relating to the genetic modification of important species.

O55

Mycorrhization of transgenic apple trees with increased resistance against fungal pathogens

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The project 'Mycorrhizal symbioses of transgenic apple trees with increased resistance against fungal pathogens' (Subproject of the framework 'Biosafety of transgenic woody plants', supported by the German Federal Ministry for Education and Research) investigates whether the transformation of apple trees with fungal chitinase encoding genes - besides increasing resistance against fungal pathogens - also interferes with mycorrhizal fungi. Pathogenic fungi such as the ascomycete species *Venturia inaequalis*, the pathogenic agent of apple scab, cause severe damage in apple cultivation. In commercial apple production, pesticides are employed frequently and in high quantities. Alternative methods such as the use of mycoparasitic *Trichoderma* fungi to control fungal plant pathogens have been explored [1]. Genetic engineering of apple cultivars with *Trichoderma* chitinases has lead to an increase of the trees' resistance against apple scab [2]. However, like many fruit tree species, apple trees form symbiotic associations with arbuscular mycorrhizal fungi (AMF) at their roots. AMF receive fixed carbon compounds, while the plants benefit from the symbiosis by enhanced uptake of nutrients and water, promoted growth, and increased resistance against biotic and abiotic stress [3]. Like in most higher fungi, the cell walls of AMF contain chitin as a major skeletal component [4]. Consequently, the symbiosis between apple and AMF may be negatively affected by the expression of *Trichoderma* chitinase genes within the plant. At the Julius Kühn-Institute in Dresden, the apple cultivar 'Pinova' was transformed by introducing the construct pBIN (Endo + Nag) into the transgenic lines. With the help of promoter CaMV 35S the transgenic lines constitutively express endochitinase gene *ech*42 and exochitinase gene *nag*70 from the biocontrol fungus *Trichoderma atroviride*. In order to study whether the constitutive expression of *ech*42 and *nag*70 has an effect on mycorrhization of plants with transgenic roots (local effect) and – as apple cultivars are propagated vegetatively by grafting– whether expression of *ech*42 and *nag*70 in a transgenic scion grafted on a non-transgenic rootstock has an effect on mycorrhization (systemic effect), a series of greenhouse experiments was conducted. In a first experiment, non-grafted clones of wildtype apple cultivar 'Pinova' and two transgenic lines were cultivated in artificial substrate and inoculated with two AMF species, *Glomus intraradices* and *G. mosseae*. After four months of cultivation, root colonization rates were significantly lower in the transgenic lines compared to the non-transformed cultivar 'Pinova' [5]. Samples were taken again after another four months of cultivation under enhanced inoculation pressure (increased inoculum mass, decreased nutrient availability in the substrate). The experimental approach was broadened by using soil from different field sites in order to supply the sample trees with complex soil matter, AMF adapted to the very soil conditions, and the appropriate soil bacteria which also play a role in the formation of mycorrhizal associations. Two sets of non-grafted sample trees (clones of apple cultivar 'Pinova' and two transgenic lines) were cultivated in the greenhouse; one set in pots with soil from an intensively managed apple plantation, the other set in pots with soil from an extensively managed apple orchard. Root sampling was conducted three and six months after transferring the plants to soil. To assess the systemic effect (expression of *ech*42 and *nag*70 in a transgenic scion grafted on a non-transgenic rootstock) on mycorrhization, two further experiments were carried out. Grafted sample trees (clones of apple cultivar 'Pinova' and two transgenic lines, all grafted on commonly grown rootstock M9) were cultivated in the greenhouse in pots with artificial substrate and inoculum of *Glomus intraradices* and *G. mosseae*. Root samples were taken after four and eight months. A second set of grafted sample trees was cultivated in pots with soil from an intensively managed plantation in the 'semi-natural' environment of a gossamer cage. Root samples were taken after 12 and 24 months. For all experiments, root colonisation rates were determined microscopically. Molecular methods – PCR with taxa specific primer sets [6], cloning of PCR products, Sanger sequencing – were applied to study the impact of the transformation on the composition of the fungal communities associated with the roots of the sample trees cultivated in soil. Chitinase activity in leaves and roots of sample trees was determined using fluorescence spectrometry and substrates 4-methylumbelliferyl N-acetyl-β-D-glucosaminide for exochitinase and 4-methylumbelliferyl β-D-N,N',N''-triacetylchitotrioside for endochitinase activity. The results of this biosafety research concerned with potential adverse effects on benefitial soil fungi will be presented and discussed.

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O56

Introduction of alkali-labile units into lignin in transgenic plants by genetic engineering

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Background: Lignin is one of major components of plant secondary cell wall. In plant cell wall, it is synthesized via radical coupling of precursors such as *p*-coumaryl, coniferyl, and sinapyl alcohols. In early stage of the lignification, 8-O-4', 8-8' and 8-5' dimers are thought to be synthesized mainly from the precursors in the wall. A gram-negative bacterium,

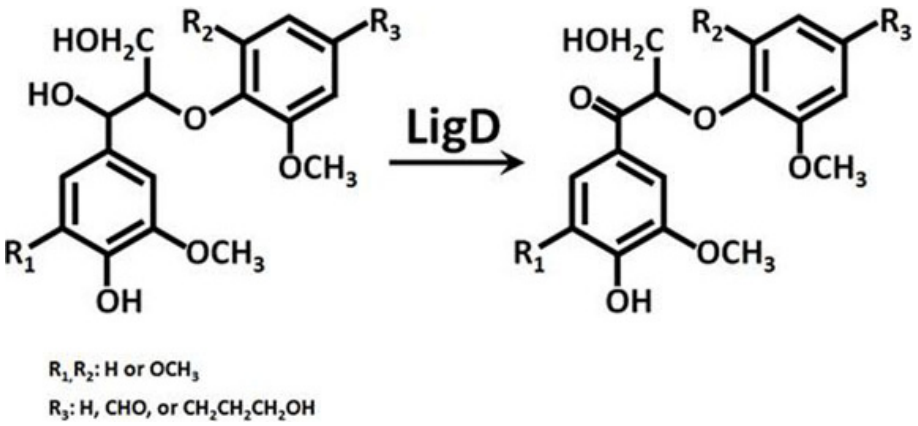

Figure 1(abstract O56) The reaction catalyzed by LigD. This bacterial enzyme catalyzes oxidation of benzyl position of 8-O-4′ lignin dimers. Both of guaiacyl-guaiacyl and syringyl-syringyl dimers can be oxidized by the enzyme.

Shingobium sp. strain SYK-6 (hereafter refer to as SYK-6) is able to catabolize a wide variety of phenolic compounds including the lignin precursors by its unique enzymatic system. One of catabolic enzymes, LigD, catalyzes oxidation at alpha (benzyl) position of 8-O-4′ dimers and forms carbonyl group at the position (Figure 1). This oxidation is the first step of catabolic pathway of 8-O-4′ dimers in SYK-6. When we express LigD polypeptide in the cell wall of transgenic plants, the oxidative dimers will be expected to be generated and then incorporated into lignin polymer. In some past studies, it has been shown that the presence of carbonyl groups at the alpha position of aryl propane units in lignin greatly speeds up the rate of cleavage of beta-aryl ether linkages during kraft pulping condition [1,2]. In order to contribute to efficient and sustainable production of kraft pulp and the other biomass-derived products such as bioethanol, we introduced the *ligD* gene into *Arabidopsis* and hybrid aspen and tried to generate transgenic plants whose lignin can be easy to remove from hollocellulose fraction under alkaline conditions.

Method: Because of codon usage is significantly different between genes in plants and SYK-6, we chemically synthesized open reading frame (ORF) of the *ligD* gene for improving its expression in the transgenic plants. After addition of nucleotide sequence for apoplast-targeting signal peptide to the synthesized *ligD* ORF, it was introduced into *Arabidopsis thaliana*, tobacco BY-2 and hybrid aspen under the control of cauliflower mosaic virus 35S promoter. LigD expression in the transgenic plants was monitored by Western blot analysis and enzymatic activity with crude extract prepared from each transgenic line. Preliminary analysis of lignin structure by 2D-NMR and nitrobenzene oxidation was also performed.

Results and discussion: At first we confirmed expression of the *ligD* transgene in *Arabidopsis* by Western blot analysis with antiserum against LigD polypeptide. Positive expressions of the *ligD* were detected in some of the transgenic plants analyzed. Enzymatic activities of LigD in crude extracts prepared from both cytosolic and apoplastic fractions of the transgenic *Arabidopsis* plants were also detected, but it was relatively higher in the latter case. As expected, 2D NMR (^{1}H-^{13}C HMQC) analysis suggests that the abundance of the alpha-keto (alpha carbonyl) structure in 8-O-4′ units of lignin in the transgenic plants is relatively higher than that in the wild-type plant. Chemical compositions of lignin (syringyl/guaiacyl ratio) and neutral sugars could not be distinguishable between the transgenic and wild-type plants. Generation of transgenic hybrid aspen and its analysis are now in progress.

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O57

Environmental and health risk assessments of genetically modified eucalypts in Brazil

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Motivated by the Brazilian Ministry of Agriculture, Farming and Supply (MAPA), we created in 2009 the "Collaborating Center in Agriculture Defense Relative to the Biosafety of Genetically Modified Eucalypts" (Project "CDA *Eucalyptus*") in order to collect information and conduct research to assess the biosafety of GM eucalypts in the Brazilian context. The Normative Resolution Nr. 5 of the National Biosafety Technical Commission (CTNBio) is the official document presenting all information needed to propose the commercial release of GMOs in Brazil. Based on this document and along with the personnel of the Suzano Paper & Cellulose Co., we conducted a series of experiments with GM and non-GM eucalypts planted in a test field in the state of São Paulo to start collecting the necessary information. Two independent groups of transgenic plants, harboring two different transgene constructions along with non-GM control plants are being assayed. The genetic traits, the identity or names of the transgenes as well as the identity of each tree individual will not be revealed due to intellectual property request still pending. Each group of plants was represented by four independent events in triplicates (2 groups x 4 events x 3 clonal trees + 3 non-GM clonal trees), therefore totaling 27 individuals under analysis. Samples were identified by random numbers and all assays were conducted in a simple-blind or a double-blind fashion. Tests concluded until now included (i) the detection of transgene regulatory sequences in purified DNA samples by conventional PCR and RT-qPCR, confirming the expected sampling conducted; (ii) extraction, chemical characterization and analysis of the antifungal effects of essential (volatile) oils extracted from leaves; (iii) pollen germination *in vitro*; (iv) flower morphology; (v) seed production; (vi) initial seedling development; (vii) leaf allelopathy; (viii) measurements of total phenolic compounds in leaves and roots; and (ix) effects of leaf extracts on the viability of human colon cells. All results obtained from experiments (ii) to (ix) revealed no statistical differences between GM- and non-GM-derived samples. A second round of experiments will be conducted to confirm these results. Proteomic and transcriptomic profiling of GM and non-GM trees are under analysis, as well as a series of experiments that include the chemical, nutritional and biological analysis of honey samples derived from bee hives located in fields of GM *versus* non-GM plants; and bee (*Apis mellifera*) population dynamics.

IUFRO WORKSHOP COST ACTION FP0905

O58

Biological characterization of genetically modified trees (GMTs)

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In the last 20 years, a large number of GMTs have been generated, characterized and field tested to evaluate their agronomic behaviour and their potential performance for technological applications.
Therefore, important knowledge has been gained from these studies on the potential of GMTs whereas biosafety concerns have been raised on the plantation of transgenic trees in the field. The European Cooperation in Science and Technology (COST) action FP0905 on the biosafety of forest transgenic trees aims to help forthe building and implementation of EU policy directives to ensure a safe development and practical use of GMTs in the future. This Action is expected to generate important benefits thanks to the experience gained by the research community, to provide general information on these studies as well as to address public concerns about biosafety issues raised by transgenic tree plantations, and finally to help for the establishment and implementation of EU policy directives in the view of a future deployment of GMTs cultivation in Europe. Within this action, the objective of the working group 1 (WG1) is to gather the existing knowledge on genetically modified forest trees (GMTs) including both field trials and greenhouse experiments. More specifically, the action of WG1 is focused on the compilation of:
- Data from literature and methods developed on gene flow and containment strategies
- Data available on the traits targeted and the function of the gene introduced, on their intended and eventually unintended effects
- Data on host species characteristics especially related to biosafety issues such as their biological cycle, their way of pollination and the characteristics of pollen and seed dispersal.
This information will be collected from published papers in books and reviews as well as from reports from different agencies available on the web. Moreover, additional data will hopefully be gained through the responses of expert scientists from public or private institutions to an ongoing survey accessible at the website of the COST Action FP0905: [http://www.cost-action-fp0905.eu/index.php?option=com_kunena&Itemid=74&func=view&catid=4&id=11]
The information collected will be used to:
- 1) establish a list of protocols for the control of transgene flow and gene containment.
- 2) establish the present state of the art in gene targeting strategies for site specific integration of transgenes.
- 3) get an appraisal on the types of construct and genes inserted in GMTs in the past; in the present and in the future.
This information will be compiled in a database that will be made available to the scientific community as well as to the European policy makers.
The results of this work will enable the participants and end-users to get a clear factual overview of the global status of GMTs and especially the status of Europe compared to non-European countries. Furthermore, the information gathered will also be useful for assessment of environmental impacts of GMTs that is the object addressed in the WG2 of the COST Action FP0905.

O59

Environmental impact assessment and monitoring of genetically modified trees

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Transgenic biotechnology can assist forest tree improvement programs but it may also raise environmental safety concerns. The environmental effects of genetically modified transgenic trees (GMTs) have been studied in many countries during the last 15 years. Today there is an urgent need of putting together this scattered knowledge to build-up a European knowledge platform for addressing GMTs in plantations. The main aims of Working Group 2 (WG2) of the European Cooperation in Science and Technology (COST) Action FP0905 "Biosafety of transgenic forest trees" (http://www.cost-action-fp0905.eu/) are (1) to discuss, based on scientific facts, whether current containment strategies are appropriate or need to be improved for GMTs, (2) to define a common protocol to track the transgene from the laboratory to the final product, and (3) to assess the possible impacts of GMTs on the environment. The potential risks of GMTs, the fate of recombinant material and the potential relevance of recombinant genes on plantÂ´s omics are other main aspects to be considered and compared to similar processes with endogenous genes in conventional breeding. The group involves experts from public research, government and independent regulatory sectors across COST and non-COST member countries. The activities of this group have been organized into three Task Groups focusing on (1) risk assessment studies and guidance documents, (2) the monitoring of the transgenes and recombinant plant material, and (3) the impact of GMTs on exposed ecosystems. As a first step of WG2 activity, a database of guidance documents from national and transnational sources dealing with impacts and risk assessments of GMTs is being created to identify common and case-specific issues on biosafety. It is expected that the information gained will facilitate (1) a science-based understanding of the impacts of GMTs on the environment in comparison with that of traditionally tree breeding, and (2) future socio-economic and cost/benefits analyses of GMTs in plantations.

O60

Dissemination of Cost Action FP0905 information by website

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Background: The Cost Action FP0905 aims to evaluate the scientific knowledge of GMT biosafety protocols coordinating the existing information generated in various European countries as basis for future EU policy and regulation for the environmental impact assessment.

For spreading the information relevant to the Action, the website http://www.cost-action-fp0905.eu/has been set up, educating the general population of the technical, social, environmental and economic aspects of this issue. The principal aims of the website are: to provide a database with the main information on forest GMTs, that should be available to the scientific community and Europe organisations; to update the website with science-based information for public interest in the utilization of GMTs in forest plantation and at the same time safeguarding the environment, using all the information collected to identify particular topics which could be useful to develop research projects collaborating with the other working groups.

Material and methods: The Website was built using the Open Source Software Joomla, a program for web site design and content management. This software has not been written for profit, but to benefit the user community. The difference between Joomla and other web design software systems is that there are thousands of fully functional, complete web site templates available in the Internet, so that all what is to do is to choose the layout you like and to add information in the templates.

However, Joomla as a "Content Management System" is not only a "simple" web site construction program, furthermore it can also be used as a database. It is used to build/design a "php/mysql database" to

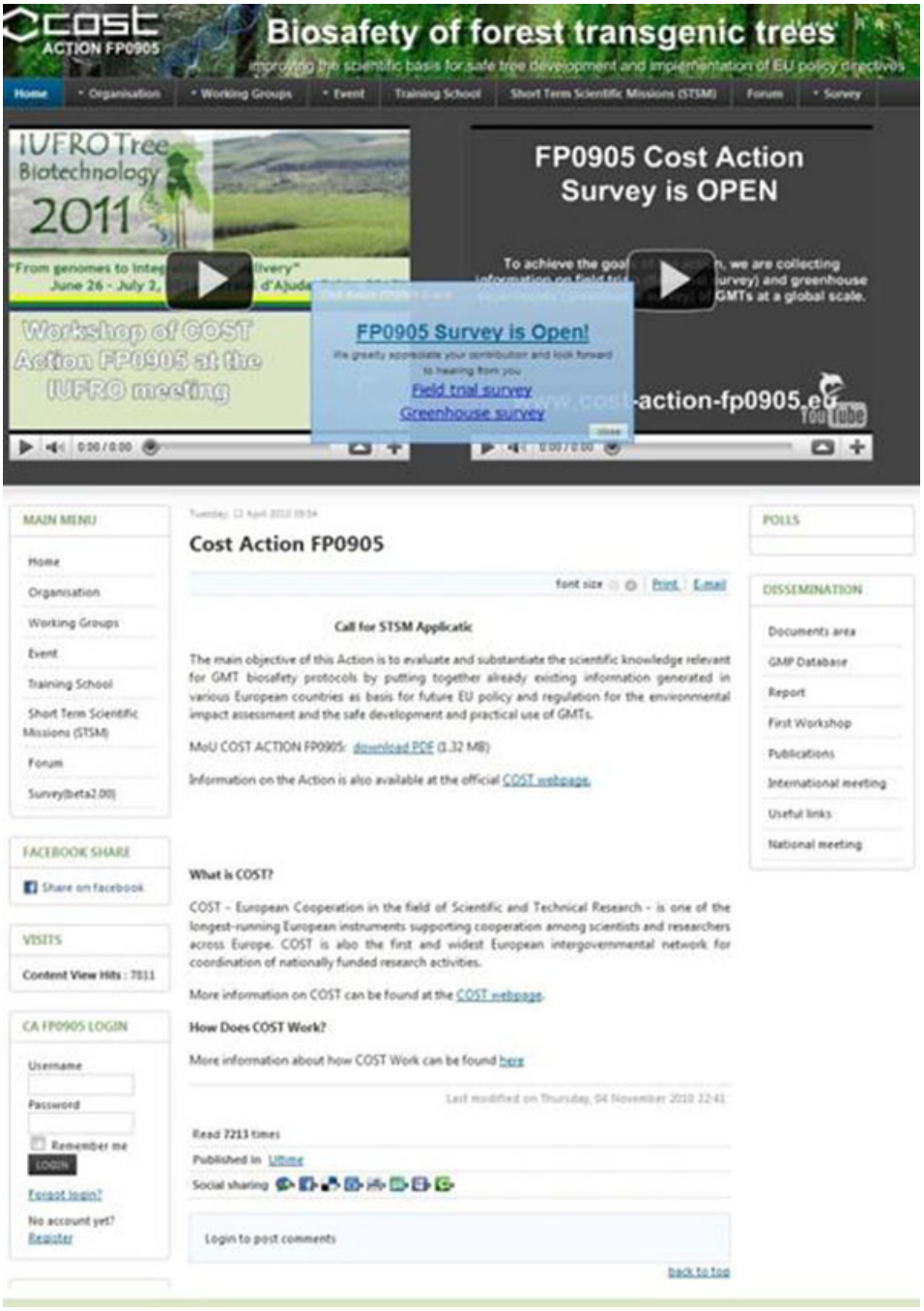

Figure 1(abstract O60) image of the COST Action FP0905 website.

collect the information. But the possibilities of the program on how to use it as a database are endless. In this way it will be simple to create the database tables and fields. The software generates the scripts to upload them into the website, to setup the MySQL tables and the scripts and to manage the tables once uploaded.
The most important features of the Joomla program are:
1. Instantly publish PHP MySQL database applications ready for uploading on the website of Action.
2. Manage easily the database.
3. Automatic generation of php forms to display and browse data content of tables, with the ability to edit, delete and add records.
4. Data navigation, sorting, searching, search filtering, modification, addition, deletion, file uploads. The website is also a very friendly searching engine. Google will regularly visit this website site, due to the frequent content changes that result from the news feeds. They are usually updated on a daily basis, even if the web site is not updated on a regular basis.
Results: Working group 4 is developing and managing the web site of the Action enhancing communication and information among the participants and with those organisations interested in the data provided. The website has provided a forum for the organizations working within and outside the consortium; discussing for example, any effect of a transgenic plantation needs in the context of already existing and accepted forestry practise. The Action has also been introduced in facebook.

O61

Field trials of GM trees in the USA: activity and regulatory developments

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Field trials are extremely important for all facets of research, breeding, and biotechnology. Tree physiology is distinctively different in greenhouses or growth chambers vs. field environments. Thus, results from non-field environments can be very misleading with respect to identification of promising biotechnologies and elite varieties. Large regulatory impediments to conduct of field tests of genetically modified (GM) trees will therefore tend to stifle scientific and technological development. This appears to be the case for genetically modified trees, where regulatory burdens to conducting field tests have grown in stringency in the USA and elsewhere in recent years [1,2]. Here, we present the record of recent field trials and some new regulatory developments in the USA.
Overview of field trials in the USA: Information Systems for Biotechnology (ISB) [3] maintains an easily used database of GM crop data in the United States. There have been nearly 600 field trials (including both "acknowledgments" and "permits") conducted since 1989, with a five-fold increase occurring in 2000-2009 compared with 1990-1999 (Figure 1).
Fifteen different genera have been tested, including *Populus*, *Pinus*, *Eucalyptus*, and *Malus*, with the first two accounting for approximately 60% of all field trials (Figure 2). Marker genes and tolerance to biotic stresses were the two most common research objectives. Private companies carried out 60% of field trials, and their activity has grown dominant in recent years.
Currently, there are more than sixty field trials covering eleven different genera, with *Populus* accounting for 35% of all field trials. *Eucalyptus*, *Liquidambar*, and *Malus* together account for another 37% of trials. Other genera being tested include *Castanea* (American chestnut), *Pinus*, *Ulmus* (American elm), *Prunus*, *Musa*, *Citrus*, and *Juglans*. Forty field trials are being conducted by private companies, with ArborGen alone accounting for 36 of the 40 active field trials. Cornell University, North Carolina State University, Oregon State University, Purdue University, the United States Department of Agriculture (ARS) and the University of California, Davis are among the public institutions currently conducting field trials. One of ArborGen's field trials with cold tolerant *Eucalyptus* covers 197.2 acres. This is the largest current field trial in terms of acres and is being conducted in six different states. In terms of research objectives of current trials, marker genes dominate in terms of frequency of trials, with modification of wood quality the next most common objective.
Regulation of field trials in the USA: In the USA, transgenic trees are regulated in the same manner as agricultural crops [4], using regulatory laws created prior to the development of transgenic biotechnology. The three agencies involved are the United States Department of Agriculture (USDA), which considers agricultural safety and economics; the Food and Drug Administration (FDA), which considers human and animal feed safety; and the Environmental Protection Agency (EPA), which considers pesticidal properties of transgenic organisms [5]. Regulations in the United States are perceived to be less stringent than those in Europe and Asia, however, a recent survey in the USA found that forest scientists—including breeders, biotechnologists, and ecologists—see regulations as major obstacles to field research and commercial development of GM trees [6].

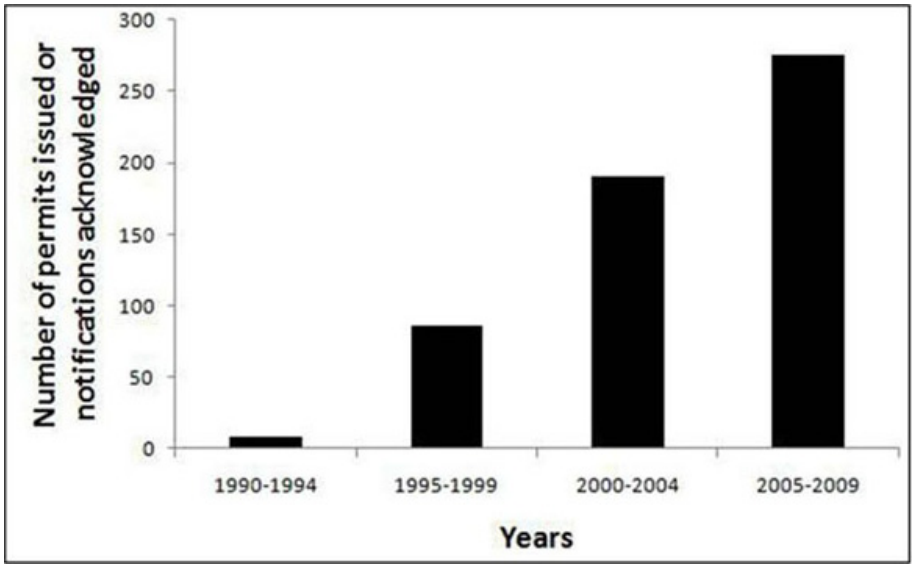

Figure 1(abstract O61) Total number of field trials with transgenic trees in the USA between 1990 and 2009.

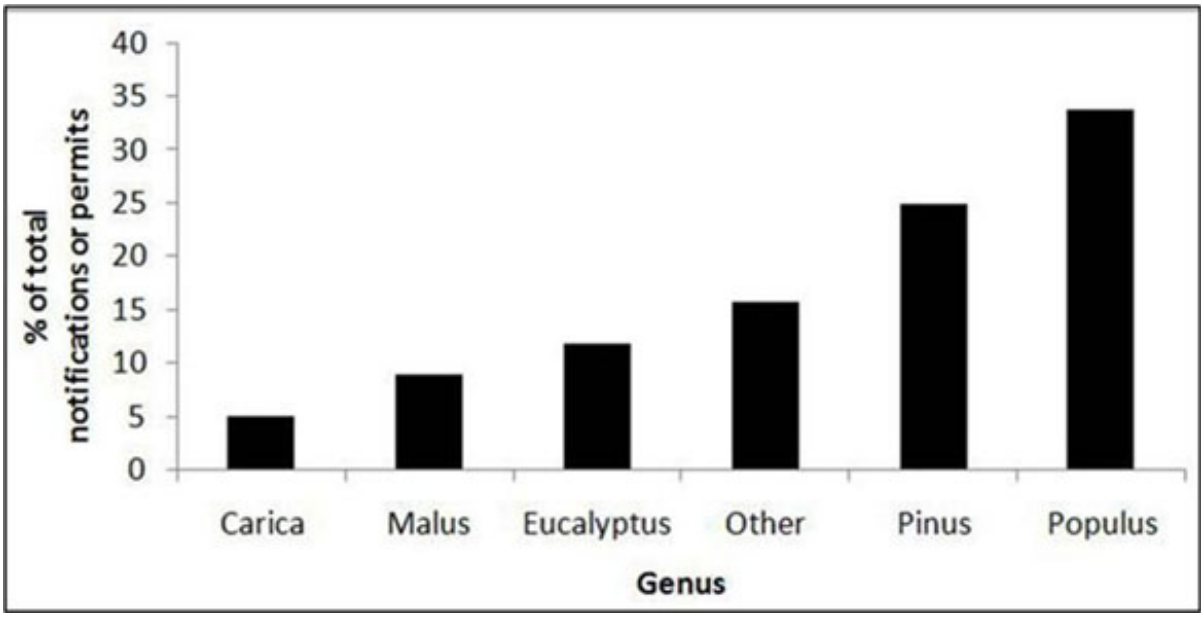

Figure 2(abstract O61) Major genera of transgenic trees in field trials in the USA, 1990 to present.

Recent regulatory developments: A company known as Okanagan Specialty Fruits has taken the lead in the production of transgenic tree varieties produced by the insertion of cisgenes and intragenes [7]. Non-browning versions of established apple varieties were developed by the company by the silencing of the polyphenol oxidase gene. These apples have been named Arctic™ apples because of the color of their skin. The company has petitioned the Animal and Plant Health Inspection Service (APHIS) for deregulation of the product in the USA [8].

Cold tolerant eucalyptus varieties were developed by ArborGen [9]. The company believes that these varieties would permit the planting of highly productive eucalypt hybrids for bioenergy and pup north of Florida in the USA. In May 2010, the USDA authorized a large scale flowering field trial of these trees, thus permitting the planting of nearly 260,000 trees over ~300 acres in 7 states [10]. However, several environmental organizations filed a lawsuit against APHIS because they believed that APHIS performed an inadequate environmental analysis, thus violating the US National Environmental Policy Act (NEPA).

A new two year pilot project has recently been announced by APHIS [11]. Under the provisions of the new project—whose aim is to speed up the review process before deregulation of biotech crops—environmental assessments of transgenic crops will be conducted by companies themselves, or by contractors authorized by the USDA. Although USDA would still need to review and approve the analyses, critics of the new policy believe that it might lead to less stringent environmental analyses.

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O62

Research in tree genetic engineering; the Canadian context for field trial of GM trees

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BMC Proceedings 2011, **5(Suppl 7):**O62

Canada is in a unique position for the regulation of GM (genetically modified) plants since it is based on the novelty of the resulting traits and not on the process to obtain it. For this reasons the term "plant with novel traits" (or PNTs) is used and these traits can be introduced into plants using biotechnology, mutagenesis or conventional breeding techniques. In Canada, less than a handful of GE tree field trials have been conducted. In all cases, poplars or spruces were used. Consistent with international guidance provided for living modified organisms, particular attention is given to a specific trait by considering both its novelty for the Canadian environment and its potential effect on human health and the environment. Our research is in line with the development of a consolidated research approach and knowledge needed for international guidance (e.g. Cartagena Protocol on Biosafety under the Convention on Biological Diversity) developed for genetically modified organisms. Important losses due to insects and fungal pathogens result in a considerable reduction of yield in Canadian forest productivity. Compounding these problems is the increased movement of pests that has been facilitated by increased international trade of agricultural and forest products as well as global climate change. More recently the increased need for biomass production and specific bioproducts to sustain crop-based biofuel production has emerged. Forest biotechnology

has the potential to maintain forest sustainability by contributing to the transformation of our forest industry so as to improve productivity in the context of tree plantation. In this presentation, we will show how genetic engineering of forest trees has been used in improving tree adaptation and pest protection. We will also describe the work being done on environmental assessment of field-grown trees and results on long-term expression of engineered genes.

O63

A brief overview of field testing and commercial application of transgenic trees in China

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Since the first report on transformation of *Bt* gene into *Populus nigra* was published in 1989, transgenic approaches have been widely used in breeding trees for high tolerance to insects and environmental stresses in China. Notably, field testing of the transgenic poplar with *Bt* was performed in 1994 and permitted to be commercialized as the first commercialized transgenic tree in the world. Up to now, nearly 50 species have been used as recipients for genetic transformation including poplar, birch, locust tree, walnut, aimed to improve their tolerance to insects and diseases, environmental stresses, prolonged storage lifetime, wood property, flowering time control, etc..

On May 5, 2006, the State Forest Administration (SFA) launched its first regulatory framework on "Management measures on the inspection and permit of forest genetic engineering" taken effect on July 1, 2006. It states that small-scale field testing, environmental release and pilot production test of each transgenic line are required before its permit of commercialization, with each phase must be evaluated by the experts. "A technical guideline on safety evaluation of the transgenic forest plants and their products" was published by SFA on June 4, 2007, which provides a standard procedure for molecular breeders to follow in order to commercialize their transgenic trees.

To the year 2010, 128 field trials have been granted by SFA, 84 of which belong to tree species while 44 to grass species. Thirty three of them were granted to transgenic poplars, with tolerance to insects and diseases, drought and salt stresses, altered wood property, etc. , and 25 to locust tree. Transgenic *Populus tomentaosa* with antisense *CCoAOMT* (coding for a key enzyme involved in lignin monomer biosynthesis) , transgenic *Robinia pseudoacacia* with *BADH*, *Sophora japonica* with *RD29A* and *P. nigra* with *Bt* are under environmental release testing.

The transgenic poplar plantation has increased to 450 hm^2 this year since two *Bt* transgenic poplars were commercialized in 2001 in China. The transgenic poplar plantations have effectively inhibited the fast-spread of target insects and significantly reduced the times of insecticide application on poplar plantation. The transgenic *Populus nigra* was also used in hybridization with non-transgenic *P. deltoides* as an insect-resistant source for breeding new hybrid clones.

S1. POPULATION GENOMICS, CONSERVATION AND ADAPTATION

P1

Identification of microsatellite loci in *Pinus tecunumanii*

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Background: *Pinus tecunumanii* has displayed good performance in tropical regions of Brazil and showed high potential for commercial exploitation. Embrapa Forestry and its partners own many of the species seed production areas. In spite of its importance, the majority of P. tecunumanii germplasm collections remain still genetically uncharacterized. Thus identifying genetic markers is an important tool to genetically characterize these collections.We describe the initial steps to develop microsatellites for *Pinus tecunumanii* by enriched library construction with the ultimate goal of characterizing accessions of the germplasm collections of EMBRAPA.

Methods: The genomic-enriched library was constructed following the protocol described by [1]. The genomic DNA of *P. tecunumanii* was digested with AFA*I* and enriched in $(CT)_8$ and $(GT)_8$ repeats. Enriched fragments were amplified by polymerase chain reaction (PCR), connected to a pGEM T-easy vector and transformed into competent XL1- blue *Escherichia coli* cells. The positive clones were selected using the B-galactosidase gene and then grown overnight in an HM/F medium with ampicillin. After PCR 95 positive clones were sequenced in both directions using the T7 and SP6 primers as well as the Big Dye terminator Kit. The sequences were assembled and edited in Seqman (DNAStar), the repetitive regions were found using the Simple Sequence Repeat Identification Tool [2]. Primer select (DNAStar) and Primers Plus were used to design primer pairs flanking the microsatellite regions.

Results and conclusion: Of the ninety five sequences cloned only eleven contained microsatellite sequences and five showed repeats and adequate flanking regions for primer design. The observed proportion of dinucleotide was 5.26% (5), while trinucleotide and tetranucleotide proportions were 1.05% (1) and 5.26% (5), respectively. Ninety one percent of nucleotides were simply perfect and 9% were compost perfect. The explanation for this low yield (11.6%) can be attributed to the genomic-enriched procedure. To overcome this problem this procedure will be repeated. The obtained sequences will be used for validation of *P. tecunumanii* microsatellite primers and used to estimate the genetic diversity from the germplasm collection located in various regions of Brazil.

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P2

Development of microsatellite markers for *Pinus maximinoi* derived from microsatellite-enriched libraries

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BMC Proceedings 2011, **5(Suppl 7):**P2

Background: Integrating the use of molecular techniques in ongoing breeding can help identifying divergent genotypes to be used in controlled crosses for the development of hybrids, estimating parentage and monitoring the efficient use of genetic variation. Breeding programs carried out at EMBRAPA have established a broad population genetic base for various species of pine, from which trees with high productivity and wood quality have been selected. *P. maximinoi* was included in this program as its timber has specific characteristics of great economic importance. One of the main features of *P. maximinoi* is its rapid growth, reaching 20 to 40 meters in height with a diameter at breast height of 100 cm. The species' straight trunk with few branches is advantageous to the sawmill, and the timber's thick fiber is necessary for quality paper production. IN this work we report on the initial steps of the development of microsatellite markers for *P. maximinoi* derived from

microsatellite-enriched libraries. These markers will be used to characterize the breeding populations and germplasm collections of EMBRAPA.

Methods: A microsatellite-enriched genomic library was constructed following the protocol described by [1]. The genomic DNA of *P. maximinoi* was digested with AFA*I* and enriched for two microsatellite motifs $(CT)_8$ and $(GT)_8$. Enriched fragments were amplified by PCR, ligated to a pGEM T-easy vector and transformed into competent XL1- blue *Escherichia coli* cells. The positive clones were selected using the B-galactosidase gene and then grown overnight in an HM/F medium with ampicillin. After PCR, 88 positive clones were sequenced in both directions. The sequences were assembled and edited in Seqman (DNAStar), and the repetitive regions were found using the Simple Sequence Repeat Identification Tool [2] Primer select (DNAStar) was used to design primer pairs flanking the microsatellite regions.

Results and conclusion: Of the eighty sequences cloned only eight contained microsatellite sequences showing repeats and adequate flanking regions for primer design. The observed proportion of dinucleotide was 6.81%, while the tetranucleotide proportion was 2.27%. Seventy five percent of nucleotides were simple perfect repeats and 25% were compost perfect. The explanation for this low yield (9.09%) can be attributed to the genomic-enriching procedure. To overcome this problem the procedure will be repeated. The obtained sequences will be used for validation of *P. maximinoi* microsatellite primers. Eight microsatellite sequences of simply perfect and compost perfect nucleotides were observed for *P. maximinoi*. These markers will be validated and used to estimate the genetic diversity from the germoplasm collection of the Brazilian Agricultural Research Corporation (EMBRAPA).

Acknowledgments: The authors would like to thank Selma Buzzetti de Moraes and Mario Luiz Teixeira de Moraes for their assistance with DNA extraction procedures and the Valor Florestal Company for collecting material used in this research.

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P3

Analysis of expression patterns of candidate genes for bud set and cold tolerance in scots pine (*Pinus sylvestris* l.)

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Conifers, like other perennial plants adapt their growth rhythm to seasonal changes in the environment. Growth cessation and bud set are among the main adaptations used by the plants to prepare for winter. These adaptive traits are mainly controlled by photoperiod, temperature and light quality and show a clinal variation in different species including Scots pine. In conifers, efforts have been made to understand the genetic basis of these traits. In Norway spruce for instance, a *Flowering Locus T* homolog (*PaFT4*) has been shown to be implicated in the control of growth rhythm.

We previously evaluated genetic variation for timing of bud set and cold tolerance in a large association population of Scots pine (*Pinus sylvestris* L.) from Punkaharju, Southern Finland, along with five other populations used as control for clinal variation. To correlate this genetic variation with the expression pattern of some candidate genes already described in other species including conifers as involved in the control of bud set (e.g. FT4, PRR1, Gi, Myb) and cold tolerance (e.g. Aba responsive, DHN3, ERD, LEA), the Punkaharju population was used together with two others from Kolari (Northern Finland) and Poland. For photoperiod treatments, seedlings of eight families from each population were grown in a greenhouse under natural photoperiod conditions and in growth chambers under constant light for three months before transfer to three different photoperiod conditions (20 h/4 h, 17 h/7 h and 8 h/16 h light/dark). For cold treatments, the same families were grown in growth chambers under constant light for two and a half months and divided into two parts. The first part was directly submitted to the cold treatments (+4°C for one week followed by -5°C for 48 hours) while for the second part, seedlings were allowed to set buds under short day conditions (8 h light/16 h dark) before treatments. The results of this expression study will be described in relation to the phenotypic variance already observed.

P4

Construction of Brazil nut (*Bertholletia excelsa: Lecythidaceae*) cambium normalized cDNA libraries for 454 next generation sequencing

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Background: The Brazil nut, *Bertholletia excelsa* (Lecythidaceae), is the most important non-timber forest resource in the Amazonian biome, where nuts for the international trade are exclusively wild-collected. The monotypic genus possesses a discontinuous distribution and is thought to have rapidly and recently radiated south and westward from a northern/eastern Amazonian origin, largely as a result of ancient human activity [1]. It's genetic variability is currently thought to be low and conserved in one or very few populations [2], and in order to better understand the population genetics of this tree, there is a need for additional, or more informative, molecular markers than is currently available.

Methods: Trunk cambium is an attractive source of material for cDNA library construction, in contrast to foliar material, where photosynthetic genes are grossly overrepresented. Furthermore, samples can be taken from mature trees at the forest floor level. Cambium samples were collected from both Acre and Amapá states of Brazil, and from both highly, and poorly-productive trees from the respective states, with the aim of detecting microsatellites (SSRs) and single nucleotide polymorphisms (SNPs) for genetic analysis. The samples were frozen in the field on dry ice and later kept at -80 °C in the laboratory. Total RNA was extracted from cambium ground with a pestle and mortar under liquid nitrogen, using either the RNeasy kit (QIAGEN) or a combination of hot CTAB pre-treatment with repeated chloroform extraction and the RNeasy kit. Messenger RNA was purified using oligo dT-magnetic bead separation (Dynabeads; Invitrogen). The Clontech SMART kit and Superscript III (Invitrogen) reverse transcriptase were used for cDNA synthesis and four normalized, PCR-amplified libraries constructed using the Trimmer Direct kit (Evrogen). The four cDNA libraries were multiplex tagged and submitted to a single 454 pyrosequencing production run, using a GS FLX-Titanium instrument.

Results and conclusions: Cambium RNA extracted with the RNeasy kit alone or with the CTAB pretreatment possessed A260/280 and A260/230 ratios above 2.0, when measured by Nanodrop spectrometry, indicating high purity, and appeared to be of high quality by agarose electrophoresis. However, the RNA extracted by RNeasy alone was not successfully reverse-transcribed and gave no significant products in RT-PCR, even after mRNA purification by magnetic bead separation, indicating the presence of recalcitrant and bound enzyme inhibitory substances. In contrast, the CTAB pretreatment yielded RNA that could be readily reverse transcribed, which was subsequently used for amplified and normalized cDNA library construction.

Data yield for the four normalized libraries averaged 73 million total bases, 270 thousand validated sequences with a mean read length of 265 bp, respectively, per library. Sequences were assembled into contigs using Newbler version 2.5 and microsatellites were searched for using Microsatellite Commander v. 0.8.2. The mean numbers of microsatellites identified per repeat class per library were: 507 dinucleotide, 897 trinucleotide, 29 tetranucleotide, 9 pentanucleotide, 41 hexanucleotide, and 138 compound/interrupted repeats, with little variation in microsatellite yield or repeat class present between libraries. These libraries appear to be a rich source of new markers, which will greatly enhance our efforts to better understand the population genetics of *B. excelsa*, to determine appropriate conservation strategies, and to work towards rational selection of productive lines.

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P5

Genetic diversity of *Bertholletia excelsa*, an Amazonian species of wide distribution

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Background: Amazonian upland forests are expansive and can comprise large continuous tracts. There have been several studies on the population genetic structure of species in this kind of forest, but there are few studies that aim to understand genetic structure throughout the Amazon [1]. The Brazil-nut tree, *Bertholletia excelsa*, is a monotypic genus, endemic to upland forests and distributed along almost the entire expanse of the Amazon [2-5].
Genetic diversity distribution through Amazon forest is an understudied issue, specially in plants. Quantifying and understanding population genetic structure, associated to gene flow and mating system studies are recognized as important tools for the development of strategies for conservation and management. Such information can also be helpful in identifying effects of habitat fragmentation [6,7].
This study aimed to evaluate genetic structure of *B. excelsa* populations in the Amazon and to verify if the structuring is influenced by distance between them.
Methods: Seven polymorphic microsatellite markers were developed from a dinucleotide-enriched genomic library. Material from 379 individuals was collected from nine Amazonian subpopulations distributed in five Brazilian states (AC: Acre, AM: Amazonas, AP: Amapá, PA: Pará, RR: Roraima). All seven microsatellite markers developed for the species were used with four others previously published markers [8], for genotyping.
Analyses within and among populations were performed to evaluate genetic diversity and population structure. All nine populations were characterized with 11 microsatellite loci for number of alleles per locus, allele frequency and observed and expected heterozygosity under Hardy-Weinberg expectation. Wright fixation indices were also estimated. Genetic distance estimates were correlated with different potential factors to find possible causes of genetic structure.
Results and conclusions: A few alleles were found in each subpopulation and considerable variation was observed in alleles found in each subpopulation and in their allele frequencies, especially when compared with very distant subpopulations. Heterozygote excess was observed in five subpopulations while in the other four subpopulations, estimates of F_{IS}, non-significantly different from zero, were found. The observed high heterozygote proportions in most subpopulations and absence of inbreeding support the existence of self-incompatibility mechanisms and selection in favor of heterozygotes. Maintenance of genetic variability is favored by the alogamous breeding system, which is an important feature to be considered in conservation and management strategies, in order to avoid fertility deficits.
Fine-scale structure, when present, was small. Estimates of inter population genetic structure varied from low (q= 0,02) to high (q= 0,244). *B. excelsa* genetic structure can be analyzed on three different scales: (*i*) within population; (*ii*) among moderately distant populations (<500km); and (*iii*) among very distant subpopulations. At all scales, significant correlations were found between genetic structure and geographic distance between pairs of individuals or populations. This may indicate that distance is an important factor in this population's genetic structure, but probably there are other factors acting in conjunction, especially on a large geographical scale.

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P6

Molecular Phylogenetics of the genus *Hexachlamys (Myrtaceae)* using chloroplast and nuclear markers

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Background: Myrtaceae family includes more than 3,800 species of trees and shrubs, distributed mainly in tropical and subtropical regions of the world. Representatives of this family have great ecological significance for forest ecosystems, and are economically important species in the pharmaceutical, food, cosmetic and perfumery industry [1]. The genus *Hexachlamys* Berg. (Myrtaceae) has about 10 species distributed from the Southern to the Southeast of Brazil, and it also occurs in Paraguay, Argentina, Bolivia and Uruguay [2,3]. Since 1968 it has been considered an independent genus. It is distinguished morphologically from the genus *Eugenia* by pentamerous or hexamerous flowers and exserta radicle, and as many genera and species of the Myrtaceae family, it has a complex taxonomic classification [2,4,5]. The goal of this study was to conduct a molecular phylogenetic analysis among species of the genus *Hexachlamys* (Myrtaceae) using chloroplast (cpDNA) and nuclear (nrDNA) markers, and to verify its phylogenetic relationships with the genus *Eugenia* in order to contribute to the systematics and taxonomy of it.
Methods: The samples were collected as leaf material(from herbaria species and samples collected in the field) of the genus *Hexachlamys* representing all described species, as indicated in herbariums and species of the *Eugenia* genus that occur in the Rio Grande do Sul state. Total genomic DNA was extracted using the CTAB method based on the protocol already described [6] and used for the PCR reactions. PCR products were sequenced on ABI PRISM 3100 sequencer (Applied Biosystem). Nucleotide sequences were aligned using CLUSTALW [7] implemented in MEGA5 (Molecular Evolutionary Genetics Analysis) version 5.0 [8], then checked visually and carefully improved manually before analysis. The phylogenetic analysis was reconstructed after nucleotide sequence alignments using three different approaches: the neighbor-joining (NJ), the Bayesian and the Maximum-Likelihood (ML) methods. The NJ, Bayesian and ML analysis were performed in MEGA 5.0, MrBayes [9] and PhyML [10], respectively.

Results and conclusions: To date we obtained DNA sequences of 21 species of the genus *Eugenia* and seven species of the genus *Hexachlamys*. Universal primers that amplify chloroplast region (*trnL-trnF*, *trnL-intron*, *ycf5*, *accD*, *rbcLa*, *psbA-trnH*, *rpoB*, *rpoC1*, *ndhJ*, *matK* and *rps16*) and nuclear (*ITS*) were tested. The regions used for the phylogenetic analisys were chloroplast *accD*, *rpoB*, *rpoC1*genes and the nuclear *ITS*, since the other chloroplast regions were not possible to amplify and sequence in all sampled species. These cpDNA and nrDNA regions presented polymorphisms among species studied. Results from Bayesian, NJ and ML tree analysis produced similar topologies and revealed that *Hexachlamys* species did not form a monophyletic clade. The *Hexachlamys* species have grouped together *Eugenia* species with high bootstrap values, indicating that *Hexachlamys* can be a synonymous of genus *Eugenia*. These results corroborate with morphological data [11]. To confirm these results, other cpDNA and nrDNA markers will be tested and *Hexachlamys* species will be collected to substitute the species obtained in herbarium, since we found difficulties in the amplification of these species with all markers previously tested. This study can contribute to taxonomic classification of this group, as well as the field of conservation, since these species have an important economically and ecologically role.

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P7

Twenty four microsatellite markers for *Aspidosperma polyneuron (Apocynaceae)*, an endangered tree species

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Background: *Aspidosperma polyneuron* (Apocynaceae), commonly known as peroba-rosa, is a native and perennial species characteristic of semideciduous forest in submontane formation of Atlantic rain forest [1]. This species is recommended for ecosystems recovery and restoration of riparian forests in areas without flooding. But, the crescent logging, agriculture and urban expansion caused the reduction of native population of *A. polyneuron*. It is now included in the Red List of Threatened Species [http://www.iucnredlist.org/apps/redlist/details/32023/0]. Little is known about the population structure of *A. polyneuron* and the effects of the spatial isolation on the levels of genetic diversity and gene flow. In this study, we developed microsatellite loci for *A. polyneuron* aiming future studies of genetic structure, gene flow and mating system.

Material and methods: Genomic DNA was extracted from leaf tissue [2] from one individual located in Cravinhos Municipality, São Paulo state, southeastern Brazil (21°17'41.8''S; 47°42'08.5''W). The Microsatellite-enriched library was constructed using the protocols described earlier [3]. The DNA was digested with *Rsa*I, bound to the adapters (Rsa21 and Rsa25) and amplified through polymerase chain reaction (PCR). The products were purified by Quiaquick PCR purification kit (Qiagen). Fragments containing microsatellite sequences were selected by hybridization with biotinylated oligo-probes $(CT)_8$, $(GT)_8$ and $(CTT)_8$ and recovered by streptavidin-coated paramagnetic beads. After that, it was carried out a second PCR in order to increase the enriched fragments number and then an aliquot of the PCR product was bound to the pGEM-T vector (Promega) and transformed into *Escherichia coli* XL-1 Blue strains. The clones were sequenced using the Big Dye Terminator Kit and ABI 377 sequencer (Applied Biosystems) and the primers were designed using PRIMER3 [http://frodo.wi.mit.edu/primer3/].

Thirty *A. polyneuron* individuals were used for screening and the amplification were carried out in 10 μL reactions containing 2.5 ng of template DNA, 0.3 μM of each primer, 0.25 mM of each dNTP, 1x PCR buffer [75 mM Tris-HCl pH 9.0, 50 mM KCl and 20 mM $(NH_4)_2SO_4$], 1.5 mM $MgCl_2$ and 1U *Taq*DNA polymerase (Biotools); and performed using a MasterCycler® (Eppendorf): 96°C for 4 min followed by 30 cycles (94°C for 40 s, 50-54°C for 1 min and 72°C for 1 min) and 72°C for 7 min. PCR products were denatured and separated on 8% denaturing polyacrylamide gels stained with silver nitrate. Allele sizes were estimated by comparison by 10 bp DNA ladder standard (Invitrogen) and the original DNA used for library development. FSTAT software [http://www2.unil.ch/popgen/softwares/fstat.htm] was used to calculate the genetic parameters per locus, alleles number, the genetic diversity (H_e) and the linkage disequilibrium. The cumulative exclusion probabilities were calculated by CERVUS 3.0 software [4].

Results and conclusions: A total of 384 clones were obtained and sequenced. Thirty-nine clones (10.2%) presented microsatellite repeats and 25 were suitable for primers design. The most common repeat motif was GA/CT as expected due the employing technique. Among the loci analyzed, one did not amplify, eight were monomorphic and 16 loci showed polymorphism in this sample. Twenty loci were dinucleotide (83.3%), one was tetra-, other one penta- and two were rare compounds loci: di/tri- (Apn8) and di/tetra- (Apn9). Mendelian inheritance analyses were carried out for each polymorphic locus using one mother tree and their open-pollinated family (26 progeny). All sibs displayed at least one of the maternal alleles, confirming Mendelian inheritance. From a total of 121 alleles presented in the sample, the genetic diversity was high (H_e = 0.65) and the linkage disequilibrium, applying Bonferroni correction for multiple comparisons, was not significant. The total paternity exclusion probabilities over 16 loci were very high for one ($P_{exclu(1)}$ = 0.9995) or both parents available ($P_{exclu(2)}$ = 0.9999) indicating that the set is suitable for paternity analysis. Our study provides a new set of variable microsatellite loci for *A. polyneuron* that may be used to estimate genetic parameters such as genetic diversity, population structure, gene flow, and mating system. These markers might also prove transferable for genetic analyses in related taxa.

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P8

Genetic diversity assessed in individuals of *Aspidosperma polyneuron* and *Cariniana estrellensis* used as seed donors in an forest gene bank

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Background: The neotropical tree species *Aspidosperma polyneuron* and *Cariniana estrellensis*, known as *peroba-rosa* and *jequitibá-branco* respectively, are characteristic of late secondary semideciduous Atlantic forest in submontane formation. Their wood is widely used for carpentry and construction. Increasing logging, intensive agriculture and urban expansion placed *A. polyneuron* in the Red List of Threatened Species [http://www.iucnredlist.org/apps/redlist/details/32023/0] while *C. estrellensis* is considered an endangered species. So, *in situ* conservation of these forest species is critical while *ex situ* collections as gene banks is an important complementary approach. Our study assessed and compared the genetic diversity of *A. polyneuron* and *C. estrellensis* in two stands aiming to provide subsidies for the *ex situ* conservation of these important genetic resources.
Material and methods: Leaf tissues of mature trees of two disturbed stands (SI and SII) of *A. polyneuron* and *C. estrellensis* were sampled and stored at -20°C: SI was composed by isolated individuals of an extensive area located among Pardo river and Mogi-Guaçu river basins used as seed donor of a Forest Genetic Bank, at the University of São Paulo in Ribeirão Preto *campus* (BG-USP/RP), SP-Brazil. SII was one of the last natural populations around Ribeirão Preto region of an isolated fragment of 7.5 ha located on Águas Claras farm in Cravinhos Municipality (21°17'47''S; 47°40'29''W).
DNA extraction was performed according to [1]. SSR markers development for *C. estrellensis*[2] and for *A. polyneuron* (Ferreira-Ramos R., unpublished)] were used for this study (Tables 1,2). Microsatellite loci were amplified individually according [3]. FSTAT software [http://www2.unil.ch/popgen/softwares/fstat.htm] was used to calculate the genetic parameters per locus and sample: mean number of alleles (*A*), the observed (H_o) and expected heterozygosities (H_e). Wright's fixation index was calculated as 1 - H_o/H_e. Deviation from Hardy-Weinberg equilibrium (HWE) was measured using GDA software[http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php].
Results and discussion: For both species, the genetic diversity was higher in SI than SII ($H_{e\ SI/SII}$= 0.65/0.47, $H_{e\ SI/SII}$= 0.72/0.61 for *A. polyneuron* and *C. estrellensis*, respectively). For *A. polyneuron*, the expected heterozygosity (H_e) was higher than observed heterozygosity (H_o) in SI and SII. For *C. estrellensis* H_e was higher than H_o in SI and the opposite was found in SII. Fixation index was higher in SI than SII for both species ($F_{SI/SII}$= 0.30/0.11, $F_{SI/SII}$= 0.29/-0.05 for *A. polyneuron* and *C. estrellensis*, respectively), suggesting inbreeding. Significant departures from HWE were observed for most loci in both species mainly in SI, which might be due to population substructure (Wahlund effect).
In summary, our study revealed a high diversity for the seed donor trees of *A. polyneuron* and *C. estrellensis* (SI), suggesting that this diversity was incorporated into BG-USP/RP. Indeed future progeny studies will be able to confirm these results. The low diversity found in SII for *A. polyneuron* highlights its threatened situation in this stand, on of the last natural populations in Ribeirão Preto (SP-Brazil). For *C. estrellensis*, a higher genetic diversity was observed which may be due to greater number of trees in the population, preferentially outcrossed mating system and probably gene flow from outside the fragment studied.
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P9

Genetic diversity and divergence applied to Enviromental services for *Araucaria angustifolia* (Brazil)

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Humans derive many utilitarian benefits from the environmental services of biotas and ecosystems.Yet ecosystem, species and genetic levels is increasingly lost from agricultural landscapes mainly due to maximisation of production [1,2]. The genetic variability is a result of several forces including mutation, recombination and gene flow. Allele frequencies are altered by natural selection and by genetic drift. Whilst mutation, recombination and genetic drift are random and independent processes, natural selection is a directional process towards evolutionary change [3]. The Araucaria Rainforest is one of the most important biomes occurring naturally in southern Brazil. The extensive logging and agricultural expansion became this forest extremely fragmented. Particularly important is the factor of ecosystem resilience, which appears to underpin many of the services. While biodiversity often plays a key role, the services can also derive from biomass and other attributes of biotas, for example, genetic diversity of a particular species. Landscape genetics are being used in this project to improve the understanding of fragmentation impact to assist an environmental services scheme. The objective of this study was to assess by microsatellites markers, the genetic diversity and dynamics in remnant patches of *Araucaria angustifolia* rainforest, with different levels of human modification. The project compared two different forest conditions: one is 1.157,48 ha of continuous forest, and the other is a fragmented forest remnant, with size from 8 ha, appraised 5 km from the continuous forest area. Genetic diversity and divergence of seedlings and adult individuals present in forest fragment was compared with the genetic composition of samples appraised in continuous forest. Cambium and seed material were collected from each recorded tree, and genomic DNA was extracted using the method described in Mazza & Bittencourt (2000). The seeds for DNA extraction had undergone a slight adjustment – Proteinase K was added to megagametophyte (maternal origin) and embryo extraction. Polymerase chain reaction (PCR) conditions used were establish by Qiagen Multiplex Master Mix protocol (1022830) and use fluorescents dye labelling. Two multiplexed systems of microsatellites were applied with the 8 primers. The eight loci used were CRCAc2, Ag23, Ag62, Ag45, CRCAc1, Ag20, Ag56 and As90. Following PCR this dilution was running in ABI sequencer 3100. Gene Scan and Genotyper software were used for data collection and alleles analysis. The average number of alleles per locus among adult trees in the continuous forest was 8.10, compared to 5.15 appraised in forest fragment. The means of expected heterozygosity were 0.623 for continuous forest and 0.579 for forest fragment, while observed heterozygosity range was respectively, 0.571 and 0.723. Subpopulations of forest fragments were more distinguished than subpopulations of continuous, due the average fixation index was 0.082 for subpopulations in the continuous forest and 0.210 in fragment. It is worth noting that the levels of genetic differentiation among all subpopulations can be considered to be high. Paternity analysis, within the continuous forest indicated that 48% of offspring were fertilized by pollen from trees outside the plot site. The average pollination distance within the continuous was 95 m. In the trees from forest fragment, the analysis showed that 42% to 65% of the offspring was fertilized by pollen from trees outside fragment. The effective number of pollen donors in the continuous forest ranged among seed-trees from 2 to 10, and in the fragment from 2 to 6. The results suggest high pollen

dispersal distance in both conditions and an absence of reproductive isolation. They also show high pollen immigration and dispersal distance among the tree groups. The results suggest that fragmentation increases divergence in *Araucaria angustifolia*.The population in continuous forest showed higher genetic diversity in the adult population than the population of trees in fragment. The reducing the heterozygosity were low, may be due the recent forest fragmentation history. There are more inbreeding in fragmented population than in continuous population. Fragmentation increased the genetic divergence among the fragmented population. However, the genetic results indicated the presence of long-distance dispersal leading to functional connectivity between isolated forest fragment. The survival of remnants of *Araucaria angustifolia* patches as well as single trees in the agricultural landscape is key factors for species conservation and could be applied to environmental payment services, these strategy for *A. angustifolia* is an integration of conservation strategies across reserves and the surrounding matrix, including productive agricultural areas, to assist gene flow movement between temporally suitable habitats.

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P10

Microsatellite variability between apricot and related *Prunus* species

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Background: Apricots, family *Rosaceae*, are economically important representatives of the genus *Prunus*. The number of apricot species ranges from three to ten, depending on the classification system adopted. The major obstacles to expansion of apricot production are irregular yields and low resistance to diseases. Compared with the high genetic variability in related species, apricots are not so variable and thus interspecific hybrids were proposed as a means to overcome deficiencies inherent in the common apricot. To estimate the extent of variation in apricot germplasm, between ecogeographical groups and related species, is very useful for planning breeding programmes, through diversity analysis, cultivar identification or marker-assisted selection.

Methods: One hundred accessions of *P. armeniaca* representing the European, Irano-Caucasian, Central Asian and North American genepool, three accessions of *P. mandshurica* and one accession each of *P. ansu*, *P. brigantiaca*, the interspecific hybrid Plumcot,*P. x dasycarpa*, *P. mume*, *P. siberica* were analysed with 10 apricot SSR loci on an ABI 3100 capillary sequencer [1]. A total of 196 alleles were detected varying from 11 to 27 with an average of 16.9. Out of them, 59 alleles occurred only oce (so-called private alleles). 34 (58%) private alleles were also found in related species. Non- amplified alleles were observed in four samples of the related species (*P. siberica* and *P.brigantina*). F_{ST} values ranged from 0.5121 to 0.3503, with an average of 0.4459 (Table 1). It is known, that F_{ST} values up to 0.05 indicate negligible genetic differentiation whereas >0.25 means very great genetic differentiation within the population analyzed. In *Prunus*, high levels of genetic differentiation could be explained by the mating system. The data indicate a low gene flow (as described by Nm; Table 1). These results could be attributed to different geographic origins of the species or the influence of the breeding strategy.

Results and conclusions: Genetic similarity among common apricots and related species was quantified using NeiÂ´s [2] genetic distance and genetic identity based on allele frequencies (data not shown). The lowest genetic identity (0.000) was found among *P. mandshurica*, *P. brigantina*, *P. mume* and *P. dasycarpa*, and between Plumcot and *P. mume*. The highest genetic identities (0.81) were found between Western European and North American accesssions and among Irano Caucasian and Eastern European groups. To demonstrate the genetic relationship between common apricots from different ecogeographic regions and related species, a neighbor joining dendrogram based on genetic distance was produced (Figure 1). The accessions are divided into two groups, one containing all common apricots and hybrids thereof, and the other containing all related species. In general, results show that the common apricots are remote from related species. The tree supports that the *P. x dasycarpa* and Plumcot, known to be a *P. armeniaca* hybrid, are intermediates between common apricot and other related species. *P. ansu* and *P. siberica* appear distant from the others accessions, and in fact they are the most distanctly related species. It is interesting to known that some authors even consider *P. ansu* as a separate species [3]. In this study *P. siberica*, the species having the largest distribution area of all apricot species, and *P. mandshurica*, being present in the very cold area, cluster together. In the analyses they appear far from the common apricot species, which confirms them as being markedly different from *P. armeniaca*. Maghuly et al. [1] described that *P. x dasycarpa*, *P. brigantiaca* and Plumcot were distant from the common apricot cluster. In fact, *P. brigantiaca* is the most distantly related species, *while P. x dasycarpa*, a hybrid between *P. armeniacax P. cerasifera*, was found intermediate between the apricot groups and Plumcot, which is a hybrid between *P. armeniaca* and *P. salicinia*.

Table 1(abstract P10) Variability parameters calculated for 10 SSR markers in 100 apricot cultivars origin of five ecogeogrphical region and related species using POPGENE

Locus	Number of putative alleles	Effective alleles per locus(Ne)	Observed heterozygosity (Ho)	Expected heterozygosity (He)	Inbreeding coefficient (F_{ST})	Gene flow (Nm)
SsrPaCITA7	18	4.6280	0.8091	0.7875	0.4252	0.3380
ssrPaCITA10	21	4.4906	0.5545	0.7809	0.4461	0.3105
ssrPaCITA19	14	4.1052	0.7636	0.7599	0.5121	0.2382
ssrPaCITA23	11	4.5846	0.5872	0.7855	0.4951	0.2550
ssrPaCITA27	12	2.9979	0.3738	0.6696	0.6130	0.1578
UDAp-407	27	7.2979	0.7182	0.8669	0.3823	0.4039
UDAp-410	15	5.5415	0.8440	0.8233	0.4493	0.3064
UDAp-414	20	5.3862	0.5000	0.8181	0.3503	0.4636
UDAp-415	15	3.7162	0.6273	0.7342	0.4072	0.3640
UDAp-420	16	3.5567	0.5909	0.7221	0.4102	0.3594
Mean	16.9	4.6305	0.6369	0.7748	0.4459	0.3107
St.Dev	4.77	1.2233	0.1471	0.0565	-	-

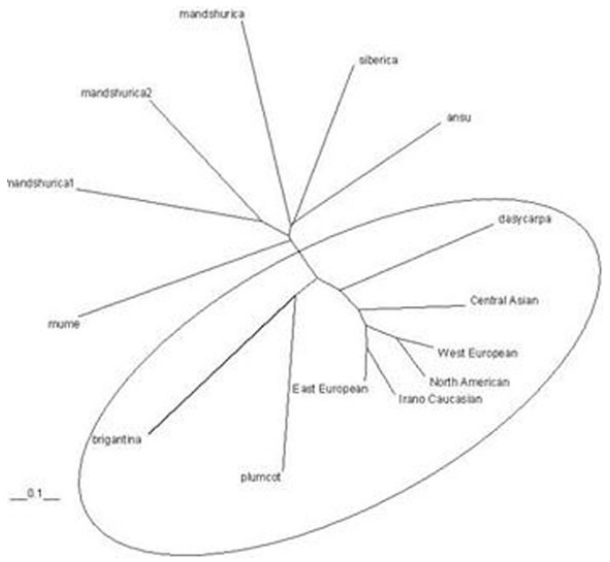

Figure 1(abstract P10) Neighbor joining dendrogram for apricots from five ecogeographical groups and related species.

Results to date indicate that crosses between apricot and apricot related species are successful, when made in either direction and the resulting hybrids are viable [3]. In this manner, *P. mandshurica* and *P. siberica* were used in common apricot breeding as a source of cold hardiness [4]. Likewise, adaptation to humid climates should be easy to transmit through hybridization with *P. mume* and *P. armeniaca* var. *ansu*. In addition, Rubio et al. [5] suggested *P. manchurica* as the possible origin of the apricot cultivars resistance to Sharka. It should be emphasiszed that in the interest of still further extending the genetic diversity available for posterity, additional efforts should be committed to the systematic exploration for unique phenotypes of apricots, and also of related species.

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P11

Development of microsatellite molecular markers and genetic diversity in *Hevea Braziliensis*

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Background: The rubber tree [Hevea Braziliensis (Willd. ex Adr. De Juss.) Muell-Arg.] is native from the Amazon region which has great economic importance for being the largest source of natural rubber in the world. Although Brazil is the center of origin and genetic diversity of this culture, the country is currently responsible for only 1% of the world production. Besides offering optimal weather conditions for crop development, the Amazon rainforest region is also favorable to the development of the fungus known as SALB (South American leaf blight), which causes the disease-of-leaves. Leaves fall sharply reducing the production of latex, limiting the production of rubber in this region. Thus breeding programs are seeking for clones which are resistant to this fungus and with high production in escape regions, which provide stress conditions such as low temperatures, high altitude, wind and other diseases [1]. The rubber tree is perennial and requires about 30 years to obtain an improved variety, starting from the controlled pollination of a clone to the final recommendation. Molecular markers such as microsatellites (Simple Sequence Repeats, SSRs) are an important tool for diversity studies and potentially to assist breeding programs. This study aimed to develop an enriched microsatellite library for *H. Braziliensis*, characterize these developed microsatellite markers and test the transferability of these markers to six other species of the genus *Hevea*.

Material and methods: For this study we used 36 accessions of *H. Braziliensis* donated by the Agronomy Institute of Campinas and one accession of each of six other species of genus *Hevea* (*H.nitida*, *H. pauciflora* (*2*), *H.camargoana*, *H.guianensis*, *H.rigidifolia* and*H. benthamiana*) provided by Embrapa. Genomic DNA samples were extracted from lyophilized leaf tissues using a modified CTAB method [2]. Trinucleotide and dinucleotide enriched genomic libraries for *H. Braziliensis* were constructed. The DNA samples were digested with AFAI and enriched using (CT)8 and (GT)8 biotinylated microsatellite probes for the dinucleotide library and (ATC)8 and (CCT)8 for the trinucleotide library. The clones obtained were sequenced and the sequences were evaluated with the Microsat program, which removes parts of the vector and the adapters and verifies the presence of restriction site within the sequence. After this step, the sequences were aligned and edited using the program SeqMan (DNAStar Inc.), which also allows analyzing the redundancy of the library. The identification of microsatellites was performed using a research tool SSRs SSRIT – "The Simple Sequence Repeat Identification Tool" avaiable at Gramene [http://www.gramene.org]and primers complementary to sequences flaking the microsatellites were designed by Primer Select Program (DNAStar Inc) and Primer 3. Amplification tests were made from a temperature gradient to know the annealing temperature and the products were evaluated and resolved on 3% agarose gels stained with ethidium bromide and in denaturing 6% polyacrylamide and silver stained [3]. The loci were characterized on the number of alleles per locus, allele frequency and the Polymorph Information Content (PIC). It was also made analysis of ancestry for de accessions of *H. Braziliensis* using the program Structure v 2.3.3 [4].

Results and discussion: A total of 384 clones from the dinucleotide library were sequenced while 288 from the trinucleotide library resulting in 133 and 62 microsatellites in each library respectively. It was possible to design 55 and 32 primer pairs for the dinucleotide and trinucleotide libraries respectively. Of the 87 microsatellite loci designed 69 were characterized. The maximum number of alleles per locus was 17 and eight loci were monomorphic. The PIC values ranged from 0.83 to 0.06, the observed and expected heterozygosity ranged from 0.86 to 0.06 to 0.90 and 0.06 respectively. A population analysis divided the 36 accessions into two groups. The cross-amplification of these microsatellites loci was tested in six other species of the genus *Hevea*. The percentage of cross-amplification in others species of *Hevea* ranged from 94.2% to 82.6%, with *H. nitida* with the highest percentage of transferability and *H. carmagoana* with the lowest one. This high percentage indicates that the regions flaking the microsatellites are well conserved between species of *Hevea*. These markers will be useful to breeding and conservation studies of *H. Braziliensis* and related species.

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P12

Phylogeography of the disjunct *Schizolobium parahyba (Fabaceae-Caesalpinioideae)*

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This work aims to analyze the phylogeography of *Schizolobium parahyba* (Fabaceae), which includes two varieties with a disjunct distribution, from Southern Brazil to Central America. Neotropical rain forests, focus on four largest wet forests: Atlantic forest, Amazonian forests, Andean forest and Central America forest. The genetic diversity and differentiation of populations among *S. parahyba* populations using sequences of three cpDNA regions (*psb*A-*trn*H, *trn*L-*trn*F and *mat*K) and one nrDNA region (ITS) were analyzed. The presence of the significant phylogeographic structure was inferred by testing if G_{ST} and N_{ST} were significantly different and a spatial analysis of molecular variance was made with both markers.Using cpDNA (*mat*K) sequences of the *S. parahyba* and other Fabaceae species and fossils, we estimated de divergent time for *Schizolobium* clade and using the average ITS substitution rate reported for a range of woody plants, w estimated the divergence time between the two varieties. The high levels of genetic diversity in some populations of *S. parahyba* and two centres of genetic diversity that correlate with the two known varieties: one in the southeast Atlantic forest, and the other in the Amazonian basin. In contrast, the populations from Northeast Atlantic forest and Andean-Central America forests showed low level of genetic diversity and divergent haplotypes, probably because the founder effect after dispersion.The results suggest dispersion from southeast Atlantic forest to Amazonian, Andean and Central America forests. We verifya high level of genetic structure, with 68% (nrDNA) and 82% (cpDNA) of the total genetic diversity due to differences among populations. Twenty-one haplotypes were found with cpDNA and four with nrDNA and no haplotypes were shared between varieties.The age for *Schizolobium*clade using *mat*K sequences was estimated ranging from 8.4-23.0 My and the divergence between two varieties using ITS sequence variation was of the 6.5 My. In conclusion, the variation pattern of cpDNA (maternally inherited) and nrDNA (biparentally inherited) markers provides different insights into the phylogeographic structure and gene flow in *S. parahyba*. This comparative analysis of cpDNA and nrDNA markerscan help a deeper understanding of the dynamics responsible for both ancient and more recent events that have shaped the current distribution of genetic variability in Neotropical plants. Theresults are relevant to conservation efforts and ongoing work on the genetics of population divergence and speciation in these Neotropical rainforests. Also, for the long-term conservation of the genetic diversity of *S. parahyba*, including the divergent lineages of the two varieties, it would be important to design strategies that aim to preserve most of its lineages.

P13

Genetic divergence in Cork Oak based on cpDNA sequence data

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Background and objectives: Cork oak (*Quercus suber* L.) is one of the dominant broadleaved woody species in the western Mediterranean Basin, defining unique open woods. These woodlands have an outstanding economical and ecological value in this region, particularly in Portugal, where they sustain a strong cork industry. In the context of a prospective management of these sustainable ecosystems and renowned reservoirs of biodiversity, it is vital to better understand how the genetic variation of *Q. suber* natural populations is spatially organized so reasonable guidelines for conservation can be provided. On the other hand, knowledge of how past climate fluctuations influenced the patterns and dynamics of *Q. suber*, shaping the ranges of the species in the Mediterranean peninsulas, is of the utmost importance for our perception of what can happen in the future. Although a great deal of details on the genetic divergence of the Mediterranean cork oak populations has been uncovered and several hypotheses have been advanced concerning its evolutionary history, there is still much to unravel. For instance, Portuguese natural population sampling included so far in previous studies has been very deficient. To achieve this goal a different and complementary analysis of cork oakÂ´s genetic diversity was initiated under a phylogeographical framework based on chloroplastidial DNA sequences. This study is the starting up of a project aiming at assessing the genetic diversity and differentiation of natural cork oak populations from the entire Mediterranean distribution, with the intent of understanding patterns of biodiversity, gene flow and population admixture, as well as to infer possible evolutionary events.
Materials and methods: We used 3-5 samples from 25 populations collected across the range of distribution of cork oak, in a total of 115 individuals. Three chloroplastidial DNA (cpDNA) intergenic spacer regions (*TrnL-F*[1], *TrnS-PsbC*[2] and *TrnH-psbA*[3]) were amplified and sequenced. Fourteen individuals of *Q. rotundifolia*, five of *Q. coccifera*, two of *Q. ilex*, and one of *Q. robur*, *Q. faginea*, *Q. pyrenaica*, *Q. lusitanicus*, *Q. rubra* and *Q. canariensis* were also analyzed as comparative references. *Castanea crenata* was used as outgroup. The alignment was developed in Clustal X 2.0.12 [4] and manually refined. The phylogenetic trees were made with PAUP 4.0d99 [5], using a Maximum Parsimony analysis method.
Results: Regarding the cpDNA regions under study, two main lineages of cork oak haplotypes were found: one lineage closely related to *Quercus rotundifolia* and *Q.coccifera*, ("introgressed lineage"), mainly present in Iberia and Morocco; and a second lineage that contrastingly seems "pure", which is the most common and does not appear to be shared with any other *Quercus* species ("pure lineage"). Three distinct sublineages are shown in the "pure" lineage, corresponding to the Eastern populations, Sicilly and the Western populations, although separated by minor differences. Some populations present cpDNA haplotypes belonging to both lineages, while others are specific for one of them. An absence of introgression in the easternmost populations of cork oak seems also apparent.
Conclusions: Although these preliminary results seem to confirm previous ones, some major differences are suggested, such as too few differences between the western and eastern groups to be explained by a Tertiary divergence pattern, as previously suggested [6], but rather more consistent with a more recent expansion from few refugia; and widespread and multiple introgression events from *Q. rotundifolia* and *Q. coccifera*, common in peripheral western populations.
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P14

Tracking down worldwide *Puccinia psidii* dispersal

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Background: *Puccinia psidii* causes rust disease on many host species in the Myrtaceae [1]. First reported in 1884 on guava in Southern Brazil [2], the rust has since been detected on several myrtaceous in South America, Central America, Caribbean, Mexico, USA: in Florida, California, and Hawaii. More recently, *P. psidii* was reported in Japan infecting *M. polymorpha* [3]. Of special note is that a rust was found infecting Myrtaceae species in Australia, where the fungus was reported as *Uredo rangelii*, based on the tonsure found on the urediniospores surface. However, DNA sequence data did not differentiate that rust from *P. psidii*[4], and the same tonsure patch, was also observed on rust urediniospores collected from several host species in Brazil [unpublished data].We have hypothesed that *P. psidii* was introduced into Hawaii through California by trade of rust infected myrtaceous plants, and that *P. psidii* populations from South America are distinct from the rust populations that became estabilished in California and Hawaii.
Material and methods: Fast-evolving microsatellite markers were used to determine genetic relationships among rust populations from South America, California and Hawaii. Such genetic markers allow inferences about the potential sources of pathogen introduction.
Results: The eight microsatellite loci analyzed revealed 14 multilocus genotypes (MGs) within the 221 *P. psidii* isolates. Isolates collected on seven different hosts in South America presented distinct MGs. In contrast, all rust isolates collected on nine myrtaceous hosts in the Hawaiian Islands were composed of only a single, unique MG. The same unique MG observed in Hawaii, was also detected on isolates from two different hosts in California,indicating a common origin of the rust genotype found in Hawaii and California.The isolates were grouped using a principal coordinates analysis (PCA). The first two axes of the PCA plot explained 89% of the total observed variation, with the first axis explaining 50% and the second 39%. According to the PCA, the *S. cumini* group is the South American group that is most closely related to the Hawaiian and Californian groups.
Conclusions: The MG comprising all isolates from Hawaii and California is distinct from the MGs found in South America so far, suggesting that the Hawaiian and Californian isolates did not come directly from South America. Isolates from Florida, Central America, and the Caribbean must be analyzed to better understand potential relationships with pathogen dispersion to Hawaii.

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P15

Genetic diversity of *Salix koreensis* based on inter-simple sequence repeat (ISSR) in South Korea

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Background: *Salix koreensisis* native to Korea, Japan and China, is a deciduous broadleaved tall tree growing throughout Korea. This species is dioecius and planted along the streets and for landscaping. Along the rivers the species grows well reaching up to 20 meters in height. Trees bloom in April and fruits ripe in May. This study was carried out to investigate the genetic diversity of *S. koreensis* within and among 10 populations throughout South Korea using ISSR marker analysis.
Materials and methods: From 10 populations identified throughout the country 8 to 11 individual trees per population were sampled, totalling 84 individual trees.Twigs with leaves were collected and used as material for genetic analysis. DNA extraction was performed using GENE ALL plant SV DNA purification Kit (Gean all, Korea) and amplified by PCR using 5 UBC (University of British Columbia) primers (#807, #834, #842, #856, #881) which showed good results in previous experiments. PCR products were electrophoresed and gels photographed. Amplified fragments were visually scored as present (1) or absent (0). Observed and effective numbers of alleles, Shannon's information index, number of polymorphic loci, Nei's genetic distance were estimated using POPGENE 3. and genetic distance was analysed using UPGMA.
Results and conclusions: The overall number of polymorphic ISSR amplicons was 80 and mean per primer was 16. The percentage of polymorphic loci was as follows: Namwon population had the highest level (61.25%), Seomjin river population had the lowest level (37.50%) and the overall mean was 48%. Shannon's Information Index indicating genetic diversity ranged from 0.17 (Seomjin river population) to 0.28 (Hanam population) with a mean of 0.24. Shannon's information index of this species was lower than the one found in other species. This result is likely due to the fact that *S. koreensis* is frequently propagated by asexual reproduction. The UPGMA dendrogram based on Nei's genetic distance did not show any clear geographic pattern. The Seomjin river population with low genetic diversity was genetically more distant from the other populations and the Upo population was the next more distant one. Hanam and Yangchon populations were the ones more genetically related.

P16

Development and characterization of microsatellite loci in *Grevillea robusta*

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Background: *Grevillea robusta* is a native tree to the subtropical coastal regions of northern New South Wales and southern Queensland in Australia. In Brazil, Embrapa Forestry and its partners have established many provenance/progeny tests to increase the species genetic basis and aim to implement a breeding program to improve timber production. Genetic variability of these tests has been monitored through the assessment of quantitative traits. However, the genetic evaluation of materials based on phenotypic traits is influenced by many environmental factors. Estimates of some genetic parameters such as gene flow and

parentage are possible using molecular tools as most common molecular marker, microsatellite (Simple Sequence Repeats, SSRs) which are codominant and highly polymorphic. Genetic markers have intensively applied for the main strategies in breeding programs, especially when economically important traits are difficult measure because of low heritability. The aim of this study was develop microsatellite markers for *Grevillea robusta* through enriched library in order to estimate the genetic diversity and structure of the species, and direct efforts for the conservation and management of its active germplasm banks.

Methods: The genomic-enriched library was constructed following the protocol described by [1]. The genomic DNA was digested with AFA*I* and enriched in $(CT)_8$ and $(GT)_8$ repeats. Enriched fragments were amplified by polymerase chain reaction (PCR), connected to a pGEM T-easy vector and transformed into competent XL1- blue *Escherichia coli* cells. The positive clones were selected using the B-galactosidase gene and then grown overnight in an HM/F medium with ampicillin. After PCR, 95 positive clones were sequenced in both directions using the T7 and SP6 primers as well as the Big Dye terminator Kit. The sequences were assembled and edited in Seqman (DNAStar) and the repetitive regions were found using the Simple Sequence Repeat Identification Tool [2]. Primer Select (DNAStar) and Primer 3 plus were softwares used to design primer pairs flanking the microsatellite regions.

Results and conclusions: Of the ninety five sequences cloned, seven contained microsatellite sequences. Only five of them showed repeats and adequate flanking regions for primer design. The observed proportion of trinucleotide was 4.21% (4), while tetranucleotide and pentanucleotide were 2.11% (2) and 1.05% (1), respectively. Of the total of nucleotides found 100% are simply perfect. The explanation for this low yield sequences (7,4%) can be attributed to the genomic-enriched procedure. These sequences will be validated and promptly used to estimate the genetic diversity, gene flow and parentage of the Brazilian germoplasm collections.

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P17

Phylogeographic patterns of *Calophyllum Braziliense* Camb. *(Calophyllaceae)* based on the psbA-trnH cpDNA locus

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Background: Past climate changes have severely influenced the current distribution of species and their genetic diversity. Phylogeography is the study of the principles and processes governing the geographic

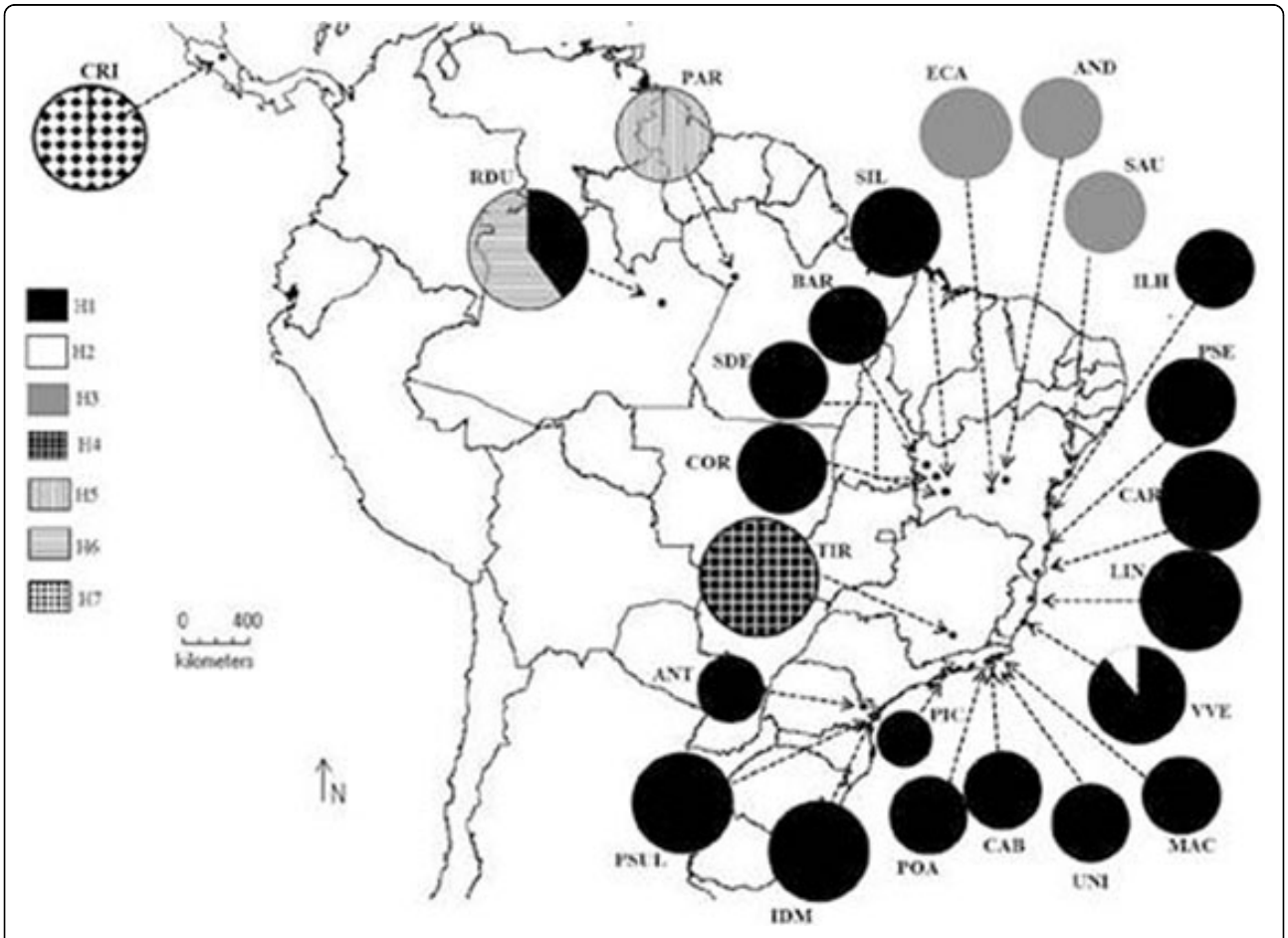

Figure 1(abstract P17) Map showing the geographic distribution of the cpDNA haplotypes in the *Calophyllum Braziliense* populations.

distributions of genealogical lineages [1]. South America has the world's largest area of swamps, floodplains and wetlands in general [2]. Brazil's major wetlands cover 2% of the country's huge territory [3]. However, from a plant ecology standpoint, Brazilian freshwater wetlands are largely unknown and the scarce data available refer mostly to flooded forests of the Amazon [4]. *Calophyllum Braziliense* Camb. (Calophyllaceae), also know as guanandi, jacareúba or landim, is a canopy tree species typical of waterlogged areas from South and Central America. This species occurs in the humid tropical forests of Central America, Amazon Forests, Atlantic Forest (including restingas); and in the riverines forests of the Cerrado biome (brazilian savannah) [5,6]. However, unlike other species typical of flooded areas, *C. Braziliense* shows none of the morphological features common to flood-adapted plants. *Calophyllum Braziliense* is a hermaphroditic tree pollinated by bees. Its seeds are animal (mainly by bats) or water dispersed. The timber has excellent characteristics and is widely used. *Calophyllum. Braziliense* is also used in vegetation restoration programs and its leaves extract presents anti-inflammatory activity [7]. This study examines the phylogeographic patterns of *C. Braziliense* based on the cpDNA intergenic region *psb*A-*trn*H.

Methods: Twenty four populations of *C. Braziliense* were sampled from Costa Rica (10° 12'N, 83° 47'W) to the Paraná State in Brazil (25° 34'S, 48° 27'W). Samples were collected from about 5-10 adult trees in each population, totaling 192 individuals. Total DNA was extracted from leaves or cambium using the CTAB procedure described by Doyle & Doyle [8]. After a screening for cpDNA amplification and polymorphism in *C. Braziliense*, the *psb*A-*trn*H intergenic regions was selected [9]. Sequences were aligned using CLUSTAL-W implemented in the MEGA 4 software. The cpDNA haplotypes were defined by analyzing the sequences with DNASP 4.01. The genetic diversity indexes were estimated in ARLEQUIN 3.01. The phylogenetic relationships among the haplotypes were estimated using the median-joining algorithm implemented in NETWORK 4.1. An analysis of molecular variance (AMOVA) was performed in ARLEQUIN. A spatial analysis of molecular variance (SAMOVA) was conduced using the SAMOVA 1.0 software. To evaluate the hypothesis of population expansion, neutrality tests were computed in DNASP and ARLEQUIN.

Results: A total of 263 aligned positions were obtained for the *psb*A-*trn*H locus. Twenty-eight variable characters were analyzed resulting in seven cpDNA haplotypes (Figure 1). The haplotype diversity (h) for each population ranged from 0.0 to 0.533 and the nucleotide diversity (π) from 0.0 to 0.01882. Similar levels of genetic diversity were observed for other tropical species [10,11]. Most of the Atlantic rain forest populations (13/14) are monomorphic and present the same haplotype (H1). Generally, the remaining populations present different private haplotypes. Spatial analysis of molecular variance (SAMOVA) identified seven phylogroups (k=7, Fct = 0,926), one consisting of 13 monomorphic populations from the Brazilian Atlantic rain forest and more three populations from de Cerrado biome. Most of the others populations constitute different phylogroups. Neutrality tests suggest expansion for the Atlantic rain forest populations. The AMOVA analysis reveals that most of the variation was found between populations (0.8788, p<0.00005). A great genetic distance was observed between Central America (Costa Rica) and the others populations, even from the Brazilian Amazon forest. Similar results were observed when *Swietenia macrophylla* (mahogany) populations from Central and South America were compared [10].

Conclusions: The genetic data obtained here for *C. Braziliense* based on chloroplast DNA diversity indicate a recent expansion for the Atlantic rain forest populations. Our results suggest that the Northeast of Brazil maintained large populations during the last glacial period and that the Southeast and South populations may have undergone a pronounced retraction process, followed by a recolonization process with a strong founder effect. Thus, the recolonization of the South and Southeast region of the Atlantic rain forest probably occurred from these more stable areas in the Northeast.

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P18

Optimizing sampling efforts for ex situ conservation of genetic variability of *Dipteryx alata* Vogel

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Background: The "Baru" tree (*Dipteryx alata* Vogel) is an endemic species from the Cerrado biome, but widely distributed within the biome and abundant in several of its habitats [1]. The species is also important in commercial terms for recover of degraded areas, and seeds are consumed *in natura* and are source of raw material for small and middle-sized food industries [2]. Thus, a more detailed knowledge of its population variability and structure is needed for better establishing both *in situ* and *ex situ* conservation efforts. Data for such analyses can be provided by new molecular markers, such as microsatellites, whereas spatial analyses and optimization procedures can be applied to analyze these data and thus improve conservation efforts. Here we investigate the genetic variability within the germplasm bank of *D. alata* based on microsatellite data and compared it with a large sample of 25 widely distributed natural populations of the species. We then used a simulated annealing algorithm to find the smallest number of these natural populations that should be sampled to conserve the full genetic variability of the species.

Methods: We described the genetic variability of 816 individuals of *D. alata* from 25 natural population widely distributed in the Cerrado biome, and from 180 individuals preserved in an active germoplasm bank, situated in the "Escola de Agronomia e Engenharia de Alimentos" Universidade Federal de Goiás, totalizing 996 individual plants genotyped for 9 microsatellite loci. The origin of the individuals in the bank is not known in detail, but it is certain that they came from several localities from Goiás State.

Overall description of genetic variability in the natural populations and in the germoplasm bank was done by percentage of polymorphic loci (P), mean number of alleles per loci (A), expected heterozigosity under H-W equilibrium (He) and observed heterosigozity (Ho). Population structure

and divergence among natural populations were analyzed using F_{ST}statistics.

A simulated annealing algorithm, implemented in the software SITES [3] was used to establish the minimum number of local populations necessary to complement the germoplasm bank so that all alleles (expressed as present or absent in each local population) are represented at least once (i.e., representation goal). The search was performed using two hundred runs and 10,000,000 iterations for each run. The problem is analogous to the ecological problem of finding a minimum number of new conservation priority areas that represent all species, after fixing a few already established natural reserves (see [4]). However, there may be frequently multiple combinations of local populations that satisfy the representation goal, so the first 100 solutions of SITES were also retained. The relative frequency of each local population in the alternative optimized solutions is an estimate of the "irreplaceability", or relative importance, of the populations to the overall goal of representing the entire genetic variablity (alleles).

Results and discussion: The genetic variability of *D. alata* for the microsatellite loci analyzed here was low compared with other forest species in the same region (e.g., [5]). Out of the 9 loci, on average 78% were polymorphic within natural population, and 67% were polymorphic in the germplasm bank. The mean number of alleles per loci were 2.7 and 4.4 for the natural populations and for the germplasm bank, respectively. The *He* and *Ho* were equal to 0.36 and 0.30 for natural populations, and 0.36 and 0.23 for the germoplasm bank. The F_{ST} was significant and equal to 0.259, indicating a relatively high differentiation among the local populations.

The simulated annealing revealed that only 4 local populations, located in Mato Grosso and Mato Grosso do Sul (Figure 1; 1-CMT. 5-AMS. 19-RAMT e 25-CAMT), are necessary to represent all alleles that appeared in the species, complementing in an optimum way the genetic variability already present in the germplasm bank. The solution is unique so that all these 4 local populations have 100% of irreplaceability.

Our analyses confirm that the germoplasm bank currently established in the Universidade Federal de Goiás preserves the genetic variability of a relatively large proportion of species' range in the Cerrado biome. To improve such representation it is important, however, to sample more local populations in the west portion of the species' distribution, in Mato Grosso region. It is important to note that these populations possess unique alleles that are not found elsewhere, so they are irreplaceable for achieving the conservation goal. If these populations are lost (i.e., extinct), there will be genetic erosion reducing total genetic variability in this species. So, an effort to sample and grow up individuals or progenies from the 4 populations, preferentially coupled with in situ conservation programs in these natural populations from Mato Grosso region, must be urgently done. Our optimization analyses reveals that only a few samples are necessary to achieve a more complete representation for the species, and this is important to reduce sampling and maintenance costs without losing efficiency in terms of establishing conservation and future breeding programs for the species.

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P19

Chloroplast DNA variation and phylogeography of *Eugenia uniflora L. (Myrtaceae)* in the Brazilian Atlantic forest

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Introduction and objectives: The Atlantic Forest (AF) is considered the second largest tropical forest in South America with high species richness and endemisms, harboring a large diversity of animals, plants and habitats types [1]. This biome covers an area of more than one million square kilometres along the Brazilian coast and extending to eastern Paraguay and northeastern Argentina [2]. AF is considered one of the

Figure 1(abstract P18) Natural populations (red arrows) of *D. alata* that must be sampled to achieve the conservation goal of representing all alleles from 9 microsatellite loci complementing, in an optimum way, the genetic variability found in the germoplasm bank reared at "Escola de Agronomia, Universidade Federal de Goiás".

most threatened ecosystems on Earth due to intense disturbance, having been reduced to only 7.5% of its original area [1]. Despite an increase in research efforts in the past few years, studies of AF species diversification and knowledge about its evolutionary history is still scarce. Vegetation changes in the Atlantic Forest related to climatic changes during the Pleistocene have been registered in paleopalinological studies, with the replacement of large areas of forests by subtropical grasslands and savannas during cooler and drier conditions [3,4]. Also, studies of paleoclimatic models, predicted the presence of historically stable areas (refugia) in the Atlantic Forest during the Late Quaternary [5]. *Eugenia uniflora* L. (Myrtaceae), a shrubby tree with edible cherry-like fruits which is locally known as pitanga or Brazilian cherry. This species is one of the key species in the Atlantic rain forest geomorphological domain, which includes the Atlantic forest and the adjacent Restinga ecosystem [6]. *E. uniflora*occurs in areas of medium and large levels of rainfall and can also be found in different vegetation types and ecosystems. This species present economic and folk medicinal applications and is an important pioneer species in the Restinga ecosystem and has been used to recover and manage disturbed and fragmented areas. Our aim in this study was to investigate the phylogeography and genetic diversity of *Eugenia uniflora* to help elucidate the evolutionary history of this species as a model for gain insights into past vegetation patterns in the Brazilian Atlantic Forest.

Methods: Forty-six populations of *E. uniflora* were sampled across the Brazilian Atlantic forest. The samples were collected as leaf material (silica gel dried) from natural populations. Total genomic DNA was isolated using the CTAB method [7]. Two cpDNA regions (*psb*A/*trn*H and *trn*C/*yc*F6) were using for population analysis based on sequence quality and degree of variation. These regions were amplified using universal primers. The individual consensus sequences were aligned through CLUSTALW [8] implemented in MEGA4 [9], then carefully improved manually. The genealogical relationships among haplotypes were estimated by using the median-joining method implemented in Network 4.2.0.1 (Fluxus Technology Ltd. at http://www.Fluxus-engineering.com). Molecular diversity estimates were calculated using Arlequin 3.1[10] and DNAsp 5.0 [11]. Genetic structure was further examined by the analysis of molecular variance (AMOVA), as implemented in Arlequin version 3.1.

