# Seasonal variation in transcript accumulation in wood-forming tissues of maritime pine (Pinus pinaster Ait.) with emphasis on a cell wall glycine-rich protein

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ORIGINAL ARTICLE

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# Seasonal variation in transcript accumulation in wood-forming tissues of maritime pine (*Pinus pinaster* Ait.) with emphasis on a cell wall glycine-rich protein

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**Abstract** Wood formation is being increasingly studied at cellular and biochemical levels; however, gene expression and regulation during wood formation remain poorly understood. Up to six types of wood can be studied within the same tree (early wood, late wood, juvenile wood, mature wood, reaction wood and opposite wood). These six types are characterized by different chemical, physical and anatomical properties. Using the cDNA-amplified fragment length polymorphism (AFLP) technique, we screened several thousand cDNA fragments from differentiating xylem of maritime pine (*Pinus pinaster* Ait.) comparing early wood vs. late wood and compression wood vs. opposite wood after 8 or 120 days of bending. About 100 transcript-derived fragments (TDFs) showed qualitative or quantitative variations between these different samples. The relative abundance of these TDFs was subsequently analyzed by reverse Northern using RNA derived from early and late wood. Analysis of variance (ANOVA) was used to identify differentially expressed TDFs ($P < 0.01$) and reverse transcription–polymerase chain reaction to confirm the differential expression of some TDFs. Among the genes with a known function, transcript expression and nucleotide sequence variation analysis showed a cell wall glycine-rich protein to be a strong candidate gene for wood properties.

**Keywords** cDNA–amplified fragment length polymorphism · *Pinus pinaster* · Reverse Northern · Seasonal variation · Wood formation

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**Abbreviations** ACC: 1-aminocyclopropane-1-carboxylic acid · AFLP: amplified fragment length polymorphism · CW: compression wood · EST: expressed sequence tag · EW: early wood · G3PDH: glyceraldehyde-3-phosphate dehydrogenase · GRP: glycine-rich protein · HSP: heat-shock protein · INDEL: insertion/deletion · LW: late wood · OW: opposite wood · SNP: single nucleotide polymorphism · TDF: transcript-derived fragment · VW: vertical wood

## Introduction

In perennial plants the successive addition of secondary xylem, differentiated from vascular cambium, gives rise to a unique tissue called wood. Wood formation (xylogenesis) includes four major steps: cell division, cell expansion (elongation and radial enlargement), cell wall thickening (cellulose, hemicelluloses, cell wall protein and lignin biosynthesis and deposition) and programmed cell death. Xylogenesis has been extensively reviewed during the past few years (Fukuda 1996; Lachaud et al. 1999; Roberts and McCann 2000; Mellerowicz et al. 2001).

In conifers, wood is comprised of two main cell types: tracheids and ray parenchyma. Despite this simplicity, it is a highly variable raw material (reviewed by Plomion et al. 2001). This variability is observed at the chemical, anatomical and physical levels. Generally six types of wood can be found within an individual tree. The largest variation observed is the transition from early wood (EW) to late wood (LW) in temperate-zone species. This variation occurs within the same growth ring with EW being formed at the start of the growing season, when temperature and rainfall are favourable to active growth, and LW being initiated toward the end of the growing season, when adverse climatic conditions slow cell division in the cambial meristem, decrease the rate and the duration of cell expansion and extend the duration of secondary wall formation (Uggla et al. 2001). At the anatomical level, LW presents narrower

diameter tracheids with thicker cell walls, resulting in a higher wood density. At the chemical level, LW is characterized by lower lignin and higher cellulose content, while at the ultrastructural level, a lower cellulose microfibril angle in the S2 layer is observed. The second main type of variation concerns the formation of reaction wood in response to a non-vertical orientation of the stem caused by environmental effects (e.g. wind, snow, slope). Reaction wood enables the stem to reorient to ensure a favourable position for the tree (Timell 1986). In conifers reaction wood is found on the underside of the leaning stem and is called compression wood (CW). At the other side of the stem opposite wood (OW) is formed. CW and OW show similar features to those described for EW and LW, respectively. Two specific anatomical characteristic of CW are the presence of rounded tracheids and large intercellular spaces in the transversal section (Timell 1986). The transition between juvenile and mature wood is the third major source of variation typically found in an adult tree (Zobel and Sprague 1998).