Results and conclusions: The total combined cpDNA matrix presented 1224 sites, which eight were variable. Eight haplotypes were found and the haplotype diversity (*h*) ranged from 0 to 0.733 and the nucleotide diversity (π) from 0 to 0.00140. Total haplotype and nucleotide diversities were 0.433 and 0.00088, respectively. The highest haplotype diversity was found in populations ITAP, PALM, CAPI and GRAV from the Atlantic forest South. Populations from Northeast were monomorphic. The most common haplotype was H1, present in 39 of 46 populations. The AMOVA analysis showed a very strong differentiation among all *E. uniflora* populations (F_{ST} =0.771, P<0.0001). These results can help a deeper understanding of the dynamics responsible for both ancient and more recent events that have shaped the current distribution of genetic variability in Atlantic Forest and also have implications for conservation efforts. For the long-term conservation of the genetic diversity of *E. uniflora*, it would be important to design strategies that aim to preserve most of its lineages. For such, the South region is a key piece, as it houses many divergent and endemic haplotypes and lineages.

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P20

Detection of genes involved in bud phenology in sessile oak (*Quercus petraea* Matt. Liebl) combining digital expression analysis and Q-PCR

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Background: In temperate zones, the seasonal cycling of perennials comprises two main steps: a growing period when environmental conditions are favourable and a non-growing period in winter. Winter dormancy consists of two phases: endodormancy and ecodormancy [1]. The former is the deepest state of the dormancy controlled by the meristem itself, while the latter is controlled by environmental factors, such as accumulated temperatures.This phenological cycle has been shown to be strongly affected by climatic change: higher temperature having a positive effect on ecodormancy phase by accelerating meristem cell growth and early bud break is particularly crucial to consider not only in respect to increasing length of the growing season, but also regarding the risk of frost damage. For these reasons, understanding the molecular mechanism controlling the shift from endo- to ecodormancy is of main importance to optimize the management of forest tree plantations to their most likely environmental conditions.

The objective of this study was to characterize the transcriptome of endo- and eco-dormant sessile oaks (a major broadleaf species in Europe) by RNA-seq and to identify differentially expressed genes between these two dormancy stages. Four cDNA libraries were generated using total RNA extracted from apical buds harvested during endo- or ecodormancy of two contrasted populations. Transcriptome characterization was performed on a Roche 454 FLX platform. Digital expression analysis leads us to identify a set of 48 genes differentially regulated between these two developmental phases (there was no differences between populations). Their function was identified by sequence homology and this result contributes to the characterization of the molecular network underlying vegetative bud dormancy, an important life history traits of these long lived organisms.

Methods: Acorns of sessile oak (*Quertus petreae* (Matt) Lieb.) were collected in 2006 on two populations from Northern France: Longchamp (LC) and Saint Jean (SJ). These populations are contrasted for their bud burst date (early vs. late flushing). We obtained 560 and 468 seedlings for the SJ and the LC populations, respectively. A forcing test experiment was performed in a greenhouse from July 30th 2007 to February 25th 2008 located at the INRA forestry research station (southwestern France). For each population, approximately 25 seedlings were selected each week. Ten apical buds from 10 seedlings were used for RNA extraction. The 15 remaining seedlings were transferred in a greenhouse with the following parameters: a 16-hours photoperiod and a day/night temperature of 25/18 °C. For each batch of seedlings, bud burst date was evaluated three times a week allowing us to determine the number of days necessary to induce bud burst. We, thus, identified periods corresponding to eco- and endodormancy.

Total RNA from apical buds harvested on September 17th, 24th and October 1st for endo-dormancy as well as on January 14th, 28th and February 11th for eco-dormancy were extracted independently, and

mixed equimolarly to obtain a composite cDNA library for each population and developmental stage. The libraries were sequenced on a Roche 454 FLX to produce 495,915 reads in total. After cleaning and removal of duplicated reads, reads were mapped onto oak unigenes [2]. A digital gene expression analysis was performed using both *r* statistics [3]and R BioConductor packages (edgeR, DEGseq and goseq). Homology searches were carried out by BlastX against SWISS-PROT and TAIR9 databases with e-value cutoff of 1e-5. Gene ontology (GO) annotations were based on the top blast hit against SWISS-PROT database.To validate bioinformatic analysis, Q-PCR was performed for 13 selected genes. One microgram of total RNA was reverse transcribed using Improm-II™ reverse transcription system (Promega) according to the manufacturer's instructions. Q-PCR reaction and quantification were performed on a Chromo4 Multicolor Real-Time PCR Detection System (Bio-Rad)

Results and conclusions: We identified 48 common contigs that showed differential expression by three bioinformatic programs (R statistics, edgeR and DEGseq) and selected 13 genes for Q-PCR analysis with the main goal to validate the result obtained in silico. One gene (heat shock protein) showed multi-banding pattern and discarded from the analysis. Among the 12 remaining genes, only one gene (cold shock protein) showed contradictory expression pattern between in silico and Q-PCR analysis. The high success rate of Q-PCR analysis (11/13 = 84.6%) indicates that the verified genes are likely to represent endo- and ecodormancy stages. Two genes (Globulin and XET) were confirmed as up-regulated in endodormancy, while nine genes (GASA, GST, DRM1, XERO2, PIP, AWPM, GID, LEA and ELP) were found to be up-regulated in ecodormancy .Analysis in terms of GO showed genes responsible to abiotic and endogenous stimulus as well as stress related were enriched in the set of 48 genes. Similar trend was observed in expression analysis for raspberry bud dormancy, where high percentage of stress-response/defense/detoxification-related genes were identified [4]. In grape also, most of genes expressed in buds at the end of dormancy were attributed to the oxidative processes and stress responses [5]. Although the number of genes indentified is small and further verification through time course change in their expression will be needed, these candidates will serve as stepping stone to dissect the molecular mechanisms involved in bud dormancy in forest trees.

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P21

Spatial genetic structure of *Stryphnodendron adstringens* (MART.) COVILLE (*Leguminosae-Mimosoideae*) using SSR markers

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Background: The forest fragments of *Stryphnodrendron adstringens* have disappeared because of the high rate of Brazilian Savannah deforestation for commercial purposes, such as eucalypt, citrus, soy bean, and sugar cane crops that have been intensely cultivated on those areas [1]. Economically, *S. adstringens* is important for manufacture industries of paints and tanneries, but also is utilized for therapy against ulcer, scurvy, and for antiseptic and healing purposes [2]. Considering the forestry sector, the species is potential for building uses because of their wood have high density and durability [3]. Aiming for germplasm preservation and for breeding programs we studied the spatial genetic structure of *S. adstringens* based on SSR markers.

Methods: Juvenile leaves of 38 individuals of *S. adstringens* were collected in natural population, of approximately 200 individuals, in Botucatu State Forest belonging to Forestry Institute, São Paulo State, Brazil. The population is located from 22° 55′ 55″ S to 22° 56′ 39″ S latitude and from 48° 27′ 19″ W to 48° 27′ 33″ W longitude, through 860 m altitude, totalizing 33 hectares of savannah. The lab procedure protocol based on Ferreira and Grattapaglia [4], and adapted to *S. adstringens*, for DNA extraction was used. We assessed the transferability of 98 primer pairs developed for different tree species of several families and genera. The amplification was performed at 92° C for 2 minutes by initial denaturation, 45 cycles of 1 minute at 92° C, 1 minute at annealing temperature, and 1 min at 72° C, and ending to 10 minutes at 72° C. The analysis of spatial autocorrelation of sampled population, with local coordinates, and variable alleles of individuals was estimated. The "Spatial Genetic Software (SGS) version 1.0 program for distance classes per each allele, and considering 1000 bootstraps to get the Moran's I Index [5] were used. The expression was:

$$I = \left(\frac{n}{W}\right) \cdot \left[\frac{\sum_i \sum_j (p_i - p)(p_j - p) W_{ij}}{\sum_i (p_i - p)^2}\right]$$

where:
I = Moran's I index, which can take values between -1.0 (negative autocorrelation) and + 1.0 (positive autocorrelation);
n = number of individuals;
pi and pj = allele frequency for i and j individuals;
p = average of p;
Wij = element of symmetric matrix square and W were nxn dimensions, which is given the value 1 for individual neighbors and 0 otherwise;
W = matrix that expresses the spatial correlation between individuals and the sum over i and j is the value iqual W.

Results and discussion: Forty eight out of 98 pairs were amplified showing 23 polymorphic primers, but only 10 of them were conserved, and presenting no null alleles: EMBRA 03, EMBRA 06 (*Eucalyptus grandis*), EMBRA 72, EMBRA 210 (*Eugenia dysenterica*) Empas 02 (*Prunus avium*) LMCH 12, LMCH 14 (*Annona cherimoya*), SCU 056, SCU 062 (*Melaleuca alternifolia*), and SP 06 (*Schizolobium parahyba*). O LMCH 12 locus was eliminated to present low accuracy. Table 1 shows the correlogram of *S. adstringens* indicating that the distances, among individuals of Botucatu State Forest, from 0 to 33 and from 99 to 132 meters have

Table 1 Correlogram using Moran's Index (D) with confidence interval (CI), probability of exclusion (P), number of comparisons (CN), and value indicating absence of spatial autocorrelation D = -0.0270 for *Stryphnodendron adstringens*

Distance (m)	D(-CI)	D(obs.)	D(+CI)	P(D)< (-CI)	P(D)> (+ CI)	CN
0-33	-0.080841	0.043351	0.034583	0.015	0.985	40
33-66	-0.078096	-0.005722	0.023789	0.190	0810	45
66-99	-0.079640	-0.010610	0.031820	0.291	0.709	41
99-132	-0.081936	-0.096614	0.032199	0.995	0.005	35
132-165	-0.079713	-0.015566	0.027000	0.355	0.645	38
165-198	-0.090142	0.025456	0.033938	0.046	0.954	37
198-231	-0.064909	-0.059062	0.007197	0.954	0.046	75
231-264	-0.086119	-0.063560	0.031723	0.867	0.133	25
264-297	-0.114183	-0.054740	0.074730	0.715	0.285	7
297-330	-0.103925	-0.058399	0.052559	0.800	0.200	10

presented spatial structure. According to Sebbenn [6]is common in tropical species, similarities until 100 meters; therefore to collect high variability of *S. adstringens* it is important obeyed distance up to 132 meters.

Conclusions: Significant results were observed for the class distances from 0 to 33 meters and 99 to 132 meters, showing there is spatial autocorrelation for *S. adstringens*.

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S2. LINKAGE AND ASSOCIATION MAPPING

P22

Development of DArT (Diversity Arrays Technology) for high-throughput genotyping of *Pinus taeda* and closely related species

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Background: *Pinus* (*Pinaceae*) is the largest existing genus of conifers, with over 100 widely recognized species. Pines are an important source of wood, paper and resins, among others. Genetic improvement of *Pinus* species is a challenging endeavor. Breeding cycles typically last for decades, significant changes take place in wood properties and growth patterns with the transition from the juvenile to the adult phase and most traits are multi-factorial with low heritability [1]. Genomic Selection (GS) could radically reduce the time required for completion of a cycle of genetic improvement by precluding the progeny testing phase, significantly increasing the selection efficiency relative to conventional breeding [2,3]. To put GS into practice, genome-wide, high-throughput and cost-efficient marker are needed to advance this genomics assisted breeding approach focusing purely on prediction of performance, precluding gene-trait association discovery. To this end we are developing a high-throughput DNA genotyping platform for Pinus taeda based on the genome complexity reduction DArT (Diversity Arrays Technology) technology that has been successfully developed for many plant species including some with very large and complex genomes such as wheat [4] and sugarcane [5].

Methods: For DArT array probe development population samples of 16 species of *Pinus*, 24 provenances of *Pinus taeda* L. and two mapping populations were used as starting material organized in pooled samples. For microsatellite and DArT mapping a set of 288 individually extracted haploid megagametophyte DNA samples of clone 7-56 were used. DArT development involved reduction of genome complexity of pooled samples by digestion with *PstI* as the primary cutter and *BstNI* as a frequently and secondary cutter followed byligation of adaptors to *PstI* overhangs and PCR amplification of intact *PstI* fragments following a protocol described earlier [6]. Genomic representations (targets) generated by PCR amplification were cloned into six separate libraries. A total of 7,680 clones were randomly picked being 2,304 clones from the 16 species libraries and 4,608 clones from *P. taeda* only libraries. A first test panel of 96 *Pinus* individuals was used to screen the 7,680 probes for polymorphic markers. Markers were declared reliable and polymorphic at a cutoff of 80% call rate, 95% reproducibility, and polymorphism information content > 0.1. Parallel to DArT marker development a linkage map of microsatellites was generated to provide a framework onto which the DArT markers will eventually be mapped. DNA from haploid megagametophyte samples of 288 seeds of Loblolly pine tree 7-56 was extracted. To immortalize the limited amount of haploid DNA of this mapping population, whole genome amplification was used. Haploid samples were genotyped with a selected set of 65 microsatellites. Genotyping was performed in an ABI PRISM 3100XL using 4-color fluorescent detection in multiplexed sets of 2 co-amplified microsatellites. Linkage analyses and map construction was carried out using JOINMAP 3.0.

Results: A total of 856 and 930 markers were found polymorphic across samples of 24 individuals of each one of two mapping populations, while 1,776 DArT markers were polymorphic in a panel of 16 species, two individuals per species and 16 *Pinus taeda* US provenances, one individual per provenance. A significant number of probes could not be adequately scored due to background signal resulting from the presence of highly repetitive DNA that the *PstI/BstNI* complexity reduction could not remove from the 23 pg *Pinus taeda* genome. Cot-1 DNA, i.e. genomic DNA highly enriched for repetitive elements was therefore isolated from *P. taeda* DNA by shearing genomic DNA in autoclave, followed by DNA denaturing and re-annealing and S1 nuclease treatment. Using the Cot-1-DNA to quench repetitive probes, the recovery of assayable DArT markers was increased by 40%. When mapping microsatellites extensive locus duplication of the 65 microsatellite primer pairs resulted in a total of 182 individually "loci" scored for presence/absence in the haploid gametes. Based on the map position 96 of these "loci" actually corresponded to 48 effective loci and the other 86 are actual individual loci totaling 134. From the 134 segregating loci 110 were confidently mapped in the 12 expected linkage groups leaving two unlinked groups of 5 and 3 markers. Locus order was essentially the same for the 50 loci derived from 31 microsatellites in common with those recently mapped [7]. Interestingly, microsatellite PtTX3021 amplified 17 individually scored "loci". Eight were allelic pairs and were consolidated in four loci while the remaining 9 were unique. Additional duplications were seen for 15 microsatellites in different linkage groups suggesting paralogous loci.

Conclusion: In spite of the complex and repetitive nature of the *Pinus taeda* genome several hundred DArT markers could be confidently genotyped indicating that DArT is a robust technology to be used for conifers. A new round of DArT probe picking and marker screening coupled to Cot-1 DNA use is now underway and should provide the target number of 7,680 selected probes for an operational array. The haploid mapping population will be genotyped with this array to provide information on distribution of the DArT markers in terms of recombination fraction and genome coverage relative to the framework of microsatellites. Additionally, probes detecting mapped markers will be sequenced to eventually aid the assembly of the 7-56 genome sequence.

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P23

Effect of population structure and kinship relationships on the results of association mapping tests of growth and wood quality traits in four *Eucalyptus* populations

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Background: In recent years, association mapping studies have been reported for growth and wood quality traits in *Eucalyptus* (e.g. [1]). One problem with association studies is that they can be sensitive to the presence of population structure. The presence of population structure may generate spurious associations between markers and traits and leading to an elevated false-positive rate (e.g. [2]). Statistical approaches that account for population structure include model-based clustering [3], principal component analysis, genomic control and linear mixed model approach [4]. The mixed model of Yu et al. (2006) accounts for both major population structure, assigning individuals to subpopulations (the Q matrix), and the relatedness among individuals within and between subpopulations (the kinship (K) matrix). The mixed model approach generally performs best [2,4].
As part of the Biotech MERCOSUR project (Marcucci Poltri et al. this volume) molecular and phenotypic data from four *Eucalyptus* populations have been obtained: three open pollinated (OP, half-sib) progeny trials of *Eucalyptus grandis* from Argentina (EgrAr) and *Eucalyptus globulus* from Uruguay (EglUy) and Argentina (EglAr) and one clonal trial of *Eucalyptus grandis* from Paraguay (EgrPy). These populations differ in the underlying substructure and genetic relatedness among individuals. It is thus important to investigate the effects of population structure and kinship on the results of associations between markers and growth and wood quality traits from these *Eucalyptus* populations.
Material and methods: A total of 612 trees were sampled from the EgrAr (188), EgrPy (121), EglAr (134) and EglUy (169) populations. The number of OP families sampled in each OP progeny trials was 132 (EgrAr), 129 (EglAr) and 70 (EglUy) from different native stand sites in Australia (from 8 to 13) and land races (from 1 to 3). The number of trees per OP family varied from 1 to 3 (EgrAr), 1 to 8 (EglUy) and 1 to 2 (EglAr). One growth trait (diameter at breast height, DBH) and three quality wood traits (extractives in ethanol, Klason lignin and syringyl:guaiacyl ratio (S:G ratio)) were studied.
All the 612 trees were genotyped using Diversity Arrays Technology (DArT) molecular markers [5]. A subset of 2816 (EgrAr), 2693 (EgrPy), 2373 (EglAr) and 2300 (EglUy) DArT markers were used in the analysis after markers with frequency greater than 0.95 or less than 0.05 were excluded.
The association mapping tests were carried out at the DArTs level using two-steps. First, for the three OP trials, the overall mean and design effects or first order autoregressive residuals for rows and columns, were fitted to deal with environmental variation. Additionally, for the clonal trial, the best linear unbiased predictions (BLUP) of clonal values were predicted. Second, the markers effects were tested on the adjusted phenotypes (OP trials) or clonal BLUPs values (clonal trial) using four models [4]: 1) Simple model, in which Q and K matrices are ignored; 2) Q model, considers only Q matrix; 3) K model, considers only K matrix; and 4) Q+K model, considers both Q and K matrices. Except for the EglAr population, the Q matrix was calculated by the software STRUCTURE [3] on basis of 400 random DArT markers. The K matrix was calculated on basis of 800 random DArT markers using the software package SPAGeDi [6].
For each of the four populations, all the association tests were carried out using TASSEL software [http://www.maizegenetics.net/].
Results: All populations showed an optimum cluster number of 4. In general, for the three OP populations the compositions of the cluster coincide with the geographical native stand sites in Australia. More than 52% of the pair-wise kinship estimates were equal to 0, whereas about the 46% of the values were less than 0.25. Without taking into account the population structure and kinship (Simple model), from 0.9 to 8.5% of the DArT markers tested were associated with the growth and wood quality traits at $P < 0.01$. These preliminary results show a high number of associated markers that might suggest that several of them are likely to be false-positives due to population structure and/or kinship relationships among trees within each population. For all populations and most traits under consideration, the controlling only for population structure (Q model) reduced the number of significant DArTs (from 0.5 to 2.6%). This effect was more pronounced for the two OP *Eucalyptus globulus* populations. However, when the relative kinship coefficients between every pair of individuals were considered (K model), a more stringent reduction with respect to the Q model was observed (from 0.3 to 1.3%). This finding suggests that, when more complex interrelationship among individuals within and between subpopulations exists, the fitting Q model was not enough to reduce the number of spurious associations. It is no clear, across populations and traits, that the Q matrix should be added to the kinship effect. The reduction of number of significant DArT markers, excluding pedigree information (K matrix) in the model, appeared to be trait dependent.
Conclusions: Both population structure and kinship relatedness between individuals had effect on the association mapping tests. The kinship had a more stringent effect on the marker-trait associations. Effect on association tests depends on the populations and traits studied.

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Construction and analysis of a leaf cDNA library from cold stressed rubber tree clones

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Background: Rubber tree [*Hevea Braziliensis* (Willd. ex Adr. de Juss.) Muell-Arg.], a native species of the Amazon Rainforest in South America, belonging to the Euphorbiaceae family, is world's major source of natural latex. Brazil, despite being the center of origin and diversity of the species and latex main producer at the end of XIX century, nowadays imports around 70% of the rubber consumed in the country. Although the Amazonian basin provides optimal conditions for rubber plantation, the occurrence of South American leaf blight (SALB) disease, caused by the ascomycete *Microcyclus ulei*, limits the rubber production in

this region. Therefore, rubber plantation has been extended to sub-optimal areas that are prominently located in northeast India, Vietnam, southern China and southern plateau of Brazil. Besides the new conditions for crop development, these new areas present stress conditions like low temperatures, dry periods and wind. It was shown that low temperatures affect the development and latex production of rubber trees. Breeding programs have been searching for clones adapted to these suboptimal areas. A complete cycle of breeding and selection takes at least 20 years to succeed in obtaining a superior clone. As such, development of new methods for early evaluation is essential to reduce and optimize the breeding management. Aiming to help in the development of these methods, in this work, we have constructed a leaf cDNA library to study ESTs (Expressed Sequence Tags) from cold stressed rubber trees. The originated sequences from this library can be used to create an ESTs databank and will provide genetic knowledge about how rubber trees cope with chilling stress, being also a valuable source of polymorphic molecular markers, such as SSRs and SNPs. Some of these markers may be associated to chilling tolerance, and early evaluation would be possible in young rubber tree clones.

Methods: The young *Hevea Braziliensis* Muell. Arg. clones GT1, IAN873, PR255 and PB217 were obtained from Agronomic Institute of Campinas (IAC). GT1, PR255 and PB217 clones are genitors of mapping populations of ongoing projects at Molecular Genetics and Analysis Laboratory and IAN873 clone showed chilling tolerance in field. The 24h cold treatment was peformed in a diurnal growth chamber (8°C, 12h photoperiod). The collected leaves were previously wrapped up in tinfoil to prevent transcript redundancy. Leaves were sampled in intervals of 6h, 10h and 24h and total RNA was extracted by LiCl precipitation. Total RNA extracted from clones were pooled in equal amounts, according to time of sampling. The In-fusion SMARTer cDNA Library Construction Kit (Clontech) was used to construct the cDNA library. A sequenced plaque (96 clones) of each sub-library (6h, 10h and 24h) was chosen for a preliminary analysis of the library. Sequences were analyzed and clustered by the DNAStar package (DNAStar, Inc.) and Blast2Go software (http://www.blast2go.org) was used to annotate the contigs and singlets. The resulting ESTs were screened for microsatellite regions using Simple Sequence Repeat Identification Tool (SSRIT) (http://www.gramene.org/db/markers/ssrtool).

Results and discussion: The cold stressed leaf cDNA library has around 6,000 clones and is sub-divided into three libraries: "cold-6h", "cold-10h" and "cold-24h", having 2,000 clones each. The average fragment lengths from these libraries are 780bp, 625bp and 930bp, respectively. In order to perform a preliminary analysis of the libraries expression profiles, 96 sequenced clones from each library were chosen randomly. Among the 288 sequences analyzed, 87 were discarded due to sequencing reaction failure or sequencing slippage. Clustering of the remaining 201 sequences generated eight contigs and 180 singlets. All contigs and singlets were annotated and distributed into functional categories. "Hypothetical and Unknown Proteins" was the major functional category, containing 52 sequences (26%). Most of these sequences (25) belong to "cold-24h" library. Second larger functional category was "Metabolic Processes", constituted by 34 sequences (17%). It was also in that category that libraries showed a visible difference among them: while about 20% of the sequences of libraries "cold-6h" and "cold-10h" is involved in cellular metabolic processes, in the "cold-24h" library this number falls to 13%. Chilling stress mainly causes alterations in metabolic processes and decrease in enzymatic activities. An exposure of 24 hours to low temperatures seems to be enough to trigger such a change in stressed rubber trees, which maybe could explain the higher number of hypothetical proteins in that library; however analysis of more sequences is necessary to confirm the results. Sequences that presented no similarity to any sequence in GenBank database were grouped in the "No Match"

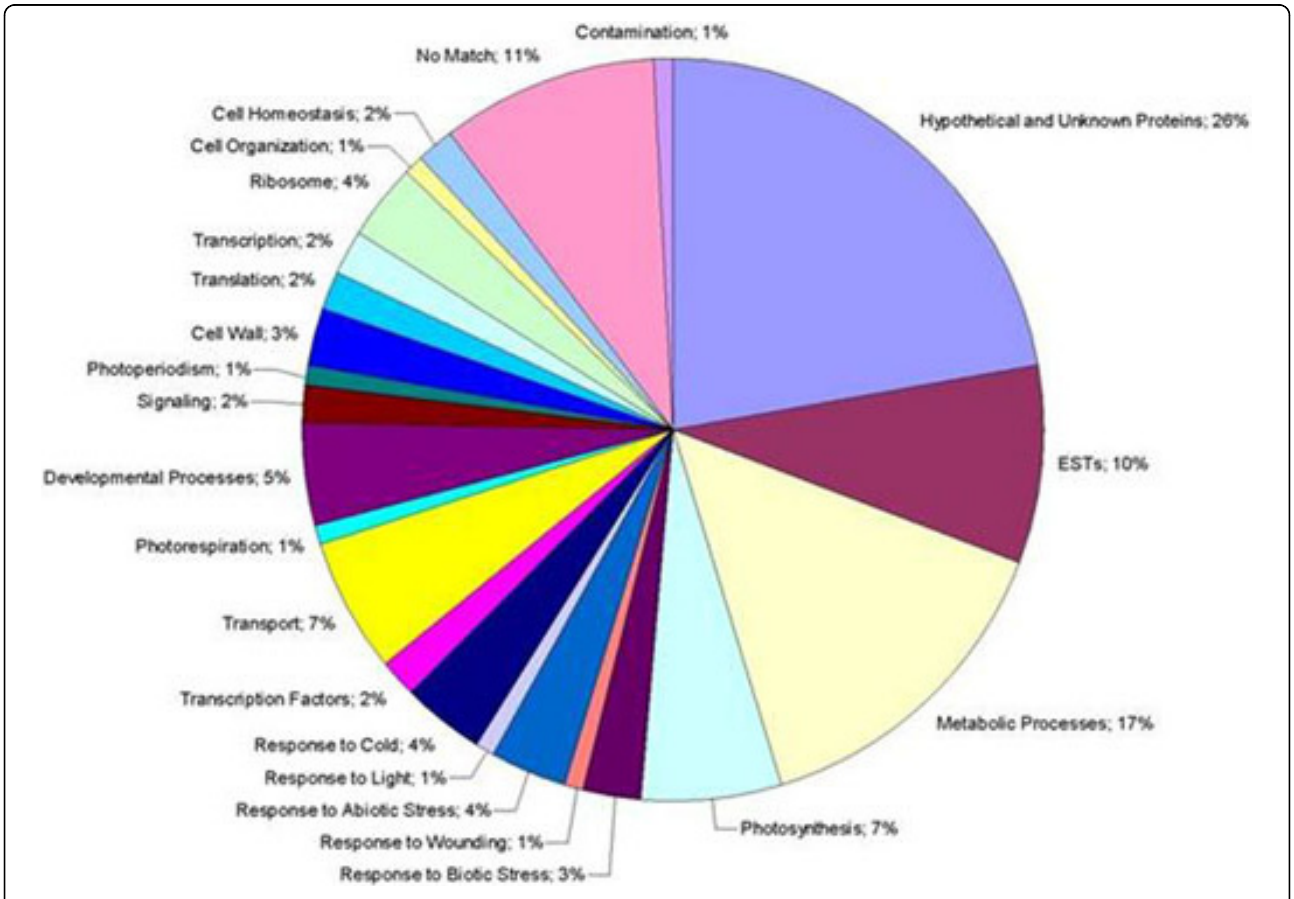

Figure 1(abstract P24) Relative distribution of annotated ESTs into functional categories.

category, which is third in number of sequences (22; 11%). These sequences may be specific to *Hevea Braziliensis* and several presented at least one ORF that could be translated. The relative distribution of all annotated ESTs into functional categories can be seen in figure 1. The presence of microsatellite regions in the sequences was also verified and 30 ESTs sequences bearing SSR regions (15%) were found: 13 dinucleotide (43%), 9 trinucleotide (30%) and 8 tetranucleotide motifs (27%). Microsatellite regions were present mostly in hypothetical protein sequences (13; 43%) and all of them were in the UTR regions. Primers for these sequences are being designed in order to evaluate their polymorphism. The sequencing process of the cold stressed leaf cDNA library is still ongoing. The comparison of a small number of sequences showed a difference in expression pattern between the sub-libraries that may be confirmed by a wide analysis of their sequences. A preliminary analysis for microsatellite regions suggested that around 15% of the sequences may have potential polymorphic SSR markers. Moreover the ESTs generated will be a rich source for polymorphic SNP markers. SNP and SSR molecular markers will be developed from these data and will be used in *H. Braziliensis* genetic mapping. These markers could provide tools for early evaluation of cold tolerant rubber tree clones, among other genetic studies.

P25

NextGen sequence analysis of two sex-linked *P. tremuloides* genomic regions on chromosome 19

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Background: Sex determination in poplars is still an open question. In the male *P. tremuloides* parent we identified several sex-linked SSR markers and mapped them to a central position on the male map of linkage group XIX.
Material and methods: Using DNA probes derived from these SSR markers, two BAC clones were isolated from a BAC library of the *P. tremuloides* male clone (Pakull B. *et al.*, 2011, Can. J. For. Res. 41, 245-253) and further analyzed by454 sequencing (GATC Biotech AG). The generated single end reads were assembled to contigs using Newbler and Mira. The set of combined contigs (Newbler and Mira) of each BAC was subjected to scaffolding by SeqMan Pro (DNASTAR Lasergene) together with the related BAC end sequences created by Sanger sequencing (Pakull B. *et al.*, 2011, Can. J. For. Res. 41, 245-253). The subsequent combination of the created scaffolds to BAC consensus sequences was assisted by Sanger sequencing of PCR amplified scaffold ends and connections.
Results: Mira created more contigs for both BACs than Newbler, where the N50-contig size was similar for both methods (~10,000 bp). The largest contig (38,518 bp) was assembled by Mira. Based on our strategy we already created a draft sequence of ~47,500 bp for one BAC clone. We expect a size of about 50 kb for the second BAC after finishing scaffolding.
Conclusions: Based on the final consensus sequences of both BACs, we will search for putative sex-determining regions or genes in the *P. tremuloides* genome and compare them with *P. trichocarpa* paralogs.

P26

A genetic linkage map for a Full sib population of *Eucalyptus grandis* using SSR, DArT, CG-SSR and EST-SSR markers

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Background: *Eucalypts* are the most widely planted hardwood trees in the world, occupying globally more than 18 million hectares, as an important source of carbon neutral renewable energy and raw material for pulp, paper and solid wood. Intensive planting programs of *Eucalyptus grandis* have been carried out in the Argentinian Mesopotamia.
Linkage maps are useful tools for quantitative trait loci (QTL) analyses and detection. Several maps for QTL analyses of growth and wood quality have been developed in this genus [1,2] and most of the *E. grandis* maps have been carried out in interspecific crosses.Improved marker density in genetic maps, high-throughput techniques and transferability across species are key aspects to increase resolution and speed for a variety of genomic applications in *Eucalyptus*.In this context, an important issue in association studies is the selection of appropriate mapped candidate genes that co-localize with QTL of interest.
As part of the Biotech MERCOSUR project (Marcucci et al., this journal), we here report the construction of a genetic linkage map for *E. grandis* in the context of a QTL study of this specie in an effort to understand the molecular basis for quantitative trait variation in wood quality. This map includes Diversity Arrays Technology (DArT) [3], microsatellite (SSR) markers [4], Candidate Genes-SSR (CG-SSR) for wood quality traits and stress responses functions and Expressed Sequence Tag-SSR (EST-SSR) for putative function related to stress responses and other functions (Acuña et al., this journal). These CG-SSR and EST-SSR were not mapped in *Eucalyptus* previously.
Material and methods: Plant material: *E. grandis* x *E. grandis* (EG-INTA-161 x EG-INTA-152) F1 population of 130 full-sib progeny cloned (3 ramets) and planted in 2007 in Entre Ríos, Argentina, was analyzed.
Genotyping: The parents of the mapping cross were initially screened with: 55 SSR, 12 CG-SSR and 37 EST-SSR markers; these last two classes of markers derive from a broad study (for details see Acuña et. al., this journal). Capillary electrophoresis and fluorescent detection were carried out on an ABI 3130xlGenetic Analyzer. A DArT Microarray of 7,860 clones was screened for useful polymorphic markers.
Linkage and bioinformatic analysis: All loci were tested for goodness of fit to expected Mendelian segregation ratios using Chi-square goodness of fit tests. The assignment of DArT sequence function was performed using the Blast2GO software [http://www.blast2go.org/]. A consensus genetic linkage map was constructed with JoinMap v3.0 [5]. Linkage parameters were set as 10 minimum LOD and 0.4 maximum recombination fractions.
Results and discussion: In this intraspecific *E. grandis* population, 78% of the SSR markers tested could be mapped. Most mapped SSR loci were fully informative, segregating in approximate ratios of 1:1:1:1 (either heterozygous in both parents four alleles in total). Seven SSR loci that followed approximate segregation ratios of 1:1 (heterozygous in only one parent) were EMBRA 47,51,101,179,676,1244,2010.
From the DArT Microarray of 7,860 clones, 31% of the marker were selected because of their high call rate (>0.80) and polymorphism between parents.
A large proportion (1,503/2,381=63%) of the DArT markers displayed a Mendelian behavior indicating that they sample single copy regions and provide markers that can be used for genetic analyses (65% segregating in 1:1 ratio and 35% in 3:1 ratio).
The map was assembled with 1032 markers, including 976 DArT, 43 SSR loci (2-6 per linkage group), seven EST-SSR and six CG-SSR. The resulting integrated map featured the expected 11 major linkage groups, yielding a genome coverage of 1358.4 cM, and an average consecutive intermarker distance of 1.3 cM in accordance to other reports [1,4]. Linkage groups were numbered following the standardized nomenclature for Eucalyptus proposed by Brondani et al [4].
The six Candidate Genes and seven ESTs include enzymes involved in lignin and cell-wall polysaccharide biosynthesis and stress responses genes, while 267 DArT (29.7%) were assigned to a gene ontology (GO) categories and 296 loci (32.9%) had significant matches with the nonredundant protein database using BLASTX. Thus, 25 enzymes in 56 metabolic pathways were represented by at least one sequence with its corresponding EC number.
The inclusion of common previously mapped SSR markers in several different eucalypt species within the subgenus *Symphyomyrtus* (*E. globulus*, *E. camaldulensis*, *E. dunnii*, *E. tereticornis*, and predominantly *E. grandis* and *E. urophylla*) allowed comparison of linkage groups among this *E. grandis* population and other species of the genus. Linkage orders previously reported in *E. globulus*, *E. grandis* and *E. urophylla* were also observed in this intraspecific *E. grandis* population, supporting the developed map.

Conclusions: In this work, 13 new functional SSR and 976 DArT (296 with assigned functions) markers are mapped in an intraspecific *E. grandis* F1 population. This map will be used to locate QTL for wood quality and growth traits in the specie. Also, this map can help to identify candidate genes and regions in the *Eucalyptus* genome useful for fine scale analysis with association studies that are being developed by our group (see Cappa et al., this volume).The putative functional approach combined with the genetic linkage mapping provides an advantage tool for future analysis in locating genes of interest in *Eucalyptus*.

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P27

QTL associated with resistance to defoliation *(Cylindrocladium pteridis)* in *Eucalyptus spp.*

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Leaf blight and defoliation caused by *Cylindrocladium pteridis* is one of the main leaf diseases found in Brazilian eucalypt plantations in warm and high rainfall regions. The inter and intraspecific variability in the intensity of defoliation indicate that your control can be achieved by planting resistant genotypes. In order to guide breeding programs and to predict the stability of resistance is essential to understand the genetic control of resistance in eucalypts to defoliation. Thus, the objectives of this paper were to map QTLs (Quantitative Trait Loci) associated with resistance to defoliation in a *Eucalyptus urophylla* x *E. camaldulensis* (EU11 x EC06) family and validate microsatellite markers linked to QTLs in four unrelated pedigrees involving different *Eucalyptus* species. Defoliation was evaluated on the branches of the lower third of the plants inoculated under controlled conditions. We identified five QTLs for resistance to defoliation (Rd), three on the map of the parental EU11, Rd-1 and Rd-2 located on linkage group (LG) 1 and Rd-4 located on LG6; and two QTLs located on the map of the parental EC06, present in LG2 (Rd-3) and LG8 (Rd-5). Markers linked to QTLRd-2 were validated in two unrelated families by simple linear regression analysis. This is the first genomic map of QTLs associated with resistance to defoliation caused by *C. pteridis* in *Eucalyptus*.

P28

Genomic of resistance for *Fusarium circinatum* in *Pinus radiata*

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The pitch canker fungus *Fusarium circinatum* was first associated with mortality of *P. radiata* in California during the 1980s. Since then it has been dispersed to several countries of the Northern hemisphere together with South Africa and Chile in the southern hemisphere. In Chile the disease caused mortality of seedlings in *P. radiata* at nursery, and in South Africa the pathogen has already completed its life cycle in plantations. This situation could be replicated in Chile, in the short-term, becoming then a major problem due to that most of the commercial plantations correspond to radiata pine. Research and breeding programs to ensure the availability of resistant trees are essential.

Controlled inoculation trials using different pine species have shown the high susceptibility of *P. radiata*[*1*]. Different families of radiata pine in Australia, New Zealand and Chile have been screened, showing a considerable genetic variation for lesion length and a high heritability depending on the population, suggesting that selection for resistance should result in useful genetic gains [2]. Based on the above findings a molecular approach of pitch canker resistance in *P. radiata* was carried out. This study focuses on the discovery of genome regions associated with resistance and the genes involved in the response mechanism(s), to develop genomic tools for breeding resistance.

The resistance to *F. circinatum* was investigated using a Quantitative Trait Locus (QTL) mapping strategy in a full-sib family of *P. radiata*. This mapping project used a double pseudo testcross approach to map each of the parents with dominant markers. Microsatellite (SSR) and Amplified Fragment Length Polymorphism (AFLP) markers were used to genotype the two parents (XO and XP) and the progeny (86 clones). Both parental maps were joined using the co-dominant SSR and AFLP markers to obtain a saturated genetic map. For each clone (approximately 8 ramets/clone) inoculations were carried out at Bioforest S.A. and lesion lengths were measured 90 days later. Phenotypic data together with genetic map allowed identified and positioned QTLs that control part of the pitch canker resistance in the progeny. Using Windows QTL Cartographer three QTLs were detected for the trait of interest for both parents.

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P29

Genetic linkage map of willow (*Salix leucopithecia*×*S.erioclada L*) based on AFLP and SSR markers

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Due to its fast growth rate and little nursery requirements, willow is largely planted as short rotation coppice species for bioenergy resources in temperate regions. Willow coppices can be easily established by vegetative propagation of cuttings and can remain productive for up to 25 years. In China, diploid species *S. leucopithecia* and *S. erioclada* L. are two promising willow species that can be used for high biomass plantation. In order to further improve the biomass yield and resistance to drought and pests, we tried to create a genetic map to assist willow breeding. 560 F_1 individuals from a cross between *S. leucopithecia* x *S. erioclada* L were used to generate a willow genetic map using AFLP and SSR markers.

Both *Populus* and *Salix* are members of the Salicaceae. They share genomic homologues with high similarity and many common biological traits. As a model species for biological studies in trees,*Populus* has considerable genetic and genomic resources, which could provide cues for willow study. Five hundred pairs of SSR primers were selected from *Populus* database for willow analysis; 88 of them successfully generated polymorphic loci between *S. leucopithecia* x *S. erioclada* L. In addition, 24 pairs of AFLP primers also succeeded in detecting polymorphic fragments from F_1 individuals.

We selected 243 individuals to construct a framework linkage maps of parents using a two-way pseudo-testcross strategy. The linkage map of *S.erioclada* L contains 98 loci, organized in 15 linkage groups with total map distances between 76.7 cM and 209.5 cM. The average map distance between markers is 18.6 cM and the total genetic distance is about 1,810 cM, covering estimated 77.13% of the genome. The linkage map of *S. leucopithecia* contains 60 loci, arranged into 9 groups with map distances between 63.5 cM and 149.1 cM. The average map distance between markers is 19.57 cM, and the total genetic distance is about 1,037 cM, covering estimated 70.3% of the genome.

P30

Quantitative trait locus analysis of growth and wood density in an interspecific pseudo-backcross population of *Eucalyptus grandis* x *E. urophylla*

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Background: F1 hybrids of *E. grandis* and *E. urophylla* are commonly grown for pulp and paper production in clonal plantations in tropical and subtropical regions. Improving tree growth [1]and wood quality [2]are important objectives in eucalypt breeding programmes. The efficiency of selection for these traits can be enhanced by molecular breeding approaches enabled by high-throughput, genome-wide genotyping technologies and the recent completion of a reference genome sequence for *Eucalyptus* (*E. grandis* V1.0, JGI, http://www.phytozome.net). In this context, interspecific hybrid pedigrees are valuable for quantitative trait locus (QTL) dissection in *Eucalyptus* as there is abundant interspecific variation in such pedigrees. Breeding for wood property traits is mainly focussed on increasing pulp yield per hectare, reducing wood specific consumption (WSC) and increasing the efficiency of downstream processing by the pulp and paper industry. In this study, we are characterizing the genetic architecture of growth and wood quality traits in an interspecific pseudo-backcross mapping population of an F1 hybrid of *E. grandis* and *E. urophylla.*

Methods: A pseudo-backcross pedigree was developed by crossing an F1 hybrid (*E. grandis* x *E. urophylla*, GUSAP1, Sappi Forest Research) clone to non-parental individuals of the two parental species. Diameter (cm) at breast height (DBH) and wood basic density were measured at three years in a set of 305 and 319 progeny of the *E. grandis* and *E. urophylla* backcross families, respectively. The backcross progeny were also genotyped with more than 1700 polymorphic Diversity Arrays Technology (DArT) markers. Framework genetic linkage maps were constructed for the *E. grandis*, *E. urophylla* backcross parents using 139 and 127 selected testcross (1:1) DArT markers distributed across the 11 *Eucalyptus* linkage groups spanning 926 cM and 1100 cM, respectively. The framework maps of the F1 hybrid contained 172 and 154 testcross DArT markers spanning 1061 cM and 1036 cM in the *E. grandis* and *E. urophylla* backcross families, respectively. The average marker interval of the parental framework maps ranged from 5.6 (F1 hybrid in the *E. grandis* backcross family) to 8.0 (*E. urophylla* backcross parent). DBH of the main stem was measured directly for three-year-old backcross progeny. For the assessment of basic density, a wood disk was taken at a height of 1.5 m. The wood disk from each tree was used to determine basic density by the water displacement method. QTL analysis was conducted using Windows QTL Cartographer using composite interval mapping (CIM) and a genome-wide significance threshold of $\alpha = 0.05$.

Results and conclusion: A total of 9 QTLs were identified for DBH and 15 QTLs were identified for basic density (Table 1). These explained 3.1 to 9.6% and 3.6 to 13.1% of the phenotypic variation in the *E. grandis* and *E. urophylla* backcross families, respectively**.** Higher numbers of QTL were identified in the F1 hybrid parent (15) compared to the backcross parents (9) congruent with the expected interspecific and intraspecific genetic variation segregating in the backcross families.

Comparative QTL mapping in the F1 hybrid and backcross parents identified common and unique QTL regions in the parental maps. On LG6 and LG10, QTLs for DBH and basic density were detected in both F1 hybrid maps (*E. grandis* and *E. urophylla* backcross families). On LG4 and LG9, QTLs for density were shared between the *E. urophylla* and F1 hybrid parent, while a QTL for DBH on LG10 was shared between the *E. grandis* and F1 hybrid parents. Overall, six unique genomic regions with QTLs for DBH and eleven QTLs regions for basic density were identified in the two backcross families. The lower number of significant QTLs identified for DBH compared to that for wood basic density (Table 1) is consistent with previous QTL reports and genetic studies showing that wood quality traits have higher heritability than growth traits [2,3]. The genetic maps constructed and QTL regions identified in this study provide a resource for identifying markers for molecular breeding of volume growth and wood density in *E. grandis* x *E. urophylla*hybrids, as well as candidate gene identification based on the recently released *E. grandis* reference genome sequence.

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Table 1(abstract P30) Putative QTL for DBH and basic density identified by CIM in *E. grandis* and *E. urophylla* BC mapping population

Parent tree	Total number of QTLs identified	Linkage group (LG) number for DBH	Linkage group (LG) number for density	Percentage of variation explained by the DBH QTLs	Percentage of variation explained by the density of QTLs
E. grandis backcross parent	3	LG9, LG10	LG2	3.6 to 4.6%	4.8%
E. grandis F1 hybrid parent	7	LG4, LG6, LG10	LG1, LG3, LG4, LG10	3.7 to 9.6%	3.1 to 5.5%
E. urophylla F1 hybrid parent	8	LG6	LG2, LG4, LG6, LG8, LG9, LG10a, LG10b	7.0%	3.6 to 13.1%
E. urophylla backcross parent	6	LG4, LG9, LG10	LG4, LG8, LG9	4.5-5.3%	4.6-7.8%
Total	24	9	15		

QTL, Quantitative trait loci; DBH, Diameter at breast height, CIM, Composite interval mapping.

P31

Assessment of SNPs for linkage mapping in *Eucalyptus*: construction of a consensus SNP/microsatellite map from two unrelated pedigrees

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Background: High throughput genotyping of SNP (Single Nucleotide Polymorphisms) based markers has been developed for an increasing number of plant and animal species. In forest trees large scale SNP development has been approached mainly by amplicon resequencing targeting specific genes for association genetics studies. This approach, although successfully employed in conifers aided by the use of haploid tissue, is technically laborious in diploids because of the very high levels of nucleotide and indel diversity in highly heterozygous tree genomes. Direct SNP development from large *in silico* sequence resources developed by next-generation sequencing is now a very efficient approach for SNP development in forest trees. We have recently developed a first set of 768 SNPs assayed by the Golden Gate Genotyping Technology for the highly heterozygous genome of Eucalyptus from a mixed Sanger/454 database [1]. We saw that a careful sequence quality assessment and the application of stringent constraints on the SNP surrounding sequences have a significant impact on SNP genotyping performance and polymorphism. With the exception of 72 SNPs specifically selected in 20 candidate genes putatively associated with relevant wood phenotypes, all remaining validated SNPs were randomly picked based solely on in silico quality. In this study we wanted to position these SNPs relative to microsatellites and assess their information content for linkage map construction. To enhance our ability of mapping SNPs we employed two eucalyptus full-sib families involving four different Eucalyptus species.
Material and methods: Two inter-specific segregating populations of *Eucalyptus*, population IP (*E. grandis* x *E. urophylla*) and DGUGL [(*E. dunnii* x *E. grandis*) x (*E. urophylla* x *E. globulus*)] were used for linkage analysis and map construction. DNA was extracted through CTAB and picogreen quantified. The DNA was used for SNP genotyping on an Illumina BeadStation 500 GX. SNP data were analyzed using GenomeStudio V2009.1 and a GeneTrain score cutoff of 0.25 and call rate ≥0.95 were initially applied to the whole dataset. After that, every single SNP was manually checked for genotyping failures and potential calling errors that would bias the overall analysis and segregation ratios. Linkage analysis, individual and consensus map construction for both segregating populations was performed using Joinmap, v. 3.0 [2].
Results: The proportion of informative segregating SNPs out of the 768 assayed were similar in the two mapping populations: 215 SNPs in DGUGL (28%) and 239 in IP (31%). SNPs were mapped on top of existing microsatellite maps. The linkage map of the IP population with 409 markers on the 11 expected linkage groups had 215 microsatellites and 194 SNPs, with an observed length of 1,581.3 cM and average distance between markers of 3.9 cM. The DGUGL map also had 11 linkage groups and 430 markers being 236 microsatellites and 194 SNPs with an estimated length of 1,252.4 cM and average distance of 2.9 cM. The consensus map, constructed using both segregating populations included 624 unique markers, of which 320 were microsatellites and 304 SNPs with 1,451.4 cM and average distance of 2.3 cM. The proportion of segregating SNPs in each population individually (28 to 31%) was consistent with a within-species level observed heterozygosity of ~50% for this set of 768 SNPs (Grattapaglia et al. 2011), reminding that none of these parents were used in the generation of the EST database wherefrom these SNP were derived. The rate of mappable SNPs was enhanced to almost 40% (304 SNPs mapped out of 768) by using two mapping populations suggesting that by sampling more full-sib families it should be possible to map most if not all SNPs in this panel. No evident clustering of SNPs was observed suggesting that these SNPs are randomly distributed in the Eucalyptus genome.
Conclusions: This study shows that large numbers of informative SNPs can be developed directly from in silico sequence databases involving unrelated individuals to the parents of mapping populations. Evidently, by being biallelic, SNPs will be less efficient than multiallelic microsatellites for linkage mapping purposes. This drawback however is clearly compensated by the much higher throughput, automation and lower cost of SNP genotyping. While this is the first reported sizeable scale SNP mapping effort in Eucalyptus, a larger number of informative SNPs mapped at regular intervals will be necessary for broader applications. For example, to implement Genomic Selection in most Eucalyptus breeding programs some 4,500 to 6,000 informative SNPs will be necessary to provide a marker density of 3 to 5 markers/centiMorgan and reach selection accuracies above 70% [3]. This would require a SNP panel of some 9,000 to 12,000 SNPs. While this goal is fully achievable, per sample genotyping cost issues, however, will have to be considered before larger scale SNP developments are undertaken in Eucalyptus. Benchmarking the Golden Gate or Infinium genotyping technologies against other high throughput systems such as DArT and emerging genotyping-by-sequencing methods will eventually define how SNP variants be will assayed in Eucalyptus and for that matter in several other highly heterozygous forest tree species.
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Identification of a novel QTL contributing to rust resistance in *Eucalyptus*

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Background: The genus *Eucalyptus* has many species that are well adapted to a wide range of environmental conditions in Brazil. However, in some areas diseases are a limiting factor, among which the Eucalyptus rust caused by *Puccinia psidii* Winter stands out as a destructive pathogen of the Myrtacea. The growth of plants with high levels of infection is severely compromised and these plants end up being dominated by adjacent plants which ultimately leads to their death. The most efficient method to control the disease is through the use of resistant genotypes. A major effect rust resistance QTL has already been identified [1,2], however a residual variance stays unexplained. In this context, our study aimed to identify genomic regions containing quantitative resistance loci to rust based on segregation data of a S_1 progeny from an inter-specific (*Eucalyptus grandis* x *E. urophylla*) resistant clone.
Material and methods: The progeny used in this study was generated by the breeding program of Fibria and is composed of 90 S_1 trees from the self-pollination of an *E. grandis* x *E. urophylla* hybrid tree. This hybrid (VCP-R) is heterozygous for a rust resistance locus [3]and highly resistant to rust. The plants were evaluated at two different sites with high incidence of rust starting in November 2005. Four disease severity evaluations were made at 90, 120, 150 and 180 days after planting by scoring the levels of disease using a four-score severity scale. Scores were used to calculate the area under the disease progress curve (AUDPC) for each plant and for BLUPs estimation. Genomic DNA was CTAB extracted from health young leaves and used in PCR amplifications of 121 microsatellite, 20 TRAP and 38 AFLP markers (totalizing 179 markers).

The Onemap software [4]was used in linkage analysis. The estimated BLUPs were used in QTL mapping [5]. The QTLs were mapped by interval mapping (IM) and composite interval mapping (CIM).

Results: Linkage analysis resulted in 11 linkage groups with 160 linked markers. The identity of the groups were as in [6]based on common microsatellite loci as references. The length of the map was 1075 cM with an average distance of 6.7 cM. IM analysis identified one QTL in linkage group 3 (LOD=7.7) with a large phenotypic effect that accounted for 28.5% of the variation (fig. 1). The negative additive effect was significant ($p = 0.05$), without dominance effects. QTL was located 1.6 cM from SSR EMBRA049 and 5.8 cM from AFLP E08M18_250. The CIM analysis mapped two QTLs, both located in linkage group 3. The peak LOD score (LOD=10.3) of QTL_1, which explained 39.5% of the phenotypic variation, mapped in the same interval of the QTL identified in the IM analysis. It was located close to Embra049 (1.6 cM) and presented significant negative additive effects and no significant dominance effect. The second QTL (QTL_2), with LOD=3.4, had a relative lower phenotypic effect (6,9%), mapped 3.1 cM close to the AFLP marker E08M18_300 AFLP. However, differently than QTL_1, QTL_2 presented both additive and dominance effects.

Conclusions: The use of the AUDPC permitted to transform discrete variables (symptoms scores) into a continuous variable, which gives greater power in QTL detection. The effect of the reduced size of the S_1 progeny in QTL detection was compensated by the BLUPs analysis, which considers only the genetic variation in QTL localization. QTL mapping was done by two different approaches (IM and CIM) that presented contrasting results. The non detection of QTL_2 in the IM analysis was probably due to the limitations of the method (e.g., non-independence in different intervals when there are more than one QTL and low precision of the position by the presence of linked QTLs). Previous studies on the inheritance of resistance to rust in eucalyptus identified a locus with large phenotypic effect, but also pointed to the existence of minor effects [1,2]. Our results confirm the hypothesis of the presence of at least one minor effect locus, by data analysis in quantitative approach and CIM, as never used before in Eucalyptus rust resistance mapping. This work revealed the existence of a second rust resistance locus in addition to one with large effect locus previously mapped in linkage group 3. The identification of markers linked to resistance loci gives perspectives of MAS of resistant genotypes, which is desirable in the case of this disease due to the technical and economical difficulties of selecting resistant genotypes based solely on phenotypes since this biotrophic requires large areas and skilled labor. In addition this study opens possibilities for the positional cloning of the resistance genes together with the availability of the genome sequence of eucalyptus.

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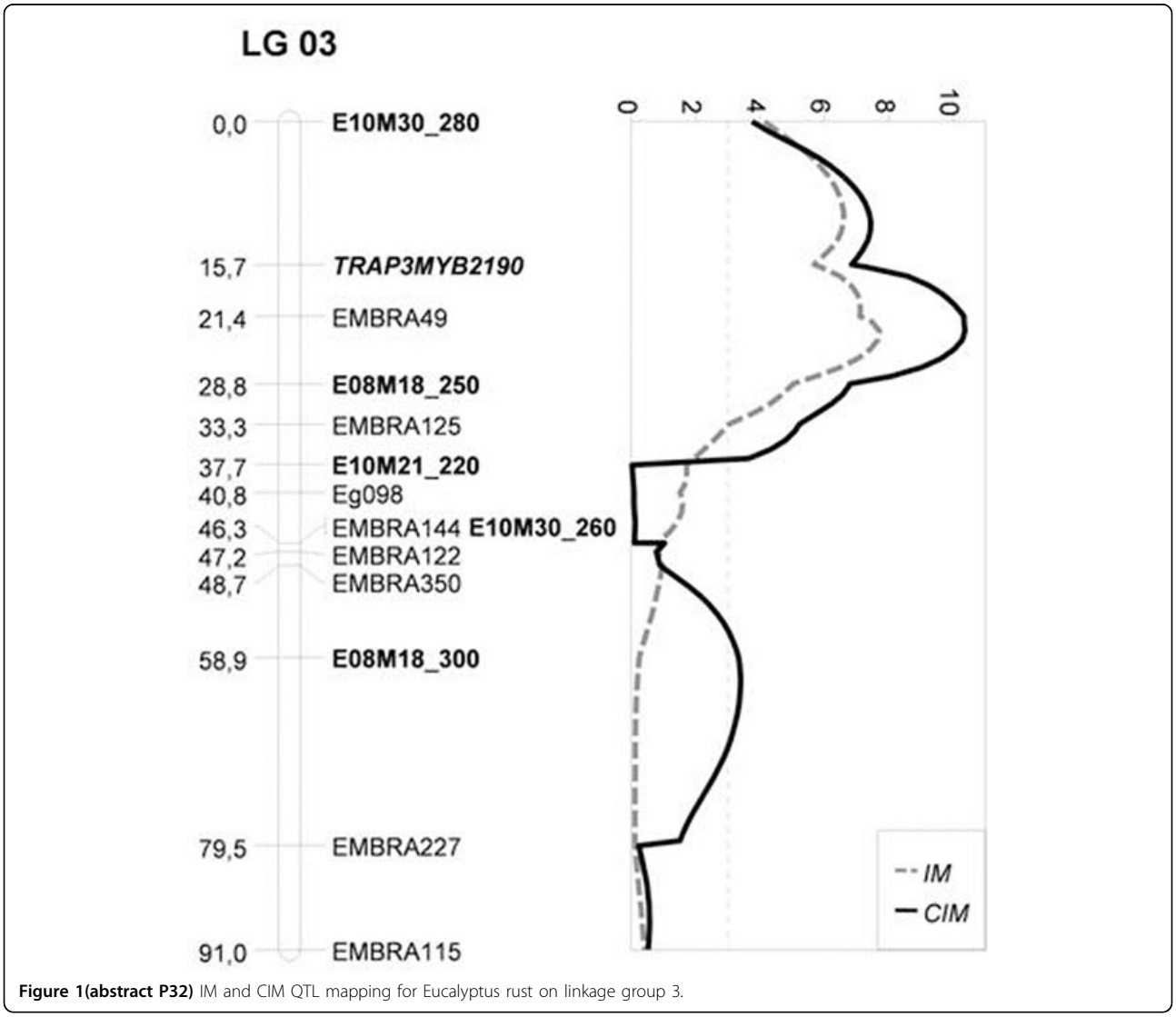

Figure 1(abstract P32) IM and CIM QTL mapping for Eucalyptus rust on linkage group 3.

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Biotech MERCOSUR project: an integrated genotyping and phenotyping platform of *Eucalyptus* germplasm for mapping purposes

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Background: The Biotech MERCOSUR project (85% founded by the European Union) launched in January 2009 and finished in December 2010. The general objective was to establish a regional scientific-technological network to develop a genotyping and phenotyping platform useful for the improvement of *Eucalyptus* in the region. Two advanced genomic strategies (genetic and association mapping) were applied to investigate the genetic basis of the industrial wood formation and energy production of *Eucalyptus* plantations. Up to date, no projects were carried out involving the four MERCOSUR countries altogether. The project brought together scientists from 3 MERCOSUR public research institutes and 2 industrial partners from 4 countries.

The project was possible thanks to the availability of five evaluated field trials of *Eucalyptus*, four segregating populations, a high-density Diversity Arrays Technology (DArT) resource of wide genome coverage and high transferability technology for the genera [1], as well as phenotypic assessment methods of physical and chemical properties of wood using Near Infrared Reflectance (NIR) [2] together with the classical measurements of growth and wood quality tree properties.

The aim of this work is to present the main inputs and results achieved in the project.

Materials and methods: Plant material: Open pollinated (OP) progeny trial of *Eucalyptus grandis* planted in Argentina: 188 trees from 132 OP families (1 to 3 trees per OP family) from 13 native stand sites in Australia and 3 Argentinean land races.

Clonal trial of *E. grandis* planted in Paraguay: 123 clonal trees most of them with unknown provenances.

OP progeny trials of *E. globulus* planted in Uruguay: 169 trees from 132 OP families (1 to 8 trees per OP family) from 8 Australian geographic races and one Chilean land race.

OP progeny trial of *E. globulus* planted in Argentina: 134 trees from 129 OP families (1 or 2 trees per OP family) from 8 Australian geographic races and 2 landraces from Chile and Portugal.

Full-sib progeny trial of *E. grandis* planted in Argentina: 130 full-sib trees (see García et al. in this journal).

E. grandis× *E. urophylla*: described in [3].

Genotyping: Over 7,600 clones of DArT *Eucalyptus* microarray were screened.

From 12 to 19 polymorphic simple sequence repeat (SSR) markers [4]were used to estimate diversity parameters as well as inbreeding coefficients for the *E. grandis* and *E. globulus* populations. For map development, more than 300 SSR markers were tested.

Growth, shape and wood quality tree measurements: Standard methods were used to measure diameter at breast height, total height, stem straightness, pilodyn penetration and basic density for all population analyzed.

Chemical wood properties for NIR spectroscopy predictions: Lignin contents was measured by Klason method, extractives by an adjusted laboratory protocol, Syringyl:Guaiacyl ratio by analytical pyrolysis.

Pulp yields were calculated based on the oven-dry weight of wood chips charged to the reactor at Kappa number 18.

The NIR spectra were obtained by diffuse reflectance on a Bruker model MPA.

Data analysis: Several tools for data analysis were applied according to the objectives of the different approaches (preliminary association mapping, see Cappa et al.; genetic mapping, see García et al., this journal and [3].

Results: 1. Germplasm characterization with DArT markers in two *E. grandis* and two *E. globulus* populations: between 2,300 and 2,816 DArT (call rate higher than 0.80, and polymorphic for more than 95% of individuals) were included in a preliminary association analysis (Cappa et al. in this volume).

2. Linkage Mapping for QTL detection with DArT (between 1500 and 2000 segregating markers per population), SSR, EST-SSR and candidate genes in *E. grandis* population (García et al., this volume) and in *E. grandis* x *E. urophylla*[3].

3. NIR-PLSR models were developed to assess extractives (ethanol and water) and lignin content (Klason and total) as well as lignin composition (S/G ratio) and pulping yield for *Eucalyptusglobulus* and *E. grandis*. All models obtained were at least good enough for screening with RPD above 2 [2]. The RPD obtained varied with trait and species (*E. globulus*: extractives-2.3; Klason lignin-3.9; total lignin-3.8; S/G-3.8; pulping yield (K18)-3.5. *E. grandis*: extractives-5.9; Klason lignin-6.5; total lignin-3.2; S/G-4.2).

4. Phenotyping analyses: between 2,000 and 16,000 trees were characterized for 2 to 5 traits (diameter at breast height, total height, stem straightness, pilodyn penetration and basic density). This data for all population is available as a product of the project. The phenotypic distribution of all continuous traits was studied. Then, to deal with environmental variation, the data were analysed using a mixed linear model with separable first-order autoregressive residuals for rows and columns (i.e., with a standard spatial analysis).

5. Generation of clonal populations in Argentina and Uruguay for future association mapping studies: two *E. globulus* and one *E. grandis* ($n > 200$ each) for a more precise and thorough evaluation of phenotypes are being planted.

6. Origin assign for clone trees of unknown provenances. This was possible due to the combination of DArT markers analysis with population structure approaches. More than 50 clones were assigned to defined clusters.

7. Training of the participants involved: 4 lab training and 2 courses were developed for different participants of the project.

Conclusions: The genotyping and phenotyping platform was successfully established and encourage the collaboration between the participants of the different countries. Likewise different strategies have established the basis for developing new works in the near future using the resources and tools generated in the context of the Biotech MERCOSUR project.

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P34

A new set of 182 microsatellites for *Eucalyptus*: characterization and mapping in a four-species consensus linkage map

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Background: *Eucalyptus* is the most widely planted hardwood crop in the tropical and subtropical world. Plantations of Eucalyptus species supply high-quality wood for industrial applications and are important sources of carbon neutral renewable energy in Brazil. *E. grandis* and *E. urophylla* and their hybrids are the most widely planted species in fast growing commercial forests in Brazil. *E. globulus* is the preferred raw material by the mills generating a pulp that is considered superior by the market. However as a pure species it does not grow adequately in Brazil but performs well in hybrid combinations. Breeding programs have increasingly incorporated *E. globulus* germplasm in fast moving elite populations. Molecular breeding in such populations will require information on markers, comparative mapping and QTL validation across pedigrees involving these different species. Highly multiallelic and transferable microsatellites not only are excellent tools for individual identification, but also provide robust and efficient framework genetic maps that serve well for mapping thousands of bialellic higher throughput markers such as Single Nucleotide Polymorphisms (SNPs) and Diversity Array Technology (DArT). Furthermore microsatellites provide a powerful way for QTL validation across species. We describe the development and characterization of 182 new microsatellites, most of them derived from ESTs and some from a genomic shotgun library. These markers, together with other previously developed ones were used to build a consensus map involving three different pedigrees derived from intercrossing four species of *Eucalyptus*.
Methods: The source of ESTs used to mine microsatellites and the methods used to select loci and design primers were described earlier [1,2]. Data from a sample sequencing experiment, carried out back in 2003 using a Sanger sequenced shotgun genomic library, was used for microsatellite discovery (R.T. Lourenço, unpublished). Microsatellite, primer pairs were initially screened for assay success, polymorphism and inter-specific transferability by colorimetric detection on polyacrylamide gels. Selected loci had their forward primer re-synthesized with a fluorescence label and used in downstream genotyping. Loci were characterized using a set of 32 unrelated individuals each of *E. grandis* and *E. globulus*. Map construction was carried out for three different pedigrees of *Eucalyptus* one of them being the pedigree used earlier for a reference map construction [3]. Mapping was conducted using JoinMap 3.0 [4]with a minimum LOD score of 10. and marker order determined with the default mapping module parameters. Parameters of genetic information content (PIC polymorphism information content; PE = probability of paternity exclusion and PI = probability of identity) were estimated for all newly developed microsatellite markers for each species separately.
Results: Data mining of 22,298 unigenes provided 1,261 microsatellite loci. Most of them were (54.71%) trinucleotide repeats. After all screening steps, 494 microsatellites amplified discrete and polymorphic fragments and 210 were selected for fluorescence based genotyping. From the single-pass genomic shotgun library 41 microsatellites were also selected following then same screening steps. Genetic maps were initially built for each pedigree separately. A consensus map was then built by combining separately the homologous linkage groups of the three maps. The total number of microsatellite markers eventually mapped was 448 including the 234 previously mapped [3]and part of those published but not yet mapped earlier [1,2]. The map had an estimated total recombination distance of 1,297 cM and an average distance between adjacent markers of 3.48 cM. The linear order of markers was conserved on most linkage groups across the individual pedigree maps and the consensus map.with no evidence of rearrangement of chromosomal blocks. For the 182 newly developed microsatellites characterized the allele size range did not vary between *E. grandis* and *E. globulus*. Only 15 loci for *E. grandis* and 17 loci for *E. globulus* were monomorphic in the set of individuals genotyped. The average number of alleles was very close in the two species, 4.6 for *E. grandis* and 4.8 for *E. globulus*. A significant estimate of null allele frequency was found for only seven loci and these coincided for the two species Average PIC, PE and PI for the set of loci were not substantially different between the two species. Furthermore the overall performance of the EST derived and genomic shotgun derived sets of microsatellite did not show any appreciable difference, somehow unexpectedly due to the generally lower polymorphism of genic microsatellites. The ascertainment bias introduced by the much larger set of EST derived microsatellites and rigorous screening applied might have contributed to this.
Conclusion: This work summarizes the development and characterization of a new set of 182 new microsatellites markers and presents a relatively dense microsatellite marker consensus map involving four different species of *Eucalyptus*. The new 182 microsatellites developed are robust and polymorphic enough to be used for applications in breeding programs that involve individual identification as well as for comparative QTL mapping and marker assisted selection.
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Genomic characterization, high-density mapping and anchoring of DArT markers to the reference genome of *Eucalyptus*

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Background: Genetic linkage maps have been essential tools to examine the inheritance of qualitative and quantitative traits, to carry out comparative mapping and to provide markers for molecular breeding applications. Linkage maps for species of Eucalyptus have been reported for several pedigrees using different molecular marker technologies [1]. However improved marker density, throughput and transferability across species are necessary to increase resolution of current maps for a variety of genomic applications. We report the development of a high density linkage map for Eucalyptus based on microsatellites and DArT (Diversity Arrays Technology) markers generated by a standardized genotyping microarray [2]. DNA probes that constitute the DArT microarray were sequenced and positioned on the reference Eucalyptus genome providing information about their sequence content, their distribution relative to annotated genes as well as the relationship between physical and recombination distance in the Eucalyptus genome.
Methods: Map construction was carried out using an F1 progeny of 177 individuals derived from a cross between *E.grandis* and *E.urophylla*. Genomic representations of both parents and their F1 offspring were produced with the same complexity reduction method used to prepare the library (PstI/TaqI) generating the 'targets' for hybridizing to the arrays. Microarray imaging, data extraction polymorphism detection and marker scoring were carried out using *DArTSoft* v.7.44 (http://www.diversityarrays.com/). Polymorphic markers were filtered according to reproducibility, quality parameter (Q) and marker call rate as described earlier [2]. An integrated linkage map was constructed with JoinMap v3.0 [3]using a framework map of previously mapped fully informative anchor microsatellites. All 7,680 DNA probes that constitute the current DArT genotyping microarray were Sanger sequenced. Redundancy analysis was carried out at the sequence level using a 50bp minimum overlap, 98% identity, and allowing for 10% mismatch and gap size of one bp. These DArT probes were mapped on the annotated Eucalyptus reference genome as available in Phytozome. Distribution of DArT probes and mapped DArT markers relative to the predicted gene models was carried out by dividing the genome in 500 kbp bins corresponding approximately to a 1 cM recombination fraction.
Results: A segregation ratio filtering (both for 1:1 and 3:1 markers) together with a relaxed call rate threshold > 50% were initially applied to include the largest number of potentially mappable markers. A total of 4,271 DArT markers segregated 1:1 and 1,572 segregated 3:1. The complete dataset with 6,065 markers (5,843 DArT and 222 microsatellites) was submitted to a linkage analysis resulting in eleven groups at LOD ≥ 15 with a total of 2,484 markers. A subset of 1,032 markers positioned at high likelihood for ordering (864 DArTs and 168 microsatellites) provided a framework map with 1,176 cM and an average recombination distance between consecutive markers of 1.15 cM. When all the 2,484 mapped markers were fitted, the total recombining genome length increased to 1,303 cM with a resolution of 0.6 cM. A redundancy analysis of the 6,918 DArT probes for which sequences could be obtained resulted in 4,583 unique sequences (66%). The estimated redundancy of 34% represents a useful resource by providing alternative probes for detecting polymorphism in the same genomic region in different individuals. A total of 6,480 probes (93.7%) were confidently mapped onto the reference genome; 4,189 of them (65%) mapped to single positions while the remaining 2,291 probes mapped to a second position with a relatively high suboptimal alignment score and were therefore considered mapped at lower confidence. About 50% of these low confidence mapping (1,026) actually mapped to a sequence position containing a repeat element which might explain the mapping result. A total of 438 DArT probes could not be mapped to the current assembly. These DArT probes might further improve the current genome version by including some yet unassembled genome contigs. A total of 1,987 linkage mapped DArT markers for which sequence was available were positioned relative to the 41,208 gene models in the current genome, distributed in 500 kbp bins providing an average of 1.6 ± 2.4 DArT markers for the 34 ± 15 gene models present in each genome bin. Interestingly a total of 4,663 DArT probes (67%) mapped at less than ten basepairs from the nearest gene model and only 76 probes mapped at more than 10kbp. The largest distance from a DArT probe to the next gene was only 156 kbp and a modest although highly significant coincidence across the whole genome was seen between the number of DArT markers and the number of gene models (Spearman rank correlation R = 0.33 p<0.00000).
Conclusions: The linkage map presented in this work was used to aid the ordering of contigs during the assembly of the *Eucalyptus grandis* genome currently available through the JGI Phytozome website. This map, together with other parallel mapping efforts that used this same genotyping platform have provided between 2,000 and 3,000 segregating markers irrespective of species of Eucalyptus. The mapping density achieved with the Eucalyptus DArT microarray provides unprecedented opportunities for comparative mapping across pedigrees, high resolution QTL analysis and molecular breeding applications across species of the genus. We have shown that not only the DArT marker sequences are highly enriched for genes, but that their distribution relative to the predicted gene models provides an extremely efficient tool to specifically tag genes at distances within the extent of LD in typical breeding populations. The use of this standardized high-throughput genotyping platform will therefore be instrumental to implement genome-wide selection strategies and positional cloning efforts in multiple Eucalyptus species.
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Development of highly-multiplexed SNP arrays in maritime pine for multi-objective genetic applications

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Single Nucleotide Polymorphisms (SNPs) are the most abundant form of genetic variation in the genome. In this poster, we reviewed what has been achieved in terms of highly-multiplexed SNP genotyping assay construction in maritime pine (*Pinus pinaster* Ait.), the main conifer used for commercial plantation in southwestern Europe. Seven custom SNP-assays (384 and 1536-plex), oriented towards broad applications, have been designed. We illustrated here the usefulness of this genotyping technology to address specific questions related to i/ genetic diversity and population structure analysis, ii/ linkage and QTL mapping and iii/ association mapping.
With respect to genetic diversity and differentiation, a custom VeraCode assay for 384 SNPs mostly based on a larger array designed for linkage mapping (see below) allowed to obtain less blurred, albeit similar, breeding zone boundaries than a set of 12 nuSSRs screened on the same individuals. Levels of diversity were also more accurately estimated, showing clear differences among gene pools. Interestingly, a relatively small subset of SNPs would be enough to develop an application tool for origin certification, which could have a notable impact on current operational practices.
In terms of linkage mapping, a custom GoldenGate assay for 1,536 SNPs detected through the resequencing of gene fragments (in vitro SNPs) and from Sanger-derived Expressed Sequenced Tags (in silico SNPs) was established. Offspring from two mapping pedigrees were genotyped. A consensus map comprising 357 SNPs from 292 different loci was constructed and the analysis of sequence homology between mapped markers and their orthologs in a *Pinus taeda* linkage map, made it possible to align the 12 linkage groups of both species. Moreover, QTL detection for different traits is underway.
In terms of association mapping, a custom GoldenGate assay with 384 SNPs was built and used to genotype 160 unrelated plus-trees from a half-sib experimental design for which breeding values (for height growth, circumference, stem straightness at 8 years, lignin content and extractives at 31 years) were available. Taking into account multiple

testing, one single SNP in a gene encoding a putative fasciclin-like arabinogalactan protein was found to be associated with growth traits.
We conclude that the VeraCode/GoldenGate assays can be used successfully for high-throughput SNP genotyping in maritime pine, a conifer species that has a genome seven times the size of the human genome. This first generation of SNP-arrays has been recently upgraded to an Infinium-array (containing 10.5k SNPs) thanks to deep sequencing based on new generation sequencing technologies. The Infinium-array also includes SNPs from comparative orthologous sequences with other major conifer species, providing a wider collection of anchor points for comparative genomics among these major groups of forest trees.

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How many genes might underlie QTLs for growth and wood quality traits in *Eucalyptus*?

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Background: QTL mapping is an unbiased approach where the phenotype reveals the location of regulatory genes or genomic regions affecting the trait of interest. The development of transferable molecular markers and the increased use of multiple pedigrees for QTL mapping have allowed comparative analysis of QTLs across independent studies thus providing validation data. Such QTL positional information, together with the availability of annotated genome sequences, now promises to identify strong candidate genes for a number of traits [1]. As large pedigrees become available and higher resolution mapping with SNPs, DArT and genotype-by-sequencing technologies becomes routine in forest trees, QTL positional information could be an alternative to the current approaches that rely on tentative candidate genes for association genetics studies. Among the several traits for which QTLs have been mapped in forest trees those that display higher heritability, such as wood chemical composition, are more likely to involve candidate genes of stronger effect although recent association studies show that even such genes explain very small proportion of the variation [2]. In this study we used a high-resolution map with over 2,000 Diversity Arrays Technology (DArT) markers to carry out an initial assessment of the number of annotated gene models in the reference genome sequence of Eucalyptus that putatively co-locate with QTLs for growth and wood quality traits.
Methods: A QTL mapping study was carried out with a clonally replicated segregating population of 171 F1 individuals derived from an *E. grandis* x *E. urophylla* cross. Individuals were genotyped with the Eucalyptus DArT microarray described earlier [3]. The DArT marker data were combined with 222 microsatellites and a linkage map for each parent was constructed using JoinMap 3.0 [4]. Six traits were measured: height growth (HG), circumference at breast height (CBH); wood specific gravity (WSG); cellulose pulp yield (%PULP); Total Lignin (TL); syringyl/guaiacyl ratio (S/G). QTL mapping was carried out using QTL Cartographer [5] on the two parental maps separately at 1 cM intervals. Empirical threshold significance levels for QTL detection were determined by 1,000 permutations considering a significance level of 5%. All the segregating DArT and microsatellite markers were mapped onto the 11 pseudo-molecules of the *Eucalyptus grandis* draft genome sequence covering 609 Mbp.
Results: QTL analyses were carried out using framework genetic linkage maps with high likelihood support for order. The maternal map had 825 markers (684 DArTs + 141 SSR) and the paternal map 511 markers (410 DArTs + 101 SSR). A total of 16 QTLs in *E. grandis* and 14 in *E. urophylla* were detected influencing growth and wood quality traits. High and significant positive phenotypic correlations were found between CBH and HG, TL and S/G, and S/G and %PULP. In the maternal *E. grandis* map, five QTLs were identified for TL (Linkage groups (LG) 1, 3, 4, 5 and 8), two QTLs for %PULP (LG 4 and 5), three for S/G (LG 1, 5 and 8), WSG (LG 6, 8 and 10) and HG (LG 1, 2 and 6). In the *E. urophylla* paternal map we detected three QTLs for %PULP (LG 1, 4 and 9), three for HG (LG7, 8 and 10), three for TL (LG3, 4 and 8), two for CBH (LG7 and 10), two for S/G (LG 8 and 9), and one for WSG (LG 8). More than two QTLs were clustered on LG 4, 5 and 8 in *E. grandis* and on LG 4, 8 and 9 in *E. urophylla* suggesting interesting genomic regions to look for candidate genes to be tested in association mapping. Several of these QTLs were syntenic to QTLs found in other studies [6,7] providing some indirect support for their validity. The sequences of DArT and microsatellite markers bracketing QTLs were used to extract the gene models from the Eucalyptus reference genome. A total of 7,125 predicted gene models are found across all maternal QTLs, with an average of 445 genes per QTL. For the paternal QTLs 5,076 gene models exist, with an average of 362 genes per QTL.
Conclusions: As in many other QTL mapping studies in Eucalyptus [6-8] we have identified several QTLs that control a modest proportion of the phenotypic variation for a number of economically important traits. In this first assessment of putative candidate genes co-locating with these QTLs we found thousands of annotated gene models, hundreds of which could be tentatively suggested as being involved in trait variation. Notwithstanding the low mapping resolution provided by the small progeny, this preliminary study shows that tens or hundreds of genes will likely be always found underlying QTLs for such complex traits. Testing and validation of such large numbers of genes will require a gigantic effort. Furthermore a large proportion of the phenotypic variation remains unexplained by the few QTLs mapped. It is therefore questionable from the applied stand point how much useful information this approach will effectively provide for the advancement of association genetics and, for that matter, of breeding practice.
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Association mapping of local adaptation traits of Scots pine in a European wide population sample

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Traits related to local adaptation by definition show high phenotypic differentiation. The underlying genetic patterns could be clines at individual loci or small effects and extensive linkage disequilibrium at the underlying loci. In any case, including many populations in an analysis provides more information, but may simultaneously induce problems due to genetic structure. Even if the neutral loci have little genetic structure, loci related to other clinally selected traits could show more structure.

Here we have developed an approach to efficiently use the information along a latitudinal environmental gradient. Scots pine populations from central Europe to the species' northern range were sampled and patterns of phenotypic variation of both timing of budset and frost tolerance were measured in common garden experiments, (10 populations, a total of 270 halfsib families, 25 trees per family). By hierarchical modelling of the phenotype's clinal variation and accounting for varying allele frequencies across the 10 populations, the statistical approach simultaneously exploits the genetic variation between and within populations to detect association signals. We apply shrinkage-based Bayesian variable selection to detect genetic associations between timing of bud set and ~450 SNPs in Scots pine.

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Development of a genetic linkage map of rubber tree (*Hevea braziliensis*) based on microsatellite markers

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Background: Rubber tree (*Hevea* spp.) is the main natural rubber producing crop, and it is cultivated in numerous equatorial, tropical and subtropical areas. *Hevea* spp. is an outcrossing perennial species and belongs to the botanical family Euphorbiaceae. Breeding of rubber tree, like for many other tree crops, is a long term process. Large scale cultivation of a new cultivar can only be reached after 20–25 years of field experiments on large areas. Breeders are thus interested in developing alternative strategies to improve and speed up the breeding scheme, using genetic markers. Molecular markers are being used for more than 15 years in *Hevea* breeding, mostly for diversity studies, assessment of genetic distance among cultivars, genetic mapping and identification of genetic loci implied in the expression of agronomical traits.
Material and methods: The mapping population was a full-sib progeny (F1 progeny) derived from a controlled cross between the cultivars PB217 and PR255. The PB217 has a high yield potential, which is expressed throughout the lifetime of its rubber production, low metabolic activity and a high level of sucrose in the latex vessels. Genotype PR255 showed tolerance to injury and cold. A set of 603 microsatellite primer pairs was tested for polymorphism the two parents and six F1 progenies genotypes. Among these, 200 genomic microsatellites have been developed by our laboratory (part published in [7]), 296 were developed by CIRAD [3] and 178 EST-SSRs were obtained from a published work [1]. The polymorphic microsatellites between the parents were selected for mapping in a F1 population. The PCR conditions were detailed by [7]. PCR products were separated using a DNA analyzer 4300 (LI-COR) through 6.5% polyacrylamide gels. Linkage map was obtained using OneMap [5]. This software generates integrated genetic maps from molecular markers with different segregation patterns; it considers multipoint technology based on Hidden Markov Models, as presented by [8]. LOD Score 4.5 and recombination fraction of 0.40 was considered to determine linkage between markers. The algorithms "compare" (until six markers) and "order" (more than six) were applied to obtain best order for each linkage groups, as presented in mapmaker/EXP [6].
Results and discussion: Out of 603 microsatellites markers evaluated, 309 (51%) showed polymorphism between the parents of the mapping population. Until now, 225 marks were genotyped (59 SSR genomic loci and 166 EST-SSR). Chi-square test was carried out on the genotyping polymorphic loci showed that 110 loci followed a segregation ratio of 1:1, 28 followed a ratio of 1:2:1 and 87 (38.7%) followed a ratio of 1:1:1:1. The map consists of 225 markers, distributed in 23 linkage groups (LG) and 2,471.2 cM in length with an average genetic distance of 11 cM between adjacent markers. The largest group has 215.9 cM (18 markers) and the smallest has 2.71 cM (2 markers). This reflects in a more realistic way the polymorphism of the full-sib cross.
In a previous work [4] a linkage map was constructed from a double pseudo testcross, and two maps were constructed separately for each parent. In the present work, instead of using only single dose markers (1:1) like [4], markers that segregate in both parents in the ratio 1:2:1 and 1:1:1:1 were also used enabling the construction of an integrated genetic map and facilitating the location of quantitative trait loci (QTL) [2]. Seven linkage groups are small, composed of two or three markers and covering a few cM. These small linkage groups probably occur because the map is not saturated enough and some chromosome regions could not be linked. The chromosome number accepted today, for most *Hevea* species, is 18 (2n = 36), as observed by [4].
Conclusions: This map is still under development and we expect to achieve a saturated map consisting of 18 linkage groups. The present map will be used for yield rubber QTL mapping and other important economical characteristics.
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P40

QTL analyses of drought tolerance in *Eucalyptus* under two contrasting water regimes

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Background: Drought stress is one of the most important abiotic factors in *Eucalyptus* sp. plantations which influences the growth and limits productivity in cultivated areas, mainly in central, northern and northeastern areas in Brazil, where large parts of these areas have limitations on water supply. The breeders are now looking for tolerant genotypes to overcome this challenge and the QTL mapping approach will help to understand the genetic control of drought tolerance. The objective of this study was to identify genetic loci controlling the phenotypic variation in drought tolerance in a *Eucalytpus* segregant progeny grown under drought and irrigation conditions.
Material and methods: The progeny used in this study was generated by the breeding program of Fibria Celulose and is composed of 184 F_1 genotypes from a cross between a tolerant and a susceptible clone to water stress, both *E. grandis* x *E. urophylla* hybrid tree. In a greenhouse condition, the progeny (seedlings with 70 days) was evaluated under two irrigation conditions (1 - control: assigned to a well-watered regime with watering equal to transpiration loss and 2: submitted to water deficit until the onset of initial drought symptoms) in 4 different experiments. Growth (the relative increase in height and stem diameter; leaf number;

leaf area; leaf, steam and root dry weight; root-shoot ratio) and physiological traits (net assimilation rate, stomatal conductance, transpiration, instantaneous and intrinsic water-use efficiency, relative water content in leaves, chlorophyll content index, photochemical efficiency and leaf water potential) were measured and BLUPs analysis were performed.Genomic DNA was extracted with CTAB protocol from young leaves and used in PCR amplifications of 121 microsatellite markers. The Onemap software [1] was used in linkage analysis. The estimated BLUPs were used in QTL mapping [2]. The QTLs were mapped by composite interval mapping (CIM).

Results and discussion: Linkage analysis resulted in 11 linkage groups with 101 markers. The groups were identified using a reference eucalyptus genetic map [3], through common microsatellite loci. The length of the map was 770 cM with an average distance of 7.2 cM between markers. The experimental design allowed analysis of genotype by drought tolerance interaction for the first time in a eucalyptus QTL mapping population, resulting in the identification of 66 loci that control traits under water restriction and 70 loci under irrigation condition, for all of 16 traits evaluated (considering LOD>3.0). Both additive and dominance effects were detected. Around 4 – 7 QTLs were identified for each trait and, in general, the QTLs identified explained from 11% to 30% of phenotypic variation, except by photochemical efficiency, where 59% of phenotypic variation was explained, mainly by 2 QTLs (25.16% and 25.17% and peak LOD score 3.46 and 18.73) mapped in linkage group 6. Most of QTLs identified were specific for each treatment and for just two traits (root and leaf dry weight) QTLs were co-localized for both irrigation conditions and they were mapped in linkage groups 2, 8, 10 and 11. Clusters of QTL for different traits were mapped close to each other at several linkage groups, indicating either a common genetic base or tightly linked QTL. The results are consistent with Ronnberg's and Yue's results [4,5],where many QTLs with minor effects are controlling drought tolerance.

Conclusions: This work revealed the existence of several QTLs that control drought tolerance in Eucalyptus and these QTLs identified may be involved in many tolerance mechanisms that plants can use to avoid this stress. Since clusters of QTL for different traits were identified, potential pleiotropic regulators could be coordinating these traits and these genomic regions can be used to identify key genes for these traits.

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S3. GENOMICS ASSISTED BREEDINGS

P41

Comparative assessment of SNPs and microsatellites for fingerprinting, parentage and assignment testing in species of *Eucalyptus*

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Background: Brazil stands on the international scenario by having one of the largest natural forest heritage and extensive sustainable planted forests. Brazilian planted forestry is based mainly on fast growing eucalyptus and pines, with the pulp, paper and steel industries as the major consumers. Introduced commercially in Brazil in the early twentieth century, the eucalypts have experienced increasing levels of genetic improvement over the years. Along with the classical breeding, the use of genetic markers has positively influenced breeding programs, contributing to quality control processes of clonal forestry and advanced breeding. Microsatellite markers have been the main tool used to date. They are multiallelic, highly polymorphic and thus very efficient for several applications that require identification and discrimination of elite clones and determination of parentage. While di and tri-nucleotide repeat microsatellites tend to be more polymorphic, tetra and higher order repeats allow a more robust allele calling [1]. SNP markers genotyped by a high-throughput system have been recently developed for Eucalyptus [2] but not yet routinely incorporated in Eucalyptus breeding programs. In spite of their lower genetic information content, many more SNPs can be simultaneously typed using automated systems, an appealing feature to operational breeding programs. The aim of this study was to comparatively evaluate the resolving power and precision of different sets of molecular markers (microsatellites and SNPs assayed by the Golden Gate technology) for the most common operational applications in *Eucalyptus* breeding programs.

Methods: Samples belonging to six species of Eucalyptus (*E. grandis*, *E. urophylla*, *E. globulus*, *E. nitens*, *E. camaldulensis and E. dunnii*) were genotyped with three different sets of markers: (1) 24 di- and trinucleotides microsatellites (2) 17 tetra-, penta- and hexanucleotides microsatellites and (3) selected subsets of SNPs from the 768 developed earlier [2]. Microsatellites were genotyped by capillary electrophoresis in an ABI3100 genetic analyser and data collected under dye set D using Genescan/Genotyper.SNP genotyping was performed using an Illumina BeadStation 500 GX at the Genome facility of the University of Florida and the data analyzed using the software GenomeStudio. Multilocus genotypes were obtained with the two microsatellite panels and a set of the 96 most informative SNPs for individual identification based on an algorithm that takes into account minimum allele frequency and observed heterozygosity within and across species. The genotype data were used to estimate the combined probability of paternity exclusion (PE) and probability of identity (PI) for the marker panels. Additionally, markers were assessed for their ability to estimate genomic ancestry of individuals, a particularly useful application for determining the origin of spontaneous Eucalyptus hybrids. All typed individuals were assigned probabilistically to a given number of populations inferred with a Bayesian approach without any prior population information using the STRUCTURE package [3]. The analyses were performed with each set of microsatellites and with a specific subset of 96 ancestry informative SNPs, selected with an algorithm that focused on SNPs that display contrasting allele frequencies among species.

Results: The panel of di- and trinucleotide microsatellites showed the fastest increase in the combined paternity exclusion with the addition of new markers. For example, a PE > 99% was achieved with only five markers in *E. grandis*. The panel of tetra-, penta- and hexanucleotide microsatellite was unable to reach a PE of 99% even with all 17 markers were used. The panel of 96 SNPs showed a slower increase in the combined paternity exclusion with the addition of new markers, but needed only 35 markers to reach a PE > 99%. The same trend was observed with the estimate of probability of identity. In the analysis of genetic structure, all three sets of markers correctly inferred the six genetically distinct populations, corresponding to the six species studied. Furthermore, both panels assigned correctly all individuals to their respective species. The SNP panel showed the highest average proportion of correct assignment for all six species, followed by the di- and trinucleotides and at last the tetra-, penta- and hexanucleotide panel, although these estimates were not statistically different between the three marker panels tested, (p= 0.327).

Conclusions: Results show that di- and trinucleotide microsatellites tested are around three times more informative than the tetra-, penta- and hexanucleotide microsatellites used and about seven times more informative than the specific SNPs employed for assessment of parentage and individual identification. Although more informative, the di- and trinucleotide microsatellites are subject to a higher inaccuracy in allele calling, which can result in inconsistencies across analyses carried out in different labs.All three marker panels showed high resolution power to detect genetic structure and carry out assignment tests in species of

Eucalyptus. We are now extending this analysis to controlled and spontaneous hybrids to compare the estimates of genomic ancestry provided by the three marker panels. Additionally we are developing much larger sets of SNPs by re-sequencing reduced genomic representations of pooled individuals from all six species. This should allow a targeted selection of more ancestral SNPs that are homogeneously polymorphic across species to be used for fingerprinting and parentage or historically more recent SNPs that display contrasting allele frequencies among species and thus be more useful for genomic ancestry determination. We anticipate that despite the lower individual genetic information content, careful selection of SNPs panels for targeted applications coupled to their accurate genotype calling and high throughput will become an increasingly attractive alternative for operational application in breeding programs.

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P42

SNP discovery in apple cultivars using next generation sequencing

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Background: Knowledge about single nucleotide polymorphism (SNP) markers is extremely important in the development of genotyping assays, allowing improvements in plant breeding through marker-assisted selection. With the emergence of next generation sequencing platforms, high-density SNP discovery in the genome of plant crops becomes more achievable. In this project, we carried out whole genome resequencing of two apple cultivars (M13/91 and Fred Hough, developed by Epagri [1,2]) using the recently released draft genome sequence of the domesticated apple genome (*Malus* x *domestica* Borkh.) [3] as a reference for SNP discovery.

Materials and methods: We sequenced four DNA samples (two from M13/91 and two from FH cultivars) on four lanes of the Illumina GA II platform (single-end sequencing), with the Illumina PhiX sample used as a control. Image analysis and base calling were performed using on-instrument real time analysis, after which the Off-Line Basecaller was used to convert per-cycle base call files (.bcl) into per read base call files (.qseq). The resulting short reads were aligned to the reference genome with the BWA software [4], with the maximum edit distance set to 3, quality threshold for read trimming set to 15, and no gap opens allowed. Next, SAMtools [5] utilities were used to convert SAM into BAM format, to remove read duplicates and indels, and also to sort reads by coordinates. SNP discovery was then carried out using the GATK Unified Genotyper SNP caller [6].

Results: Alignment of the M13/91 and FH reads to the reference genome resulted in a total of 37,757,897 and 60,400,252 reads mapped, respectively. Considering only reads with root-mean-square mapping quality greater than 20, and occurring at raw read depths (DP) greater than 6 and lower than 20, a total of 143,468 and 474,483 heterozygous putative SNPs were identified when comparing the reference genome with the M13/91 and FH cultivars, respectively. A total of 80,554 heterozygous putative SNPs are shared by both M13/91 and FH cultivars. When considering only homozygous putative SNPs, a total of 20,296 (M13/91) and 70,659 (FH) SNPs were identified. A search was also made between the M13/91 and FH cultivar genomes, resulting in a total of 2,631 SNPs which are homozygous in FH and heterozygous in M13/91, and 4,768 SNPs which are homozygous in M13/91 and heterozygous in FH. In order to determine whether the differences in SNP frequencies between these cultivars are due to differences in read coverage obtained from sequencing, we set up a cut-off value above which all SNP calls in both cultivars had the same coverage, and it showed that their SNP frequency is similar.

Conclusions: We have used next generation sequencing data combined with high-density SNP detection methods to discover large numbers of putative SNPs in apple cultivars, which can be used in the development of genotyping assays.

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P43

Improvement of *Eucalyptus* sp for biomass and bioenergy production in the north of Spain

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Eucalyptus globulus Labill. has been used in Spain for decades as a cellulose source for the paper and textile industry. Since 1997, Sniace group has tested new provenances and families from 29 different *Eucalyptus* species to explore their capacity for biomass and bioenergy production.

Plus trees for growth, wood quality, rooting capacity and tolerance to *Mycosphaerella sp* have been identified by mass selection, and more than 300 controlled crosses among those trees have been carried out. However, the restrictions caused by the high susceptibility to *Mycosphaerella* leaf

disease and the low rooting capacity of the species *Eucalyptus globulus Labill.* delay the application of the gains to a commercial scale.

The objective of this project is to improve the production of *Eucalyptus* in the North of Spain focused on the improvement of two traits of economic importance: clonal/rooting capacity and tolerance to *Mycosphaerella sp.* To achieve this goal three partial objectives have been approached:

Mass propagation of selected adult clones and rooting improvement by in vitro tissue culture: Clonal propagation by sequential subcultures of axillary buds in proliferation and elongation media and further rooting of elongated shoots will be developed. This will further optimize a simple *in vitro* protocol for the micropropagation of identified elite mature trees for raising plantations. Micropropagation can maintain selection gains, developed in improvement programmes, to be transmitted directly to plantations or to seed orchards, adding value to the subsequent products and reducing production costs in the long term. For this purpose, it is required to test differences between in vitro produced trees and seed-derived trees, regarding some important traits such as juvenility, growth, productivity, uniformity and morphological traits.

Certification of clones and varieties by the use of specific molecular markers: A collection of 97 elite clones have been genotyped using 22 SSRs [1,2], and four multiplex PCR reactions. This analysis has allowed the genetic characterization of each individual clone, the analysis of genetic similarity and inferring tentative relatedness among clones. This information will be used to improve crossing designs as well as to certify clonal material.

Genetic dissection of rooting capacity and tolerance to Mycosphaerella sp: A strategy combining transcriptomics, genetic mapping, phenotypic characterization of the targeted traits and QTL analysis has been established. For this purpose a mapping progeny was obtained using two contrasting progenitors: A tree with high rooting capacity, tolerant to *Mycosphaerella* sp, and a tree with low rooting capacity, susceptible to *Mycosphaerella* sp. The steps to reach this goal include: 1) Construction of a cDNA library made of pooled RNAs, from different tissues collected from the progeny plants grown under different conditions. This cDNA library is used as template for Roche GS-FLX Titanium high throughput sequencing. Once the unigene is identified, SNPs from selected genes are chosen to design a 1536 Golden Gate array which is used to genotype both progeny individuals and progenitors. Segregating SNPs are used to construct genetic maps. 2) Evaluation of rooting capacity and response to infection during two consecutive years. 3) Identification of association between SNPs and trait parameters using QTL analysis followed by identification of favourable alleles (SNP variant).

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P44

Genome wide selection for *Eucalyptus* improvement at international paper in Brazil

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Background: The efficiency of plant breeding depends mainly on two actions of the breeder: creation and subsequent identification of superior genotypes. In both actions, selection plays a fundamental role in the definition of crosses to be performed, with interest in creating new genotypes and identifying superior trees to be used commercially. The great attraction of molecular tools for plant breeding is the direct use of DNA information in selection, allowing higher efficiency, quickness in obtaining genetic gains with relatively low costs, when compared to the traditional selection based on phenotypic data. All these objectives can be reached through a new approach: genome-wide selection (GWS) or just genomic selection (GS). Genomic selection can be applied in breeding programs of any species. Results obtained in simulated data indicated that GWS can be very profitable in eucalyptus breeding [1]. GWS is based on selection exclusively by molecular markers, after having their genetic effects estimated based on phenotypic data, in a breeding population sample [2]. The present work aims to characterize and estimate genetic parameters of a hybrid progeny test, the population that will compose the genome-wide selection study at International Paper in Brazil.

Material and methods: The International Paper Brazil population chosen for genome-wide selection purposes is installed in a Hybrid Progeny Test, comprising 58 crosses from controlled pollination of *Eucalyptus grandis* and *Eucalyptus urophylla* and five common checks (commercial clones of the company), totaling 63 treatments. These 58 families are derived from 56 different parents crossed. The test was installed in July 2006 in Brotas (São Paulo State, Brazil) in randomized complete block design, with six plants per plot and eight blocks, corresponding to a total of 3,024 plants. In 2011 the test was evaluated, performing the measurement of diameter at breast high (DBH) and plant height of the progenies. The obtained data were analyzed in Selegen-REML/BLUP software for the estimation of genetic parameters. The analysis, will allow the identification of the elite individual trees, which comprised the GWS population, ranking the best living trees of the test by estimating the annual average increment and morphological aspects (removing dead, forked and broken trees). Following the selection of the GWS 1,000 trees population, xylem samples were collected and sent for DNA extraction and genotyping with DArT and SNP markers.

Results: Three variables were analyzed to characterize the test population: DBH, plant height and wood volume (calculated as function of plant diameter and height). DBH had general mean of 13.8 cm and genotypic variance between progenies of 1.61 cmÂ². The individual narrow-sense heritability of the character was 0.255 ± 0.039, very similar to the plant height heritability of 0.236 ± 0.037. Plant height presented general mean of 21.3 meters and genotypic variance of 2.17 mÂ². The estimated wood volume had general mean of 0.168 m3 per plant and genotypic variance of 0.0012 (m3)Â². The individual narrow-sense heritability was slightly higher than for DBH and plant height, namely 0.297 ± 0.042. All estimates were typical of quantitative traits controlled by many loci of small effects. Based on the analysis of the experiment, the best trees were selected, as potential clones and parents for crossing. This analysis revealed 44 crosses with elite clones, to be used as training/discovery population for GWS purposes. The superior individuals of these crosses were selected to complete 1,000 trees, dispersed through the entire test. The number of crosses (44) selected and the different parents identified (45) are important informations for estimating easily the effective population size (Ne), an important factor in GWS [1].

Conclusions: The information and data generated will lead the studies of the next steps into GWS approach. With the deep understanding of the training population that we have, the genomic breeding values (GBV) can be estimated and the GWS applied using phenotypic and marker data simultaneously. A correct choice of a breeding population is essential for traditional plant breeding and is not different in integrated molecular approaches. Subsequently, the wood phenotyping will be performed and the GBV also estimated for wood quality traits. These data will start up the implementation of GWS in International Paper breeding program in Brazil, as operational procedure, looking at quick selection and, consequently, cost decreases.

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P45

Genome-wide genotyping and SNP discovery by ultra-deep Restriction-Associated DNA (RAD) tag sequencing of pooled samples of *E. grandis* and *E. globulus*

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Background: The availability of next generation sequencing (NGS) technologies has opened the door to new strategies of SNP discovery and genotyping. Rapid genome-wide SNP detection via deep resequencing of reduced representation libraries of restriction digested pools of genomic DNA combined with a reference genome has been successfully used for SNP discovery in microorganisms [1], plants [2]and domestic animals [3]. Taking a step further from using NGS for SNP discovery, Baird et al [1]showed that NGS of short tags derived from barcoded multiplexed genomic representations generated with restriction enzymes could be used for direct genotyping of individuals, calling this method RAD (Restriction-site associated DNA) sequencing. RAD sequencing involves cutting a genome with at least one restriction enzyme and NGS the ends of the resulting fragments. We have recently developed a first set of SNPs for high-throughput genotyping of species of *Eucalyptus*. Although SNP assay success was high, the proportion of polymorphic SNPs declined as phylogenetic distance between species increased, down to <20% when contrasting *E. grandis* and *E. globulus*, the two main worldwide commercially planted species were considered [4]. In this work we used RAD sequencing to discover polymorphic SNPs across these two species. Additionally we were interested in assessing the potential of RAD for direct genotyping-by-sequencing in *Eucalyptus*.

Methods: DNA was extracted separately from 18 unrelated individual trees of *E. grandis* and 18 of *E. globulus*. For each species three bulks of six individuals were prepared with equimolar amounts of picogreen quantified DNA. DNA samples were delivered to Floragenex who carried out the RAD reduced representation library preparation using PstI and Illumina 75 bp single-end sequencing on a GAIIx. Raw sequence data was filtered for quality and mapped onto the 11 chromosomes of the *E. grandis* reference genome available in Phytozome. SNPs in the short sequence tags were called for nucleotides with quality Q> 30 at the position and a minimum of 6X coverage.

Results and conclusions: The average sequencing depth exceeded 28X for all bulked samples, providing a minimum estimated ~5X coverage for each individual present in each bulk providing a 93.75% probability of detecting a heterozygous SNP position. With 18 individuals per species (36 chromosomes), the probability of detecting a SNP allele with a Minimum Allele Frequency (MAF) > 0.1 is > 95% [5] therefore providing good power to select informative SNPs in each species separately and even more so in both species simultaneously. RAD sequence tags may be present or absent in specific individuals depending on the presence of the PstI restriction site providing large numbers of dominant markers; SNPs detected within the aligned tags provide additional co-dominant markers (Figure 1).

Out of a total of 200,712 SNPs declared with high confidence, 42,300 were simultaneously polymorphic in the two species while the remaining were fixed in one or the other. These 42,300 SNPs provide an average density of one SNP every 14 kbp in the Eucalyptus genome. These SNPs could be immediately used to select an evenly spaced set of SNPs for the development of a high density SNP genotyping platform.

Taken together, the RAD tags plus the SNPs into them provide excellent marker density for applications such as Genomic Selection [6]. Besides the RAD method, Elshire et al. [7] recently described a straightforward method of genotyping-by-sequencing. Additionally the DArT complexity reduction protocol has also been streamlined based on NGS for a number of plant genomes including *Eucalyptus* (see Sansaloni et al. this meeting). All these NGS based genotyping methods will cause a paradigm shift in our ability to carry out high density, high throughput and low-cost genotyping of large numbers of samples, unlocking incredible opportunities in forest tree genetics and breeding in the years to come.

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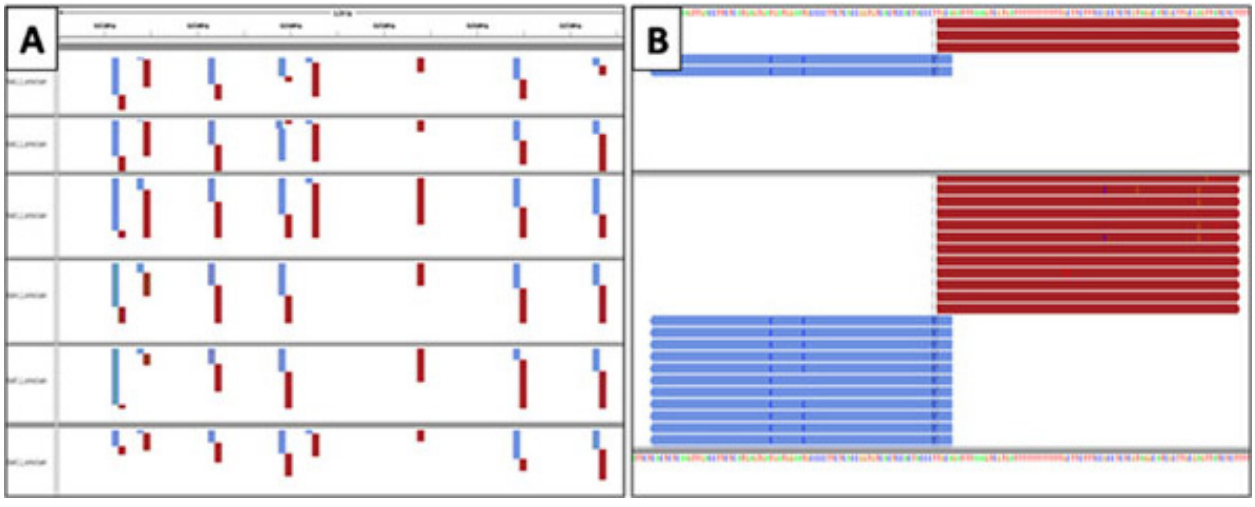

Figure 1(abstract P45) Screenshots of RAD sequencing tags mapped onto the Eucalyptus genome. (A) RAD tags generated from the six bulked samples (top to bottom) from 8 sequential PstI sites showing the variability in the presence or absence of tags across samples and in different directions (red = forward; blue=reverse); (B) close-up screenshot of SNPs detected in the RAD tags of two bulked samples.

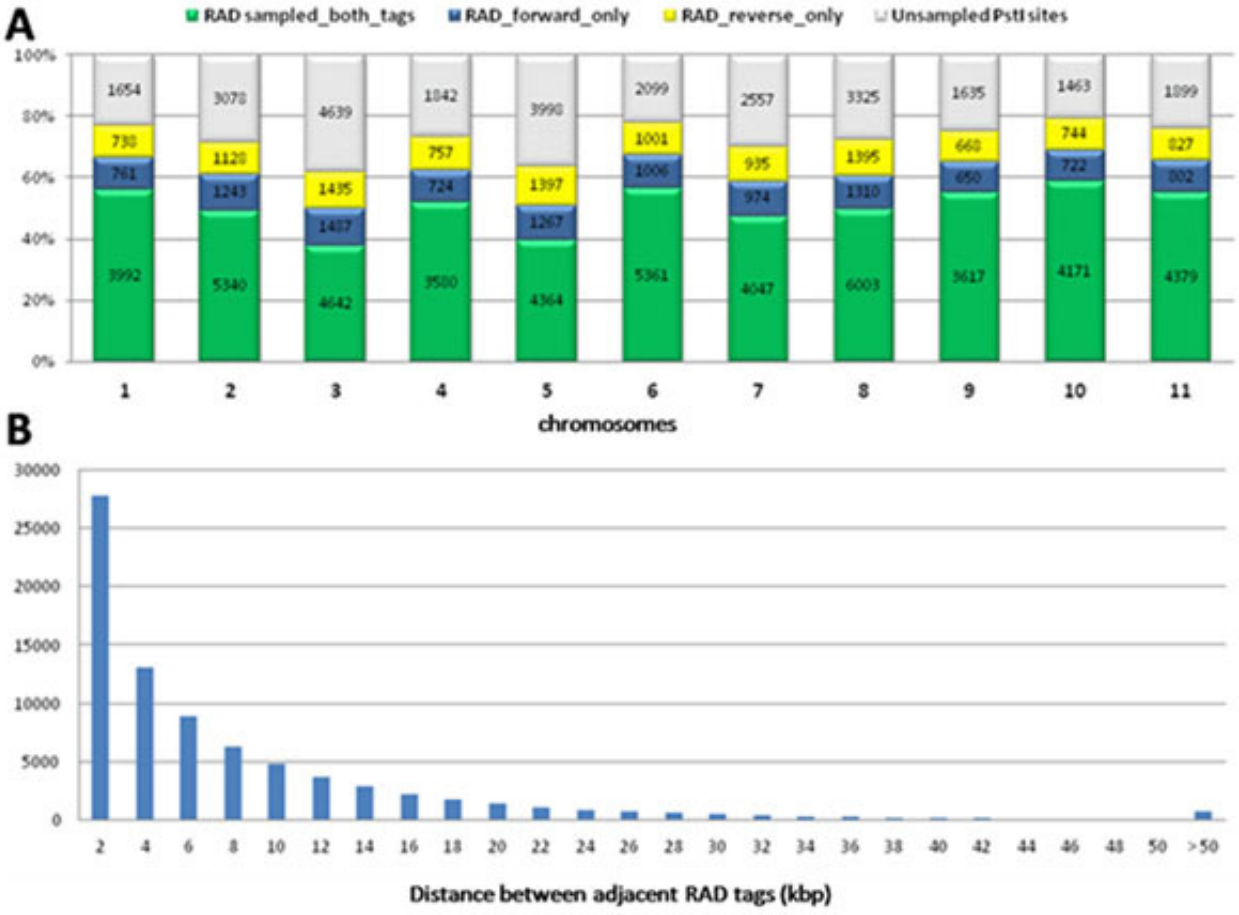

Figure 2(abstract P45) (A) Sampling of the PstI sites in the 11 chromosomes of *Eucalyptus* provided by RAD sequencing. Counts (into bars) and percent (Y axis) of unsampled PstI sites, RAD sequence tags generated in both directions out of the PstI site or in single directions (fwd or rev); (B) Distribution of the distances between adjacent RAD sequencing tags across the 605 Mbp of the *Eucalyptus* genome.

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P46

Identification of five commercial *Eucalyptus* species by SCAR markers development

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The genus Eucalyptus native to Australia, had some species introduced in Brazil between the nineteenth and early twentieth century and is currently planted in tropical and subtropical regions of the world. Currently, species of the genus have been highlighted as some of the most important in reforestation projects to provide wood for pulp, paper, energy and solid wood products. Eucalyptus breeders frequently face difficulties to identify closely related species and their hybrids that share common morphological traits, some of them expressed only later in the breeding cycle [1]. Molecular markers could be useful for early identification of species and hybrids. A strategy to identify species specific molecular markers is the use RAPD (Random Amplified Polymorphic DNA) combined with bulked segregant analysis [2]. We used RAPD markers and BSA to find putative species specific markers for five pure species of Eucalyptus: E. tereticornis, E. saligna, E. grandis, E. brassiana and E. urophylla. We tested 112 RAPD primers which generated 187 candidates polymorphic bands, of which 44 have proved reliable after validation carried out by genotyping individually all plants that composed the bulks.A good species-specific candidate RAPD marker was defined as being exclusive to one bulk, have a size between 300 bp and 2000 bp; provide easy visualization in agarose gel and display repeatability in independent assays. The best candidate bands were provided by the Operon primers: AD01, H03, H19, H20, K10, X06, W03, W05 and W07. As expected, although polymorphic between species, none of the RAPD markers were totally exclusive to one species and absent in the others. However by combining sets of two or more RAPD markers with contrasting frequencies among species it was possible to discriminate all species with high confidence. The most informative polymorphic RAPD amplicons were isolated, purified and cloned into pGEM ®-T Easy Vector System I (Promega) and cloned into competent cells of strain DH5α-UltraMAXTM FTTM (Life Tecnlogies, GibcoBRL) for subsequent sequencing and primers design. Based on the sequences obtained a set of SCAR markers was derived after carrying out a temperature gradient test ranging between 52°C and 69°C. The SCAR marker CXT1 allows the identification of E. tereticornis individuals with 80% efficiency. A combination of markers CAS1 and CAS2 or CAS 1 and

CWB 1 or CAS2 and CWB 1 allows identifying 90% of the E. saligna individuals. SCAR markers CAG1 combined with CHG1 can identify 100% of the E. grandis individuals; markers CXG1 and CWB1 combined provide 90% identification of E. urophylla and CWB1 is a strong candidate to uniquely identify E. brassiana. These results indicate that the development of SCAR markers from intensive RAPD marker screening can be an inexpensive way to discriminate different species and possibly hybrids.

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P47

QTL tools applied to forest breeding

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Background: In last decades, the progress of molecular techniques, bioinformatics tools and genome analysis equipment allowed genomes to be sequenced and analyzed for several plants. For Eucalyptus, the combination of quantitative trait loci (QTL) mapping with genomic analyses, has allowed the identification and validation of a few genomic regions involved in growth and wood quality characters. However, several forest companies still have difficulties to apply Marker Assisted Selection (MAS) at the operational level in their genetic improvement programs. Using as case study of a Eucalyptus hybrid progeny, which had QTLs mapped for growth and wood quality characters, the present study compared the possible outcomes of traditional selection used in forest breeding programs, where individuals are classified by genetic values (eBLUP), with selection based only on superior QTL genotypes (MAS).

Methods: Genotype selection included 30 trees as the maximum number selected by both selection methods, an amount that corresponds to a 15% selection intensity in relation to the mapping population size used in this study. About 200 individuals of an interespecific hybrid progeny had 3 growth characters evaluated in the field and 5 wood quality characters predicted by Near Infra-Red Spectroscopy (NIRS). This progeny was genotyped with 76 microsatellite markers (SSR). An integrated genetic map was built at LOD 3 and 0.40 recombination fraction, composed by 65 SSR markers distributed in 12 linkage groups with total length of 1,365 cM and average distance among markers of 21 cM [1,2]. The genetic map built covered approximately 83% of the Eucalyptus genome estimated by Brondani et al. [3]. Due to the low density and large distance among markers obtained, QTL mapping was only carried out by single marker analysis using R, at p-values smaller than 0.01.

Results: Out of the 65 mapped loci, 10 of them (~15%) showed association with a total of 12 QTLs; 10 loci were associated with only one character and 2 loci with two different growth characters. Three characters showed association to only one QTL. The average volume growth of the 30 individuals selected by MAS was 55% lower than the average of the 30 best individuals selected by phenotypic eBLUP values. One QTL was found for syringil/guayacil lignin ratio and the average of the 30 selected trees by MAS was 17% lower than the average of the best individuals selected by eBLUP. Two QTLs were found for wood density, diameter and lignin content. For density and lignin content, multiple selection for superior alleles at two QTL didn't restrict the number of selected trees, and the average values of individuals selected by MAS were respectively 45% and 83% lower than the average of the best individuals selected by eBLUP. The simultaneous selection for superior alleles at two QTL for diameter restricted from 30 to 23 the number of selected trees. The simultaneous selection for these two QTLs reduced the average value of the 23 individuals selected by MAS to almost half of that of the 23 best individuals selected by eBLUP. For cellulose yield, the simultaneous selection for superior alleles at three QTL reduced the number of selected trees from 30 to 29. The average value of MAS selected trees was 78% lower than the one of the 29 trees selected by eBLUP. In conclusion, for all traits evaluated, selection based on eBLUP was significantly more efficient than MAS. This result was expected, due firstly to the low marker density of our experiment and hence low power to detect all QTLs involved in the trait. Secondly due to the overestimated QTL effects that bias the real value of each QTL used in selection and finally to the fact that eBLUP based selection captures the final effect of all genes involved in trait expression, while MAS based on a few QTLs captures only a small portion of the variation. These results indicate that MAS for a few QTLs will hardly be useful as the sole selection criteria in forest breeding programs.

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Eucalypt pulp yield QTL from Raiz as compared to the literature

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Background: RAIZ is a Portuguese private non-profit research institute owned by the Pulp & Paper Portucel Soporcel Group (http://www.raiz-iifp.pt). RAIZ *E. globulus* genetic improvement program is managed in order to generate trees with increased economic value, through gains in forest productivity and wood properties. Molecular markers have been used for clonal identification and genetic diversity management in the program since 1990. Moreover, RAIZ has been engaged in a longer term genomics project aiming to identify quantitative trait *loci* (QTL) for wood properties. The ability to detect QTL depends on sample size, genetic background, environment and genetic interactions. Most importantly, the ability to use detected QTL depends on their adequate map location and the identification of the molecular variation behind them. This in turn is determined by linkage map quality, the choice of phenotypes and the precision of phenotyping. There are many reports in the literature on QTL detection for economically important traits in *Eucalyptus*, but very few present data on QTL verification (at the statistical and/or biological levels). We illustrate the importance of this issue for pulp yield related traits by comparing available results from QTL mapping studies in the literature with those obtained from a QTL detection and verification experiment pursued by RAIZ.

Methods: An F_1 full-sib family with 361 progeny from an intraspecific *E. globulus* cross was planted in a field trial and phenotyped (at age $4_{1/2}$ years) for pulp yield using near infrared spectroscopy. Phenotypic data was adjusted for spatial variation in the field trial using a first-order separable autoregressive model. The NIR-PLSR spectra were recorded using a *Bruker* equipment (*vector 22N model*), and the calibration model to estimate pulp yield (RTIK16) was constructed using partial least-squares regression as implemented in with the *QUANT2 software*. DNA extraction and microsatellite genotyping were carried out as described in [1,2]. *Linkage* analysis and map construction were performed on independent

male and female datasets using *MAPMAKER/EXP® 3.0*[3] under the F_2 backcross model. Framework maps with evenly distributed selected SSR and gene markers were used for QTL detection [4-8]. The selection of SSR markers took into consideration the possibility to establish synteny between available eucalypt maps in the literature. Interval Mapping and Multiple Interval Mapping QTL detection results were compared with the *MultiQTL vs 2.6 software*. QTL detection was repeated in data from 100 different simulations (using the *R software*), after adding to the original phenotypes randomly selected values from a normal distribution with mean zero and a standard deviation that accounted for the reference essay error and the prediction error of the NIR calibration model.

Results: Many QTL for pulp yield related traits have been reported for every eucalypt chromosome, in the literature. There is insufficient mapping information to infer if these QTL where detected in similar genomic locations. In 6 of the 11 eucalypt linkage groups (LG2, LG4, LG5, LG6, LG9 and LG10), QTL for pulp yield related traits were detected in different species (*E. globulus*, *E. grandis* x *E. urophylla* and/or *E. nitens*) (Figure 1). RAIZ reports two verified QTL for pulp yield, in an *E. globulus* intraspecific cross, in linkage groups 3 and 11, detected in all simulations. Although these results can reflect the polygenic nature of the trait (as suggested by [9]) we cannot exclude the putative biases resulting from current limitations of existing studies in terms of experimental designs, phenotyping and/or data analysis.

Conclusions: QTL studies in some plant species have proved useful to target genomic regions for subsequent genomics investigation [10]. Forest trees experience a variety of environmental conditions and it is expected that some QTL will be age/environment specific and some will be consistently detected over multiple growing seasons. In order to raise the scope of inferences that can be drawn from QTL research in *Eucalyptus* and the prospects of delivering breeding tools from gene sequences, future QTL detection studies should account for experimental phenotyping error, in order to reduce putative false positive results, as we have done in RAIZ QTL detection experiment. Existing QTL mapping studies from different institutions could be upgraded in this perspective, in order to allow comparative mapping and the identification (or not) of specific genomic regions, detected in multiple *pedigrees* and environments, that could be explored further.

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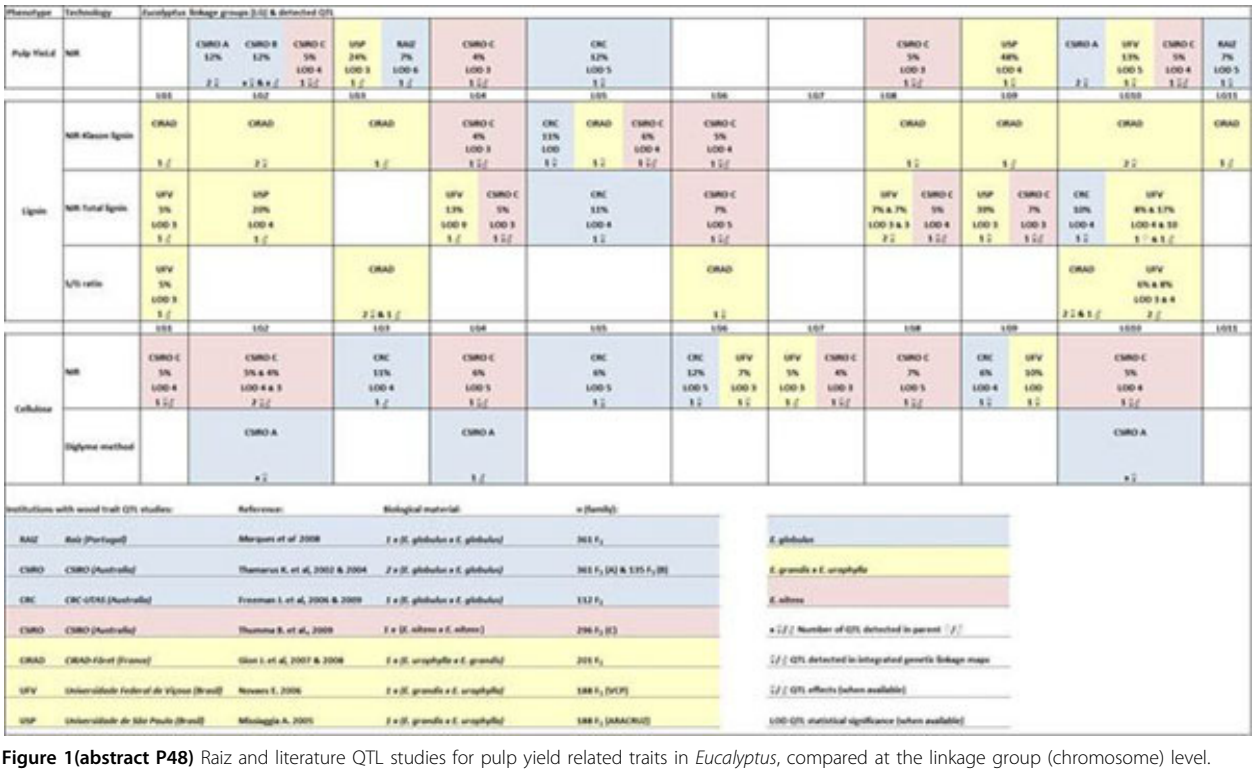

Figure 1(abstract P48) Raiz and literature QTL studies for pulp yield related traits in *Eucalyptus*, compared at the linkage group (chromosome) level.

P49

Effect of BLUP prediction on genomic selection: practical considerations to achieve greater accuracy in genomic selection

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Background: Prediction of breeding values (BV) using only genotypic information is the final goal of Genomic Selection (GS) [1]. Commonly, BV prediction from traditional BLUP analysis is the input for constructing GS prediction models, and GS predicted BVs are correlated with traditional BLUP BVs to estimate the accuracy of GS models. The use of GS in plant breeding depends on the accuracy of the GS models to predict the BVs. Therefore, better accuracy and less bias in traditional BLUP BVs should improve the final accuracy of GS predictions. Such improvements in GS predictions are not due to GS modeling itself, but rather to the reduced noise in the BLUP BV used as input.

Improvements in BLUP BV can be obtained simply by correcting errors in the pedigree [2] or using more complex approaches, such as applying a realized relationship matrix (RRM) in the BLUP prediction as an alternative to the relationship matrix (A) based on expected values derived from the pedigree [3]. Misspecification of effects in BLUP models tends to produce upward bias in the BV estimates, which also impact GS accuracy [4]. In addition, not correcting with the additive-genetic relationship information in the GS prediction model leads to overestimates in accuracies due to inadequate accounting for confounding genetic relationships found in the training population [5]. The inflated accuracy cannot be exploited in future generations and should be guarded against.

Our objective was to use real data to study the effect on the GS accuracy from 1) pedigree errors, 2) incorporation of the RRM in the BLUP analysis, 3) misspecification of non-additive effects in the BLUP analysis and 4) the effect of ignoring the additive-genetic relationship in the GS prediction model.

Methods: Height (HT) was measured in one field test containing 860 clonally propagated loblolly pine trees (~8 ramets per genotype) derived from 32 parents crossed in a circular mating design. The population was genotyped using the Illumina Infinium™ assay (Illumina, San Diego, CA) with 7,216 SNPs. A total of 3,938 SNPs were selected for use in GS based on frequency of polymorphism across genotypes, quality and reliability of the reads. SNP markers were used to estimate the RRM following a recently published method [3] where identity by descent is determined relative to a base population. RRM values were adjusted as recommended [6] to obtain less biased variance estimations. Based on the RRM, a new pedigree was constructed.

Several BLUP models were fit in ASReml to study the following effects:

Model 1: Additive + non-additive effects model – original pedigree

Model 2: Additive + non-additive effects model – new pedigree (expected A matrix)

Model 3: Additive model – new pedigree based (expected A matrix)

Model 4: Additive model – RRM (observed A matrix)

The BVs obtained from models 1-4 were deregressed and used to construct GS prediction models with GBLUP [1]. Additionally, two GS prediction models were constructed based on the raw BVs (not deregressed) from models 3 and 4 to study the effect of ignoring the additive-genetic relationships in the training population when constructing the GS model.

Results and discussion: The RRM among 6475 full-sib pairs (Figure 1a) showed a normal distribution of relationship coefficients around the expected value.

As expected, when the RRM was used to correct the original pedigree [3] the accuracy of the BLUP predictions increased from 0.80 to 0.85 (Table 1), and GS accuracy improved from 0.64 to 0.77 [4]. When the RRM was used directly, instead of the corrected pedigree accuracy of the BLUP, the BVs improved (Figure 1b). Improved BLUP BV estimates also resulted in the improvement of the accuracy of GS predictions from 0.58 to 0.60. The same results were obtained when the additive model was compared with the full model, indicating that misspecification of effects in the BLUP model will cause a decrease in the GS accuracy [5]. In addition, as shown [6] ignoring the additive-genetic relationship dramatically inflates GS accuracy from 0.58 to 0.87 and from 0.60 to 0.88 for Models 3 and 4 respectively.

Conclusions: To maximize the true accuracy of GS, it is recommended: 1) construct a RRM for the training population that should be used to correct the pedigree and to predict the BLUP BVs, 2) correct for non-additive effects if using a family related training population, and

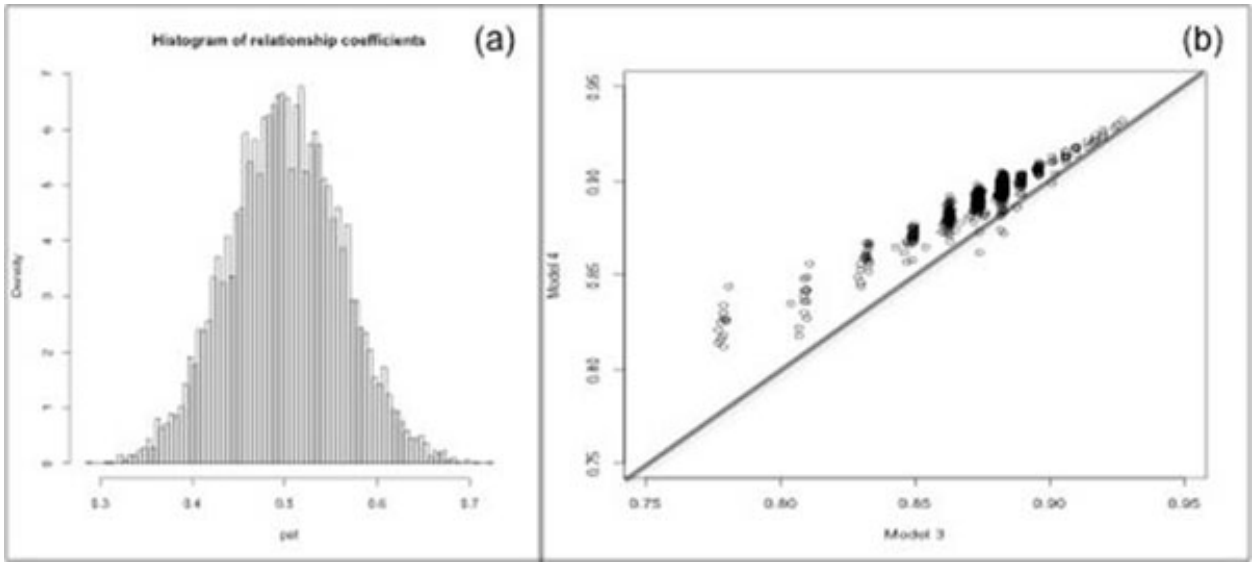

Figure 1(abstract P49) (a) Distribution of relationship values around the expected mean (0.5) for full-sib individuals n=6475, SD=0.06; (b) Accuracy of BLUP prediction in pedigree-based (Model 3) and RRM-based (Model 4) analysis.

Table 1(abstract P49) Heritability, BLUP and GS accuracy for models 1, 2, 3 and 4

ModelNumber	Heritability[1]	BLUP[2]Accuracy[2]	Deregressed	GS[3]Accuracy[3]
1	0.26	0.80	Yes	0.64
2	0.31	0.85	Yes	0.77
3	0.34	0.87	Yes	0.58
3	0.34	0.87	No	0.87
4	0.36	0.89	Yes	0.60
4	0.36	0.89	No	0.88

[1] Narrow sense heritability, [2] Average of accuracy among all clones, [3] correlations between BLUP BVs and GS-BVs prediction.

3) deregress BVs prior to use as input for construction of GS prediction models.

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P50

Recent application developments in genomic research using the AdvanCE™ FS capillary electrophoresis platform

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Central to genomic research is the analysis of nucleic acids, which requires versatile, sensitive and high throughput technologies that can deliver accurate results in a short time. Reported herein are recent developments for sizing and/or quantifying nucleic acids (dsDNA and RNA) on a parallel capillary electrophoresis platform with LED-based fluorescence detection (AdvanCE™ FS system). This flexible multi-capillary platform has been employed for applications such as SSR, gDNA analysis, RNA analysis, quantification of next generation sequencing (NGS) libraries, and the separation and sizing of large cDNA from *Populus spp*,This poster will discuss recent validation of the platform for a subset of these applications in detail. The AdvanCE™ FS system successfully scored all SSR samples with resolution as low as 2 bp. Assessment of the quality/concentration of gDNA is also demonstrated with a new separation matrix, for separation of large DNA fragments. Separation and sizing of large cDNA was also demonstrated with good resolution and accuracy. A method for rapid and sensitive detection of total RNA concentration and quality will be discussed, as well as the sizing and quantification of NGS libraries.The AdvanCE™ FS platform offers rapid separation and ample resolution with excellent sensitivity and dynamic range, to benefit a variety of applications in genomic research.

P51

Novel design and deployment of orthologous genic SSR markers in *Eucalyptus camaldulensis* Dehnh

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Background: *Eucalyptus camaldulensis* is a widely planted tree species in India, because of its rapid growth and adaptability to dry regions. Genetic improvement through informed breeding of *E. camaldulensis* largely depends on availability of molecular markers, linkage maps and genome information. Microsatellite markers, also called as simple sequence repeats (SSRs) have wide application due to their unique advantages over other marker systems. In Eucalyptus, SSR markers have been used in various breeding applications from DNA fingerprinting to QTL mapping [1,4]. Despite of their advantages, the major drawback is the time required for their development [6]. There are less than twenty five *E. camaldulensis* specific SSR markers available in the public database [5]. Although, there are large number of SSRs available in other *Eucalyptus* species [2,3], their species transferability in *camaldulensis* is questionable for practical use. Nevertheless, the existing *camaldulensis* specific SSRs are insufficient for developing linkage maps, QTL and comparative mapping studies. Besides, EST based markers are handicapped due to exclusion of introns, which sometimes lead to compromise on product sizing. Therefore we have modeled a novel strategy that targets highly conserved domain in the genic region using both publicly available ESTs as well as genome sequences.

Methods: Targeting conserved genic region: A set of publicly available *E. globulus* ESTs were assembled as unigenes using CAP3 algorithm and mined for repeat motifs using MISA program [http://pgrc.ipk-gatersleben.de/misa/]. The repeat rich unigenes were marked and mapped on the whole genome scaffolds of *E. grandis*. The sequence of mapped co-ordinates were extracted from EUCAGEN database [http://web.up.ac.za/eucagen/] and further validated for repeat motifs and the presence of exons using MISA and GENSCAN (http://genes.mit.edu/genscan.html). A total of 300 ± base pairs were marked in the genic region of scaffolds flanking the repeat motifs and further primers were designed on the flanking region of repeat motifs using the Primer3 tool [http://primer3.sourceforge.net/], following the appropriate parameters (Figure 1). The entire pipeline was automated and multi-threaded using a set of in-house PERL programs.

PCR, multiplexing and genotyping: We adopted an innovative two-tier polymerase chain reaction (PCR) system to reduce the cost 12 times on labeled oligo synthesis. The first PCR was performed with M13 tagged *forward* primer at 5′ end whereas the second PCR was performed with fluorescently labeled M13 as *forward* primer and SSR *reverse* primer. Multiplexing was carried out at post PCR stage. The first PCR consisted of template DNA (5 ng), primers (2 pM), 10X PCR buffer, dNTPs (1 μM), $MgCl_2$ (1.5mM) and Amplitaq Gold *Taq* DNA polymerase (0.25 U). The PCR profile consisted of denaturing the template DNA at 94°C for 5 min, followed by 35 cycles, each at 94°C for 30 sec, 50-65°C for 30 sec and 72°C for 1 min, followed by 72°C for 8 min. The first PCR products were resolved on 2% high resolution agarose. The second PCR consisted of template DNA (2 ng), 2 pM each of forward (labeled M13) and reverse (SSR) primer, 10X PCR buffer, dNTPs (1 μM), and 0.5U of *Taq* DNA polymerase. The PCR conditions remained same except reduced number of cycles to 20. The amplicons were resolved using ABI 3730 sequencer and each amplicon was manually validated for their allele size.

Results and conclusion: About 13,441 *E. globulus* ESTs and 13,380 *camaldulensis* EST/genomic sequences were collected from public database and mined for repeat motifs. A total of 2,330 repeat motifs were identified on 1873 ESTs of which, 1159 were di; 1128 tri and 43 were tetra repeats. There were more than 290 repeats found to be in compound form. The repeat containing ESTs were assembled to a total of 301 unigenes. These ESTs were mapped on *E. grandis* genome scaffolds.

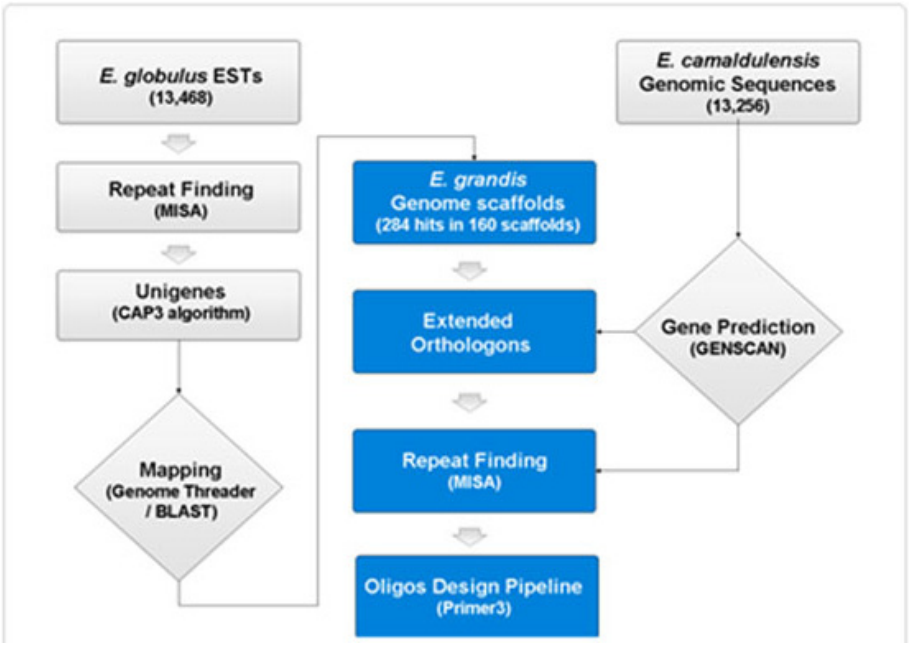

Figure 1(abstract P51) Strategy summarizing the in silico development of orthologus SSRs.

A total of 124 SSR positive scaffolds were identified and used for designing 230 primer pairs. Primers were standardized using gradient PCR at appropriate annealing temperatures. Of the 230 primers, 179 were successfully amplified and validated in *E. camaldulensis* resulting in 77.82% success. About 95% of the primers were amplified as single and clean product, indicating their locus specificity (Figure 2). These markers were validated in 4 *Eucalyptus* and 2 *Corymbia* species with multiple alleles ranging from 4 to 12. Surprisingly, 92% of cross species amplification was observed within the genus Eucalyptus, while only 21% in *Corymbia*. Higher species transferability in *Eucalyptus* genus shows the power of design as they originate from conserved domain. Further to validate these markers in *E. camaldulensis*, some of the selected primers

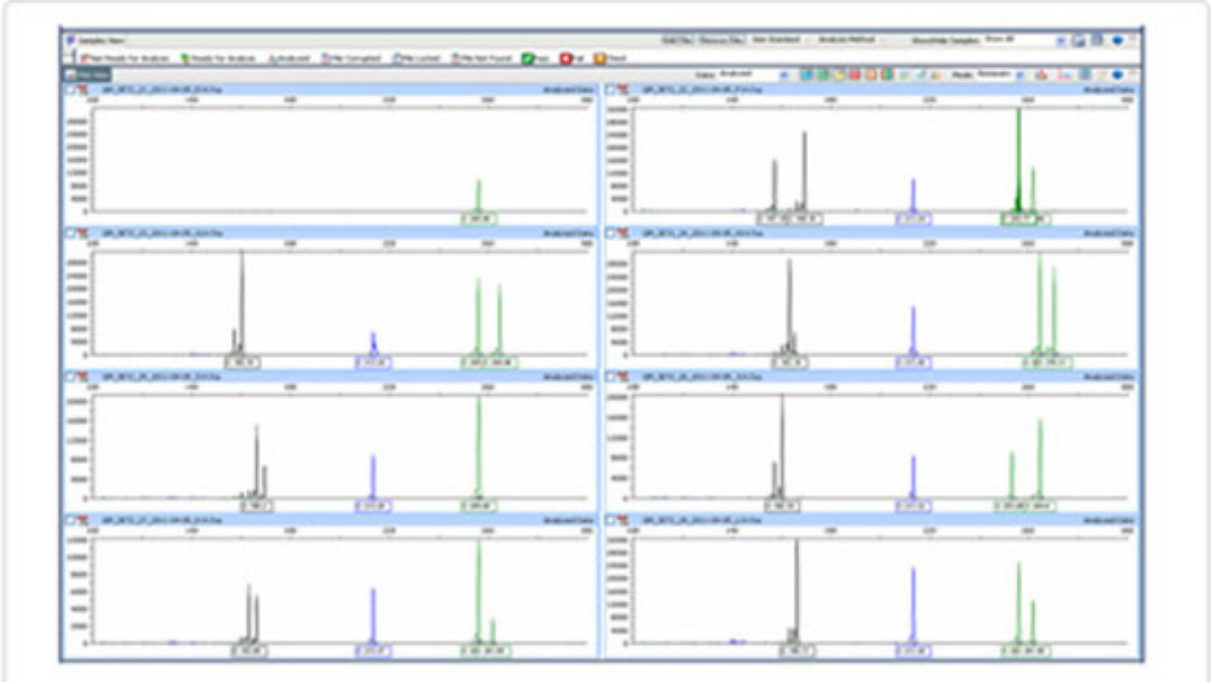

Figure 2(abstract P51) Electropherogram of multiplexed PCR products of primers IME3126 (black), IME3130 (blue) and IME3285 (green) in eight clones of *E. camaldulensis*.

were successfully utilized for parentage analysis, confirmation of interspecific hybrid and genotyping of seedling seed orchard in *E. camaldulensis*. Unlike SSR markers developed from conventional *in silico* methods, orthologous SSRs resulted in very high success rate in *Eucalyptus* species due to targeted repeats in conserved domain. Our present strategy successfully demonstrated the power of orthologous SSR makers and its application in informed breeding in *E. camaldulensis*.

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P52

Identification of SNPs in candidate genes related to water stress in *Eucalyptus*

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Background: Forestry companies have occupied Northern and Northeastern of Brazil with plantations of *Eucalyptus* species; however, limitations on water supply are affecting the biomass production and reducing the yield significantly [1]. Tolerant plants develop defense mechanisms like hormone production of abscisic acid (ABA) and osmoprotector glycine betain (GB) when submitted to drought conditions [2]. Identifying and studying genomic regions related to water stress tolerance are important for tree improvement programs. In this work, SNPs in candidate genes, 9-cis-epoxy-carotenoid dioxygenase (NCED) and choline monooxygenase (CMO), of ABA and GB biosynthetic pathways related to drought tolerance in a population of *Eucalyptus* were identified, as well as, a possible molecular marker associated to genomic regions related to plant response to water stress through AFLP technique [3] combined with bulk segregant analysis method [4].

Materials and methods: Contrasting plants of *E. grandis*, *E. urophylla,* and their hybrids were selected according to their physiology. Specific primers were designed from homology sequences from *Eucalyptus* ESTs databank and amplification products submitted to sequencing which allowed the identification of SNPs and the genotyping of these plants. For the identification of the AFLP marker, contrasting DNA bulks were digested with restriction enzymes combination and routine protocols were followed. The selective amplification products were separated on 6% denaturing polyacrylamide gel and seen in the silver nitrate staining [5].

Results and conclusions: Seven SNPs were identified in a region of 1230 bp on NCED gene from which five were in codified regions and generated synonymous mutations. For the CMO gene, 49 SNP were identified in a region of 3885 bp, which 12 were in codified regions and 37 in UTRs and intron regions. Especially for these codified regions; 83,3% of the mutations were synonymous and 16,7% were non-synonymous. Through the genotyping of the SNPs, the NCED and CMO genes presented respectively, seven haplotypes with 15 different genotypes and 18 haplotypes with 16 diverse combinations. Nevertheless, CMO gene showed some unique genotypes for some species. Then, the genotyping of individuals by the allele-specific extension technique demonstrated to be efficient; moreover the SNPs primers designed can decrease costs and permit the genotyping of these mutations in large scale of contrasting populations to water deficit and in population studies. From the analysis of the DNA bulks, 50 AFLP primer combinations were tested from which 27 generated polymorphic bands between the bulks and just one primer combination was confirmed in all susceptible plants of the bulk by now. This fragment sequence will be compared and converted into PCR-based markers.

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P53

Identification of genomic regions related to early flowering in *Eucalyptus*

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Background: The genus *Eucalyptus* is the most important planted tree to the Brazilian economy. Besides being a source of timber, its wood pulp is mostly used for papermaking. There is a considerable interest in its genetic improvement due to the economic importance of the gender to forestry companies. The time required to obtain ideal populations, after selective breeding, is a limiting factor of classical breeding in these species. However, the breeding program of Suzano Papel & Celulose company obtained a plant of *E. grandis* with the early flowering character, whose first flowering took place between 60 and 90 days. This plant was grown and its seeds were collected and germinated. Progeny segregated for early flowering after approximately 60 to 90 days.

Materials and methods: AFLP markers [1] combined with bulk segregant analysis method (BSA) [2] were used to search for specific DNA polymorphisms in the genome to produce markers which could identify the character at any stage of development. AFLP markers showed polymorphisms based on the distribution of restriction sites and differential amplification of the fragments. The methodology followed the routine protocols for the AFLP technique and BSA method. Contrasting bulks were prepared, one bulk with ten normal flowering plants and another with ten early flowering plants. DNA was extracted and digested using two restriction enzymes, a frequent cutting (*Mse*I) and a rare cutting (*Eco*RI), followed by connection of the adapters.

Results and conclusion: The pre-amplification and selective amplification were performed to test 32 primer combinations, which were seen in 6% polyacrylamide gel stained with silver nitrate [3]. As a result, 13 primers amplified and showed polymorphic bands. These amplified fragments are candidates to be specific molecular markers but still need confirmation. Hence, new analyses are being conducted using DNA from each individual separately in order to verify its co-segregation with the early flowering phenotype. Moreover, confirming its effectiveness as a marker in these plants, new strategies will be adopted for validation, such as cloning, sequencing and the development of primers for testing in other populations of *Eucalyptus*. These results indicate the feasibility of using AFLP technique to detect polymorphisms, which might contribute to the improvement of the species.

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P54

Diversity Arrays Technology (DArT) and next-generation sequencing combined: genome-wide, high throughput, highly informative genotyping for molecular breeding of *Eucalyptus*

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Background: Wider genome coverage and higher throughput genotyping methods have become increasingly important to meet the resolution and speed necessary for a variety of applications in genomics and molecular breeding of forest trees. Developed more than 10 years ago [1], the Diversity Arrays Technology (DArT) has experienced an increasing interest worldwide for it has efficiently satisfied the requirements of throughput, genome coverage and inter-specific transferability for over 40 different plant species to date, including *Eucalyptus*[2] and recently *Pinus* (Dione Alves-Freitas, this meeting). DArT is based on genome complexity reduction using restriction enzymes, followed by hybridization to microarrays to simultaneously assay hundreds to thousands of markers across a genome. Genome complexity reduction for genotyping has now been taken to another level when combined to next generation sequencing (NGS) technologies. Such a strategy has been used for rapid SNP discovery in different organisms [3], and proposed as a way to genotype with RAD (Restriction-associated DNA) sequencing [4]and recently by a similar method generally termed GbS (Genotyping-by-Sequencing)[5]. In this work we assessed the power of the now well established DArT marker platform in combination with Illumina short read sequencing to generate a linkage map for a segregating outcrossed F1 population derived from *E. grandis* BRASUZ1, the donor of the *Eucalyptus* reference genome.

Methods: A segregating population of 89 individuals derived from the intra-specific cross BRASUZ1 x M4D31 was provided by Suzano company. Correct parentage of all individuals was certified by microsatellite genotyping. DNA samples of parents and progeny were processed for the conventional array-based DArT genotyping as described earlier [2] to generate marker data for comparative analysis with the NGS based DArT data. For the sequencing based DArT genotyping two complexity reduction methods optimized for several other plant species at DArT PL were used: PstI_ad/TaqI/HpaII_ad and PstI_ad/TaqI/HhaI_ad with TaqI restriction enzyme used to eliminate a subset of PstI -HpaII and PstI-HhaI fragments, respectively. PstI-site specific adapter was tagged with 92 different barcodes enabling encoding a plate of DNA samples to run within a single lane on an Illumina GAIIx. PstI adapter included also a sequencing primer, so that the tags generated were always reading into the genomic fragments from the PstI sites. After the sequencing run the FASTQ files (full reads of 77 bp) were quality filtered using the threshold of 90% confidence for at least 50% of the bases and in addition filtered more stringently for barcode sequences. The filtered data were split into their respective target (individual) data using barcode splitting script. After producing various QC statistics and trimming of the barcode the sequences were aligned against the reference *Eucalyptus grandis* genome available in Phytozome. The output files from alignment (generated using Bowtie software) were processed using an analytical pipeline developed by DArT PL to produce "DArT score" tables and "SNP" tables. A linkage maps was constructed with JoinMap 3.0 [6]using the microarray-based DArT markers, DArT NGS markers, and 40 microsatellites of known map position as anchors. A parallel analysis exclusively meant to estimate the total number of potential SNPs within the short read tags was carried out using CLC genomic workbench v4.6 software [7]with a minimum read coverage of 6 and minimum variant frequency of 25%.

Results: The microarray-based DArT platform yielded 1,088 high quality markers of which 505 (46.4%) segregated in a 1:1 pseudo-testcross while the remaining 583 (53.6%) segregated 3:1. This relatively lower number of markers when compared to other Eucalyptus mapping populations was expected. Not only it is an intra-specific cross but also involves BRASUZ1, a know self (S1) individual with a lower level of sequence heterozygosity. DArT genotyping using NGS technology yielded 2,835 polymorphic presence/absence markers, almost three-fold the number produced by the microarray platform. Of these, 2,449 markers mapped to the 11 chromosome scaffolds with an average of 222 markers per scaffold, while the remaining 386 markers fall out of the 11 scaffolds, potentially allowing the localization of a fraction of the still unassembled smaller genome scaffolds. In total, an integrated linkage map with 564 DArT markers, 1,930 DArT-NGS and 29 microsatellites was preliminarily built. Furthermore, from the 148 million reads generated (~10.5 Gb), 83.6 million (6.1 Gb) were successfully mapped on the Eucalyptus reference genome. Although a very large number of SNPs can be identified when all reads combined are mapped, only a fraction that displays sufficient coverage allows robust scoring at the individual level. Still, over 1,500 SNPs could be confidently genotyped providing a further advantage of adding co-dominant markers to the already large number of dominant markers obtained.

Conclusion: These initial results show that the combined use of DArT as a robust genome complexity reduction method with optimized barcoded NG sequencing protocol provides at least three fold more dominant markers than the conventional microarray-based DArT method and an additional set of co-dominant SNPs. We are now genotyping a much larger set of distantly related individuals of a training population to be used for Genomic Selection (GS). The possibility of delivering large numbers of both dominant and co-dominant markers with the same platform will enable fitting dominance effect in predictive models therefore increasing the selection accuracy.

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P55

Evaluation of the genetic diversity of a set of parents of *Eucalyptus spp.* by using microsatellite markers to direct matings

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Background: To obtain genetically superior cultivars in a breeding program, methods and procedures are necessary to allow the identification

of selected individuals over several cycles of selection while at the same time maintain broad genetic base of the breeding populations. This is crucial to guarantee continuous genetic gains along the program. The establishment of efficient breeding strategies depends on methods and analytical tools. The assessment of genetic diversity with molecular markers of parents used in mating designs could aid optimizing the recombination phase. Microsatellites provide good information content and require small amounts of DNA and may be transferable between species of the same genus. In this study we evaluated the genetic diversity in a set of *Eucalyptus* parent trees and indicated those to be preferentially crossed in a recombination process to potentially maximize variation in the offspring for individual selection of clones.

Materials and methods: A total of 20 genotypes were selected from 44 families obtained by crossing 24 parents in different types of hybridizations of populations of *Eucalyptus grandis* and *E.urophylla*. Genomic DNA was extracted from leaves and amplified with microsatellites developed during the Genolyptus project and previously used for *Eucalyptus spp*[1]. The amplifications were performed using the GeneAmp PCR System 9600 (Applied Biosystems) thermal cycler using the following amplification cycle: 96 ° C for 2 min, 30 cycles at 94 ° C for 1 min, specific primer annealing temperature for 1 min, 72 ° C for 1 min and a final extension step at 72 ° C for 7 min. Loci were amplified in single and duplex system. Tocher clustering were developed using the genetic distance matrix with the software Genes.

Results and conclusions: This study showed wide genetic diversity among the 20 genotypes under evaluation; all nine SSR loci analyzed were polymorphic, with a total of 77 alleles. The number of alleles per locus ranged from 5 (EMBRA 646) to 10 (Embra 645) averaging 9.1. The lowest value of polymorphism was obtained for marker EMBRA 915 (PIC = 0.59) and higher for the marker EMBRA 645 (PIC = 0.89). The expected heterozygosity ranged from 0.66 to 0.89, averaging 0.80. Based on genetic distances, the 20 genotypes were clustered into six distinct groups. The greatest genetic distance was observed between genotypes C022H (GIV) and M4320G (GVI), and the smallest among genotypes P023H (GI) and C407 (GII). Group II clustered the largest number of genotypes (C386H, C407H, C053H, C051H, TC10H, P044H, VR3748H, VR3709, P4295H), consistent with the fact that these genotypes are considered to belong to the same "heterotic" group by the breeders. Based on the genetic distances, the following trees were selected to be preferentially crossed: M4320H, P4295H, P082G, VR3709H, C053H, C219H, C041H and C022H. It remains to be seen if the progenies derived form these crosses will in fact display a wider segregation and allow, as expected, effective selection of extreme phenotypes for growth and wood quality.

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P56

Chloroplast SNP-marker as powerful tool for differentiation of *Populus* species in reliable poplar breeding and barcoding approaches

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Background: The genus *Populus* is one of the world's most important tree genera. High growth rates, particularly of some interspecies hybrids, and a broad range of applicability from wood and paper to energy production, led to their widespread cultivation in Europe and North America. For the use in SRCs (short rotation coppices), in particular interspecies-hybrids are well suited because of their superior growth and advanced resistance traits. However, *Populus* hybrids are often morphologically extreme variable and can show either more criteria of the one or the other parental species. Thus, species identification within the genus *Populus* using morphological characters can sometimes be difficult. Furthermore, systematically performed records during breeding or vegetative propagation of poplar hybrids and/or clones are not available to date. For breeding activities with the priority of registration of new high-efficiency clones, clear species identification is inevitably necessary. Therefore, we evaluate the usability of already published plant barcoding regions ("barcoding"; Barcode of Life [http://www.barcodeoflife.org/]) for their efficacy to differentiate seven often used poplar species. We present data on amplification success of the barcoding regions by using the already published primers. Moreover, novel primers were established in promising chloroplast regions to differentiate *Populus* species.

Methods: Twenty three published barcoding primer combinations were used for PCR amplification of coding and non-coding (intergenic spacers) regions. Additionally, 17 primer combinations have been newly designed taken advantage of the sequence of the *Populus trichocarpa* chloroplast genome. Obtained sequences were aligned and screened for presence of SNPs by using either the software SeqMan 7.1.0 from DNAStar (Lasergene, GATC Biotech, Konstanz, Germany) or Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, USA). The sequences around the SNPs were checked for restriction sites using the software NEBcutter V2.0 from New England BioLabs Inc (Ipswich, USA).

Results: Twelve of the 23 used barcoding primer combinations and fifteen of the seventeen newly designed primer combinations resulted in PCR amplification products in all individuals for the seven species tested. Twenty four of these amplification products have been sequenced and checked for species-specific SNPs or indels. Three chloroplast regions revealed species-specific SNPs using the original barcoding primers and within seven chloroplast regions species-specific SNPs or indels were identified when using the newly designed primers. A ranking, taking into account the percentage of variable sites for the ten chloroplast regions, reveals the first five places being occupied by intergenic spacers: *trnH-psbA*, *psbK-psbI*, *trnG-psbK*, *ndhE-ndhG* and *rbcL-accD*, followed by two coding regions: *rpoC* and *rbcL*, again one intergenic spacer, *rps2-rpoC2*, and finally two coding regions *rpoB* and *matK*. Besides the usability of the identified SNPs and indels as contribution to the "barcoding of life" project, they should also be used to identify *Populus* species for breeding purposes. For an efficient application, rapid test methods without the need for sequencing or capillary electrophoresis facilities are required. Thus, all sequences with SNPs were checked for restriction sites for use as PCR-RFLPs. For five of the 21 identified SNPs suitable restriction enzymes were found. Thus, we can identify five of the seven used species by using PCR-RFLPs.

Discussion and conclusions: Altogether, the use of intergenic spacers seems to be more successful to differentiate closely related species as given within the genus *Populus*, because of the higher overall variability. Thus, the choice of suitable barcoding regions is obviously different for different groups of land plants. Our results support the recommendation of some authors of focussing the examinations of the plastid genome on the barcoding potential of the *trnH-psbA* spacer. We recommend focusing even more on intergenic spacers in general (for example *trnG-psbK*). And, therefore, our results also follow the idea of several authors in using multi-locus combinations, because differentiation of only seven species within the genus *Populus* requires three chloroplast regions.

Our study clearly show that SNP markers combined with methods as PCR-RFLPs and length polymorphisms are convenient, easy to use, and - necessary in most breeding programs – fast and of low cost for application in breeding projects.

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Genome-wide SNP discovery from a pooled sample of accessions of the biofuel plant *Jatropha curcas* based on whole-transcriptome Illumina resequencing

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Background: *Jatropha curcas*(JC) is an oil-rich, drought-tolerant perennial shrub of the *Euphorbiaceae* family widely dispersed throughout the world. Thought to be native to Central America, it has been the object of an increasing number of studies in recent years for it exhibits a number of

appealing attributes as a promising source of biodiesel. Although its undomesticated nature and preferential outcrossed mating system would suggest a high degree of genetic variation to be exploited in breeding, studies have shown limited genetic diversity in the existing germplasm collections [1]. In spite of the increased interest in this bioenergy plant, challenges still exist to turn this species into a genuine crop and improved varieties that consolidate desirable traits are not yet available, making JC large scale plantation an uncertain business [2]. Genomic studies to potentially assist JC breeding efforts have started in the last few years. JC is diploid (2n=22), with a haploid genome size estimated at 416 Mbp [3]. EST databases focusing on gene discovery were constructed [4]and a draft genome sequence was recently published covering 285 Mbp (~68%) of the genome in 120,586 contigs with 40,929 predicted gene models [5]. The focus of our work with JC is to provide effective tools to accelerate breeding through Genomic Selection (GS) [6] and to help assess the levels, organization and enrichment strategies of genetic diversity in germplasm banks and breeding populations. To this end we have started the development of SNP markers. Available EST databases built from single individual plants do not provide the necessary sequence diversity for SNP discovery.In this work we report on the discovery of a set of SNPs for JC derived from a pool of genetically diverse accessions using Illumina sequencing and a SNP selection pipeline recently described [7].

Methods: Genetic diversity data was used to select twelve JC accessions that maximized genetic diversity out of a germplasm collection currently serving as the foundation of a breeding program [1]. Total RNA of young expanding leaves was extracted from each individual plant and a pool of equimolar quantities of RNA was prepared. Two Illumina GAIIx single end lanes were sequenced following standard protocols. Raw reads were processed and aligned on the mapped reference genome using GSNAP [9]. GATK Unified Genotyper [10] was used to estimate the allele frequency in the pooled samples and to provide an accurate posterior probability of there being a segregating variant allele at each locus using a Bayesian genotype likelihood model. SNPs were then specifically selected to design Illumina Golden Gate Genotyping Technology (GGGT) assays based on an *in silico* estimated minor allele frequency MAF >0.10 and at least 60 bases available on each SNP flank with no additional SNPs following a procedure described earlier [7].

Results and discussion: The two lanes yielded a total of 74 million reads from which 66.5 million were filtered providing 11.8 Giga bases of high quality sequence. Upon mapping on the JC draft genome sequence 28,110 unigenes were sampled covering 22.1 Mbp of the 39.7 Mbp total unigene length, i.e. 56% of the transcribed portion currently predicted in the draft genome. From the 66.5 million reads, 60.8 were aligned on the genome with an average coverage of 152X of the unigene sampled. A large percentage of these reads (73%) were identical, derived either from abundant transcripts or more likely from amplification bias introduced by the PCR enrichment step during library preparation a standard occurrence in NGS [8]. After removing read amplification bias a total of 16.4 million de-replicated reads were aligned providing an average coverage of 26X of the sampled unigenes and a much more reliable substrate for SNP discovery. The distribution of percent coverage levels attained for each sampled gene was estimated (Figure 1). If a gene was completely covered by reads from the first base to the last at a depth >1X, then this gene was given a value of 100%. With no MAF and flanking sequences filtering a total of 18,225 SNPs were detected.When a MAF>0.10 was applied, 9,164 SNPs in 2,907 genes survived. When 60 bases with no SNPs on both flanks were required 1,574 high quality SNPs were recovered sampling 895 genes while 561 SNPs had coordinates not falling into any predicted gene model. These results corroborate the low level of sequence polymorphism in the breeding material and further highlight the need to widen the current germplasm base for successful breeding [1]. While a set of 768 high quality

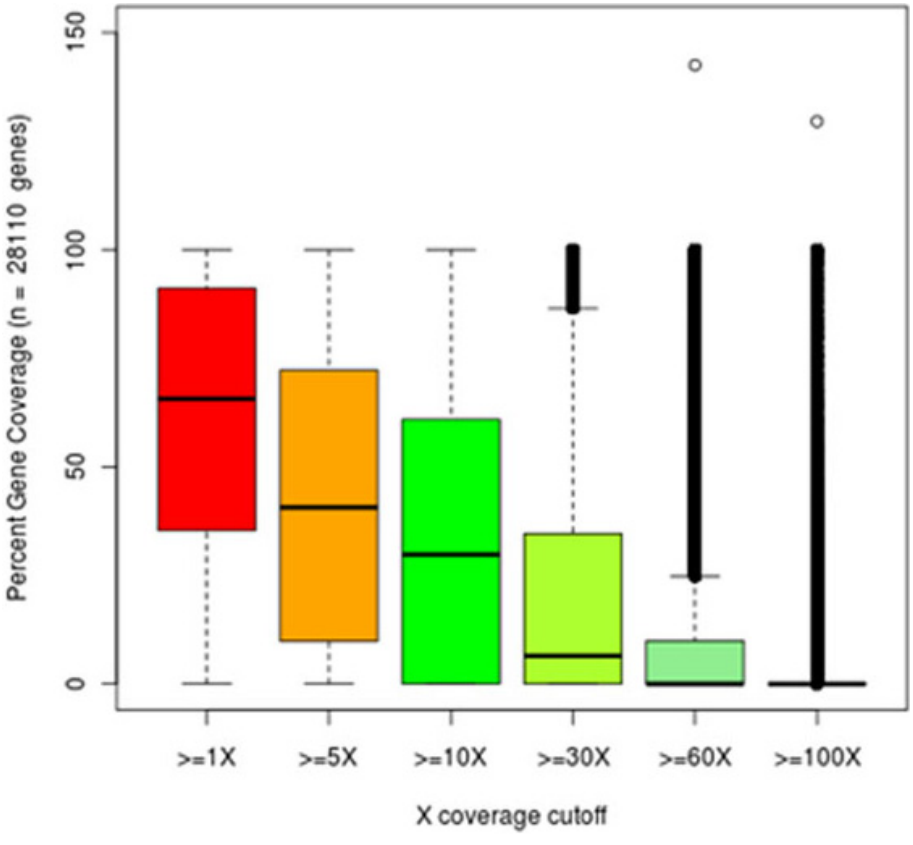

Figure 1(abstract P57) Distribution of percent gene coverage. Distribution of percent gene coverage attained by the transcriptome sequencing at various coverage depth levels using dereplicated reads.

SNPs likely to show high conversion rate with the GGGT can now be developed, alternative genotype-by-sequencing technologies might provide wider genome coverage and thus assay a larger number of sequence polymorphisms.

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Use of SSR-Tools for clone certification in Uruguayan *Eucalyptus grandis* and *Eucalyptus dunnii* breeding programs

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Background: Over the last two decades, commercial plantations in Uruguay have increased exponentially, particularly those destined for pulp and paper industry. Although the about 700,000 ha of commercial plantations of Eucalyptus are a continuous source for the selection of elite genotypes, there is no national registration system for Eucalyptus clones in this country. The traditional procedure of clonal identification accepted by the UPOV (International Union for the Protection of New Varieties of Plants) involves the evaluation of morphological descriptors such as leaf shape, bark texture, fruit shape, etc. [1]. The assessment of these characters varies from one observer to another, potentially leading to ambiguous results [2,3]. This restriction has led countries such as Brazil to include the use of microsatellite markers as additional descriptors, in their legislation. The hypervariability and single inheritance of microsatellite markers provide a powerful clonal characterization system through fingerprinting. The identification of elite clones is nowadays one of the most widely used applications of molecular markers. This could generate a labeling system to follow the material traceability in companies with large-scale production of clonal nurseries. Proper identification makes new clones releasing easier and improves the management of seed orchards and controlled pollination breeding programs [4-6]. The aim of this study was to characterise 24 elite clones of several breeding programs in Uruguay, by the use of microsatellites. This was achieved by verifying the potential for discrimination of these molecular tools by assigning a specific molecular pattern of fingerprinting to each tested clone.

Methodology: Plant material was collected from 24 elite clones of Eucalyptus, provided by INIA Uruguay and Forestal Oriental. They were subdivided in 14 samples of E. grandis and 10 of E. dunnii. The tested clones were analyzed by PCR with 5 microsatellite loci using EMBRA 4, EMBRA 5, EMBRA 10, EMBRA 11 and EMBRA 16 markers under the same conditions as previously reported [7,8]. The molecular weight of the alleles was estimated with an automatic sequencer Macrogen Koerea [http://www.macrogen.com/eng/macrogen/macrogen_main.jsp] that employed Peak Scanner software V1.0 [https://products.appliedbiosystems.com]. The data obtained for each microsatellite was subjected to statistical assay to determinate the following parameters: the probability of identity (PI), the paternity exclusion probability (Pe), the heterozygosity (He) and the observed heterozygosity (Ho). This analysis was performed with the software IDENTITY 1.0 [9].

Results and discussion: The generated data was sufficient to assign a specific profile for each tested clone. The five EMBRA markers amplified a total of 90 alleles and a maximum of 23 alleles for the EMBRA 11 marker. The minimum was found for the EMBRA 16 marker with 14 alleles. The remaining three markers showed 20, 18 and 15 alleles per locus.. The probability of identity (PI) was 5.43 x10-9 and the paternity exclusion (Pe) 0.999. The expected heterozygosity (He) for all loci ranged from 0.894 to 0.958 while the observed heterozygosity (Ho) showed values ranging from a minimum of 0.471 to a maximum of 0.936 for every loci. A likelihood value of complete identity of 5.43 x10-9, guarantees the individual identity of each clone, as the probability of two being equal by chance is 1 in 184 million. A precise molecular pattern for each of the 24 genotypes could be assigned and the information produced is sufficiently robust to ensure the molecular traceability for each of these materials at the different stages of nursery production and/or field trials. However, the five markers derived from genomic sequence containing dinucleotide repeats. These markers, even when making a great discrimination, have reduced precision of genotyping, necessary for comparative multilocus profiling across laboratories or even at different times in the same equipment [10]. Considering a National System of Clones Registration, it proves necessary to increase the number of markers employed by including tetra and pentanucleotide markers to strengthen the precision and reproducibility among different laboratories. In addition, special emphasis should be laid on their selection, focusing on those microsatellites with high interspecies transferability. The testing of different species of Eucalyptus with the same molecular methodology and standardized procedures, will contribute to the registration of elite material and the protection of intellectual property.

Conclusions: The results show that using five microsatellite markers it is possible to discriminate among 24 clones and assigning a molecular fingerprint characteristic for each of them. The transferability of the five markers used is also verified, since they amplified both *E. grandis* and *E. dunnii*.

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Selection of SSR markers for population studies in *Eucalyptus globulus* seed orchards

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Background: *Eucalyptus globulus* is a widely planted species in temperate regions of the world for pulpwood production. Its good characteristics for kraft pulping in addition to a broad adaptability to different site conditions have led this species to be employed in commercial plantations and to be included in breeding programs [1]. Uruguay has approximately 300.000 hectares forested with *E. globulus* being the most cultivated species in the country and representing 45% of the total forested area. The main objectives of many Uruguayan breeding programs for *E. globulus* are the increase of volume per hectare, basic density and pulp yield. The strategies for genetic improvement used in many breeding programs in Uruguay require seed multiplication in seed orchards to obtain genetic gain [2].
In long-term breeding programs, the strict selection of reproductive populations restricts the number of genotypes involved in the final orchard, thus reducing genetic diversity and increasing the risk of depression through inbreeding in the following generations. The use of SSRs (simple sequence repeat) markers as selecting strategies of seed orchards in other species such as *Eucalyptus dunni*, has proved to be an excellent tool to reduce inbreeding [3,4]. The transferability of SSR markers across Eucalyptus species has been widely studied [5-8]. The aim of this work was to select highly polymorphic SSR markers of *E. globulus* to assist breeding programs.
Methodology: Eighteen *E. globulus* samples were collected from the breeding program of the National Agricultural Research Institute of Uruguay .DNA was extracted with CTAB 2X buffer [9]. Eight SSR markers, previously reported for *E. grandis* and *E. urophylla* were used: EMBRA 8, EMBRA 11, EMBRA 18, EMBRA 32, EMBRA 47, EMBRA 51, EMBRA 58 and EMBRA 155 [6,7] The annealing temperature was specifically adjusted for *E. globulus* using a temperature gradient of 52°C – 56°C. The other reaction parameters did not differ from those previously reported. The results were visualized in polyacrilamyde gels 8% under denaturing conditions at 80 W constant power over 3h 30min and were stained with silver nitrate. The estimation of the molecular weight of the alleles was made by comparison with a ladder. The matrix information was analyzed with the software Identity v 1.0 [10] and the following parameters were calculated: alleles number per loci, expected heterozygosity (He), observed heterozygosity (Ho), identity probability (Pi) and paternity exclusion probability (Pe).
Results and conclusion: From the eight tested markers, EMBRA 8, EMBRA 18 and EMBRA 13 did not amplify at any of the tested temperatures. EMBRA 11 and EMBRA 47 showed good amplification at 52°C of annealing. The rest of the primers worked optimally in the conditions previously described for *E. grandis* and *E. urophylla*[7]. The five SSRs markers amplified a total of 42 alleles. With a maximum of 14 alleles for EMBRA 11, 9 alleles for the markers EMBRA 47 and EMBRA 58 and 5 alleles for the markers EMBRA 51 and EMBRA 155. The range of heterozygosity expected for all the loci ranged from 0.64 to 0.88. However, the observed heterozygosity showed values of 0.1 to 0.9. With this information the probability of identity (IP) was 17 x 10^{-5} and the paternity exclusion probability was 0.99. The considerable number of SSR markers currently published [6-8] confirm how necessary the availability of these tools is to carry out precise population analyses. The five identified markers turned out to be promising candidates to be used in *E. globulus* seed orchards population studies.

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Genomic selection using a realized genomic relationship matrix in a *Pinus taeda* L. cloned population

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Genetic merit can be considered the finite sum of thousands of allelic effects, each physically located at some place on the genome, whose transmission can be traced through molecular markers. Traditionally, best linear unbiased prediction (BLUP) of breeding values relies on average additive genetic covariances (the numerator relationship matrix **A**) derived from pedigrees to utilize information from relatives. For example, all pairs of full-sib offspring of a cross are assumed to share 50% of alleles in common. Such assumptions ignore variation in Mendelian segregation of alleles among progeny within family. With advances in marker genotyping technology and reduction in genotyping cost, it is now feasible to estimate genetic covariances from markers.
Linear mixed models that utilize realized genomic relationship matrices could predict genomic estimated breeding values (GEBV) more accurately than those that use expected average genetic covariances derived from pedigrees. Dense markers can be used to trace identity by descent probabilities at each locus, and those probabilities used to construct an incidence matrix. The incidence matrix is used to estimate the genomic relationship matrix (**G**), which is used in place of the **A** matrix in solving the mixed model equations. This may allow more accurate estimation of individual breeding values than the traditional model based on average genetic covariances.
We estimated realized genetic covariances between cloned progeny of a *P. taeda* population. There were 165 cloned progeny derived from nine

full-sib families. The realized genomic relationships were based on a set of 3,461 biallelic SNP markers. We used the following linear mixed model **y** = **Xb** + **Zu** + **e** to estimate GEBV. In the model **X** and **Z** are incidence matrices, **b** is the vector of fixed mean, **u** is the vector of additive genetic effects that correspond to allele substitution effects for each marker with Var (**u**) = $\mathbf{I}\sigma^2_m$; where σ^2_m is the marker variance and **I** is the identity matrix. The term **e** is the vector of residuals. The dimension of **Z** is the number of individuals (n) by the number of loci (m). The regression method used to construct our **G** matrix did not require allele frequencies; instead, the inverse of the **G** matrix was generated by regressing **ZZ'** as a dependent variable on the **A** matrix as the independent variable. Therefore, the expected value of **G** is **A** plus a constant matrix.

Different cross-validation methods were used to test performance of the **G** matrix. Clones were divided into a training group with both marker and phenotypic information and a validation group for which only marker genotypes were used. In one scenario ~90% of the clones (148) were sampled for training, either randomly selecting within each of the nine families or at random without family consideration. The remaining ~10% were used for validation (17 clones). In the second scenario, ~50% of clones (84) were sampled either within family or randomly from the whole population for training, and the remaining ~50% were used for validation (81 clones). The model parameters estimated in the training set were used to predict GEBV in the validation set. For each scenario, six independent samplings were carried out. The mean correlation between the GEBV based on **G**-BLUP and breeding values based on **A**-BLUP were determined for each scenario, along with the accuracy of the BLUP predictions for both **G** and **A** based models. The mean correlation varied from 0.37 to 0.61 across the four validation methods. The accuracies of the predictions for any validation scenario were always higher for **G**-BLUP (range of 0.65 to 0.75) than **A**-BLUP (0.60 to 0.62), which is related to the smaller standard error of the predicted **G**-BLUP for the validation clones (17 or 81) under the different scenarios.

Estimating realized genetic covariances based on the genotypes of biallelic markers and incorporating those estimates into G-BLUP helps to more accurately characterize Mendelian segregation of alleles, and could allow more accurate selection within family. Such a method would increase genetic gains in forest tree breeding. The major impact would be on reducing the need for expensive field testing, but it may also be possible to shorten the breeding cycle and thus increase genetic gain per unit time and cost. The impact of genomic selection applications in forest tree breeding may be greater than for other crop or animal species, because of the biology of trees and their much longer breeding cycles.

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Imputing missing genotypes: effects of methods and patterns of missing data

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Costs of high-throughput genotyping have decreased to the point where it appears economically feasible to use molecular genetic marker information in applied breeding programs. Some practical questions remain to be addressed about how best to deal with missing data in the resulting genotype datasets, to minimize the impact of the missing data on the accuracy of breeding value prediction. Data can be missing for two reasons – first, genotyping assay failure is likely for at least some loci in some samples; and second, it may prove economically desirable to invest more resources for high-density genotyping of a few individuals and fewer resources for lower-density genotyping of many individuals [1]. The proportion of missing genotypes may range from less than one percent due to genotyping assay failure, to over 80% if a selective genotyping strategy is used. Many methods for predicting genetic merit of trees using marker genotype data require complete genotype information for mathematical reasons. It is therefore important to use efficient statistical methods to accurately impute missing genotypes. In species with complete reference genome sequences available, the map order of markers and linkage disequilibrium (LD) information can be used to guide imputation of missing genotypes. Completely sequenced reference genomes are available for only two forest tree species, so these methods are not suitable for most forest trees.

Gengler et al. [2] described a method to impute missing genotypes using mixed linear models and BLUP. We determined the effect on accuracy of BLUP estimated breeding values of imputation with different levels (10%, 20%, 40%, 60% and 80%) of missing genotypes. Analyses were conducted both with empirical data (3461 SNP markers in a cloned loblolly pine population of 165 genotypes) and simulated data, using missing data created by random sampling (some loci missing in all individuals) or by structured sampling (all loci missing in some individuals). Simulations were used to examine the effect of family and progeny size, mating design, proportion of missing genotypes, genotyping strategy and the method for imputation on the accuracy of breeding values. Imputed genotypes were obtained using the numerator relationship matrix (the **A** matrix) and solving the mixed model equations of **y = Xb + Mu + e**, where **y** is the vector of gene content predictions, **X** is the design matrix (vector of 1s) for the mean, **M** is the design matrix connecting trees to the gene content vector **y**, **u** is the individual tree effect and **e** is the error variance. The solutions of mixed model equations produce predicted SNP genotypes for trees with missing genotypes. The solutions would be continuous, centered on 1 because the gene content values are 0, 1 or 2.

Imputation of missing genotypes in empirical data from an unbalanced mating design with family sizes ranging from 1 to 35 was more powerful for data with structured missing genotypes at all levels of missing data than for data with random missing genotypes with same proportions of missing data. The accuracy of imputation for 10% and 80% missing genotypes ranged between 0.96 to 0.23 and 0.96 to 0.16 for structured and random missing genotypes in the data, respectively. As the proportion of missing genotypes increased in the data, the power of imputation decreased. With simulation, we found that the imputation was less affected by the distribution of missing genotypes in a balanced mating design with families of equal size. The accuracy of imputation ranged between 0.97 to 0.75 for the 10% and 80% missing genotypes in the data, respectively.

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S4. REPRODUCTION, GROWTH AND DEVELOPMENT

P62

Variation in gene expression profile with aging of *Pinus radiata* D. Don

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Phase change in higher plants, from juvenile to mature, affects the reproductive competence, morphology and growth rate, as well as the regenerative potential of tissue explants. However, in conifers these changes can be achieved before the onset of flowering and at the beginning of the seed production. Early maturational changes in conifers are very obvious, severe and overall irreversible [1], and are characterized by lost of morphogenetic capacity. This may affect clonal multiplication programs and genetic manipulation, due mainly to the decline in adventitious rooting capacity which is the most marked maturation and aging effect. Despite the serious effects of these processes, little is known about their basic regulation in forest clonal propagation programs. The latest researches regarding this subject are related to auxins changes during maturation [2], epigenetic variations during phase-change, like DNA methylation [3], and proteomic changes during needle maturation [4].

This work has been performed to gain deeper insight into the genetic mechanisms that regulates the loss of morphogenetic capacity with the aging process in *P. radiata*.

P. radiata ortets of different age were brought from La Posada nursery at BioBio region, Chile. Needles were collected in August, frozen in liquid nitrogen and stored at -80°C until its RNA extraction. RNA was isolated from 100 mg of frozen tissue according to Chang [5], the RNA extracts were purified with RNeasy Clean up and treated with RNase-free DNase (both from QIAGEN). Gene expression analysis was done by a reverse dot blot hybridization technique by comparing the transcription levels of 174 genes in 1, 3 and 5 years old ortets using the methodology described by Valledor *et al.*[4]. The 174 genes analyzed were classified into categories being the most important: Chloroplast and photosynthesis, protein translation folding, modification and degradation and proteins involved in transcription and DNA replication. Gene expression was quantified as signal intensity using the Gel Pro-analyzer 3.1.The values were normalized dividing every spot intensity by the average of the intensity of each membrane.

From the 174 studied sequences only 56 were differentially expressed among ortets of different ages. From the 56 genes differentially expressed, 27 showed a clear variation tendency with age, from which 21 were down-regulated and 6 were up-regulated, reflecting a higher number of active pathways in the younger ortets. GBBS1, PPi-phosphofructokinase and α-L-fucosidase (Figure 1) genes are related with carbohydrates and carbon metabolism that were affected by the ortet age; all were down-regulated with the increasing age, showing a significantly lower level of transcription in ortets of 5 years old. Both PPi-phosphofructokinase and α-L-fucosidase have been correlated to a higher level of expression in younger and metabolically active tissues. Specifically the *Arabidopsis thaliana* fucosidase (*At*FXG1) has shown a higher expression level in younger leaves than in the older ones, and it has been proposed that it has a role as a growth regulator. The previous results are consistent with the higher expression level in RUBISCO activase (figure 1) in the younger ortets (1 and 3 year old), which in turn agree with different studies, indicating that photosynthesis is reduced with increasing age of shrubs and trees [7] and higher in the younger ones.

Transcription regulated protein family were mainly over expressed in 1 year old ortets. It is the case of the chromosome condensation gene regulator (RCC1) (figure 1), which inhibit the chromosome condensation until G2 phase of the cell cycle and is required for the formation of the mitotic spindle [7].This result indicates that 1 year old ortets are more transcriptionally active. Likewise, proteins like Putative valyl-tRNA synthase and T-complex polypeptide protein like (TCP-1) (figure 1), involved in processes of protein modification and translation, were up-regulated in 1 year old ortets, indicating that juvenile plants are more active at protein synthesis and modification level. In contrast 5 year old ortets presented a down-regulation in these genes expression. Additionally, in 5 year old radiata pines genes like histidine-rich glycoproteins (figure 1) were up-regulated, this type of protein have been found to be a component of the cell wall, like histidine-rich extensin in *Zea mays* [9], this is consistent with the increase in cell wall synthesis in older plants.

Thus, this work has provided an initial insight of the pathways and mechanisms that may be involved in the loss of morphogenetic capacity caused by aging in *P. radiata* ortets. Juvenile ortets showed higher expression in proteins related with transcription activators, photosynthesis and carbon and carbohydrate metabolism, while older *P. radiata* ortets showed a down-regulation in the same genes and a increase in genes related to cell wall synthesis, indicating that these might be candidates genes to be markers of the aging and maturation process, results that will be complemented and validated with qRT-PCR and proteome studies.

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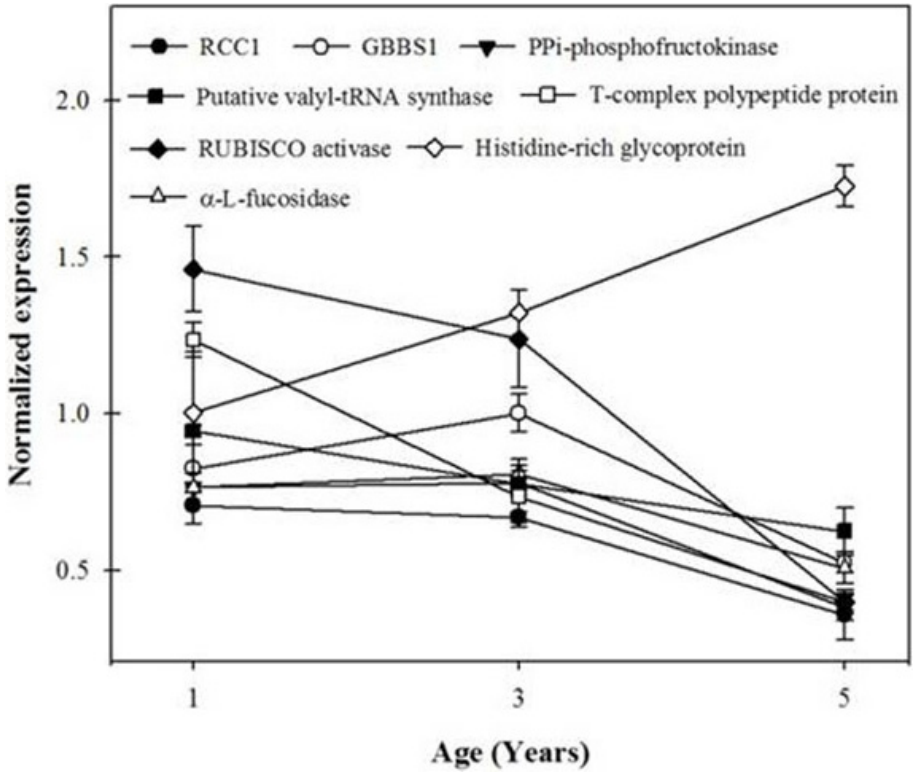

Figure 1(abstract P62) Normalized gene expression of: regulator of chromosome condesnation (RCCI), granule-bond starch synthase (GBBS1), putative valyl-tRNA synthase, T-complex polypeptide protein (TCP-1), RUBISCO activase, histidine-rich glycoprotein and α-L-fuocosidase *Pinus radiata* genes. Values are means ±SE.

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Competing MYB networks as switches in primary and secondary metabolism in spruce

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Background: R2R3-MYB transcription factors are regulatory genes that have been linked to key aspects of plant development and secondary metabolism. They have been implicated in the transcriptional control of phenylpropanoid, and flavonoid metabolic pathways in model plant systems. However, in trees, knowledge is still limited about the role of R2R3-MYBs in such processes. Microarray transcript profiles (9K custom cDNA array) obtained from comparison between wild type and transgenic spruces that constitutively overexpressed *Pinus taeda* MYB1, MYB8 [1], and MYB14 [2] identified putative targets in flavonoid and phenylpropanoid metabolism.Cross-comparison of these data sets identified 70 sequences that were common to the three transgenic backgrounds: interestingly, 66 of these 70 sequences were co-expressed between PtMYB1 and PtMYB8 transgenics but produced opposite profiles with PtMYB14 transgenics. Pairwise comparisons between these transgenic data sets identified 121 additional sequences showing opposite profiles. Predicted annotations and KEGG classification showed that many of the sequences were linked to the metabolism of amino acids and carbohydrates as well as flavonoids, phenylpropanoids, and terpenoids.It was hypothesized that MYB1/MYB8 and MYB14 may be part of competing transcriptional networks for the regulation of primary metabolism and secondary metabolism. The opposite transcriptional responses might also support structural or defensive oriented response, respectively.
Material and methods: A qPCR based experimental approach was used to test these hypotheses by evaluating transcript profile of the negatively co-expressed sequences together with MYB1, MYB8 and MYB14. Transcript levels were monitored during a diurnal cycle in three year-old wild type spruces, based on the fact that some genes associated to phenylpropanoid metabolism [3] and stress [4] were reported to follow a diurnal transcript variation. Our analysis also included a set of 10 additional MYBs putatively linked to secondary cell wall deposition [1] and stress oriented response [2]. A Spearman correlation rank test was used to estimate significant correlation (adjusted P val ≤ 0.01, FDR 1%) between MYB transcript profiles and selected co-expressed sequences. A functional assay system developed in embryogenic spruce cells was used to screen for possible interactions between transcription factors and promoters of putative target genes.
Results: Among the co-expressed sequences tested, five sequences coding for 4CL, DHS2, OMT1, SMT4, and a Lipase thioesterase displayed transcript profile that positively correlated with those of PgMYB1, PgMYB8 but negatively with those of PgMYB14 and PgMYB15, the closest homologue of PgMYB14 [2]. Interestingly, these oppositions were observed in secondary xylem differentiating tissues but not in bark/phloem. Significant correlations were also observed between the co-expressed sequences tested and the others MYBs. A separate analysis of large scale expression profiles for spruce transcription factors gave some additional evidences that these putative competing MYB networks may be tissue-preferential. Transient expression results obtained with our functional assay system further suggested interactions between these MYBs and some of their proposed targets. More extensive testing is in working progress.
Conclusions: The results of the present study strongly suggest that putative MYB networks may compete to allow rapid metabolic switch within wood forming tissue in order to direct metabolic flux towards specific aspects of primary and secondary metabolism for structural, or defensive-oriented response.

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Expression of auxin carrier genes during adventitious rooting in *Eucalyptus globulus*

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Background: *Eucalyptus globulus* and its hybrids are important for the cellulose and paper industry, mainly due to their relatively low lignin content. However, rooting of cuttings of this species is often recalcitrant and exogenous auxin application is necessary for adventitious root development. Auxin plays a central role in rooting capacity, which is particularly affected by its endogenous content and transport rate. The shoot apex is a major source of endogenous auxin, which is mainly transported by both influx (*AUX1*) and efflux (*PIN*) carriers in a specific basipetal active transport through the vascular parenchyma in stems. As part of a larger study to investigate the causes of low rooting in *E. globulus* microcuttings without exogenous auxin, we evaluated the expression profiles of *AUX1* and*PIN1* during the process of adventitious rooting using qPCR.
Material and methods: *E. globulus in vitro* tip microcuttings obtained from 14 week-old seedlings were submitted to a culture system consisting of a two-step protocol: an initial step of induction, which lasted 96 h (induction medium composition: 0.3x MS salt concentration, 0.4 mg l^{-1} thiamine HCl, 100 mg l^{-1} inositol, zero (control) or 10 mg l^{-1} indolyl-butyric acid (IBA – root promoting auxin), equivalent to 49.3 μM, 30 g l^{-1} sucrose and 6 g l^{-1} agar, followed by a formation step (same composition of induction medium, but devoid of auxin and supplemented with 1 g l^{-1} activated charcoal). The expression analysis of the selected genes was monitored along the rooting process and the harvest of microcuttings for RNA extraction was done at 6, 12, 24, 48 and 96 h of exposure to induction medium and 24 and 48 h after transfer to formation medium (formation step), for both treatments (with and without auxin in the first step). For the formation step harvest, the microcuttings remained for 96 h in the induction medium before transfer to formation medium. Total RNA was extracted, and the first strand cDNA synthesis was performed for all of the samples starting from about 500 ng total RNA, using oligo-dT primers and

reverse transcriptase M-MLV (Invitrogen) in a final volume of 20 µl. The final cDNA products were diluted 50-fold in RNAse-free distilled water prior to use in qPCR. The analysis was carried out using specific primers for *Arabidopsis thaliana* orthologue genes in eucalypt and both *Histone H2B* (*H2B*) and *Alpha-Tubulin* (*TUA*) genes were used as references [1]. The data were analysed with the comparative Ct method [2].

Results and conclusion: The gene encoding the auxin influx carrier (*AUX1*) did not show differences in expression profile between treatments (with and without exogenous auxin), suggesting that *AUX1* is not critical to the process of adventitious rooting promoted by exogenous auxins in microcuttings. This would be in line with the fact that the rate of endogenous auxin transport is probably not limiting under exogenous auxin supply. The auxin efflux carrier gene encoding *PIN1* showed an expression increase during the first 24 hours of the induction step in microcuttings exposed to exogenous auxin when compared with the control treatment (without exogenous auxin supply). This result seems to indicate a requirement of *PIN1* to redistribute and perhaps concentrate auxin, possibly IBA-derived IAA (indolyl-3-acetic acid), in specific areas of the base of microcuttings, in order to allow root development. Although IBA is a natural auxin recognized as an IAA precursor, its use as exogenous auxin instead of IAA, a common practice in clonal propagation by cuttings, may have also involved the expression of other sets of recently described IBA-specific transporters [3]. In conclusion, despite the unusual exogenous auxin entrance pathway into the microcutting compared to the endogenous auxin fluxes, *PIN1* likely takes part in the process of auxin concentration required to program founder cells involved in the establishment of new root meristems.

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Genome characterization of a *Eucalyptus* natural mutant

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Background: *Eucalyptus* genetic improvement is time-consuming mainly because its long time to reach reproductive maturity along with its mixed mating system [6]. As a result, the production of pure lines as in maize is impracticable. The heterozygous status hides deleterious recessive genes that, when in homozygosity can reduce seedling survival due to physiological changes, resulting in considerable loss in seedling production [6]. Therefore, it would be important to identify the gene(s) associated with inbreeding depression. There are very few studies involving the characterization of deleterious recessive genes in Eucalyptus. We detected an anomaly in the offspring obtained from a controlled cross of *Eucalyptus grandis*. The anomaly appears in a ratio of one abnormal to three normal seedlings, suggestive of a Mendelian segregation. Based on the segregation ratio, we hypothesized that the character is controlled by a single gene, with a homozygous genotype for the recessive allele showing abnormal seedlings. The parents showed normal phenotype and they have no relationship, excluding the possibility of inbreeding depression due to identity by descent. The abnormal seedlings die in a few months and show different characters: high shoot branching, height reduction, leaf area reduction, and changes in leaf shape (Figure 1). In this scenario, we aimed to characterize the genomic and genetic causes of the observed anomaly.

Methods: The full-sib individuals generated at Suzano Papel and Celulose SA company were divided into normal and abnormal seedlings. The ratio between normal and abnormal seedlings was tested by chi-square analysis. DNA was extracted according to Ferreira and Grattapaglia [1]. The DNA concentration was quantified using a spectrophotometer (Thermo Scientific NanoDrop™ 1000), and subjected to electrophoresis in 1% agarose gel and stained with ethidium bromide. DNA from ten normal individuals were randomly selected and mixed, creating the normal bulk, and the same was performed to create the abnormal bulk. The bulks were amplified with random primers (kits AD, AE, AF, AJ, and AK – Operon Technologies, Inc.). Amplicons were submitted to electrophoresis in 1% agarose gel stained with ethidium bromide. Polymorphic markers detected by RAPD were converted to SCAR (Sequence Characterized Amplified Regions) markers. DNA fragments from polymorphic marker were purified from agarose gel using the illustra GFX PCR DNA (GE Healthcare), cloned into vector pGEM-T Easy (Promega) and inserted into UltraMAX DH5α-FT competent *Escherichia coli* cells (Life Technologies). DNA was sequenced in an ABI3100 Genetic Analyzer (Applied Biosystems). SCAR primers were designed based upon the above mentioned sequencing and further used to amplify DNA fragments from bulks and six individuals: three anomalous, one individual from normal bulk; and two normal individuals of unrelated populations (BAC and Brasuz). Amplicons of the six individuals were purified, cloned into vector pGEM-T Easy (Promega) and inserted into DH5αcompetent *E. coli* cells (Life Technologies). Six colonies from each individual were amplified with the SCAR primerss. Purified product of amplification was

Figure 1(abstract P65) Normal (left) and abnormal (right) seedlings.

sequenced by ABI3100 Genetic Analyzer (Applied Biosystems). SCAR derivedsequences were compared among themselves and with the eucalypt genome (Phytozome v7.0 database - http://www.phytozome.net/) using BLAST. The genomic region identified was analyzed by FGENESH tool (Softberry, Inc. – http://www.softberry.com) to find predicted genes in the region. Predicted genes were translated by EXPASY translate tool (http://expasy.org), and the protein analyzed by PFAM database (http://pfam.sanger.ac.uk) for domain identification.

Results: Chi-square analysis confirmed Mendelian segregation of 3 normal: 1 anomalous seedlings in the tested progeny. The data indicate that the anomaly is a monogenic character that manifests when the deleterious recessive allele is in homozygosity. Among the random primers tested only one showed polymorphism with the segregation pattern. This marker appears in all abnormal and in 31% of normal individuals. The RAPD marker was converted into a SCAR marker and its segregation pattern confirmed. All abnormal individuals and 22.2% of normal individuals showed the marker. Sequencing of the SCAR marker was done to identify the possible genomic region where it is linked and detected a polymorphism of two base pair between anomalous (mutant) and normal individuals. Our *in silico* analysis showed the polymorphism in an intergenic region, localized between two protein-coding with a Bet v1-type domain belonging to the PR10 family (Pathogenesis-related protein 10). In general, this is associated with plant defense function in response to biotic and abiotic stresses [3-5]. However, there are reports that PR10 has important roles in biological processes [4,5].