The formation of these different wood types is the result of profound changes during xylogenesis. These changes concern cell division, cell expansion and cell wall thickening and are triggered by external (e.g. temperature, photoperiod) and/or endogenous factors (e.g. phytohormones, sugars). In Scots pine (*Pinus sylvestris* L.) and hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx) a steep radial concentration gradient of indole-3-acetic acid (IAA) across developing tissues of the cambial region was observed by Uggla et al. (1996) and Tuominem et al. (1997), suggesting that IAA could provide positional information during xylogenesis and therefore regulate the rate of cell division and duration of cell expansion. Uggla et al. (2001) recently studied the transition from EW to LW in Scots pine. They showed that the radial distribution pattern of IAA across the cambial zone is altered during this transition, resulting in an increased concentration of IAA in the cambial meristem and its most recent derivative. The sucrose synthase activity was also shown to increase in tracheid-forming secondary walls of LW. These data are consistent with previous observation that LW formation is under developmental rather than metabolic control.

External and internal factors involved in the formation of CW, OW, juvenile wood and mature wood have also been extensively studied (e.g. Timell 1986; Zobel and Sprague 1998).

Understanding the molecular mechanisms that coordinate the response to these exogenous and endogenous factors remains in its infancy (reviewed by Plomion et al. 2001). The recent development of high-throughput genomics methods has allowed gene and protein expression patterns to be studied during the development of these different wood types. These approaches will improve our understanding of the molecular mechanisms involved in wood formation and will indicate the main targets for selection or genetic manipulation for the provision of superior wood quality. Random expressed sequence tag (EST) sequencing (Allona et al. 1998; Sterky et al. 1998), serial analysis of gene expression (SAGE; Lorenz and Dean 2002) and micro-array analysis, (Hertzberg et al. 2001; Whetten et al. 2001) have all been used to investigate differential expression between different tissues and wood types. These experiments have been used only to identify differentially expressed genes between CW vs. OW or mature wood vs. juvenile wood. The cDNA–amplified fragment length polymorphism (AFLP) assay (Bachem et al. 1996) is an efficient and cheaper alternative to detect differentially expressed genes between contrasted samples (e.g. Milioni et al. 2002; Dubos and Plomion 2003). In this study, we report on the identification of transcript-derived fragments (TDFs) in differentiating xylem associated with different wood types in maritime pine (*Pinus pinaster* Ait.) with the primary objective being to detect TDFs differentially expressed between EW vs. LW. In order to increase the power of detection and also to study the possible interactions with other wood types, differentiating xylem associated with vertical wood (sampled from straight trees), OW and CW (sampled from a bent tree), from the same genotypes, were sampled early and late in the growing season. Differentially expressed TDFs were cloned, sequenced and spotted onto nitrocellulose membrane and hybridized with cDNA probes corresponding to developing xylem of EW and LW. Finally, we confirmed the differential accumulation of some selected genes by reverse transcription–polymerase chain reaction (RT–PCR).

## Materials and methods

### Sampling differentiating xylem

Compression wood was induced in four 14-year-old maritime pine (*Pinus pinaster* Ait.) clones by artificial bending. Differentiating xylem tissues associated with CW and OW were collected after bark, phloem and cambium were peeled from the stem following the procedure described by Plomion et al. (2000). Samples were taken after 8, 35, 66, and 120 days of bending. Two control clones (no bending) were sampled for vertical wood (VW) xylem associated with EW and LW at days 8 and 120, respectively.

### mRNA fingerprinting

Differentiating xylem samples corresponding to VW, CW and OW at days 8 and 120 were used for mRNA fingerprinting using the cDNA-AFLP assay described by Bachem et al. (1996). Total RNA was extracted following Chang et al. (1993) with modifications described by Dubos and Plomion (2003). The extracts were digested at 37 °C for 30 min with 50 U of Rnase-free DNase RQ1 (Promega, Madison, WI, USA). Total digested RNA was cleaned using the RNeasy plant kit (Qiagen, Valencia, CA, USA). Concentration was estimated spectrophotometrically. One microgram of cleaned RNA was used for cDNA synthesis using the Smart PCR cDNA synthesis kit (Clontech Laboratories, Paolo Alto, CA, USA) according to the manufacturer's protocol. The number of PCR cycles corresponding to the exponential phase was determined by sampling the PCR reactions after 15, 17, 19, 21 and 24 cycles (Fig. 1). The steady state was reached after 21 cycles, indicating that the amplification reaction should be performed over 19 cycles

to allow the recognition of quantitative expressed genes. The non-radioactive cDNA–AFLP procedure described by Dubos and Plomion (2003) was then used to identify differentially expressed fragments in differentiating xylem associated with the different types of wood.