Conclusions: According to our results, we propose that the anomalous character might be caused by a major effect gene. The marker found is related with the recessive; therefore, normal individuals with the marker are likely heterozygous. The polymorphism detected in the SCAR sequence, suggests that the mutation might be related to the two base substitution observed. However, at this point, it is unclear whether these base substitutions are involved affecting close genes (such as the identified PR10 proteins) or any other unknown genomic region. Expression analysis of the PR10-coding genes might provide clues to answer this biological question.

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P66

Apparent coordination of isocitrate dehydrogenase and glutamate decarboxylase expression in early stages of tree development

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The biosynthesis of 2-oxoglutarate and glutamate are key steps in the biosynthesis of nitrogen compounds and plant development. The reaction catalyzed by cytosolic isoenzyme of NADP+-linked isocitrate dehydrogenase (IDH) is also considered as the main route in the production of 2-oxoglutarate. According to its expression pattern during development, IDH is also involved in other, yet unknown, processes [1,2]. In addition to the importance of glutamate in the biosynthesis of nitrogen compounds, glutamate also serves as precursor of GABA, a molecule that is currently considered as a signal in higher plants. GABA is produced by the action of glutamate decarboxylase (GAD), a cytosolic enzyme that is regulated by Ca2+/calmodulin and pH. In contrast to IDH, that it is encoded by just one gene in most of plant genomes [2], GAD is encoded by a small family of nuclear genes [3]. The expression of IDH and GAD has been investigated during the differentiation of hypocotyl and stem in tree species. Our results indicate a coordination of the expression of IDH and GAD in developmental processes suggesting a role for 2-oxoglutarate supply and GABA synthesis during early stages of organ differentiation in trees.

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NAC regulation of embryo development in conifers

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Background: In most gymnosperms the cotyledons develop as a crown surrounding the incipient shoot apical meristem (SAM), which maintains the radial symmetry of the plant throughout embryogenesis. This is in contrast to the *Arabidopsis* embryo in which a symmetry-breaking event (from radial to bilateral symmetry) is associated with the emergence of the SAM and the two cotyledons [1]. We have previously shown that the differentiation of cotyledons and the establishment of the SAM and the root apical meristem (RAM) in somatic embryos of Norway spruce (*Picea abies*) is dependent on polar auxin transport (PAT) [2]. In *Arabidopsis* PAT restricts the expression of several genes belonging to the large plant specific NAC family of transcription factors [3-5], which in turn induce developmental processes involved in booth shoot and root formation. Members of the NAC family have a conserved DNA-binding domain in the N-terminal region known as the NAC domain, and a more diverse domain in the C-terminal region, proposed to be responsible for the trans-activation capacity of the protein [6]. Despite the diversity of the NAC gene family, and the importance of NAC proteins in fundamental processes, no NAC genes have to our knowledge been characterized in conifers.

Methods: Phylogenetic analyses of NAC domain containing genes from *Picea glauca*, *Arabidopsisthaliana*, *Medicago truncatula* and *Physcomitrella patens* were performed using PAUP* and MrBayes. Full-length cDNAs of two NAC genes were isolated from maturing somatic embryos of Norway spruce and cloned using standard protocols. The promoters were amplified using the Genome Walker™ kit (Clontech). The motif discovery tool MEME was used to find conserved motifs in the C-terminal regions and in the promoters of the NAC sequences from different species. The expression pattern of the two isolated NAC genes was analyzed in Norway spruce somatic embryos using quantitative real-time PCR. Their expression was also analyzed in embryos that had been treated with the PAT inhibitor NPA as described earlier [2]. Promoter::GUS fusions were inserted into embryogenic cultures of Norway spruce using *Agrobacterium* mediated transformation.

Results and discussion: We identified several different NAC sequences from white spruce in GenBank and deployed them to different sub-clades within the NAC gene family. Two full-length cDNAs (*gene 1* and *gene 2*) were cloned from Norway spruce. Both genes have elements in their promoter region which are significant for specific NAC genes. Furthermore, *gene 1* harbors previously characterized motifs that have been shown to have functional importance in NAC genes in *Arabidopsis*.

The relative expression of *gene 1* increased as early embryos differentiated, and remained at a high level until separated cotyledons were clearly visible. However, the up-regulation was blocked in embryos that formed fused cotyledons and lacked distinct SAM and RAM after being treated with NPA. The relative expression of *gene 2* was high in proembryogenic masses and early differentiating embryos but decreased as the embryos developed and matured. Interestingly the down-regulation occurred at about the same time as the cotyledons were delineated. The expression of *gene 2* was not dependent on PAT. Spatial expression analysis using promoter::GUS fusions revealed that the spruce genes have specific expression patterns, distinct from the expression patterns of their most closely related *Arabidopsis* counterparts.

Conclusion: Taken together, our results show that NAC genes with distinct signature motifs existed before the separation of angiosperms and gymnosperms approximately 300 million years ago. However, through evolution the expression of closely related genes has diversified leading to induction of distinct developmental programs in angiosperms and gymnosperms.

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P68

An *Arabidopsis thaliana* (Ler) inbred line exhibiting stacked stem/inflorescences mainly due to the reduced AP1 expression

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Background: Bolting is regarded as the initiation of reproduction stage in the growth and development of an Arabidopsis plant, when a set of floral integrator genes activate the expression of floral meristem identity genes *LFY* and *AP1* to initiate flowering transition[1,2]. However, how the expression of key genes, such as *AP1*, responds of diverse signals during flower development remains largely unknown. Here we have obtained an inbred line exhibiting an abnormal stem/inflorescence and flower development.

Methods: The morphological variations were carefully observed visually or with a Zeiss SteREO Discovery V8 stereomicroscope. Hybridization of GeneChip arrays was done in an Affymetrix Hybridization Oven 640 following the manufacturer's protocol (Affymetrix) and expression levels in seedlings and flower buds were calculated from Affymetrix intensity data. Real-time PCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Shanghai, China) with SYBR GreenPCR Master Mix (Applied Biosystems) as the fluorescence source.

Results: The inbred line exhibits a flower phenotype similar to the *ap1*, such as homeotic conversion of sepals (first whorl) to leaf-like, petals often absent (second whorl) and complete flower-to-inflorescence conversions (Figure 1, B). However flower meristem was replaced by emerging inflorescence meristems thus leading to a stacked stem/inflorescences before final flowering (Figure 1, D). Position and degree of stacked stem/inflorescences are varied differently (Figure 1, E and F).

Microarray and quantitative real-time PCR analysis revealed that the expression of *AP1* was significantly reduced, while the expression of its interacting genes *TERMINAL FLOWER 1* (*TFL1*), *OVEREXPRESSION OF CONSTANS(SOC1)*, *AGAMOUS-like 24* (*AGL24*), *SEPALLATA* (*SEP*) and upstream genes *FLOWERING LOCUS M* (*FLM*) were increased in flower buds (Figure 2).

AP1 sequence analysis showed that the promoter, coding sequences and intron splice sites of *AP1* genomic sequence in this inbred line were unchanged comparing to that in wildtype, suggesting the complexity in the regulation of AP1 in the line. Therefore these synthetic contributions caused the development of this unique phenotype. For instance, TFL1 was found to be highly expressed, and this gene can negatively regulate AP1 and specify inflorescence meristem identity leading to a delay of floral meristem formation[3]. On the other hand, the low levels of AP1 in flower buds cannot repress expression of *AGL24* and *SOC1*, which promote inflorescence fates rather than flower formation in the meristem and result in more abundant and longer inflorescences[4]. This expression variation of these genes is subjected to a threshold[4], leading to an ON/OFF expression pattern of the master regulatory gene(s) (like *AP1*) to specify a floral or a stem/inflorescence meristem.

Conclusions: The inbred line identified in this study is phenotypically similar to *ap1* mutants with noticeable deviations. The abnormal stem/inflorescence of the inbred line was mainly caused by significant

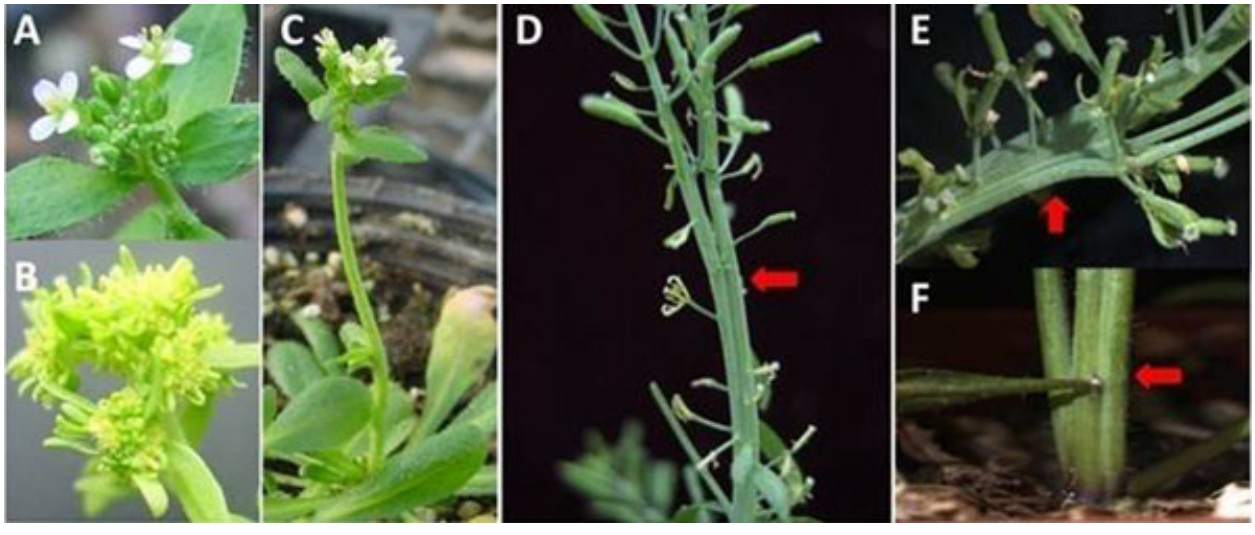

Figure 1(abstract P68) Phenotype of Wt and An inbred line.A, C: Wt; B, D-F: An inbred line).

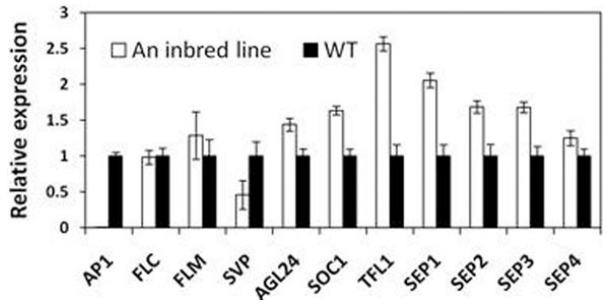

Figure 2(abstract P68) Expression of flowering regulatory genes in flower buds using qRT-PCR.

downregulation of *AP1*, but also is attributable to crosstalk among key genes like *TFL1*, *AGL24, SOC1*, etc. to control the transition of vegetative growth to the flowering phase. The inbred linemerits further molecular characterization to understand better the regulatory molecular network.

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Identification of GPAT acyltransferases in cork oak

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Background: Acyltransferases are enzymes with an important role in the synthesis of both cutin and suberin which are part of the lipophilic barriers, such as epidermis and periderm that protect terrestrial plants against water loss and other external aggressions. During secondary growth in woody plants such as cork oak (*Quercus suber* L.), the epidermis is replaced by a suberized periderm that includes the phellem (cork), phellogen (cork cambium) and phelloderm tissues. In *Q. suber* the successive formation of phellem following removal at periodic intervals (every 9 years) allows for exploitation of cork oak on a sustainable basis. The main component of cork (45-50%) is suberin, a complex polymer comprising both aliphatic and aromatic domains and associated waxes [1,2]. Despite the physiological importance of suberin, its biosynthetic pathway as well as its deposition remains largely unknown. Since cork oak is a unique species among terrestrial plants due to its remarkable capacity for cork production, it is expected that suberin biosynthesis and deposition are tightly controlled mechanisms.

As a first step to start unraveling these control mechanisms we intend to identify and characterize genes coding for the acyltransferases of the GPAT (glycerol-3-phosphate acyltransferase) family, involved in suberin and cutin synthesis in cork oak. Two ESTs highly similar to *GPAT5* (EE 743864 and EE 743865) and one EST (EE743668) highly similar to *GPAT4* shown to be strongly up-regulated in the suberin-rich phellem of cork oak tree (*Q. suber*) were first identified by Soler et al. [3].

Material and methods: In this work, phellem tissues from small branches with increasing age (1 to 7 years old) were harvested from cork oak and holm oak (a related but cork non-producing species)at the Instituto Superior de Agronomia (Portugal). Tissues collected during different growth periods were also used for analysis: samples collected during a period of high phellogen activity (April – June, 2009 and 2010) and samples collected during the inactive growth period (January, 2010). Total RNA was successfully extracted from these tissues using a protocol described by Reid et al. [4], with minor modifications. cDNA was synthesized using standard procedures and 5′- and 3′-RACE are being performed in order to determine the full-length of putative *GPAT* coding sequences from *Q. suber* transcriptome. The expression level of *GPAT4* and *GPAT5* genes was assessed by quantitative RT_PCR in two different seasonal stages (April and June) in periderm cells from 3 year old branches of *Q. suber*. The Cp values were converted into relative quantities, using the formula, $Q=E^{\Delta CP}$, where E (the efficiency of the gene amplification for each primer pair) was calculated using the Real-time PCR Miner algorithm.

Results and conclusions: A cDNA fragment with 1265bp of the putative *Qs_GPAT5* was obtained. The predicted amino acid sequence displays the glycerol-3-phosphate acyltransferase (PLN02499) conserved domain. Based on a database search we have identified putative orthologs of *AtGPAT5* in *P. trichocarpa* (Pt_8s_AT, accession number 002312108; Pt_10s_AT, accession number 2315213) and *R. communis* (Rc_ERGPAT, accession number 2531580) genome, which show high similarity to the putative *Q. suber GPAT5* gene. The *Rc_ERGPAT* is most similar to the *Qs_GPAT5* with an identity of 83% at the amino acid level.

The expression profiles of *GPAT4* and *GPAT5* were successfully analysed during phellem differentiation in periderm collected from cork oak tree. The relative expression level of *GPAT4* gene was similar in April and June. However, the relative expression level of *GPAT5* gene was higher in June, which corresponds to a period of higher phellogen activity, when compared to April.

Further information on the expression of these genes in several tissues of cork oak under different developmental stages and stress conditions will also be gathered as a result of the recent effort of the Portuguese research community involved in the transcriptome sequencing of cork oak. With this work we expect to contribute to elucidate basic aspects of the molecular networks involved in cork formation.

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Identification of a putative molecular regulator of cork cambium

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Background: Cork oak (*Quercus suber*) is a long-living species of the Fagaceae family that highly contributes for the economy of several countries such as Portugal. It is the only plant species with a phellogen capable of a sustainable production of cork with properties suited for industry applications. Phellogen, or cork cambium, usually initiates in the

subepidermis and differentiates during the first year of growth [1]. Its meristematic activity gives rise to phellem (cork) cells to the outside and to phelloderm to the inside.
It is likely that genes involved in the regulation of other plant meristems such as shoot apical meristem and vascular cambium are also involved in the regulation of phellogen. Based on its role in other meristems, and on specific expression patterns in poplar stems, we hypothesize that *SHORT-ROOT* (*SHR*), a transcription factor from the GRAS family, may be involved in the regulation of phellogen. This gene has been well described in the *Arabidopsis* root where it plays a key role in the radial patterning and in regulating the specification of the root stem cell niche [2-4]. Recently, its involvement in the root vascular system to control patterning processes [5] and in the control of proliferative cell division in developing leaves [6] has also been reported. In this work we report the cloning of *SHR* from cork oak transcriptome and the characterization of *SHR* expression patterns in the stem with the aim of investigating its putative function in the phellogen.
Materials and methods: Through a search in public databases we have retrieved putative orthologs of *ArabidopsisSHR* gene from species with a sequenced genome including *Populus trichocarpa*, *Vitis vinifera* and *Medicago truncatula*. Based on multiple alignments at the cDNAand deduced aminoacid sequence levels, we have designed degenerate primers in order to clone and characterize *SHR* from cork oak transcriptome. The amplified PCRfragment was inserted into pCR2.1 (Invitrogen) and sequenced to determine the percent identity to known *SHR* gene sequences.
To analyze *SHR* expression patterns poplar is being used as a model tree species. Transgenic poplar plants carrying the *SHR* promoter fused to the *GUS* reporter gene have been previously generated. Stem sections collected from 1-2 year-old transgenic plants have been subject to the histochemical GUS assay and embedded in Technovit 7100 resin for detailed analysis of gene expression localization under the microscope.
Characterization of *SHR* transcript abundance in cork oak developing stems taking into account different developmental stages and collection seasons has also been performed.Transcript abundance was analysed in stem tissues from the first to the third year of growth collected between January and October from a cork oak tree in order to cover periods of active growth and dormancy. Reproduction cork was collected in July which is the period during which the debarking process is performed from cork producer trees. RNA was extracted according to a protocol described by Reid *et al.* (2006) and cDNA was successfully synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Quantitative RT-PCRwas performed with LightCycler LC480 (Roche).
Results: While *Arabidopsis* has one *SHR* gene sequence in its genome, at least two additional *SHR*-like sequences are present in the poplar genome. Also, more than one *SHR*-like sequence seems to be present in the cork oak transcriptome and, at the moment, one of those sequences, *QsSHR1*, consisting of 1455bp and showing strong homology to the *Arabidopsis SHR* gene, was identified, and cloned. This putative *SHR*-like gene from cork oak was found to be expressed in stems at different developmental stages as well as in reproduction cork. Furthermore, evidence of specific expression of one of the poplar *SHR*-like sequences has been found in the phellogen and lenticels of stems from transgenic poplar carrying a promoter fusion to the *GUS* reporter gene.
Conclusions: *SHR*-like genes may be involved in even more developmental processes than previously thought. It seems plausible that in woody species such as poplar and cork oak, additional copies of *SHR* may have evolved to acquire new functions. We expect that these results, as well as results from further ongoing studies, will contribute to elucidate the putative involvement of *SHR* in the regulation of phellogen. Additionally, these studies may provide tools to be used in future strategies aiming at the improvement of cork production from cork oak.
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Molecular characterization of pine embryogenesis: pursuing the role of a putative non-specific lipid-transfer protein

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Background: Embryogenesis in Gymnosperms presents unique characteristics when compared to Angiosperm embryogenesis. However, little is known about the molecular regulation of embryo development in Gymnosperms because most studies have been conducted in Angiosperm model species. Due to the economic relevance of some forest species included in this group of plants, namely pines, efforts have been made worldwide for the establishment of efficient clonal propagation methods, of which somatic embryogenesis is an example. A deeper knowledge of the molecular players regulating zygotic embryo development in pines will likely contribute to a better understanding and control over somatic embryo development *in vitro*.
We have previously performed a global gene expression analysis during maritime pine (*Pinus pinaster*) embryo development [1] and have selected a set of transcripts for further characterization based on their expression profile. One of such transcripts (*PpAAI-LTSS1*) putatively encodes a protein of the Alpha-Amylase Inhibitors (AAI)/Lipid Transfer (LT)/Seed Storage (SS) protein family, non-specific lipid-transfer protein (nsLTP)-like subfamily.The AAI-LTSS family of proteins is unique to higher plants and includes cereal-type alpha-amylase inhibitors, lipid transfer proteins, seed storage proteins, and similar proteins. Proteins in this family are known to play important roles, in defending plants from insects and pathogens, lipid transport between intracellular membranes, nutrient storage, as well as in developmental processes. *PpAAI-LTSS1* is up regulated in zygotic embryos at a pre-cotyledonary stage of development [2]. The aim of this work was to characterize in detail the expression patterns of *PpAAI-LTSS* along embryo development and its upstream regulatory sequence.
Materials and methods: Zygotic embryos were isolated from immature cones and separated into five groups according to the developmental stage/collection date. The embryo staging was based on the system of Pullman and Webb [3] as described by Gonçalves *et al.* [4]. In each collection date, one or several consecutive embryo developmental stages were pooled as follows: Day 0 (stages T0, T1 and T2); Day 5 (stages T3 and T4); Day 11 (stage T4B); Day 15 (stage T5) and Day 25 (stage T7). Quantitative real-time RT-PCR (RT-qPCR) was performed using a iQ5 Real-Time PCR detection system (BioRad) to quantify PpAAI-LTSS1 transcripts in each of the five embryo groups to confirm differential expression along embryo development and developmental stage showing maximum up-regulation. Cloning of the upstream genomic region putatively corresponding to the promoter region, as well as localization of this transcript within the embryo tissues using in situ hybridization, are also being pursued.
Results: Confirming previous results using microarrays, *PpAAI-LTSS1* transcript showed a peak of expression in developmental stages corresponding to pre-cotyledonary and early cotyledonary embryos decreasing onwards until embryo maturation. Preliminary *in situ* localization results point to specific expression of this gene in suspensor tissues but additional experiments are still running to confirm these observations. In addition, a 900bp genomic DNA fragment that putatively belongs to the promoter region of this gene has been cloned. The analysis of this DNA sequence revealed the presence of several conserved promoter motifs and no significant homology to any sequence present in public databases has been found. Further experiments will allow us to isolate the complete promoter sequence to be used in functional analysis studies.
Conclusion: The transcript *PpAAI-LTSS1* putatively coding for a protein of the Alpha-Amylase Inhibitors (AAI)/Lipid Transfer (LT)/Seed Storage (SS) protein family may play an important role during embryo development in maritime pine, specifically at the pre-cotyledonary/early cotyledonary

stage, as supported by quantitative gene expression data. The ongoing experiments for cloning the promoter region and for localizing specific expression in embryo tissues will provide additional clues for functional characterization.

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In search for the role of thermospermine synthase gene in poplar vascular development

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Background: Plant polyamines are preferentially detected in actively growing tissues and have been implicated in growth and developmental processes such as embryogenesis, floral developmental, fruit ripening, senescence and stress responses [1]. Recently it has been established a link between polyamines and vascular development as it was found that, in *Arabidopsis,* the loss-of-function mutants of *ACAULIS5* (*ACL5*) gene, encoding thermospermine synthase, exhibit a severe dwarf phenotype, suggesting that thermospermine acts as a regulator of stem elongation [2,3]. However, in trees, no studies have yet been reported. Due to the relevance of vascular development in wood formation we are investigating the role of thermospermine in vascular tissues of poplar.

Materials and methods: A search for *ACL5*-like sequences in *Populus trichocarpa* genome allowed us to identify three putative *ACL5* orthologous genes. Based on the degree of sequence similarity, we have selected one of them, *PtACL5*, to generate transgenic plants bearing the constructs for overexpression and silencing of this gene in poplar.

Results: High expression levels of *PtACL5* in overexpression transgenic lines have been found to be correlated to higher thermospermine content in leaves and young stems, but not to a higher level of other polyamines, suggesting that *PtACL5* encodes a thermospermine synthase in poplar, and it is most probably an ortholog of *ACL5* in poplar. Interestingly, these plants display altered and arrested shoot development in the early stages following p35S::Pt*ACL5* transformation, as well as severe dwarfism. Anatomical changes associated to the lack of elongation include arrested development of the root system and no elongation of the stem from the first internode onwards. Because *Arabidopsisacl5* loss-of-function mutants show accelerated vessel cell death and *ACL5* expression is confined to xylem vessel elements [4], we further looked for alterations in the vascular pattern of the poplar stem, and observed the development of a wider stem in dwarf plants, composed of primary vascular tissues only, lower number of metaxylem cells and with no secondary growth.

Conclusions: Overall, our results suggest that thermospermine has a regulatory role in xylem differentiation/maturation in poplar. Although a feedback control of thermospermine synthesis seems to be present in *Arabidopsis*, in our transgenic poplar the high levels resulting from overexpression of thermospermine synthase gene seem to overcome any turn-over that might be occurring of the excess thermospermine being produced. Currently we are pursuing the spatial localization of the *ACL5* transcript in poplar plants through *in situ* hybridization, and by taking advantage of the generated transgenic lines we hope to understand the role of thermospermine in the vascular tissues formation in this woody species.

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P73

A direct stimulatory role of mobile gibberellin in *Arabidopsis* hypocotyl xylem expansion

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Background: Can Arabidopsis research contribute to our understanding about wood development? Does the function of vascular cambium in a herbaceous weed resemble that of a tree? Despite its diminutive size as compared to a tree, Arabidopsis still displays cambial driven secondary thickening in several organs, including the hypocotyl. Hypocotyl is a good model organ for wood development studies, as in this organ the radial secondary development can be uncoupled from the apical primary growth. This is due to the fact that the hypocotyl elongates only for five days after germination; thus, the radial secondary growth starts only after the elongation has ceased. This is in contrast to the other Arabidopsis organs displaying cambial growth, where it is accompanied by the simultaneous activity of the shoot and root meristems.

Two phases can be identified in the hypocotyl secondary development: 1) an early phase of proportional radial growth, where the cambium produces both xylem and phloem at a similar rate, and 2) a later xylem expansion phase, where more xylem than phloem is produced (Fig 1A) [1] . Notably, the composition of xylem is different between these two phases: the xylem produced during the first phase consists of xylem vessels and parenchyma cells, and of xylem vessels and fibers during the second phase. Especially the later phase, characterized by extensive wood formation, resembles the secondary growth in tree species.

We have previously shown that in Arabidopsis hypocotyl the transition from the first to the second phase is triggered through the onset of flowering, when the identity of shoot apical meristem changes from vegetative to reproductive [1] . Upon this transition, instead of new leaves, an inflorescence stem emerges from the middle of rosette leaves. What could be the nature of this signal [2]?

Results and discussion: Does flower differentiation trigger the onset of xylem expansion? To study this, we analyzed Arabidopsis null mutants for key flowering regulators. We were able to confirm that xylem expansion took place in all our mutants, suggesting that neither floral specification nor bolting (the emergence and elongation of the inflorescence stem from the rosette) are required for this process.

Thus, our signal appeared to be upstream of flowering; possibly the same signal activated both flowering and xylem expansion? Since gibberellin hormone (GA) has been shown to be an important regulator of flowering initiation, we wondered what effect it would have on the secondary growth. Indeed, both xylem expansion and flowering were initiated upon

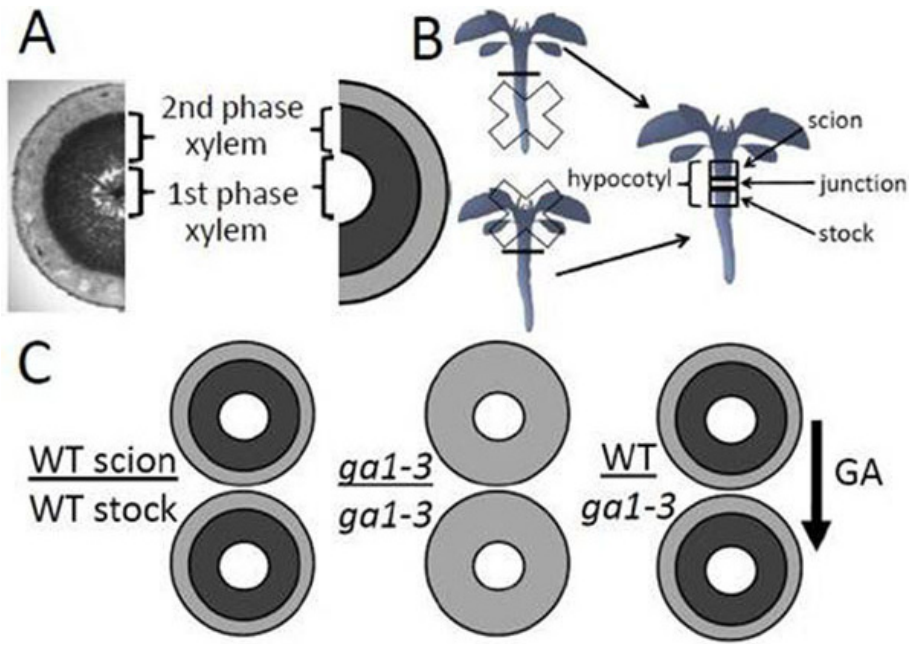

Figure 1(abstract P73) A) Cross-section of Arabidopsis hypocotyl showing the two phases of secondary development. B) In Arabidopsis grafting, young seedlings are cut into two through hypocotyl, and the hypocotyl of shoot (scion) part and the root (stock) part are then re-attached in the desired combination. C) Schematic picture of the *ga1-3* vs wild-type (WT) grafting experiment. GA from the WT scion was able to rescue xylem expansion in the hypocotyl of the GA deficient *ga1-3* stock.

GA treatment. To study if GA biosynthesis was required for xylem expansion, we analyzed *ga1-3*, a null mutant for the *GA REQUIRING 1* gene encoding a key GA biosynthesis enzyme. As expected, these plants displayed strongly reduced xylem expansion.

Next we wanted to study, if not only the GA content, but the actual level of GA signaling, was important for the regulation of xylem expansion. We observed that the transgenic and mutant lines with elevated GA signaling displayed increased and the lines with impaired GA signaling accordingly reduced xylem expansion, thus confirming the rate-limiting role of GA in this process.

Does GA signaling stimulate xylem expansion at the cambium, or does it function at the shoot apex to launch the production of the mobile signal? To study this, we performed grafting experiments (Fig 1B). We could see that the wild-type level of GA signaling in either part of the graft could not rescue the xylem expansion if the other part was dominantly inhibited in GA signaling. Accordingly, elevated GA signaling level enhanced xylem expansion only locally; the effect was not graft-transmissible to the wild-type part of the graft. Thus, GA signaling cascade appears to act as a local regulator of cambial activity, downstream of the mobile signal.

Could the GA hormone itself be mobile? We studied this by reciprocally grafting the GA deficient *ga1-3* mutant with a wild-type plant. Strikingly, in these plants, the wild-type scion (shoot part) restored xylem expansion in the hypocotyl of the *ga1-3* stock (root part) (Fig 1C). To sum up, an impaired GA signaling did not affect xylem expansion systemically, suggesting that it acts downstream of the mobile cue. By contrast, the GA effect was graft transmissible, confirming that GA itself is the mobile shoot-derived signal.

Conclusions: Our study shows that GA acts as a mobile signal that activates the onset of extensive xylem production in Arabidopsis hypocotyl. It would be interesting to study if a similar GA driven process takes place also in tree species upon the seasonal or age-related activation of their cambial growth. We think that Arabidopsis research has the potential to contribute novel hypotheses into secondary development research, and that it can complement tree studies in some areas of this research field. However, due to the sheer extend of secondary development present in tree species, and their other special characteristics (the seasonal activity-dormancy cycle etc.), many processes of secondary growth remain to be best studied in trees.

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P74

Deep sequencing combined with microarray hybridization to identify novel and conserved microRNAs during somatic embryogenesis of hybrid yellow-poplar (*Liriodendron chinense* (Hemsl.) Sarg. X *L. tulipifera* Linn.)

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Background: Hybrid yellow-poplar was a man-made cross between *Liriodendron tulipifera* Linn.× *L. chinense*(Hemsl.) Sarg.. Heterosis was strong both on growth and adaptation, no matter what, the crosses or the reciprocals. During the last 12 years, somatic embryogenesis systems were successfully established in our lab, in order to meet the needs of planting materials for expanding plantations in Southern China. Synchronous development of high quality embryos was always required both for the study of embryo development, and the supply of commercial emblings. The functions of plant microRNAs (miRNAs) in post-transcriptional gene regulation by targeting mRNAs for degradation, or the translational repression by hybridizing to complementary sequences within target mRNA molecules were well reported [1]. One of the major roles of miRNAs in regulating plant development has been obtained from a number of studiesbut molecular mechanisms regulating the development of embryos are not well understood, especially during the early stages of somatic embryos [2]. Here we present some results on the discovery of novel and conserved miRNAs during somatic embryogenesis of hybrid yellow-poplar using Illumina sequencing and miRNA microfluidic chip in order to

understand how the embryogenic somatic mass is developed into embryos and plants.

Materials and methods: The pre-embryo mass (PEM) and different stages of somatic embryos (SE) (Figure 1) were collected sequentially from the hybrid yellow-poplar somatic embryogenesis system. All the callus and embryo tissues were staged by microscope and immediately forzen in liquid nitrogen and stored at -80C until used. Total RNA was isolated from different developmental stages of somatic embryogenesis using *Total RNA Purification Kit* (Norgen Biotek Corporation, Canada). All the RNA samples from different somatic embryo tissues were mixed equally to form a single RNA pool. Following the instruction of LC Science, small RNA sample was prepared and then sequenced using Illumina Technology. Processing and analysis of raw data was done following Sunkar et al [3,4]. Conserved and candidate novel miRNAs in hybrid yellow-poplar were identified . A mixed small RNA microarray hybrid was used to validate the sequencing result and detect new miRNAs in hybrid yellow-poplar; 299 sequences detected by sequencing and 1,450 plant miRNAs sequences in miRBase 15.0 were ordered to serve as probes. A well-developed stem-loop strategy [5]and SYBR Green master mix were used to perform real time PCR to validate the miRNAs expressed in both sequencing and microarray analysis. The potential targets of miRNAs were predicted using the psRNATarget program (http://bioinfo3.noble.org/psRNATarget/) with default parameters. The biology and molecular functions of these predicted targets were categorized by the Gene Ontology program (http://www.Geneontology.org/).

Results: A total of 17,214,153 reads representing 7,421,623 distinct sequences were obtained from a short RNA library generated from small RNA extracted from all stages of somatic embryogenesis tissues. Hybrid yellow-poplar has a complex small RNA population and the length of small RNAs varied. The 24-nt and 21-nt are the predominant length for the majority of the small RNAs. Combining deep sequencing and bioinformatics analysisthere were 82 sequences, with perfectly matched known miRNAs from 32 conserved miRNAs families and 273 species-specific candidate miRNAs whose precursors were generated from *Liriodendron tulipifera* ESTs and form good hairpin structure. Among these miRNAs, both miR156 and miR166 which have been reported to be important during plant early embryonic patterning, had a large family members expressed in this process. Microarray hybridization and qRT-PCR technology were used to demonstrate that most conserved and species-specific miRNAs expressed in hybrid yellow-poplar. In addition, another set of 149 miRNAs included in 29 conserved families was found, which were not discovered by deep sequencing analysis but have obvious detectable signals in the microarray. Potential targets of biological processes and molecular functions of these miRNAs were predicted by blasting with *Arabidopsis thaliana* and analyzing with Gene Ontology. These predicted target genes participate in many metabolic and biological processes such as transport, signal transduction, response to stress, cell organization and many other cellular or developmental processes.

Conclusions: By combining Illumina deep sequencing with microarray hybridization we discovered 231 miRNAs from 61 conserved miRNA families and 273 species-specific candidate miRNAs. The potential miRNA target functions related to various biological and metabolic processes. These results indicate that microRNAs play wide-ranging and important roles during all stages of somatic embryogenesis in hybrid yellow–poplar. Additional experiments on the functional verification of interesting candidate miRNAs are now under consideration.

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P75

Phytohormone targeting in plant tissues

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Background: The identification and quantification of plant hormones in plant tissues are necessary for physiological studies of their metabolism and mode of action. The major problem associated with plant hormone analysis is that the amount of phytohormones present endogenously in plant tissues is very low, usually in the range of fmol to pmol/g fresh weight.

Methods: Homogenization and extraction with organic solvents was done in one microcentrifuge tube and accelerated by crushing the plant material in a vibration mill. The extracts from minute amounts of fresh plant material were immediately purified using a solid-phase extraction (C18, C8, Oasis™ HLB cartridges)in combination with ion-exchange and/or immunoaffinity purification. A fast chromatography technique, the ultra performance liquid chromatography (Acquity^{TM} UPLC,Waters) was coupled to triple quadrupole mass spectrometer (Xevo^{TM} TQ MS, Waters) equipped with an electrospray interface (ESI) and the unique performance of collision cell – ScanWave^{TM}. The mass spectrometric conditions were optimized for each analyte and quantification was obtained by multiple reaction monitoring (MRM) of precursors and the appropriate product ions.

Results and discussion: We found that a combination of different sorbents, reverse phases and ion-exchange phases, was the best tool in the one-step purification, giving a total extraction recovery ranging between 50-80% for all studied biologically active compounds. In MRM mode, the detection limit for most of phytohormones (cytokinins [1,2], auxins [3], abscisic acid [4], gibberellins, brassinosteroids) as well as phenolic acids [5] and mammalian steroids [6] was close to 1 fmol and achieved linear range was at least five orders of magnitude. Use of our procedures can allow the quantification of plant hormones and their derivatives (in total 145 compounds) in very limited amounts of material, ca. 100 mg FW. The methods provide substantial improvements in terms of robustness, sensitivity, selectivity, convenience, through-put and cost-effectiveness over previous methods published.

Conclusions: The application of new analytical approaches based on UPLC separation makes possible a new direction in plant hormone research. We believe that UPLC-ESI(+)MS/MS technology can be used for fast and sensitive quantitative analysis showing reproducibility in the plant hormones profiling in different tree extracts.

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P76

Ectopic expression of C-terminal tubulin variants alters wood composition and structure in *Populus*

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Cortical microtubules are cytoskeletal components that are relevant to the bioenergy and forest products industry due to their postulated role in orchestrating cellulose microfibril deposition during cell wall formation. The microtubule component proteins α- (TUA) and β-tubulins (TUB) are encoded by multi-gene families with very high overall sequence homology across species. To advance our initial characterization studies (Oakley et al., 2007), we have developed a suite of transgenic *Populus* that exhibit perturbed *TUA* to *TUB* transcript ratios, or that express tubulin PTM mimics. Most of the construct combinations resulted in abnormal organogenesis and vascular development, and failed to produce viable plants. Only three of the combinations led to whole-plant regeneration, and interestingly, all three featured the C-terminal variants. This is significant because the C-terminus is the post-translational modification (PTM) hot-spot in animal tubulins. One of the PTM mimics that we developed lacks a C-terminal tyrosine, thought to be the target of a tyrosine cleavage and re-ligation cycle important for tubulin regulation in animal cells. The transgenic trees appeared morphologically normal, but exhibited a range of epinasty and twisting phenotypes in mature leaves. Bark color was noticeably lighter in the transgenics. Microfibril angle, wood density, lignin content, lignin structure and metabolic profiles were altered in the transgenic wood. The results are consistent with a function of microtubules and microtubule PTMs for plant development and cell wall biogenesis in *Populus*, and offer novel strategies to manipulation of wood properties.

P77

Isolation and characterization of hybrid poplar galactinol synthases

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The raffinose family of oligosaccharides (RFOs) likely fulfill at least few major physiological roles in plants, translocation of carbon in the phloem, storage in sink tissues, and as putative biological agents to combat both abiotic and biotic stress [1-3]. The synthesis of galactinol from *myo*-inositol + UDP-galactose, by galactinol synthase (GolS), is considered a key regulatory step in RFO synthesis [4].To investigate the functional roles of this class of compounds in trees, two cDNAs that encode galactinol synthase (GolS), were identified and cloned from hybrid poplar (*P. alba* × *grandidentata*). Phylogenetic analyses of the *Populus* GolS isoforms with other known galactinol synthases suggested a putative role for these enzymes during biotic or abiotic stress in hybrid poplar. The predicted protein sequences of both isoforms (PaxgGolSI and PaxgGolSII) showed characteristics of galactinol synthases from other species, including a serine phosphorylation site at position 266 and the pentapeptide hydrophobic domain ASAAP [5]. Kinetic analyses of recombinant Pa×gGolSI and PaxgGolSII resulted in K_m values for UPD-galactose of 0.79 and 0.65 mM and V_{max} values of 660.4 and 1245 nM min^{-1}, respectively. PaxgGolSI inherently possessed broader pH range and temperature sensitivity when compared to Pa×gGolSII. Interestingly, spatial and temporal expression analyses revealed that *PaxgGolSII* transcript levels varied seasonally, while *PaxgGolSI* did not, thereby implying a temperature-regulated transcriptional control of this gene in addition to the observed thermosensitivity of the respective enzyme. Based on this evidence, we suggest that Pa×gGolSI may be involved in basic metabolic activities (*i.e.* storage), while Pa×gGolSII is likely involved in seasonal mobilization of carbohydrates.

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P78

Important processes during differentiation and early development of somatic embryos of Norway spruce as revealed by changes in global gene expression

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Background: Although most morphogenic events in plants occur in the sporophyte following seed germination, the embryonic phase is crucial as it is then that the meristems are specified and the shoot-root body pattern of the plant is established. We are using somatic embryogenesis in Norway spruce (*Picea abies*) as a model system for studying embryology in conifers. A deeper understanding of the genetic regulation can provide clues on how to improve the culture conditions in order to propagate economically important conifers via somatic embryos. The model system includes a well-characterized sequence of developmental stages, resembling zygotic embryogeny, which can be synchronized by specific treatments, making it possible to collect a large number of somatic embryos at specific developmental stages [1]. Here, we have extended the study of early events during embryogenesis in Norway spruce, by using microarray slides spotted with 12,536 cDNA clones from loblolly pine cDNA libraries. We have focused on the first stages of embryogenesis: the differentiation of early embryos from proembryogenic masses (PEMs) and the beginning of the development of late embryos. Few such studies have been undertaken and the work, which we describe here, is the first comprehensive analysis of gene expression of a conifer species during early stages of somatic embryogenesis. The analysis revealed the importance of previously unknown molecular events regulating putative processes associated with pattern formation.

Methods: The developmental stages of somatic embryogenesis in Norway spruce have been described previously [2] . The stages used in this study included proliferating PEMs on medium containing PGRs, early embryos one week after withdrawal of PGRs and developing late embryos after one week on medium containing ABA.Alteration in gene expression pattern during embryo differentiation and development was analyzed by comparing gene expression of samples from sequential developmental stages. In addition, to identify rapidly occurring transient changes, gene expression in proliferating PEMs was compared with gene expression in PEMs 24 hours after withdrawal of PGRs. Amplified RNA molecules were labeled and expression profiling was conducted using the TIGR loblolly pine microarray [3] with 12,536 validated cDNA clones originating from whole megagametophytes and zygotic and somatic embryos from different stages of development.

Results: Testing for significant differential expression identified 106, 208 and 464 mRNAs that showed different abundance 24 hours after withdrawal of PGRs, during differentiation of early embryos and development of late embryos, respectively. 53 mRNAs were present in more than one set, giving a total of 720 unique transcripts. The reliability of the microarray results was confirmed by qRT-PCR.
The relative abundances of Gene Ontology (GO) terms within the sets of differentially expressed genes were compared against the GO distribution of all clones on the array.We observed a general over-representation of genes involved in response to stress, both during the transition from proliferation to differentiation of early embryos and from differentiation of early to development of late embryos. During transition from early to late embryogeny, genes involved in catabolic processes, in carbohydrate metabolic processes and in gibberellin (GA) -mediated signaling were over-represented, while genes involved in metabolic processes were under-represented.
Our data show that already 24 hours after withdrawal of PGRs, the elimination of cells by PCD has been initiated as PCD-related genes like *METACASPASE 9* (*MC9*, AT5G04200) were up-regulated.
During differentiation of early somatic embryos, the first week after withdrawal of PGRs, genes related to cell wall modifications were down regulated. An increase in transcript accumulation was observed for genes coding for developmental regulators like MATERNAL EFFECT EMBRYO ARREST 49 (MEE49, AT4G01560), and a decrease of transcripts coding for LOB-DOMAINS containing proteins, indicating that pattern formation has started. The analysis revealed a putative increase in the content of both auxin and gibberellin.
At the developmental switch to late embryogenesis, several genes of importance for cell organization, developmental processes, and other biological processes were up- or down-regulated. The relative transcript abundance of *L1L*, *ROXY1* (AT3G02000) and *WRKY6* (AT1G62300) decreased while the abundance of transcripts such as *ABI3*, *ABI4* (AT2G40220) and *GROWTH-REGULATING FACTOR 1* (*GRF1*, AT2G22840) increased. In addition, genes coding for the LEA proteins were up-regulated. These changes in transcript levels suggest that pattern formation continued and that maturation had initiated at this developmental stage. The transcript levels of genes encoding a fatty acid elongase, FIDDLEHEAD (FDH, AT2G26250), which regulates epidermal cell differentiation, and for an EXTRACELLULAR DERMAL GLYCOPROTEIN (EDGP, AT1G03230) decreased. Transcripts encoding SUR1, MYB77 (AT3G50060), the auxin-induced protein INDOLE-3ACETIC ACID INDUCIBLE 11 (IAA11, AT4G28640), the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1, AT3G62980) and a positive regulator of brassinosteroid signalling (AT1G78700) were up-regulated, indicating that the auxin-responsive machinery was up-regulated. Furthermore, genes encoding the GA signaling repressors were up-regulated, while a GA induced and a gene encoding for a GA inactivating enzyme were down-regulated.
Discussion: By examining changes in global gene expression, we have been able to determine the timing of molecular events regulating putative processes during embryogenesis based on the assumption that the conifer genes are homologous to their Arabidopsis counterparts. We recognize notable changes in the expression of genes involved in regulating auxin biosynthesis and auxin response, gibberellin-mediated signaling, signaling between the embryo and the female gametophyte, tissue specification including the formation of boundary regions, and the switch from embryonic to vegetative development. In addition, our results confirm the involvement of previously described processes, including stress, differentiation of a protoderm and programmed cell death. Our analyses of genes and putative processes that take place during differentiation of early embryos and development of late embryos in a conifer can now serve as a basis for further studies of the processes.

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S5. BIOTIC AND ABIOTIC INTERACTIONS

P79

Identification of genes that contribute to drought stress tolerance in *Populus*

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Background: The cultivation of poplars (*Populus* spp.) is favored for forestry and reclamation purposes all over the northern hemisphere where they represent a commercially important resource. Poplars may become a component of programs to optimize carbon sequestration however; poplars are generally regarded as drought sensitive. The patterns of episodic drought over the last decade suggest that the development of drought tolerant poplar genotypes could be a useful tool to achieve sustained forest productivity [1]. Previous reports have shown that expression of hundreds of poplar genes changes in response to drought, presenting a problem in the identification of genes that are more important than others in counteracting the harmful effects of drought [2,3].
The genus *Populus* contains many fast growing hybrids that show varied drought tolerance according to genotype [4]. Hence, there is genetic variation among poplar hybrids that can be used to identify genes that contribute to drought stress tolerance. Despite extensive physiological and morphological descriptions of the response of Populus to drought, little work has been undertaken to explain genotype differences at the gene level. Therefore, this research has been undertaken and its major objective is to identify the genes that contribute to drought stress tolerance in poplar by correlating the physiological responses to gene expression. These genes may potentially be used as molecular markers in the drought tolerance breeding programs.
Materials and methods: Hardwood cuttings of 9 poplar hybrids (Green Giant, Assiniboine, AP-36, Canam, Katepwa, Hill, Walker, WP-69 and WP-86) were obtained from Dr. Barb Thomas (Alberta Pacific Forest Industries) and Bill Schroeder (Agriculture & Agri Food Canada, Indian Head, Saskatchewan). Cuttings were established in the greenhouse and plants were obtained for the drought stress trial. At the start of the trial, water was withheld from plants used for drought experiment whereas control plants were watered regularly. During the drought stress trial, a plastochron index was established that helped to collect the data from similar leaves of all drought stressed and control plants. Data were collected from 9 different poplar hybrids at 3 time points (mild stress, severe stress and recovery). Split plot design was used to collect the data from drought stressed and control plants using genotypes as main plots (9) and time points as sub-plots (3). Physiological data were collected for height growth, new leaf formation, water potential (ψ) and relative water content (RWC) from drought stressed and control plants. Young leaves were also collected for gene expression analysis from both stressed and control plants. Quantitative polymerase chain reaction (Q-PCR) was used to analyze the expression of several candidate drought responsive genes.
Results: Drought significantly affected the growth of the trees (Figure 1). Data from 3 drought stress trials in the greenhouse showed that different hybrids responded drought stress differently. Some hybrids behaved as tolerant whereas others as sensitive and some hybrids showed intermediate response to drought. By statistical analysis of physiological data, we ranked these 9 poplar hybrids according to their drought tolerance ability. Statistical analysis showed that Walker maintained significantly better height growth, leaf formation, ψ and RWC during mild, severe stress and recovery as compared to all other hybrids; therefore Walker was identified as the most drought tolerant hybrid genotype. On contrary, Green Giant showed poor maintenance of physiological parameters at all time points and hence was regarded as the least drought tolerant hybrid genotype. An intermediate response of all other hybrids was recorded during 3 drought stress trials in the greenhouse. Further, we focused only on 2 genotypes (the most and the least drought tolerant) to analyze the expression of several candidate drought responsive genes, which were obtained from microarray studies in poplar. Q-PCR results revealed that selected 2 genotypes had no significant effect

on the stress response of some genes, however, other genes showed marked and significant differences in expression between the two genotypes.

Conclusions: • Three trials in the greenhouse showed that physiological response to drought varies among different poplar hybrids.

• Walker was identified as the most whereas Green Giant as the least drought tolerant genotype among a group of poplar hybrids.

• Q-PCR analysis showed that among a set of candidate drought responsive genes, some genes showed differential expression between the most (Walker) and the least (Green Giant) drought tolerant genotypes.

• The genes showing differential expression in the most and the least drought tolerant genotypes might be playing an important role in drought tolerance of poplar..

• We have identified *Populus* genes those expression correlate with drought tolerance, providing candidate genes for drought tolerance breeding.

• These genes may also be used as molecular markers for drought tolerance in *Populus*.

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P80

Are there any *Pinus pinaster* trees resistant to *Bursaphelenchus xylophilus*? Studies implemented in Portugal to address this question

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The Pinewood nematode *Bursaphelenchus xylophilus* (PWN) was found to be responsible for maritime pine (*Pinus pinaster*) death for the first time in Europe in 1999, in a Portuguese forest south of Lisbon. It is the causal agent of the Pine Wilt Disease (PWD) and a quarantine organism in the European Union with approximately €80 million spent from 1999 – 2009 for attempting its eradication, which must also be added to direct losses from tree mortality. During this period, important progress has been made in understanding the relationships between PWN, its insect vector and the host tree/environmental factors that result in pine wilt in all countries affected but, especially, in Portugal.

Although PWN can cause devastating tree mortality, there is also evidence that some tree species or individual trees within a species are either tolerant (i.e. support the nematode, but are not killed immediately) or resistant to the nematodes. In order to investigate the susceptibility of *Pinus pinaster* to *Bursaphelenchus xylophilus* as a first step for the establishment of methodologies for the improvement of its resistance, a research program was implemented recently in Portugal for this species, combining genomic and quantitative genetics approaches. Until now, more than 500 trees were phenotypically selected in a highly affected stand and monitored during two years, regarding their phytossanitary status. The percentage of mortality observed in the first year was about 10%, with this value decreasing to less than 5% in the second year. Around 200 trees (including 150 of the selected trees) were genotyped for 6 microsatellite loci and three combinations of AFLP markers. The genotyping results show a high diversity of genotypes, which may open good prospects for the selection of resistant plants to *Bursaphelenchus xylophilus*.

At the same time, genomic and transcriptomic studies have been initiated with the final goal to discover candidate genes, loci and molecular markers related with disease resistance to PWN. With regards to the effects of the disease at a transcriptional level, the SSH technique was utilised to identify ESTs in *P. pinaster* and *P. pinea* when inoculated with PWN. ESTs were isolated, cloned, sequenced and identified using BlastN and BlastX, and clearly indicated that at an initial stage of the disease there is activation of a defence response at a molecular level, mainly related to oxidative stress, production of lignin and ethylene and post-transcriptional regulation of nucleic acids. 58% of the isolated sequences are not yet described, which shows the lack of genomic information currently available for pine. The results obtained are presented and discussed.

P81

Isolation, characterization and genes expression analysis of three dehydrin genes during cold acclimation of *Eucalyptus globulus*

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Eucalyptus globulus is an important species for pulp production in Chile; however it has a high sensitivity to frost. During the last few years, many studies have directed their efforts to elucidate the molecular mechanisms that regulate plant response to cold stress through the analysis of gene expression.This work reports the isolation and characterization of coding and non coding sequence of three dehydrin genes of *E. globulus* which allowed comparing the gene expression of these genes during cold acclimation, the type of dehydrin and the presence of regulatory elements in their promoters which provided valuable information about the possible signaling pathways and regulation of these genes. The three dehydrins identified in *E. globulus* showed a high transcript accumulation in stem and leaf tissue of acclimated plants, compared to non-acclimated, and the highest transcript accumulation was observed after the exposition of plants to night frosts (-2°C). Furthermore, the freezing resistant genotype exhibited a higher transcript accumulation after frost exposition compared to the sensitive genotype. These results support the idea that dehydrin proteins have an important rol during cold acclimation and frost tolerance in *E globulus*.

P82

Induced resistance and associated defence gene responses in *Pinus patula*

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Background: Plants are able to incite a type of broad spectrum resistance against pathogens upon pre-treatment with biological or chemical inducers. Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) are two types of induced resistance which lead to the accumulation of specific pathogenesis-related (PR) proteins. Non-pathogenic rhizobacteria are inducing agents for ISR and increased levels of ethylene (ET) and jasmonate (JA) are associated with this pathway, whereas SAR is associated with an increase in salicylic acid (SA) levels [1]. *Pinus patula* and *P. radiata* are commercially planted conifer species in South Africa, but are both highly susceptible to the causal agent of pitch canker, *Fusarium circinatum*. Annually, the forestry sector suffers substantial economic losses due to this disease which affects 20-30% of

Table 1(abstract P82) Disease progression represented as percentage live stem in 6 month old *P. patula* seedlings during an eight week period post inoculation with *F. circinatum*. The significance levels are relative to the relevant control

Week	2	3	4	5	6	7	8
Chitosan (1mg/ml)	89.73	82.31	76.75	71.43	60.81	61.16	36.10
Chitosan (10mg/ml)	91.12*	84.89*	80.60*	72.80*	64.28*	60.32	44.18
Control	89.23	82.10	74.48	69.04	58.65	55.15	34.53

*p<0.05, Kruskal-Wallis test.

the planting stock. Bonello *et al*. (2001) showed that repeated mechanical inoculation of *P. radiata* with *F. circinatum* activated induced resistance, enhancing the protection of the tree against subsequent pathogen challenge [2].

Detailed knowledge of the molecular mechanisms underlying induced resistance may be useful to develop strategies to control diseases of pine trees. This study aimed to compare the efficiency of ten biological and chemical inducers in inciting resistance against *F. circinatum*. Additionally the molecular basis of this induced resistance was investigated by analyzing the response of selected putative defence response genes.

Methods: Ten activators of induced resistance (Bion®, Messenger®, Chitin, MeJA, *Fusarium oxysporum*, *Pseudomonas fluorescens*, SA, Kannar, *Ralstonia solanacearum* and potassium phosphate monobasic) were compared. A set of 80 *P. patula* seedlings were used per treatment. Inducers were applied at four and six months of age and *F. circinatum* spores ($1x10^4$) were used to challenge the seedlings a week after the booster application (six months). Disease severity was assessed six weeks after inoculation by comparing the size of the lesions on treated plants to water control plants. Three inducers that curbed symptoms most successfully were selected for further analysis. A set of 116 plants per treatment were screened weekly for eight weeks. Aerial parts of the six month old plants were harvested for RNA isolation at 24 hrs after the second application. For each treatment, three replicates, with 12 plants per replicate, were harvested. Subsequently, RNA was extracted for the reverse transcriptase quantitative PCR (RT-qPCR) analysis, where four putative defence genes were profiled using the Roche LightCycler® 480 instrument.

Results and discussion: MeJA, Messenger and chitin treatment resulted in the reduction in symptom severity (results not shown). MeJA, Messenger and the deacetylated version of chitin, chitosan were then tested under stringent inoculation conditions with *F. circinatum* to verify the effectiveness of the inducers. The most promising treatment was chitosan at a concentration of 10 mg/ml, which resulted in a significant reduction in lesion length over a period of 6 weeks. Lesion lengths were converted to percentage live stem (calculated as lesion length divided by plant height multiplied by 100) and are displayed in Table 1.

The defence response elicited by chitosan application was investigated. Four putative genes, representing the onset of SAR and ISR were analyzed (Table 2). Phenylalanine ammonia lyase (*PAL*) had a three-fold accumulation in transcript expression in comparison to uninduced plants (Table 2). The expression level of the 1-deoxy-d-xylulose-5-phosphate synthase (*DXS*) gene, which catalyses the methyl erythriol–phosphate pathway, was down-regulated in comparison to the control. This pathway is important for the production of terpenes, which are building blocks for resin [3]. In previous studies, *PAL* and *DXS* were both shown to be responsive to chitosan. Using the *PAL* gene as a diagnostic marker of the phenylpropanoid pathway, the up-regulation of the transcript suggests that chitosan treatment induces the phenylpropanoid pathway which is known to lead to the production of secondary metabolites to elicit resistance.

Conclusion: The potential of priming *P. patula* to defend itself against pathogen attack was explored. We tested the application of ten different inducers to enhance tolerance to *F. circinatum*. The application of chitosan reduced pitch canker symptoms. Reduced lesion length was observed for a period of six weeks, indicating the activation of induced resistance. Further molecular analysis suggests that the treatment may activate the phenylpropanoid pathway, which is involved in the production of secondary metabolites that have antifungal properties [4]. Entire defence response pathways influenced by chitosan application in *P. patula* will be investigated in subsequent expression profiling assays.

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P83

Local and systemic defense response in Aspen clones: contrasting defense response to biotrophic and necrotrophic pathogens

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Trees are exposed to a variety of pathogenic fungi. The defense response toward a biotroph may require a different strategy that toward a necrotroph. To understand the key processes of defense responses toward pathogenic fungi in aspen (*Populus tremulae*) at the transcript level we inoculated clones of this species with a foliar rust on the leaves and a necrotroph in the bark. Leaf samples were collected from above the inoculation site to examine the long distance (systemic) defense responses and bark tissue around the site of inoculation examined for the local response as early as day1 post treatments. We performed microarray experiments on the biotrophic and necrotrophic interaction and between healthy controls of two SwAsp clones. Selected candidate genes were also examined in more detail by qRT-PCR and chemical analysis for phenols and tannins was also performed. We found that the two clones

Table 2(abstract P82) Log2 expression levels of putative defence response genes in *P. patula* 24 hrs after booster treatment with 10 mg/ml chitosan. Three biological replicates were used to calculate significance using the *t*-test

Gene symbol	Gene Name	Log2 Expression	P-value (t-test)
PAL	Phenylalanine ammonia lyase	1.773	0.028
DXS	l-deoxy-d-xylulose-5-phosphate synthase	-1.142	0.041
FMO	Flavin-dependent monoxygenase	0.486	0.173
PR-3	Chitinase	-0.997	0.199

respond in a very different in fashion at the transcriptional level to both the biotrophic and necrotrophic pathogen. The more resistant clone responded systemically within 24 hours while little response at the transcriptional level was detected in the more susceptible clone in response to the biotroph, while indications of suppression in response to the necrotroph was found.

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The reaction zone is a unique plant defense found in trees: differentially expressed genes and cell wall changes

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Heterobasidion annosum senso lato is the most devastating pathogen of conifers such as Norway spruce in Europe. This pathogen enter Norway spruce trees trough the roots or wounds and colonizes the tree from within, growing as a saprophyte when established within the dead heartwood and acting as a necrotroph when in contact with living host tissue. We have examined the host response in Norway spruce at the molecular level as well as the responses of the pathogen.

We have studied the defense reactions toward pathogenic fungi in the ecological and economic important conifer Norway spruce from both a molecular and anatomical perspective. We have studied the host responses of the tree as well as the attack modes and genes indused by its pathogens. The disease caused by this *H. annosum* s.l. is complex as it is can act both a necrotroph and saprotroph as well as a broad host range. Twenty percent of the trees in Norwegian spruce stands tend to be infected by this pathogen and *H. annosum* s.l. typically colonize and decay the economically important wood inside the trunk. However, Norway spruce have defences against this and other pathogens and the attack can be fought off by the living bark and sapwood but not the hearthwood being composed of dead zylem.

The bark has effective defense reactions that can be induced and we have seen indications of systemic and primed defense responses but also the sapwood has defensive capabilities. The tree has a unique defense against this internal attack by forming a reaction zone; in this case the host defense is directed inwardly by the still living sapwood toward the central colonized wood. We have studied the host defense at the transcriptional level, changes in phenols and lignification and found that the speed of the host response appear to be crucial in fending off the pathogen.

P85

Transformed Hairy Roots of the actinorhizal shrub *Discaria trinervis*: a valuable tool for studying actinorhizal symbiosis in the context of intercellular infection

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Background: Nitrogen is a major limiting factor of plant growth in many ecosystems. Root nodule symbiosis (RNS) is one of the most efficient adaptations allowing plants to cope with nitrogen deficiency by establishing a symbiotic association with diazotrophic bacteria able to produce ammonium from atmospheric nitrogen. Nevertheless RNS is restricted to two groups of plants: legumes and Parasponia (*Celtidaceae*), that interact with a group of gram-negative proteobacteria collectively called rhizobia, and actinorhizal plants, a group of 220 species, mostly shrubs and trees distributed in the orders Fagales, Cucurbitales and Rosales, that interact with gram-positive actinomycetes of the genus *Frankia*[1]. All these plants belong to the Rosid I clade, suggesting a common origin for the ability to establish RNS [2].

In recent decades a strong research effort focused on model legumes lead to the identification of key molecular actors involved in nodulation, including the bacterial signalling molecules, the Nod factors and several genes involved in the symbiotic signalling pathways [3]. Much less is known in non model legumes and actinorhizal plants, particularly in species that are not infected like model legumes through root hairs but show more ancestral infection mechanisms like crack entry or intercellular infection. Yet important cues regarding the diversity and evolution of RNS are being found precisely in these more primitive non-model systems [4,5].

Among infection mechanisms leading to root nodule symbiosis, the intercellular infection pathway is probably the most ancestral but also one of the least characterized [6,7]. Intercellular infection has been described in *Discaria trinervis*, an actinorhizal shrub belonging to the Rosales order [8]. To decipher the molecular mechanisms underlying intercellular infection with *Frankia*, we set up an efficient genetic transformation protocol for *D. trinervis* based on *A. rhizogenes*.

Methods: We analyzed the susceptibility of *D. trinervis* to two strains of *A. rhizogenes*: A4RS, and ARqua1; both strains contained a pHKN29 plasmid with a 35S::GFP fusion [9]. The classic *in-vitro* inoculation was compared to an *ex-vitro* method reported to be successful in several plant species [10]. The functionality of the symbiosis was tested on composite plants by performing nodulation tests and acetylene reduction assays. Using this technique, we introduced the promoter of *MtEnod11*, a nodulin gene from *M. truncatula* widely used as a marker for early infection-related symbiotic events in model legumes [11].

Results: Transgenic roots showing strong levels of GFP were obtained for all treatments. The *ex-vitro* method using Arqua1 was the best compromise to obtain a good co-transformation efficiency while minimizing the impact on root system architecture. Co-transformed roots were specifically and efficiently nodulated with *Frankia* and the resulting nodules were undistinguishable from non-transgenic nodules in terms of developmental timing, anatomy, nitrogen fixation and feedback control by nitrogen. The expression of reporter genes such as *GUS* and *GFP* could be easily detected within transgenic *D. trinervis* root systems. The promoter of *MtEnod11* retained its symbiotic activation in transgenic *D. trinervis* nodules. Similar results were obtained in *C. glauca*[12].

Conclusions: These findings open new avenues to study the genetic mechanisms of intercellular root invasion and single cell infection, allowing detailed characterization of genes involved in *D. trinervis* nodulation and a better understanding of the most ancestral infection pathways leading to RNS. In addition, because *D. trinervis* belongs to the Rosales order, evolutionary comparisons can be made with plants belonging to the same clade but unable to nodulate (most *Rosaceae*), or with Parasponia sp., the only non-legume able to enter RNS with rhizobia. The transformed roots in *D. trinervis* with appropriate reporter genes would be a powerful tool to explore signaling mechanisms in symbioses with this ancestral infection mode [13].

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DNA methylation and adaptive response in forest tree species

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Progressive increase of temperatures as well as longer seasonal drought periods revealed by climate studies correspond to fast environmental changes that forest species face with their actual genetic background. Natural selective processes cannot develop an adaptive response within this time frame. Thus the capability of forest tree species to adapt to the new environments will depend on their genetic background, but also rely on their phenotypic plasticity. Several reports have shown the involvement of epigenetic modifiers as the basis of the phenotypic plasticity, and in particular to the adaptation to abiotic stresses. DNA methylation (methylation of cytosine residues)is one the most important epigenetic modification in eukaryotes. Itis involved in specific biological processes such as gene transcription regulation, gene silencing, mobile element control or genome imprinting.Therefore, there is a great interest in analyzing cytosine methylation levels and distribution within the genome.
In order to analyze methylation-sensitive anonymous CCGG restriction sites we used MSAP technique (Methylation-Sensitive Amplified Polymorphism), an AFLP-based technique for the analysis of cytosine methylation. The technique is based on the use of isoschizomers that show differential cleavage sensitivity to cytosine methylation. *Hpa*II and *Msp*I are isoschizomers that are frequently used to detect cytosine methylation. *Hpa*II cannot cleave if one or both cytosines are fully methylated (in both strands), whereas *Msp*I cleaves $C^{5m}CGG$ but not $^{5m}CCGG$ sequences). For each sample, MSAP analysis is performed using both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I digested samples. Comparative analysis between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I fragment patterns allows the identification of two types of polymorphisms: (1) "Methylation-insensitive polymorphisms" that are associated with genetic variability and will show common *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I profiles among samples; and (2) "Methylation-sensitive polymorphisms" that are associated with epigenetic variability and detected as amplified fragments differing in their presence or absence or in their intensity between *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I patterns of the same sample. Thus, full methylation of the internal cytosine at the assayed CCGG sites will be associated with fragments detected in *Eco*RI/*Msp*I pattern which are absent or less intense in *Eco*RI/*Hpa*II profile. Hemi-methylation of the external cytosine will be associated with fragments observed in *Eco*RI/*Hpa*II pattern which are absent in *Eco*RI/*Msp*I profile.
We have optimized DNA methylation analysis for two forest tree species, including both angiosperm (*Fagus* sp.) and gymnosperm (*Pinus* sp.) species. The set-up of the MSAP technology has allowed study intra-specific variability of DNA methylation in *Pinus pinea* L. a specie which is characterized by a low genetic variability and high level of phenotypic plasticity. Representative populations spanning the whole distribution of this specie within Spanish geography were chosen for this study, including coastal and interior populations. MSAP patterns from vegetative propagated trees were compared among and within populations. Percentage of methylation-sensitive polymorphisms was calculated and selective markers were identified.
To test a possible role of DNA methylation in the plasticity of *Fagus sylvatica* L. in response to water deficit we analyzed two populations of beech from Spain and Sweden which differ in the response to water stress. Patterns of DNA methylation clearly differed between populations. Although the rate of DNA methylation was similar between irrigated and non-irrigated trees in each polpulation after AMOVA, some specific MSAPs associated with water stress response were identified.

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Analysis of adaptive responses of *Pinus pinaster* to changing environmental conditions in the Mediterranean region

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Recent climate evolution studies highlight the progressive temperature increase and prevalence of seasonal drought, with specially incidence in the Mediterranean region. Although conifers are very important species regarding forest conservation, sustainability and productivity, given the large forest surface they cover in Spain and their active role in preventing soil erosion and desertification, we know little about the molecular mechanisms which control adaptation in this ancient taxonomic group.
The work is focused on the study of adaptive responses of maritime pine (*Pinus pinaster* Ait.), one of the most important gymnosperm species in the Mediterranean region. To better understand these processes, we designed a strategy which integrates several approaches:
- Phenotypic characterization in response to drought, evaluating functional (physiological) and morphological parameters in a full-sib family originated from a controlled cross between two progenitors showing an *a priori* contrasting response to drought. In order to improve the phenotypic estimates, clonal material was generated by vegetative propagation and several ramets per clone were analyzed per experiment. Two experiments have been already developed: a) Plants from a complete full-sib family were exposed to severe drought for a short time; b) Selected clones showing contrasting behavior in their response to drought were subjected to a moderate drought for long time. The latest experiment was designed to better understand the functional mechanisms of drought tolerance. In 2011 a third experiment will be developed to evaluate survival capacity.
- Molecular characterization of the adaptive response, including:
1. Transcriptome analysis. ESTs collections associated with different tissues and growth conditions. We developed: a) Subtractive cDNA libraries to study the response of clonal material subjected to controlled hydric stress. A total of 386 unigenes have been identified from the SSH library (Sanger sequencing), 351 of them presumably corresponding to nuclear sequences. We have selected a set of 67 reliable candidate genes significantly upregulated by PEG-induced water stress, according to a microarray analysis: 45, 29 and 29 from roots, stems and needles, respectively. Functional classification of the unigenes, based on the BLAST homology analysis, showed that the largest group corresponds to genes involved in metabolism (16%). Up to 42% of these are related with carbohydrate metabolism, consistently with the role played by sugar accumulation in drought tolerance. b) cDNA library made of mRNAs extracted from buds collected from January to April, to dissect bud burst in this species. A total of 9,000 ESTs were sequenced by Sanger (CT574594

- CT583294) in collaboration with C. Plomion, UMR BIOGECO. c) A cDNA library made of mRNAs from multiple tissues (needles, stems and roots) and growing conditions (including drought stress and hormone treatments – auxin and cytokinin) collected from full-sib progeny individuals (collaboration with C. Díaz-Sala, UAH). The cDNA library was constructed by EVROGEN and used as template for Roche GS-FLX Titanium high throughput sequencing (Lifesequencing). A total of 22,427 isotigs/contigs were assembled from 1,218,000 reads. 15,722 isotigs/contigs were annotated and a total of 9,085 proteins detected.

2. Characterization of full-length cDNAs (FLcDNA). Complete and accurate annotation of functional genes rely largely on the information of FL-cDNAs since eukaryotic genes often have differential splicing of introns and unpredictable transcription starting sites, making gene prediction less accurate. In order to characterize FLcDNAs, a pool of approximately 5.000 PCR amplified inserts from selected cDNA clones (based on cDNA length versus annotation) from different EST libraries was generated. This pool of inserts was sequenced using Roche GS-FLX Titanium. A total of 3,259 isotigs from 573,242 aligned reads were obtained. This work was carried out in collaboration with S. Fluch (ARC) and C. Plomion (UMR BIOGECO).

3. SNP identification and analysis. Two sources of SNPs were used: 1) SNPs detected in a set of re-sequenced candidate genes (*in vitro* SNPs) used to design a 384 and a 1536 Golden Gate arrays for diversity analysis (to be used in association studies) and construction of genetic maps, and 2) SNPs detected from the third cDNA library described in the transcriptome analysis section, to design a 1536 Golden Gate array for construction of genetic maps as well as a 7600 Infinium assay for diversity and mapping purpose (the latest in collaboration with C. Plomion).

4. Construction of genetic linkage maps using dominant (SAMPLs), as well as co-dominant (microsatellites, ESTPs and SNPs) markers. Two INIA and two INRA mapping progenies will be used to construct species genetic map of *Pinus pinaster*. For this purpose, a set of common markers including SSRs, ESTPs and SNPs from three Illumina assays (384 Vera Code; 1536 Golden Gate; 7600 Infinium assays) will be performed to genotype 100 individuals per mapping progeny. Comparative mapping with other conifers will also be addressed.

5. Association studies. Association analyses are in progress by looking for association between phenotypes (growth, drought stress response) and genotypes (SNP genotypes) of 22 populations (each represented by 20 to 30 trees), spanning the natural distribution of the species (collaboration with C Plomion, UMR BIOGECO) and a metapopulation from Central Spain which consists of 5 populations (each represented by 20 to 30 trees; collaboration with C. Plomion, UMR BIOGECO and Reiner Finkeldey, UGO). QTL analysis is carried out to identify genome regions involved in the genetic control of maritime pine response to hydric stress, growth and biomass production based on association studies between genotypes of mapping populations and their corresponding phenotypes.

6. Maritime pine genome sequence. De novo sequencing of *Pinus pinaster* genome using haploid DNA will be carried out using current next-generation DNA sequencing technologies and a shotgun strategy. This approach includes: 1) >30x genome coverage based on paired-end (short-insert libraries) and additional >10x genome coverage base on mate-pairs (long-insert libraries) using HiSeq2000 sequencing; 2) use of mate-pairsequenced with GS FLX Titanium to construct longer scaffolds; 3) use of transcriptomics data described in sections "a" including FLcDNAs (section "2") to confirm results; and 4) genome assembly and annotation. This research results from the collaborative work between Spanish groups from UMA, PAB, UV, UPM, CNAG, BSC, Lifesequencing and CIB-CSIC coordinated by CIFOR-INIA and UAH and it is supported by the Spanish Ministry of Science and Innovation.

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The effect of heterologous VHb expression to the functioning of stress-related genes in hybrid aspen lines exposured to biotic stress

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Background: The hemoglobin produced by *Vitreoscilla* bacterium, VHb, is capable to promote the respiratory activity and ATP production under hypoxic conditions [1]. In micro-organisms grown under oxygen-limited conditions, the heterologous *vhb* expression improves the production of primary and secondary compounds, growth as well as stress tolerance [2,3]. VHb has also been shown to protect plants against deleterious effects of nitric oxide (NO), the important signalling molecule produced during pathogen challenges [4]. We have previously studied the *vhb* expressing hybrid aspen lines (*Populus tremula* L. x *tremuloides* Michx.) under elevated UV-B illumination and showed that the VHb lines had increased secondary metabolite production and also enhanced accumulation of starch in chloroplasts when grown in standard greenhouse conditions [5]. In order to reveal the effect of VHb on the expression of stress-related genes in hybrid aspen exposed to biotic stress, we conducted experiments with pathogenic fungus and larvae of insect herbivore.

Materials and methods: In the herbivory experiment, two-year-old VHb line and non-transgenic hybrid aspen line were exposed to the feeding of chestnut moths larvae (*Conistra vaccinii* L.) for 24 hours. Four VHb hybrid aspen lines and two non-transgenic hybrid aspen lines were infected with pathogenic fungus *Venturiatremulae*, *in vitro*. Samples for the RNA and protein extractions were collected after 10 and 21 days of the pathogen inoculation. In both experiments, the transcriptome changes were studied with microarrays and real-time RT-PCR. The cDNA slides contained 8256 stress-related oligos of *Populus euphratica*. The relative expression of selected genes were conducted with the LightCycler® 2.0 (Roche) and LightCycler® 480 instruments according to the manufacturer's chemistry. The amount of VHb protein during pathogen fungus experiment was analysed by Western blots.

Results and conclusions: The consumption of the leaves by the chestnut moth larvae was comparable on the non-transgenic and the VHb line. The effect of herbivory to the gene expression levels of wounded and systemic leaves was similar in the VHb and non-transgenic hybrid aspen lines, but in the non-transgenic line, the detected changes were more severe than in the VHb hybrid aspen line. The genes encoding bark storage proteins (BSPs), copper chaperone (CCH), and nitrate transporter (NRT) were among the limited number of genes which showed different expression changes between the VHb and non-transgenic hybrid aspen line.

The hybrid aspen lines infected with *V. tremulae* showed symptoms of shoot blight i.e. blackened stems and leaves, but the amount of VHb protein did not correlate with the severity of the disease. At the transcriptome level, the studied lines responded to the pathogen treatment individually in the first sampling (10 days) whereas the expression profiles of the lines resembled each other in the second sampling (21 days). The functional grouping of the genes with known mode of action resulted most representatives in protein metabolism, photosynthesis, stress as well as RNA metabolism groups.

In general, the transcriptome profiling of fungus- and herbivory-induced responses did not reveal any biological or molecular process altered in all of the VHb lines due to the biotic interactions. Moreover, based on the real-time RT-PCR results, the *vhb* expression did not show induction after the 24-hour herbivore treatment or the inoculation with the *V. tremulae*.

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P89

Increased *Bacillus thuringiensis* δ-endotoxin Cry3Aa toxicity against longhorned beetle by fusing to peptide specifically binding to beetle Cx-cellulase

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Background: *Bacillus thuringiensis* (Bt) Cry toxins have specific toxicity to susceptible insects. They are being used in transgenic plants or spray to control insect pests in agriculture [1, 2, 3]. Cry3A toxins are used extensively for biological control of coleopteran larvae [4, 5]. A Bt886-*Cry3Aa* gene that exhibited a high activity against Coleoptera insects isolated Our laboratory. Insect bioassay performed on *Anoplophora glabripennis* Motsch and *Apriona germari* Hope showed that the mortality of larvae fed with the product of this gene was over 60% [6]. However, both transgenic poplar with native *Cry3Aa* and withmodified-*Cry3Aa* by using poplar-prefered codons did little effects on longhorned beetles probably due to its low expression level in poplar.A peptide (LPPNPTK) named PCx that specifically bind to cellulase from midgut of longhorned beetle larvae was screened out from a phage display library previously in our laboratory[7].

Materials and methods: Fused *Cry3Aa* genes with PCx coding sequence at 5′ or 3′-end were amplified using pET-30a(+)-*Cry3Aa* [6] as a template and designed primers, and. used to construct three recombinant plasmids (pET-30(+)-PCx-*Cry3Aa*, pET-30(+)-*Cry3Aa*-PCx and pET-30(+)-*Cry3Aa*). Target proteins were characterized by Western Blot, ELISA and LC-MS/MS methods. To analyze the activity of PCx-Cry3Aa, Cry3Aa-PCx and Cry3Aa proteins against longhorned beetle larvae, bioassays were performed on *A. germari*Hope larvae by artifical feed with toxins. The retaining time of target proteins in midgut and the cellulase activity of longhorned beetle larvae were measured in order to elucidate the potential mechanism of the fused toxins of PCx and Cry3Aa against longhorned beetle larvae.

Results: Expression products contained target proteins were characterized by SDS-PAGE, Western-blot and ELISA after induced by IPTG. The target bands were digested and analyzed by LC-MS/MS to further confirm them as Cry3Aa proteins. The bioassay showed that the mortality of larvae fed with the two fused Cry3Aa proteins (PCx-*Cry3Aa* and *Cry3Aa*-PCx) was up to three times higher than that fed with Cry3Aa (Figure 1). Retaining time analysis was performed on excretas that collected at different times after feeding. The result showed that the fused Cry3Aa was concentrated in excreta collected at 6 h, whereas Cry3Aa at 4 h. Meanwhile, the Cry3Aa concentration in midgut juice after fed with fused Cry3Aa was higher than that with Cry3Aa alone. This indicated that the retaining time of fused PCx-Cry3Aa in midgut of larvae is longer compared to that of Cry3Aa alone (Figure 2). In addition, we also analyzed the cellulase activity when bond with fused Cry3Aa or Cry3Aa alone and showed that the fused protein did not affect the activity of cellulase. Therefore, the remainning time of fused Cry3Aa is prolonged after binding with cellulase, thus the enhanced toxicity of fused Cry3Aa is due to the prolonged retaining time.

Conclusions: These data demonstrate that the cellulase-binding peptide could enhance the toxicity of *Bacillus thuringiensis* Cry3Aa against the longhorned beetle.We also confirmed that the increased lethality in larvae fed with PCx-Cry3Aa or Cry3Aa-PCx was attributable to the ability of the toxin to bind Cx-cellulase, thereby increasing toxin retention in the midgut. These uniquely modified Cry3Aa proteins have potential use for pest control. The significantly enhanced activity of Cry3Aa fused with the Cx-cellulase-binding peptide provides a new strategy for increasing δ-endotoxin efficacy against the longhorned beetle.

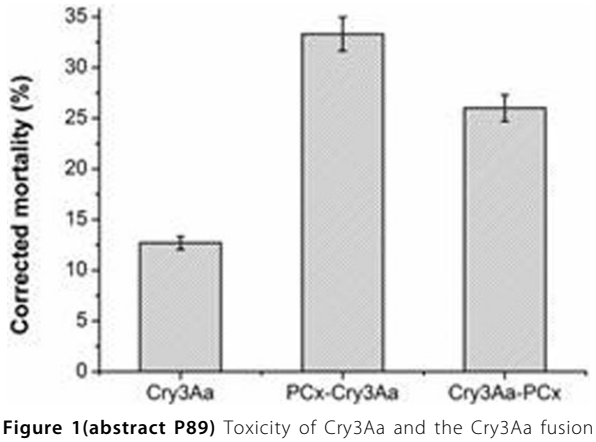

Figure 1(abstract P89) Toxicity of Cry3Aa and the Cry3Aa fusion proteins against *A. germari*Hope larvae. Corrected mortality rates are shown for larvae fed with Cry3Aa, PCx-Cry3Aa, or Cry3Aa-PCx. Both Cry3Aa fusion proteins exhibited increased toxicity toward the larvae, compared with non-modified Cry3Aa.

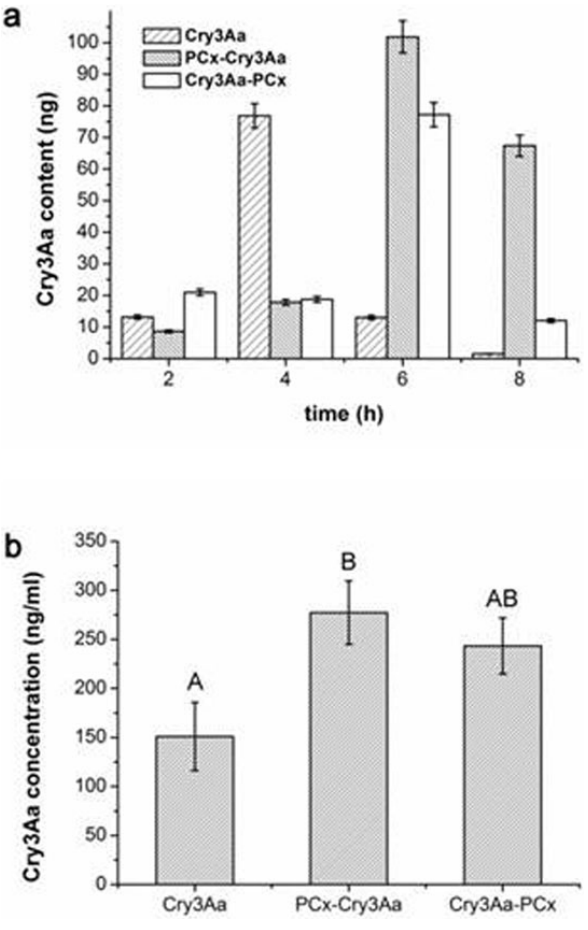

Figure 2(abstract P89) Concentration of Cry3Aa in *A. germari* Hope larvae after feeding with Cry3Aa and the Cry3Aa fusion proteins. a, Cry3Aa was measured in larval excretions collected at 2, 4, 6, and 8 h after feeding. The Cry3Aa content peaked at 4 h after feeding with Cry3Aa, whereas the peak was observed at 6 h after feeding with the Cry3Aa fusion proteins. b, Cry3Aa was measured in larval midgut extractions at 4 h after feeding. The concentration of Cry3Aa in the midgut extracts was higher after feeding with the fusion proteins, compared with non-modified Cry3Aa.

P90

Metrology of morphological response of Siberian elm to drought stress: increased stomatal pore depth

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BMC Proceedings 2011, **5(Suppl 7):**P90

Background: Structural characteristics of stomata consist of stomatal shape, density, depth, and pore dimension. Commonly called Siberian elm, the species *Ulmus pumila* L. is a fast-growing and small to medium-sized tree. Due to the superb adaptations to the harsh conditions of the Gobi Desert, the trees have been preferentially planted in Mongolia. It is worthwhile to investigate the morphological characteristics of the tree species that are tolerant to drought stresses in such arid areas.
Materials and methods: Based on average annual precipitations, two types of leaf specimens were collected from Korea, China, and Mongolia: (i) leaves under normal environmental conditions and (ii) leaves under arid conditions. Leaf stomatal characteristics of Siberian elm were investigated by electron microscopy and white light scanning interferometry [1].
Results and conclusions: Field emission scanning electron microscopy revealed stomata on the lower leaf surface of the tree species. Measured as ca. 30 micrometers in width, the stomata appeared to be oval in shape. In-lens secondary electron imaging by a coaxial annular type detector showed a difference in depth from epidermis to pore between the two types of leaves. Leaf stomata under arid conditions appeared to have higher levels of depth from epidermis to pore than ones under normal conditions (Figure 1). Line profile analysis by white light scanning interferometry allowed for the nondestructive measurement of the stomatal dimension (Figure 2). The depth from epidermis to pore of stomatal complexes under normal conditions was ca. 1.79±0.13 micrometers, whereas that under arid conditions was ca. 2.12±0.08 micrometers. In addition, higher levels of surface roughness were observed in stomata under arid conditions than those under normal conditions. These results suggest that increased stomatal pore depth would be responsible for the adaptations of the tree species to arid conditions. Furthermore, such architectural differences in

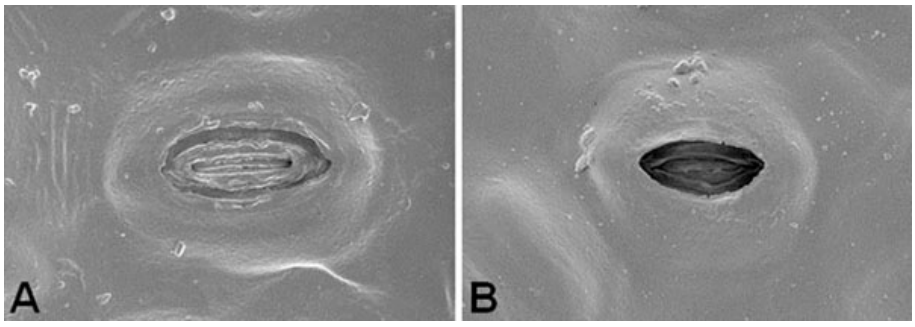

Figure 1(abstract P90) Field emission scanning electron micrograhs of stomata of Siberian elm. (A) Stoma under normal conditions. (B) Stoma under arid conditions.

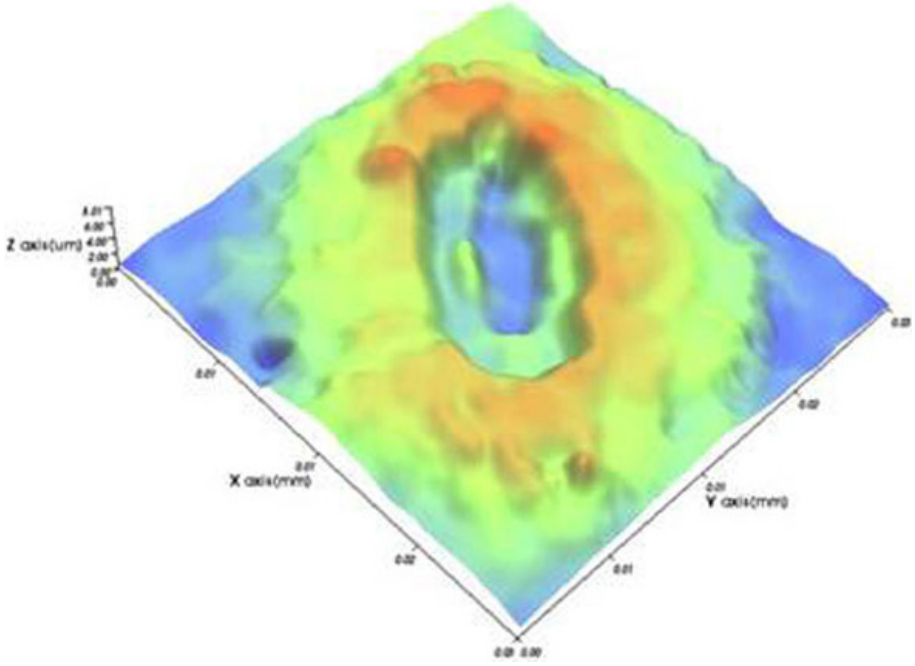

Figure 2(abstract P90) Three-dimensional surface plot of a stoma under arid conditions by white light scanning interferometry.

stomatal dimension could be quantitatively analyzed by complementary microscopy.

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P91

Regulation of the boron transporter EgBor1 in *Eucalyptus globulus*: a plausible model

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Boron is a micronutrient that plays an important role in plant cell wall biosynthesis. Nevertheless, an excess of boron in the soil causes severe damage to the respiratory tissue of the plant. In *Arabidopsis thaliana*, the *Atbor1* gene encodes a boron transporter that distributes this element throughout the plant.

A cDNA sequence encoding a *bor1* transporter was isolated from a *Eucalyptus globulus* cDNA library. This sequence contains several stop codons within the coding region. Initial bioinformatic analyses suggest that this interruption corresponds to an intron that may generate a truncated protein.

*Egbor1*was overexpressed in *Saccharomyces cereviseae* to assess whether it was capable of restoring the phenotype of a mutant strain that lacks the boron transporter. It was also overexpressed in a wild type strain as a control. In both cases a significant increase in boron tolerance was observed, suggesting that the encoded transporter is functional. Subsequently, a western blot analysis showed that the expressed transporter corresponds to the product of the full-length protein, rather than the truncated protein.

Additional bioinformatic analyses showed that the intron presents several regulatory elements, therefore it may function as a promoter for a small protein encoded in the 3' region of *Egbor1*. Transient expression of GUS under the control of the intron sequence proved its capability to activate gene expression. Thus, we have identified three ORFs in the *Egbor1* sequence: a full length protein encoded by the spliced mRNA named fragment C, and two smaller proteins encoded by the 5' and the 3' regions of the non spliced mRNA named fragments A and B.

We are currently challenging the yeast mutant strain with all the putative encoded proteins to understand their specific role in boron transport.

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P92

The expressed genes of Japanese red pine (*Pinus densiflora*) involved in the pine wilt disease severity

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Background: Japanese pine trees have been severely damaged by the pine wilt diseases, and hardly been keeping the productive forest in Japan. The pine wood nematode,*Bursaphelenchus xylophilus,* is a virulent pathogen in Japan, while it is originated and is an endemic one in the United State [1]. The diseases have now been spread not only in East Asia but also in a part of Europe. The Japanese Government has been developed the resistant pine breed varieties which had selected the survived-tree lineages obtained from the damaged pine forests. The developed varieties vary in the disease severity depending on the individual trees even in the same variety, partly because of the wind-pollination. Thus it needs molecular indices to select the resistant individuals without nematode infection.

Shin et al. [2] reported up-regulated genes in a non-selected, or susceptible, Japanese red pine, *Pinus densiflora*, after the nematode inoculation . Defense responses distinct from the susceptible trees may be expressed in the resistant bleed variety against the pine wood nematodes. By comparing the expressed gene profiles between the resistant and susceptible varieties against the diseases, a molecular clue for the resistant response against the diseases will be provided. Here, we have compared gene expression profiles between the susceptible and resistant breed varieties of Japanese red pine at the stage when the defense response had

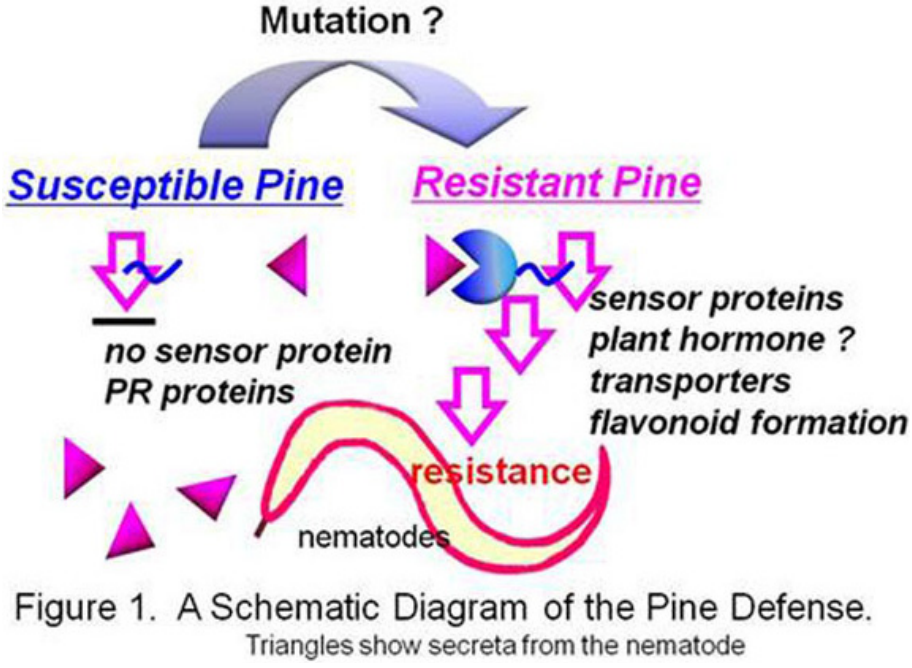

Figure 1(abstract P92) A schematic diagram of the pine defense. Triangles show secreta from the nematode.

just established after the nematode inoculation. We also discuss on the gene components involved in the disease severity or resistance.

Methods: Pine wood nematodes were inoculated on 2-year-old seedlings of a non-selected susceptible and one of the strongest resistant breed variety, respectively. The five samples were harvested at every 7 days and counted the nematode numbers of the samples. They are also stored at -80°C for RNA extraction. The nematodes were collected by Baermann funnel technique and counted under a stereomicroscope to judge the resistance. RNAs were extracted from the resistant and susceptible stems judged at the stage when the nematodes had just started to propagate, by Quagen RNeasy Plant Mini Kit with minor modification. The cDNAs obtained from the resistant and susceptible breed varieties, were differentially screened by Megasort beads technology. The 1507 and 1329 cDNAs, or ESTs, from the respective breed varieties, were effectively sequenced and assembled as the contigs. They were subjected to Blast retrieval against various public databases for their assignments. The data sets obtained were analyzed *in silico*.

Results and conclusions: A contig sequence is composed of ESTs, and it approximates an expressed gene species, while the numbers of ESTs in a contig approximate the expressed level of the gene. Comprehensive expression analyses show that the contig numbers, i.e. gene species, were more abundant in a resistant breed variety than those in a susceptible one. The contigs expressed more than 4 ESTs, being composed of 803 ESTs, were three times abundant in the resistant variety than in the susceptible one. The defense response might be triggered by the nematode invasion in a resistant variety but not in a susceptible one. Alternatively, it may be caused by the suppressed gene expression in the susceptible one. The latter is more probable because the signal transduction involved in the resistant response had blocked in the susceptible variety (Figure 1).

The flavonoid biosynthesis had been transcriptionally more expressed in the resistant variety than in the case of the susceptible one. One of the authors had suspected that nematicidal stilbenoids, which are abundantly localized in pine heartwood, might be involved in the disease resistance [3]. The stilbenoid biosynthesis was activated by methyljasmonate or salicylate application but did not detect in this experiment at the stage when the resistant variety had just established defending response. The phenolic metabolites were more produced in the resistant variety comparing to the case of the susceptible one, supporting the results from the transcriptional observation. Some pathogen related (PR) proteins were not observed in the resistant breed but were up-regulated in the susceptible one, suggesting such proteins may be induced by stress and not directly involved in the defense response. A cluster, being composed of the contigs, approximates a gene family and represents a hot spot in the mutation which may or may not be involved in the defense response. We will also discuss those gene families.

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P93

Occurrence of African cassava mosaic virus (ACMV) and East African cassava mosaic virus – Uganda (EACMV-UG) in *Jatropha curcas*

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Background: *Jatropha curcas* is a drought resistant shrub native in tropical America, now widely grown in many tropical and subtropical regions for biodiesel production [1]. First reports on virus infections in *Jatropha* indicated the occurrence of viruses closely related to *Cassava mosaic virus* in India, reaching a disease incidence from 25 to 47%. This might represent a major constrain to the production of *Jatropha* in large scale [2]. The genome of *Cassava mosaic geminiviruses* (CMG) consist of two components termed DNA A and DNA B (~ 2.7 – 3.0 kb) [3]. Furthermore, *Jatropha* has been described as host of *Cucumber mosaic virus* (CMV) [2].

Methods: In this study we attempted to detect and molecularly characterize viruses infecting *Jatropha* in Eastern Africa (Kenya and Ethiopia). Detection methods will be valuable tools for early screening of plant viruses in order to make appropriate decisions and selection of planting material.

A total of 127 *Jatropha* samples from Ethiopia and Kenya (districts: Kakamega, Siaya, Busia and Nakuru showing typical virus symptoms and symptomless plants were used in this study. ELISA was performed to detect the presence of three RNA viruses: CMV, *Cassava common mosaic virus* (CsCMV) and *Cassava brown streak virus* (CBSV). PCR was performed using newly designed primers based on multiple alignments of full length DNA A sequences of geminiviruses available in the NCBI Genbank, reported to infect either *Jatropha* or cassava. This allowed to amplify the variable regions of full length (2800 bp) and shorter sequences (380-1085 bp). PCR products were sequenced. A phylogenetic tree was constructed from multiple alignments by performing a heuristic search. Multiple alignments were analyzed by maximum parsimony with full-length DNA A using Phylogenetic Analysis Using Parsimony (PAUP) and a bootstrap analysis with 1000 replicates.

Results and conclusions: None of the *Jatropha* samples analysed was infected with the RNA viruses CBSV, CMV and CsCMV. PCR primers amplifying a 380 bpfragment of AC1, AC2 and AC3 yielded positive results with 75% of the symptomatic samples from Kenya and further detected 20% of asymptomatic samples as positive. Furthermore, 61% of symptomatic *Jatropha* samples from Ethiopia were positive. Full length primers were able to detect 69% symptomatic *Jatropha* samples from Kenya, and also in 67% of asymptomatic samples. PCR analyses of sample K1J5 amplified the expected 2.8 kb of a near full length DNA A component of the Begomovirus sequence and an additional shorter fragment.

Complete nucleotide sequences of 34 DNA A components typical of Begomoviruses were determined in the Kenyan samples. Thirty three sequences ranged from 2770 bp to 2816 bp while one (K1J5) consisted only of 1416 bp and termed as a defective (Def) DNA. Phylogenetic analyses indicated that the defective molecule belongs to geminiviruses involved in CMG, representing a Def from DNA A of the bipartite Begomovirus ACMV. All viruses characterized in this study grouped with two previously identified Begomoviruses found in cassava in Western Kenya, namely EACMV – UG and ACMV. The Def DNA showed 96.6% sequence identity with the ACMV reference sequence [GenBank NC001467.1].

In this study, we report for the first time the detection of Begomovirus: ACMV and EACMV – UG in *Jatropha* from Kenya. From an evolutionary perspective, the phylogenetic data indicate that the virus isolates from the study were closely related to those isolated previously in Western Kenya from cassava [4,5]. Recombination and synergism that have long occurred in cassava [6,7] could have led to the recent spread of the virus in the field to infect *Jatropha*. Presence of EACMV – UG and ACMV on different *Jatropha* plants in the same field indicates the opportunity for mixed infections, hence offering good opportunities for more recombination to occur. EACMV – UG and ACMV are associated with severe synergistic epidemics on cassava. Synergism lead to a 10 - 50 fold increase in viral DNA accumulation which substantially increases the potential for a higher efficiency of vector transmission to even infect non cassava host plants [6,7]. This explain why EACMV - UG is the predominant virus in *Jatropha*. The deletions occurring in the Def DNA found in the study might affect the replication of the molecule and it might depend entirely on its helper virus for replication.

There is a possibility of *Cassava mosaic virus* in *Jatropha* being more wide spread than anticipated, since we have detected it also in *Jatropha* samples from Ethiopia.

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Characterization of responses to flooding and post – flooding recovery in two *Populus deltoides* clones: physiological and biochemical aspects

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Background: In Argentina, most poplar plantations are located at the Paraná River Delta. Climate change models indicate an increase of flooding events for the next decades in this area. Therefore, poplar genotypes with a higher flooding tolerance will be needed for the forest activity. Whether this aim is achieved through traditional breeding or biotechnological developments, it will be important to gain a better understanding of poplar responses to flooding at physiological, biochemical and molecular levels.
In a previous work, we characterized the growth and morphological responses of several poplar clones to 35 days of flooding of the root system [1]. From this group, we selected two *Populus deltoides* clones with different growth responses to flooding: Alton and Stoneville 67. In clone Alton, above ground growth is not affected by flooding, while in Stoneville 67 growth is reduced after the second week of flooding. In the present work, our aim was to identify mechanisms underlying the differences in growth responses to flooding between the two clones. We characterized physiological and biochemical responses in roots and leaves, during a flooding period of 28 days, followed by an after flooding period of three weeks.
Materials and methods: Two *Populus deltoides* clones were used, Alton and Stoneville 67. Plants were obtained from 20 cm length one year old cuttings. The cuttings were planted in 3.5 L pots filled with a mix of topsoil and sand 50:50, and grown in a greenhouse in a totally randomized design; pots were watered daily to field capacity until the start of the treatment. The flooding treatment was started when the plants reached 50 cm of height. Flooding was imposed by covering the pots with tap water up to 5 cm over the surface soil, during 28 days, after this period flooding was ended and pots previously flooded were removed from the water and left to drain.
Growth in height was measured weekly with a graduate stick. Stomatal conductance was measured in the last expanded leaf with a Decagon SC1 porometer. Electrolyte leakage was measured in root tips. Samples were taken from leaves and roots to measure oxidative damage by thiobarbituric acid reactive substances (TBARs) method and enzymatic and non-enzymatic antioxidants. Leaf discs were taken from the last expanded leaf to measure protein content using the Bradford method. This destructive sampling was carried on in the following dates: day 0 (before the start of flooding); 2 weeks after the start of flooding, 4 weeks after the start of flooding; 1 day after the end of flooding, and 1, 2 and 3 weeks after the end of flooding.
Results and discussion: After 28 days of flooding, growth in height was reduced 14% in flooded plants of Stoneville 67 compared with non-flooded control plants, but it was not affected in Alton. This was a lasting difference; three weeks after the end of flooding the height of previously flooded Stoneville 67 plants still was 15% lower than the control plants.
Stomatal conductance was reduced by flooding in both clones, but in Stoneville 67 to a greater extent: 81% compared with only 63% in Alton. The recovery of the stomatal conductance to the control levels after flooding was slow and occurred only after two weeks, and was faster in Alton than in Stoneville 67.
Protein soluble content per unit leaf area of the last expanded leaf increased in flooded plants of both clones, in Alton the increase was two fold, and 2.5 times in Stoneville 67, 28 days after the start of flooding. This increase is due to changes in the structure of the leaves of flooded plants; these leaves have a lower area, and a higher number of stomata and epidermal cells than the control leaves per unit area (Rodriguez et al. unpublished results). Likely, these alterations in the leaf structure will have effects in the photosynthetic activity of leaves.
In several species, the sudden exposure of tissues to oxygen after a period of anoxia can cause oxidative stress; this damage is known as post-anoxic injury [2]. We measured electrolyte leakage to estimate membrane damage. Electrolyte leakage was measured in roots, since these were the organs that were submerged and experienced lack of oxygen. The value was higher in Stoneville 67 than in Alton after 28 days of flooding, but there were no differences between clones or treatments 24 h after the end of flooding and thereafter. Since electrolyte leakage is a general measurement of membrane damage, it cannot be attributed solely to oxidative stress. Therefore, we are measuring TBARs content to confirm if this damage is caused by oxidative stress.
As a preliminary conclusion, it seems that clone Alton is less sensitive to flooding than Stoneville 67 because its leaf stomatal conductance and root membrane integrity are less affected by flooding, and its recovery after the end of the stress period is faster.

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P95

Molecular integration of light responses in *Arabidopsis*

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Growth habit and developmental phase transitions in plants are acutely responsive to the quantity and quality of incident light. Information about neighbouring plants is primarily perceived by changes in the light environment. Arabidopsis is able to distinguish these changes through the phytochrome (PHYA-E) red light receptors that perceive changes in the red to far red (R:FR) ratio, and the cryptochrome and phototropin blue light receptors. Phytochrome, in its biologically active far-red light-absorbing form (Pfr), mediates its effects by directly binding with the basic helix-loop-helix (bHLH) phytochrome-interacting factors (PIFs). High R:FR ratios initiate phytochrome (Pfr)-PIF binding and PIF protein degradation. Low R:FR ratios photo-convert Pfr into the inactive form Pr

Figure 1(abstract P95)

allowing the gene expression that leads to a range of adaptations, including the shade avoidance responses. PIF1 regulates the expression of a number of genes by directly binding to a G-box element in their promoter. T-DNA insertional mutagenesis of a number of these genes results in pleiotropic effects associated with an altered capacity to respond appropriately to environmental cues, including those signalling changes in soil water status and the ambient light environment. The data show evidence of a perturbation of light regulated response processes, including germination, the shade avoidance responses, stomatal conductance, flowering and the circadian clock perturbation in the PIF-regulated gene mutants. The *pif1,3,4,5* quadruple and one of the PIF-regulated gene mutants fail to show shade avoidance responses in older plants grown under shade inducing conditions. This suggests that the encoded proteins mediate a parallel pathway in the induction of the SAS. The implications of our findings for an understanding of how plants appropriately respond to changes in their environment will be discussed.

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Expression profiling of putative *Eucalyptus grandis* defence marker genes in response to treatment with methyl jasmonate and salicylic acid

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Background: *Eucalyptus* species and their hybrids encompass approximately 40% of forestry plantation area in South Africa and contribute significantly to the paper pulp industry due to their favourable wood fibre properties. Eucalypt plantation trees are affected by numerous pathogens during their lifetime, some of which can cause severe losses such as *Phytophtora* spp and *Chrysoporthe* spp. Plant defence mechanisms against pathogens is currently better understood in the model plant *Arabidopsis thaliana* where it has been shown that the salicylic acid (SA) and jasmonic acid (JA) signalling pathways enhance resistance against biotrophic and necrotrophic pathogens, respectively [1] . This process involves the up-regulation of specific defence genes which are considered to be marker (diagnostic) genes for the two signalling pathways [2,3].

Methods: The aim of this study was to utilize the draft (8X) *E. grandis* genome sequence that has recently become available (http://eucalyptusdb.bi.up.ac.za) to identify *Eucalyptus* orthologs of defence marker genes (e.g. *PR2*; *PR3*; *PR4*; *PR5*; *LOX2*) specific for the SA and JA signalling pathways [2,3]. Bioinformatics tools were used to identify putative orthologs of these marker genes in *E. grandis* based on sequence information from other plants. This was followed by a co-phylogenetic analysis in which a neighbour-joining tree with 10 000 permutations was constructed to add confidence that the correct orthologs had been identified. In the phylogenetic tree analysis, closely related family members of a particular gene were added to increase the certainty and accuracy of selecting a specific ortholog.

The expression profile of the putative marker genes was assessed via Reverse Transcription quantitative PCR (RT-qPCR) analysis of transcript levels following treatment with various concentrations of the inducers (SA and JA) as well as different time points. This was done to confirm that the putative orthologs respond to the appropriate pathways in *Eucalyptus*. Additionally the expression profile of these putative orthologs was analyzed in response to the causal agent of *Eucalyptus* stem canker, *Chrysoporthe austroafricana*. The defence response of *Eucalyptus* to this nectrotrophic pathogen was investigated in both a tolerant (*EgrTOL*) and

Table 1(abstract P96) Selected results from the dose response and specificity trial

	SA (5mM)		MeJA (100μM)	
Marker	**Exp Ratio***	**P – value**	**Exp Ratio***	**P – value**
PR2	4.05	0.01	-0.34	0.28
PR3	-4.09	0.03	0.9	0.05
PR4	1.3	0.25	1.5	0.00005
PR5	0.5	0.3	1.6	0.09
LOX2	-4.6	0.04	0.5	0.17

*Expression ratios are represented as LOG2 values relative to the control samples.

susceptible (*EgrSUS*) species. Changes in the level of transcript expression of the putative marker genes (*PR2; PR4; PR5; LOX3*) were assessed at three time points using RT-qPCR.

Results and discussion: A dose response experiment of the putative marker genes was conducted with various concentrations to elucidate which would elicit the most paramount response in gene expression. It was found that amongst the tested candidates, 5mM and 100μM displayed the most significant change in gene expression for SA and JA respectively (Table 1). The specificity of the putative markers was also determined by profiling the putative marker genes with material induced by the opposing pathway, i.e SA markers were assessed with MeJA induced material.

A time course experiment was done to investigate how the expression profiles of the genes respond over a period of time. This would shed light on a possible window period that one could focus on for enhancing resistance as the timing of defence is crucial in determining the outcome of a pathogen interaction. For example the *PR2* gene, a marker for the SA pathway, was shown to be drastically induced at 24hrs followed by a decline at 48hrs. This could be due to the fact that high levels of SA are toxic to the cell so the plant needs to closely monitor SA levels. On the other hand *PR4,* a marker for the JA pathway displayed a gradual increase over time beginning at 6hrs and peaking at 48hrs.

When the putative marker genes were assessed in tissue infected with *Chr. austroafricana,* it was observed that role of SA could potentially have a crucial role in determining the outcome of the infection. It is interesting to note that at two weeks there is no significant difference in lesion length between *EgrSUS* and *EgrTOL*. In *EgrTOL*, the expression level of *PR2* was significantly up-regulated at two weeks post-inoculation whereas *EgrSUS* had significantly altered levels of expression only at six weeks. However in *EgrSUS*, the level to which PR2 is induced is still lower than in *EgrTOL*. At two weeks and six weeks there is an increase in *PR4* transcript levels in *EgrSUS*. This could explain the inability of *EgrSUS* to accumulate SA due to the antagonistic relationship between the two pathways which is in accordance to what is currently known in Arabidopsis [1].

Conclusion: The genes identified in this study were tested as a diagnostic tool for the screening of pathogen challenged eucalypt plant to determine which signaling pathway(s) were playing a role in defence against various pathogens. It was found that SA could potential play a role in enhancing resistance to *Chr. austroafricana*. Future work would involve studying the expression profile of these genes in response to various other pathogens as well as to elucidate more putative marker genes. This research provides a platform from which to expand our knowledge of plant defence in *Eucalyptus* and work towards curbing tree diseases.

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Investigating *Eucalyptus* – pathogen and pest interactions to dissect broad spectrum defense mechanisms

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Background: *Eucalyptus* species, hybrids and clones are attacked by various fungal and bacterial pathogens and pests during their life-time. Global climate changes are predicted to create favourable environments for such pathogens and pests and increase incidence of host jumping from other crops, resulting in increased losses to the forestry industry [1]. The use of tolerant or resistant plant varieties as part of an integrated disease management strategy is recognised as a desirable means to curb disease incidence. Vertical resistance mediated by resistance (*R*) genes, may be easily overcome by a pathogen and is thus not adequate on plantation species such as *Eucalyptus*, which would be exposed to various pathogens during its life-time. Broad spectrum resistance on the other hand, would be desirable to provide resistance against multiple challenges [2].

The aim of this study is to investigate mechanisms involved in host resistance with an emphasis on broad-spectrum resistance. The availability of the complete genome sequence of *Eucalyptus grandis* (http://www.eucagen.org) and the transcriptome sequence of a *E. grandis* X *E. urophylla* (GU) hybrid [3] has provided resources to investigate defense responses in the natural host. When a pathogen attacks a plant, the plant launches a sophisticated defense response involving phytohormones such as salicylic acid (SA), methyl jasmonate (MeJA) and ethylene (ET). These responses are finely tuned and tailored to the invader [4]. Down-stream of the signalling cascade is the production of pathogenesis related (*PR*) genes and antimicrobial genes which serve to limit the pathogen and afford protection. *PR* genes, such as *PR-1* and *PR-5,* are known markers of the salicylic acid defense pathway, while *PR-3*, *PR-4* and the lipoxygenase (*LOX*) genes are known markers of the MeJA and ET signalling pathways. The discovery of *PR* genes in *Eucalyptus* is desirable as these genes have previously been shown to afford broad spectrum resistance in other crops. We present our progress in exploiting the *Eucalyptus* genomic and transcriptomic data for the discovery of tree defense genes and explore the application thereof in determining which pathways are activated in response to various pathogens.

Materials and methods: Infections and infestations: *E. grandis* clones were treated with *Chrysoporthe austroafricana* in the following manner: wounds were created using a 0.3cm cork borer to expose the cambial tissue and an agar plug containing fungal mycelia were applied to the wound and sealed with parafilm. As a control, plants were mock inoculated. *E. nitens* plants were inoculated with *Phytophthora cinnamomi* using a 0.3cm cork borer, and a mycelial plug applied. The wound site containing the pathogen was sealed with moist cheese cloth and parafilm. A set of plants received no inoculumn. *E. grandis* clones were maintained in the FABI nursery and were naturally infested with *Leptocybe invasa*. A set of plants were maintained under similar conditions but were not exposed to the insect pest.

Expression analysis: RNA was harvested from from stem tissue (from *C. austroafricana* and *P. cinnamomi* interactions) and leaf tissue (for *L. invasa* interactions) at two time points after challenge in order to detect early and late responses. RNA was isolated using the CTAB method [5] and cDNA synthesized. Reverse transcriptase quantitative PCR (RT-qPCR) was performed using the Roche 480 LightCycler instrument.

Results and discussion: Reliable pathosystems were established for *Eucalyptus* with *P. cinnamomi* and *C. austroafricana*. Figure 1A and 1B show the lesions which developed after pathogen challenge compared to mock inoculated plants. Figure 1C shows the results of natural infestation of young leaves of *E. grandis* clones under nursery conditions compared to plants not exposed to the pest. The oviposition sites are evident on the leaf midrib (indicated by the white arrow).

Using a bioinformatic and phylogenetic approach, the putative orthologs for *PR-1*, *PR-2*, *PR-3*, *PR-4* and *PR-5* were identified. The basal expression level of *PR-3* in the GU hybrid transcriptome is indicated in Figure 2 as an example.

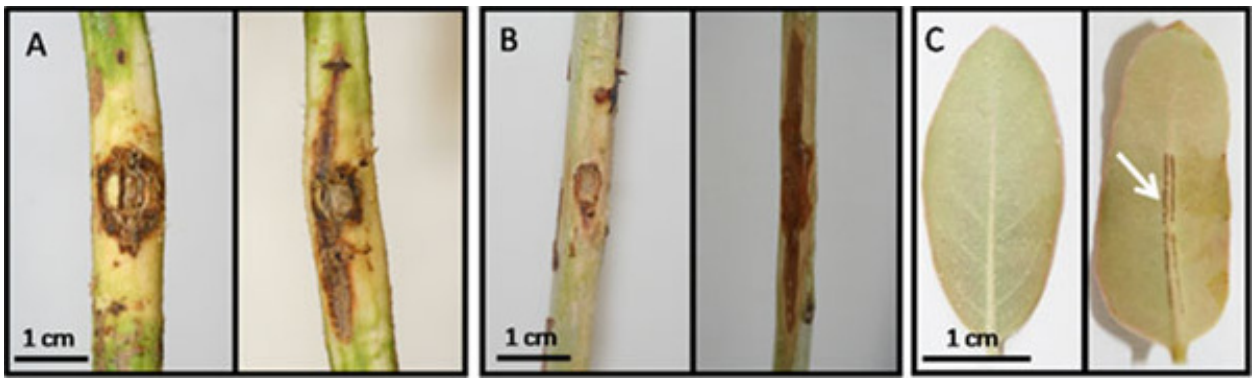

Figure 1(abstract P97) Results of infections and infestations on *Eucalyptus* species and clones. A) *E. nitens* challenged with *P. cinnamomi*, B) *E. grandis* clones challenged with *C. austroafricana* and C) *E. grandis* clones infested with *L. invasa*. Control, unchallenged plant material is indicated on the left and challenged plant material is indicated on the right of the respective panels.

The high basal expression of *PR-3* in the GU hybrid may indicate that the MeJA and ET pathway is activated in this genotype. *PR-3* genes are chitinases, enzymes that are able to hydrolyze chitin, a component of fungal cell walls [6]. Further expression profiling of the diagnostic marker genes for the two main defense pathways during *C. austroafricana* challenge suggests that the SA pathway is important for defense against the pathogen in the tolerant interaction.

Conclusions: We have established important pathosystems between *Eucalyptus* and a fungal pathogen, *Eucalyptus* and an oomycete pathogen and between *Eucalyptus* and an insect pest. Genes diagnostic of the main defense signaling pathways have been identified and are being exploited to determine which pathways are activated in tree-pest/pathogen interactions. It is expected that transcriptome sequencing of each of these interactions will not only reveal the suite of genes important for defense against specific pathogens and pests, but the overlap of the responses at the molecular level, would be informative for broad spectrum resistance. These genes are potential future candidates for genetic improvement of disease tolerance in eucalypts.

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P98

Identification of insect-specific target genes for development of RNAi based control of the *Eucalyptus* gall pest *Leptocybe invasa* Fisher & La Salle (Hymenoptera: Eulophidae)

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Background: *Eucalyptus* is grown in around 3.9 Mha in India. *Leptocybe invasa* Fisher & La Salle (Hymenoptera: Eulophidae) has emerged as a serious pest in *Eucalyptus* causing considerable loss of quality planting materials besides loss in productivity and quality of timber. Current strategies for its control include selected deployment of *Eucalyptus* clones tolerant to the pest resulting in several productive *Eucalyptus* genetic

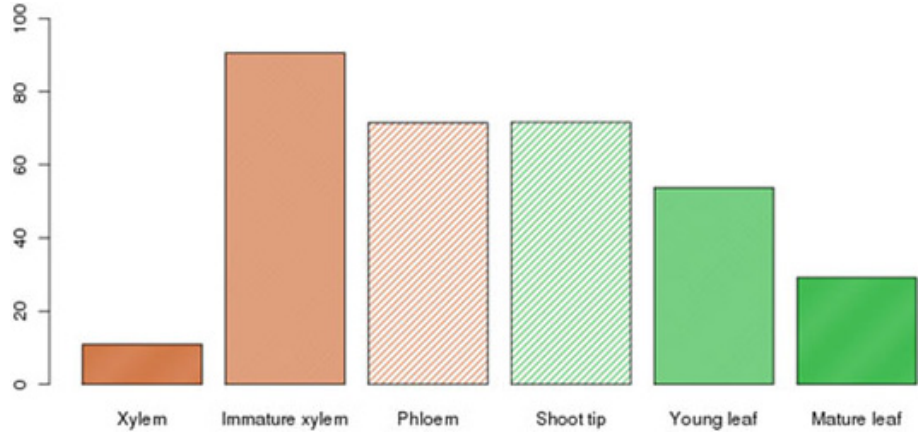

Figure 2(abstract P97) Relative expression levels of the putative *Eucalyptus PR-3* ortholog in various tissues of *E. grandis* x *E. urophylla* (data from Eucspresso [3]).

resources, not being considered for plantation programmes, as in the case of the widely planted *Eucalyptus* clone, ITC 10, and ITC 271. The securely ensconced grub within the gall for a period of around 4 months post oviposition makes it refractory to pesticide applications. This feature, however, makes plant- incorporated protectants, via RNAi approaches a potential strategy for engineering resistance [1-3]. However, application of RNAi technology requires determination of sequence information of insect-specific genes so that off-target effects in plants as well as human beings are avoided.

Methods: Grubs were collected from the galls of *E. camaldulensis* clones, APNP 1.1 and ITC 351, infested with *L .invasa* from the nursery of the Institute of Forest Genetics and Tree Breeding, Coimbatore, India. Genomic DNA was isolated from the grubs homogenized in liquid nitrogen using a modified CTAB protocol [4]. RNA was isolated from the grubs using Qiagen RNeasy plant mini kit, followed by cDNA synthesis using SmartScribe reverse transcriptase.

Grubs were also directly used for PCR amplification circumventing DNA isolation. Furthermore, to enable multiple PCR analysis, a single grub was collected into 50 µl of sterile distilled water, vigorously vortexed for 2 min followed by brief denaturation at 95^{0}C for 10 min and centrifugation at 10,000 rpm for 10 min. The supernatant was used for PCR analysis.

The PCR mix consisted either 100 ng genomic DNA or 100 ng cDNA or a single grub or 2 µl of supernatant from denatured grub, in 1X PCR buffer, 0.8 mM dNTPs, 1 µM of each primer, 2.5 U *Pfu* polymerase (Fermentas). The PCR conditions used were initial denaturation at 94^{0}C for 5 min, cycle denaturation at 94^{0}C for 40s; annealing at 60^{0}C for 40s; extension at 72^{0}C for 2 min for 30 cycles, and a final extension of 72^{0}C for 5 min. In case of grub PCR, initial denaturation was for 10 min at 95^{0}C. The PCR products were resolved on a 1.0% Agarose gel in 1X TAE. When multiple bands were obtained, the products were gel eluted and either directly sequenced or cloned into pGEMTeasy vector prior to sequencing. Sequencing was done using the ABI PRISM 3130 XL Genetic Analyzer using Big Dye Terminator version 3.1" Cycle sequencing kit through the commercial sequencing services available at Chromous Biotech India Pvt Ltd.

Results and discussion: Chitin synthase gene was partially amplified using the degenerate primer sets, CSF3 (5'-TGYGCGACHA TGTGGCACGARAC-3')/ CSR1 (5'-GTCCTCSCCYT GRTCGTAYTGCAC-3') and CSF1 (5'-YTGGAY GGMGACATMGAYTTC-3')/ CSR1 (5'- GTCCTCSCCYTGRTCGTAYTGCAC-3') [5] from genomic DNA and cDNA. CSF3/R1 primers yielded multiple bands of which the expected 1KB size amplicon was sequenced. CSF1/R1 primers yielded an amplicon of 370 bp, which overlapped with the sequence from CSF3/R1. The sequence data obtained were aligned, annotated, and a sequence of 624 bp was submitted to NCBI (Accession number: JF772551/ to be published). The sequence comprising 2 exons and 2 introns, corresponded to 138 amino acids on conceptual translation. BLAST analysis of the nucleotide sequence showed 85% homology to *Nasonia vitripennis* chitin synthase 1 and 76% to *Apis mellifera* krotzkopf verkehrt (chitin synthase1) sequences. Furthermore, a rapid method of PCR amplification directly from grubs was developed. The amplicons generated using the primers CSF1/CSR1 yielded the same sequence, indicating its utility for rapid PCR screening of *L. invasa* grubs. In order to identify housekeeping genes for use as reference genes during quantitative PCR analysis, Elongation factor-1 alpha (EF-1 alpha) was partially amplified using primers EF1-F (5'-GGTATCGACAAA CGT ACCATCG-3') /EF1-R (5'- AATCGAGCACAGG TGTGTAACC-3') and the sequence data of 534 bp was submitted to NCBI

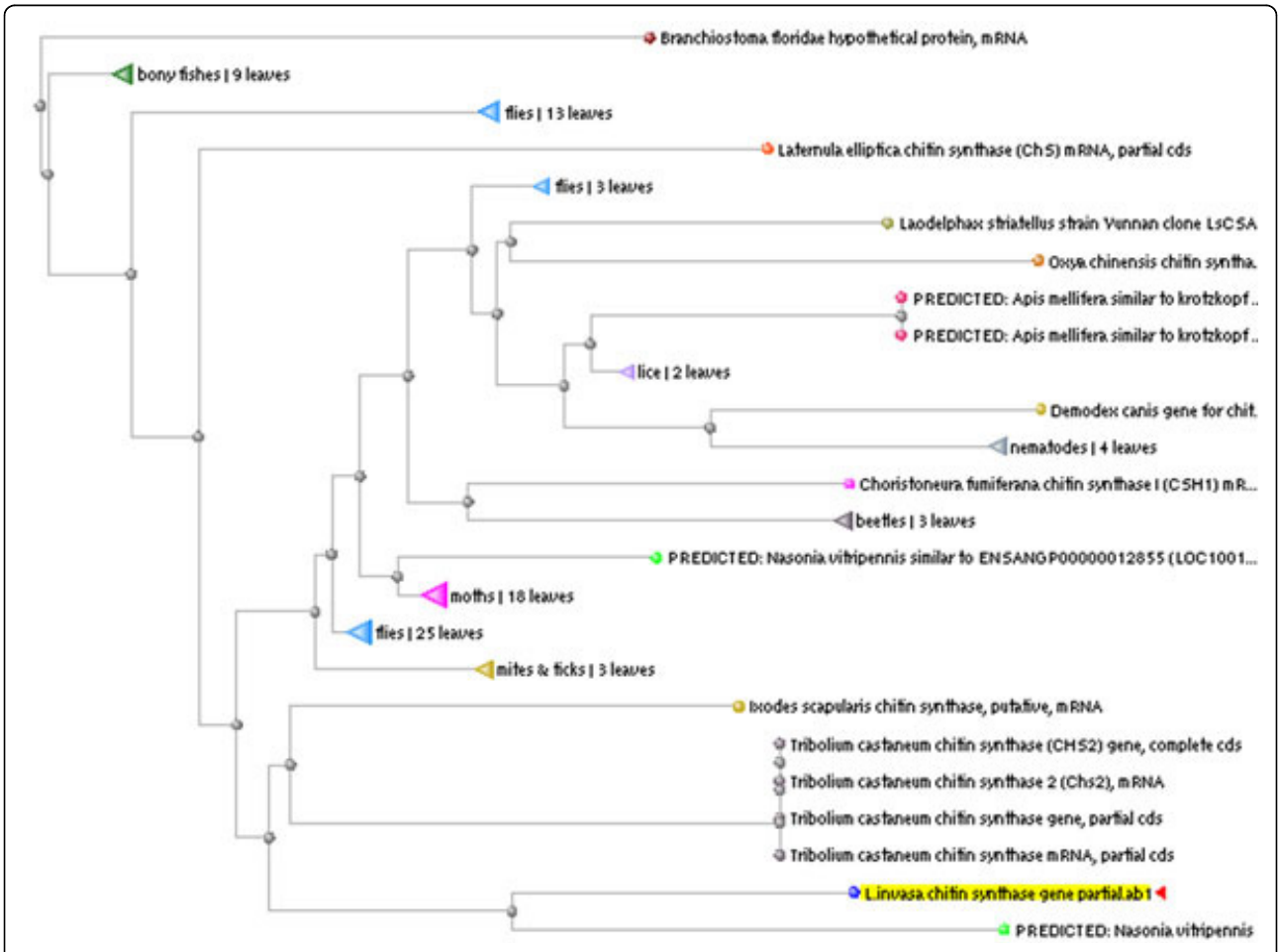

Figure 1(abstract P98) Tree view produced using BLAST pairwise alignment for the partial sequence of *L. invasa* chitin synthase1 gene.

(Accession number: JF772552/to be published). Blast analysis of this sequence showed 91% similarity to *N. vitripennis* EF-1 alpha.

Conclusion: Basic techniques like nucleic acid isolation and PCR amplification were adapted for this less studied insect pest. A rapid method for PCR amplification directly from the insect grub was developed for use in *L. invasa*. Our efforts have unraveled the partial gene sequence of *L. invasa* chitin synthase, a processive enzyme involved in chitin biosynthesis during different stages of insect development. The sequence of chitin synthase and EF-1 alpha represent the first genome sequence information for *L. invasa*. Chitin synthase offers a potential target for RNAi based pest control owing to its crucial role in growth and development of insect. Following full length sequence determination of *L. invasa* chitin synthase gene, RNAi target regions will be chosen for development of RNAi constructs. EF-1 alpha will be used as the reference gene in RT-PCR studies to quantitate the effect of RNAi on the transcript levels of the target gene. The effect of *Eucalyptus*-expressed dsRNA molecules cognate for chitin synthase, will be evaluated on the growth and development of *L. invasa*. Towards this end, protocols have been optimized for generation of transgenic *Eucalyptus*[6].

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Gene expression responses of black spruce (*Picea mariana*) to global climate change conditions

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Global climate change conditions (elevated CO_2 and atmospheric temperatures) are subjecting our forests, especially Boreal and temperate forests, to significant abiotic stresses, such as drought. This can affect health, productivity and fitness of our forests. Therefore, it is imperative to understand genomic and eco-physiological responses of forest trees to global climate change. We are addressing this aspect in black spruce (*Picea mariana*) - a transcontinental, ecologically and economically important tree species of the North American Boreal forest. Our objective was to determine gene expression and physiological responses and their inter-relationships in black spruce to elevated CO_2, drought and co-stressed conditions.

We have used NGS whole transcriptome sequencing, cDNA-AFLP and qPCR analyses to identify, annotate and characterize genes expressed differentially in response to elevated CO_2, drought and combined elevated CO_2 and drought conditions in black spruce using the cloned material. Photosynthetic rate and stomatal conductance were measured simultaneously with tissue collection for RNA extraction. Thousands of transcripts (genes) showed differential expression (no expression, up-regulation or down-regulation) in response to elevated CO_2, drought and/or their combined conditions, with over 1600 genes from several pathways showing >10-folds gene expression differences between control and treated plants. A number of genes showed 100 to 500 folds up or down regulation in response to elevated CO_2, drought or their combined conditions. Responses to each treatment at the gene expression and physiological levels were correlated well among different genotypes. We will present these results which contribute significantly to our understanding of tree's responses to global climate change.

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Oleoresin yield and carbon stocks in tapped subtropical *Pinus elliottii* forests

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Background: Low-cost methods to mitigate the increasing levels of carbon dioxide in the atmosphere and their implications on global climate change have received considerable attention in the last years [1,2]. Afforestation is an important alternative to reduce the rise in atmospheric CO_2 concentration due to the system's ability to fix carbon in forest biomass and soil [3]. Several studies have been performed to estimate carbon sequestration in temperate, tropical, and mediterranean forests ecosystems [4,5]. However, there are no reports related to carbon balance in pine forests used for oleoresin tapping grown under subtropical climate. The main goal of this research is to estimate the effect of resin tapping on C sequestration by *Pinus elliottii* forests.

Material and methods: Resin tapping operation: In June 2009, 90 slash pine (*Pinus elliottii* Engelm.) trees of a 14 year-old forest were selected based on a previously determined DBH interval (between 23.48 ±1.12 and 22.77 ±0.88). Since the beginning of the essay, pine trees have been biweekly stimulated to produce oleoresin [6]. Three treatments were evaluated for pine biomass increase and oleoresin yield: bark streak (mechanical wound), paste (mechanical wound + chemical stimulation) and control (intact trees). At the end of each season, the released oleoresin was collected and weighed in a digital balance. The results shown below were obtained between Spring 2009 and Winter 2010.

Biomass production and carbon accumulation: In November 2010, fifteen pine trees (5 intact tree control, 5 mechanically wounded, and 5 paste stimulated tapped trees) were felled and weighed in the field to estimate the above and belowground total fresh biomass of trees. Sub-samples of each part of trees were collected and dried in an oven at 105° C until reaching constant dry weight. The carbon content per unit dry weight present in the aboveground and belowground biomass was estimated using a carbon content of 50% [7]. Data were analyzed for differences between resin treatments (bark streak vs. paste) by comparison of means by ANOVA and Tukey test (Systat Software Inc., Richmond, CA USA); significance was set at P £0.05).

Results and conclusions: The seasonal oleoresin production did not show the same pattern previously observed [8]. The highest oleoresin yield was observed in Spring (data not shown). Statistical differences between oleoresin yields of paste-treated and control (bark streak) trees were observed (Figure 1). These results are in agreement with the fact that besides genetic traits, physiological status, season and environmental conditions [9-11], inducible oleoresin biosynthesis consistently responds to exogenous chemical stimuli [6,8].

The production of aboveground biomass expressed on a dry weight basis was significantly higher than that of belowground biomass. This was observed for all treatments, and no significant differences were detected in biomass production and partition for tapped (both plain wound and chemical stimulation) or control trees (Figure 2). The aboveground biomass

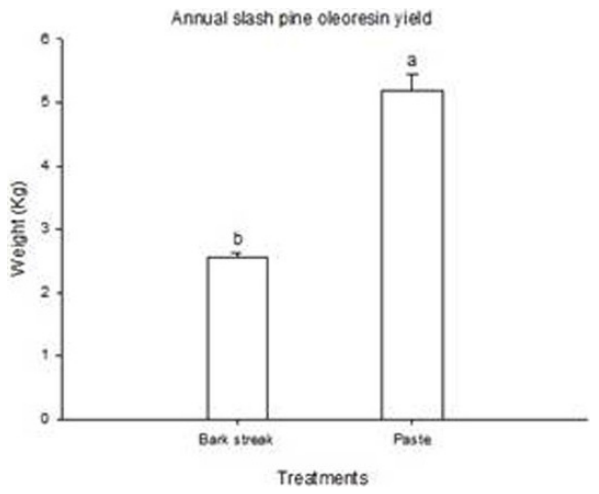

Figure 1(abstract P100) Annual production of oleoresin by pine trees. Standard errors of the means are indicated on top of bars. Bars with different letters indicate significant difference by a Tukey test ($P \leq 0.05$). Each mean was calculated with 30 individual trees.

showed a carbon accumulation between 221.3 and 235.9 Mg C ha^{-1} and in the roots, the carbon accumulation is in the range between 35.7 and 48.3 Mg C ha^{-1}.

These results indicate that carbon stored in aboveground biomass appeared to represent the main carbon pool of tree biomass and that resin tapping had minor impact on C allocation in wood biomass, considering the time frame and stand age examined. Further studies and sampling times are ongoing in order to better characterize carbon stocks in subtropical slash pine plantations and to elucidate the contribution of oleoresin production in carbon stocks and its relation with C in wood biomass.

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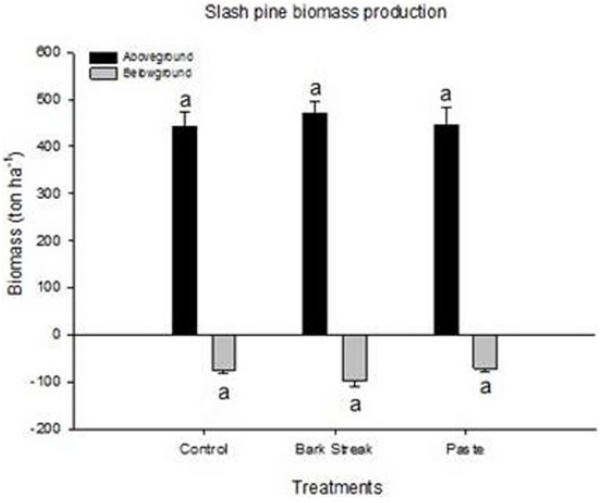

Figure 2(abstract P100) Aboveground and belowground biomass among the treatments (control, bark streak and paste). Standard errors of the means are indicated on top of bars. Each mean was calculated with five individual trees. Identical letters indicate no significant difference by a Tukey test ($P \leq 0.05$).

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P101

Tree-insect interaction - defence response against herbivorous insects

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Background: The defoliation of oaks is an urgent problem for forestry in Central Europe. During the last outbreak of the green oak leaf roller(*Tortrix viridana*) in 2003-2005, we observed fundamental differences in the defoliation level of individual *Quercus robur* trees in Germany. Some of the trees seem to be somehow "tolerant" (T oaks) against the insects grub while some seem to be conspicuously "susceptible" (S oaks). Within this study we aim to identify the underlying molecular and biochemical mechanisms in oaks responsible for the behavioural preference of *T. viridana*. By means of combined behavioural experiments and biochemical as well as molecular analysis of preformed and induced defence mechanisms in T and S oak phenotypes, we will identify the metabolic/chemical basis of the observed differences as prerequisite for the selection of candidate genes differentially expressed in tolerant and susceptible trees, respectively, after insects feeding.

Methods: For this purpose we until now grafted each four of the identified T and S oaks, and used them for our further experiments. The methods used at this time are as following:

1. Establishment of a "bioassay": Larvae of the green oak leaf roller have been tested for feeding preferences on T or S oaks in a feeding choice experiment. Furthermore first olfactometer experiments with adult females were performed and the developmental performance of the larvae has been measured.
2. Preliminary analysis of emission pattern of plant volatile organic compounds (VOC) during the bioassay on T and S oaks has been done.
3. Biochemical analysis of phenolic compounds (e.g. tannins, soluble phenolic substances) and constitutive and inducible emissions of plant volatiles in T and S oaks during relevant stages of leaf ontogenesis and insect feeding and oviposition are ongoing
4. Transcriptom sequencing analysis of T and S oaks: Overall gene expression differences between the T and S oaks have been tested using biochemically defined leaf material harvested after the bioassay by next generation sequencing analysis of the complete mRNA (transcriptom). Candidate genes involved in the defence response of oaks will be identified and compared to differences in constitutive and induced patterns of phenolic compounds and VOC emissions.

Results: We will present preliminary results of the following aspects of our study:
1. Feeding choice experiments with larvae of *Tortrix viridana*: The larvae had the choice between a leaf from T-oak and a leaf from S-oak. After 24h, the amount of feeding at each leaf was documented. We found a significant preference of the larvae for the leaves from S-oaks.
2. Olfactometer experiments with adult females: Adult paired moth had the choice between a grafted T- and S-oak in an olfactometer. After 30 minutes the decision of the moth was documented. A highly significant number of females chose the S-oaks instead of the T-oaks.
3. Performance experiment: Larvae had been fed with leaves of either S- or T-oaks only. The larvae fed with T-leaves needed much more leaf material to end up with the same weight of pupae than the larvae fed with S-leaves.
4. VOCs: In an extensive experiment the volatile substances emitted by T and S-oaks during feeding of larvae of *T. viridana* were measured online. We found clear differences in the amount of e.g. sesquiterpenes emitted by S- and T-oaks. Furthermore the S-oaks seem to emit attractants which leads to a higher amount of larvae feeding on them.

Conclusions: We started the project with an observation, thus, we first identified phenotypes. Now, concerning the behavioural and biochemical results, we conclude that there are other factors than only environmental ones which lead to tolerant and susceptible oaks. There are physiological differences between S- and T-oaks and the identification of the candidate genes responsible for these differences is ongoing.
This approach will give us an insight into the functional genomics of *Q. robur* relating to the feeding of herbivorous insects. The question still remains unsolved whether the *Tortrix*-oak interaction is highly specific, or basics of the "*Tortrix* tolerance" can be transferred to other host-pathogen interactions. With the identification of molecular and biochemical markers of "*Tortrix* tolerance" in oaks we can contribute to decision support in sustainable forest management.

P102

Low temperature stress and changes in the lignin content and composition in *Eucalyptus globulus*

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It is known that many abiotic stresses, such as mineral deficiency, drought, UV-B radiation, wind and low temperatures, alter the quantity and composition of lignin in several species [1]. The aim of this work was to verify if the cold stress may cause changes in the quantity and composition of lignin in *E. globulus*. Additionally, we analyzed whether these changes can also be beneficial from the industrial point of view, that is, if this would allow a better extractability of lignin in this species, which is a desirable feature in the manufacture of paper. During the day, all plants were kept in a greenhouse at room temperature but at night, half of plants was transferred to growth chamber at 12 °C and half to 25 °C. These conditions were kept during 20 days. The stems of plants from the two groups were collected and analyzed for total lignin with thioglycolic acid [2] and submitted to thioacidolysis and GC-MS analysis for the determination of the monomeric lignin composition [3]. Part of the material was used to determine the digestibility of cellulose [4].
It was observed that cold reduced the accumulation of lignin in *E. globulus*. GC-MS analysis showed that the proportion of the S/G was reduced in plants subjected to low temperatures and it was also observed a lower digestibility of cellulose in these plants, indicating that this lignin could be more difficult to be removed in industrial processes of papermaking.
These results have been related with gene expression studies for the enzymes of the lignin biosynthesis pathway and may contribute to understand the processes controlling lignin deposition in eucalyptus.

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P103

Drought stress and changes in the lignin content and composition in *Eucalyptus*

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It is known that many abiotic stresses, such as mineral deficiency, drought, UV-B radiation, wind and low temperatures, alter the quantity and composition of lignin in several species [1]. The aim of this work was to verify if the drought stress may cause changes in the quantity and composition of lignin in *Eucalyptus globulus* Labill and in the hybrids *E. urograndis* (*E. urophylla* x *E. grandis*) and *E. uroglobulus* (*E. globulus x E. urograndis*). In the experiments the plants were divided in three groups (control, drought and drought recovered.) The control plants were irrigated daily. The plants from the group "drought" were not irrigated and were collected when wilt symptom was observed. The plants from the group "drought recovered" were irrigated when wilt was observed and were collected after recovery.
Samples of basal and apical regions of the stem were collected and analyzed for total lignin with thioglycolic acid [2] and analyzed by GC-MS to determine lignin monomeric composition [3].
E. urograndis subjected to drought decreased the amount of lignin in the stem apical regions and increased lignin in the basal region. *E. globulus* showed opposite behavior in apical regions and showed no significant changes in the basal regions. *E. uroglobulus* showed a pattern similar to *E. urograndis* in apical regions and similar to E. *globulus* in basal regions.
Although *E. urograndis* and *E. uroglobulus* reduced lignin and *E. globulus* increased in the apical part of the stem, it was observed that these different adjustments of lignin deposition eventually result in an increased proportion of S/G in both species. Moreover, when the amount of lignin is increased in the basal regions of *E. urograndis* there is a decrease in the proportion S/G.
Increasing the proportion S/G, either by increasing the amount of lignin-rich syringyl units or reduction of coniferyl units can be an important aspect in the adaptation of both species to drought stress.

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S6. WOOD FORMATION

P104

A novel Aaproach to increase cell wall saccharification for efficient biofuel production

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The conversion of biomass to biofuels requires heat, pressure and acid treatments to overcome the recalcitrance of the cell wall and remove lignin that hinders access to cellulose. Economic production of biofuels from lignocellulosic biomass and efficient sugar release depends on the ability to reverse and overcome this natural recalcitrance of the plant cell wall.

Most of the current ideas and methods to generate lignocellulose feedstock which is more amenable to saccharification, are limited to the modification or down-regulation of the lignin biosynthesis. This often results in dwarfing, xylem vessel collapse or reduced fitness to stress. In order to overcome these limitations, we present here a novel approach for cell wall remodeling. By introducing new trans-genes from algae, fungi and viruses we create **"Trojan Horses":** polymer pockets of enhanced solubility within the cell wall, thus increasing cellulose accessibility to solvents and enzymes. Two different methodologies are presented. The first includes transgenic introduction of soluble polysaccharides such as **hyaluronan** into the cell wall during its development, leading to its intercalation with the cellulose microfibrils. Upon processing, these soluble intercalated polysaccharides are washed away, resulting in highly porous cell walls with increased accessibility to hydrolytic enzymes. The second methodology involves modification of the cellulose by expression of recombinant **cellobiose dehydrogenase (CDH)** in plant cell walls, leading to disruption of the cellulose microcrystalline lattice and thus increasing hydrolytic enzymes accessibility. Both methodologies result in transgenic plants that exhibit **significantly enhanced cellulose hydrolysis**, while maintaining their physiological and structural integrity.

This novel concept provides new prospects for more economical production of liquid biofuel to substitute fossil fuels.

P105

Characterising the role of the *Eucalyptus grandis SND2* promoter in secondary cell wall biosynthesis

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Background: NAC and MYB transcription factors (TFs) have been shown to play prominent roles in the regulation of plant developmental processes. Two *Arabidopsis thaliana* NAC domain TFs (AtSND2, AtSND3) and one MYB domain TF (AtMYB103) were shown to be downstream targets of two master regulators of xylem fibre cell development, NST1 and SND1 [1,2]. These TFs were able to induce the expression of a GUS reporter gene under the control of the *AtCesA8* promoter [3], indicating that they may be involved in the regulation of cellulose biosynthesis in the secondary cell walls of *A. thaliana* xylem fibres. It is hypothesized that putative orthologs of these TFs will also play important roles in regulating fibre secondary cell wall biosynthesis in woody plants such as *Eucalyptus grandis*. The transcriptional network regulating wood fibre development is uncharacterized in *E. grandis*, therefore it would be beneficial to identify upstream components of this transcriptional network in *E. grandis*. In this ongoing study we aim to identify TFs which bind to the promoter of the putative ortholog of *AtSND2* in *E. grandis* (*EgSND2*) and possibly also to the promoters of the orthologs of *AtSND3* and *AtMYB103*. In parallel, we are characterizing the detailed heterologous expression patterns of the *EgSND2*, *EgSND3* and *MYB103* promoter regions in *A. thaliana* plants. This work forms part of an effort to elucidate the transcriptional network regulating wood fibre development in *E. grandis*.

Methods: A reverse BLAST approach was used to identify candidate orthologs of *AtSND2*, *AtSND3* and *AtMYB103* in *Eucalyptus*, *Populus* and *Vitis*. *In silico cis*-element analysis was performed on the promoter regions of the putative orthologs to identify previously characterised and novel *cis*-elements. The 1.5 kb regions upstream of the translational start site (TSS) of *EgSND2*, *EgSND3* and *EgMYB103* were isolated from *E. grandis* genomic DNA. The amplified fragments were cloned into pMDC162, a GUS reporter vector, and introduced into *A. thaliana* Col-O plants for heterologous GUS expression analysis. The same reporter constructs were also transformed directly into the vascular cambium of potted *E. grandis* plants to determine endogenous promoter activity by Induced Somatic Sector Analysis (ISSA, [4]). Qualitative GUS reporter analyses were performed on the *EgSND2promoter::GUS*, *EgSND3promoter::GUS* and *EgMYB103promoter::GUS* constructs in *Arabidopsis* plants at 1, 3 and 6 weeks. A 500 bp truncation upstream of the TSS of *EgSND2* was generated and cloned into the pHIS2.1 vector along with the 1.5 kb *EgSND2* promoter sequence for use in yeast one-hybrid (Y1-H) screening. Candidate proteins which bind to the cloned promoter sequences will be identified using Y1-H analysis and further characterised.

Results and discussion: A number of previously described (e.g. [5]) and novel *cis*-elements were present in the three cloned *Eucalyptus* promoters (*EgSND2*, *EgSND3* and *EgMYB103*)suggesting regulation by an overlapping set of upstream transcription factors. The 1, 3 and 6-week GUS analyses of the *EgSND2promoter::GUS* and *EgSND3promoter::GUS* constructs revealed strong GUS expression in vascular tissues, but the GUS expression was not specific to vascular tissues as reported for the endogenous *AtSND2* and *AtSND3* genes [3]. This suggests that the 1.5 kb region upstream of the translational start site may not be sufficient for fibre-specific expression in a heterologous system. Similarly, the *EgMYB103promoter::GUS* construct was expressed in stems and leaves, in contrast to strong stem specificity reported by Zhong et al., [3] for the *Arabidopsis* ortholog. ISSA (ongoing) results of endogenous expression in *Eucalyptus* may clarify the possible regulatory divergence in these promoter sequences. We hope to soon identify and functionally annotate a number of protein candidates binding to the *EgSND2* promoter using Y1-H analysis.

Conclusions: The *EgSND2*, *EgSND3* and *EgMYB103* promoters were found to contain common *cis*-regulatory elements, which suggests at least partial co-regulation in *E. grandis*. The 1.5 kb upstream regions of *EgSND2*, *EgSND3* and *EgMYB103* induced strong heterologous GUS expression in vascular and non-vascular tissues of *A. thaliana* suggesting either that these promoter sequences have functionally diverged in *Arabidopsis* and *Eucalyptus*, or that the 1.5 kb upstream regions alone are not sufficient to induce fibre-specific expression as previously reported in *Arabidopsis*.

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P106

Effects of nitrogen fertilization on global xylem transcript profiling of *Eucalyptus urophylla x grandis* evaluated by RNA-seq technology

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Background: Eucalyptus species are the most widely planted hardwood trees in the world representing more than 4.75 million ha in Brazil. Their high productivity, valuable wood properties and wide adaptability could allow sustainable and cost-efficient production of lignocellulosic bioenergy. The main limitation to this objective is wood recalcitrance to degradation which is linked to the structure and composition of lignified secondary cell walls. Lignin, for example, impairs the accessibility of cellulose during kraft pulping as well as during saccharification, a key step of bioethanol production.

The application of nitrogen fertilizers is one strategy to increase growth rates and productivity since nitrogen is one of the most limiting nutrient for tree growth and carbon sequestration. However, the effects of nitrogen availability on wood properties and related gene expression are poorly understood.

In poplar, it was recently reported that N fertilization increased aerial biomass, while in wood, fibre morphology and secondary cell wall structure and composition were modified. An increase in cellulose coupled with a decrease in lignin was observed and the mRNA profiles evaluated by microarray showed that nitrogen and tension wood have overlapping effects [1]. Moreover, a highly significant genetic correlation was observed between plant growth and lignin/cellulose composition. Quantitative trait loci co-localization identified the genomic position of potential pleiotropic regulators [2].

In order to get an insight on the regulation of nitrogen availability on wood formation in Eucalyptus, we have studied the effects of nitrogen fertilization on xylem transcriptome profiles using RNA-seq technology.

Methods: An experimental system was set up in which rooted cuttings of *Eucalyptus urophylla* x *grandis* were fertilized during 30 days with three different amounts of N (limiting, -N; adequate, CT; excess, +N). For the treatment with excess of N fertilization, we used two different nutrient solutions with different concentration of NO_3^- and NH_4^+.

Histochemical analyses were performed on stem transverse sections (80 μm thick) obtained using a Vibratome (LEICA VT 1000S). The Weisner reagent (phloroglucinol- HCl) was used in order to detect lignified cell walls, and calcofluor reagent to evaluate the cellulose content by fluorescence. The samples were observed in confocal microscopy (SP2-AOBS, Leica) and under bright-field microscopy (DM IRBE, Leica) coupled with a CCD camera (DFC 300 FX, Leica).

The construction of the RNA-seq libraries and sequencing were performed accordingly to Illumina's protocols. Prior to analyze the RNA-seq data, we have done an assembly of Eucalyptus ESTs (GENOLYPTUS and NCBI). The 53,412 unigenes produced were automatically annotated using BLAST (e-value cutoff of 1e-5) against different sequence databases.

The RNA-seq reads were aligned against the assembled unigenes using the SOAP2 aligner [3] configured to allow up two mismatches, discard sequences with "N"s and return all optimal alignments. In order to perform the differential expression analysis between libraries, a normalization and statistics pipeline were applied using DEG-seq software [4] considering 99% of confidence rate (cut-off value of 0.01).

Results and discussion: The intensity of staining with phloroglucinol was higher in stem sections from samples grown under -N treatment as compared to control, whereas it was lower in both +N treatments. This suggests that lignin biosynthesis is decreased in presence of excess of nitrogen fertilizer. We could also observe in the +N samples, an increase in cellulose staining intensity with the calcofluor reagent specially noticeable when comparing with -N samples suggesting that nitrogen also influence cellulose biosynthesis.

The RNA-seq analysis generated 123,121,154 sequence reads after filtering. Of the total reads, about 86 millions matched either to a unique (47,9%) or to multiple (21,8%) EST locations. Each nitrogen treatment was represented by at least 28.8 million reads, a tag density sufficient for quantitative analysis of gene expression.

The sequence reads were aligned on the new *Eucalyptus* EST assembly resulting in 36,125 unigenes expressed (15,293 contigs and 20,832 singlets). After statistics analysis, we determined 14,400 differentially expressed genes, that for a preliminary analysis were divided in two scenarios: the genes down-regulated in -N and up-regulated in both +N treatments (8,967 genes), and the genes up-regulated in –N and down-regulated in both +N treatments (5,433 genes).

To facilitate the global analysis for each scenario, gene ontology (GO) classification (http://www.geneontology.org) was performed and showed differences in some biological process categories. For instance, we observed that genes involved in the biosynthesis of cell wall main components (lignin, cellulose and hemicelluloses) were differentially expressed between the treatments. For example, some genes of the lignin biosynthetic pathway were up-regulated in the treatment with less nitrogen (-N) and down-regulated in both +N treatments.

Conclusions: This first analysis in Eucalyptus allowed us to show an effect of nitrogen fertilization on cell wall composition both at the histological and at the gene expression level. We believe that further experimentscompleted by chemical analysis of wood samples will give more insights in the mechanisms of nitrogen fertilization on wood formation. This knowledge will be important to address new demands on better suited biomass quality for industrial applications.

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In silico and functional characterization of the promoter of a *Eucalyptus* secondary cell wall associated cellulose synthase gene (EgCesA1)

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Background: Cellulose is an important biopolymer produced by all plants and is used in a number of different industries, including for pulp and paper production. Cellulose is deposited into the plant cell wall by a large membrane-bound protein complex, which is composed of different cellulose synthase (CESA) proteins. The cellulose content and pattern of deposition in plant cell walls is highly variable depending on the function of the cell. All plant cells have a thin primary cell wall, but a number of plant cell types, including xylem cells, also deposit a secondary cell wall to give these tissues mechanical strength required to perform their function. Different cellulose synthase (*CesA*) genes have been shown to be involved in the deposition of primary and secondary walls. In *Arabidopsis*, three *CesA* (*AtCesA4*, *7* and *8*) genes have consistently been associated with cells depositing secondary cell walls, while a different set of *CesA* genes have been shown to function during primary cell wall formation [Reviewed in 1]. These findings have been mirrored by studies of *CesA* gene orthologs in

Populus and *Eucalyptus*[2-4]. While there have been a number of studies on *CesA* genes and their functions, much less is known about the regulation of these genes. In a previous study, we investigated the promoters of *CesA* genes involved in primary and secondary cell wall formation by performing a phylogenetic footprinting analysis to identify cis-elements conserved in the promoters from orthologous *Arabidopsis*, *Populus* and *Eucalyptus* cellulose synthase genes [5]. We identified a number of putative cis-regulatory elements that may play a role in the regulation of cellulose biosynthesis during primary and secondary cell wall formation. In the current study our aim is to further validate the cis-elements identified in the *CesA* gene promoters by investigating their conservation across different *Eucalyptus* species and to determine the regulatory function of these promoter regions and the proteins which bind to them.

Methods: A number of different methods are being employed to investigate the regulatory functions associated with the *EucalyptusCesA* promoters. Firstly, to validate the cis-elements previously identified, we cloned and sequenced the promoters of six *CesA* genes from 13 different *Eucalyptus* species. The promoter sequences were analysed on the nucleotide diversity level. The cis-elements identified in the previous study were mapped onto the cloned promoter sequences and analysed for conservation in sequence and position. Next, we studied the possible roles of promoter regions harbouring conserved cis-elements in spatio-temporal regulation of *CesA* genes. We tested regions of the *EucalyptusgrandisCesA1* (*EgCesA1*) promoter for involvement in spatio-temporal regulation by cloning the full-length (2 kb) promoter and a series of truncates thereof upstream of the β-glucuronidase (GUS) reporter gene. These constructs were used to transform *Arabidopsis* (floral dipping) and *Eucalyptus* (Induced Somatic Sector Analysis, [6]). The GUS expression patterns were compared to the pattern produced by the 2 kb *EgCesA1* promoter. Finally, promoter regions identified as functionally active and harbouring conserved elements of interest are being used to screen a *Eucalyptus* immature xylem cDNA expression library for yeast-1-hybrid interactions to identify proteins which interact with these regions.

Results and discussion: Studying the diversity of *CesA* promoter sequences and cis-elements in 13 *Eucalyptus* species provided us with valuable insight into the relative conservation of specific promoters regions and the cis-elements within these regions. We found that the overall nucleotide diversity of the promoter sets varied greatly from promoter to promoter, but we could identify regions in the promoters that were as conserved as coding regions. We found that in many cases these localized decreases in nucleotide diversity corresponded to clusters of conserved cis-elements which were identified previously [5]. This was particularly noticeable at the transcriptional start site for most genes and in this region we noticed a repeat element in all of the promoters investigated. Some elements were also shown to be specific to either the primary or secondary cell wall associated promoters. The cis-element information obtained from this study was used to create seven truncates of the *EgCesA1* promoter. Using GUS expression analysis in *Arabidopsis* we identified a number of repression and activation sites within the promoter. We also observed a loss of leaf (vein) expression 800 bp upstream. One of the repeat elements fused to the 5'UTR greatly enhanced overall GUS expression in a non-specific way. In a *Eucalyptus* background (ISSA), six of the seven truncates showed xylem-specific expression, but the 5'UTR and repeat element fusion showed GUS expression in phloem and xylem. These regions have been used to construct bait vectors for Yeast-1-hybrid screening which is still ongoing.

Conclusion: In this study we addressed three main objectives, (1) investigate the evolution of the *EucalyptusCesA* promoters and cis-elements associated with primary and secondary cell wall formation, (2) investigate the expression patterns of truncated versions of the *EgCesA1* promoter using the GUS reporter system and (3) test the *EgCesA1* promoter regions affecting gene expression in a yeast-1-hybrid assay to identify possible regulators of this gene. We have identified regions in the promoter that were conserved and corresponded to previously identified cis-elements. Using this information we produced seven promoter truncates and discovered several regions and cis-elements in the *EgCesA1* promoter which affect GUS expression patterns. These results will aid in understanding and elucidating transcriptional networks regulating xylogenesis in woody genera such as *Eucalyptus*.

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P108

Generation and analysis of expressed sequence tags (ESTs) from cambium tissue cDNA libraries of contrasting genotypes of *Eucalyptus globulus* Labill

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In the present study, reported the generation and analysis of ESTs of cDNA libraries from cambium tissue of secondary xylem obtained of two genotypes of *Eucalyptus globulus* contrasting in wood density and pulp yield. The sequences were blasted and annotated to compare with genome and ESTs database of other plant species. The goal of study was to determinate wich genes was differentially expressed and compare levels of transcript, validated by qRT-PCR, in each genotype involved in wood formation, to explain the differences founding in wood traits of contrasting genotypes.Sequences obtained was 450,000 ESTs of which approximately 21,000 sequences showed homology with genes of different vascular plants, mainly *Vitis vinifera*, *Populus sp*, *Eucalyptus sp*, *Ricinus sp* . Moreover, it was determined that 265 genes differentially expressed in both genotypes, and 41 genes were directly involved in wood formation process (xylogenesis). Of the 41 differentially expressed genes could be determined that mainly correspond genes involved in lignin biosynthesis pathway which HCT, C3H, CAD, PAL, COMT and F5H and lignin polymerization like laccase and peroxidase. Otherwise we found genes involved in carbohydrate biosynthesis (cellulose and hemicellulose) among which Sussy, UDP glucose dehydrogenase, UDP-mannose dehydrogenase and β-xylosidase. Also we described genes involved in morphological characteristics of fiber such FLA, XTH and transcription factors such MYB and LIM related to fiber length, microfibrillar angle and extensibility of cell wall. Finally some of these genes were validated by the qRT-PCR technique to determine the level of transcripts in each genotype.

P109

Eucalyptus transcriptome analysis revealed molecular chaperones highly expressed in xylem

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Background: Plant development is very plastic, being coupled to environmental cues. As sessile organisms, plants must be able to respond

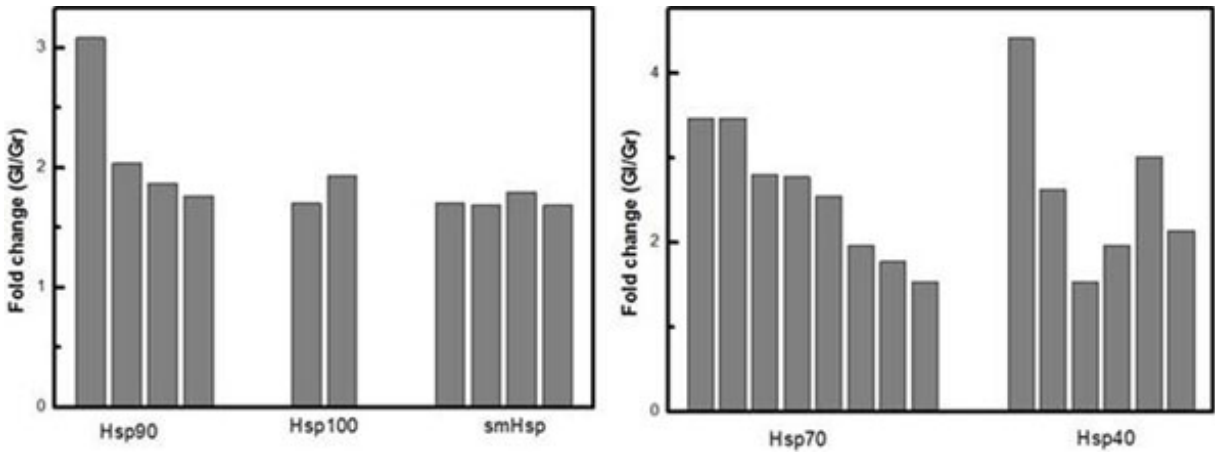

Figure 1(abstract P109) Molecular chaperones up-regulated in *E. globulus* xylem in comparison to *E.grandis* xylem.

rapidly to environmental stresses such as changes in temperature and salinity, heavy metals and water deficit. Efficient stress response systems are prerequisites for plant survival and productivity [1].

Molecular chaperones (or Heat Shock Proteins – HSP) compose a ubiquitous class of proteins involved in cellular protein quality control (PQC) and homeostasis. They play a critical role in folding and degradation of polypeptides, and therefore, in maintenance and modulation of cellular pathways, which are dependent of function (correct folding) and availability (stability and degradation) of involved proteins, under normal and stress conditions [2].

Genetics and proteomics studies of wood formation have highlighted some chaperones up-regulated in xylem of *Eucalyptus*, *Pinus* and *Populus* species, stating that they may play an important role in cell wall formation and xylem development [3,4].

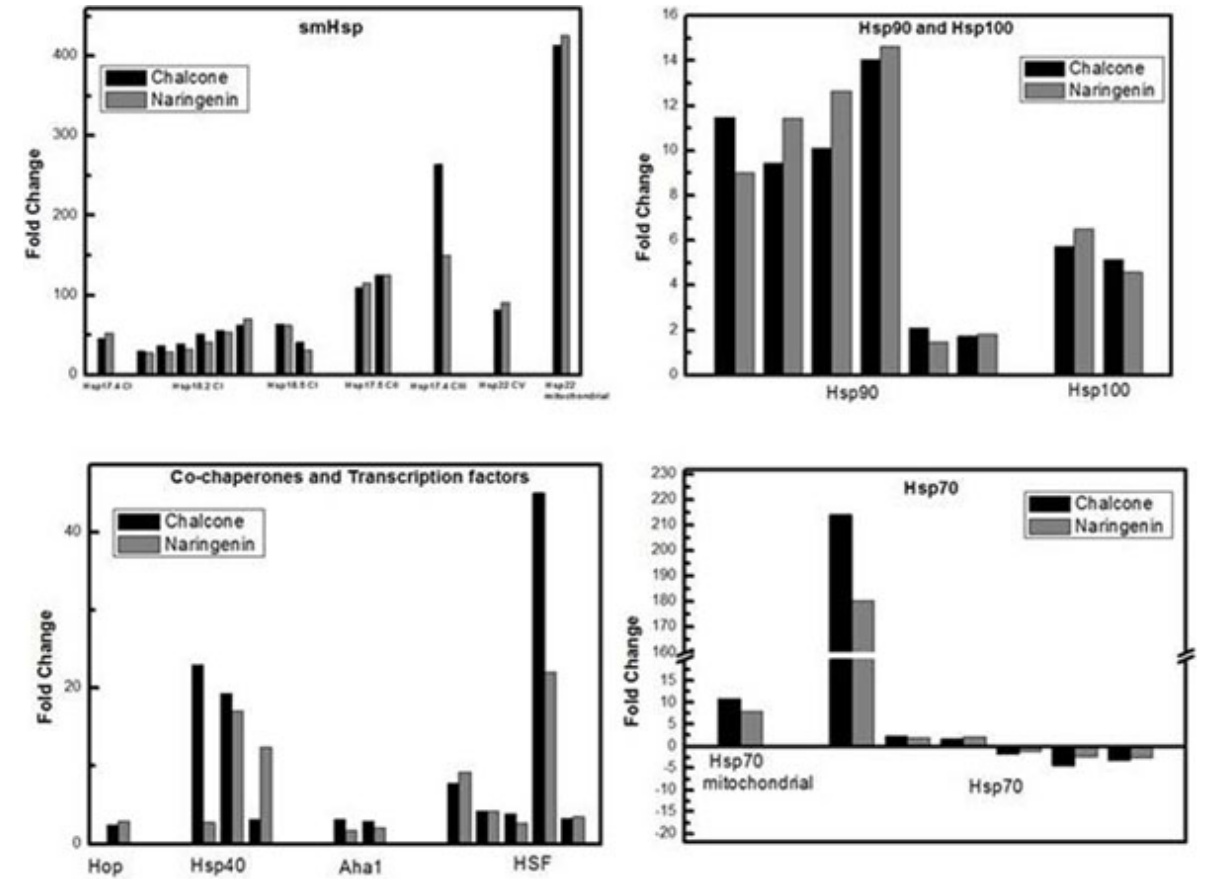

Figure 2(abstract P109) Molecular chaperones, co-chaperones and Heat shock transcription factors are up-regulated in *E. urograndis* narigenin-chalcone (black bars) and naringenin-supplemented (gray bars) in comparison to control group.

Different species of *Eucalyptus* are known for their superior performance in growth, wood quality and resistance to different types of stress [5]. Such characteristics are probably driven by distinct gene expression coordination in xylogenesis. *Eucalyptus grandis* is one of the most planted species in the world due to its rapid growth, wide adaptability and wood quality. *Eucalyptus globulus* wood has higher S/G ratio which provides high yields in cellulose extraction [6].

Lignin extraction consumes large quantities of chemicals and energy, and many efforts have been made to improve this process by modifying lignin content or composition in trees, in order to reduce lignin content or make it easier to extract. Results have been achieved by supplementation and genetic modification [7,8].

This study aims to identify chaperones possibly involved in wood formation and quality of wood for pulp and paper industries.

Methods: The RNA-Seq reads were produced from two xylem libraries for comparison between species (*Eucalyptus globulus* and*E. grandis*), and from two libraries for evaluating flavonoids supplementation (*E. urograndis* supplemented with naringenin and narigenin-chalcone).

Reads were aligned against the assembled unigenes using SOAP2 aligner [9] configured to allow up two mismatches, discard sequences with "N"s and return all optimal alignments. To perform the differential expression analysis between libraries, a normalization and statistics pipeline were applied using DEG-seq software [10] (confidence rate: 99%; cut-off: 0.01). RNA-Seq libraries and Gbrowse are available at http://bioinfo03.ibi.unicamp.br/eucalyptus/.

Results and discussion: In this study, we identified chaperones differentially expressed between *Eucalyptus* species. RNA-seq analysis revealed chaperones as smHsp, Hsp40, Hsp70, Hsp90 and Hsp100 between 1 and 3 fold up-regulated in xylem of *E. globulus* (Figure 1).

Eucalyptus urograndis narigenin-chalcone or naringenin supplemented also presented molecular chaperones highly expressed (Figure 2). Recent findings reported that supplementation with these flavonoids can inhibit lignin biosynthesis, and change lignin content and composition [10].

SmHsp family is active in a wide range of environmental stresses, including heat, cold, drought, salinity and oxidative stress [11]. Gion et al [4] noted smHsp accumulation in wood forming tissues, and suggested a role in extending the cell wall thickening phase during xylogenesis.

Hsp70 family acts in protein refolding, translocation, and facilitating the degradation of unstable proteins, directing them to lysosomes or proteasomes [12].

Hsp90 family has a select group of substrate proteins as polymerases and kinases [13]. In*A. thaliana* some kinases are required for optimal cell elongation, which is important for plant growth and vascular system formation [14].

We have successfully identified chaperones with higher expression on *E. globulus* xylem and in *E. urograndis* flavonoids-supplemented plants, which provide evidences to link xylogenesis and chaperone expression. Those findings are crucial on helping to elucidate the role of chaperones on plant development, stress response and wood formation.

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P110

Master regulators of wood formation in *Eucalyptus*

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With the current global focus on bioenergy, forest plantations are increasingly becoming important sources for second generation biofuel, where the whole plant lignocellulosic biomass is to be mobilized. The lignocellulosic biomass is mainly composed of secondary walls (SW) possessing unique characteristics (biochemical composition and tridimensional association of polymers), which govern the intrinsic properties of wood. They especially contain high amounts of lignins, hydrophobic phenolic polymers which constitute an obstacle to the optimal utilization of plant species in paper industry and for saccharification prior to bioethanol production. Among perennial species, *Eucalyptus* species grow very fast and produce high yields of lignocellulosic biomass. They represent the main industrial plantations in the worldand one of the most appealing lignocellulosic feedstock for bioenergy production. Dissection of the molecular switches controlling the coordinated lignin biosynthetic genes is therefore of utmost importance to understand the molecular mechanisms underlying tissue specific deposition of lignin and be able to improve secondary cell wall properties.

With the objective of improving Eucalyptus wood quality to better-fit industrial applications, we are focusing our efforts towards the identification and functional characterisation of regulatory genes controlling the biosynthesis of the cell wall polymers (mainly lignins).

We performed a precise mapping and functional characterization of the *cis*-regulatory elements contained in the promoters of two genes encoding key and consecutive steps of the lignin biosynthetic pathway *i.e.* Cinnamoyl CoA reductase (CCR) and Cinnamyl Alcohol dehydrogenase (CAD) (Rahantamala *et al*, 2010). Our results supported a major role for the MYB transcription factors (TF) consensus sites in the control of the coordinated expression of these two genes. The functional analysis of two MYB factors (EgMYB1 and EgMYB2) preferentially expressed in *Eucalyptus* xylem revealed that they are able to bind specifically to these promoters and regulate transcription*in vivo*. EgMYB1 behaves as a repressor whereas EgMYB2 is an activator (Goicoechea *et al*, 2005, Legay et al, 2010) of the lignin biosynthetic genes but also of the secondary wall biosynthesis. Indeed, both MYBs were shown recently to be master genes regulating the entire secondary wall biosynthetic program including cellulose, xylan and lignin genes (Zhong *et al*, 2010; Legay *et al* 2010). The presence of both positive and negative regulators in *Eucalyptus* xylem offers the possibility of a combinatorial control of gene expression that could provide the necessary flexibility to ensure tight temporal and spatial regulation of lignin biosynthesis or secondary cell wall.

To address this question and get a deeper insight the complex regulation of the SW formation in *Eucalyptus*, we are now studying the regulation of these two MYBs including fine spatial and temporal expression,

identification of their direct targets genes and of their protein partners. We have constructed a yeast-two-hybrid library from *Eucalyptus* xylem that will also be instrumental for deciphering the interactants of landmark genes for the International *Eucalyptus* community. Thanks to the recent release of the *E. grandis* genome (*Eucalyptus grandis* Genome Project 2010, http://www.phytozome.net/eucalyptus), we have performed a genome-wide survey of the large R2R3-MYB superfamily. The phylogenetic comparison of this family with *Arabidopsis*, rice, poplar and grapevine showed a marked expansion of some clusters putatively involved in wood-related processes. Some R2R3 MYB genes seem to be specific of woody plants. The spatiotemporal expression patterns of members of such clusters are currently being studied. Although Auxin is known as a key regulator of cambium activity and wood formation, the Auxin response mediators [Auxin/Indole-3-Acetic Acid (Aux/IAA) and Auxin Response Factor (ARF) transcription factors] extensively characterized in model plants, are still largely uncharacterized in tree species. We have identified 23 *Aux/IAA* and 17 *ARF* in the *E. grandis* genome. Comparative phylogenetic analysis revealed that several *Aux/IAA* and *ARF* subgroups have differentially expanded or contracted amongst the three dicotyledonous plants studied (*Arabidopsis*, *Populus* and *Eucalyptus*). Expression analysis and EST database surveys are currently underway to explore the transcript levels of each member in the different organs and tissues of *Eucalyptus* at key developmental stages as well as in response to hormonal treatments and to environmental stresses. Further functional genomics studies conducted on new candidate transcription factors, their regulation under the developmental or environmental stimuli will help identifying major factors underpinning the physicochemical properties of cell walls, the recalcitrance of which remains a key scientific challenge for establishing highly efficient, sustainably produced, second-generation biofuels.

P111

Partial suppression of a strongly expressed tonoplast sucrose transporter affects water use and carbon partitioning in *Populus*

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Sucrose export from source organs, and its subsequent distribution among differentiating organs in wood-forming stems and elsewhere depends on the activity of sucrose transporters (SUTs). There are no comprehensive reports on SUT function in temperate tree species valued for their lignocellulosic biomass. To begin to address this gap, the SUT gene family was characterized and functionally analyzed in transgenic *P. tremula x alba*. The *Populus* SUT family features the three major groups characteristic of other dicots. In general, functionally distinct SUTs fall into different phylogenetic groups. Group-1 *PtaSUT3* transcripts localize to leaf vascular traces and stem developing xylem; Group-4 *PtaSUT4* to leaf spongy mesophyll, stem developing xylem, cambium and phloem; Group-2 *PtaSUT5/6* to all leaf cells, stem developing xylem and phloem fibers. The SUT4 ortholog of *Populus* differs from that of other model plants in encoding a vacuolar transporter that is unusually well expressed in source leaves compared to Group-1 and 2 SUT genes. SUT4-RNAi transgenic plants demonstrated a shift of biomass allocation from stem to leaf in both nitrogen (N)-replete and N-limited plants. In those plants, sucrose exhibited a complex pattern of hyper-accumulation in exporting leaves and vascular tissues of the stem, and decreased accrual in the shoot tip and sink leaves. RNAi silencing of SUT4 reduced water uptake during drought simulation without significantly affecting overall shoot biomass accumulation.

P112

Time influence in the enzymatic saccharification of cellulose pulp samples

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Background: With growing concern in obtaining clean and renewable energy, many works in bioenergy has been developed in order to obtain substantial gains for the environment.
Due to the large environmental impact associated to the use of fossil fuels, ethanol production from lignocellulosic materia lhas been widely explored, aiming environmental, economic and social benefits.
Lignocellulosic biomass, composed primarily of cellulose, hemicellulose, and lignin, can be submitted to enzymatic hydrolysis in order to produce reducing sugars, which in turn can be converted into ethanol. However, for the whole process to become economically feasible, optimization of the conditions is necessary [1].
To find innovative solutions for the use of lignocellulosic wastes, the aim of this study was to evaluate the ability of converting cellulose bleached pulp into reducing sugars from enzymatic hydrolysis, at different exposure times.
Methods: Cellulose pulp samples were weighed in the amount of 0.2 g and transferred to Falcon tubes, in which were added 40 mL of 50 mM sodium citrate buffer with pH 4.8. For hydrolysis, 0.05 mL of cellulase (Celluclast 1.5 L) and 0.05 mL of cellobiase (Novozyme 188) enzymes were transferred to each tube. The Falcon tubes were incubated in shaker at 50±1 °C and 250 rpm for 24, 48, and 72 hours. Subsequently, reducing sugars were quantified through the dinitrosalicylic acid (DNS) method [2].
Results and conclusions: The results were very similar for all incubation times. The average reducing sugar concentrations were 4.15 g/L, 4.14 g/L and 4.16 g/L, for 24, 48, and 72 hours, respectively. The composition of substrate was: 98% cellulose, 1.7% humidity and 0.3% ashes. Cellulose conversion into sugar was calculated considering that cellulose was converted mainly into glucose, in a proportion of 1.11 g glucose/g cellulose. The conversion percentages obtained were: 76.3%, 76.1% and 76.5%, for 24, 48 and 72 hours, respectively. These results show that the hydrolysis incubation time higher than 24 hours does not improve the conversion of cellulose into glucose. One possible explanation would be the fact that the enzymes mainly hydrolyze amorphous or less ordered crystalline cellulose. Therefore, remains just highly crystalline cellulose molecules, hindering the access of the enzyme. In other words, this highly crystalline cellulose prevents the total conversion, even over long periods of time. X-ray diffraction (XDR) measurements will be done to check this hypothesis.
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P113

The EnergyPoplar project

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BMC Proceedings 2011, **5(Suppl 7):**P113

Energy Poplar (Enhancing Poplar Traits for Energy Applications) is an EC Seventh Framework Programme project aimed at further improving poplar trees as an energy crop. The work is directed to understand and improve traits such as yield and wood properties coupled to Bioethanol production. The project also addresses environmental and economical sustainability questions.
The final goal of ENERGYPOPLAR is to develop poplar as a bioenergy short rotation coppice crop, suitable for large-scale deployment in Europe in areas unlikely to be used for food agricultural production. All will be placed in an environmental framework to ensure environmental sustainability with respect to land use, inputs and soil status
World primary energy consumption increased by 2.4% in 2007. With the worlds growing energy consumption, the development and use of renewable, sustainable liquid bio fuels has become a strategic priority for

the EU. Bio fuels can minimize energy import dependence, reduce greenhouse gas emissions and assist rural and agricultural development.
Bio ethanol can be produced from energy crops that do not compete with food crops for land use and can be directly used by current transportation vehicles. This alcohol can be produced from biomass feedstock and in particular from cellulose, a sugar based polymer present in the cell wall of plants. Such crops are known as 'second generation' bio fuel crops.
To develop a new bio ethanol industry competitive with fossil fuels, the quality of the biomass feedstock, the methods to produce ethanol from cellulose and the yield per hectare must be improved. Energy trees must also support an environmentally sustainable agriculture that uses less agrochemicals, develops rural economies and spares natural forests from agricultural expansion.
Poplar is an economically and ecologically attractive energy crop because it displays a wide range of growth habits and can grow on marginal lands unsuited for food crops with reduced input costs and optimized land management. Additionally, poplar is a commercial crop and the model organism for hardwood tree genomics and physiology.
http://WWW.Energypoplar.eu for more information.

P114

The role of *SND2* in the regulation of *Arabidopsis* fibre secondary cell wall formation

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Background: Transcription factors (TFs) play important roles in the regulation of secondary cell wall (SCW) biosynthesis in herbaceous and woody plants. In *Arabidopsis*, the onset of SCW deposition is initiated by a nexus of NAC, MYB, homeodomain and several other families of TFs, which function in a transcriptional network regulating SCW biosynthetic genes. NAC family members SND1/NST1 and VND6/VND7 have been identified as functionally redundant master regulators of SCW formation in fibres and vessels, respectively [1,2]. *Arabidopsis* plants overexpressing *SND2*, an indirect target of fibre master regulator SND1, exhibited increased SCW thickness in inflorescence stem fibres, whilst dominant repression lines exhibited a decrease in fibre SCW thickness associated with a reduction in glucose and xylose cell wall sugar content [3]. The ability of *SND2* to transactivate the *CesA8* promoter [3] suggested that *SND2* may regulate cellulose biosynthetic genes during fibre SCW formation. The evaluation of this hypothesis necessitates the identification of all downstream genes potentially regulated by *SND2*, and the analysis of SCW chemistry and morphology in overexpression lines. The aim of this ongoing study is to further elucidate the role of *SND2* in fibre SCW formation through microarray analysis of over-expression lines and by independent confirmation of the effect of *SND2* overexpression on fibre SCW chemistry and morphology in *Arabidopsis* plants.
Methods: We generated *2x35S::SND2 Arabidopsis thaliana* Col-0 plants, screened them for *CesA8* upregulation and phenotypically assessed several homozygous (T4) transgenic lines. Inflorescence stem fibre SCW thickness was measured from light and scanning electron micrographs, and the cell wall monosaccharide and Klason lignin composition of stems was determined relative to the wild type. We performed microarray analysis of the inflorescence stem transcriptomes of wild type and transgenic *Arabidopsis* plants using the Agilent 4x44k transcriptome array and confirmed the expression profiles of differentially expressed genes with RT-qPCR.
Results: Transgenic *Arabidopsis* (T4) lines showed no significant external phenotype, and we were unable to reproduce the increased fibre SCW thickness phenotype reported by Zhong *et al.*[3]. We identified a single homozygous line with *CesA8* upregulation and moderate *SND2* overexpression. Whole-transcriptome analysis of the line revealed the upregulation of several TFs and genes associated with SCW biosynthesis, which were reproducibly upregulated in an independent trial. We additionally observed possible ectopic artifacts and gene dosage effects associated with excessive constitutive expression of the *SND2* gene. Chemical analysis revealed only minor changes in SCW monosaccharides, despite the upregulation of SCW biosynthetic genes.
Conclusions: Our results implicate *SND2* in the regulation of cellulosic and non-cellulosic components of fibre cell walls, and we provide a model for the position of *SND2* in the transcriptional network regulating fibre SCW formation.

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Wood quality-related gene expressions of *Eucalyptus globulus* grown in a greenhouse

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Eucalyptus species constitute the most widely planted hardwood trees in temperate and subtropical regions. Their wood is used as a raw material for the production of cellulose. Eucalyptus species have fast growth rates and the ability to adapt to a broad range of geographic locations. Most importantly, Eucalyptus has been listed as one of the candidate biomass energy crops [1]. *Eucalyptus globulus* is the main hardwood species grown in pulpwood plantations in temperate regions of the world.
In this study, we focused on four kinds of key genes, 4-coumarate-CoA ligase (4CL), LIM domain transcription factor (LIM) [2], coniferaldehyde 5-hydroxylase (CAld5H) and the three catalytic units of cellulose synthase (CesA1, CesA2 and CesA3) influencing wood quality . We investigated correlation between relative expression levels of these genes and wood qualities.
We have cloned the genes encoding LIM, 4CL, CAld5H and the cellulose synthase (CesA1, CesA2 and CesA3) from *E. globulus* by the method of cDNA library screening (Fig.1). A cDNA library was constructed using mRNA purified from stems of four-month old *E. globulus* grown in the greenhouse. The expression levels of LIM in basal stems of ten independent *E. globulus* lines showed similar patterns to those of 4CL, indicating that the LIM may control 4CL expression. We investigated the correlation between gene expression levels and wood qualities such as Klason lignin (KL) content, syringyl/guaiacyl (S/G) ratio and holocellulose (HC) content.Expression of the LIM and 4CL were positively correlated with KL content. A highly significant positive correlation was observed between CAld5H expression and S/G ratio. Furthermore, a ratio of the sum of the expression levels of three CesA1, CesA2 and CesA3 to 4CL showed positive correlation with a ratio of HC/KL content that positively correlated to the chemically extracted fiber content in this woody plant. Overall, our results provide a strong foundation for manipulating candidate genes such as LIM, 4CL, CAld5H and CesA towards the production of desirable wood qualities in an extremely important biomass plant, the Eucalyptus species.
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P116

Pre-treatment of eucalypts biomass towards enzymatic saccharification

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Background: There are a few possible ways to produce ethanol from lignocellulosic biomass, for instance, thermochemical and acid hydrolysis. However, enzymatic hydrolysis of carbohydrates is considered the greenest process for saccharification, followed by sugar fermentation into ethanol.

The main challenge of the enzymatic saccharification process is that cellulose is not exposed to the enzyme action in the lignin matrix. The cellulose molecules are arranged in semi-crystalline nanofibrils immersed in lignin matrix with hemicelluloses (polyoses) and extractives between them acting as coupling agents. These nanofibrils are placed together to form helical microfibrils inside the cell wall. Thus, a pre-treatment is necessary to make room for the enzymes to reach the cellulose fibril surfaces in order for the whole process to become economically feasible.

There are many pre-treatments proposed in specialized literature, but their efficiencies are dependent on the biomass composition [1-4]. Moreover, these treatments have to address some constraints such as the recyclability of the chemicals used, low consumption of energy, and sustainability concerns.

We devised a future possibility of a cellulose pulp mill to be transformed into a biorefinary, where besides cellulose pulp, ethanol could also be produced. In the Brazilian pulp mill industry, the process most commonly used is the Kraft process, so the digestion with green and white liquors can be adapted for pre-treatment towards enzymatic saccharification. Also, the industry had already tackled recycling of the black liquor – obtained after wood chips digestion –, recuperating thermal energy by burning lignin and recovering the green liquor.

This work is part of our research to evaluate some modifications on the green liquor digestion towards enzymatic saccharification. We evaluated efficiencies of some pre-treatments with green liquor through enzymatic hydrolysis for holocellulose saccharification.

Methods: A wood log from a 7-year old tree of *Eucalyptus benthamii* was cut into discs. They were cut into 30° wedges and were then grounded using a Willey mill and sifted to be between 20 and 40 Tyler mesh.

Composition of the *E. benthamii* wood obtained by NBR standards was: 3% of total extractives; 28.4% of Klason lignin; 0.03% of ash and 3.5 of syringyl:guaiacyl molar ratio.

Table 1 shows the characterization of the green liquor acquired from Iguaçu Celulose Papel S.A.

The pre-treatment conditions used in this study were two levels of temperature – 120 and 180 °C – and three levels of reaction time – 1, 2, and 3 hours. Cylindrical stainless steel reactors (Parr, USA) with volume capacity of 50 mL were used. The reactors were heated in an aluminum dry block furnace (Marconi, Brazil) with a temperature and time controller. The ratio of solid wood and green liquor was 1:4 ($g.mL^{-1}$). Typical values were 4 g of dry biomass in 16 mL of green liquor per reactor.

The pre-treated biomass was digested using Falcon tubes with enzymes in a buffer solution of sodium citrate (pH of 5.0) in an incubator (Tecnal TE-420, Brazil) at 50 °C and 250 rpm during 24 hours. The enzymes used were cellobiase from *Aspergillus niger* (Novozyme 188) and cellulase from *Trichoderma reesi* (ATCC 26921), acquired from Sigma Aldrich, USA. Typically, the proportion was 0.2 g of dry biomass, 40 ml of buffer solution, 46 mL of cellobiase and 90 mL of cellulase (both used as received). After saccharification, the hydrolyzate was filtered and the reducing sugars were quantified through the dinitrosalicylic acid (DNS) method.

Results: Table 2 shows the solid mass loss after treatment, the total reducing sugar after enzymatic hydrolysis, and the global yield of saccharification.

As can readily be seen on Table 2, temperature plays a crucial role in exposing cellulose for enzymatic hydrolysis, as does the length of cooking. For lowest temperatures, (120 °C) increasing time causes increased sugar release and conversion yield. On the other hand, for the highest temperature level (180 °C), the time of cooking plays a more complex role. The treatment at 180 °C after 1 hour reaches the highest sugar conversion based on holocellulose content of untreated biomass.

The above results can be explained by the following suppositions. Increasing treatment time to 2 and 3 hours results in hemicellulose and amorphous cellulose losses, which decreased conversions yield. However,

Table 1(abstract P116) Characterization of the industrial green liquor used in this work

Analysis of green liquor	Content ($g.L^{-1}$)
Content of sodium sulfide (X) in Na_2S	14.59
Content of sodium sulfite (Y) in Na_2SO_3	13.9
Content of sodium thiosulfate (Z) in $Na_2S_2O_3$	3.16
Total alkali (concentration of alkaline constituents: $OH^-+HS^-+HCO_3^-$) expressed in NaOH	114.1
Active alkali (sum of concentrations of hydroxyl and hydro-sulfite ions including ions formed by hydrolysis of sulfides: OH^-+HS^-) expressed in NaOH	43.3
Effective alkali (concentration of hydroxyl ions including those formed from sulfides by hydrolysis: OH-) expressed in NaOH	32.5
Sulfidity	33.7%

Table 2(abstract P116) Mass loss after pre-treatment, total reducing sugar released after enzymatic digestion, and the yield of conversion based on holocellulose content in the untreated biomass

Treatment	Mass loss (%)	Total reducing sugar ($kg.kg^{-1}$ of treated biomass)	Yield of sugar conversion based on holocellulose of untreated biomass (%)
untreated	0.0	0.04	5.6
120°C, 1h	26.5	0.10	10.6
120°C, 2h	26.4	0.13	14.1
120°C, 3h	34.3	0.16	15.6
180°C, 1h	47,3	0.92	70.7
180°C, 2h	52.5	0.78	53.9
180°C, 3h	50.8	0.91	65.7

when the treatment time was increased to 3 hours, besides amorphous holocellulose loss, there was also the occurrence of crystalline modification of part of the crystalline cellulose type I into amorphous and cellulose type II. Both amorphous and crystalline type II cellulose are enzymatic digested easier than crystalline type I cellulose. This could explain why sugar conversion yield after 2 hours of treatment at 180 °C are smaller than after 3 hours. XRD and chemical characterizations of the pre-treated biomass have to be done in order to corroborate with the above suppositions.

Conclusion: Simple modifications of the Kraft process can be considered as an alternative route for pre-treatment of woody biomass towards enzymatic saccharification.

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Evaluation of the enzymatic digestibility of paper industry byproducts

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Background: Researches concerning renewable energy sources and wastes recycling are strategic to ensure energetic and environmental sustainability of the planet. In this scenario, biofuels (including bioethanol) have attracted increasing attention.

Most ethanol is currently produced from sugar- and starch-based feedstocks, such as sugar cane in Brazil and corn in the U.S.A. However, lignocellulosic biomass also presents great potential for being used as an alternative raw material. Unlike ordinary feedstocks, sugars from cellulosic biomass are not readily available for fermentation and conversion to ethanol. A preliminary pretreatment step is necessary in order to remove the lignin that involves the structures of cellulose and hemicellulose, acting as a barrier. In the following step, fermentable sugars can be released by enzymatic hydrolysis of polysaccharides. Finally, monosaccharides can be converted into ethanol by anaerobic fermentation [1,2].

This process allows the use of forest wastes to produce ethanol. Besides adding value to these byproducts, this action could contribute to reducing environmental problems related to waste disposal.

Several solid byproducts from pulp and paper industry have a high conversion potential into ethanol [3-7], since they have high carbohydrate content and susceptibility to hydrolysis process. This is due to the fact they have already undergone a previous treatment of the fibers. One example is the sludge generated in wastewater treatment of paper recycling industries, which is composed primarily of cellulose and ash. Although studies have been conducted focusing recycling and reusing, currently its primary destination has been the disposal in landfills, causing high costs and requiring large storage areas. As this product usually has high content of moisture, the production of wet sludge can reach one ton per ton of paper produced [8]. Therefore, suitable utilization of such waste would result in significant economic benefits to the pulp and paper sector.

The aim of this study was to evaluate the enzymatic digestibility of byproducts generated by paper industry, including samples of recycled paper sludge and bleached and unbleached pulps.

Methods: The "recycled paper sludge" was a pressed solid material resulting from the wastewater treatment process of a paper recycling mill. Two samples obtained from Kraft pulping process were also used: an unbleached pulp and a bleached one. All materials were ground in a blender before use. Water and ash contents were determined gravimetrically, according to international standards.

Samples were suspended in 0.05 M sodium citrate buffer, pH 4.8, to obtain an initial solids concentration of about 5 $g.L^{-1}$ (dry basis). A mixture of two commercial enzyme preparations was added: cellulase 2.25 $mL.L^{-1}$ (Celluclast 1.5 L), and cellobiase 1.13 $mL.L^{-1}$ (Novozyme 188). Samples were incubated at 50 °C in an orbital shaker at 250 rpm for 24 h. This hydrolysis time was selected based on a recent study that has demonstrated little effect of time on the process yield over 24 hours [9]. Tests were performed in triplicate for each sample and the concentrations of reducing sugars were estimated by the dinitrosalycilic acid method.

Results and conclusions: Recycled paper sludge presented high contents of moisture (66.2%) and ash (20.6%, wet basis). Inorganic fraction probably includes additives such as kaolin, calcium carbonate and titanium dioxide, as well as metals from printing inks. Moisture and ash fractions were around 7.0% and 0.3% for bleached pulp and 22.5% and 1.1% for unbleached pulp.

Cellulose conversions estimated from the hydrolysis trials were: 82.1% for the bleached pulp, 69.5% for the sludge and 27.2% for the unbleached pulp. As expected, the sample that was not subjected to the bleaching process showed lower susceptibility to hydrolysis, probably due to its higher residual lignin content that hinders the enzyme action. The other materials showed relatively high saccharification efficiencies because they have already been "pretreated" during the production process itself. Although paper sludge have presented lower conversion than the bleached pulp, the presence of inorganic compounds seemed to cause no significant inhibition of the saccharification process.

Therefore, the present study suggests the technical feasibility of using the materials tested as a source of monosaccharides for ethanol production, without requiring an additional step of pretreatment. Special interest is focused in the use of sludge from the paper recycling process, adding value to this byproduct and minimizing environmental impacts.

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mRNA sequencing of *Eucalyptus urograndis* trees supplemented with flavonoids shows changes on metabolic process and decrease of lignification

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Background: The flavonoids, naringenin-chalcone and narigenin, are intermediates in phenylpropanoid metabolism in plants, occupying the central position as primary intermediates in flavonoid biosynthesis and are synthesized by *chalcone synthase* (CHS) and *chalcone isomerase* (CHI) respectively.[1] It has been reported that supplementation of narigenin-chalcone and narigenin can inhibit the activity of 4CL in vitro, and suppress the growth and reduce lignin content in gramineous plants, while 4CL suppression affected plant phenotype and resulted on dwarfed trees on *Pinus*[2], and its down-regulation promoted enhanced growth phenotypes on transgenic aspens trees [3], also CHS expression controls flavonoid synthesis and reduced size phenotype on arabidopsis. [4] Eucalyptus is the main source of biomass for pulp and paper industries therefore it's imperative to study the influence of flavonoid supplementation on Eucalyptus and what kind of overall impact it can have on plant development, especially wood formation and gene expression.

Methods: Plant Material: 1 month *Eucalyptus* plantlets were divided into 3 groups with 25-30 individuals per group. Groups were given normal root induced nutritive solutions (Group 1) and added narigenin-chalcone (group 2) and narigenin (group 3). Treatment lasted 5 months with regular supplementation. Samples were then collected and properly stored.