Briefly, in the selective amplification step, primers complementary to the adaptors *Pst*I and *Mse*I or *Eco*R1 and *Mse*1, with one or two additional selective 3′-end nucleotides (*Pst*I primers: 5′-GACTGCGTACATGCAGX-3′ where X corresponded to C, G, AG, AT, CA, CC, GA, CG, CT, TG; *Eco*R1 primers: 5′-GAT GAGTCCTGAGTAAATX-3′ primers where X corresponded to AA, CA, CT, GT, AT, GA; *Mse*I primers: 5′-GAT GAGTCCTGAGTAAX-3′, where X corresponded to A, C, G, AC, AT, CC, GA and GT). Following electrophoresis, gels were silver-stained and dried.

TDFs showing quantitative and qualitative variations were eluted in 5 µl of distilled water as described by Chalboud et al. (1997) and used as template for PCR re-amplification, using the same conditions as in the selective PCR reaction. These TDFs were separated on a 2% agarose gel and purified using a PCR product purification kit (Promega). TDFs were cloned using the TOPO TA-cloning kit (Invitrogen, Carlsbad, CA, USA) and purified plasmids were used as template for sequencing using the DNA sequencing kit (Perkin Elmer, Foster city, CA, USA). Sequencing was carried out using a Li-Cor 4000 sequencer (Li-Cor, Lincoln, NE, USA). Nucleotide sequences were loaded onto the NCBI Resource Centre (http://www.ncbi.nlm.nih.gov/) form, for BLAST searching (Altschul et al. 1997). Score and *E*-value from BLAST-X were considered when determining significant similarities: a score greater than 80 and an *E*-value lower than $2\ e^{-20}$. Sequences were also compared to maritime pine ESTs (1,975 entries available in the EMBL database) and loblolly pine ESTs (59,859 entries available at http://pine.ccgb.umn.edu/) using BLAST-N software (Altschul et al. 1997).

## Characterization of differentially expressed TDFs

*Reverse Northern and data analysis* Inserts corresponding to the differentially expressed TDFs were re-amplified by PCR in 50 µl of 1× PCR buffer containing 2 µM of each of the primers (M13 forward: 5′-TGACCGGCAGCAAAATG-3′ and reverse: 5′-GGAAACAGCTATGACCATG-3′) that were complementary to the vector sequence flanking the insert, 2 mM $MgCl_2$, 400 µM dNTP and 0.15 U of *Taq* DNA polymerase (Invitrogen). PCR reactions were performed using a GeneAmpPCR system 9700 (Perkin Elmer) with the following cycling parameters: preliminary denaturation (4 min, 94 °C), then 35 cycles of denaturation (30 s, 94 °C), annealing (1 min, 55 °C), extension (1 min, 72 °C) and final extension (4 min, 72 °C). The quantity and quality of each PCR product was determined on a 2% agarose gel using Low DNA Mass Ladder (Invitrogen) as a control. PCR products (20–30 ng $\mu l^{-1}$) were arrayed onto nitrocellulose filters (Eurogentec, Liège, Belgium) using a *Micro*Grid Robot (Biorobotics, Cambridge, UK). The 0.4-µm tips deposited 500 nl of each product with spacing of 500 µm on a filter (7 cm × 10 cm) saturated with 0.1 M NaOH at a density of 12 clones per $cm^2$. Internal controls were spotted at specific positions on the filters. These contained distilled water, polylinker, desmine and nebuline (human proteins kindly provided by H. Höfte, INRA Versailles, France) and receptor tyrosine kinase/RAS from *Pisolithus trinctorius* (kindly provided by S. Duplessis, INRA Nancy, France). Four replicated clones were spotted on each filter.

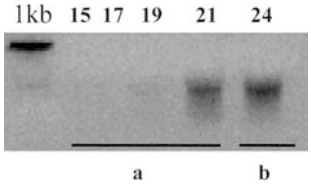


**Fig. 1** Determination of the number of PCR cycles required for the recognition of quantitatively expressed genes in differentiating xylem of maritime pine (*Pinus pinaster*). Five microliters of PCR products were collected after 15, 17, 19, 21 and 24 PCR cycles. *a* Exponential phase, *b* plateau phase

Total RNA (prepared as described above) from differentiating xylem associated with EW and LW was used to prepare complex full-length cDNA probes by reverse transcription using the Smart PCR cDNA synthesis kit (Clontech). cDNA probe labelling (400 ng) was done in the presence of 1,480 kBq of α-[$^{33}$P]dATP and 1,480 kBq of α-[$^{33}$P]dCTP using the Prime-a-Gene labelling system kit (Promega). Unincorporated nucleotides were removed using the Qiaquick PCR purification kit (Qiagen).

After purification, the specific activity of each probe was determined using a scintillation counter. Filters were pre-hybridized in 30 ml of hybridisation buffer (5× SSC, 5× Denhardt's, 0.5% SDS, 10 µg $ml^{-1}$ sheared salmon sperm DNA) for 5 h at 65 °C. Filters were then incubated in 20 ml of fresh hybridisation buffer with denatured radiolabelled probes at 65 °C for 20 h. After hybridisation, filters were washed twice for 5 min in 2× SSC, 0.5% SDS at room temperature, twice for 15 min in 2× SSC, 0.1% SDS (65 °C), twice for 15 min in 1× SSC, 0.1% SDS (65 °C) and twice for 15 min in 0.1× SSC, 0.1% SDS (65 °C). Washed filters were wrapped in plastic film and exposed to PhosporImager screen (Bio-Rad Laboratories, Hercules, CA, USA) for 72 h, after which the imaging screen was scanned using Personal Molecular Imager FX (Bio-Rad) at maximum resolution screen of 50 µm per pixel.

Signals were detected and quantified using the imaging software ArrayVision (Imaging Research, St. Catharines, Ontario, Canada). A two-level grid template was generated and automatically superimposed on the data image for spot signal quantification with few manual adjustments. Median-density quantification was used, as this measurement is less dependent on extreme values, allowing partial elimination of artefacts resulting, for example, from dust particles or membranes defects. Local background for each spot was calculated on the basis of four small areas (totalling the spot area) surrounding each spot (corner-background method) using a median-density method. The worksheet generated by the software contained several quality and quantity measurements, such as median density or "signal/noise" ratio, which were exported to Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA, USA).

Each spot density signal value was corrected by subtraction of the corresponding local background value and the subtracted data from each filter were then adjusted by dividing the corrected density value of each spot present on one filter by the mean density value of that filter. Further analyses were performed on the relative value of each TDF present in the complex target. Analysis of variance (ANOVA) was then performed between EW vs. LW to determine the significantly differentially expressed genes ($P < 0.01$). The following linear model was applied to each spot $Yij = \mu + Ci + \epsilon ij$ with i standing for the seasonal effect (I = EW vs. LW), and j the number of replicates (j = 1–4). Prior to ANOVA, the distribution of the corrected spot intensity was transformed ($Log_{10}$). All statistical analyses were performed using a published routine in Splus (Becker et al. 1988).

*Reverse transcription PCR* For each xylem sample (EW, LW, CW, OW), contaminating DNA was removed from total RNA by digestion with RNase-free DNase RQ1 (Promega) for 1 h at 37 °C in the presence of RNase inhibitor (RNasin; Promega). Following digestion the DNA-free total RNA was purified through an RNeasy mini-column (Qiagen). Purified RNA (2 µg) was reverse-transcribed at 42 °C for 1 h in 20 µl of 1× buffer containing 500 pmol oligo(dT) primer, 500 pmol DTT, 200 µM dNTPs and 200 U of SUPERSCRIPTII RnaseH Reverse Transcriptase (Invitrogen). First-strand cDNA was diluted 4-fold and amplified using PCR in 50 µl final volume, containing 1× PCR buffer (Invitrogen) with 2 µl of template, 0.1 mM dNTPs, 0.2 µM of each primer pair [one TDF primer pair (Table 1) and one control primer pair (*trans*-cinnamate-4-hydroxylase, C4H, EC 1.14.13.11)], 2 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Invitrogen) (Dubos and

Plomion 2003). The PCR was performed in a Perkin Elmer 9700 thermocycler with the following cycling parameters: preliminary denaturation (4 min, 94 °C), denaturation (30 s, 94 °C), annealing (30 s, 58 to 61 °C), extension (45 s, 72 °C) and final extension (4 min, 72 °C). TDF primer pairs (Table 1) were designed in the 3′ region of the gene using Primer 3 (http://wwwgenome.wi.mit.edu/genome_software/other/primer3.html). Ten microlitres of each PCR reaction was sampled at 15, 20 and 35 cycles. Part of the 35-cycle PCR product was run on a 1.2% agarose gel in order to confirm the PCR quality. PCR products corresponding to 15 and 20 cycles were also run on a 1.2% agarose and transferred onto nylon membrane using standard capillarity techniques. The remaining 35-cycle PCR product was purified using MicroSpin S 300HR columns (Amersham Bioscience) and its concentration was estimated using the low DNA Mass Ladder and labelled in the presence of 1,110 Kbq α-[$^{32}$P]dCTP and random hexamers using the Prime-a-Gene labelling system kit (Promega). Pre-hybridisation and hybridisation were performed as described in the previous section. The hybridized membranes were washed for 5 min in 2× SSC, 0.5% SDS at room temperature, 10 min in 2× SSC, 0.1% SDS (65 °C), 10 min in 1× SSC, 0.1% SDS (65 °C), 10 min in 0.1× SSC, 0.1% SDS (65 °C). Membranes were exposed to Konica medical X-ray film (Konica, Tokyo, Japan) for 1–3 days.