Histhochemical analyses: Stem samples were harvested and fixed in FAA for at least 24h. Sections were double stained with 1% alcoholic Safranin O and 1% aqueous Astra Blue. Hand sectioned tissue samples from the same material were stained with Mäule reagent: 1% KMnO4 for 15 min, 2% HCl for 5 min, and 2N NH4OH solution. Sections were observed with an Olympus BX51 microscope under white light, and the images were obtained with DP-72 digital camera and Image Pro Plus 6.3 software.

mRNA Sequencing: RNA was extracted from xylem tissue according to [5] and mRNA libraries were prepared following instructions from the Illumina mRNA-Seq Prep Kit. Three distinct libraries were generated: *E.urograndis* plantets supplemented with narigenin-chalcone; narigenin; and control solution, containing 34,157,958 (65,75% mapped); 33,743,449 (64,80% mapped); 32,076,198 (65,09% mapped) reads, (reads size approximately 36pb).

Assembling and annotation: We assembled 167,271 ESTs (130,290 from Genolyptus and 36,981 from NCBI) from several species and tissues using the program CAP3. [6] The assembly produced 53,412 unigenes (18,098 contigs and 35,314 singlets).

All unigenes were automatic annotated using the BLAST [7], including: non-redundant (NR) database of NCBI, pfam [8]. Moreover, we performed a functional annotation using the BLAST2GO software. [9]

The RNA-Seq reads were aligned against the assembled unigenes using the SOAP2 aligner [10] In order to perform the differential expression analysis between libraries, a normalization and statistical pipeline were applied using DEG-seq software [11] considering 99% of confidence rate (cut-off of 0.01).

Results and discussion: Dual staining with Astra-blue and safranin-O histochemical analysis show, that both groups of plants treated with flavonoids exhibit a much greater blue stain, typical of celluloses content, when compared to control plants, while exhibiting a lesser amount of the red/purple stain related to phenolics compounds. The Mäule color reaction which provides an effective method for the detection of syringyl units, show an increase of S lignin content on both group of plants supplemented with narigenin and narigenin-chalcone. mRNA sequencing shows drastic changes on genes related with flavonoid sinhtesys, annotated contigs for those genes presented a much lower expression than control groups. Almost all genes from the phenylpropanoidpathway presented lower expression on treated groups, with genes at the beginning of the pathway presenting the most noteworthy reduction, while genes at the end of the pathway presented higher expression on treated groups which is interesting as it can suggest a shift from guaiacyl to syringyl monolignols increasing lignin solubility. Gene Onthology annotation shows significant differences among a number of metabolic process, including cell growth, cell division and response to stress. Among the most expressed genes were found heat-shock proteins, dehydrins and many no-hits. It is clear that supplementation with flavonoids alter gene expression especially regarding lignin biosynthesis.

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The transcriptome of a *Populus* pseudo-backcross identifies genes and pathways co-expressed with monolignol biosynthesis

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Background: Lignin is the main impediment for efficient cellulose extraction and ethanol production from woody tissues. Even though, the biosynthesis of the lignin constituents - the monolignols - is well characterized, little is known about the genetic control of the natural variation in lignin content and composition. The few association studies performed to date in forest species only uncover 5-20% of the heritable variation of quantitative traits such as wood composition. The "missing heritability" can be explained in part by the low resolution of these pioneering association studies in forest species. However, as observed in genome-wide association studies with humans, a large proportion of the "missing heritability" is likely to occur due to other factors, such as the

abundance of rare alleles observed in forest species and complex epistatic interaction between genetic elements. Causal rare alleles and genetic interactions remain undetectable with current statistical methods. In order to shed light on possible interactions between lignin biosynthesis genes and other pathways, we analyzed the transcriptome of 181 genotypes of a pseudo-backcross family of *Populus*. The analyses allowed identification of genes and pathways that were highly co-expressed with genes involved in the biosynthesis of monolignols. Correlations at the transcript level offer initial evidence of possible interactions between genetic elements for the production of monolignols.

Methods: A previously published microarray data [1] was utilized. Briefly, this microarray contains a gene expression probe for every gene annotated in the genome of *Populus trichocarpa* (v1.1.). Microarray chips were hybridized with cDNA synthesized from xylem tissue of 181 genotypes from a pseudo-backcross pedigree of *Populus trichocarpa* x *P. deltoides*. This family was also analyzed for lignin content on xylem tissue [2]. Genetic differences in gene expression allowed us to correlate the transcript abundance of 23 previously identified xylem-specific monolignol biosynthetic-genes [3] against the cDNA levels of all annotated genes of *Populus*. Correlation was estimated based on Spearman's rank method in R. The top correlated genes ($r > 0.75$, p-value <0.001) were clustered with the 23 monolignol biosynthetic-genes based on a "Modulated Modularity Clustering" method [4]. The genes clustered with the 23 monolignol biosynthetic-genes were GO annotated based on the BlastP best hit against the *Arabidopsis* gene models. A Fisher's exact test was applied to identify GO terms enriched within these clusters.

Results: Utilizing a correlation threshold of 0.75 (p-value < 0.001), 1369 genes were correlated with at least one of the 23 monolignol biosynthetic-genes. These genes were clustered together with the lignin phenotypic trait and 78 genes that were correlated with lignin ($r > 0.45$). The 23 monolignol genes were clustered in four groups, containing a total of 936 genes (Figure 1). Surprisingly, the lignin trait itself did not cluster with any other gene, and the vast majority (75%) of the top 78 lignin-correlated genes were grouped on clusters 12 and 15. Clusters 14 and 16 did not have lignin-correlated genes. We analyzed the GO annotation among genes within the four monolignol-related clusters. A Fisher's exact test was utilized to find GO terms enriched within these clusters when compared to all annotated genes in the genome of *Populus*. Sixty-nine GO terms were significantly enriched (FDR < 0.01) among genes clustered with the 23 monolignol genes: 11 terms are from the ontology Cellular Component (CC), 26 from Molecular Function (MF) and 32 from Biological Process (BP). As expected, most of these MF and BP terms (>65%) are related to lignin, cellulose and hemicellulose biogenesis. This high specificity indicates that even the genes with unknown MF and BP (260 of the 936 monolignol clustered genes) might be involved in cell-wall biogenesis. Enriched GO terms not directly related to cell-wall biogenesis include "drought-recovery", "salicylic acid catabolism", "response to cadmium ion" and "response to zinc ion". In addition to the significantly enriched GO terms, we also constructed a co-expression network including the core, most highly correlated genes ($r > 0.85$) within each of the four identified, monolignol biosynthetic clusters. These networks, representing the most likely interacting genes, will be presented.

Conclusion: This work presents a set of approximately 900 genes highly co-expressed with xylem-specific monolignol biosynthesis genes. Many of these genes are currently annotated with unknown function, or are not known to be involved in cell-wall biogenesis. We offer initial evidence towards a role or interaction of these genes in the biosynthesis of lignin and possibly other cell-wall components.

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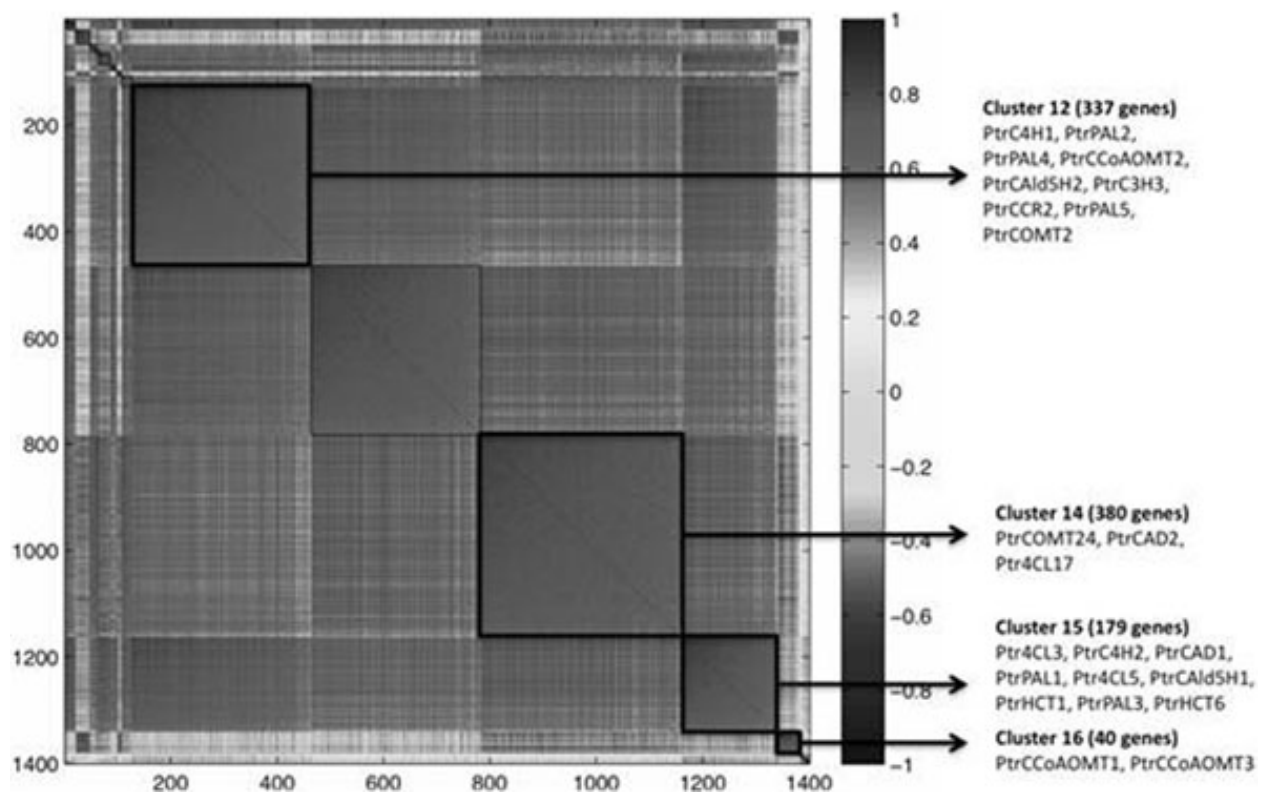

Figure 1(abstract P119) Symmetrical correlation matrix depicting clusters of genes highly co-expressed ($r > 0.75$) with 23 xylem-specific monolignol biosynthetic-genes. Clusters 12, 14, 15 and 16 contain the 23 monolignol genes, as shown. Genes within these clusters were analyzed for gene ontology term enrichment. Additionally, co-expression networks were built within these four clusters.

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P120

Regulatory network in lignin biosynthetic pathway through small RNAs in *Acacia mangium*: implications to the pulp and paper industry

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Background: Lignin, after cellulose, is the second most abundant biopolymer accounting for approximately 15-35% of the dry weight of wood. As an important component of wood, lignin is indispensable for plant structure and defense. However, it is an undesirable component in the pulp and paper industry. Removal of lignin from cellulose is a costly and environmentally hazardous process. Tremendous efforts have been devoted to understand the role of enzymes and genes controlling the amount and composition of lignin to be deposited in the cell wall. However, studies on the impact of downregulation and overexpression of monolignol biosynthesis genes in model species on lignin content, plant fitness and viability have been inconsistent. Recently, non-coding RNAs have been discovered to play an important role in regulating the monolignol biosynthesis pathway genes [1-3]. Non-coding RNAs represent an emerging class of riboregulators, which are processed to shorter miRNAs or siRNAs. The current paradigm indicated that plant system use small RNAs (miRNAs and siRNAs) as guide for post-transcriptional gene silencing and epigenetic regulation. Although miRNAs and siRNAs result from different biogenesis pathways but both interact with target transcripts for direct cleavage or translation repression, effectively shutting down that genes' functions. However, much less is known about the mechanism of gene regulation governed by these small RNAs in lignin biosynthesis pathway in *A. mangium*.
Methods: Total RNA was isolated from secondary xylem tissue with contrasting lignin content using mirVana microRNA Isolation Kit (Cat. AM1561, Ambion, Austin, TX, USA) following manufacturer's protocol. Thin cookies were first ground in a blender and then further ground to fine powder using mortar and pestle. Integrity of the isolated Total RNA was analyzed using Bioanalyser 2100 (Agilent Technology, Palo Alto, USA) and only Total RNA with RIN value above 7 was selected for library construction. Library construction, sequencing and bioinformatics pipeline analysis was done by Gene Pool Sequencing Centre, United Kingdom. Small RNA libraries were generated using DGE small RNA Sample Preparation Kit (Cat. # FC-102-1009; Illumina, San Diego, CA, USA). Illumina sequencing libraries were prepared using the 'long' Illumina protocol according to the manufacturer's direction and two libraries were sequenced on an Illumina GA-II following manufacturer's instructions. After masking of adapter sequences and removal of contaminated reads, the clean reads were filtered and the resulting oligos were totalled. Clustering based on relative lengths from 7 nt to 35 nt were done using in house perl scripts. The targets were extracted by in house python script and annotated using BLAST to GO database. Only highly conserved miRNAs with strong differences in their expression level between high and low lignin secondary xylem were selected for validation using IQ5 real time – PCR technology (BioRad, Hercules, USA) (Table 1).
Results: A total of 14,582,383 reads were generated in Am54 and 10,281,313 reads in Am48.We have identified several conserved and novel small RNAs that may serve as an important regulatory role during secondary wall formation. Majority of these small RNAs emerged as critical regulators for normal growth and developmental processes in *A. mangium*. Only a few small RNAs were postulated to play an important role during epigenetic silencing. We found that the expression level of these miRNAs belong to four different families was up regulated in tension wood (Fig. 1). Tension wood is composed almost entirely of cellulose while compression wood is rich in lignin. We will further investigate the effects of over expression of these four highly conserved miRNAs in tension wood on the expression level of monolignol biosynthetic pathway genes.
Conclusions: From the sequence results, we concluded that *A. mangium* small RNAs consist of a set of 14 highly conserved miRNAs families found in plant miRNA database, 82 novel miRNAs and a large proportion of non-conserved small RNAs with low expression levels. Out of these 14 highly conserved miRNAs families, only four miRNAs families were selected for validation in compression wood and tension wood and their total relative counts between Am54 and Am48 are shown (Table 1). Although these four miRNAs belong to different families, all of them were up regulated in tension wood, a region composed entirely of cellulose. The results obtained can be used to better understand the roles of small RNAs and for the development of a gene constructs for silencing of specific genes involved in monolignol biosynthesis with minimal effect on plant fitness and viability.
Acknowledgements: Special appreciation to IUFRO Tree Biotechnology Conference 2011 organizing committee for awarding Ong Seong Siang a Student Travel Grant to attend this conference. The author wish to thank Gene Pool Sequencing Centre for small RNA library preparation, sequencing and bioinformatics analysis. This work was funded by Ministry of Science, Technology and Innovation, Government of Malaysia through eScience grant 05-01-02-SF1020.

Table 1(abstract P120) Total Counts of the four selected highly conserved miRNA families isolated from secondary xylem of low lignin *A. mangium* 54 (Am54) and high lignin *A. mangium* 48 (Am48)

miRNA family	AM54	Am48	Predicted Target
amg-miR159	3158	1008	MYB Trabscription Factor
amg-miR168	79040	33447	Agrgonuate
amg-miR172	32743	18204	APETALA 2-LIKE transcription factor
amg-miR394	2037	226	F-box

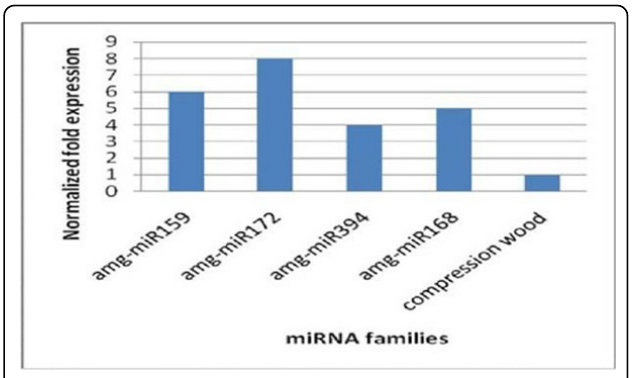

Figure 1(abstract P120) Relative normalized expression of four selected conserved miRNAs in secondary xylem tissue of *A. mangium* Tension Wood (TW) using real time PCR technology. 5.8S rRNA was selected as references genes.

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P121

Toward identifying molecules responsible for the peculiar properties of the G-layer in tension wood fibres

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Background: Due to its peculiar properties, tension wood formation constitutes a remarkable adaptation mechanism, that makes possible for the tree to reorientate its axes (stem and branches) in response to environnemental cues. In poplar, tension wood fibres harbour an extra cell wall layer, the G-layer, responsible for the peculiar mechanical properties of tension wood. This G-layer is very thick, most likely devoid of lignins and strongly enriched in highly cristalline cellulose. In addition, cellulose microfibril orientation is almost parallel to the fibre axis.
We aim to identify molecular actors responsible for the tensioning of cellulose microfibrils and we choose as candidate, molecules containing complex carbohydrates, such as pectin and the glycosylated part of arabinogalactan proteins. Indeed, a wide array of different carbohydrates has been recently evidenced in the G-layer, suggesting the occurrence of complex polysaccharides other than cellulose within this layer (1, 2).
Material and methods: As a first step, we realized a comparative study between tension and opposite wood fibres using immunochemistry. A number of antibodies raised against different polysaccharide epitopes were assessed.
Results: The study revealed important differences in the distribution of the labeling with the kind of wood, the cellular type and within a single fibre between the different cell-wall layers. When using AX1 antibody directed against arabinoxylans, the secondary cell wall layers exhibit a very strong labeling whereas G-layers were completely devoid of labeling (3). With LM5 antibodies (directed against β(1-4)galactans, opposite wood is mainly labeled at the primary wall (Figure 1A), whereas in mature tension wood the G-layer is strongly labeled (Figure 1B) as already observed by (4). With JIM14 antibody directed against cell surface arabinogalactan-proteins, a uniform but moderate labeling was visible on the middle lamella and primary cell wall of fibers, ray-cells and vessels from both opposite and tension wood. In addition, a strong labeling appears at the inner surface of the G-layer (5). The labeling of antibodies directed against the protein moiety of poplar fasciclin-like arabinogalactan proteins are also detected in the G-layer forming fibres, and mainly at the inner surface of G-layers whereas this labeling is hardly present on primary walls which were labelled with JIM14. With CCRC-M7 antibodies directed against RhamnoGalacturonan I, the labeling is restricted to the G-layer of young tension wood fibres and more specifically to the innerside of the G-layer.
Conclusion: Our results strongly suggest the involvement of pectin and arabinogalactan proteins in the building of the G-layer.
Acknowledgements: The PhD thesis of Fernanda TP Guedes is funded by Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior – CAPES.

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P122

Regulation of biomass growth and carbon partitioning in poplar – molecular characterization of a candidate gene

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Improvement of plant feedstock for bioenergy production can be achieved by modifying wood chemical properties and increasing biomass productivity. We previously identified a candidate gene for carbon partitioning and growth on chromosome 13 (cpg13) of poplar. Cpg13 was identified as the regulator of carbon partition and growth within a QTL interval that explains 56% of the variation in cellulose to lignin ratio, as well as 20-25% of the heritable variation in biomass. Putative homologues of cpg13 in Arabidopsis are annotated as proteins of unknown function; therefore, the functional characterization of cpg13 is essential. At present, evidence of the functional role of cpg13 is being obtained by the analysis of poplar transgenic plants transformed with RNAi, overexpression and GFP-fused – cpg13 constructs. Preliminary data indicates that down-regulating cpg13 positively impacts growth, while a negative effect is detected in lines overexpressing cpg13. Transgenic poplar 35S::cpg13:GFP shows localization in cell wall, consistent with *in silico* predictions. Comparative genomics indicate moderate similarity with methyltransferase. Analysis of the impact of differentially regulating cpg13 on lignin and cellulose is currently under way in the mutants. Furthermore, attempts to purify the cpg13 protein are in progress, to define its role through a series of biochemical function assays.

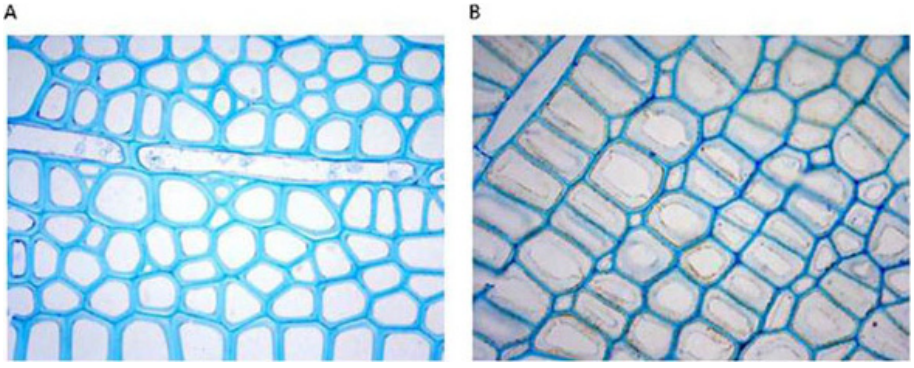

Figure 1(abstract P121) A: in opposite wood, LM5 labeling is restricted to the primary cell wall. B: strong labeling of the G-layer in mature tension wood fibres.

P123

Formation of woody biomass is regulated by class III HD Zip genes

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Secondary growth and the development of woody tissue is a key process in the formation of woody biomass. The gene family of Class III HDZip genes has been shown in the herbaceous Arabidopsis model to play a central role in regulating polarity and vascular development. While Arabidopis is a poor model for investigating processes of wood formation, in this project all poplar Class III HDZip genes were cloned and expressed in hybrid aspen as a tree model system. To circumvent an endogenous regulation mechanism involving microRNAs the sequences were also mutated to render them microRNA resistant. Lines expressing the mutated Poplar ortholog of the Arabidopsis Revoluta gene (Populus Revoluta PRE) show a spectacular phenotype with stunted growth, radialized and rolled leaves, and a double and at times triplication or quadruple layer of the xylem, suggesting the formation of multiple layers of cambium. ClassIII HDZip genes have thus been shown to be crucial for the formation of lignified tissue in trees.

P124

Transgenic aspen and birch trees for Russian plantation forests

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BMC Proceedings 2011, **5(Suppl 7):**P124

Background: Aspen(*Populus tremula*) and birch (*Betula pubescens*) are the fast-growing trees successfully used for the aim of plantation forestry. These species have the great potential in Russia to meeting the need for paper, timber and other wood-based products. However, enhanced growth rate, decreased lignin content and herbicide resistance are the required properties of new trees for plantation forestry. Breeding of forest trees is a slow process due to the long generation intervals typical of most forest trees and because many traits can only be properly assessed at rotation age. Genetic modification is an alternative method that can be used for new trees creation.

Methods: Genetic transformation experiments were carried out using vectors pBI-4CL, pBI-Xeg, pGS and pBIBar and supervirulent *Agrobacterium tumefaciens* strain CBE21. pBI-4CL plasmid contains the expression cassette harboring inverted fragments of 4-coumarat-CoA-ligase gene (GeneBank AY043494). pBI-Xeg vector was constructed using cDNA of xyloglucanase gene cloned from *Penicillium canescens*. pGS and pBIBar vectors contains pine glutamine synthetase gene (*GS*) and phosphinothricin acetyltransferase gene (*bar*). For transcription control of expression 35S promoter used in all cassettes harboring the gene of interest.

Internodes from *in vitro* aspen plants (*Populus tremula*) and leaves from *in vitro* birch plants (*Betula pubescens*) cultivating on WPM medium were used as an explants for *Agrobacterium*-mediated transformation [1,2]. Transformed shoots selected on the modified MS medium with 25 (aspen) or 50 (birch) mg/l kanamycin.

To detect DNA fragments from binary vectors transformants were analysed by PCR. Expression of recombinant genes was detected by RT-PCR. Lignin content determination performed by using the Klason method.

Results and conclusions: As a result of two *Agrobacterium*-mediated transformation experiments with pGS vector 56 and 27 transgenic lines of birch and aspen have been produced respectively. Presence of recombinant glutamine synthetase *GS* gene confirmed by PCR in 52 birch and 23 aspen lines. *GS* gene expression at the RNA level demonstrated RT-PCR analysis (Figure 1).

Most of GS lines have resistance to subletal dose of phosphinotricin as well as in the in vitro and greenhouse conditions. Comparative biometric analysis of GS plants in a greenhouse makes it possible to select 7 and 3 superior lines of birch and aspen respectively. Growth rate enhancement of selected lines was 20-25%.

Genetic transformation of aspen plants for 4-coumarat-CoA-ligase gene down-regulation and xyloglucanase overexpression has been performed

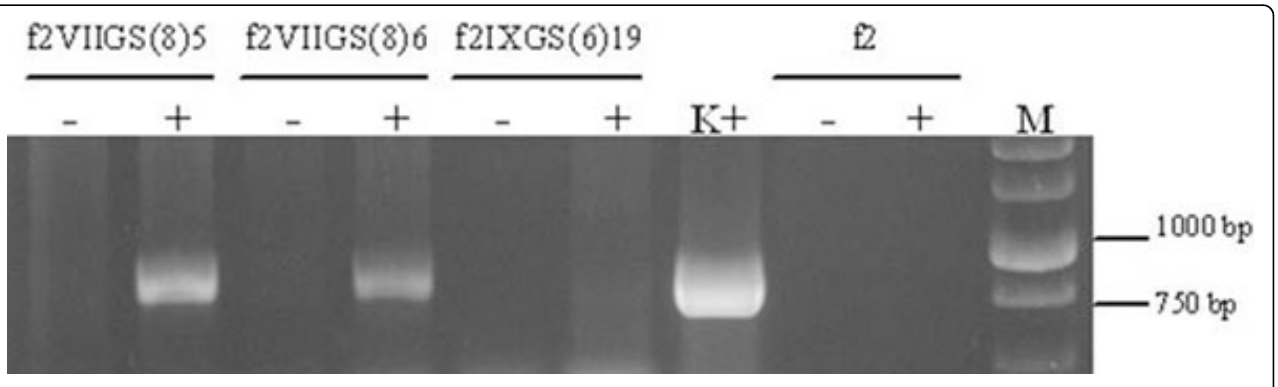

Figure 1(abstract P124) RT-PCR analysis of a glutamine synthetase gene expression in transgenic aspen plants: + and – are RNA preparations with or without the treatment with reverse transcriptase, respectively; K+ is pGS plasmid; f2 is nontransformed control; M - molecular marker.

Table 1(abstract P124) Lignin content of extract-free wood from control and transgenic aspen plants

Line	Acid soluble lignin, %	Acid insoluble lignin, %	Total lignin, %	Lignin reduction, %
Pt (control)	21,0	3,4	24,4	-
PtXIII4CL1c	21,4	3,0	24,3	0,2
PtXIII4CL2c	15,2	3,6	18,7	23,2
PtXIII4CL3a	22,5	3,0	25,5	-4,7
PtXIII4CL3c	17,9	3,7	21,7	11,1
PtXIII4CL4a	18,5	3,2	21,7	11,1
PtXIII4CL4c	17,4	3,8	21,2	13,0

Figure 2(abstract P124) Stem wood coloration of control (right) and transgenic 4CL plants (left).

using pBI-4CL and pBI-Xeg binary vectors respectively. 11 transgenic lines with inverted fragments of 4CL gene and 15 lines harboring Xeg1 gene have been produced. DNA fragments of 4CL RNAi-construct were detected in all lines. 14 out of 15 transformants produced with pBI-Xeg vector harboring Xeg1 gene. Chemical analysis of stem wood of 4CL lines demonstrated decreased lignin content (Table 1). Lignin reduction from 11 to 23% was achieved. Line PtXIII4CL2cdemonstrated minimum lignin content 18,7%. Color modification of stem wood was observed for 8 out of 11 4CL lines (Figure 2).

Aspen and birch transgenic plants with *bar* gene coding resistance against herbicide phosphinotricin were also created. Transformation experiments were carried out using vector pBIBar. 19 aspen and 9 birch lines were selected as a result of transformation. Presence of *bar* gene was confirmed in all lines. Herbicide resistance was observed at 16 and 7 lines of aspen and birch respectively. Lines tested in a greenhouse demonstrated normal phenotype without somaclonal variations and may be recommended for the field trials.

To combine several new properties in one plant the co-transformation method was developed. This method allows transferring recombinant DNA fragments from three T-DNAs of two plasmids independently [3]. Efficiency of co-transformation was up to 100%. Currently we attempt to apply this method for the production of superior aspen and birch plants with enhanced growth rate, decreased lignin content and herbicide resistance at the same time.

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P125

Selection of reference gene in *Eucalyptus camaldulensis* for real-time qRT-PCR

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Background: Real time quantitative reverse transcription PCR (real-time qRT-PCR) is an established technique for quantification of mRNA and has been extensively used for gene regulation studies in plants. However, there are inherent challenges associated with the technique such as variability of RNA and extraction protocols, different rates of reverse transcription and PCR efficiencies. This demands an accurate method of normalization to obtain reproducible results. Among the various methods, normalization to a reference house keeping gene is the most commonly used method. If inappropriate reference genes are used for normalization, the experimental results can vary significantly leading to false results [1].

Eucalyptus camaldulensis Dehnh. is a widely distributed tree species used for planting in arid and semi-arid areas. The wood is composed of mainly cellulose and lignin and the pathway involved in lignin formation is fairly understood. The lignin biosynthetic genes*viz Ferulate 5 Hydroxylase* (*F5H*), *4 Coumarate CoA Ligase* (*4CL*), *Cinnamoyl CoA Reductase* (*CCR*) and *Cinnamoyl Alcohol Dehydrogenase* (*CAD*) are highly conserved across the tree species and have been utilized as targets for manipulating lignin

content. The present study describes validation of a reference gene in various tissue types of eucalyptus and its subsequent use for the expression analysis of lignin biosynthetic genes.

Materials and methods: Samples were collected from three different tissues of *E. camaldulensis* namely xylem, young leaf and mature leaves and three developmental stages *viz* one, one and half and three years. The samples were named as X (1-3) for all the xylem samples coming from three developmental stages and Y(1-3) and M(1-3) for young leaves and mature leaves respectively (Figure 1). Total RNA was extracted with RNAqueous Kit (Ambion, Austin TX, USA) following manufactures protocol. cDNA was synthesized from 1 μg of RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with random hexamer primers. Primers for house keeping genes and lignin biosynthetic genes were designed based on the EST data from Eucalyptus using Primer Express 3.0 Software (Applied Biosystems). The nucleotide sequence of *CAD*, *CCR*, *4CL* and*F5H* from *E. camaldulensis* were deposited in NCBI database (HR309064, HR309065, HR309066, HR309067). The real time PCR efficiency of each primer pair was determined with the slope of a linear regression model and all PCRs displayed efficiencies between 90 and 95%. Comparative C_T method was followed for quantification of lignin biosynthetic genes with 18S rRNAas the internal control as suggested by Schmittgen and Livak [2]. The stability analysis of each reference gene was carried out by the method given by Brunner *et al* [3].

Results: The choice of internal control is usually straight forward especially for analysis of samples from same tissues. However, when samples are derived from different tissues or developmental ages, as it is seen in our study, required validated reference gene/ genes. Six different housekeeping genes were chosen for the analysis which included NADP isocitrate dehydrogenase, 18S ribosomal RNA, Histone H4, 60S ribosomal protein L7, Translational initiation factor (TIF) and Glyceraldehyde 3 phosphate dehydrogenase. Among the genes used in the study, 18S rRNAshowed the least C_T indicating higher abundance while the highest C_T was observed for TIF. Further, 18S rRNA showed maximum stability when C_T was plotted against samples (Figure 1). A gene with low stability index is considered to have highest stability [3]. In the present study with E. camaldulensis, 18S rRNA had the lowest stability index while GAPDH showed maximum variation among the samples.

Expression analysis of four lignin biosynthetic genes *viz CCR*, *CAD*, *F5H* and *4CL* were carried out in different samples by using 18S rRNA as the reference gene. Lignin analysis of samples showed higher lignin content in three-year-old samples estimated by klason lignin analysis compared to one-year old. All genes under study showed increased expression in three year old samples except for *CAD* gene. The results also showed increased amount of *F5H* transcripts in older tissues indicating higher syringyl units in older tissue. Chen *et al* .[4] reported syringyl type lignin content and S/G ratio increased from younger internode to older internode while G and H decreased in parallel.

As the expression profile showed variations in different age samples, further experiment was under taken to evaluate the expression levels of these genes in same age trees (Three year old) in correlation with lignin content. Based on klason lignin content, trees with lignin content of 26± 0.3 and 24± 0.2% were chosen for the studies. All genes under study *viz4CL*, *CAD*, *CCR* and *F5H* showed increase in expression in high lignin sample in comparison with low lignin.

Conclusions: Among the house keeping genes under study, 18SrRNA exhibited lowest stability index and we are suggesting it as a reference gene for expression studies in Eucalyptus. The reduction in *CAD* transcript level and higher levels of *F5H* in older tissues indicated a possible shift towards an increased syringyl lignin where as when analysis was done at same age, expression levels of all selected lignin biosynthetic genes were high in high lignin tree. The study opens up the possibility for using these genes as candidate genes for the selection of desired genotypes.

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P126

Genomic of *Eucalyptus globulus* for pulp and biofuels

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Eucalyptus is a species of great interest for the pulp and paper industry. Worldwide there are 20 million hectares planted and within Chile a total of 700.000 ha, being the main species grown for short-fibre pulp *Eucalyptus*

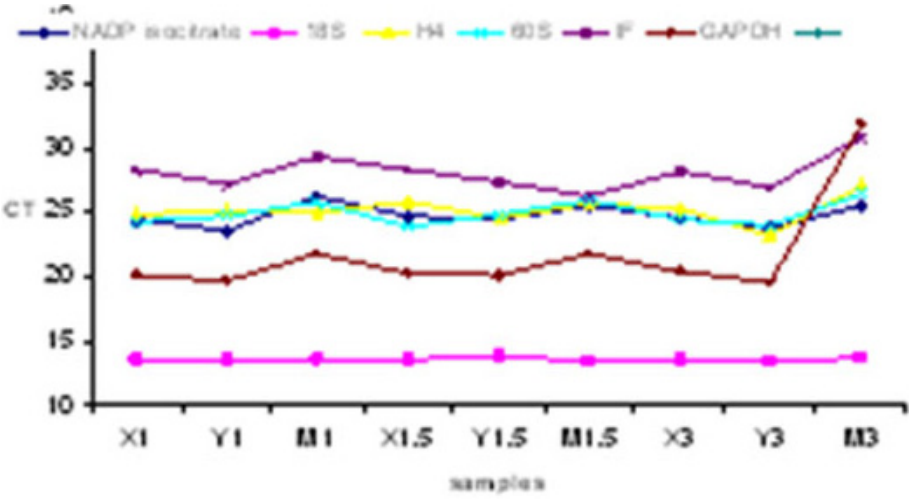

Figure 1(abstract P125) Distribution of average C_T values of various house keeping genes under study.

globulus, which is extensively used for pulping due to its fast growth, high pulp yield, and good fiber properties. Genetic programs of this specie have been oriented in improving commercial traits as volume, growth and form and recently traits as pulp ability have been included. Understanding the genomics of wood formation and identifying genes that are responsible for the traits of interest is a major challenge. An increase in 2% of pulp yield in trees can translate in a large economical gain, but most importantly there will be less pressure for land. There are two ways of reaching this goal, one through the use of genetic engineering and the second by genetic improvement, in both cases it is important to understand and identify the main genes involved in conferring the traits of interest. To identify these genes, different studies have been carried out, mainly by using EST libraries, finding a large number of sequences. Although there is large information regarding the enzymes and genes involved in the lignin pathway, little information is available regarding other metabolic pathways as cellulose and hemicellulose, as well as genes responsible for traits as density, cell wall, among others. In this study a detailed phenotypic characterization of 100 different genotypes was made, determining pulp content, lignin, cellulose, hemicellulose and Syringyl/Guaiacyl (S/G) ratio, among others. By employing NIR models a larger number of *E. globulus* clones were characterized for the same properties. From the 300 genotypes studied, "contrasting genotypes", with high (good) and with low (bad) density and pulp yield were selected. It was found that clones with high density and pulp yield had high glucan content, lignin rich in S units, high β-O-4 linkages and low lignin, and xylan content. Out of these contrasting genotypes, one of each, low and high pulp yield and density, were further studied to find candidate genes involved in pulp ability. An EST library was made for each genotype, which was sequenced by the 454 platform, giving a total of 21,000 sequences, out of which 250 were differentially expressed. Due to the low amount of Eucalyptus sequences available, 28% of these sequences blasted to ESTs from wine grape, 22% to poplar and less than 5% to eucalypts. From these sequences, genes involved in lignin pathway, cellulose biosynthesis as well a transcription factors were identified. F5H was one of the main genes studied, which was characterized at a biochemical and expression level.

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S7. PROPAGATION AND IN VITRO TECHNOLOGIES

P127

Somatic embryogenesis for mass propagation of elite Spruce families: effect of storage time on somatic embryogenesis initiation

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Background: Somatic embryogenesis (SE) is the only clonal propagation method that has potential for large scale production of elite conifer plants from the breeding programs. Methods that support bioreactor-based methods for SE propagation are developed [1,2]. Samples of somatic embryos can be stored indefinitely under liquid nitrogen for future plant production. Somatic embryo cultures are also studied as model systems for conifer embryo development to address fundamental research questions, or used as material for genetic transformation to study gene function in conifers.

In addition to utilizing SE for masspropagation of known elite clones previously tested in field tests, the SE technology offers an opportunity to directly capture and increase the value of small samples of elite seeds from the breeding programs. Furthermore, by direct masspropagation of families through SE, the value of the elite seed is increased; however without the cost of clonal testing. This is arguably an alternative approach to the traditional approach of only utilizing clonal field-tested material for SE masspropagation [3].The aim of this project was to investigate the effect from seed storage time on the rate of somatic embryo initiation for the purpose of optimizing the use for SE over time of small valuable seed samples. This was done by isolating ZE from seeds of Norway spruce that had been stored for various times, and were collected from different parts of Sweden.

Material and methods: Plant material: Nineteen batches of Norway spruce (*Picea abies*) seeds from commercial seed orchards in southern, middle and northern parts of Sweden were provided by the forest companies supporting the project.

Initiation of Somatic Embryogenesis: The spruce seeds were sterilized with 95% ethanol followed by 30% (v/v) commercial bleach and Tween 20. The bleach was discarded and the seeds was rinsed three times with sterile distilled water and left to imbibe overnight at room temperature. After imbibition, ZE were dissected from the female gametophyte under a dissecting microscope and cultured on half-strength LP medium supplemented with 10 μM 2, 4-Dichlorophenoxyacetic acid and 4.4 μM Benzyladenine for SE initiation. In total 90 ZE were isolated from each seed batch. The SE initiation was monitored on weekly basis.

Maturation of Somatic Embryos: One cell line per seed batch was tested for embryo differentiation from pro-embryogenic masses (PEMs) on DKM containing no plant growth regulators (PGRs) and maturation on DKM supplemented with 30 μM Abscisic acid.

Results: In total we tested 19 seed batches of Norway spruce where 90 ZE were isolated per seed batch and placed on ½ LP medium containing PGRsfor SE initiation. Three weeks after isolation of ZE, callus formation was observed. Embryogenic callus is composed of PEMs that have a white and translucent appearance (Fig. 1) and are mostly produced from the hypocotyl region of the ZE. When whitish callus reached a size of 5x5 mm, it was isolated from primary explants and placed on proliferation medium for continuous growth.

All 19 seed batches showed SE initiation however at different frequencies (Fig. 2). The initiation frequency did not vary notably between the seeds from different parts of Sweden. There was also no difference in initiation frequency related to the time in storage. The seeds tested had been collected between 1984- 2007; the highest initiation frequency was observed in seed batch FP 444 collected in 1992 and the lowest initiation rate was observed in seed batch Saleby collected in 2006.

One of the initiated and established cell lines from each seed batch was subjected to maturation medium to examine whether the cultures of

Figure 1(abstract P127) SE initiation from zygotic embryo of Norway spruce. ZE extracted from spruce seeds produced PEMs after 3-4 weeks in culture.

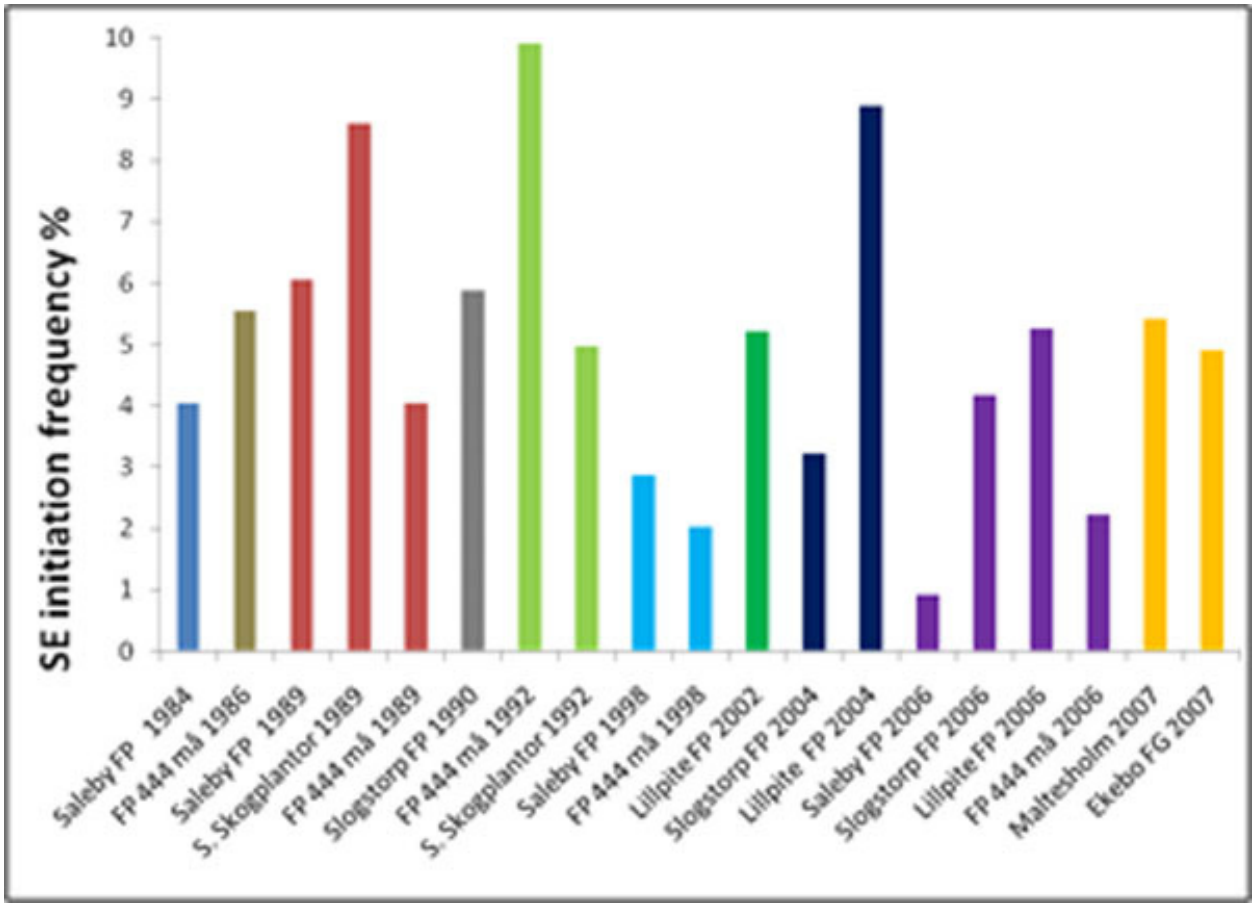

Figure 2(abstract P127) SE initiation rates from seeds from different seed batches and collection years. The SE initiation rate for each seed batch is shown in percentage of seeds tested (90 for each batch) that produced PEMs that could be isolated and cultured. Only one cell line per seed was recorded.

PEMs could produce mature somatic embryos. We observed that 11 out of 19 tested cell lines produced mature somatic embryos (data not shown). Since only one cell line from each seed batch was tested for maturation, we cannot exclude that the remaining 8 seed batches were capable of producing mature somatic embryos. However, similar to the initiation process, the maturation stage did not appear to be related to the storage time and the geographical origin of the seed.

Conclusion: We have demonstrated that it is possible to propagate small batches of Norway spruce seeds stored for up to 25 years through somatic embryogenesis. All initiated cell lines established cultures of PEMs and most cell lines tested produced mature somatic embryos. Thus we conclude that SE can provide a promising method for amplifying small valuable batches of elite seeds even if the seeds have been stored for up to 25 years.

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P128

Somatic Embryogenesis as a tool for forest tree improvement: a case-study in *Eucalyptus globulus*

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Background: Somatic embryogenesis (SE) technology is expected to play a significant role in future forest tree improvement programs. Methods for efficient SE propagation in softwood species based on bioreactors are underway [1]. Furthermore, genetic transformation through SE is also the most promising approach to generate and propagate elite recalcitrant genotypes of forest trees [2], or clonal propagation of selected endangered high-value forest trees [3,4].

Somatic embryogenesis induction was first reported for the genus *Eucalyptus* in 1980. Mass production of SE plants however remains

difficult although procedures designed for plant regeneration have been reported for *E. citriodora*, *E. grandis*, *E. tereticornis*[5]. Recent data on *E. globulus* are promising and show the potential of this technology for application in clonal forestry [6,7].

A service platform for routine transformation of forest trees has been established at the Berzelii Center at Umeå Plant Science Centre using Norway spruce as the primary model species for gene function analyses. Other target species for future studies include hardwood species such as Poplar and Eucalyptus. We present here a brief summary of a case study for introduction of a recalcitrant forest species to the platform as demonstrated by the establishment of the SE technology for *E. globulus* following protocol previously established in Portugal [6,7] and preliminary results aiming to improve the induction of SE.

Material and methods: Half-sib seeds of *E. globulus* collected from trees in Portugal (Altri Florestal Breeding Program) were used in this study. Seeds were surface-disinfected with a mixture of 1:1 absolute ethanol: hydrogen peroxide 30% (v/v) for 15 minutes. Two sources of explants were tested: (a) cotyledons and hypocotyls isolated from 15 day-old *in vitro* seedlings germinated on standard MS medium and (b) zygotic embryos (ZE) isolated after removal of the seed coat. Briefly, using a standard protocol [7], explants were inoculated on induction medium (MS_{3NAA}) consisting of MS with 3 mg/l NAA, 30 g/l sucrose, 2.5 g/l Gelrite, pH 5.8. Cultures were incubated in the dark at 24°C for 25 days. After this period, explants were transferred to the same MS medium without NAA (expression medium MS_{WH}) under the same conditions.

To evaluate the effect of factors indicated from previous studies as essential for the process of SE induction, we then tested the effect of media composition using only ZE as explants: induction and expression using MS salts but containing other vitamins (MS, B5 and RP) and 5 days induction on MS containing 5 mg/l NAA or 2 mg/l 2,4-D and the remaining 20 days on MS_{3NAA} .

Embryogenic potential was analyzed under the microscope and the results expressed as the percentage of explants showing somatic embryos. Callus production and root formation were also scored.

Results: Adopting a standard protocol, the source of explants affected the SE induction as previously reported for other species: no embryogenic response was observed and only callus and root formation occurred when cotyledons and/or hypocotyls was used as source of explants (data not shown); 5-20% of the explants showed formation of globular somatic embryos after transferring to expression medium within 5-8 weeks after induction when ZE was used. The present study included the same OP families previously tested in Portugal. The induction rates rated in a similar way to the previous study, confirming the importance of the genotype.

Preliminaries results from the evaluation of factors affecting SE inductions show that root and root hair formation was observed on most of the explants in all tested treatments. Adding different vitamins to the MS medium promote the same embryogenic response. When ZE were cultured for short period on medium containing 2,4-D or 5 mg/l of NAA, almost 100% of the explants showed callus formation. However, the embryogenic response was similar to the control treatment indicating that concentration/induction period should be adjusted and/or other PGRs combinations tested in the future.

Individual or small clusters of globular embryos were isolated and transferred to MS_{3NAA} for capture of embryogenic cultures. The most developed somatic embryos isolated displaying a shoot and tap root were transferred to MS_{WH} for plant conversion.

Conclusion: The data reported here show the reproducibility of the previously published protocol using different genotypes of *E.globulus*. The results also indicate the importance of genotype over culture condition for the induction success. New experiments are in progress aiming to increase the induction of SE and establish an efficient protocol that can form the basis for genetic transformation of hardwood SE cultures.

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Practical testing of Scots pine cutting propagation - a joint Metla-Skogforsk-Silava project

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Testing of candidates as clones would greatly benefit breeding of Scots pine (*Pinus sylvestris* L.), but has not been applied because of vegetative propgation of the species is difficult. With a common interest in Scots pine breeding, forest research institutions from Sweden, Finland, and Latvia (Skogforsk, Metla, and Silava, respectively) joined in a collaborative project to develop pine cutting propagation for breeding purposes. The main objective of this effort was to find protocols for sufficient shoot production, and at the same time, maintain a high rooting response. Secondly, the aim was to increase the knowledge on the influence of different rooting agents, watering regimes and substrates on the rooting of cuttings.

From Finland and Latvia, respectively, 15 full-sib families from parents with high breeding values were included. From Sweden, 15 families from each of two research stations, one northern and one southern, were included. These sets of the families were used locally. Another collection of five families was shared among the participants, which means that 5 families extra were included at each location.All donor plants were pruned according to the same principles. However, the timing of the pruning varied in the five propagation models tested:

A. One-year-old donor plants pruned to yield 2x winter cuttings / 4-year method

B. One-year-old donor plants pruned to yield winter cuttings and late summer cuttings/ 3-year method

C. "Turbo line" i.e. one-year-old donor plants pruned 2 x cuttings in one year/ 2-year method

D.One-year-old donor plants pruned to yield 2x late summer cuttings/ 3-year method

E. Two-year-old donor plants pruned to yield winter cuttings/ 4-year method

According to the results, the average production of cuttings with propagation models including two harvests in consecutive years on the same donor plant can be predicted to be 10-15 cuttings per a donor plant, with a substantial variation among families and donor plants. Even though rooting responses above 50% can be achieved, this could not be repeated for a large number of propagations. The generally low and erratic rooting response leads to conclusion that the project was unsuccessful in developing improved and reliable protocols for Scots pine cutting propagation. However, a propagation model enabling two harvests on a 1-year-old donor plant within the same year is a promising option but needs further studies.

More detailed information on the project and its results is available at: http://www.metla.fi/julkaisut/workingpapers/2011/mwp198.htm

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Gene expression patterns associated with developmental transitions during somatic embryogenesis in pine

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The low regeneration capacity of forest species is one of the major limitations for vegetative propagation [1]. The molecular mechanisms that determine the efficiency of clonal propagation programs via either adventitious organogenesis or somatic embryogenesis have not been established. For clonal propagation via somatic embryogenesis, the success of the process depends on an initial reprogramming step and on further developmental transitions involved in the maturation of somatic embryos [2] . The identification of candidate genes involved in the regulation of key steps of the regeneration processes is essential to generate tools and strategies to improve the success of clonal propagation programs in forest species.

The aim of this work is to identify new candidate genes potentially involved in the regulation of developmental transitions in somatic embryogenesis in pine. For that purpose, samples of embryogenic tissue from *Pinus radiata* D. Don at different stages of development were used: proliferative tissue (after 7 and 14 days from the last transference to fresh proliferation medium), somatic embryos at the beginning of differentiation and somatic embryos at cotyledonary stage [3,4].

Large-scale expression analysis using a microarray containing an EST collection enriched in auxin-induced genes, and several tissue-specific cDNA libraries from meristematic and embryonic tissues, were used for the identification of phase-specific candidate genes. Genes related to auxin signaling, regulation of gene expression, signal transduction, proliferation and embryo development were selected for further analysis. The expression of these candidate genes was confirmed by QRT-PCR. The information obtained from this work will open new ways of research on molecular mechanisms involved in developmental processes in conifers.

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P131

Development of micro-propagation and mini cutting protocol for fast growing Melia, Dalbergia and *Eucalyptus* clones for pulpwood and bio-energy plantations

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Per capita paper consumption is only 9.2 kg in India, which is much lower when compared to other neighboring developing economies, such as, China (42 kg) & Indonesia (23 kg). Currently Indian economy is growing by 7.5% per annum. Along with economical growth, the paper demand is also expected to grow and cross 20 million tonnes per annum by 2020 from current 10 million tonnes. Availability & quality of raw material, non availability of land for pulp wood plantations, alternate use of pulp wood for other uses like bio-energy and regulations to increase green energy use in mill operations would become major challenge for Indian paper industry's survival and growth. To face these changes effectively, paper industries in India initiated many social forestry models and brought 4,00,000 hectors under pulp wood plantations. This has helped pulp wood availability and reduced the fibrous raw material shortage to some level but it is not enough to meet the growth.

Tamil Nadu Newsprint and Paper Limited (TNPL) is state owned integrated pulp and paper industry situated in southern most state of India and produce around 4,00,000 tons of paper per annum. TNPL has 300 Tonnes per day (TPD) hardwood fibreline along with 550 TPD bagasse fibre line to meet its pulp requirement. TNPL had setup biotechnology and bio-energy research facility under the existing R & D Department to work on identification, selection and multiplication of improved pulpwood clones of *Eucalyptus* and other alternate fast growing hardwood species through modern biotechnological methods to meet pulpwood and also bio-energy need. The plant tissue culture facility has one million plants per annum capacity and optimized protocols for many improved *Eucalyptus* pulp wood clones to use as clonal mother plants at Clonal Production and Research Centre of Plantation Department using mini cutting process. TNPL clonal production facility has capacity of 15 million seedlings per annum and already covered 52,000 acres of pulp wood plantation and plan to cover additional 15,000 acres every year.

Table 1(abstract P131) Results of wood pulping and pulp properties of selected pulpwood and bio-energy clones

Parameters	Units	*Melia dubia*	*Dalbergia sissoo*	*Eucalyptus urograndis*	*Eucalyptus tereticornis*	*Eucalyptus camaldulensis*	*Reference*
Bulk density	kg/m3	136	230	184	213	220	225
Basic density	kg/m3	318	547	402	502	520	510
Chemical addition	%	15.0	15.0	15.0	15.0	15.0	15.0
Screen rejects	%	0.8	0.7	0.2	0.2	0.8	0.8
Screened pulp yield	%	46.5	47.0	46.6	47.3	47.5	44.2
Kappa Number		21.6	18.5	20.4	19.1	19.7	25.3
Brightness	% ISO	27.8	30.7	29.1	34.9	30.5	24.2
Unbleached Strength properties at 300ml CSF							
Tensile index	Nm/g	94.2	79.0	98.0	92.0	95.0	74.0
Tear index	mN. m2/g	11.9	9.3	8.7	10.3	9.5	8.2
Burst index	kPa. m2/g	6.4	5.6	6.9	6.5	5.6	5.1

As a part ongoing research activity, one *Melia dubia* clone for bio-energy/ pulpwood and one *Dalbergia sissoo* clone and three clones of eucalyptus, (each one from *E. urograndis*, *E. tereticornis*, and *E. camaldulensis*), were identified based on its biomass productivity, as well as, pulp quality and yield. Micro-propagation protocol was optimized for all the above five clones with modified MS medium with various concentrations of cytokinin and auxin. The seedlings produced using micro-propagation were used as mother plants for clonal mini garden in the concrete sand beds where the nutrient requirements where optimized for further multiplication using mini cutting process. The results of above study is presented and discussed in this paper.

Wood and pulp properties of all selected *Melia dubia*, *Dalbergia sissoo*, *E. urograndis*, *E. tereticornis*, and *E. camaldulensis* clones are presented in the Table 1 along with reference *Eucalyptus* pulp wood used in the mill. All the selected clones show high yield and strength properties. However, *Melia dubia* is not preferred as pulpwood due to low bulk density which would result in less through-put in fibreline. But it can be exploited for bio-energy applications especially for biomass gasification to generate producer gas and use in Lime Kiln to replace fuel oil and also for other wood product applications. All the selected clones give approximately minimum 2.5% more pulp yield with improved pulp properties when compared reference pulpwood currently used in the mill. Higher pulp yield would definitely give better economy and environmental performance of mill operations. For example, 1% yield increase would result in annual savings of INR 35.0 million in pulp wood cost for mill of our size i.e. 300 TPD. Therefore, for 2.5% yield increase would result in annual saving of around INR 87.5 million apart from chemical saving and other environmental benefits.

To take all the selected clones from lab to land in a short period, micro-propagation and mini cutting protocols were optimized. The results are presented in the Table 2. *E.tereticornis* clone found to perform well during initiation, elongation and hardening.On the other hand , *E. urograndis* found to perform well during multiplication and gave maximum number of shoots per clump. All the *Eucalyptus* clones found to perform well during the rooting. *Melia dubia* and *Dalbergia sissoo* are found to perform poor in micro-propagation compared to *Eucalyptus*. Seedlings produced by micro-propagation are used for mini cutting experiments and results are presented in Table 2. *E. urograndis* produced more number of cuttings per plant per month followed by*Dalbergia sissoo*, *E.tereticornis E. camaldulensis* and *Melia dubia*. Rooting and survival rate is also high for *Eucalyptus* clones when compared to*Dalbergia sissoo* and*Melia dubia*.

Micro-propagation followed mini cutting protocol for propagation of all the above clones has been successfully adopted by mill for commercial production of quality seedling for mill's pulp wood and other plantation programme.

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Optimization of a plant regeneration and genetic transformation protocol for *Eucalyptus* clonal genotypes

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Background: *Agrobacterium* mediated gene transfer technology offers the potential to introduce novel, high-value traits into selected, elite

Table 2(abstract P131) Results of micro-propagation and mini cutting studies of selected pulpwood and bio-energy clones

Parameters	Units	*Melia dubia*	*Dalbergia sissoo*	*Eucalyptus urograndis*	*Eucalyptus tereticornis*	*Eucalyptus camaldulensis*
Imitation						
Optimum Benzylaminopurine	mg/lit.	1.0	1.0	1.5	0.5	0.5
Initiation rate	%	56	36	48	84	64
Average shoot length after 30 days	cm	1.0	1.5	1.0	3.0	1.5
Multiplication						
Optimum BAP	mg/lit.	0.25	0.15	0.15	0.15	0.15
Average Number of shoots per clump	no	2.0	3.0	32	24	15
Average shoot length	cm	3.0	2.5	0.5	1.5	1.0
Elongation						
Optimum Auxin	mg/lit.	0.0	0.0	0.0	0.5 (IAA)	4.0 (NAA)
Average Number of shoots per clump	no	2.0	2.0	4.0	6.0	4.0
Average shoot length	cm	5.0	5.0	3.0	7.0	4.0
Rooting						
Optimum IBA	mg/lit.	1.0	1.0	1.0	1.0	1.0
Average Number of roots per shoot	no	4.0	4.0	8.0	7.0	5.0
Average shoot length	cm	2.0	1.5	2.0	5.0	3.0
Rooting	%	90	40	100	100	100
Hardening						
Survival	%	62	45	85	95	80
Mini cutting						
Number of shoots per plant/ cutting	no	4.0	7.0	8.0	6.0	5.0
Rooting and survival	%	70	63	79	86	73

tree genotypes. Significant progress has been made in the regeneration and genetic transformation of *Eucalyptus* trees, but transformation efficiency has generally been low for eucalypts [1], especially for elite clonal genotypes. As a preliminary step towards the regeneration of explants derived from clonal material and the production of transgenic plants from commercially important *Eucalyptus* genotypes, there is a need to identify genotypes exhibiting high regeneration capacity. In this ongoing study, we are comparing eight *Eucalyptus* genotypes for callus induction and shoot regeneration potential. Browning of callus tissue and surrounding culture media is a common obstacle limiting regeneration of shoots in eucalypts. Hence, a second aim is to minimize the oxidation of phenolic compounds released from the wounded tissue and improve shoot regeneration rates.

Methods: Shoot buds collected from potted ramets of eight *Eucalyptus* clones (Sappi and Mondi, South Africa) were established under *in vitro* conditionson MS [2] basal medium containing BAP (Benzyl adenine purine). The established clones consisted of four *E. grandis* (G1, G2, G3 and G4), one *E. grandis* x *E. nitens* (GN1), two *E. grandis* x *E. camaldulensis* (GC1 and GC2) and one *E. grandis* x *E. urophylla* (GU1) genotype. Leaf explants were excised from *in vitro* shoot cultures and cultured on MS basal medium containing BAP, NAA (Naphthalene acetic acid) and TDZ (Thidiazuron). Browning and necrosis of the callus as well as surrounding culture media was observed in all cultures and PVP (polyvinylpyrrolidone) was therefore added to the media to reduce oxidation.

A standardised regeneration protocol was used for the optimization of *Agrobacterium* mediated genetic transformation of selected *Eucalyptus* clones. The *Agrobacterium tumefaciens* strain AGL1 containing pMDC162 with the *uidA* gene (β-glucuronidase; GUS) and *hpt* (hygromycin phosphotransferase) gene was used for transformation. To minimize extensive exudation of phenolic compounds from the wounded explants, the leaves were subjected to different wounding methods. Intact leaves were excised from *in vitro* shoot cultures and preconditioned for 24 hrs on callus induction medium followed by wounding with a surgical blade prior to *Agrobacterium* infection. The leaves were wounded in two different ways: (a) removing the edges of the leaf, making 8-10 small wounds and cutting the leaf transversally into two equal halves and (b) making 5-6 small wounds in the intact leaf without damaging the rest of the leaf. Alternatively, the leaves were wounded prior to 24 h preconditioning using the first wounding method (a). The explants were immersed in a bacterial solution (A_{600} = 0.8) for 1 hr followed by blotting with sterile filter paper. The explants were co-cultivated for 24 hrs and stained for GUS activity after two days to determine the percentage of explants showing transient GUS expression.

Results and discussion: Callus initiation was observed at the cut edges and wounded regions of the leaf explants after 20 days of incubation in complete darkness at 25 ± 2°C. Shoot regeneration was also observed on the same medium after 30 days of culture. Callus induction percentages of 76% (GU1) to 100% (G3) and shoot regeneration percentages of 3.4% (G4) to 75% (G1) were obtained from the leaf explants (Figure 1).Tissue oxidation was observed in all of the cultures when exposed to light, which hampered shoot bud development and elongation considerably. Addition of PVP to the media resulted in reduction of tissue browning and increased shoot regeneration rate ranging from 6.7% (G4) to 86.7% (G1 and GN1) (Figure 2). Browning of the tissue and surrounding media occurs due to oxidation of the phenolic compounds by polyphenoloxidase, peroxidise or exposure to air which can be reduced by the addition of antioxidants such as PVP to the culture media [3]. Based on the modified protocol including the addition of PVP, *Eucalyptus grandis* (G1) and *E. grandis* x *E. nitens* (GN1) exhibited the highest shoot regeneration potential (86.7%) and may serve as suitable starting material for further genetic transformation studies.

Different wounding methods did not have a significant effect on transient GUS expression in leaf explants. However, good GUS activity was detected in the transformed callus induced from the leaf explants wounded prior to preconditioning.

Conclusion: We have achieved efficient and rapid callus induction, as well as shoot regeneration for selected *Eucalyptus* clones. The information presented here forms the basis for ongoing optimization of a protocol for the generation of transgenic plants of clonal *Eucalyptus* genotypes.

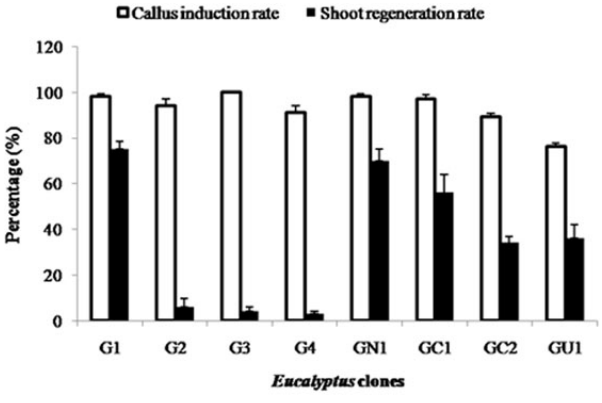

Figure 1(abstract P132) Effect of genotype on percentage of callus induction and shoot regeneration after one month of culture on regeneration media without PVP. Callus induction rate= Number of explants inducing callus / Total number of leaf explants cultured x 100; Shoot regeneration rate= Number of calli inducing shoots/ Total number of leaf explants cultured x 100. Error bars indicate the standard error of the mean of five replicates.

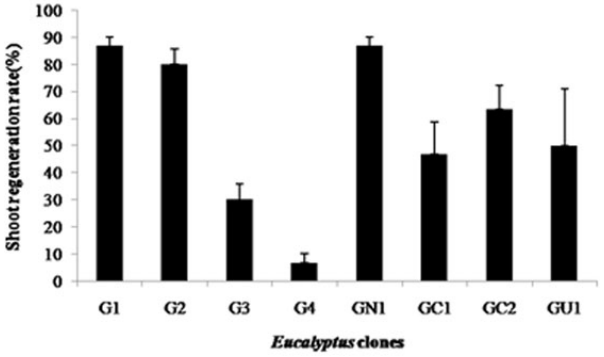

Figure 2(abstract P132) Percentage of leaf explants exhibiting shoot bud induction after one month of culture on shoot regeneration medium containing PVP. Error bars indicate the standard error of the mean of three replicates.

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Screening of genes associated with early stages of adventitious root formation from progenitor adult cells of pine

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Background: In plants, the possibility to regenerate adventitious roots, shoots or embryos directly from adult tissues has been known for more than 60 years and has been exploited in horticulture, agriculture and forestry [1]. However, little is known about the mechanisms that enable a

somatic differentiated cell to switch its fate into a multipotent, pluripotent or totipotent cell that can develop a root, shoot or embryo or repair damaged tissues [2]. Although apparent dedifferentiation and respecification of cells seems to occur, whether acquisition of competence to regenerate organs occurs, as in animal cells, through dedifferentiation, or whether it is via transdifferentiation or by the use of pre-existent totipotent, pluripotent or multipotent cells in adult tissues remains unknown. In either case, cell fate switches are characterized by remarkable changes in the pattern of gene expression, as cells switch from an expression pattern typical of a somatic cell to a new one directing a new developmental pathway [3]. Thus, determining the way by which cells reset their gene expression pattern,especially for the timetable and repertoire of gene expression characteristic of the earliest stages of normal development, is crucial to understand cellular plasticity.

In forest species, a loss of regeneration capacity is associated with tree age and maturation, which makes forest species representative and reliable systems to study how cell fate becomes fixed during development and how plant cells can manage to retain developmental plasticity [4]. The decline in the capacity to regenerate roots from cuttings is one of the most dramatic effects of tree maturation and has been the subject of investigations on the basic nature of the process [5].

Methods: An experimental design based on the analysis of temporal and spatial expression of genes during the earliest steps of adventitious root formation, when cell dedifferentiation and reorganization takes place but before the onset of cell division, was set up in pine cuttings to provide additional clues to the process [6,7]. Two approaches were followed: 1. Expression screening using cDNA collections, and 2. Analysis of the expression pattern of candidate genes potentially involved in root meristem specification, such as genes encoding GRAS proteins, transcription factors involved not only in root patterning but also in the establishment of the quiescent centre identity and in the maintenance of the stem cell status of the surrounding initial cells during the embryonic pattern formation and postembryonary development.

Results and conclusions: For the first approach, the expression pattern of regulatory genes identified from cDNA collections enriched in auxin-responsive, meristem specific and regeneration capacity specific genes was analysed by cDNA microarray and by qRT-PCR. Gene clustering by functional classification and expression pattern revealed the existence of auxin-dependent and auxin-independent pathways potentially involved in the initial phases of regeneration. In addition, genes specifically expressed either in competent or in non-competent tissues were identified.

For the second approach, fifteen *GRAS* genes have been identified in the conifer genome using *in silico* analysis of conifer gene databases. Expression analysis in rooting competent and non-competent pine cuttings revealed different patterns of expression during adventitious root induction: 1. genes that were induced in the presence of exogenous auxin within the initial 24 of the root induction process in rooting competent cuttings only (*PrSCL1*), 2. genes that were induced within the initial 24 of the root induction process in rooting competent cuttings only, and whose expression was not dependent on the presence of exogenous auxin (*PrSHR*), 3. genes that were not induced during the adventitious rooting but their level of expression wasassociated with the developmental stage of the cutting (*PrSCR*), 4. genes that showed a transient short-term expression in both types of cuttings, and 5. genes that did not show an expression pattern related to neither developmental stage nor adventitious rooting competence. Spatial analysis of the expression of two of these genes, *PrSCL1* and *PrSHR*, during adventitious root formation showed a transient increase of mRNA levels asymmetrically localized to the cambial area and rooting competent cells during the earliest stages of adventitious root induction, before the resumption of cell division leading to the root formation pathway, but not in non-competent cuttings. These results suggest the presence of specific cellular signalling pathways or specific factors, perhaps distributed in a localized- or developmental-specific manner, in the tissues involved in rooting. Since auxin transport, accumulation and metabolism do not account for the difference in the rooting capacity of pine cuttings, other factors such as an asymmetric auxin distribution or other determination factors could be involved in the control of age dependent cellular plasticity. Auxin distribution has been analyzed in rooting competent and non-competent cuttings during the initial 24 h of the root induction process. Results showed an asymmetric auxin distribution localized to the cambial area and rooting competent cells in rooting competent cuttings. The spatiotemporal colocalization of auxin accumulation and *GRAS* gene expression was observed in rooting competent cuttings only. This result suggests that signaling pathways involving *GRAS* transcription factors and auxin distribution could be related to the regulation of age-related changes in regeneration capacity.

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In vitro shoot organogenesis from *Eucalyptus* sp. leaf explants

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Background: *In vitro* organogenesis is one of the key techniques associated with genetic transformation, as it determines the successful regeneration of transgenic plants after co-cultivation with bacteria. Therefore, the development of efficient regeneration protocols is the most critical step in developing genetic transformation. Protocols have been developed for several species of eucalyptus. In most of the studies cotyledon, hypocotyl and leaf segments of plants cultivated *in vitro* are used as explants [1]. Several eucalyptus functional genomics projects have used *Populus* species as a model for it counts with well established regeneration and transformation protocols. However, the use of *Eucalyptus* clones as model plants could be more adequate, if clones with high regeneration rates as those obtained for *Populus* could be found. The aim of this study was to evaluate several variables in the *in vitro* organogenesis from leaves of an *Eucalyptus* sp. clone maintained *in vitro* at Embrapa Forestry.

Methods: The experiments were performed at the Laboratory of Tissue Culture of Embrapa Forestry, Colombo, PR. Leaf explants were collected from *in vitro* grown plants maintained on MS medium [2] containing 30 g L^{-1}sucrose, 0.88 μM BAP, 0.05 μM NAA, 0.5 g $L^{-1}$and 7 PVP g L^{-1} agar. The youngest leaves were cut longitudinally and placed with the adaxial side facing the media. In the first experiment, the effect of 0.5 μM thidiazuron (TDZ) was compared with different concentrations of zeatin (2.28, 4.56, 9.12 and 13.68 μM). The plant growth regulators were added to the basic medium, composed of MS salts with half of nitrogen concentration (N/2), supplemented with vitamins of Morel and Wetmore, 30 g L^{-1}sucrose, 0.1 g L^{-1}myoinositol, 0.1 μMNAA and 7 g L^{-1}agar. The second experiment compared the effect of MS salts N/2, WPM [3] and B5 [4] on the same basic medium described above, containing 0.5 μM TDZ. In the third experiment the effect of different concentrations of TDZ (0.25, 0.5, 0.75, 1 and 2 μM) added to the basic medium, replacing the MS N/2 by WPM was evaluated. The explants were kept in growth chamber with

controlled temperature at 23 ± 2 °C in the dark for four weeks, and then transferred to the same medium after 2 weeks. After this period the explants were transferred to basal medium with WPM, 20 g L^{-1}sucrose, 0.1 g L^{-1}myo-inositol, 5 μM BAP, 0.05 μM NAA and 7 g L^{-1}agar and placed under a photoperiod of 16 hours. The pH of all media was adjusted to 5.8 before autoclaving. After two months explants oxidation, callus formation, shoot formation in explants with callus and number of shoots per explants with callus were evaluated. Each treatment consisted of five Petri dishes with 10 explants. Data were analyzed by an analysis of variance. Comparisons between treatments were made by orthogonal contrasts.

Results and conclusions: In the first experiment, there was no callus formation or shoot regeneration on media containing zeatin in any tested concentration. These results contradict those found by [5] who observed shoot formation on leaf explants on medium containing the combination of zeatin and NAA. However, these authors have used salts formulations other than MS. In the treatment with TDZ, 10% of explants regenerated shoots. The number of shoots per callus ranged from one to more than 10. In the second experiment, B5 medium was not favorable for shoot induction. All explants oxidized, although they formed small calli. In the comparison between WPM and MS N/2, in both media 6% of explants regenerated shoots. However, the WPM showed less oxidation than MS N/2. In the third experiment, where several concentrations of TDZ were compared, the number of explants with callus induction was lower in treatment with 0.25 μM TDZ (83%). In all other treatments all explants formed callus. The number of explants with shoots ranged from 7.5% in the treatment with 0.75 μM to 14% in treatments with 0.25 and 0.50 μM TDZ. Treatments with 1 and 2 μM TDZ showed 11% and 12% of calli with shoots, respectively. In *E. globulus*[6] observed buds induction on hypocotyls when placed on medium containing 0.05 μM TDZ and 5 μM NAA or 0.2 μM 2,4-D in. In *E. saligna* 40% of the calli induced on cotyledon explants regenerated shoots on medium containing 1 μM TDZ and 0.1 μM NAA [7]. Among the media tested, the WPM medium containing 0.5 μM TDZ and 0.1 μM NAA was the most suitable for *in vitro* organogenesis of the *Eucalyptus* sp. clone tested. However, regeneration rates (14%) are still low and further research is needed.

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Characterization of herbicide-resistant *Eucalyptus* plants expressing phosphinothricin acetyltransferase gene

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Herbicide resistant crops are commercially advantageous for efficient field productivity by enabling use of non-selective herbicides for weed management. Herbicide resistant trees could be used to improve productivity and reduce the costs of forest management through to the first and second years post tree establishment. We describe the introduction of the *phosphinothricin acetyltransferase* (*bar*) gene, which confers resistance to the herbicide glufosinate, into *Eucalyptus pellita* (clone EP11) and hybrid *Eucalyptus* (*E. grandis x E. dunni* - clone SP1383) under the control of a constitutive promoter. The transgenic lines produced showed a copy number ranging from 1 to 3 copies by Southern blot analysis. Herbicide resistance of the transgenic clones was assessed in the greenhouse by application of 200g/L phosphinotricin at the level of 6.0 L/ha and 4.0 L/ha (commercial rate). The effect of the two herbicide treatments was tested on 4-week and 6-month old plants derived from several transgenic events from the two different *Eucalyptus* clones. All 4-week old non-transgenic control plants, as well as whole shoots of 6-month old control plants were killed by the herbicide by fifteen days after application. The same herbicide treatment effectively killed all the weeds in field conditions. All the transgenic plants showed strong resistance to the herbicide in all treatments with no observed negative effects. The use of herbicide resistant trees may provide an additional means for improving the economic efficiency of plantation forest management.

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Expression pattern of the GRAS gene family during somatic embryogenesis in pine

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The GRAS protein family of putative transcription factors, which includes SHORT-ROOT (SHR), SCARECROW (SCR) and SCARECROW-LIKE (SCL) proteins, is involved in root development in *Arabidopsisthaliana* and other plant species [1]. In forest species, genes with homology to the *A. thalianaSCR* gene have been involved in the induction of somatic embryogenesis in *Picea glauca* (Moench) Voss [2] and *Pinus taeda* L. [3] as well as in the development of radial patterning of roots in *Pinus sylvestris* L. [4]. Schrader *et al* [5] also reported the expression of genes with homology to the *A. thalianaSHR* gene in cambial region of *Populus tremula x tremuloides*. Increased levels of mRNA of *Pinus radiata SHR* (*PrSHR*), *Pinus radiata SCARECROW-LIKE1* (*PrSCL1*) and *Castanea sativaSCARECROW-LIKE1* (*CsSCL1*) have been associated with the early stages of adventitious root induction in *Pinus radiata* D. Don and *Castanea sativa* Mill., respectively [6-9].

In addition to *PrSHR* and *PrSCL1*, we have identified 13 new *GRAS* genes belonging to the different *GRAS* clades in the pine genome. The objective of this work is the analysis of the spatiotemporal expression patterns of the pine *GRAS* gene family during somatic embryogenesis in *Pinus radiata* D. Don. Somatic embryogenesis has become the first biotechnology showing great potential for mass propagation of conifers for application in forestry, allowing the implementation of multivarietal forestry (MVF) [10,11]. Despite major advances in clonal regeneration by somatic embryogenesis or organogenesis, many forestry species are recalcitrant [12]. More knowledge of the regeneration process regulation is necessary to improve the capacity of vegetative regeneration.

The expression pattern of the genes was analyzed by qRT-PCR following the methodology described by Sánchez *et al*[6] and Solé *et al* [7]. For expression analysis, total RNA was extracted from four stages of the somatic embryogenic process: proliferative tissue after 7 and 14 days from the last transference to proliferation medium, somatic embryos at the beginning of differentiation and cotyledonary somatic embryos.

In general, the transcripts of the pine *GRAS* genes accumulated at the highest levels in cotyledonary somatic embryos. In addition, the transcript levels of *PrSCR*, *PrSHR*, *PrSCL1*, *PrSCL6*, *PrSCL8*, *PrSCL11* and*PrSCL12* showed an increase in somatic embryos at the beginning of differentiation. No differences in *PrSCL10* transcript levels were found between the four stages analyzed. Transcript levels of *PrSCL16* were undetectable at all stages. *In situ* hybridization for spatial expression analysis will confirm differential expression domains.

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Tissue culture of two medicinal trees native to Japan

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Wadatsuminoki (*Nothapodytes amamianus*) is an endangered tree species observed in only Amami Oshima Island located in southern part of Japan. According to the Red list published by Ministry of Environment, it is classified as 1A (Critically endangered) and naturally remaining number is only 20. It contains camptotesin which is used for anti-cancer drugs. Kagikazura (*Uncaria rhynchophylla*) is an medicinal tree species observed widely in Japan and China. It contains alkaloids (rhynchophylline, iso-rhynchophylline, hirstine and so on) which are good for remedy of high blood pressure and dementia. For the purpose of micropropagation and development of basis of useful substance production by cell culture as well as conservation of endangered species, tissue culture procedure was developed for those two species.

Excised shoots of 2 years old seedling of Wadatsuminoki rooted in the 1/2DCR medium containing 3 g/l activated charcoal powder. Newly shoots were induced from in vitro root segments subcultured to 1/2MS medium containing 2uM BAP. This cycle can be used for micropropagation of Wadatsuminoki. We have succeeded in micropropagation by tissue culture of Wadatsuminoki (Figure 1). Callus proliferation from stem or root segments was observed on the 1/2LP medium containing 0.5uM BAP and 1 uM 2,4-D, this subculture cell line may be used for the possible production of secondary metabolites in vitro.

Shoots were induced from stem spine (thorn) of Kagikazura in the 1/2MS medium containing BAP or Zeatin. Regenerated plants were obtained by rooting of these shoots on 1/2MS medium containing 1 uM IBA. Callus induced around the stem segments were continuously subcultured in the fresh 1/2LP medium containing 0.5 uM BAP and 1 uM 2, 4-D. These cell lines can be used for the possible secondary metabolite production and for breeding by somaclonal variation or genetic engineering.

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Establishiment of molecular markers for early selection of embryogenic cultures with high embryogenic potential in brazilian pine (*Araucaria angustifolia* (BERT) O. KTZE)

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Background: Brazilian pine (*Araucaria angustifolia* (Bert) O. Ktze) is the only native conifer species with economic importance in Brazil. Recently, due to intensive exploitation Brazilian pine was included in the official list of endangered Brazilian plants. Biotechnology tools, like somatic embryogenesis (SE), may become a potentially useful tool for mass clonal propagation and *ex situ* conservation of commercial and endangered plant species, especially conifers. SE involves the coordinated execution of four steps (embryogenic culture (EC) induction, proliferation, maturation, and plant regeneration). As observed for other conifers, the presence of well-developed early somatic embryos (SE) in EC of Brazilian pine can be considered the pre-requisite for embryo maturation in a medium supplemented with abscisic acid (ABA) and osmotic agents. However, in some genotypes even the presence of bipolar SE does not guarantee embryo maturation. Since SE morphology cannot be used as the only factor for EC selection, the development of molecular markers for early detection of embryogenic cultures responsive to maturation promoters (ABA and osmotic agents) is highly desirable. Polyamines (putrescine (Put), spermidine (Spd), and spermine (Spm)) have been classified as plant growth regulators and hormonal second-messengers playing a critical role in various growth and developmental processes in plants, such as the differentiation and development of somatic embryos. The relation Put/Spd has demonstrated the best answers for predicting embryogenic potential in different plant species. Apart from polyamines quantification, the analysis of gene expression has been used to detect the expression of embryogenesis regulating genes like somatic embryogenesis receptor kinase (SERK), wuschel-related WOX (WOX), and ABA insensitive-1 (ABI1) during conifer somatic embryogenesis. In order to develop molecular markers for early detection of EC with high embryogenic potential in Brazilian pine, we measured the polyamine content (free and conjugate) and the expression of three embryogenesis-regulating genes (SERK, WOX and ABI1) during the proliferation phase of ECs with different maturation capabilities.

Methodology: For induction of ECs, immature zygotic embryos were inoculated in MSG medium free of growth regulators supplemented with 1.46 g L^{-1} filter-sterilized L-glutamine, 30 g L^{-1} sucrose, 3 g L^{-1} Gelrite® (Sigma) in the dark at 25 ±2°C. After one year of EC proliferation, maturation tests were performed using MSG semi-solid medium supplemented with 120 mM abscisic acid, 9% (w/v) maltose, 7% (w/v) PEG 4000, 3% (w/v) sucrose, and 0.15% (w/v) active charcoal. All ECs were maintained in the dark at 25 ±2°C and subcultured every four weeks by transferring ECs to fresh maturation medium. Total RNA from ECs with different maturation capabilities (0.3 g fresh weight) were extracted with Trizol® (Invitrogen, Carlsbad, CA). cDNA was synthesized using 2 μg of total RNA, digested with DNAse I (Fermentas), and reverse transcribed with 500 ng oligo-dT25-ancored primer (5'-T(25)VN-3') using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Primers were designed from nucleotide sequences of Brazilian pine cloned cDNA fragments. The template cDNA were synthesized and the dilutions adjusted with Ubiquitin 1 as an endogenous normalization factor. PCR reactions were carried out in a final volume of 25 μL and the PCR products had an average length of 175 - 200 bp. The RT-PCR products were resolved on 2% (w/v) agarose gels stained with ethidium bromide

and photographed.The methodology for the determination of free PAs and conjugate was based on that developed by [1]. Samples (0.2 g fresh weight) were ground in perchloric acid (PCA) 5%. The conjugated forms of the PAs were obtained by hydrolysis (18 h at 110°C) in 12 N chloridric acid (HCl). The samples were then derivatized using dansyl chloride and partitioned with toluene. PAs levels were obtained by means of HPLC using a C18 reverse phase column.

Results and discussion: Despite of the maturation capability and as observed in other conifer species, the levels of free PAs in all Brazilian pine ECs tested were higher than the conjugated form, and the most abundant PA found was Put followed by Spd and Spm. However, ECs responsive to maturation conditions (with development of mature somatic embryos) showed significantly lower Put/Spd ratios, when compared to non-responsive ECs. A similar profile was observed in embryogenic cultures of *Oryza sativa* L. [2]. In somatic embryos of *Vitis vinifera*, an abnormal growth and a disorganized cellular proliferation were associated to an inadequate Put/Spd ratio [3].

Concerning gene analysis, only the expression of ABI1 gene could be detected during proliferation phase of the ECs cultures. Although ABI-1 gene is normally associated to events mediated by ABA [4], both ECs responsive or not to ABA showed the expression of ABI1. No expression of SERK and WOX could be detected during the proliferation phase of ECs tested, although the expression of these genes was already detected in somatic embryos, late stage zygotic embryos and seedlings of Brazilian pine.

Conclusions: Based on our results, we can suggest that the Put/Spd ratio can be used as a molecular marker for early selection during proliferation phase of Brazilian pine ECs with high embryogenic potential. However, selected embryogenesis regulating genes (ABI1, SERK-1, and WOX) did not show any association with the embryogenic potential in the ECs tested.

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Chemical screening for promotion of adventitious root formation in *Eucalyptus globulus*

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*Eucalyptus globulus*is one of the most profitable trees for pulp and paper industries due to its fast growth and short harvesting cycle. This species is easily pulped with high yield, and its fiber qualities are among the best for paper production. However, it is difficult to vegetatively propagate *E. globulus*.So far, we have developed "photoautotropic culture method" that promotes rooting percentage by feeding approximately three times higher level CO_2 level (1000 μmol mol^{-1}) and suitable culture condition [1]. However, several lines showed poor rooting percentage even in the higher CO_2 conditions. Therefore, it was necessary to develop novel method for promotion of adventitious root (AR) formation in *E. globulus*.

First, we measured endogenous levels of 20 kinds of hormones such as abscisic acid, auxins, cytokinines and gibberellins, at basal part of stem of easy-rooting line and poor-rooting line by UPLC-ESI-qMS/MS. As a result, the Indole-3-acetic acid (IAA) level of easy-rooting line was two times higher than that of the poor-rooting line, suggesting that endogenous IAA level may regulate ability of AR formation.

Next, we focused on the cytochrome P450s that are involved in a vast array of reactions of many different metabolic pathways. Several triazole-containing chemical compounds have previously been shown act as efficient inhibitors of cytochrome P450 monooxygenases. A chemical library of triazole derivatives to find chemicals which have the effect of promoting AR formation was screened. Consequently, five compounds effectively promoted AR formation.

Finally, we investigated how these chemicals affected the growth of *Arabidopsis thaliana*. *Arabidopsis* seedlings were grown on agar medium containing 1 μM selected chemicals. One of the selected chemicals, MA65 increased the number of roots in wild-type *Arabidopsis* seedlings and this phenotype was similar to a mutant *superroot2* (*sur2*) [2]. The *SUR2* gene encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. The amounts of endogenous IAA in 14-d-old Arabidopsis grown in the presence of 1 mM MA65 were analyzed. The IAA content was increased two-fold in the presence of MA65 as compared with untreated *Arabidopsis*. In addition, 1.0 cm explants of Arabidopsis stems were incubated for 7 d on MS medium containing 1 mMMA65. Stimulation of an AR formation was observed as compared to untreated samples. Taken together, MA65 may increase endogenous IAA level in a plant cell and promotes an AR formation.

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Gene expression in cultured primordial shoots of adult white spruce (*Picea glauca*) in somatic embryogenesis responsive and non responsive genotypes

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Background: Modern forest management relies on extensive breeding and reforestation programs to sustain forest productivity and conservation of natural forests. Plantation forestry, with its increased productivity and improved wood quality, is likely to become an important source of wood products in the future. Vegetative propagation of superior coniferous forest trees through tissue culture has the potential to deliver a stable supply of superior seedlings for forest plantation. To clone adult (mature) conifer trees by means of tissue culture has been a cherished goal for the last several decades. The benefits derived from improving the genetic make-up of planting stock would be significant if such clonal propagation could be achieved at a high efficiency and without growth abnormalities of regenerated plants. Although, somatic embryogenesis (SE) technology has worked well for many conifer species using zygotic embryos as starting material, attempts to achieve the same in adult conifers have failed. The basis of this failure is not exactly understood.

Results and discussion: Recently, we have been successful in inducing SE from primordial shoots (PS) of 10 years-old somatic white spruce, genotype 893-6 [1]. We have also identified a few genes (VP1, WOX2, CHAP3A, SAP2C) that were expressed exclusively or significantly up regulated in embryonal mass (EM). In an attempt to gain some understanding of the underlying general molecular events occurring during SE induction phase, we used microarray technology to examine gene expression in responding PS of 893-6 genotype and in non responding PS of 893-12 genotype. Shoot buds were collected from trees in early spring. Some of them were immediately frozen in liquid nitrogen (day 0) and others were disinfected. PS were excised, subdivided into two or four sections and cultured on a standard medium that is used for SE induction from zygotic embryos of white spruce [1]. After 3, 7, 15 and

21 days of culture the PS explants were collected and frozen in liquid nitrogen. RNA was extracted from shoot bud samples at day 0 and from PS explants after 7 days of culture. cDNA was then subjected to microarray analysis using an oligo-based microarray developed by the Arborea project that contains 32,000 probes.
Microarray analysis lead to identification of a number of genes that was up-regulated in each genotype (1-45 fold) in response to day 7 of culture on SE induction medium. Absolute quantification qPCR of the four most up-regulated genes in each genotype confirmed the microarray results, although the magnitude of up-regulation determined by qPCR tended to be greater than that predicted by microarray analysis. In order to provide a more detailed perspective into the dynamics of gene expression, qPCR analysis of these eight genes was expanded to include 3, 15 and 21 days samples. This revealed that while differences in the magnitude of up-regulation for most of these genes differed, the overall trend in expression dynamics were very similar for both genotypes. The one exception was a gene of unknown function, which was rapidly induced in the responsive genotype, reaching a maximum at day 3 followed by progressive reduction over the remaining 18 days. In contrast, expression of this gene remained very low in the non-responsive genotype throughout the entire culture period. The expression level for three other genes peaked between 7-15 days, followed by a reduction at 21 days in both genotypes, whereas the remaining four genes showed a progressive increase up to the last day in culture in both genotypes.This project also included analysis of nine candidate reference genes, which demonstrated that expression of five were highly stable (<±20% CV).

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Effect of inorganic nitrogen concentration in co-culture and regeneration media on *Agrobacterium tumefaciens* growth and on the regenerative capacity of transformed *Pinus radiata* embryonal mass

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The Genetic Engineering program at Genomica Forestal SA, Chile (GFSA) has a goal of generating stably transformed radiata pine for *in planta* evaluation of candidate genes. Regeneration of transgenic plants depends mainly on two factors: regeneration ability of transformed cells and stable transgene integration and expression.
In several conifer species, including radiata pine, transgenics have been regenerated through cocultivation of *Agrobacterium tumefaciens* with embryogenic cells [1-3]. However, in our first experiments using MSG [4] culture medium we found that radiata pine embryonal masses did not recover easily after co cultivation and that there was an excessive overgrowth of bacterial cells in spite of using bacteriostatics in the medium. This impediment prompted our study on testing other culture medium formulations, routinely used in conifer somatic embryogenesis, on the growth of *A. tumefaciens* GV3101. The tested media were: MSG, DCR [5], and modified Litvay [6] MLV. Of the three media MSG supported significantly higher bacterial growth than the other two media. One of the major differences in the composition of these media is inorganic nitrogen concentration (NH_4NO_3 and KNO_3). Compared with MLV and DCR, MSG has the lowest concentration of inorganic nitrogen (100 mgl^{-1} compared with 340 in DCR and 950 mgl^{-1} in MLV) provided in the sole form of KNO_3. Based on our results and the work of others, we concluded that low nitrate concentration in MSG medium promoted *A. tumefaciens* growth and this had a deleterious influence on the viability of radiata pine cells during co cultivation, and also rendered eradication of bacterial cells difficult. Comparison of growth of radiata pine embryonal mass on the three media did not show statistically significant differences. A strategy for producing transgenic radiata pine for *in planta* transgene expression and stability study will be presented.

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"Vegetative propagation – knowhow and technology for enhancing bioeconomy " - A new project launched in Finland

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A three-year project, financed by EU, European Regional Developmental Fund, will be carried out in 2011-14 as a collaborative effort with Finnish Forest Research Institute as a main performer, and Eastern Finland University and a commercial company Taimityllilä Oy as partners. Three new researchers are engaged by the project. The aim of the project is to deepen knowhow and develop technology for vegetative propagation of forest trees in Finland, and to enhance collaboration among research institutions and practical plant producers. The project strengthens infrastructure for research and development, and benefits industrial and commercial activities in the field. A target group of the project includes forest owners, forest and ornamental nurseries, professionals in landscaping and home gardeners, wood product industry, and research sector. The objectives of the project are divided into five theme areas:
New solutions for producing forest regeneration material: The main aim of the project is to develop propagation methods for Nordic conifers, especially Norway spruce with a goal set for commercial mass-propagation. This will be done in collaboration with practical plant producers. Tissue culture approach using somatic embryogenesis, potentially combined with cutting technology, will be studied. Also issues related to user rights and royalty fees when using selected clones or bred families will be touched.
Production of special forms of forest trees for ornamental use and landscaping: Methods of vegetative propagation for special forms of forest trees are developed to meet the increasing demand of hardy conifers for ornamental purposes and landscaping in Northern conditions. Both cutting and tissue culture technology will be tested, and commercial plant production piloted together with a company partner. User right and royalty issues for both natural mutants found or special forms created by breeding are also covered.
Potentials of vegetative propagation in improving the quality of Scots pine heartwood: The success of tissue culture propagation of Scots pine families producing abundantly extractives, and thus resistant to wood decay, will be compared with the success of families producing only small amounts of extractives. Furthermore, analyses of secondary compounds induced in tissue cultures will be carried out and their use in selection *in vitro* will be studied.
In vitro –testing of wood quality using mould and decay fungi: Methods of *in vitro* –testing of wood decay resistance will be developed and used to enhance the selection of materials more resistant against fungal degradation. Better decay resistance leads to longer service life of wooden constructions and thus improves the environmental performance of wood.
Vegetatively propagated materials in enhancing research activities: Clonally propagated materials would greatly benefit e.g. pathological and entomological studies in Nordic conifers. In addition, developed technologies and knowhow can be utilised in forest tree breeding programmes by adopting clonal testing options.

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Novel BAP degradation pathway during adventitious caulogenesis in *Pinus pinea* L. cotyledons

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Background: Cytokinins (CKs) are a group of phytohormones which probably regulate the growth, development, and metabolism of all plants. The aromatic CK benzyladenine (BA) has been widely applied in *in vitro* culture for inducing shoot organogenesis. Our study of endogenous cytokinin profiles during the caulogenic process based on mature cotyledons of stone pine (*Pinus pinea* L.) showed a novel metabolic pathway of aromatic cytokinins based on modification of purine skeleton.
Methods: Three-year-old mature seeds from two half-sibling selected families and open-pollinated trees of *P. pinea* were used [1] and the samples were collected following the Alonso *et al.*[2] procedure. Extraction and purification of cytokinins was based on the method described by Novák et al. [3], including modifications published later [4]. The samples were purified using a combination of a cation (SCX-cartridge) and anion [DEAE-Sephadex/C18-cartridge] exchangers. Combination of high performance liquid chromatography (HPLC) with quadrupole-time of flight mass spectrometry (QqTOF) was used for accurate and sensitive identification and quantification of cytokinins.
Results and discussion: Cortizo et al. [5] published the dynamics of BA uptake and metabolism in *P. pinea* cotyledons excised from embryos precultured for 2 and 4 days and cultured *in vitro* in modified Le Poivre media with 4.4 μM BA. In our experiment, we used 44.4 μM concentration of BA and samples were collected at different periods (0; 1; 2; 6; 16; 24 h and 2; 4; 6 d). Using high-resolution MS, the naturally-occurring BA metabolites as well as new BA forms were identified. In comparison with previously published profiles of the BA metabolite pool [5,6], the ribosyl and glycosyl forms were quantified as the most abundant metabolites. Moreover the biological activity of identified BA and its derivatives were compared in various CK bioassays. The results indicate that BA uptake during the caulogenic process may be possible to regulate not only by known cytokinin pathways. Finally, the feeding experiment with stable isotope-labelled standard, $^{15}N_4$-BA, confirmed our identification of the novel metabolic CK pathway.
Conclusions: The identification of the novel BA forms demonstrates that the novel cytokinin pathway is used as a control mechanism of BA uptake from the bud induction medium during adventitious caulogenesis in cotyledons of *P. pinea*. Our results help to understand the processes associated with embryo germination in plant tissue culture.

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Micropropagation of *Pinus taeda* L. via axillary buds

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Introduction: *Pinus taeda* stands for productivity and quality of its timber [1]. Researches using biotechnology are of great importance and have been applied to the improvement of its timber and plantation [2]. The main method of *Pinus* propagation is by seeds, once the minicuttings depends on the season of the year or depends of juvenile material [3-5]. Thus, researches on micropropagation of Pinus taeda are currently a priority in Brazil [6]. Micropropagation is the best method for mass production of superior genotypes and represents a strategy for tree improvement and capture of genetic gains [7]. Studies on *Pinus taeda* micropropagation by axillary bud proliferation are quite few. The purpose of this study was to develop a protocol for micropropagation of this species from juvenile material.
Materials and methods: For *in vitro* establishment two to four month old seedlings were used. Apical shoots and nodal segments of 3 cm length were inoculated in MS [8], DCR [9], WV3 [10] or WV5 [11] medium. For axillary shoots induction, the explants were inoculated in WV3, WV5 or DCR medium, with BAP (0, 0.12, 0.25 and 0.50 μM). For the induction of roots, we tested the effect of double-layer medium, with semi-solid phase consisting of agar and water or GDm/2 [12] medium and the liquid phase containing water or GDm/2 medium. Both phases were supplemented with 2.69 μM NAA and 0.44 μM BAP for 9 days, followed by transfer to growth regulator-free GDm/2 medium. The rooted plants were planted in Plantmax® Forestry substrate and maintained in a greenhouse.
Results and Discussion: Nodal segments showed better responses during *in vitro* establishment, with up to 100% of explants forming

Figure 1(abstract P144) Nodal segments of *Pinus taeda* inoculated in WV5 medium, after nine weeks in the *in vitro* establishment. Bar: 1 cm.

axillary shoots and an average of 4.3 to 5.8 shoots per explant. The WV5 media proved better and presented the highest survival rate (86.0%) and highest elongation percentage (85.2%) (Figure 1).

The balance of salts in WV5 and WV3 culture media favored an optimal development of *in vitro* cultures of *Pinus taeda* due to its lower concentration of N in comparison with MS medium and to higher concentrations of thiamine and inositol, which are growth promoters. Elongated shoots were subdivided into segments, increasing the multiplication rate to 3 segments per shoot. The majority of BAP treatments did not promote better multiplication when compared to control. However, the alternate use of 0.12 μM BAP added to WV5 culture medium during initial culture and a BAP-free medium during the 1st subculture can increase the multiplication rate. The estimated production was 1024 shoots from 100 explants, in seven months of cultivation. The best rooting percentage (37.5%) was obtained in shoots treated with 2.69 μM NAA and 0.44 μM BAP for 9 days in culture medium composed of water and agar without liquid phase, followed by transfer to growth regulator-free GDm/2 medium. The double-layer medium did not increase the rooting percentage. This result was higher than that found in *Pinus virginiana*, when the same combination of plant growth regulators was used [13]. The roots originated directly and indirectly from the stem with callus formation. After 90 days of acclimatization, the survival rate was 90% and an average of 4.6 roots per plant was obtained (Figure 2). This result was better than that obtained in other study with *Pinus taeda* that reported 38% of necrosis five weeks after transplantation [14]. Micropropagation of *Pinus taeda* from axillary buds and juvenile material is feasible, but requires further studies to optimize the rooting stage.

Figure 2(abstract P144) Micropropagated plants of *Pinus taeda*, 60 days after transplanted and acclimated. Bar: 5 cm.

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P145

Indirect organogenesis from leaf explants of *Eucalyptus benthamii x Eucalyptus dunnii* and shoot multiplication

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Background: In Brazil, especially in the Southern region, stresses caused by cold and eventual frost are those that exert the most negative effect on the productivity of *Eucalyptus* spp. The genetic transformation techniques may contribute to forestry improvement programs in order to obtain genotypes expressing new interesting characteristics. They allow shortening the long breeding cycles and avoiding manipulation of adult trees. Their efficiency depends on establishment of regeneration procedures that allow the development of shoots from the transformed tissues.

*E. benthamii*x *E. dunnii* hybrids have shown superiority to their parents concerning growth and frost tolerance [1], but no information about their *in vitro* organogenesis has been reported in the literature.

The objective of this study was to evaluate the effect of some factors of culture medium on indirect organogenesis and shoot multiplication of an*E. benthammi* x *E. dunnii* clone.

Methods: *In vitro*established shoots,provided by EMBRAPA-Florestas (Colombo, PR, Brazil), were used as explant source. Cultures were maintained under white fluorescent tubes providing a photon flux density (PFD) of approximately 20 μmol m^{-2} s^{-1}, a 16-h photoperiod and a temperature of 25±2 °C.

The cultures were performed in glass flasks containing 25 mL MS [2] medium supplemented with 1.11μM BA and sealed with rigid polypropylene caps. For the indirect organogenesis, leaves were excised from shoots at the petiole base, split into two halves and inoculated in culture media. The cultures were done in Petri dishes kept in a growth chamber in the dark throughout the experiment.

The statistical design was performed in a factorial scheme (2:2:2) and a comparison was done between two culture media (MS-N/2, with half concentration of potassium and ammonium nitrates, and JADS [3] with 0.1 μM NAA, with and without PVP-40 (250 mg L^{-1}) and two TDZ concentrations (0.1 and 0.5 μM).

After 70 days, the percentages of explants forming callus, oxidized explants, explants producing anthocyanin, explants forming buds and shoots, and the number of shoots per explant were evaluated.

For the multiplication test, the statistical design was performed in a factorial scheme (3:2) with three culture media (MS, WPM [4] and JADS, with 1.11μM BA) and two subcultures (28 and 56 days after the initial culture period). The analyzed variables for each subculture were: percentage of oxidation, of explants showing chlorosis, fresh weight and number of shoots.

Results and discussion: Regarding the oxidation, the higher rates (100%) were observed on JADS medium in presence or absence of PVP-40 and on MS-N/2 medium (68.3%) in presence of PVP-40. However, the JADS medium showed the highest percentage of callus formation (83.3%). In MS-N/2 medium the highest percentage of callus formation (55%) was found in the presence of PVP-40 and 0.5μM TDZ. Similar results were observed [5] with *E. saligna* leaf explants cultured on MS-N/2 medium supplemented with 0.1μM NAA and 1.0μM TDZ. Anthocyanin was only observed in explants cultured on MS-N/2 medium without PVP and containing 0.5μM TDZ. The lowest percentage of explants presenting anthocyanin was observed on MS-N/2 with PVP-40 that gave the best result for organogenesis (8.3%).

For the multiplication experiment, the variance analysis did not reveal significant interaction between the factors. During the first subculture MS medium with 1.11μM BA promoted the highest number of shoots/explant (9.2).The explants cultured on MS medium showed the highest fresh weight (0.384g) and highest percentage of chlorotic explants (88%). The percentage of oxidized explants was the same on JADS and MS media (40%). In a study on *E. urophylla* x *E. globulus* shoot multiplication [6], an increase of oxidation was also observed on JADS medium in comparison with MS medium.

With regard to the second subculture, the number of shoots per explant and percentage of oxidation did not differ among the treatments. The explants cultured on MS medium showed the same behavior for the chlorosis than during the first subculture and the percentage of chlorotic explants was superior to that observed in others treatments (64%). On WPM medium, the mean number of shoots/explant was the highest (9.08).

Conclusions: *E. benthamii*x *E. dunnii* hybrid has a low organogenic response to the tested combinations of growth regulators and high tendency to oxidation even when an antioxidant is present in the culture medium. However, the concentration of PVP-40 used in this study may have been low and other types of antioxidants and concentrations need to be compared.

The MS medium with 1.11 μM BA promoted the highest number of shoots per explant after a 28 day culture period, and after 56 days, there were no differences among the treatments.

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P146

Characterization of metabolic differences between embryogenic and non-embryogenic cells in forest trees

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Somatic embryogenesis (SE) in forest trees is considered as one powerful approach in cloning elite genotypes. However, SE is difficult to achieve in tissue or cell beyond the mature embryo phase in trees and its physiological process involved are also poorly understood. Recently metabolic profiling studies have been contributed to understand the mechanism involved in SE process of plant [1-3]. In addition, there are also a number of reports indicating that polyamines (PAs) play a crucial role in SE process [4,5]. As part of an ongoing study of the transition of somatic cells to an embryogenic state in adult trees, embryogenic (EC) and non-embryogenic cells (NEC) in various forest species were investigated for its metabolic compositions including PAs. A comparison of metabolic compositions of NEC and EC using gas chromatography/mass spectrometry (GC/MS) identified around 50 compounds, partly displaying significant changes in metabolite levels, e.g., highly elevated levels of xanthosine and methyloxazole in EC compared to NEC of broadleaves and conifer species (Fig.1). Changes in the polyamine content were also analyzed in both cell types. Free polyamine contents varied according to the species and cell types, the highest levels occurring in the NEC on proliferation medium, when putrescine and spermidine were most abundant. However, the putrescine/spermine+spermidine (put/spm+spd) ratio was higher in EC of yellow poplar and hybrid pine. Analysis of PAs in both cell types indicated that total polyamine concentration was always higher in NEC than in EC, and spermine was present in only minute quantities and showed only a small change. From this analysis, we have identified numerous compounds involved with embryogenic state, and could characterize its differences between broadleaves and conifer trees.

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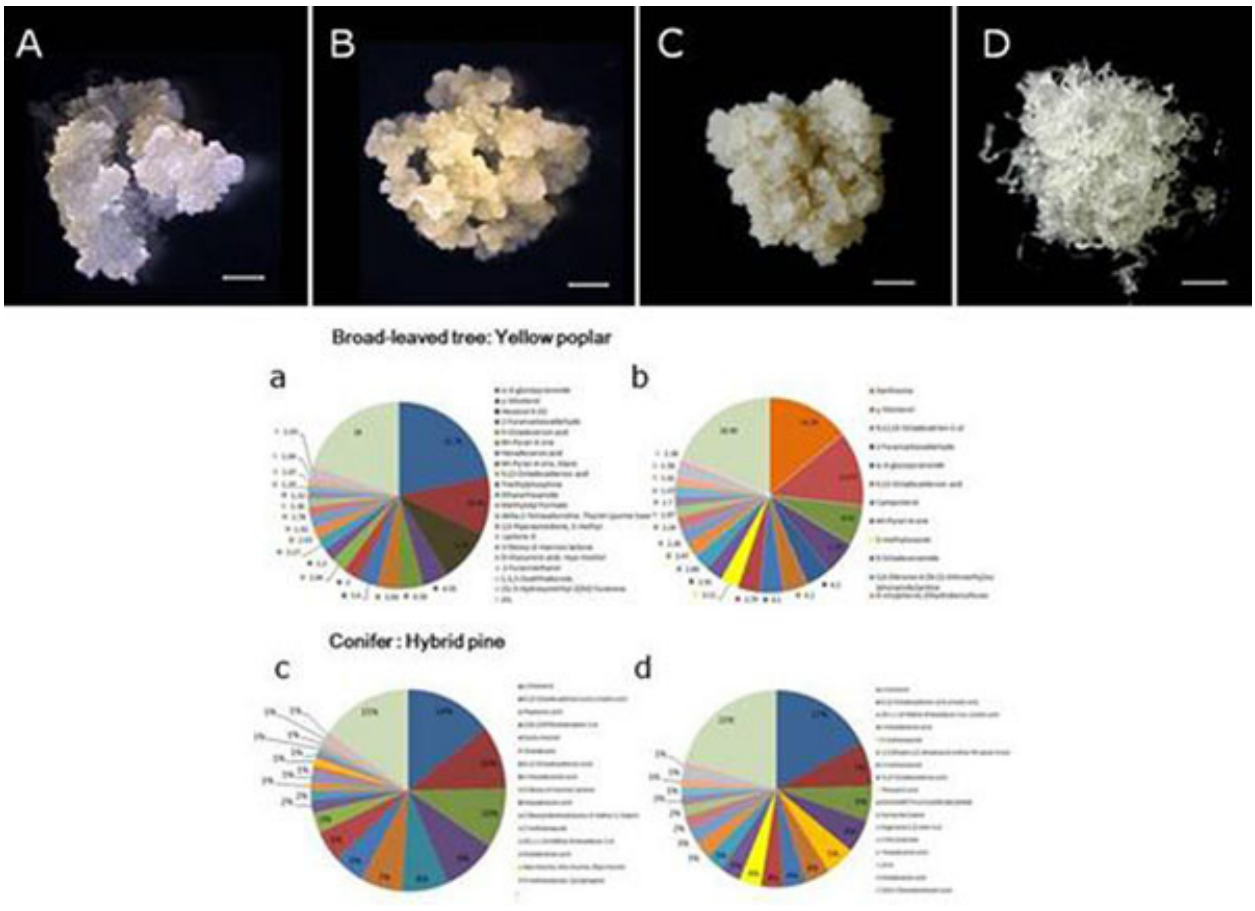

Figure 1(abstract P146) Non-embryogenic (NEC; A, C) and embryogenic cells (EC; B, D) of yellow poplar and hybrid pine 'rigida x taeda' respectively, and its metabolic compositions (NEC: a, c and EC: b, d).

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P147

Use of kanamycin for selection of *Eucalyptus saligna* genetically transformed plants

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Background: Several factors may affect the genetic transformation efficiency of woody species. One factor is the use of an efficient selective agent that inhibits the development of non-transformed cells and just allows the development of transformed tissues. The most used selection agent is the neomycin phosphotransferase II (*NPTII*) gene, which confers resistance to aminoglycoside antibiotics kanamycin, neomycin and G-418 [1].

The selective agent concentration in culture medium may have influence on shoot regeneration and high concentrations may promote adverse effects on organogenic potential [2]. The kanamycin effects in *Eucalyptus* are variable and depend on the species and genotypes [3]. The purpose of this study was to evaluate the effect of kanamycin concentration on transformation efficiency for *Eucalyptus saligna* cotyledons after co-culture with *Agrobacterium tumefaciens*.

Methods: The bacterial strain was EHA 105, containing a binary vector carrying the *GUS* gene under control of CaMV35S promoter and *NPTII* gene under control of the same promoter. Cotyledons from twelve days old *E. saligna* plantlets were co-cultured for 30 min in the bacterial suspension (OD_{600nm} = 0.5) followed by a 5 day co-culture on MS culture medium containing 2.7 μM NAA + 4.4 μM BAP in the dark. The explants were then transferred on the same medium supplemented with (1) 12.5 mg L^{-1} kanamycin (Km) + 300 mg L^{-1} Augmentin (Aug); (2) 25 mg L^{-1}Km+ 300 mg L^{-1} Aug and (3) 50 mg L^{-1}Km+ 300 mg L^{-1} Aug. The explants were subcultured in the same culture medium every 15 days and, after 60 days, the percentage of oxidation, callus and shoot formation, and shoot number per explant were evaluated. DNA was extracted from fresh young leaves and processed according to the specific protocol [4]. The presence of *GUS* gene in the putative transformed plants was confirmed by PCR 10 months after inoculation.Gus expression was studied byhistochemical test for ß-glucuronidase, three and 120 days after inoculation.

Results: With regard to the percentage of oxidation, percentage of callus formation and percentage of shoot formation, there was no significant difference among the three treatments. The percentages of explants

regenerating shoots were 24, 15.9 and 14.6 respectively for the three Km concentrations, after 60 days of co-culture period. The lower concentration of kanamycin (12.5 mg L^{-1}) showed best results for shoot regeneration (24%) and number of shoots per explant (3.9) and these results were statistically differents of those obtained with other treatments. Three gus positive events were regenerated from explants cultured on medium containing 12.5 mg L^{-1}Kmand the transformation efficiency was 0.0075%.

Conclusion: The concentration of 12.5 mg L^{-1}Kmallowed the shoot induction from genetic transformed tissues and was considered satisfactory for selection of transformed tissues. The information presented here may constitute the basis for optimization of the genetic transformation of other *E. saligna* genotypes.

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P148

Induction and repetitive embryogenesis of *Ocotea porosa*

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Introduction: *Ocotea porosa* (Liberato Barroso) known as "imbuia" belongs to the Lauraceae family and is native to the Mixed Ombrophilous Forest (Araucaria Forest) where it was heavily exploited due to the high quality and worldwide value of his hardwood that is exported in large quantities for luxury furnishing manufactures [1]. The sexual propagation of *O. porosa* at its natural occurrence area is difficult, due to the strong tegumentary dormancy and its irregular germination. Moreover, the seed viability is short by virtue of being a recalcitrant species, showing high level of humidity [1]. Another limiting factor for its vegetative propagation is the low response of cuttings to the induction of adventitious roots (4%) [2]. The aim of this study was to establish repetitive cycles of secondary somatic embryogenesis from *O. porosa* embryonic axes.

Material and methods: Zygotic embryonic axes from immature seeds were used as explants. The seed disinfestation was performed in a laminar flow chamber, through immersion in ethanol 70% (v/v) for 5 min, followed by 20 min in NaOCl 4% (v/v) supplemented with 0,1% of Tween® 20. After that, the seeds were rinsed five times with sterile water. The WPM [3] culture medium was used in all stages. Embryonic axes were inoculated on culture medium supplemented with sucrose (20 g L^{-1}), activated charcoal (1.5 g L^{-1}), agar Vetec® (4 g L^{-1}) and2,4-D (200 μM) combined or not with hydrolyzed caseinor glutamine (0.5 or 1 g L^{-1}) during 90 days. The somatic embryos obtained in the induction phase were multiplied on medium with sucrose (20 g L^{-1}), agar Vetec® (3.5 g L^{-1})and 2,4-D (22.62 μM) combined with 2-iP (2.46 μM) for 90 days followed by transfer to culture medium with 0.5 g L^{-1} of hydrolyzed casein combined with 0.5 g L^{-1} glutamine or 1 g L^{-1} hydrolyzed casein or 1 g L^{-1} glutamine for 30 days. The maturation of somatic embryos was tested in WPM culture medium containing sucrose (20 g L^{-1}), agar Vetec® (3.5 g L^{-1})and polyethylene glycol (PEG 6000) (control, 3.5 and 7%) during 30 days. The experiment was repeated twice. All the cultures were maintained in the dark at 27±2°C (day) and 18±2°C (night).

Results and discussion: Patterns of direct and indirect induction of somatic embryos were observed with low frequency in culture medium containing 200 μM 2,4-D alone or combined with 1 g L^{-1} hydrolyzed casein or glutamine. The mean percentage of calli with somatic embryos varied between 4.2% and 8.3% for primary somatic embryogenesis without differences between treatments (after 90 days). In the present study, the maximum percentage of somatic embryos induction was 8.3%. Similar results were found with *O. odorifera* that presented a mean percentage of 6.5% [4]. On the culture medium containing 200 μM 2,4-D, the percentage of calli with somatic embryos was 6.3% and the formation of one globular embryo was visible. When 200 μM 2,4-D was combined with 1 g L^{-1} hydrolyzed casein this percentage was 8.3% and two globular somatic embryos were developed per callus. However, in the medium containing 200 μM 2,4-D and 1 g L^{-1} glutamine, after 90 days the percentage was smaller (4.2%) and two globular and one cordiform shape embryos were observed. After three months, the primary somatic embryos subculturedin culture media without plant growth regulators with or without activated charcoal did not progress to torpedo and cotyledonary stages. In repetitive embryogenesis the combination of 0.5 g L^{-1} of hydrolyzed casein and glutamine promoted an average of 46.6 new globular embryos per callus and 75% of callus presented embryogenic mass com pro-embryos. The subculture in media containing 1 g L^{-1} hydrolyzed casein promoted an average formation of 58 globular embryos, 2 cordiform, 2.5 torpedo and 1 cotiledonary per callus at the end of every subculture and 72.5% of explants formed mass containing pro-embryos. When 1 g L^{-1} glutamine was added into the media, 57.5% of the mass presented pro-embryos and the induction of globular somatic embryos was lower (23.3 globular embryos per explant). During maturation phase, the somatic embryos develop from initial to late stages. However, in the present conditions of maturation, the percentage of embryos developing to cordiform, torpedo and cotyledonary ontogenetic stages was low, revealing an asynchronous development. In both treatments (3.5 and 7% PEG) as well as in the control, the first response was the formation of pro-embryogenic masses in 100% of calluses. In the control and in the medium supplemented with 3.5% PEG, an average of 32.7 and 27 new globular embryos were formed per explant, respectively. When PEG concentration in culture medium was 7%, the formation of new globular embryos decreased (12.7), but the development of cordiform (1.5), torpedo (2) and cotyledonary embryos (2) was observed.

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P149

Establishment of endomycorrhizal fungi on micropropagated teak (*Tectona grandis* L.f.)

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Background: Commercial micropropagation of teak (*Tectona grandis*L.f.) has been achieved for a number of years in countries such as Thailand, India and Malaysia. This has led to the availability of elite genotypes for large scale plantation production. Teak has been shown to develop arbuscular mycorrhizal (AM) fungal associations [1], and this has been attributed to increasing productivity [2]. It is likely that the establishment of mycorrhiza, through the introduction of *Glomus*species, on the roots of micropropagated plantlets will improve the productivity of selected

clones. Established hyphal networks can speed up plant colonization [3] and in the long term may also increase carbon storage through glomalin production [4].Our research is pursuing the development of a mycorrhization protocol for micropropagated teak at the acclimatization stage.

Methods: Shoots of two clones of teak were multiplied on media containing Murashige and Skoog [5] nutrients and organics, 30 gL^{-1} sucrose, 0.5 μM benzyl amino purine, 0.5 μM kinetin, 2.5 gL^{-1} agar, 2.5 gL^{-1} gelitre and a pH of 5.8. Individual shoots were subsequently exposed to a medium containing ¼ strength M&S macronutrients, full strength M&S micronutrients, 60 mM sucrose, 2.5 gL^{-1} of agar and 2.5 gL^{-1} of gelrite [6], and a range of indole butyric acid (IBA) concentrations (0-160 μM) for varying lengths of time (4-28 days) to produce roots *in vitro*. Rooted plantlets were transferred to three pasteurised soil types (1sand:1perlite;1sand:1peat;and 1sand:1perlite:1peat) under the following conditions: they were maintained under mist (covered 75-95% humidity) for 5 weeks followed by hardening through gradual reduction in humidity (50-65%) on greenhouse benches for a further 5 weeks. Plant survival, height and root area were measured.

Two clones of *T.grandis*were exposed to different inoculum sources and various inoculation techniques during the acclimatization period. Unprocessed commercial AM inoculum 100 g kg^{-1} from a legume/grass pot culture (chopped mycorrhizal roots and soil) or processed commercial AM inoculum 10 g kg^{-1} were both mixed with pasteurised inert soil (1sand:1perlite) and watered to field capacity with ½ strength sorghum nutrient solution [7]. When the processed inoculum was used, roots were also dipped into the inoculum powder before being planted. All plants were acclimatised under high humidity as above and periodically fertilized with ½ strength sorghum nutrient solution. Plantlet height, root area and mycorrhizal development were assessed at 10 and 20 weeks and means compared using analysis of variance and Tukey's multiple range test.

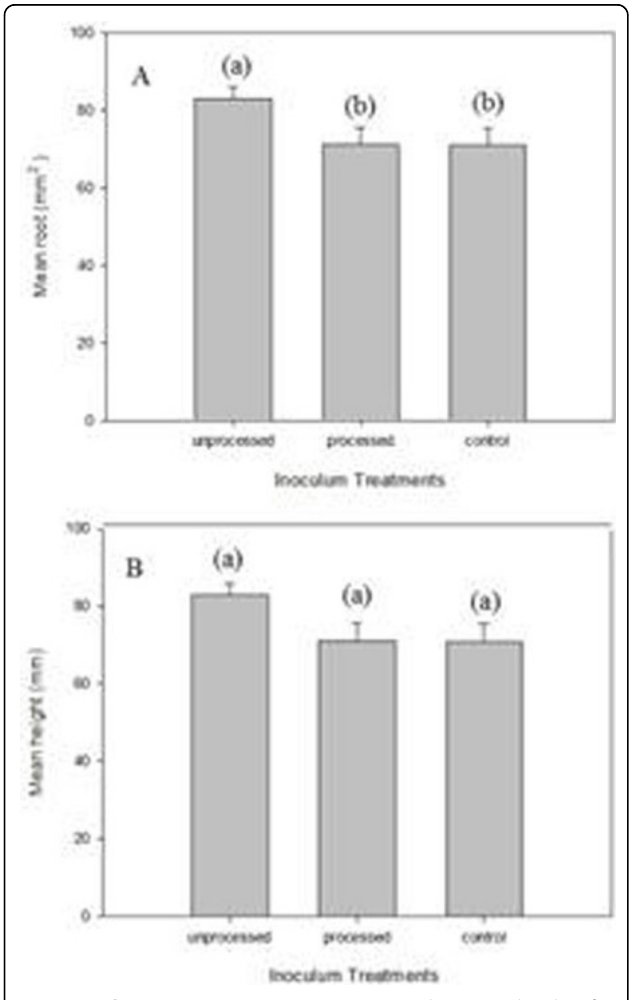

Figure 1(abstract P149) A) Mean root area and B) mean height of a single teak clone inoculated with: unprocessed AM commercial inoculum, processed commercial inoculum and control, 10 weeks after inoculation. Different letters above bars represent differences (P<0.05); error bars= standard errors.

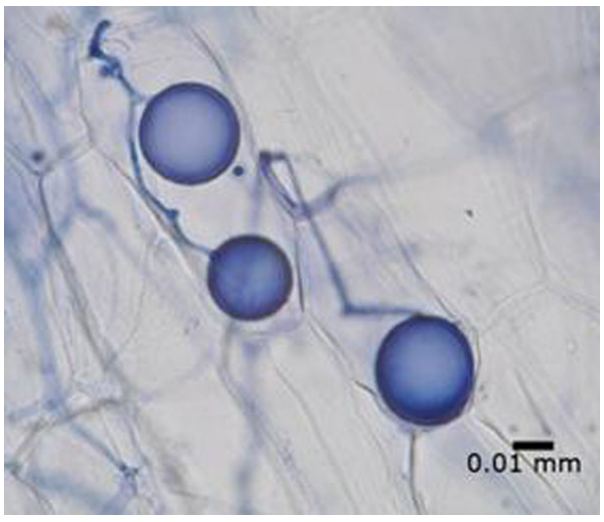

Figure 2(abstract P149) Glomoid mycorrhiza in a cleared teak root and stained with trypan blue, (X400) scale bar = 0.01 mm.

Results and discussion: Optimum root production, (8.6 ± 0.7 roots per shoot) and tallest plants were obtained from shoots exposed to 80 μM IBA for 8 days. However, shoots from higher IBA (160 μM) concentrations had lower survival rates but there was no effect on the growth of the plantlets that survived. Soil type used for acclimatisation did not affect plant height and root area measurements. This allowed the acclimatisation to be conducted in a soil type that is considered most appropriate for mycorrhization development [7].

At 10 weeks, 100% of inoculated plantlets had survived. Mycorrhizal inoculation using unprocessed AM commercial inoculum increased root area (609.9 ± 46.2 mm^2; Fig 1 A & B), a similar result to that of [8] for micropropagated grapes. This was achieved, however, for one clone only. Teak root infection was also achieved using the high concentration of the unprocessed AM commercial inoculum 20 weeks after inoculation. Control plants were not infected. A preliminary comparison of the inocula used showed that early evidence of mycorrhization can also be achieved using soil pot culture (Figure 2) which agrees with [3].

Conclusions: *T. grandis*can be inoculated and *exvitro*mycorrhization achieved during the acclimatization phase of micropropagation. The time required for the mycorrhization appears to be dependent upon inoculum source, and perhaps other factors that are currently being investigated such as inoculum concentration and substrate phosphorus contents. In addition, early results indicate that successful mycorrhization may vary between host genotypes.

The availability of clonal, mycorrhizal teak may lead to greater plantation sustainability through more appropriate fertilizer regimes and greater carbon storage.

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P150

Effect of PGR pulse treatments and ethylene precursor and inhibitors on Scots pine micropropagation

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Background: Conifer clonal forestry as a form of plantation forestry has great potential advantages. However, vegetative propagation of conifers, especially that of mature trees, is quite problematic. These species also are most recalcitrant objects for cultivation in vitro. For this reason the development of an effective system for clonal micropropagation of some conifers is still an important aim. Scots pine (*Pinus sylvestris* L.) is one of the most widespread conifers in the world and in Russia. This important species has high economic value. Among *Pinus* species, Scots pine is especially difficult to deal with in culture [1]. We investigated influence of pulse treatment with BA, GA and auxins on clonal micropropagation of Scots pine. In addition effects of ethylene precursor and inhibitors were analyzed.

Methods: Seeds were collected in 2008 from open pollinated trees growing in the Republic of Belarus (Gomel Region) and were stored at 4-5 °C until used. Seeds were washed under running tap water for 24 h, surface-sterilized in 15% H_2O_2 for 30 min, rinsed in sterile water and germinated on wet filter paper in plastic containers. The germinating seeds were incubated for 10-11 days at 23±1 °C in the dark. Hypocotyls of the seedlings were trimmed to 5-7 mm upper cotyledons, sterilized in 0.2% $Hg(NO_3)_2$ for 4 min, rinsed in sterile water and placed vertically on hormone-free medium PM1 [2], containing 3% sucrose, 0.25% Gelrite gellan gum (Sigma, USA). After one week (BA treatment) or 6-7 weeks (auxin treatment) explants were used for experiments. For shoot induction explants were treated in an aqueous 50 or 100 mg/l BA solution buffered with 0.1 M MES during 0.5, 1.5 or 4 h. For elongation newly formed shoots were treated in 0.2, 1 or 5 mg/l GA3 during 0.5, 1, 2 or 4 h. For rooting pulse treatment by 50 or 100 mg/l NAA and IBA for 6 or 24 h were used. After treatments explants were transplanted on hormone-free medium: MS and PM1 (BA treatment), PM1 (GA treatment), 1/2 DCR (auxin treatment). Effects of various ethylene inhibitors, including $AgNO_3$ (10 and 40 μM) and $CoCl_2$ (10 and 100 μM), and the ethylene precursor ethephon (3 and 10 mg/l) also were studied in our experiments. Culture conditions were 23±1 °C and a photoperiod of 16 h. Rooted shoots were transferred to a peat-perlite (3:1) mixture and acclimatized in a greenhouse under gradually decreasing humidity conditions during 3-4 weeks.

Results and conclusions: The best results after BA pulse treatment were obtained at 4 h exposure to 100 mg/l solution (Table 1). Using this time, about 74.2% of explants formed an average of 3.5 buds. The proportion of vitrified explants was higher on MS medium (up to 76%) than on PM1 medium (up to 33%). It is interesting that we observed shoot regeneration on tips of cotyledons.

The previous studies showed that optimal treatment duration and the concentration of cytokinin are different for each conifer species. For instance, for explants of *P.canariensis* the best exposure time was 4-8 h in 22.5 mg/l BA solution [3]. High concentrations or prolonged exposure can completely suppress shoot formation. Bud induction in *P. wallichiana* was not observed at concentrations above 135 mg/l BA and time more than 3 h [4].

Pulse treatment with 0.2, 1 or 5 mg/l GA solution did not improve shoot elongation. Addition of $AgNO_3$ to medium decreased vitrification and death of explants in comparisons with $CoCl_2$ and ethephon. The treatment with 50 mg/l NAA during 6 h resulted in 44% rooted shoots after 12 weeks of culture, whereas pulse treatments with IBA resulted in 13-19% root formation (Table 2). An average of 1.5-3.2 roots per shoot were produced after NAA treatment and only single root per shoot after IBA treatment.

Similar results were obtained when auxin pulse treatment was applied on other *Pinus* species. When shoots of *P.ayacahuite* were incubated in 18.6 mg/l NAA for 8 h, up to 40% of the shoots were rooted [5]. An exposure of 6 h to 203 mg/l IBA solution resulted in 27.4% rooted shoots of *P. wallichiana*[4]. Rooted plants of Scots pine were successfully acclimatized in the greenhouse with the survival rate 80-90% (Figure 1).

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Table 1(abstract P150) Effect of BA pulse treatment on shoot formation in Scots pine

Treatment, mg/l - hours	Medium	Vitrification, %	Necrosis, %	Explants with shoots, %	Shoots/explant
0-1.5	PM1	33.3	16.7	0.0	-
	MS	63.6	50.0	0.0	-
50-0.5	PM1	20.5	12.2	75.6	3.1
	MS	75.5	15.5	71.4	3.1
50-1.5	PM1	12.8	2.1	53.2	2.6
	MS	73.5	11.0	40.8	2.8
50-4	PM1	5.7	3.6	62.3	3.0
	MS	50.0	12.5	69.0	2.4
100-0.5	PM1	27.8	3.6	64.8	3.2
	MS	44.6	6.7	60.7	2.9
100-1.5	PM1	6.3	11.1	56.3	2.8
	MS	45.2	13.9	54.8	2.6
100-4	PM1	3.2	8.8	74.2	3.5
	MS	23.8	12.5	50.0	1.7

Table 2(abstract P150) Effect of auxin pulse treatment on root formation in Scots pine

Auxin	Treatment, mg/l-hours	Rooting, %	Root/shoot	Root length, mm
control	water-24	3.1	1.0	2.0
NAA	50-6	43.8	1.5	16.5
	50-24	34.4	1.8	17.5
	100-6	37.5	3.2	14.9
	100-24	21.9	2.6	15.8
IBA	50-6	15.6	1.0	40.8
	50-24	12.5	1.0	21.5
	100-6	18.8	1.0	14.2
	100-24	12.5	1.0	12.0

Figure 1(abstract P150) Acclimatized plants of Scots pine in the greenhouse.

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P151

Embryogenic potential and expression of embryogenesis-related genes in conifers are affected by treatment with a histone deacetylase inhibitor

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Background: Somatic embryogenesis is a useful method to propagate conifers vegetatively. In many coniferous species embryogenic cultures can be established from zygotic embryos, while the embryogenic potential decreases during germination. Embryo formation is thought to require a signal that induces a somatic cell to dedifferentiate and gain embryogenic potential as well as the expression of an appropriate cellular environment for the response of the inductive signal. In *Arabidopsis thaliana* (*Arabidopsis*) *LEAFY COTYLEDON* (*LEC*) genes are expressed during the embryonic stage but must be repressed to allow germination [1]. Treatment with the histone deacetylase inhibitor trichostatin A (TSA) causes de-repression of *LEC* genes. In addition, ectopic post-embryonic expression of *LEC1* is sufficient to induce differentiation of embryo-like structures from vegetative cells [2]. *ABSCISIC ACID3* (*ABI3*) and its maize (*Zea mays*) orthologue *VIVIPAROUS1* (*VP1*) act together with the *LEC* genes

to promote embryo maturation [3]. Knowledge about the molecular mechanisms underlying embryogenic competence in conifers is largely uncharacterized, although this is the foundation for propagation of conifers through somatic embryos. However, we have recently shown that TSA-treatment affects both the embryogenic potential and the expression of embryogenesis-related genes in Norway spruce [4].

Materials and methods: Conifer sequences of LEC1-type *HAP3* and *ABI3/VP1* homologues were retrieved from public databases and isolated in Norway spruce (*Picea abies*) and Scots Pine (*Pinus sylvestris*). Phylogenetic analyses were done on nucleotide alignments of conserved domains made using Baysian inference (MrBayes) and maximum parsimony (PAUP*). Embryogenic cell lines 06.28.05 of Norway spruce and 12:12 of Scots pine were used in this study. To analyze the effect of TSA (Sigma-Aldrich) during maturation and germination of somatic embryos of Norway spruce, the growth media were supplemented with 10μM TSA. Expression levels of the conifer *HAP3a* and *VP1* genes were assessed using quantitative real-time PCR.

Results and discussion: We isolated two conifer LEC1-type HAP3 genes, *HAP3A* and *HAP3B*, from Norway spruce and Scots pine. A comparative phylogenetic analysis of plant HAP3 genes suggests that *HAP3A* and *HAP3B* are paralogous genes originating from a duplication event in the conifer lineage. The angiosperm ABI3/*VP1* genes belong to the plant specific B3 gene family and phylogenetic relationship position the conifer homologs closest to ABI3 and VP1. The expression of *HAP3A* is high during early embryo development but decreases during late embryogeny, while the expression of *VP1* is initially low and increases during late embryogeny, in both Norway spruce and Scots pine. The expression levels for both genes are similar during somatic and zygotic embryogenesis. When embryogenic cultures of Norway spruce were exposed to TSA during embryo maturation, maturation was arrested and the expression levels of *PaHAP3A* and *PaVP1* were maintained. Furthermore, when germinating somatic embryos of Norway spruce were treated with TSA, the germination progression was partially inhibited and the embryogenic potential was maintained at a similar level as embryos before germination.

Conclusions: Taken together, our results suggest that important regulators of embryogenesis are conserved between angiosperms and gymnosperms, and, assuming that TSA affects histone acetylation in conifers, our results indicate a connection between between chromatin structure and expression of embryogenesis-related genes in conifers.

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P152

Transgene copy number estimation and analysis of gene expression levels in *Populus* spp. transgenic lines

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Background: The genus *Populus* has certain important features, such as a relatively small nuclear genome, it can be easily regenerated easily *in vitro* and genetically transformed by *Agrobacterium* vector system, which make it ideal for gene transfer and molecular genetic studies in forest trees [1]. Insect-tolerant poplars have been obtained using several types of insecticidal genes coding for *Bacillus thuringiensis*-toxins. Regenerated plants with insect-resistance were obtained in different studies. *Agrobacterium*-mediated transformation has been the favored method for the introduction of foreign genes into plants. The effectiveness of insect-resistance in transgenic plants is related to the side effects of gene transfer (site of gene insertion, copy number, gene silencing etc.). Moreover intransgenic plants, transgene copy number can greatly affect the expression level and genetic stability of the target gene, making estimation of transgene copy numbers an important area of genetically modified plant research [2]. Thus molecular biological analysis of transgenic plants, like real time PCR, widely used to detect and quantify DNA and cDNA [3], could represent an useful tool to investigate the genetic stability of transgenic forest trees having a long life cycleas well as for determining copy number in transformed plants.

Trangenic line	*cry*	*nptII*	Rearrangements
37-2	1	2	yes
80-1	1	1	no
81-1	1	3	yes

Figure 1(abstract P152) Copy number values estimated by real-time PCR. Data are expressed as mean ± 95% confidence limit.

Material and methods: The present study was undertaken to investigate *Populus alba* and *P. tremula* x *P. tremuloides* transgenic lines, obtained via *Agrobacterium*-mediated transformation, carrying *cry1Ab* and *nptII* genes in the T-DNA region. The plants were vegetatively propagated in growth chambers over 2 years. Ten individuals from each clone were planted in containers with "forest soil", and grown in a climate chamber.
Extraction of genomic DNA and RNA from leaves was performed for PCR and Real Time PCR (RT-PCR) analysis to estimate the transgene copy number [4] as well as expression of the inserted gene [5]in transgenic poplar, respectively.

Results and discussion: All lines contained one copy of *cry* gene and two of them showed that the copy number was different for the *cry1Ab* and *nptII* genes, suggesting rearrangements or multiple but incomplete copies of the transferred DNA (Figure 1). The copy number was concordant among the 3 individuals of each lines analysed and with those determined from the same transgenic lines kept in micropropagation for 2 years.

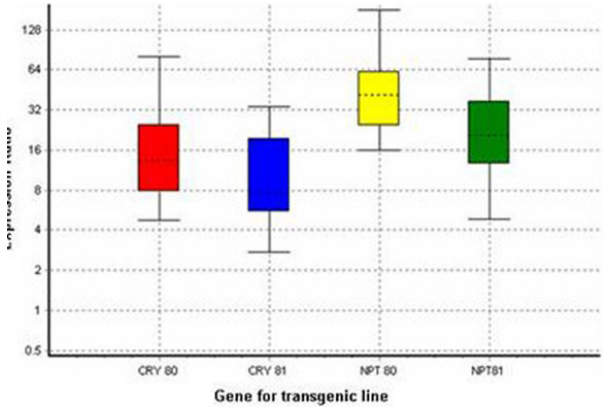

Figure 2(abstract P152) Relative expression obtained through qRT-PCR for the transgenes *cry1Ab* and *nptII* in transgenic *Populus* lines. The data are shown relative to the endogenous *actin* gene. Boxes: interquartile range, or the middle 50% of observations; dotted line: median gene expression; whiskers: minimum and maximum observations. Data are expressed as mean ± 95% confidence limit after 10000 permutations and have a $p = 0.000 - 0.002$.

The transcript levels from both genes were determined in 3 individuals for each line growing in climatic chambers. High levels of mRNA expression were detected with respect to the stable endogenous *actin* gene for both transgenic lines (Figure 2). Comparing the transcript level of inserted genes among lines, a significant low level of *nptII* gene (p = 0.005) in the line carrying 3 copies was observed.

Preliminary results indicate a differential expression of endogenous genes among transgenic lines and towards their isogenic form.

Conclusions: The evaluation of the copy number of the inserted genes has indicated their stability after 2 years of micropropagation. The lower expression level of the *nptII* inserted gene in one line could suggest that factors like position effects or DNA rearrangements lead to differential expression.

The screening of the transcriptomic variations in transgenic plants carrying the *cry* gene and the comparison with position effects or DNA rearrangements is in course. The final aim is to unravel possible pleiotropic transcriptomic effects following *cry* gene expression in *P. alba* and *P. tremula x P. tremuloides* transgenic lines.

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P153

Development of *Eucalyptus* tissue culture conditions for improved *in vitro* plant health and transformability

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Background: Despite its importance as a widely-planted crop tree, eucalypt species and hybrids are relatively difficult to micropropagate, culture and genetically transform *in vitro*. Compared to other plant species, few non-commercial laboratories are proficient at *Eucalyptus* tissue culture and transformation. We have undertaken to establish and transform several eucalypt clones in the laboratory. Our main aims include the identification of clones amenable to culturing and transformation, and the development of robust and transferable micropropagation, organogenesis and transformation protocols to enable routine production of transgenic eucalypts for public sector research. Efficient transformation protocols are essential to take full value of the eucalypt genome for functional genomics, ecophysiology, and biotechnology.

Methods: Five different clones of *E. grandis* (including the clone of which the genome was recently sequenced – Brasuz1) as well as a single *E. grandis* x *urophylla* hybrid were established in the laboratory. Light intensity was studied as a means to reduce browning and promote callus growth and shoot regeneration. Gibberelic acid (GA_3) concentrations in the micropropagation medium (Murashige and Skoog's (MS) basal medium containing 0.05 mg/L benzylaminopurine – BAP) were studied to help produce long internodes to aid transformation *in vitro*. To mitigate phenolic production we used several different antioxidants including ascorbic acid, PVP and PVPP. Shoot regeneration rates were studied by testing a concentration range of cytokinin (zeatin, benzyl aminopurine – BAP and thidiazuron - TDZ). We tested different *Agrobacterium* transformation protocol enhancements including the use of acetorsyringone and two different co-cultivation techniques (whole explant immersion versus pipetting of *Agrobacterium* suspension onto cut leaf edges). We also tested the suitability of kanamycin and hygromycin as selectable markers during transformation.

Results and discussion: Light conditions during shoot regeneration were critical to the rate of organ regeneration, with more than 50% of explants producing shoots in the presence of reduced or indirect light, compared to approximately 30% incubated under normal light conditions. In addition, shoot differentiation occurred earlier (10-14% at 40 days) under reduced or indirect light compared to normal light (1%; Fig. 1).

We have also begun to test the use of muslin screens to identify the best level of light exposure. The optimal level of GA_3 in the micropropagation medium for the production of sturdy elongated internodes was found to be 0.05 mg/L. It was also apparent that the recovery of micropropagated plantlets on medium without GA_3 for 21 days prior to internode harvesting improved plant health and produced better quality stem sections for transformation. Ascorbic acid, used as a means of reduction of phenolic browning, proved to be ineffective when used alone, even though it produced no adverse effects on callus health. By comparing several shoot induction media (SIM) for shoot regeneration from stem and leaf explants of the *E. grandis* x *urophylla* containing different cytokinin combinations, we found that the highest rates of shoot regeneration (11.8%) occurred when using stem explants incubated on SIM containing 1 mg/L NAA (nicotinamide) and 10 mg/L zeatin. Of the five tested *E. grandis* clones, we found that the highest rates of shoot regeneration (52%) were from leaf explants of clone p207 in the presence of SIM containing 3 μM TDZ and 0.1 μM NAA. Neither the use of two different acetosyringone concentrations (250 and 750 μM) during co-cultivation, nor either of the two co-cultivation techniques (immersion and pipetting) had a significant effect on transient gene expression measured shortly after cocultivation. Testing of different kanamycin and hygromycin concentrations ("kill curve"), revealed that 30 mg/L kanamycin or 5 mg/L hygromycin can be used for the selection of putative transgenics after transformation (Fig. 2).

Conclusions: There is significant variability in the performance and response of different clones of *Eucalyptus* during micropropagation, organogenesis and transformation. Testing of multiple *Eucalyptus* clones confirmed that the genus is relatively unwieldy in tissue culture compared, for example, to *Populus*. We found that explant type (stem vs leaf for example), and quality (age, general health, absence callus browning) as well as finely-tuned phytohormone concentrations, will play critical roles in enhancing the probability of the successful generation of stable transgenic lines.

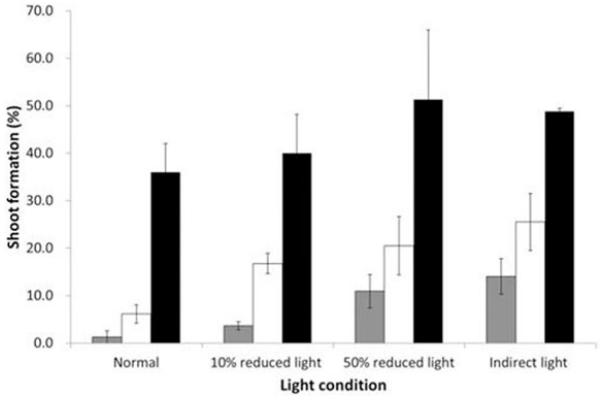

Figure 1(abstract P153) Percentage of shoot regeneration from co-cultivated leaf explants of a *E. grandic x urophylla* clone under different light conditions.

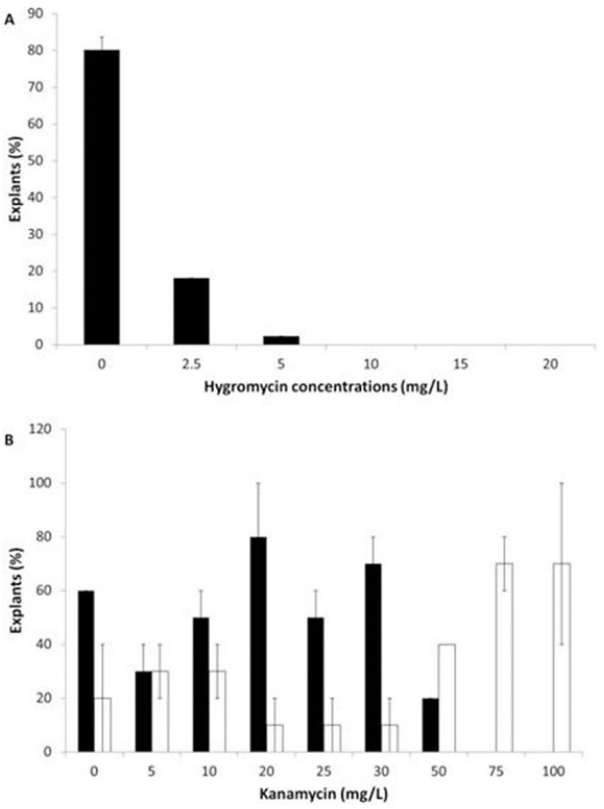

Figure 2(abstract P153) A. Percentage of non-transgenic explants forming callus after 30 days on shoot induction medium (SIM) with different concentrations of hygromycin. **B.** Percentage of explants forming roots (black bars) and exhibiting shoot yellowing (white bars). This experiment was conducted on a single kanamycin-resistant transgenic line of a *E. grandis x urophylla* clone after 30 days on rooting medium.

S8. GENOMIC, PROTEOMIC AND METABOLOMIC TECHNOLOGIES

P154

Functional markers development and genetic diversity analysis in *Eucalyptus globulus* with emphasis in wood quality candidate genes

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Background: *Eucalyptus globulus* is the most planted hardwood species for pulpwood in temperate regions. Genomic researches in *Eucalyptus* have increased the information available in DNA sequences public databases and several structural and regulatory genes involved in the cellulose and lignin pathways are known.
Functional genetic markers, while frequent in crop, are still scarce in forest species. Hence the detection and validation of SSRs in interesting genes to be used in future projects of marker-assisted breeding are needed.
The present study aimed the development of novel functional markers (SSRs) in ESTs and wood quality candidate genes (CG) from *Eucalyptus globulus*, and analyzes their potential for genetic diversity and individual identification studies.
We report the design of SSR primers flanking simple sequence repeats in ESTs and CG, the validation of a subset of randomly selected EST-SSRs using eight *E. globulus* genotypes and the screening of a sample of 60 trees with the polymorphic SSRs. Also, SSRs cross-transferability was tested in seven *Eucalyptus* species coming from three sections: *E. grandis*, *E. saligna* (section *Latoangulatae*); *E. globulus*, *E. dunnii*, *E. viminalis* (section *Maidenaria*); and *E. camaldulensis*, *E. tereticornis* (section *Exsertaria*).
Material and methods: Plant material: A total of 60 trees, each from a different OP family of *E. globulus*, were analyzed for their variability. These sixty trees represented major geographical races of the species' natural distribution that were grown in a field trial in the Province of Buenos Aires, Argentina, between 1995 and 1997. For validation analyses one individual of each race (except Furneaux) was included.
A total of 47 individuals from seven species (including *E. globulus*) of the genus were sampled for the transferability analyses.
Methods: Novel microsatellites were identified mainly by two different methods:
-SSR Mining software (GDR Server, http://www.rosaceae.org/bio/content?title=&url=/cgi-bin/gdr/gdr_ssr) on selected candidate gene sequences identified at the GenBank.
-SSRs detection from non redundant ESTs of *E. globulus* from GenBank: Annotations of these SSR-ESTs were based on the Gene Ontology (GO) (http://www.geneontology.org/) using Blast2GO (http://www.blast2go.org/) [1].
For validation and diversity analyses, amplification products were silver-stained or analyzed through an ABI3100 Genetic Analyzers (Applied Biosystems, USA) with fluorescent dyes respectively.
SSR statistics for determining number and frequency of alleles, effective number of alleles (N_e), observed heterozygosity (H_o) and unbiased expected heterozygosity estimates (UH_e), fixation index (FI) and probability of identity (PI) were determined with the GenAlEx 6.4 program [2]. Tests for Hardy-Weinberg equilibrium were conducted using GENEPOP 4.0.10 [3]. Null allele frequencies were estimated with INEST software (Inbreeding/Null allele estimation) [4], using and Individual Inbreeding Model (IIM) with 10,000 iterations.
Structure analysis was explored through UPGMA and DAS (Shared allele distance) indices as well as Structure [5]software.
Results: From 12,690 updated *E. globulus* EST database published in National Center for Biotechnology Information a total of 4,924 non-redundant sequences were identified. From these ones, 952 unigenes (19.3%) contained 1,140 SSRs. A new set of 979 primers was designed. The predicted functions of these EST-SSRs were adjudged, including biological process, molecular function and cellular component Gene Ontology (GO) categories.
Twenty four structural and regulatory candidate genes for wood quality carrying 29 SSR were indentified. Microsatellite sequences were located in UTR, introns and exons from candidate genes (CG) from: phenylpropanoid biosynthesis, cellulose biosynthetic process, hemicellulose metabolism, shikimate pathway, methionine metabolism, tubulin genes and the transcriptor factor LIM1.
Sixty five percent out of atotal of 85 SSR (56 EST-SSRs and 29 SSR containg GC) detected in this study were validated for actual PCR amplification of tree DNA samples in eight genotypes of *E. globulus*. From this assessment a total of 17 polymorphic EST-SSRs and 12 polymorphic CG-SSRs markers were obtained. These ones were selected for further analyses, so as to accurately estimate genetic information content in a larger sample of 60 non related trees represented major geographical races of the species' natural distribution.
PIC, H_o and UH_e values varied over a wide range from around 0.02 to 0.9, whereas the allele number ranged from 2 to 16, with and average of 7.55. A set of 49 *loci* (37 validated EST-SSRs (polymorphic and monomorphic) and 12 polymorphic CG-SSRs) were also tested for cross-transferability to other six *Eucalyptus* species (*E. grandis*, *E. saligna*, *E. dunnii*, *E. viminalis*, *E. camaldulensis*, *E. tereticornis*).A total of 33 out of the 49 validated markers in *E. globulus* amplified in the six other species and six markers amplified in at least other five.
Finally, the analyses of polymorphism and transferability of functional markers, enabled the selection of a set of 13 (7 EST-SSRs and 6 GC-SSRs) highly informative and transferable to six other species of *Eucalyptus*.
Conclusions: The set of highly informative markers developed here will have potential use in studies of genetic diversity, taxonomy, gene mapping and will help the improvement of *Eucalyptus* trough the assisted selection.

Thirty percent of EST-SSRs (17 from 56) are expected to be polymorphic in *E. globulus* and 25% (14 polymorphic from 56) are expected also to be transferable to other six species (*E. grandis*,*E. saligna*, *E. dunnii*,*E. viminalis*, *E. camaldulensis* and*E. tereticornis*). Under these proportions, potentially more than 200 new EST-SSRs described here may contribute to the verification of synteny and collinearity between different *E. globulus* maps, and would allow the validation of gene and QTL positions in multiple pedigrees in the botanical sections *Maidenaria*, *Exsertaria*, and *Latoangulatae*, to which most of the commercially planted eucalypt species belong.

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Proteomics reveals proteins linked to the quality of wood in contrasting xylem of *Eucalyptus* clones

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Background: Eucalyptus spp. is a genus widely planted in many tropical and subtropical regions of the world as a major source in the production of pulp and paper. Aiming to meet the demand required by the world market papermaker, with the need for greater productivity in forests, as well as higher quality of wood, reducing the amount of lignin and extractive, is that proteomics emerges as an additional tool to help accelerate progress in the selection of elite clones.
Materials and methods: In this study, using the combination of the two-dimensional gel electrophoresis (2D-PAGE) and electrospray mass spectrometry on quadrupole-time of flight (ESI-QUAD-TOF) identify differentially expressed proteins between four genotypes of eucalyptus, brothers siblings, contrasting with differences for components of wood quality. The components evaluated in this study for four xylems were: wood density, which was higher in clones X1 and X2, total lignin content, with levels higher in the X3 and X4 clones, extractive content, with higher values for the clones X2 and X4, and gross yield in pulp, which was higher in clones X1 and X3. Whereas the desirable characteristics for application in pulp and paper industry is the clone that best combines the highest density of wood and pulp yield, with lower lignin content and extractive, the X1 is the clone that best meets these criteria, the most suitable for such application, and X4 clones, considered inadequate for the same process.
Results: The profile followed by 2D gel analysis software with 3D Image Master Platinum xylem protein extracts of these clones resulted in identification of 30 spots differentially expressed, with 3 proteins in clone X1, 4 proteins in clone X2, 11 proteins in clone X3 and 12 proteins in clone X4. These differential spots were excised, subjected to tryptic digestion and processing, followed by mass spectrometry analysis.The identified proteins are involved in various biological processes, including polyphosphate biosynthetic process, catalytic activity, nucleotide-excision repair, cellular metabolic process, cell redox homeostasis, response to salt stress, response to heat, oxidation-reduction process, potassium ion transport, electron transport chain, response to wounding, superoxide metabolic process, response to water deprivation, protein phosphorylation. It is noteworthy that the proteins involved in oxidative stress were up regulated in clone X4, followed by clones X2 and X3, and to a lesser extent in clone X1. Among the proteins differentially expressed in clone X4 can highlight catalase, superoxide dismutase, annexins, heat shock protein (chaperone) and ABC transporter. Catalase is an enzyme that converts 2H2O2 in O2 + 2H2O, primarily by preventing the potential damage caused by changes in H2O2 homeostasis [1]. The superoxide dismutase is the first line of plant defense against reactive species of O2, O2- removing the cellular compartments where this radical is formed [2]. Annexin peroxidase may play a role as the process of oxidative stress in plants [3]. The first reviews of plant ABC transporter showed that they participate in detoxification processes, but evidence already exists of their participation in the flow of ions, and being involved in the process of growth and development of plants [4]. The molecular chaperones known as heat shock proteins (Hsp) are stress proteins. Under stress conditions, Hsp facilitates protein folding and help stabilize polypeptides and membranes [5].
Conclusions: The proteins identified in the study involved in redox process in the plant, as well as the extractives, including polyphenols, tannins and resins, components characterized as inadequate in the pulp and paper industry, are induced as a defense response to pathogen attacks as well as to abiotic stress [1,6]. Furthermore, it has been demonstrated that oxidative stress can also induce lignification in plant tissue [7,8].Given this, we infer that the presence of proteins involved in oxidative stress in the xylem of a eucalyptus is a strong predictor of inappropriate clones in the pulp and paper industry.
Selection of candidate genes and validation study of gene expression through genetic materials in the wood quality will be known the next step in the selection of genetic markers to identify elite clones of eucalyptus for the pulp and paper production.

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Oxidative stress as an indicator of lower quality eucalyptus for pulp and paper industry

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Background: Wood is the most important natural and renewable source of infinite energy, but this is not their only function, it also has great significance to the buildings and other products [1]. Eucalyptus, the genus that includes more than 500 species, is among the key forest species planted around the world to solid wood, and mainly for the pulp and paper industries. Aiming at the sustainable use of timber, supplying the

demand and meeting the requirements of the paper industry, genetic breeding programs looking for, among other agronomic traits of interest, improve the quality of wood, that is, lower lignin and extractive, and increase the density wood and pulp yield. Therefore, it is interesting to identify genetic markers that characterize the major events of xylem differentiation, as well as stress response, because it is key factors to determine some properties of wood, affecting their performance and industrial value. Thus, this study aimed to identify genes specifically involved in oxidative stress response and characterization of genes in determining the quality of the wood.

Materials and methods: To this end, we constructed a Suppression Subtractive Hybridization 3 – SSH3 using two samples of xylem. These samples were extracted from trees of a population of sib eucalyptus from a commercial plantation Suzano with contrasting characteristics to wood quality, among them, lignin content and total amount of extractive, higher for the clone X4, and basic density and cellulose yield, for the largest clone X1, the latter considered as higher for the production of pulp and paper. After identification and selection of genes of interest was the analysis of the expression for Real Time PCR with the same clones of Eucalyptus used in making the library.

Results: From the sequencing and bioinformatics analysis of the library has been possible to identify a total of 449 sequences and 90 contigs in SSH3. Among the most representative unigenes, annotated by the program Blast2GO versão1.2.7 in SSH3 were identified proteins involved in oxidative stress response and heat shock protein, ABC transporter and glutamine synthetase. After analyzing for real-time PCR for validation of these sequences more representative, was unable to confirm the decreased expression of all genes in clone X1 compared with the X4 clone.

Conclusions: The result was expected for these clones, since the plants when in oxidative stress caused by abiotic and biotic agents, activates the defense mechanism through the expression of genes involved in stress response, as well as accumulated extractives as polyphenols, tannins and resins [2]. So, the ABC transporter, for example, between genes possibly involved in this process,carries lignin monomers to enhance the cell wall making it difficult the entry of pathogens [3,4]. The small heat shock proteins, also known as molecular chaperones, although not expressed constitutively, are abundant in plants, especially when under stress, as observed by Harndahl and collaborators, in transgenic Arabdopsis thaliana [5]. As glutamine synthetase, this protein is involved in improving the assimilation of nitrogen by the plant [6]. Other words, in a soil poor in nitrogen, such as soil Formation Barriers/Spodosol where these clones were grown, these proteins must play important role in plant growth,once the availability of this nutrient is limiting factor growth of eucalypts [7]. In addition, this protein has an important role in maintaining the health of the plant toattack by pathogens [8]. Considering this, it was note worthy that the signaling proteins were oxidative stress in Eucalyptus clone, and that the result reinforces the idea that proteins involved in oxidative stress response is related to the high concentration of extractive and lignin.

Preliminary tests in Real Time PCR with the juvenile offspring of these clones are already underway. But in order to reaffirm the presence of these genes involved in oxidative stress to the condition of poor quality for the production of pulp and paper, the expression analysis performed in the progenies of appropriate age and assessment of components involved in wood quality are needed.

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Development, characterization and use of microsatellite markers for genetic analysis of Cashew tree (*Anacardium occidentale*)

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Background: The cashew tree (*Anacardium occidentale*) is found throughout the Brazilian territory, although it is better adapted to the northeastern coast climate. This fruit crop is essential to the agroindustry of the States of Ceará, Piauí and Rio Grande do Norte, where around 95% of the production is harvested and where the whole processing of the chestnut is done. It currently represents 157 million dollars in exports of nuts. Despite its socio-economic importance it still lags behind in the adoption of breeding technologies. There is very little information about the genetics of this species and no molecular tools yet developed. In this work we developed and characterized a set of microsatellite markers for *Anacardium occidentale* from the construction of genomic libraries enriched for repetitive sequences.

Material and methods: The markers developed were used in studies of genetic relationships among samples of the germplasm bank, and saturation of existing genetic maps of the species. Genomic libraries enriched for microsatellites were constructed and out of the 5,472 selected clones, 540 were sequenced and analyzed for the presence of microsatellites. A total of 117 sequences containing microsatellites were selected to design PCR primers and screen the resulting markers. One hundred pairs of primers were synthesized and initially tested for amplification in agarose gel. Fourteen markers were selected and characterized by fluorescent detection in an ABI 377 sequencer based on genotyping a representative set of 35 samples derived from the germplasm collection. Genetic parameters such as observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC) and power of exclusion (PE) for paretage testing were estimated.

Results: Great variability in analytical performance was seen among the tested microsatellite markers.Marker AOBR48, for example, proved to be the marker with the highest polymorphic information content. Analysis of accessions from the germplasm collection using microsatellite markers indicated that cashew and a commonly used dwarf clone likely have distinct origins, since they were separated into different groups based on similarity coefficients. Additionally, A. microcarpum, used as an outgroup control, clustered with accessions of the giant type. An F1 population derived from a cross between the dwarf cashew variety (CCP1001) and the giant type (CP 96) was used to map these microsatellites. Among the hundred new markers developed in this study, 11 showed segregation of alleles from the female parent (CCP 1001) in the F1 population, while 21 showed segregation of alleles from the male parent (CP 96). Only three of the 100 tested markers were fully informative showing segregation of alleles from both parents in the F1 population. In total 29 markers could be mapped in this population and 11 markers were positioned in both, the male and female maps.

Conclusions: An initial set of microsatellites was developed for the Cashew tree. These new markers can be useful for a number of applications in germplasm analysis and breeding. The identification of duplicate accessions in the current germplasm collection, the study

of genetic diversity in natural populations, represent immediate potential applications to better understand the existing diversity available to breed better clones. Genetic mapping of QTLs for some economically important traits such as increased yield and quality of fruits and cashew, and resistance to diseases could be future targets provided a higher marker density is achieved.

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Promoting a functional and comparative understanding of the conifer genome- implementing applied aspects for more productive and adapted forests (ProCoGen)

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In the midst of a climatic change scenario, the genetics of adaptive response in conifers becomes essential to ensure a sustainable management of genetic resources and an effective breeding. Conifers are the target of major tree breeding efforts worldwide. Advances in molecular technologies, such as next-generation DNA sequencing technologies, could have an enormous impact on the rate of progress and achievements made by tree breeding programmes. These new technologies might be used not only to improve our understanding of fundamental conifer biology, but also to address practical problems for the forest industry as well as problems related to the adaptation and management of conifer forests. In this context, the FP7-KBBE-2011-5 project "Promoting a functional and comparative understanding of the conifer genome- implementing applied aspects for more productive and adapted forests" (ProCoGen), granted in 2011 by the European Commission, will address genome sequencing of two keystone European conifer species. Genome re-sequencing approaches will be used to obtain two reference pine genomes. Comparative genomics and genetic diversity will be closely integrated and linked to targeted functional genomics investigations to identify genes and gene networks that efficiently help to develop or enhance applications related to forest productivity, forest stewardship in response to environmental change or conservation efforts. The development of high-throughput genotyping tools will produce an array of pre-breeding tools to be implemented in forest tree breeding programmes. ProCoGen will also develop comparative studies based on orthologous sequences, genes and markers, which will allow guiding re-sequencing initiatives and exploiting the research accumulated on each of the species under consideration to accelerate the use of genomic tools in diverse species. ProCoGen will integrate fragmented activities developed by European research groups involved in several ongoing international conifer genome initiatives and contribute to strengthening international collaboration with North American initiatives (US and Canada). Partners involved in this project are:
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Phylogenetic comparative and expression analysis of genes encoding dof transcription factors from *Eucalyptus grandis*

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Dof proteins are a family of transcription factors specific to the plant kingdom that contain a particular class of zinc finger DNA binding domain. Members of this family are involved in the regulation of genes related to a plethora of metabolic processes including stress or hormone response, seed and endosperm development, flowering, carbohydrate metabolism, and cell or tissue specificity. Dof proteins and encoding genes were characterized in several plant species. Nevertheless poplar is the only woody species whose *Dof* genes were better characterized. The recent availability of the *Eucalyptus grandis* genome and transcriptome, along with transcription factor databases of several plant species allowed us to identify and run a valuable comparative analysis of the Dof protein family in this tree. These species included *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Carica papaya*, *Populus trichocarpa*, *Vitis vinifera*, *Sorghum bicolor*, *Chlamydomonas reinhardtii*, *Oryza sativa indica* and *Zea mays*.The phylogenetic relationships among Dof proteins from *E. grandis* and *Arabidopsis thaliana* - a popular model for studying the genomics of many plants - is a fundamental step to unravel functionality of new *Dof* genes not yet characterized. Twenty-three distinct DNA sequences were predicted to belong to the *Dof* gene family after the analysis of the complete available genome of *E. grandis*. The deduced protein sequences of 22 members do contain a conserved Dof domain. One sequence seemed to have lost the conserved Dof domain, suggesting it to be a pseudogene or to present an activity not directly linked to the Dof family. Gene structures, including exon/intron positions, and amino acid sequences were predicted for each gene based on the available *E. grandis* transcriptome. In order to determine the relationship and function of the genes putatively encoding Dof proteins, we carried out a phylogenetic analysis with 43 Dof protein sequences from *A. thaliana*. Our analysis allowed us to classify the *E. grandis Dof* sequences into five groups of orthologous genes. Gene expression analysis via real time, quantitative PCR was also conducted with ten of the *E. grandis Dof* genes, using samples obtained from flowers, leaves and vascular tissue. Generally, *Dof* steady-state mRNA levels were higher in *E. grandis* vascular tissues, with more reduced levels in flowers. *Dof* genes showed an increase in steady-state mRNA levels after hormone signaling, and reduced levels following abiotic stress. This is the first study that aimed the identification of *Dof* genes in *E. grandis* which are possibly involved in numerous plant metabolic processes. The phylogenetic relationship to *A. thaliana* counterparts and the patterns of mRNA accumulation in *E. grandis* allowed us to speculate on possible roles for some of the Dof-encoding genes.
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Mitochondrial iron-sulfur cluster genes in *Eucalyptus*

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Iron-sulfur [Fe-S] clusters are prosthetic groups required to maintain life processes including respiration, photosynthesis, metabolic reactions, sensing, signaling,and gene regulation. In plants the biogenesis of Fe-S proteins is compartmentalized and adapted to specific needs of the eukaryotic and photosynthetic cell. Although critical to so many fundamental metabolic pathways and drastically affecting plant adaptability and productivity, Fe-S proteins were never investigated in woody species. *Eucalyptus grandis* is an important economical tree widely cultivated in subtropical regions which suffers under low temperature stress. Here we describe a transcriptional analysis of the *E. grandis NFS1*, *ISU1* and *ISA1*, three genes involved in the biogenesis of [Fe-S] clusters. Microarray analyses were carried out for the comparison of global gene expression in leaves and vascular tissues (xylem) of E. grandis and vascular tissues of E. globulus. In general, leaves from *E. grandis* demonstrated higher expression of these genes than xylem. *EgrISU1* had a constitutive expression in E. grandis, but its expression pattern was higher in this species than in *E. globulus* xylem. Differences observed in the relative gene expression profile between xylem tissues from the two Eucalyptus species suggest that these genes may be implicated in the contrasting phenotypic characteristics of their wood. The response of these genes to a series of hormonal and stress signals over *E. grandis* seedlings was also evaluated by RT-qPCR. After the chilling treatment of seedlings, *EgrNFS1* and *EgrISU1* showed 6 to 8-fold and 0.6 to 1.7-fold increase respectively; and*EgrISA1* exhibited a drastic 69 to 114-fold increase. These data are the same observed in *Arabidopsis* microarrays available in GeneVestigator database. These results suggest that (i) NFS1 and ISA1 may be related to the cellular response to stress caused by chilling, and (ii) the increase in the expression is probably due to sulfur metabolism. A time-course chilling experiment was also carried out. The ISU1 gene expression was higher in the first two hours of treatment and decreased right after that period. The ISA1 gene, which showed the highest expression in the previous experiment, didn't show significant differences in the expression pattern during the 16 hours of chilling, as well as the NFS1 gene. Our data indicated that Fe-S proteins are possibly involved in the recovery of plants after chilling stress.

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Gravitropic response in radiata pine seedlings. Searching molecular keys

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Coniferous trees develop compression wood in response to gravitropic stimuli. In nature this response can be generated by growth in slope, exposure to snow or high winds [1]. However, genes and molecular mechanism involved in this phenomenon are still unknown. We studied gene expression in response to gravitropic stimulation induced by 45° inclination in *Pinus radiata* D. Don one year old seedlings. To characterize the gravitropic response, whole seedlings were inclined and transversal cuts were performed in order to identify morphological wood characteristics. Xylem cells were visualized by optical microscopy in a time course experiment (fig. 1). On the other hand, a transcriptomic approach was assayed generating libraries based on the Suppressive Subtractive Hybridization (SSH) strategy [2]. This technique allows the isolation of genes differentially expressed between two samples. The libraries were generated from total RNA extracted at 2.5, 10, 24 hours and 30 days from the inferior and superior stem half. The sequences obtained were assembled, analyzed and ontology classified by biological process, molecular function and cell components. The information give clue about the molecular mechanism related to this phenomenon. To validate the differential gene expression by qPCR analyses, housekeeping genes were evaluated in order to have normalization genes for the gravitropic stress response. We could identify a large number of genes activation involved in different initial process, previous to the anatomical hallmarks of compression wood formation.

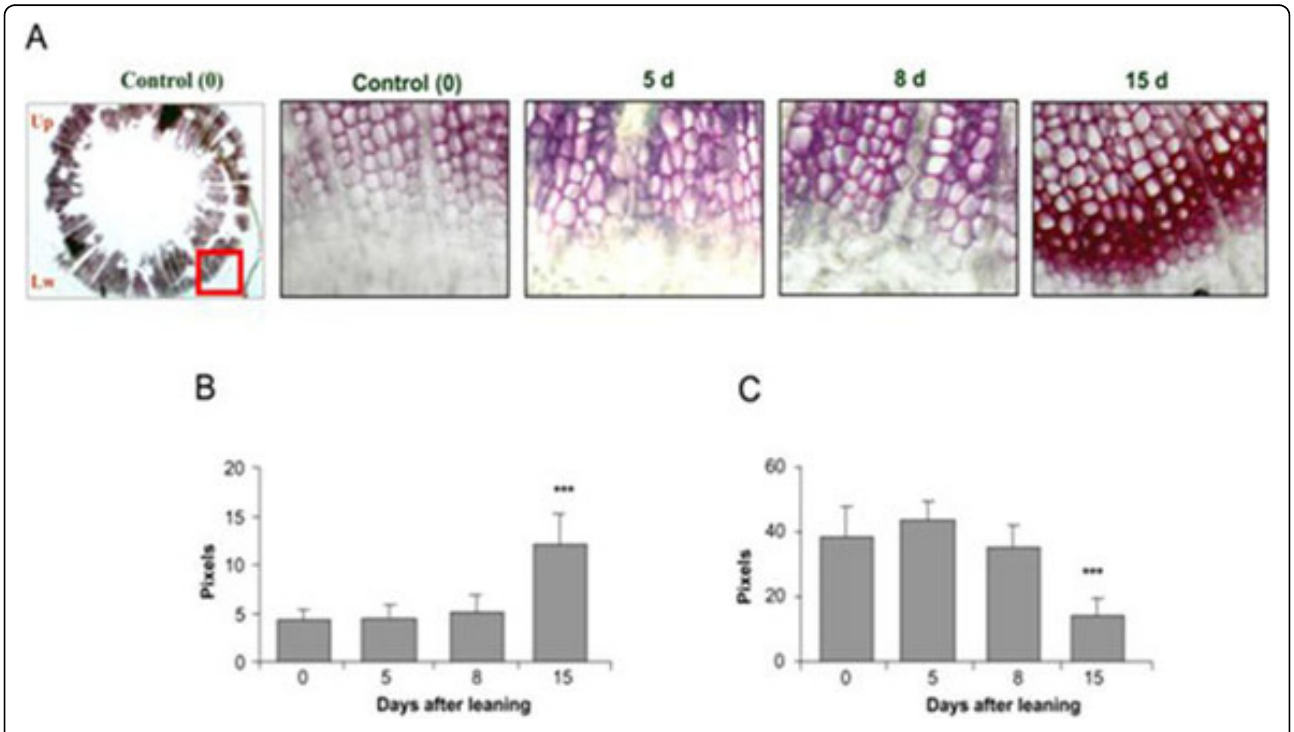

Figure 1(abstract P161) Anatomical modification on lignin deposition and xylem cell shape on inclined seedlings.

One-year old *Pinus radiata* seedlings were inclined during 15 days (d) and transversally cut at 5d, 8d and 15d. Stem slices were stained using a solution of phloroglucinol (2A). From these preparations, wall thickness (2B) and cell diameter (2C) of xylem cells were measured.

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P162

Proteomics, a systems biology based approach to investigations of *Jatropha curcas* seeds

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Background: *Jatropha curcas*(*Euphorbiaceae*) is cultivated for harvesting the unique oil contained in its seeds, which can be used as raw material for the production of biodiesel [1]. The seed cake remaining after pressing can not be used for animal feed, because it contains toxic proteins and other compounds, e.g. curcin, phorbol esters. The development of genotypes better suited for the production of biodiesel showing lower levels of toxic/allergenic proteins is being hampered by a lack of understanding the a) metabolic pathways and enzymes leading to the production of fatty acids during seed development and b) role of proteins deposited during seed development. In recent years the field of proteomic research has become a fast growing discipline with high relevance to biological sciences.

While genomic and transcript-profiling studies have provided a wealth of information about different plant developmental processes, there is growing awareness that the abundance of mRNA transcripts is not always representative of protein levels and that mechanisms of post-translational regulation must also play an important role.

Proteomics is a systems biology based approach investigating the whole expressed proteins at a given time point and under certain condition [2]. It has been shown to be a valuable tool for studying the biology of living organisms and their interaction with the environment in post genomics area. Proteomics has been shown to have potential values to deliver knowledge about complex biochemical processes and is being used in various fields of modern botany and agriculture like plant biomarker discovery related to resistance as well as drought and water stress. Furthermore proteomics offer major advantages linked to its high throughput capacity and its ability to perform simultaneously the analysis of hundreds of proteins from the same samples.

Methods: The method of choice for proteome analysis is the combination of high resolution protein separation like two dimensional gel electrophoresis (2-DE) with tandem mass spectrometric (MS/MS) identification of proteins. The two dimensional gel electrophoresis separates proteins based on their isoelectric points (isoelectric focusing) and in a second dimension based on their molecular weight [3].

In this study a proteomic approach was conducted in order to identify the expression patterns of interesting proteins during seed development, as well as toxic and/or allergenic proteins in Jatropha, 2-DE coupled with mass spectrometry and *de novo* sequencing, were employedto analyze whole seed proteins of six developmental stages, covering the essential ontological phases of these important plant organs.

Prominent spots identified in 2-DE analyses were excised, washed, reduced with DTT and alkylated with iodoacetamide. The in-gel digest with trypsin was carried out for 8 hours. The peptides were de-salted using µZip-Tips C18 (Millipore) and 0.5 µl were spotted onto a disposable AnchorChip MALDI target plate pre-spotted with a-cyano-4-hydroxycinnamic acid (PAC target, Bruker Daltonics). Data were acquired on a Matrix Assisted Laser Desorption Ionisation Tandem Time-of-Flight (MALDI-TOF/TOF) mass spectrometer (Ultraflex II, Bruker Daltonics) in MS and MS/MS modes. Spectra processing and peak annotation were carried out using FlexAnalysis and Biotools (Bruker Daltonics).

In order to enhance the quality of tandem mass spectrometry (MS/MS) spectra for de novo sequencing, N-terminal chemical modification using 4-sulfophenyl isothiocyanate (SPITC) was carried out for 1 h at 56°C [4]. The derivatised peptides were de-salted a second time. Again 0.5 µL were spotted onto a PAC target and analyzed as described above.

For standard database searches, processed spectra were searched via Mascot2 in the Swiss-Prot database and in NCBInr. Identifications were considered statistically significant where $p < 0.05$. Peptide deNovo-sequencing was carried out manually using FlexAnalysis. The sequences were then used for a homology search using MS-homology with the following search parameters: taxonomy Viridiplantae using pre-search parameter 9VIRI (organism code from the UniProt Knowledgebase); Database UniProtKB.2011.01.11; Score Matrix BLOSUM62; 30% amino acid exchanges allowed.

Results and conclusions: In 2-DE analyses it was possible to identify 100 non-redundant protein spots. Identified proteins belonged to different functional classes, including proteins involved in metabolism, protein destination and storage, and energy metabolism, which were highly representative.

The significant change in abundance of protein spots during the different developmental stages of seeds indicated that several pathways were involved in the biosynthesis of these interesting compounds, but vary during the growth phases of plant organs, in this case seeds.

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P163

Cloning and Functional characterization of the promoter of a high affinity potassium transporter gene from *Eucalyptus grandis*

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The characterization of organ/tissue-specific promoters is of great interest to transgenic production. The construction of expression cassettes containing tissue-specific promoters is a viable alternative to limited transgene expression to specific organs and cell types. In this context, the purpose of this study was to functionally characterize the promoter of a *Eucalyptus grandis* gene encoding a high affinity potassium transporter (named EgHAK) shown to be specifically expressed in roots. For that, the 5'-flanking region of *EgHAK* (1,3 kb) was cloned and transcriptionally fused to the b-glucuronidase reporter gene (GUS), and then used to transform tobacco leaf discs. Histochemical analysis of GUS activity in transgenic plants showed that GUS staining was mainly detected in vascular tissues of leaf and root. To investigate the response of the

studied promoter to potassium starvation, a hydroponic system was employed. In this case, enhanced GUS staining was observed in the roots of plants starved for 6 days when compared to control ones. Moreover, a weak induction of the promoter at low potassium conditions was observed using fluorimetric assays. Thus, our results indicate that, in a heterologous system, the studied promoter shows preferential expression in roots in the absence of potassium.

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Identification of potential transcriptionally active Copia LTR retrotransposons in *Eucalyptus*

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Background: Long Terminal Repeat retrotransposons (LTR-RTs) represent the most abundant genomic component in all plant genomes thus far investigated. They are transposable elements that replicate through a "copy/paste" mechanism that relies on reverse transcription and integration of a RNA intermediate. Plant LTR-RTs can be divided in two major superfamilies: *Copia* and *Gypsy*[1]. LTR-RTs have impact on genome size variation, as well as in the expression of adjacent genes in their host genomes, providing a "genomic plasticity" [2]. Their transcription was believed to be extremely repressed in plants. However, despite their potential mutagenic and deleterious effects, LTR-RTs were proven to be transcriptionally active in several plant species [3].
Eucalyptus is one of the most commercially important forest genus in the world, due to their superior growth, broad adaptability and multipurpose wood properties. Most molecular studies in *Eucalyptus* are focused on cellulose production and wood development, and there are few works on genome composition, structure and evolution. *Pinus* and *Populus*, the tree genera with most available genomic resources, have several works analyzing their repertoire of LTR-RTs [i. e 4, 5], but only one study characterized LTR-RTs in *Eucalyptus*[6], with no detailed manual checking or phylogenetic analysis. Here, we used FOREST database as a starting point to identify transcriptionally active *Copia* LTR-RTs in *Eucalyptus*, that were further analyzed regarding their *in silico* expression, evolutionary diversity, and distribution in public genomic databases.
Methods: A previous survey with 88 *Copia*LTR-RTs from diverse plants defined six major common evolutionary *Copia*lineages [7]. The 22 *Arabidopsis thaliana* families analyzed in that study were used as queries to the identify *Eucalyptus*EST sequences related to *Copia*elements in FORESTS database [8], by tBLASTx (e-value >1e-50). Sequences were then analyzed in RepBase [9] to confirm their similarity to *Copia* LTR-RTs. *Eucalyptus*ESTs with >200bp of *copia*-like retrotransposon fragments were used to identify complete copies in *Eucalyptusgrandis* genome v 1.0 in a BLASTn search (identity >80%; in a region >250bp). We picked up 10000bp surrounding the aligned region, that were analyzed using LTR-Finder [10] and LTR_STRUC [11]. Full-length LTR-RTs were then used as queries in GenBank to retrieve related *Eucalyptus*EST sequences (>200bp; >80% identity). Phylogenetic analyses using the reverse transcriptase of these elements (alignment in MUSCLE, Maximum Likelihood method, bootstrap 1000 replicates) were done using MEGA 5.01 [12].

Table 1(abstract P164) Overall features of LTR-RTs analyzed

Family	Lineage	Genomic copy number	FOREST cDNA libraries	GenBank cDNA libraries
RTE_*copia*_Eu_ 1	*Ale*	28	seedlings	xylem
RTE_*copia*_Eu_ 2	*Ale*	262	roots, leaves	xylem
RTE_*copia*_Eu_ 3	*Ale*	24	root	xylem, cold-stressed
RTE_*copia*_Eu_ 4	*Angela*	243	seedlings, calli	xylem
RTE_*copia*_Eu_ 5	*Ivana*	54	leaves, root, calli, wood	xylem
RTE_*copia*_Eu_ 6	*GMR*	63	leaves, seedlings	xylem

Figure 1(abstract P164) Phylogenetic tree of Copia LTR-RTs elements. Based on Du and collaborators (2010).

Results: Stem, calli and seedlings were the cDNA libraries from FOREST database with most EST sequences, in this *Copia* LTR-RT search. We identified 20 consensus sequences (total: 36 ESTs) from 3 tissues, roots, leaves and flower-buds. We also identified 29 ESTs in GenBank from xylem, root apex and cold-stressed plants (Table 1). Using EST data, we identified six full-length retrotransposons families that had different copy number in the *Eucalyptus* genome, estimated by BLAST searches (cutoff 1e-50). Copy number ranged from 24 to 262 (Table 1). Phylogenetic analyses showed that they are members of the *Ale*, *Angela*, *GMR* and *Ivana* evolutionary lineages (figure 1). *Ale* was the evolutionary lineage encompassing families with highest and lowest copy number (Table 1).
Conclusion: In summary, the present data demonstrate the potential impact of future studies about functional and genomic analysis of LTR-RTs in *Eucalyptus*. This is the first characterization of full-length *Copia* LTR-RTs families in *Eucalyptus* genome with potential transcriptional activity, giving insights about phylogenetic diversity and copy number variation of retrotransposons in this tree.
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P165

Bioinformatic prediction of the AP2/ERF family genes in *Eucalyptus grandis*: focus on the CBF family

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Background: Due to their very high economic importance, Eucalyptus tree species are among the most planted hardwoods in the world with over 20 million hectares. However, as long-lived evergreen species, this genus is particularly exposed to cold. Frost tolerance varies among species and is inversely correlated to productivity. The AP2/ERF gene family includes developmentally and physiologically important transcription factors characterized by the presence of the AP2/ERF DNA-binding domain. AP2 proteins contain two AP2-like domains and RAV family proteins contain one AP2 domain and one B3 domain. ERF family proteins exhibit only one AP2 domain and are further divided into the DREB subfamily and the ERF subfamily [1]. The CBF/DREB1 protein differ from the other DREB proteins by the presence of "signature sequences" (PKK/RPAGRxKFxETRHP and DSAWR) flanking the DNA-binding AP2 domain [2]. The DREB factors recognize the C-repeat or dehydration response element (DRE) in the promoters of low temperature and/or water deficit responsive genes and would play a crucial role in response to abiotic stresses. CBF/DREB1 are the key regulators of the cold-responsive (COR) genes. So far CBF transcription factors have been mainly characterized in model plants such as Arabidopsis, but lately they were identified in several tree species including Eucalyptus [2]. The Eucalyptus cold tolerance was greatly improved in our hands when two genes from the four CBF members isolated from a tolerant species *E. gunnii* were individually constitutively overexpressed in the frost sensitive *E. urophylla* x *E. grandis* hybrid [3]. In the present study *E. grandis* AP2/ERF family genes were identified based on the presence of putative encoding AP2-domain(s) and were studied with regard to the model herbaceous Arabidopsis as well as the main sequenced woody plants. Within this family, a part of the study focused on the CBF/DREB1 subfamily which was compared to the four genes already characterized in *E. gunnii*[*2*].
Methods: We used the available annotated *E. grandis* genome sequence at phytozome (http://www.phytozome.net/eucalyptus.php), the *Arabidopsis thaliana* AP2/ERF family downloaded from the DATF (Database of Arabidopsis Transcription Factors) database website (http://datf.cbi.pku.edu.cn/), and the sequences of AP2/ERF gene family from the grapevine, and the poplar obtained from several publications. In order to get the exhaustive gene AP2 family and the location of the genes of the *E. grandis* genome, we used Scipio [4]. Every identified protein was analyzed for structural motifs by scanning them against PROSITE patterns and profiles (http://www.expasy.org/prosite) and against Pfam (http://pfam.sanger.ac.uk/) to make sure of the presence of AP2 and/or the B3 domains. A fine correction of the predicted AP2/ERF proteins was performed. In order to identify all the members of CBF subfamily, a search within the *E. grandis* annotated AP2/ERF family was performed using consensus sequence including the two highly conserved signatures (PKKPAGR and DSAWR) surrounding the DNA-binding AP2-domain. As a check, we confirmed by blasting the sequences on the NCBI blast page. From a multiple alignment analysis performed with Clustal W, the phylogenetic trees were generated using MEGA version 5. The resulting phylogenetic trees were based on the maximum likelihood (ML) method [5].
Results and main conclusions: Based on the sequence alignement, the phylogenetic analyses, and the known criteria described in litterature, this study revealed that the annotated AP2 genes from *E. grandis* were divided into the four subgroups already described for Arabidopsis (DREB, ERF subfamilies, AP2 subfamily and RAV subfamily). The two subfamilies DREB and ERF were separated according to the similarity of the sequences of the AP2/ERF domain and to the amino acid at 14 and 19 positions. Distribution of the DREB members in the four dicotyledonous plant genomes (Vitis, Arabidopsis, Populus and Eucalyptus) shows similarities between grapevine and *E. grandis* except for the A1 subgroup which corresponds to the CBF genes. Interestingly this group is much larger in *E. grandis* compared to the three other plant species. The 17 CBF genes (including one pseudogene) identified on *E. grandis* genome exhibit a very good conservation when compared to the *E. gunnii* CBF genes. The *E. grandis* CBF genes could be classified into the four groups (a, b, c et d) described for *E. gunnii* CBF. Most of the *Egr*CBF were localized on the chromosome 1, one member is on the chromosome 4 and the last two on the chromosome 5.
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P166

Exploring a new model of cell wall regulation: identification and expression of two putative SHINEs transcription factors in *Eucalyptus*

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Background: *Eucalyptus* forests are a competitive and efficient alternative to convert carbon from the atmosphere in cellulose, an important source for paper manufacture and bioenergy production.
To obtain transgenic *Eucalyptus* with important traits improved it is necessary to make modifications in genes that affect the final phenotype. One interesting gene that follows this requisite was recently found: this is the *AtSHN2* gene (*Arabidopsis thaliana SHINE 2*).
AtSHN2 codifies to a Transcription Factor known as "Arabidopsis *SHINE/ WAX INDUCER*". Instead of inducing drought tolerance in transgenic rice (*Oryza sativa*), *AtSHN2* overexpression causes: i) 34% increase in the cellulose content; ii) 45% reduction in lignin content and iii) increase in wood digestibly (elevated S:G ratio) with no compromise in plant strength and performance [1].
The discovery of *AtSHN2* function in plant cell wall formation, led Ambavaram and collaborators [1] to perform other studies and ultimately to propose the following model: *AtSHN2* regulates positively *MYB* transcription factors (TF) related to cellulose synthesis and it down-regulates *MYBTF's* related to lignin formation. At the same time, *SHINE* can repress *NAC TFthat controls MYB expression*[*1*].
As a consequence of the interesting phenotype achieved through *AtSHN2* overexpression in rice, this work focused on the identification and analyses of *AtSHN* orthologues in *Eucalyptus*. Bioinformatics tools were used to search for *AtSHN* similar genes in *Eucalyptus*. Moreover, the expression profile of the corresponding genes in *Eucalyptus* was evaluated to prove their role as *AtSHN*. To carry it on, the expression experiments were done with flower, leaf and xylem. If the *Eucalyptus* putative*SHINE's* has the same function of the *AtSHN's*,, gene expression in flower tissues will be the highest [2]. This is because it is known that *AtSHN's* genes are preferentially expressed in abscission and dehiscence zones, a phenomenon that usually occurs in lots of flower tissues.

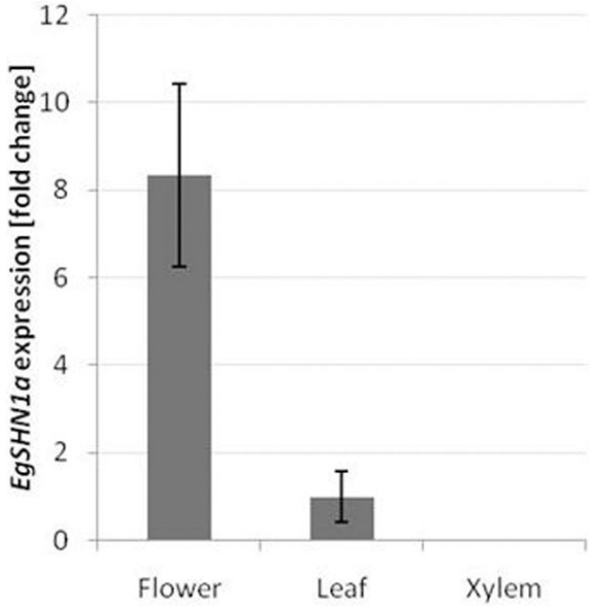

Figure 2(abstract P166) *EgSHN1a* expression ratio in three different tissues: flower (rich in abscission and dehiscence zones), leaf and xylem assessed trough qRT=PCR. Data are expressed as fold change and leaf was chosen as reference condition. Error bars representSE(n=3).

Material and methods: Putative transcription factors of the SHINE family were searched in *Eucalyptus* by comparing the *AtSHN* amino acid sequences with the *Eucalyptus* genome assembly (obtained from *Eucalyptus grandis* – http://eucalyptusdb.bi.up.ac.za).
This analysis revealed the existence of two sequences with high similarity to *AtSHN* proteins. Quantitative RT-PCR assays were carried out to verify the expression profile of these genes in different tissues (leaves, flowers and xylem) of the hybrid variety *E. urograndis* (*E. uroplhyla* x *E. grandis*). The RNA extraction was carried out following the protocol described by Zeng & Yang (2002) [3]. The qRT-PCR was performed with the *SYBR® Green PCR Master Mixkit* from *Applied Biosystems*. The Results were analyzed according to the mathematical method described by Pfaffl, 2001 [4].
Plant materials were provided by the International Paper Company, Brazil.
Results and discussion: The comparison of the *AtSHN* amino acids sequence with *E. grandis* genome revealed the existence of two putative *SHN* genes in *Eucalyptus* (named *EgSHN1a* and *EgSHN1b*). A phylogenetic

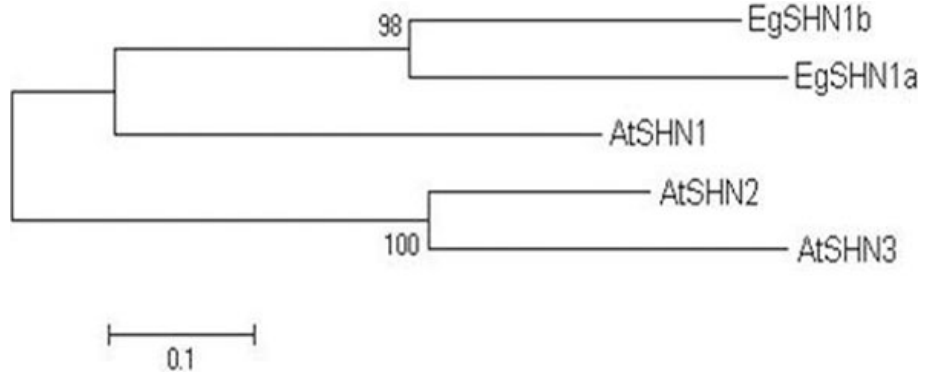

Figure 1(abstract P166) Phylogenetic analysis of SHN genes from *A. thaliana* (*AtSHN1, AtSHN2 and AtSHN3*) and the new SHN genes identified in *Eucalyptus grandis* (*EgSHN1a* and *EgSHN1b*). The scale bar of 0,1 corresponds to 10% sequence divergence. Bootstrap values are given for nodes and are considered as value of significance of the branches.

analysis showed that these *Eucalyptus* SHN genes are orthologs to the *AtSHN1* of*Arabidopsis thaliana* (Figure 1).

The proteins encoded by *EgSHN1a* and *EgSHN1b* share approximately 58% identity to *AtSHN1*and a conserved gene structure is found between *AtSHN* and both *EgSHN* genes: a single intron is present and located approximately 80 bp from the start codon. In addition, as in *AtSHN1*, *EgSHN* genes contain an AP2 DNA binding domain, which is in accordance to their putative role as transcription factors. More importantly, the domains "mm" and "cm", which are exclusive of SHN genes, could be identified in the *Eucalyptus* sequences. In the same way that the gene sequence is very correlated between the species, it's probable that their function are the same two.

Gene expression analysis revealed a higher expression of *EgSHN1a* in flowers (Figure 2). This result is similar to the *AtSHN1* expression profile [2], suggesting a similar function for *EgSHN1a*.

The expression pattern of the gene *EgSHN1b* is being carried on.

Conclusions: There are two SHN genes in *Eucalyptus* (*EgSHN1a* and *EgSHN1b*), which are orthologs to *AtSHN1*;

The *EgSHN1a* function might be the same of *AtSHN1* as suggested by their similar expression patterns. It is possible that, in *Eucalyptus*, biosynthesis of cuticle and cell wall in abscission and dehiscence zones is regulated by *EgSHN*'s, as already described in *A. thaliana* [1].

The new genes described here are interesting candidate for the development of transgenic *Eucalyptus*. Overexpression of SHN genes in *A. thaliana* increased tolerance to drought and pathogen attacks and greatly improved cell wall quality.

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P167

Transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* overexpressing the *Eucalyptus grandis* Cellulose Synthase 3 and its expression pattern in different *Eucalyptus* species and tissues

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Background: In Brazil, the forest industry accounts for 4,5% from the Gross Domestic Product and the country is the biggest *Eucalyptus* cellulose exporter. That's really good news because *Eucalyptus* forests are a competitive and efficient alternative to convert carbon from the atmosphere in cellulose, an important source for paper and bioenergy production.

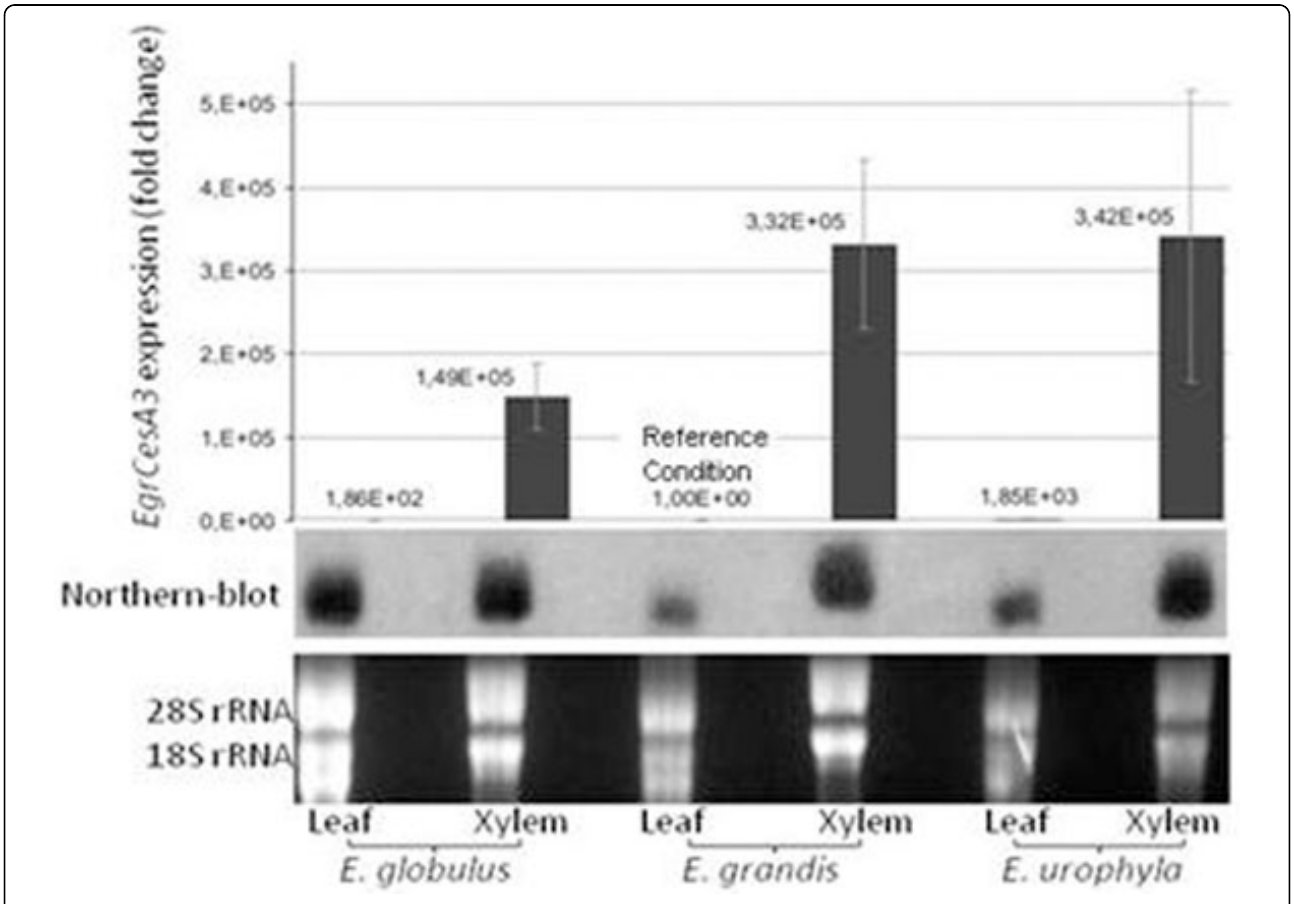

Figure 1(abstract P167) *Eucalyptus Cellulose Synthase 3* expression pattern. On the top, expression pattern analyzed trough qRT-PCR and, below, the expression analyzed trough Northern-Blot. The tissues studied were leaves and xylem from three different *Eucalyptus* species: E. *globulus*, *E. grandis* and *E. urophyla*.

The cellulose biosynthesis happens through the *Cellulose Synthase Complex* activity. This complex is composed by different *Cellulose Synthase* genes (*CesA*) that work together in a non redundant way [1]. It is also known that some of these isoforms act in the primary cell wall synthesis while others, in the secondary cell wall. In this last group there is the gene *Eucalyptus Cellulose Synthase 3* (*EgCesA3*), the most expressed *CesA* gene during xylogenesis [2]. Besides, knockout experiments proved that the *AtCesA7* (*EgCesA*3 ortholog in *Arabidopsis thaliana*) activity is essential for the xylem formation and for plant vertical growth [3].

In front of these evidences, the *EgCesA3* gene had it expression pattern evaluated in leaf and xylem tissues among the three most economic important *Eucalyptus* species in Brazil: *E. grandis*, *E. globulus* and *E. urophyla*.

As demonstrated in this work, the expression experiment provided enough information about the *EgCesA3* function, that's why this gene was chosen to be overexpressed in model plants (*Arabidopsis thaliana* and *Nicotiana tabacum*). It's expected increased cellulose content in the transgenic plants xylem.

Material and methods: The *EgCesA3* expression pattern was examined trough qRT-PCR and Northern-Blot in xylem and leaf from the tree most commercially important *Eucalyptus* species: *E. grandis*, *E. urophyla* and *E. globulus*. The qRT-PCR was performed using SYBR Green and amplicons with approximated size of 100pb. On the other hand, the Northern-Blot was made with probes containing phosphorous-32 radioactive.

To create transgenic plants with improved cellulose content this work overexpressed the gene *EgCesA3* under the control of the CaMV 35S promoter in *A. thaliana* and *Nicotiana tobacum* trough *Agrobacterium* transformation by floral-spray.

Results and discussion: The results show that the *EgCesA3* is strongly more expressed in xylem than in leaves among the three most important economical *Eucalyptus* species for the Brazilian forest industry (Fig. 1). This data corroborate to the theory that this gene is related to the secondary cell wall formation during the xylem development.

However, it's necessary to mention that the gene expression data obtained through the northern-blot related to the *E. globulus* leaf can't be analyzed. This because the amount of RNA used in this sample was different from the others. It can be seen through the rRNA amounts in the RNA electrophoresis (Fig. 1).

Besides, comparing the*EgCesA3* expression pattern between the xylem from the three species studied, it's possible to conclude that should exist a difference: the *EgCesA3* gene is apparently more expressed in *E. grandis* and *E. urophyla* xylem than in *E. globulus* xylem (Fig. 1). However, this conclusion isn't distant from doubts: the problem is the large error bars that happened in the experiment. In order to solve it, the qRT-PCR repetition is being carried on.

In front of the *EgCesA3* rolein the secondary cell wall synthesis, this gene was overexpressed in model plants (Fig. 2). The genetic modified plants were successfully obtained. Thus, after the generation advance, the homozygous transgenic plants will be evaluated to measure the cell wall chemical composition and morphology.

Conclusion: The *Eucalyptus Cellulose Synthase 3* function in the secondary cell wall synthesis was confirmed by this work;

The cellulose content variation trough the species studied should be a consequence of the *Eucalyptus CesA3* expression level;

Transgenic *A. thaliana* and*N. tabacum* overexpressing the *EgCesA3* gene were successfully obtained. The transgenic plants analyses are being performed; however it's already possible to notice, by empirical view, growth improvement in the transgenic plants in comparison to the wild type.

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P168

In silico comparative analysis of glycoside hydrolase (GH) family 10 endo-(1-4)-beta-xylanase genes from *Eucalyptus grandis* and *Arabidopsis thaliana*

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Background: The hemicellulose xylan constitutes the major non-cellulosic component of plant secondary cell walls. It has been shown

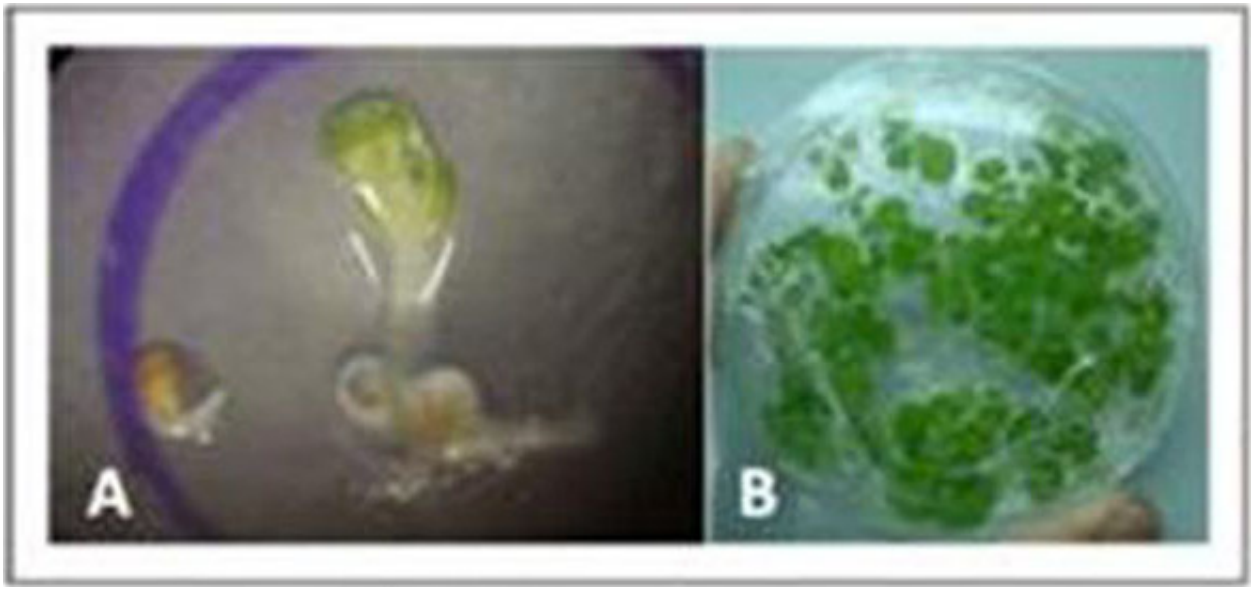

Figure 2(abstract P167) Transgenic model plants overexpressing the *Eucalyptus grandis Cellulose Synthase 3* gene. In "A" it's possible to see one transgenic *A. thaliana* near a nother non-transgenic seeds being selected in the media containing hygromycin. In "B" it's shown transgenic *Nicotiana tabacum* growing in selection media with hygromycin.

that xylan adsorbs to cellulose fibres and also covalently binds a carbon moiety of lignin [1,2]. *Eucalyptus* is an important hardwood tree genus used in the pulp and paper industry and has potential as biofuel feedstock. Xylan removal is expensive and uses environmentally harsh chemical treatments [3]. Previous studies have shown that endo-(1-4)-β-xylanase enzymes belonging to glycoside hydrolase (GH) family 10 internally attacks the xylan backbone resulting in shorter xylo-saccharide chains [4]. The recently sequenced *Eucalyptusgrandis* genome (DOE-JGI, http://www.phytozome.net) provides a unique opportunity to analyze the native endo-(1-4)-beta-xylanase proteins involved in xylan modification in

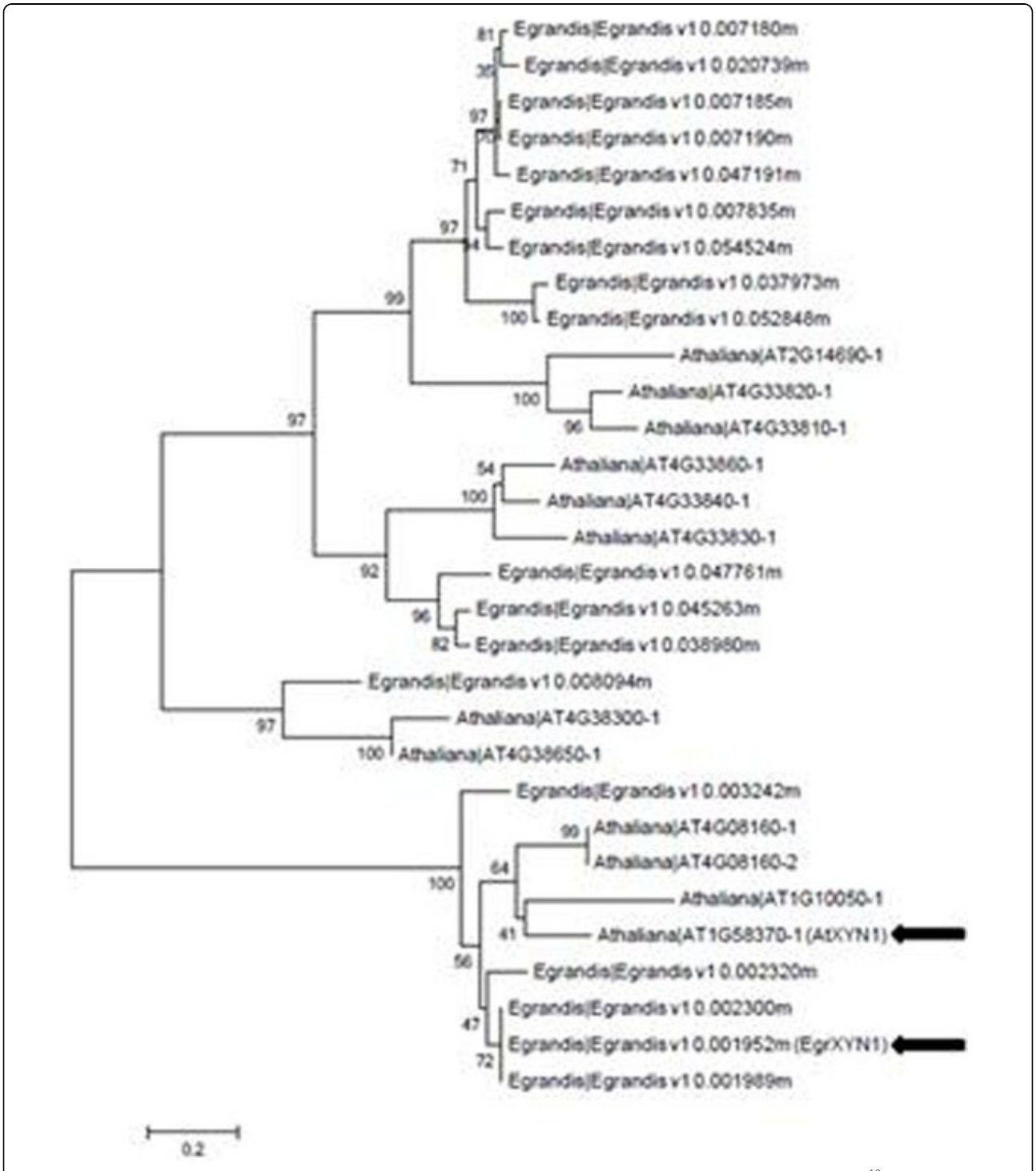

Figure 1(abstract P168) Co-phylogenetic analysis of the predicted protein sequences of GH10 family members (E score < $1e^{-10}$) in *E. grandis* and *A. thaliana*. Neighbor-joining and 1000 bootstrap replicates conducted in MEGA5. EgrXYN1 refers to Egrandis_v1_0.001952m while AtXYN1 refers to At1g58370 (indicated with bold arrows).

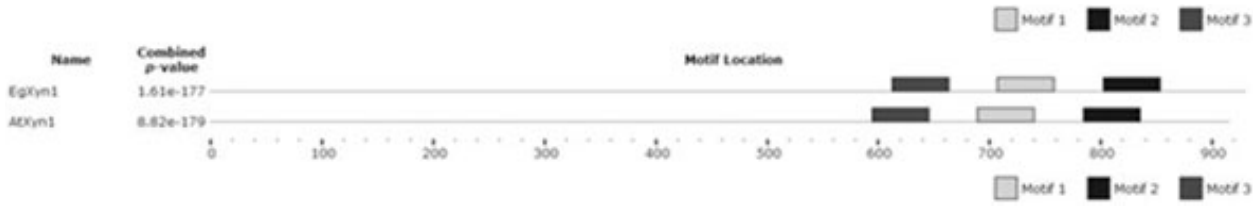

Figure 2(abstract P168) Mining of EgrXYN1 and AtXYN1 for novel motifs using MEME resulted in three additional motifs [6].

eucalypt fibre cell walls. Detailed knowledge of endogenous xylanolytic enzymes from *Eucalyptus* could facilitate the development of strategies to enhance the processing of woody biomass for cellulose and biofuel production. The aims of this study are to identify xylem secondary cell wall-related endo-(1-4)-β-xylanase genes in the *E. grandis* genome and to perform a comparative analysis of the *Eucalyptus* xylanasepeptide sequences with those of previously studied *Arabidopsis* orthologs to provide a framework for assigning function to the *Eucalyptus* enzymes.

Results: Analysis of the *E. grandis* genome sequence on Phytozome v7.0 (http://www.phytozome.net) for putative endo-(1-4)-β-xylanase genes resulted in the identification of 18 putative GH10 family members. The expression profile of each family member was assessed (via mRNA-Seq analysis, http://eucspresso.bi.up.ac.za/) to identify members with putative roles in xylem secondary cell wall metabolism. Egrandis_v1_0.001952m (designated *EgrXYN1*) showed the highest xylem to phloem and xylem to leaf expression ratios of the expressed *E. grandis* GH10 genes [5]. BLAST analysis (<1e-10) of the *A. thaliana* genome for putative orthologs to *EgrXYN1* and co-phylogenetic analysis of all 18 *E. grandis* enzymes with the putative *A. thaliana* xylanases revealed that *AtXYN1* (At1g58370) [4] was one of the closest putative orthologs to *EgrXYN1* (Figure 1). Alignment of the predicted amino acid sequences of *EgrXYN1* and *AtXYN1* Jalview 2.6.1 revealed 68.76% identity between the two sequences.

In silico biochemical analysis predicted that EgrXYN1 has a molecular weight of 103 kDa with a pI of 6.08. This is very similar to AtXYN1 which is 102 kDa with a pI of 6.1. The protein domain view in Phytozome (http://www.phytozome.net) revealed that EgrXYN1 contains three successive N-terminal β-sandwich carbohydrate binding modules IV (at amino acid positions 53-185, 216-357 and 387-532) which were also observed in AtXYN1. A protein motif search (http://motif.genome.jp) revealed that EgrXYN1 also contained a conserved and identical C-terminal GH10 active site sequence "GLPIWFTELDV" at amino acid position 802-812. Finally, *de novo* motif search of both AtXYN1 and EgrXYN1 using MEME revealed the presence of three additional novel C-terminal motifs present within both enzymes (Figure 2).

Conclusion: The *E. grandis* genome contains 18 putative GH10 family members (at a BLAST threshold of $1e^{-10}$). One of these, *EgrXYN1* is highly preferentially expressed in *Eucalyptus* xylem tissues and shows highest similarity to *AtXYN1*. The similarities between AtXYN1and EgrXYN1 suggest similar biochemical properties and biological functions. Previous studies showed that AtXYN1::eGFP localized to the cell wall providing support for its function in cell wall modification. AtXYN1prom::GUS constructs expressed predominately in the vascular bundles suggesting that AtXYN1 (and therefore putatively EgrXYN1) is involved in secondary cell wall modification [4,7]. Future work will involve experimental validation of the biochemical properties and enzyme kinetics of EgrXYN1.

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P169

Expression analysis of DNA methyltransferase and co-repressor genes in *Quercus suber* phellogen: an attempt to correlate with cork quality

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Background: Cork oak (*Quercus suber*) is one of the most important forest species in Portugal. Cork oak produces a thick cork bark which is harvested for industrial uses. Cork quality is the most important factor that affects its uses technical performance and economic value. Cork quality is associated with many features, the most relevant being the porosity resulting from the phellogen's differentiation in filling tissue which will degenerate to lenticels. Good cork has few pores of very thin diameter being the opposite valid for low cork quality. Aiming to understand the mechanisms responsible for controlling of the molecular machinery involved in cork production, namely at the epigenetic level, it is relevant to study the expression profiles of the enzymes involved in DNA methylation in the phellogen.

Methylation of cytosines in the DNA, achieved by DNMT methyltransferases, is one of the most important factors in the regulation of gene expression [1]. In plants three DNMT classes have been identified, each one with its function: the CMT (chromomethylase) class found only in plants, is responsible for maintaining the methylation in CpHpG sequences [2]; the DRM (Domain-Rearranged-Methyltransferase) class is associated with *de novo* methylation in any context [3]; and the MET class, responsible for the maintenance of methylation in CpG zones [1]. Proteins, such as the DNA methyltransferase-associated protein (DMAP1), are known to form stable complexes with DNMTs and act as co-repressors of gene expression.

In this work, we report the transcriptional profile of three putative DNA methyltransferase genes from the CMT, DRM and MET classes, and one DMAP in *Q. suber* phellogen of trees producing good or bad quality cork.

Methods: Three *Quercus suber* potential producers of good quality cork and three producers of bad quality cork were selected. Samples of cork and phellogen were collected from these trees to further estimate cork quality and perform a transcriptional analysis of selected genes. Previously an *in silico* analysis was performed on a cork oak EST library (COEC, Cork Oak ESTs consortium). Three EST sequences, referred as DNA methyltransferases, and one as DMAP1 were selected. Each one was identified and characterized against available databases. Primers were designed to specifically amplify each sequence in real-time-PCR analysis. The actin gene was chosen as internal reference. RNA was extracted from phellogen

samples, using Spectrum Plant Total RNA kit and cDNA was obtained with Retroscript kit. Gene expression was analyzed in the six phellogen samples, using qRT-PCR in triplicate reactions performed for each cDNA template with each primer pair. The NormFinder algorithm was used to evaluate the gene expression's stability using the sample 75 as reference.

Results: Measurement of cork density allowed forming two groups of trees: (1) three individuals with the lower average density (0.289 g.cm-3 ± 0.037) classified as good cork quality producers, (2) three trees with the highest average density (0.388 g.cm-3 ± 0.036)classified as bad cork quality producers.

Homology search using BLASTP algorithm against available databases showed significant similarities between selected our putatively translated ESTs and known DNMTs (CMT3 - ABW96889, DRM2 - ABW96890 and MET2 - XP_002874265) and DNMT associated proteins (DMAP1- XP_002515237) with e-values of 2e-50, 5e-95, 1e-92, 9e-64, respectively

The expression of the three putative DNMTs and DMAP1 genes was evaluated in the phellogen of both tree groups.

The gene expression stability, evaluated through the NormFinder algorithm, showed values ranging from 0.4 for QsDRM2 to 1.5 for DMAP1. Actin also showed an appropriate value (0.7) to be used as internal control. Bad cork quality tree #75 was used as reference. QsMET2 and QsDMAP1 genes showed the highest expression values, while QsCMT3 and QsDRM2 presented the lowest. Comparison of both group trees revealed that, in average, the expression level of each gene is higher in the bad cork quality group than in the good cork quality (Fig1).

Conclusions: In this work we identified one functional DNA methyltransferase of each class (QsCMT3, QsDRM2 and QsMET2) and the MET1 associated protein (QsDMAP1) in cork oak phellogen. This protein has been described in humans [4] as a co-repressor of gene expression, capable of binding with other regulatory proteins such as histone deacetylases [4] which are also associated with gene silencing.

The general tendency of DNMTs to express less in trees able to produce good cork quality suggests a globally lower methylation level and therefore a lower phellogen gene repression. We can hypothesize that gene silencing is higher in bad quality producers, leading to a higher weight of genes whose expression originates defects.

Gene stability analyses lead us to consider QsDRM2 as the most stable, as was already reported in other species. Therefore, this gene may be a good candidate to be used as reference gene, in future real-time-PCR analyses. This high stability seems to indicate that *de novo* methylation although occurring, has no influence in good or bad cork quality production. Conversely, the more variable expression, and therefore the genes that can potentially affect cork quality are QsMET2 and QsDMAP1 genes.

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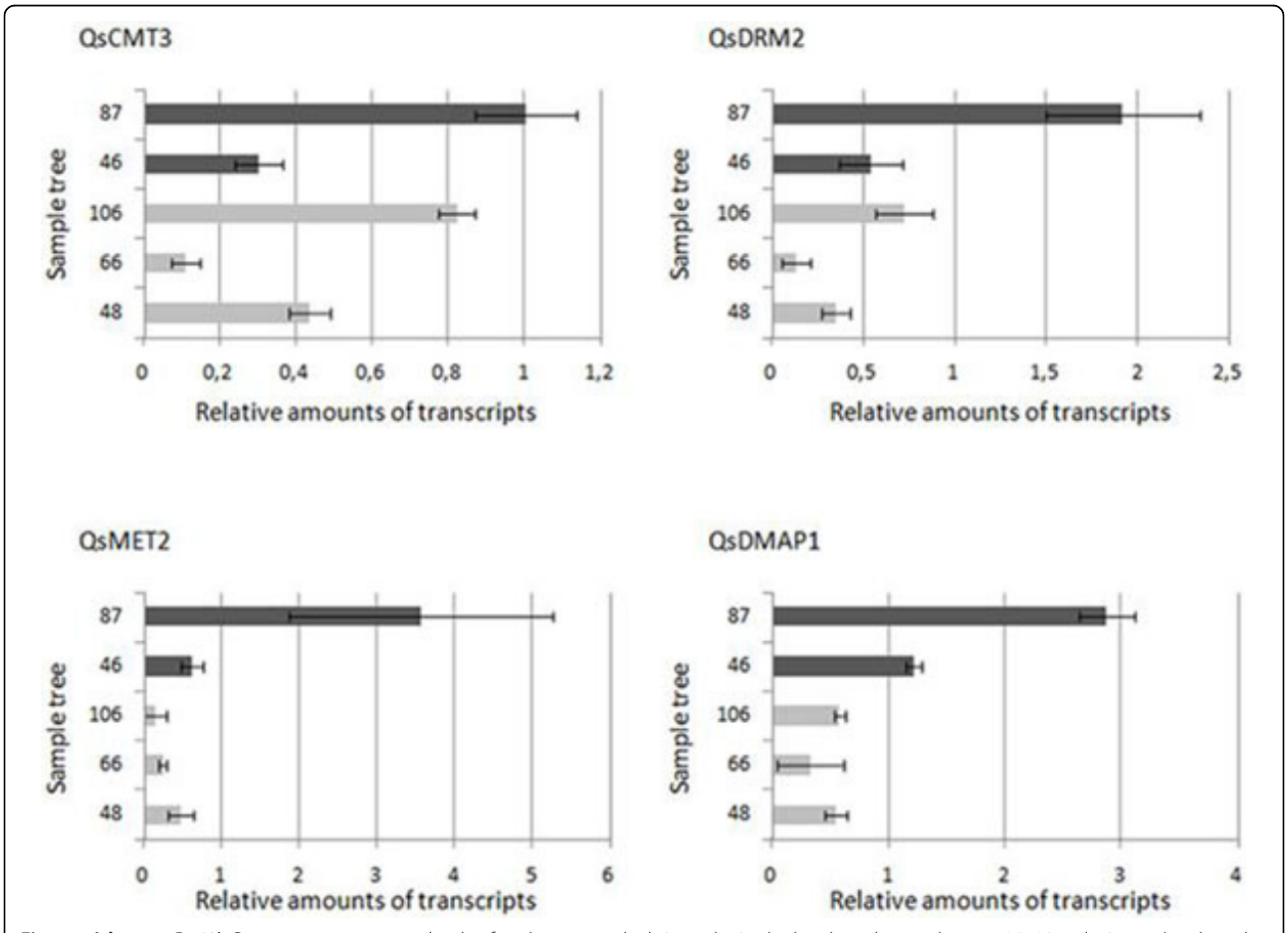

Figure 1(abstract P169) Comparative expression levels of each gene studied. 87 and 46 – bad cork quality producers; 106, 66 and 48, good cork quality producers.

P170

An integrated database of *Eucalyptus* spp. genome project

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BMC Proceedings 2011, **5(Suppl 7):**P170

Background: The species of the genus *Eucalyptus* are the most planted for the fiber crop in the world. They are mainly utilized for timber, pulp and paper production. Brazil, helped by the favorable weather conditions, appears as a big producer and exporter of eucalyptus derivates. In 2002, the Brazilian network research of the *Eucalyptus* Genome (Genolyptus) was established with the goal of integrating several academic and private institutions currently working with eucalyptus genomics in Brazil. This project generated around 200.000 ESTs from several tissues and conditions. Consequently, several individual projects have been implemented generating other transcriptome databases, in special, using RNA-Seq technology. In 2010, a draft genome (http://eucalyptusdb.bi.up.ac.za) of the specie *E. grandis* was produced by researches of the Joint Genome Institute (DOE-JGI) and the *Eucalyptus* Genome Network (EUCAGEN). The main goal of this work is to develop an *Eucalyptus*database (http://www.lge.ibi.unicamp.br/genolyptus) integrating public and private data in a friendly and secure web interface with bioinformatics tools that allowing the users perform complex searches.

Results and discussion: First, the public and private ESTs (130,290 from Genolyptus and 36,981 from NCBI) were assembled producing 48,760 unigenes (17,795 contigs and 30,765 singlets). Basically, the bdtrimmer [1] and CAP3 [2] programs were used to perform sequence trimming (exclude vector, ribosomal, low quality and too short reads) and sequence assembly, respectively.

The autofact pipeline [3] was used to perform an automatic annotation of the assembled unigenes based on BLAST [4] searches, e-value cutoff of 1e-5, against some protein databases, including: non-redundant (NR) database of NCBI, uniref90 and uniref100 – databases containing only curated proteins [5], pfam – database of proteins families [6], kegg – database of metabolic pathways [7] and Gene Ontology (GO) – database of functional annotation [8].

The Genomic and Expression Laboratory at State University of Campinas (http://www.lge.ibi.unicamp.br) sequenced ten RNA-Seq libraries from four species (*E. Urograndis*, *E. globulus*, *E. grandis* and *E. urophylla*) using the Illumina/Solexa technology. Additionally, three RNA-seq libraries [9] were downloaded from NCBI (SRA – sequence read archive). All RNA-seq reads were aligned against the assembled unigenes and genome assembly using the SOAP2 [10] and TopHat [11] aligners, configured to allow up two mismatches, discard sequences with "N"s and return all optimal alignments.

In order to perform a differential expression analysis between ESTs or RNA-seq libraries some normalization pipelines and statistical tests have been implemented.From ESTs, the differentially expressed genes between libraries were performed applying AC test [12] in assembled unigenes. The results are available to the users by a web interface (called Electronic Northern) that allows searches by gene or library name. Furthermore, it is possible to compare the gene expression between two or more libraries. From the RNA-seq libraries, the DEG-seq software [13] was used to perform normalization and statistical analysis considering 99% of confidence rate (cut-off of 0.01).

To integrate all data described above, we developed a web site (Fig. 1) hosted in a Fedora Linux machine with MySQL database server. The web interface is based on a combination of CGI scripts using PERL language (including BioPerl module) and the Apache Web Server. The site contains many bioinformatics tools allowing the user perform keyword or local BLAST search in assembled unigenes. Also it is possible to connect these results with gene expression analysis. Moreover, the Gbrowse software (Generic Genome Browser) (Fig. 2) was used to visualization the data in a genomic context, integrating the different information by clickable tracks. The top track is the reference genome assembly and the other tracks correspond to assembled unigenes and RNA-seq data mapped into reference.

Figure 1(abstract P170) Home-page of the *Eucalyptus* database, hosted at http://www.lge.ibi.unicamp.br/genolyptus.

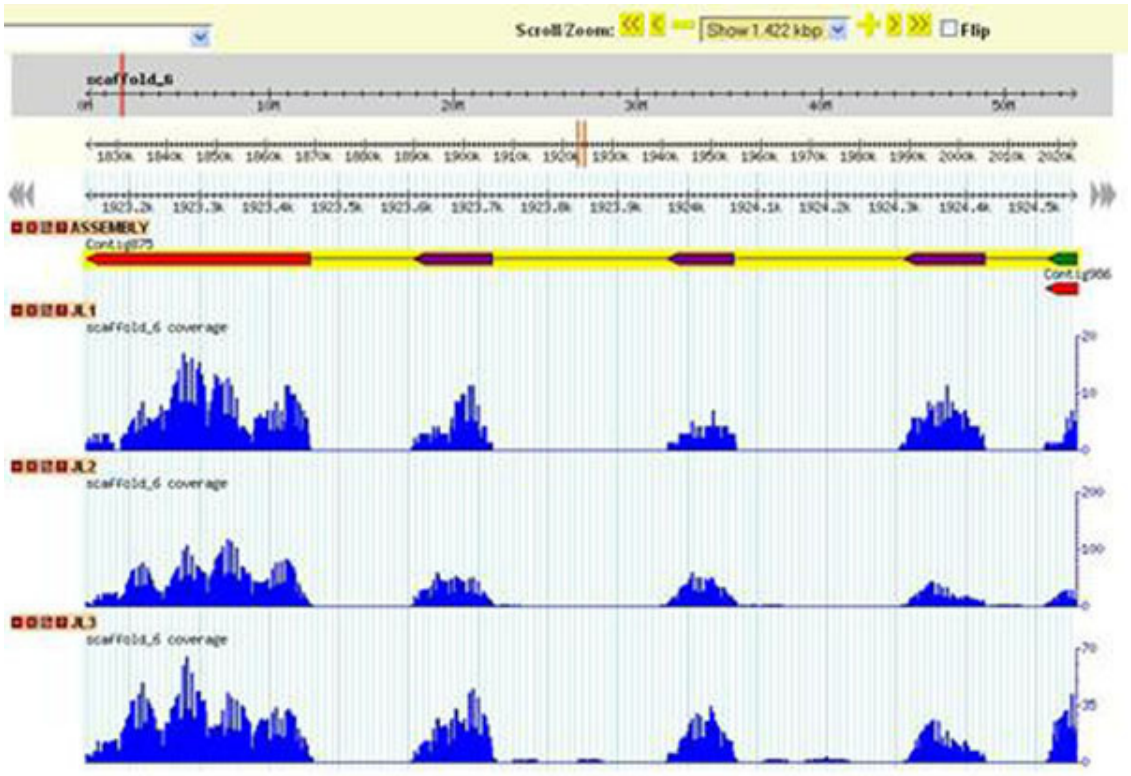

Figure 2(abstract P170) Gbrowse interface of the *Eucalyptus* database. Using Gbrowse is possible to compare gene expression between the RNA-Seq libraries.

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P171

Effects of high levels of CO2 on gene expression in two different genotypes of *Fagus sylvatica*

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Background: The scenario of a changing environment, widely termed as global change is mainly caused by human activities. Oil and carbon

combustion, the use of chlorofluorocarbons, and deforestation are one of the main factors responsible for increasing CO_2 concentration and for an increase of air temperature. In addition global changes will affect precipitation patterns, nitrogen concentration in the atmosphere and enhanced UV-B radiation. Forest trees constitute a relevant economic and ecological resource that is under severe pressure by such environmental changes.

However, the response of forest trees and in particular of the important forest tree species *Fagus sylvatica* to elevated CO_2 levels on a gene expression basis is unknown so far. The principal aim of this study is the investigation of two different genotypes of *Fagus sylvatica* upon increased CO_2 by microarray and gene expression analyses.

Materials and methods: Shoots of *F. sylvatica* (Germany) and *F. sylvatica* "purpurea tree" (Germany) were grafted on *F. sylvatica* rootstocks. Plants were kept for 10 days under controlled conditions in climate chambers using the same temperature and light parameters, whereas the CO_2 concentration was approx. 380-400 ppm (ambient) in the control chamber and 1000 ppm (high) in the CO_2 chamber. Five leaves were taken from arbitrary chosen plants in control and high CO_2 chamber at 2 different time points (T2=2 days and T10=10 days) with 3 biological replicatesper each genotype per each sampling time point. The leaves were immediately frozen in liquid nitrogen and stored at -80°C. Extraction of total RNA was according to Kiefer *et al* [1] and microarray analyses were carried out as described by Olbrich *et al* [2]. Real Time PCR (RT-PCR) [3,4] analyses were carried out for selected genes to evaluate changes in gene expression.

Results: The microarray resulted in differentially expressed gene at T2 and T10 in each beech genotype analyzed. In particular, genes involved in photosynthesis and chloroplast biogenesis were up regulated at T2. A similar trend was been for genes involved in sugar metabolism. This indicates that, at high CO_2 concentration, the activity of the photosynthesis machinery will be accelerated and, therefore, also the one of the glycolysis pathway which brings to an accumulation of carbon. After 10 days of high CO_2 level, few of these genes were down regulated but most of them are expressed at normal level. This effect has previously been described in most of the studies on pot-grown other C3 plants under elevated CO_2 which have indicated photosynthetic acclimation [5]. This response was particularly evident for the *F. sylvatica*"purpurea tree" showing a different behaviour to the second beech genotype.

RT-PCR for genes involved in photosynthesis, chloroplast biogenesis and sugar metabolism are in progress for both genotypes.

Conclusions: A lot of studies have reported contradictory responses of higher C3 plants to elevated levels of CO_2. This observation has also been made in the two German beech genotypes analyzed in this work confirming high expression variability between different genotypes of the same plant species. Therefore, considering the importance to preserve the forest ecosystems under global climate change, further investigations are necessary to understand the regulatory events associated with the adaptive acclimation responses of trees.

A lot of studies have reported contradictory responses of higher C3 plants to elevated levels of CO_2. This observation has also been made in the two German beech genotypes analyzed in this work confirming high expression variability between different genotypes of the same plant species. Therefore, considering the importance to preserve the forest ecosystems under global climate change, further investigations are necessary to understand the regulatory events associated with theadaptive acclimation responses of trees.

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Deciphering cork formation in *Quercus suber*

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The family of *Fagaceae*, comprises about 900 species, among them the best-known group of this family is the oaks, genus *Quercus*, that are commonly used as timber or for cork production. Cork is produced by *Q. suber*, an evergreen oak with major economic and environmental importance for Mediterranean region, in particular for Portugal that is the leading producer of this material.

Besides, the economic importance of cork production and cork manufacturing, little attention has been paid to the molecular mechanisms underlying wood and cork formation as well as cork quality. To overcome this constraint, an international partnership (SuberGene) was established 2008, involving Portuguese and French research institutions and one Producer's Association organization (FJLF). The main driving force of this partnership is to join efforts and complementary skills to unravel to the molecular mechanisms underlying cork formation and decipher the structural polymorphisms and regulation network that determine cork quality.

A non-normalized cDNA library of developing phellem (DP) was produced and 5,000 clones were sequence both ends using Sanger technology. More than 6500 good quality ESTs were deposit at GeneBank. DP transcriptome was also assessed by pirosequencing (454FLX Titanium, Roche) generating more than 200,000 reads. Sequencing data (238,911 ESTs) data were assembled into 69,559 contigs. Suberin biosynthesis genes such as Glycerol-3-phosphate acyltransferase and Omega-hydroxypalmitate O-feruloyl transferase are among the more expressed genes in DP tissues. More than 2,800 putative SNPs were detected in 1,121 contigs. As a complementary strategy, the proteome of DP are being assessed, by 2D-PAGE, and mass spectroscopy. In order to get more information about the gene structure of genes related with the suberisation of cork cell-wall, one *Q. suber* BAC library have been constructed, and are being characterized. Clones harboring genes of interest were identified by screening high-density filters of this BAC library.

These resources provides us with valuable tools to study the nature of the molecular machinery involved in cork formation,and most importantly with the players involved in the variability of cork characteristics. Future ongoing approaches and the impact of these finding will be discussed.

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Interspecific discovery and expression profiling of *Eucalyptus* micro RNAs by deep sequencing

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Background: Micro RNAs (miRNAs) are a class of small (~21 nucleotide) non-coding RNAs that recently gained much attention due to their perceived role as master regulators of gene expression in Eukaryotes, responsible for fine tuning gene expression regulation and, in plants, has been shown to be involved in a diverse range of biological processes such as plant development and architecture, flowering, cell differentiation and response to biotic and abiotic stresses [1]. The repertoire of expressed miRNAs differs among cell types, tissues, development, environmental condition, etc [2]. Notwithstanding, the exact function of thousands miRNAs sequences present in miRBase [http://www.mirbase.org/] is not elucidated. At this point, discovery and profiling of new and conserved miRNAs are critical in the attempt to understand their function and mechanism. Deep sequencing through next generation sequencing is the methodology of choice for this purpose as its ultra high throughput permits a comprehensive interrogation of the small RNA transcriptome, permitting *de novo* identification and relative quantification of different small RNA species [3].
Due to its economic importance the *Eucalyptus grandis* genome has been sequenced by JGI and the annotation of miRNAs is pivotal. In order to provide the first large scale experimental characterization of *Eucalyptus* miRNAs we performed an Illumina deep sequencing run that allowed us to discover and quantify the miRNA levels in two different tissues – xylem and leaves. Additionally, to get insights of the observed phenotypic differences in wood quality among *Eucalyptus* species, we characterized the xylem small RNA transcriptome of two different *E. globulus* individuals and integrated the results to catalog conserved and *Eucalyptus* specific miRNA gene families.
Materials and methods: Four biological samples were used: xylem from two *E. globulus* genotypes, xylem and leaf from BRASUZ1 *E. grandis*, the one currently being sequenced by JGI. Total RNA extraction was performed with CTAB protocol to a total amount of 10 mg per sample [4]. Fraction of small RNAs were barcoded to be sequenced in a single flow cell in Illumina GA II Sequencing System by Fasteris [http://www.fasteris.com]. A computational pipeline was specifically developed to process the deep sequencing data. The pre-processing step cleans the sequences by quality screening, adapter sequence removal, contaminant checking. Cleaned reads were sorted according to size, quantified (tag counting) and used to create an additional set of non-redundant sequences (using uclust). Bowtie was used to map sequences against the 8X *E. grandis* – BRASUZ1 genotype – genome sequence draft. Mapped positions in the genome were extended by 150 bases to be used as input to predict secondary structure (miRDeep) to test for stem loop structure of miRNA precursors. Northern blot hybridization is being used for experimental validation of some conserved and potentially new *Eucalyptus* miRNAs sequences.
Results and conclusions: Total number of reads was 6,104,498 ranging from 1,115,404 to 1,766,355 per sample – 36 nt average size. After pre-processing, total number of sequences was reduced to 1,980,958. As expected, read size distribution has two main peaks at 21 and 24 nt. Comparative analysis of size distribution interestingly shows higher abundance of the 24 nt fraction for all samples, being up to 3,75 times higher than 21 nt. The 24 nt small RNAs are predominantly small interfering RNAs (siRNAs) which are involved in RNA-directed DNA methylation resulting in gene and transposon silencing. Putting it all together, reads from four samples resulted in 169,642 unique sequences mapped against the genome. From that, 70,55% had at least one alignment to the genome reported, 23,54% failed to align and 5,91% mapped to multiple loci, indicative of repeat regions.
Mapping 20-22 nt reads against the reference genome revealed that BRASUZ1 had more reads mapping to its own genome than the other samples, totaling 95% for leaves sample and 91% for the xylem. *E. globulus* samples showed a reduced percentage of mapped reads, around 85%, corroborating the existence of interspecific variability. Besides that, the relative abundance among the three xylem samples reinforces the variability also in the expressed repertoire of small RNAs.
Annotation of plant miRNAs was done meeting a set of strict criteria, particularly a proper secondary structure of the precursor [5]. The positions in the genome with a mapped reads were extended to 300 bases and fed to the program miRDeep [6] to test for the compatibility of precursor hairpin. At least 38 sequences mapped were positive under these premises.
Conserved *Eucalyptus* miRNAswere identified by similarity searches against the miRBase. A total of 206 distinct *Eucalyptus* sequences showed significant similarity (at most one mismatch) to an orthologous sequence, confirming the presence of 36 different mir genes families, including many of its isoforms.
Quantitative differences in miRNA abundance were probed by pairwise comparison of tag counts contrasting intraspecific, interspecific and tissue-specific analysis. Results revealed that the most similar small RNA repertoire are between intraspecific samples and, the least, between tissue specific. Tissue specific differential expression analysis shows that around 36% of the conserved miRNAs sequences observed in each tissue was mutually exclusive and the ones present in both samples vary up to two-fold (p-value=0.05).
Experimental validation is being carried out by northern blot hybridization and preliminary results validated new and conserved miRNAs, such as mir 156 and mir 172.
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Next-generation gene catalogues and genomics tools focused on forestry research

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Gydle is developing reference gene catalogues and genomics resources targeted at forestry research, leveraging its unique bioinformatics expertise in next-generation sequencing.
Next-generation sequencing technologies have revolutionized the field of genomics and have reached the capacity to generate enough quality data to sequence and assemble thousands of genomes and gene catalogues per year. These extraordinary opportunities are currently limited by challenges in data analysis which hinder the translation from raw sequencing data to applications in research and in the field. There is an enormous gap between an instrument capacity to produce millions of reads per day and the ability to analyze and translate quickly this data into usable information and value-added sets for genomics research. One particular limitation stems from the current state of assemblers which produce crude contigs and scaffolds that need considerable editing in order to obtain contiguous, gene-oriented, full-length, artifact-free

annotated sequences desirable for the production of quality tools for genomics research. Researchers also lack tools to rapidly integrate and visualize their own data and collaborate in a user-friendly way. Finally, the ability to produce gene catalogues for thousands of samples calls for a new generation of tools to conduct large-scale comparative genomics studies between and within species.

Gydle has developed a complete suite of proprietary bioinformatics tools to address many of the challenges associated with next-gen sequence analysis. A survey of these tools will be presented, which include innovative raw data filtering, rapid and accurate DNA alignment and gene-oriented assembly software, automated and interactive visualization tools for sequence correction and annotation. These tools enable users to process from start to finish a typical next-gen sequencing run in a user-friendly way in a matter of minutes to hours instead of weeks. Users can then visualize their results, edit sequence assemblies and annotations and identify features such as sample-specific expression patterns, SNPs, unspliced introns and alternative splicing events, handling information from hundreds of millions of reads on their workstation or laptop computer. We intend to make these bioinformatics tools available commercially in the near future.

Applying these bioinformatics tools, we combined sequencing data generated by Gydle with publicly available data to produce curated reference gene catalogues for species of interest to the forestry industry, which currently 35 tree species including 7 pinus, 3 picea, 3 eucalyptus, 2 poplar and 5 oak species. Gydle gene catalogues also include over 50 other plant species of agricultural and medicinal interest as well as gene catalogues for species ecologically related to trees such as insects, endophytes, fungi, and microbiota. We intend to make these catalogues available commercially in addition to offering the possibility for researchers to integrate their own data with these catalogues privately.

We used these reference genes catalogues to produce species-specific and cross-species value-added genomics resources such as:

- reference gene sequences covering biochemical pathways involvedin growth, wood formation, climate adaptation, biofuel production, and secondary metabolites of interest to pest resistance and human health
- curated amino-acid databases to improve proteomic and metabolomic identifications
- SNP sets and designs of high-throughput genotyping assays such as Illumina GoldeGate/Infinium for marker discovery and genomic selection.
- reference sets and tools for gene expression studies by RNA-seq and microarrays.

Examples of gene catalogues, cross-species analysis and value-added sets will be presented.

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Comparative transcriptome analysis of tree *Eucalyptus* species using RNAseq technology: analysis of genes interfering in wood quality aspects

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Background: The *Eucalyptus* wood is one of the most important raw materials for pulp and paper industry. Brazil is currently the first producer of short-fiber pulp and sixth in total production of cellulose. To maintain the industrial competitiveness, investment in genomic research started in 2002 with the *GENOLYPTUS* project (Brazilian Network of Eucalyptus Genome Research). Recently, a new transcriptome library was generated using Next Generation RNA Sequencing by Illumina's sequencing by synthesis technology.

Different species of *Eucalyptus* are recognized for their superior characteristics in terms of growth, wood quality and resistance to different types of stress (1). Such features are probably driven by the coordinated expression of numerous genes involved in processes of structural and regulatory genes in xylogenesis. Therefore, the main purpose of this study is to identify genes and key metabolic compounds directly involved in wood quality, as well as transcription factors involved. An extensive data mining in the RNAseq database was conducted to identify sequences over expressed in xylem and those that were differentially expressed between species.

Methods: Genolyptus Sanger sequenced ESTs (167,271) and NCBI *Eucalyptus* ESTs (36,981) were assembled using the program CAP3 (2). All unigenes were automatically annotated using BLAST (3) (e-value cutoff of 1e-5) against protein databases, including: non-redundant (NR) database, uniref (4), pfam (5) and keg (6). Moreover, a functional annotation using the BLAST2GO software was performed (7). The RNA-Seq reads produced from three different xylem libraries (*Eucalyptus globulus*, *E. grandis* and *E. urophylla*) were aligned against the assembled unigenes using the SOAP2 aligner (8) configured to allow up two mismatches, discard sequences with "N"s and return all optimal alignments. In order to perform the differential expression analysis between libraries, a normalization and statiscal pipeline were applied using DEG-seq (9) software considering a 99% confidence rate (cut-off of 0.01). From this analysis we obtained

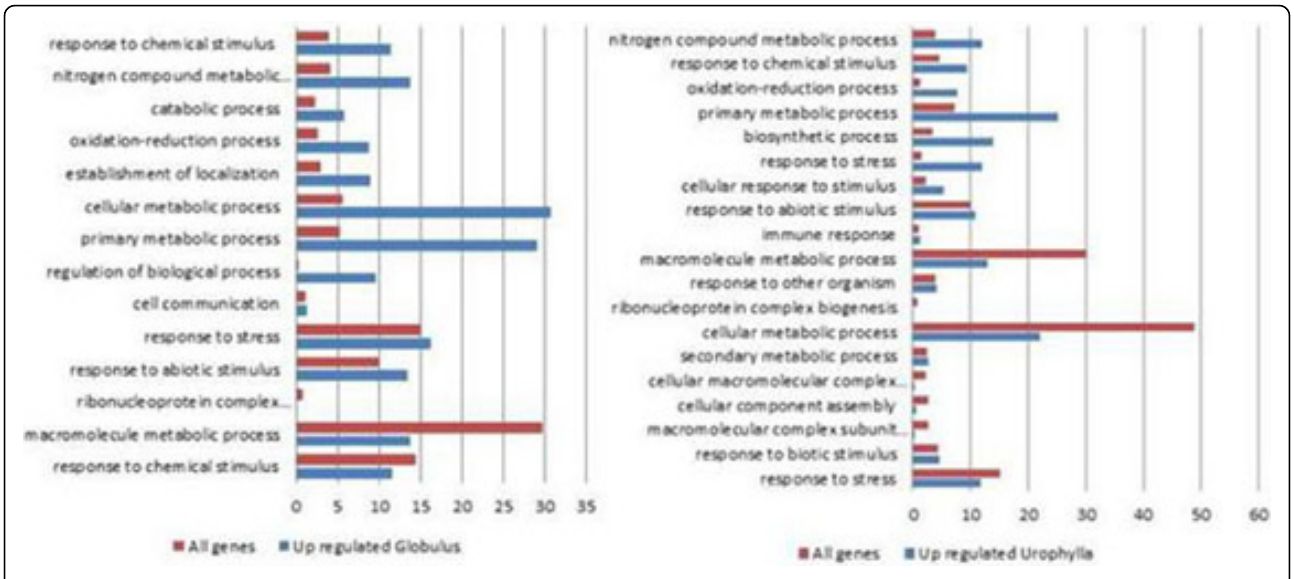

Figure 1(abstract P175) Functional annotation using the BLAST2GO software.

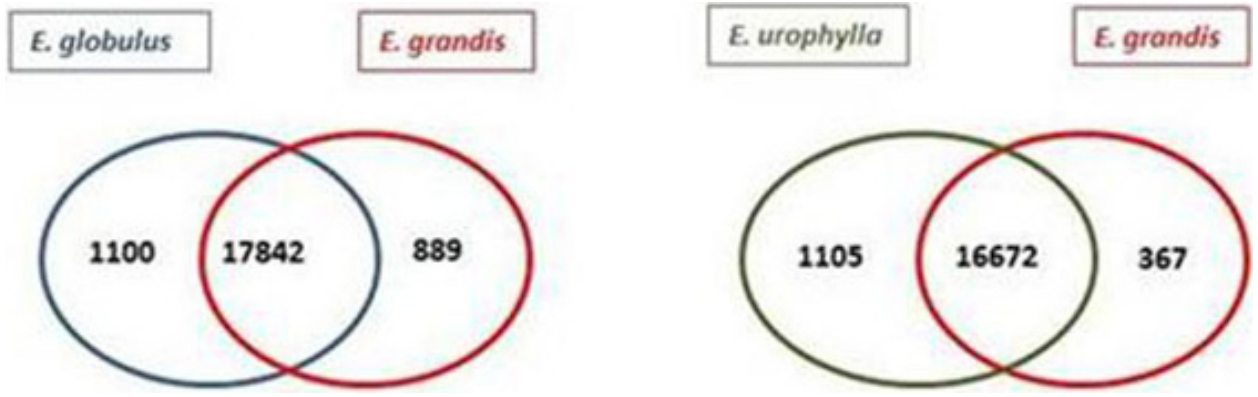

Figure 2(abstract P175) Venn diagrams showing different expression between three distinct xylem libraries.

xylem genes and transcription factors differentially expressed between the three species.

Results and discussion: The assembly produced 53,412 unigenes (18,098 contigs and 35,314 singlets). The xylem libraries produced a large number of RNAseq reads (35bp). About 28 million reads were produced for the *E. globulus* library, 25 million for *E. grandis* and 25 million for *E. urophylla*. About 2% of reads were discarded after filtering. Most part of RNAseq reads mapped into the new EST assembly: 69.27% for *E. globulus*, 71.97% for *E. grandis* and 67.90% for *E. urophylla*. As a result, 33,599 unigenes were aligned to the RNAseq libraries. The functional annotations (Figure 1) show percent of genes related to the most relevant GO categories represented in each of the species pairs syudied for Biological Process, level 3.

In the *E. globulus* X *E. grandis* comparison, most genes are in the macromolecule metabolic process category that includes genes for pectin, cellulose and hemicellulose metabolism and also transcription factors involved in such pathways. Over 10% of these genes are over-expressed in *E. globulus*. Over 30% of the genes are over-expressed in *E. globulus* in the category metabolic cellular process. *In the E. urophylla X E. grandis* comparison, the metabolic cellular process category is representative of the total number of contigs, however, the number of genes over-expressed in *E. urophylla* is much lower. This may be an indicative that genes that participate in such pathways can contribute to the differential wood qualities found in *E. globulus*.

The new assembly, RNAseq libraries and Gbrowse are available at www.lge.ibi.unicamp.br/eucalyptus. *E. globulus* and *E. urophylla* libraries were compared against *E. grandis* library in order to access differentially expressed genes (considering 99% of confidence rate - cut-off of 0.01). As a result, 19,828 genes were differentially expressed in the *E.gl* X *E. gr* comparison (51.43%) and 18,142 (49.27%) in *E.ur* X *E. gr*. Also in these groups there were genes not expressed in one of the species, as can be seen in Venn diagram below (Figure 2).

These results may contribute to the understanding of wood formation processes and possibly help guide its improvement. The increase in wood quality and productivity has significant economic impacts especially in the pulp and paper industry.

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P176

Proteomic analysis of *Clostridium acetobutylicum* in butanol production from lignocellulosic biomass

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Background: Plant biomass is an abundant and renewable source of energy rich carbohydrates that can be efficiently converted by microbes into biofuels [1]. Butanol is considered as a second generation biofuel when it is produced from lignocellulosic biomass comprising of agricultural and garden wastes that does not compete with the food supplies [2]. *Clostridium acetobutylicum* is a gram positive, spore forming, obligately anaerobic bacteria capable of converting different sugars from lignocellulosic biomass to butanol through acetone – butanol – ethanol (ABE) fermentation process [3]. However, the production of butanol from ABE fermentation process is not economically viable and studies have been performed to understand the utilization of lignocellulosic biomass and regulation of butanol production to improve butanol productivity [4]. Successful industrial butanol production process through ABE fermentation requires complete understanding of the *C .acetobutylicum*. Shotgun proteomics provides a direct approach to study the whole proteome of an organism at molecular level in depth. Therefore, this paper focuses on shotgun proteomic profiling of *C. acetobutylicum* ATCC 824 from butanol fermentation process, elucidating the molecular functional mechanisms of *C.acetobutylicum* in butanol production.

Materials and methods: The microorganism *C.acetobutylicum* bacterial strain ATCC-824 was cultured [5]and ABE fermentations were carried out in batch mode using glucose substrate which is the most abundant compound present in lignocellulosic biomass. Samples of 10ml were harvested at late exponential phase from the start of the inoculation and proteins were extracted followed by digestion to peptides. The complex peptide solution was desalted through C18solid-phase extraction, concentrated, filtered and for each LC-MS/MS analysis, ~1/4 of the total sample was used based on the protocol used by [6]. Samples were analyzed in technical duplicates through a 2D nano-LC MS/MS system with a split-phase column (RP-SCX-RP) with 12hr runs [7-9]. All MS/MS spectra were searched with the SEQUEST algorithm [10] against

C. acetobutylicum Uniprot proteome databases [11] and filtered with DTASelect/Contrast [9]at the peptide level. Only proteins identified with two fully tryptic peptides from a 12hr run were considered for further biological study.

Results: A total of 479 proteins were identified in the proteome analysis of *C. acetobutylicum* from a single data point during the ABE fermentation process and 372 proteins were found to be present in both the first and second MS runs and identified as common proteins. This analysis confirms that 12 proteins were involved in the butanoate metabolism and about 32 uncharacterized proteins were found to be present during the ABE fermentation using glucose substrate. Overall, this is the first study which represents an extensive survey of whole proteome analysis of *C. acetobutylicum* from a single data point by multidimensional protein identification technology (MudPIT) and provides a valuable dataset of *C. acetobutylicum* proteins for a better understanding of the butanol production.

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P177

Developmental variation in DNA methylation in poplar (*Populus trichocarpa*)

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Using methylated DNA immunoprecipitation followed by high-throughput sequencing (MeDIP-seq), we analyzed DNA methylation patterns in the *P. trichocarpa* genome in relation to four biological processes: bud dormancy and release, mature organ maintenance, in vitro organogenesis, and methylation suppression. Here, we report results from Illumina sequencing of nine sampled tissues, each representing 1 to 2 biological replicates. We sequenced 26M – 97M reads per tissue type, and validated our MeDIP-seq results using bisulfite sequencing of selected targets.

Unique MeDIP-seq reads covered ~30-60% of genome space at an average depth of 4 to 12 reads/nucleotide. Transposons and other repeat elements were enriched within the methylated fraction of the genome. The pattern of gene model methylation showed higher methylation at promoters, in the middle of coding regions, and 3' to ends of genes, similar to that observed in other plant and animal species. Numbers of methylated genes identified varied widely by tissue type.

We produced summary data for genome methylation in *P. trichocarpa*, including the distribution of methylation across chromosomes (Fig. 1) and in and around genes. The intensity of methylation was highly heterogeneous within and between chromosomes. One-third of the genome, analyzed as 1 kb tiled windows, was differentially methylated among tissues. An example of a chromosomal region with differential methylation is shown in Fig. 2. Promoter methylation was more frequent than gene body methylation. Male catkins differed from the other tissues in that gene body methylation was more prevalent than promoter methylation, and two transposable element categories that were methylated in all other tissues were unmethylated in male catkins. We also analyzed the association of methylation intensity to gene expression data from an existing microarray study of the same tissues. At a whole genome scale, both promoter-

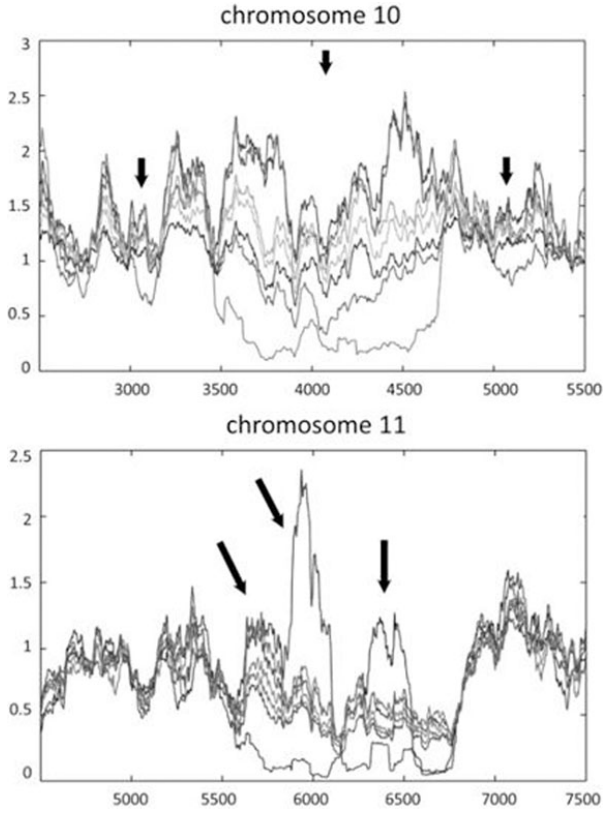

Figure 1(abstract P177) Two examples of section of poplar chromosomes showing tissue-specific methylation. Each line represents one tissue type, including roots, buds, xylem, phloem, leaves, male catkins, and female catkins. Arrows point to regions showing strong tissue-specific differentiation. X-asix is 1 Kb tiled windows. Y-axis is RPKM.

methylated and body-methylated genes had lower expression than unmethylated genes. We will report on our continued studies of tissue methylation/expression relationships.
We have developed a customized genome browser (Gbrowse version 1.69), compatible with the most recent (v2.2) *P. trichocarpa* genome assembly, at which our data can be explored: http://poplar.cgrb.oregonstate.edu.

P178

A drought stress transcriptome profiling as the first genomic resource for white teak - Gamhar - (*Gmelina arborea* Roxb) and related species

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Background: Gmelina (*Gmelina arborea* Roxb), also known as *white teak* is a tropical deciduous tree native from moist tropical forests of Asia. It has been successfully introduced in equatorial Africa, as well as central and south America. Gmelina is known for its very fast growing rate, its intrinsic disease, fire and drought tolerance, as well as the quality of its wood which is suitable for different types of uses such as paper pulping, plywood or particle board industry, furniture and light constructions. In addition, gmelina is considered a pioneer plant species capable of rapidly colonizing eroded or low nutritional quality lands, which makes it interesting for reforestation or landscape restoration programs. Despite its ecological and increasing economic importance, very little is known about the biology of this species and its remarkable field behavior such as drought tolerance, at the genetic, molecular and biochemical levels.
Genomic tools have recently increased the numbers and volume of genomic resources for several crop plants and trees and have contributed to enlarge our knowledge on basic aspects of plant biology, population dynamics or phylogeny; furthermore, they represent valuable sources of candidate genes and new molecular markers to assist breeding programs. Biological sequences reported to date in public databases and belonging to Gmelina do not exceed 20 entries: this very narrow availability of genetic information is the main bottleneck to initiate molecular breeding programs in *Gmelina arborea*.
We report here the first genomic resource for this tropical tree based on an RNAseq transcriptome profiling approach.
Methods: Two subtracted cDNA libraries were constructed from both leaf and root tissue of gmelina seedlings submitted to a water deficit stress. Subtracted cDNA sequencing was completed using 454 GS FLX Titanium sequencing. After sequence trimming, de novo assembly and clustering were completed using TGICL assembler and contigs were functionally annotated using BlastX against high quality curated protein reference databases. Both Gene Ontology (GO) terms and KEGG metabolic pathways assignments were used to further characterize transcripts. SSR motives were identified using MISA. Except for the KEGG pathway assignments, all the sequence analysis was compiled in the previously developed bioinformatic pipeline ESTTIK [http://esttik.cirad.fr/].
Results: A preliminary collection of 10.528 contigs and 10.661 singletons, enriched in drought related transcripts, was obtained from both root and leaf subtracted libraries. Contig sequence sizes ranged from 97 to 2.187 bp with an average of 456 bp and a mean coverage depth of 38-fold. Functional annotation was completed only for contigs: up to 65% of these assembled sequences had significant Blast hits and about half of all assembled contigs could be assigned to one or more GO terms (Table 1).
Top hit species for homology based annotations of gmelina unigenes were: *Arabidopsis thaliana* (20-27%), *Vitis vinifera* (10-14%), *Populus trichocarpa* (4-7%) and *Ricinus communis* (4-7%). Among the different biological processes, cellular and metabolic processes, biological regulation, localization and response to stimulus were the most highly represented GO term categories, involving several genes related to drought or general stress, transport, transcription regulation and signal transduction, as expected. Analysis of KEGG metabolic pathway assignments revealed that our gene catalog covers all major plant metabolic pathways, with a certain dominance of enzymes of the carbohydrate, amino acid and energy metabolism in leaves, indicative that those pathways, seemingly impaired in response to water deficit stress, tend to recover by means of an active transcriptional rate (Table 2). The metabolic response in roots was slightly different with dominance of enzymes related to lipid, amino acid and carbohydrate metabolism, supporting important membrane and osmotic adjustment mechanisms in these tissues. Importantly, our gene catalog comprises many genes encoding proteins involved in signal perception and transduction, effector proteins as well as proteins with regulatory functions, allowing to cover the whole molecular response to drought stress. On the other hand the presence of several unigenes with no blast hit or with homology to unknown hypothetical proteins opens the possibility to uncover either previously unknown or less characterized protein functions related to drought stress.
Finally, we also identified microsatellite motifs within 428 unigenes, from which, 255 primer pairs have been designed for further experimental validation as functional SSR markers.
Conclusions: In this survey, we present the first unigene resource of this economically important tropical timber for which almost no prior genetic information existed. We identified drought stress related genes in different functional categories ranging from membrane bound sensor proteins, signal transduction proteins, transcription factors to metabolic or effector proteins. The SSR motifs found are good candidates for drought-related functional marker development in this species. Altogether, the results represent a first contribution to a better knowledge of the biology of *white teak* as well as the molecular mechanisms underlying its drought tolerance, which is essential to further encourage breeding program developments.

Table 1(abstract P178) Summary of Gmelina arborea transcript sequencing and functional annotation

		LIBRARY	Leaves	Roots	TOTAL
ASSEMBLY		Input sequences	401.181	87.078	488.259
		Clean sequences	340.654 (85%)	74.480 (86%)	415.134 (85%)
		Singleton	6.045	4.616	10.661
		Contigs	**5.696**	**4.832**	**10.528**
		Size range (average) (bp)	100-1731 (413)	97-2197 (499)	97-2197 (456)
		Depth coverage (fold)	59	14	38
Annotation	I	**Blast Hit**	**4.185 (73%)**	**2.623 (54%)**	**6.808 (65%)**
		No Blast hi	1.511	2.209	3.720
	II	**GO annotated contigs**	**3.274 (57%)**	**1.743 (36%)**	**5.017 (48%)**
		GO terms	20.965	10.474	31.439
		Contigs with Enzime Codes	1.252	509	1.761
	III	**KEGG pathway assigned contigs**	**738 (13%)**	**292 (6%)**	**1.030 (10%)**

[a] Percentages are related to total number of contigs

Table 2(abstract P178) Principal KEGG metabolic pathway assignments

Metabolic pathway	Leaves	%	Roots	%	Total	%
Carbohydrate	348	20.5	99	15.0	447	18.9
Energy	362	21.3	87	13.2	449	19.0
Lipid	119	7.0	106	16.1	225	9.5
Nucleotide	60	3.5	45	6.8	105	4.4
Amino Acid	299	17.6	118	17.9	417	17.7
Other Amino Acids	67	3.9	28	4.2	95	4.0
Glycan Biosynthesis	13	0.8	19	2.9	32	1.4
Cofactors and Vitamins	124	7.3	33	5.0	157	6.7
Terpenoids and Polyketides	66	3.9	19	2.9	85	3.6
Other Secondary Metabolites	128	7.5	46	7.0	174	7.4
Other	114	6.7	60	9.1	174	7.4
Total Assignments	**1700**		**660**		**2360**	

S9. BIOSAFETY, CERTIFICATION AND ECONOMICS OF TREE BIOTECHNOLOGY

P179

***Eucalyptus* transgenic plants: from genetic transformation protocols to biosafety analysis**

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Eucalyptus is an exotic plant in Brazil, being originally from Australia. There are about 700 *Eucalyptus* species described and over 3,000 hybrids. It is one of the most planted tree genus in the world. The great economic interest on *Eucalyptus* trees is due to their fast growth, high productivity, great adaptability to different types of soils and climates, and also to the high versatility of their wood. *Eucalyptus* timber has applications for many different purposes such as cellulose pulp and paper production, electric poles, energy, charcoal, lumber and furniture. Given the economic importance of *Eucalyptus* in Brazil, it is of great interest to generate trees with superior characteristics that may result in considerable gains for the sector, particularly with regard to productivity and wood quality. One main goal of the present work is the definition of an efficient protocol for the genetic transformation and regeneration of transgenic *Eucalyptus* trees. We have so far obtained callus derived from leaves of *E.globulus* showing high capacity of *in vitro* regeneration. These calli were transformed with *Agrobacteriumtumefaciens* LBA4404 harboring the binary plasmid pGfpKan, containing the *green fluorescent protein* (*gfp*) gene as reporter. Regenerated plants were transferred to culture pots with MS medium. Leaves derived from each regenerated plant were collected and analyzed using confocal microscopy to investigate the presence of fluorescence, indicating successful transformation. Molecular assays are also being performed to confirm the independence of transformation events via the pattern of transgene integration into plant genomes. The commercial release of GMOs and its derivatives in Brazil is regulated by the National Technical Biosafety Commission (CTNBio) in terms of the Regulatory Resolution No. 5, March 12, 2008. A series of experiments must be performed in order to prove the equivalence between GM and non-GM plants concerning the effects on human and animal health as well as on the environment. We are conducting the evaluation of global gene expression among different lines of transgenic and non-GM *Eucalyptus* adult plants, already available in test-fields belonging to FuturaGene in Brazil. Leaf and stem samples of GM and controls were collected and stored at -80 °C. Total RNA and proteins from leaves and stems were extracted and quantified. Messenger RNAs are under sequencing in Illumina platforms, according to the mRNA-Seq protocol (Fasteris S.A.) Our idea is to compare transcript profiles among GM and non-GM tree samples, checking for possible pleiotropic effects of the transgenes. Protein profiles of the same individuals will also be analyzed to verify if the presence of the transgene influences the expression of other proteins in *Eucalyptus*. In order to do so, total proteins were extracted from samples and fractionated by 1D SDS-PAGE, cut off from gels and processed for posterior Mass Spectrometry sequencing.

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P180

Towards male sterility in *Cryptomeria japonica* using the male strobilus-specific genes of *C. japonica*

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Background: *Cryptomeria japonica* D. Don (sugi) is one of the most important Japanese conifer species. Rapid improvement of the wood trait (e.g., growth speed and wood quality) using a conventional breeding approach is not possible, because breeding of coniferous tree requires a very long time. Genetic modification might be a powerful tool to shorten the time needed to breed trees compared with traditional breeding methods because it is able to induce the favorable traits by introduction of specific genes in trees without unnecessary genetic transitions. But the transfer of foreign genes from GM plants to related plant species by pollen has been cited as an environmental concern. For the purpose of creating male sterile GM *C. japonica*, we attempted to identify genes related to male flower formation.
Methods: To isolate the male strobilus specific genes, we constructed male strobilus specific SSH libraries based on three different stages according to male strobilus development; early stage, tetrad stage and mature stage. The microarray were designed using 19,259 genes consisting of isolated genes from SSH libraries and ForestGen(FORest EST and GENome database [http://forestgen.ffpri.affrc.go.jp/en/index.html]).We analyzed expression profiling associated with male strobilus development. To isolate the male strobilus specific promoter of male strobilus specific genes, *1009-C47* and *1009-C96*, TAIL-PCR methods were performed. To confirm the tissue specific activity of the promoter regions, the promoter::*GUS* fusions were introduced into *Arabidopsis* and *C. japonica*. Furthermore, we introduced *1009-C47::Barnase and 1009-C96::Barnase* to *Arabidopsis* and *C. japonica*. We evaluated the ability of pollen formation of the transgenic *Arabidopsis* that introduced *1009-C47::Barnase and 1009-C96::Barnase* construct.
Results and discussion: The microarray analysis was performed using cDNA of different developmental stages of male strobilus; early stage, microspore mother cell stage, tetrad stage, free spore stage and mature pollen stage. We showed that the strongly expressed genes in each developmental stage were markedly different. The GUS assay revealed that the *1009-C47::GUS* showed anther-specific GUS activity in *Arabidopsis* and *C. japonica*. The introduction of *1009-C47::Barnase and 1009-C96::Barnase* into *Arabidopsis* led to male sterility phenotype. We showed probability that the *1009-C47* promoter and *1009-C96* promoter are useful for male sterilization of *C. japonica*.

P181

Molecular Certification Laboratory of Arauco

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Objective: The main objective of the Laboratory is the Molecular Certification of the genetic material of Arauco, through the use of microsatellites (SSR).

Methodology: For the selection of the markers, a reference population of 30 unrelated parents for each species of interest (Eucalyptus globulus and Pinus radiata) was used. In these populations, we tested 25 SSR for pinus and 38 SSR for eucalyptus and the best 12 markers were selected for each species. With the selected markers, a pattern or fingerprint of each operational clone was obtained, out of samples taken from the original material (tree or embryogenic mass). Against these patterns, a total of over 20,000 samples were compared for validation of various operational processes. The sampling is planned to get 90% confidence of detecting operational errors (contamination) greater than 10%. Since the laboratory should process a large number of samples, protocols have been developed to be simple and short. DNA is extracted with 96 commercial Qiagen kit, for different tissues (embryogenic cells, embryos, leaves, bark, etc.). Sometimes, extractions are doubled by mixing leaf tissue of P. radiata and E. globulus, which is possible because SSRs amplified in one species do not interfere with the other. The amount of sample used for the extraction process varies from 50 to 200mg, which yields to concentrations of 30 to 100ng/ul of DNA. Since the amount is enough for several analyses, there is no need to store the original samples. The amplification is performed in thermal cyclers AB 9700 with a single PCR program for each species, using 10 ng of DNA for P. radiata and 2-4ng DNA for Eucalyptus sp. The reading of the fragments is performed in AB 3130xl Genetic Analyzer, while the analysis and correction of the electropherograms is done with the GeneMapper 4.0 program. The fingerprint comparison is made with self developed software, which reduces the noise of size variation observed between samples from the same clone and the appearance of false alleles or silent alleles. All these noises are more noticeable in some clone-marker combinations.

Results: Currently, Arauco has determined the pattern of 400 molecular clones of P. radiata and 200 clones of E. globulus. Thanks to the massive use of fingerprinting in the production and multiplication of clones, steps where mistakes are most likely have been identified and in many cases, errors have been repaired. During 2010, out of 4600 P. radiata samples corresponding to 170 clones, the rate of mistaken identity found was 5.6% in genetic field trials, 5.3% in the embryogenesis laboratory process and 8.4% in nurseries. In E. globulus for 3,000 samples analyzed, of 135 clones, the mistaken identity rate was found to be 7.4% in genetic field tests, 8.3% in clonal orchards and 7.1% in nursery samples.

P182

Evaluating impact of possible transgenic poplar cultivation on protected areas

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Background: Plant biodiversity studies have been performed in the Migliarino-San Rossore-Massaciuccoli Regional Park in Tuscany (Italy) within the framework of the European project LIFE08 NAT/IT/342.

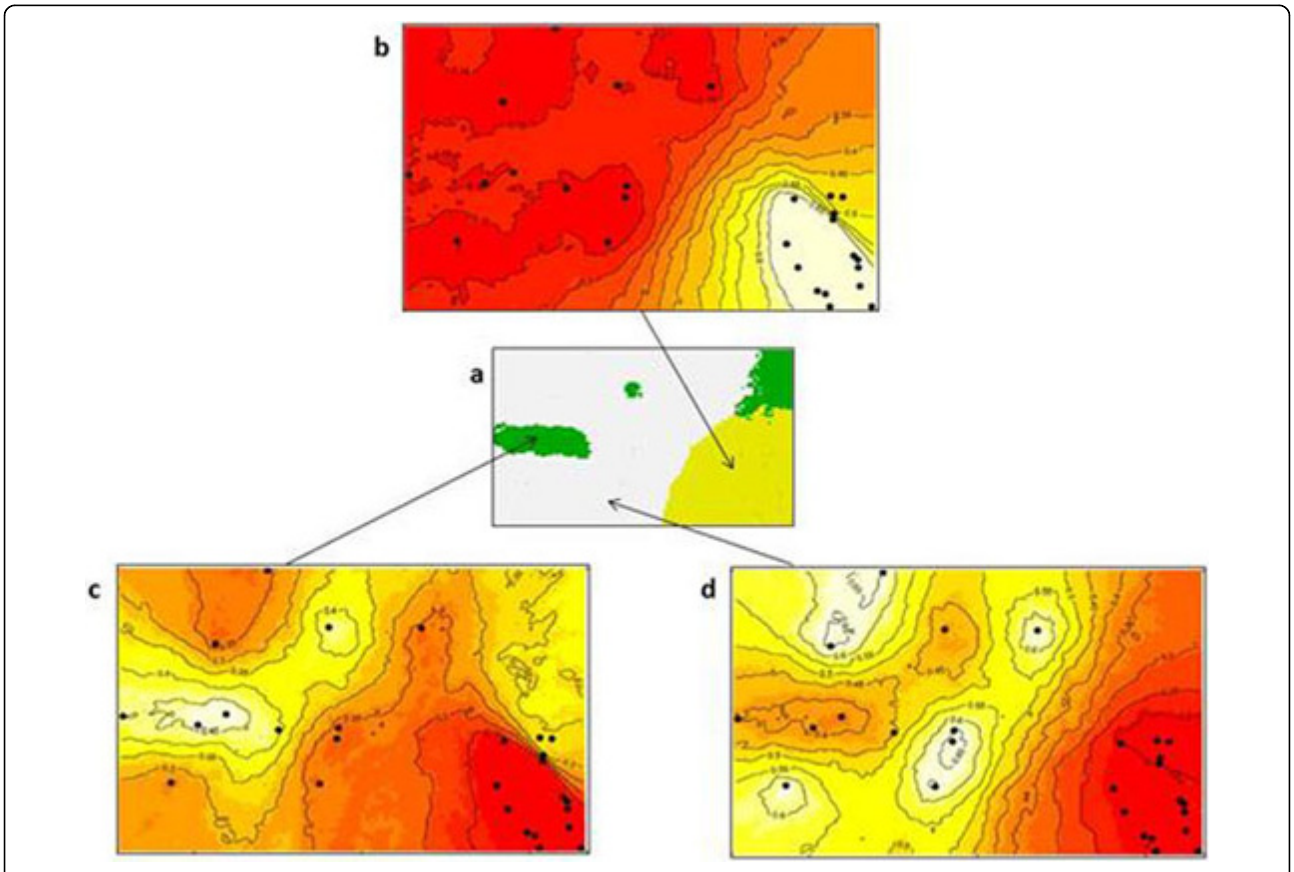

Figure 1(abstract P182) Results of Geneland analyses of area A showing: (a) Spatial organisation into three clusters; (b), (c), and (d) are maps of posterior probabilities of each cluster.

This project aims at developing a quick monitoring index (QMI) to rapidly assess the potential risk generated by transgenic plants in characterized ecosystems or biotopes. For this reason test areas have been selected inside the protected area to evaluate plant (weeds and trees), animal, and soil microoganisms biodiversity. The proximity of the selected test area to cropped surfaces where Genetically Modified Plants (GMPs) might be cultivated has been taken into account. GMPs could spread pollen and contaminate natural populations. To avoid this risk, an efficient monitoring system is required taking into account genetic diversity and breeding study. As far as tree biodiversity concern, *Populus* species were identified in the test areas. Two populations of *Populus* present into two different ecosystems (forest and wetland areas) were examined together with two cultivated varieties. The two ecosystems were characterized for the vegetation. Nuclear microsatellites were used to evaluate genetic diversity of poplar populations and level of breeding between natural and cultivated *Populus*. In addition the insect populations present on male and female poplars during flowering period have been studied.

Materials and methods: The selected *Populus* test areas are: A) a mixed forest stand in the Tenuta di San Rossore; B) a scattered *Populus* population in the wetland area of "lake of Massaciuccoli". Test areas A and B are 8 Km apart.

Test area A is a naturally-originated mixed forest stand. The prevailing tree species are *Populus alba*, *P.* x *canescens*, *Fraxinus angustifolia* and *Alnus glutinosa*. In the test area B single trees and small groups of *Populus* spp. are scattered along the lake.

In the test area A an experimental subplot 2500 m^2 large has been designed and the position has been acquired by GPS. All *Populus* trees within the plot have been identified and their position have been collected by GPS. The stand structure analysis has been performed using spatial functions.

In the test area B the position of *Populus* has been acquired by GPS.

The poplar populations present in areas were examined. From literature, six nuclear microsatellites were selected, all markers carried dinucleotides repeats except two with trinucleotides repeats [1,2].

Total DNA was extracted from the samples to perform microsatellite analysis [1,2]. Sizing of the PCR products was carried out using software Gene Mapper ver. 4.0 (Applied Biosystem).

In order to better define the species density and composition of the herbaceous stratum, some sampling subplots have been designed within the experimental plot by using the standardized multi-scale approach proposed by Dengler [3]. To define possible variations of distribution and/or density of plant species, their relative abundance has been evaluated according to the Braun Blanquet approach (1964).

During poplar tree blooming period inflorescences of male and female trees were collected. Weekly sampling was carried out with the aid of an elevator truck that permitted to collect samples out of reach.

Results: In relation to the herbaceous stratum, the observations made so far show some variability in the distribution of species to a plot to another, probably due to the micro topological characteristics of the site.

The biodiversity analysis of the insects present on poplar trees indicates an evident difference in the faunal community between male and female trees and a relatively low number of species. Dipteran larvae were most abundant followed by coleopterans such as coccinellids and curculionids, araneids and lepidopteran larvae.

The analysis of spatial and genetic structure of the two poplar populations was performed using Geneland [4].

The results of Geneland clearly showed that three distinct clusters can be identified in the area A (Figure 1a) indicating the presence gene flow barriers (Figures 1b, 1c, 1d). The cluster represented in Figure 1b comprise individuals with the same genotype as indicated by microsatellites data (not shown). In fact, it is highly probable that these individuals are root suckers. The second and third clusters show the occurrence of gene flow within and among the two clusters (Figures 1c and 1d). This indicates that a minor barrier is present because the other tree species of the stand block only in part the pollen flow. The same analysis indicates two clusters in area B (Figure 2) with a gene flow among them higher respect to the area A. In fact, this population is in an open area (lake) and therefore the pollen flow is favored.

Conclusions: In general the level of biodiversity within the selected test area is high, and in particular the level of gene flow inside area B. Therefore, the possible cultivation of transgenic poplar close to the protected areas could influence their biodiversity. Especially the level of gene flow can determine a contamination of the autochthon poplar populations. The development of a QMI using the experimental data is in course.

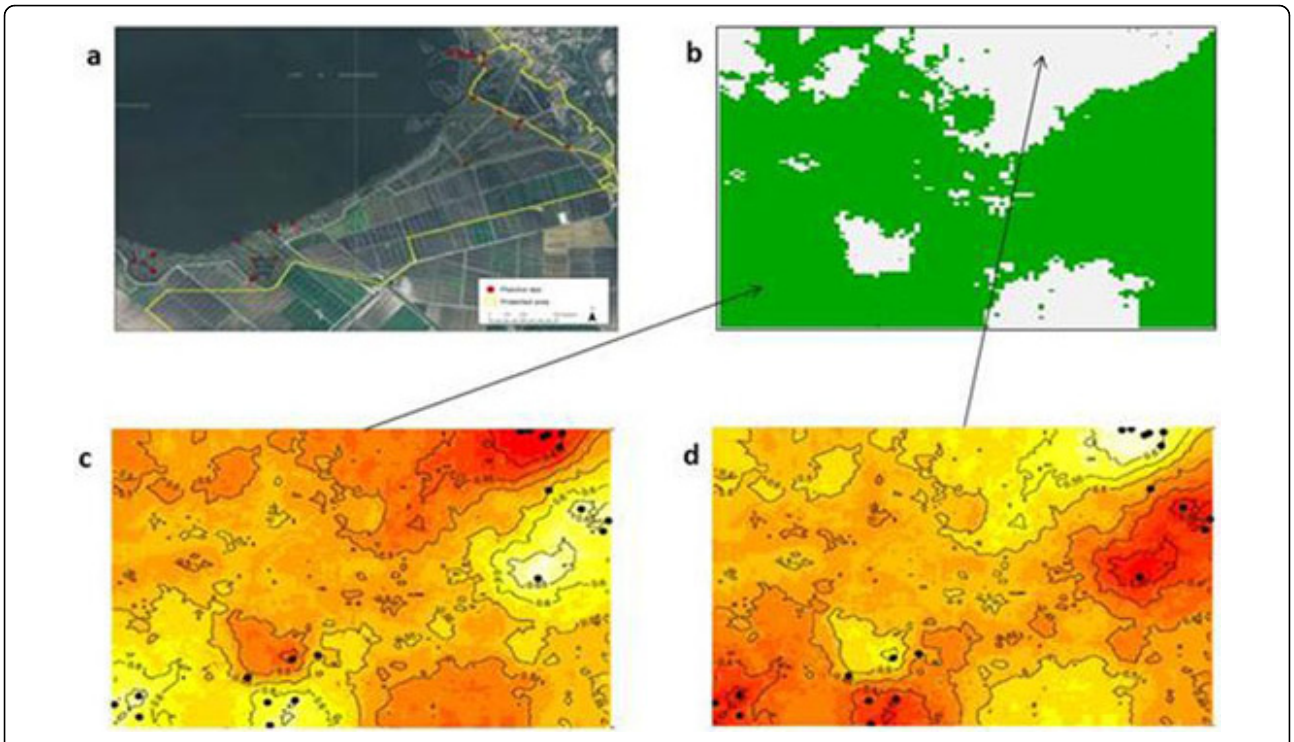

Figure 2(abstract P182) Map of area B (a), and results of Geneland analyses of area B showing: (b) Spatial organisation into two clusters; (c), and (d) are maps of posterior probabilities of each cluster.

The availability of the QMI to rapidly assess the potential risk generated by transgenic plants in characterized ecosystems, is a useful tool to assess the releasing or not of the permission of cultivation close to protected areas.

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Evaluation of different versions of the gene encoding green fluorescent protein (GFP) in plants

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The main purpose of the "CDA *Eucalyptus*: Collaborating Center in Agriculture Defense Relative to the Biosafety of Genetically Modified (GM) Eucalypts" is to gather, assess and validate the existing information concerning GM and non-GM *Eucalyptus* and its derivatives in the Brazilian environment, both in laboratories and field test experiments. Accordingly, the purpose of the present research activity is to provide a binary plasmid collection containing different versions of the gene encoding green fluorescent protein (GFP), originally from *Aequorea victorea*, for the future generation of easily detectable GM plant phenotypes. Using commercially available plasmids encoding blue (ECFP) and yellow (EYFP) versions of GFP for expression in bacteria, the initial strategy was to transfer the coding sequences to the intermediate plasmid pSport1 (Invitrogen) and, subsequently, to pART7 plasmid, which contains the promoter and terminator sequences for gene expression in plants. Although recombinant bacteria have been obtained for both genes, DNA sequencing showed that success was achieved only with pSport1-*eyfp*. So far, pART7 versions were not obtained. A new cloning strategy was proposed, which involves the binary plasmid pCAMBIA1302 (Cambia). This plasmid already contains one version of the *gfp* gene for expression in plants. The intention is to replace the *gfp* gene by *ecfp*, *eyfp* and *mCherryFP* versions. As soon as plasmids are finished, plants will be transformed via *Agrobacterium tumefaciens*, and their transgenic state will be confirmed by the fluorescence of the encoded proteins. Thus, the fluorescence of the GFP different versions will be used to monitor seeds, pollen, leaves and transgenic plants as a whole in the environment.

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IUFRO WORKSHOP COST ACTION FP0905

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Plant biotechnology research at forest fields in South Korea

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This paper is to give an outline of the tree biotechnology research in South Korea.

Tree Breeding, we call Traditional Tree Breeding later compared to Tree Biotechnology, started in 1954 in South Korea. Tree Biotechnology Research started as Tissue culture in the late 1970 in South Korea. Micropropagation was the first field adopted. Embryo culture, Bud culture, Callus culture, Cell culture are the methods for Micropropagation. Many tree species and herbaceous plants have been subjected to micropropagation and successfully established. Tall tree species, *Populus alba* ×*P. glandulosa*, *Quercus*, *Betula et al* are successful for plantation, rare woody plants such as *Forsythia saxatilis*, *Abeliophylum disticum*, *Berchemia berhemiaefolia*, *Hovenia dulsis*, *Lagerstroemia indica* for. *alba*, *Empetrum nigrum* var. *japonicum were* also subjected to in vitro culture. For somatic embryogenesis many species were successful in *Laxix leptolepis*, *Liriodendron tulipifera*, *Aralia elata*, and *Schisandra chinensis* et al. For Practical purpose a few species such as Mountain ginseng(root inductio from callus culture and subsequent root culture by liquid culture) , thornless *Aralia elata*(somatic embryogenesis form petioles), *Liriodendron tulipifera*(somatic embryogenesis from immature zygotic embryo) are under way of commercialization. Mountain ginseng is wild ginseng grown in mountain area. In mountain area they live very long time , even as long as over 100 years meanwhile the plants of it can grow only up to 6 years and produce healthy secondary products for human body. After Multiplication the produced roots are utilized for liquor, cosmetics, medicines *et al*. Yellow poplar**(***Liriodendron tulipifera***),** introduced from north America**,** shows good growth performance and is planted widely throughout the country. Through mass propagation of superior trees by somatic embryogenesis clonal forestry is planned in the forest area.

Biosafety policy of south Korea is very strict. Cultivation of transformed plants is not allowed.

The following list shows the outline of the species which have been reported to be successful to the category in south Korea

Bud culture; *Populus alba* ×*P. glandulosa***,***Kalopanax septemlobus*, *Corylopsis coreana*, *Eucalyptus pellita*, *Actinidia deliciosa* × *A. arguta*, *Prunus yedoensis*,*Diospyros kaki* . *Phellodendron amurese*, *Robinia pseudoacacia*, *Quercus acutissima* .

Forsythia koreana for. aureoreticulata, Salix hallasanensis, Sorbus commixta, Betula schmitti, Machilus thunbrgii, Aconitium koreanum, Juglans regia, Betula dahurica, Crataegus pinnatifida,

Callus cuture; *Pinus koraiensis*, *Ailanthus altissima*, *Medicago sativa*, *Lycium chinense*, *Robinia pseudoacacia* wild Panax ginseng**, Populus koreana** × **P. nigra var. italica,**

Cotyledon culture;*Pinus rigida* × *P. taeda*

Cell culture; *Salix koreensis*, *Gardenia jasminoides*, *Taxus cuspidata*, *Hibiscus syriacus*,

Embryo culture; *Pinus rigida*× *P. taeda***,***Camelia sinensis*, *Pimpinella brachycarpa*, *Populus glandulosa*,

Leaf culture; *Camelia sinensis*

Cambial tissue culture*; Larix decidua*

Somatic embryogenesis; *Populus nigra*× *P. maximowiczii*, *Zizyphus jujuba*, *Oplopanax elatus***,***Eleutherococcus koreanum*, *Kalopanax pictus*, *Aralia elata"Zaoh"*, *Liriodendron tulipifera*, *Larix kaempferi*, , *Lycium chinense*, *Camelia sinensis*, *Orostachys japonicus*, *Quercus variabilis*, *Pimpinella brachycarpa*, *Juglans regia*

Anther culture; *Populus glandulosa*

Protoplast isolation or culture; *Populus alba* ×*P. glandulosa*, *Populus eurameriсana Populus alba*, *Populus glandulosa*, *Populus nigra*×*P. maximowiczii*

Bioreactor culture; *Ganoderma applanatum***,** , *Eleutherococcus koreanum*, *Tricholoma matsutake*(mycelium), *Lilium 'Casa Blanca'*, *Acanthopanax semticosus***,** *Acanthopanax koreanum***, *wild Panax ginseng***

Molecular markers such as I-SSR marker, cpSSR marker have been developed in*Quercus acutissima*, *Pinus densiflora*, *Salix koreensis***for forensic medicine**

Functional genomics: *Populus alba* ×*P. glandulosa*, *Magnolia kobus*

Quantitative Traits Loci for root growth; *Populus davidiana*

Root culture*; Albizzia kalkora*, *Eleutherococcus koreanum*, *Tripterospermum japonicum*

Transformation*: Populus alba*×*P. glandulosa*(Model tree), *Aralia elata*, *Populus koreana* × *P. nigra*, *Camellia chinensis*, *Populus nigra*, *Populus davidiana*, *Pinus densiflora*, *Quercus acutissima*,

I-SSR analysis*; Stewartia koreana*, Genus*Juglans*, *Vaccinium uliginosum*, *Oplopanax elatus*,*Eleutherococcus senticosus*, *Ginkgo biloba*, *Abies holophylla'*

Torreya nucifera, *Ginko biloba*, *Rhododendron schlippenbachii*,*Pinus densiflora*, *Taxus cuspidate*, *Thuja koraiensis*

Cryopreservation*; Bursaphelenchus xylophilus*(*Pine Wood Namatode*), ***Populus koreana* × *P. nigra var. italica***, *Populus alba*× *P. glandulosa*

Expressed Sequence Tags*; Populus alba* × *P. tremula var. glandulosa*

Microsatellite markers; *Quercus acutissima*
Secondary Metabolites: *Eleutherococcus chiisanensis*(*eleutherosides*), *Cornus walteri*
Amorpha fruticosa, *Eleutherococcus senticosus*, *Piper nigrum*, *Kalopanax pictus*, *Grifola umbellate*(Sclerotium)*Populus alba* ×*P. glandulosa* (anthocyanin), Taxus cuspidate, *Phellodendron amurense*, *Camelia chinensis*
Organogenesis; Orchard grass
Phytoremediation; *Populus alba*×*P. glandulosa*, *Populus nigra*×*P. maximowiczii. Populus euramericana*
Allelopathy; *Phellodendron amurense*,
Cryopreservation; *Sapindus mukorossi*, *Lycium chinense*, *Melia azedarach Acer mono*

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COST Action FP0905: Biosafety of forest transgenic trees: improving the scientific basis for safe tree development and implementation of EU policy directives

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Background: The potential for unintended consequences of the spread of foreign genes (either via vertical or horizontal transfer) and of pleiotropic effects following transgene expression may be enhanced in long-lived forest trees. The COST Action FP0905 will focus on four key aspects related to the biosafety of genetically modified trees (GMTs): (a) analyses of the efficiency of existing gene containment strategies to avoid or if not possible to minimize gene flow; (b) facilitate efforts to develop site-specific integration of transgenes in tree genomes to minimize variability of transgene expression and pleiotropic effects, (c) evaluate possible methods to monitor GMTs in the whole production chain, and (d) conduct socio-economic and cost/benefit analyses in relation to the use of GMTs in plantations.
This Action combines multidisciplinary knowledge generated with transgenic lines of different forest genera (such as, *Populus* spp., *Pinus* spp., *Eucalyptus* spp., *Betula* spp., *Castanea* spp., *Picea* spp., etc.) as well as extensive expertise in correlated topics. The information gained should contribute to strengthen the scientific basis for the execution of the EU policy directives related to transgenic trees intended for cultivation in Europe. The main objective of the COST Action is to evaluate and substantiate the scientific knowledge relevant for GMT biosafety protocols by putting together already existing information generated in various European and Non-EU countries as basis for future EU policy and regulation for the environmental impact assessment and the safe development and practical use of GMTs.

Work plan and organisation: To reach its aim, the work plan of the Action is organised in 4 Working Groups (WGs) to implement collaboration of scientists.
WG 1 - Biological characterization of GMTs: to characterize the GMTs in respect to their genetic and phenotypic features relevant for gene flow, gene containment and gene targeting.
WG 2 - Environmental impact assessment and monitoring of GMTs in the whole production chain from plantation to final products: to study environmental risk assessment strategies and monitoring the GMTs along the whole production chain.
WG 3 - Socio-economic implications of and recommendations for the use of GMTs: to make socio-economics analyses of the use of GMTs considering the concerns and acceptance by the public, the economic potential for GMTs and R&D efforts to be invested, as well as cost/benefit analyses, and propose recommendations for the use of GMTs.
WG 4 - Management of intranet - internet websites and dissemination: through a website (www.cost-action-fp0905.eu), provide science-based information and increase public awareness in the utilization of GMTs in forest plantation and at the same time safeguarding the environment
The knowledge gained will be summarised in a book as a final output of this Action which will report the state of art of knowledge and research on GMTs with suggestion on how to effectively implement present EU directives on GMO considering the problematic of forest trees and their environmental impacts.
The Action started the 12th of April 2010 and it will end the 11th of April 2014. Actually, 23 COST countries (Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Denmark, Estonia, Finland, France, Germany, Greece, Israel, Italy, Latvia, Netherlands, Norway, Romania, Serbia, Slovak Republic, Slovenia, Spain, Sweden, and United Kingdom) have signed the Memorandum of Understanding (MoU). Seven NON-COST countries (Albania, Australia, Canada, China, New Zealand, South Africa, USA) are participating to the Action.
Conclusion: With integration of all the countries listed above, the EU COST Action FP0905 is expected to generate important benefits as it also foresees a strong collaboration among R&D bodies and legislative directives. This kind of collaboration will be fundamental, on the one hand, to address policy-making efforts and, on the other hand, to allow the scientific community to discuss to public concerns in a responsible way, particularly concerning socio-economic implications and biosafety issues of transgenic tree plantations.