### Polymorphism detection and diversity structure in the glycine-rich protein (GRP)

*Amplification, sequencing and polymorphism detection* Nucleotide polymorphism was detected using genomic DNA extracted from 27 megagametophytes (haploid tissue surrounding the embryo), representing the whole geographic range of maritime pine (13 populations). A gene-specific primer pair in the 3′-end region of the full-length cDNA for TDF PP.PM16 was designed using Primer 3 to amplify a 439-bp genomic fragment. PCR reactions were performed in Thermal Cycler GenAmp 9700 in a final volume of 30 µl containing 1× buffer, 30 ng of DNA, 3 mM of $MgCl_2$, 300 µM of dNTPs, (0.2 µM of each primer (Upper primer: 5′-GAGTTCTCAAGGGATGTCGG-3′and Lower primer: 5′-TAACACACCAAGAGGCCACC-3′) and 0.5 U of Amplitaq Gold (Invitrogen). In order to avoid non-specific PCR products a five-step PCR programme was used: (i) a preliminary denaturation (3 min, 94 °C), (ii) two highly specific cycles including a denaturation step (1 min, 94 °C), an annealing step (1 min, 60 °C) and an extension step (35 s, 70 °C), (iii) 18 cycles of touchdown (45 s at 94 °C, 45 s at 59 to 50.5 °C and 45 s at 70 °C) followed by (iv) 20 cycles (30 s at 92 °C, 30 s at 50 °C, 1 min at 70 °C) and then (v) a final extension step (5 min at 70 °C).

PCR products were sequenced using the Big Dye terminator kit (Amersham Bioscience, Uppsala, Sweden) according to the specifications of the supplied protocol and run in an ABI 3100 automatic sequencer (Applied Biosystems, Foster city, CA, USA). One sequence was obtained for each individual. Sequences were aligned and nucleotide polymorphism detected using the Sequencher software (http://www.Genecodes.com). Chromatograms were checked for all polymorphisms in order to distinguish true polymorphisms from scoring errors. Only clear polymorphisms (single peak) were accepted. The polymorphisms involving double peaks were discarded.

*Sequence analysis and statistical tests* The number of single nucleotide polymorphisms (SNPs), insertion/deletions (INDELs), synonymous (S) and non-synonymous (NS) mutation transitions and transversions, as well as nucleotide diversity in coding and non-coding regions, was calculated using SITE software (Hey and Wakeley 1997). Determination of S and NS mutations was based on the definition of the coding frame obtained by Blast-X (Altschul et al. 1997).

Haplotype number and frequency were estimated by analysis of all polymorphisms. Due to our sampling strategy with several populations from different provenances, and the previous results obtained with neutral markers (Mariette et al. 2001; Derory et al. 2002) showing a geographic structure of the genetic diversity, no linkage disequilibrium calculations were performed.

A neutrality test based on the relative abundance of S and NS substitutions having occurred in the gene sequence (dN/dS test: Hill and Hastie 1987; Huges and Nei 1988), that does not require assumptions about the population structure, was performed using MEGA v2.1 (Kumar et al. 2001). A *Pinus taeda* consensus obtained using 13 ESTs presenting significant ($<10^{-50}$) homology with PP.PM16, was used as an out-group to run this analysis.

## Results and discussion

### Identification of putatively differentially expressed genes using the cDNA–AFLP assay

cDNA fragments were amplified using 155 primer–enzyme combinations (PEC: *Pst*1/*Mse*1 or *Eco*R1/*Mse*1). An average of 35 transcript-derived fragments (TDFs) per PEC was obtained after silver staining, resulting in approximately 5,500 screened fragments. cDNA–AFLP fragment size ranged from 50 to 800 bp with a mean of approximately 400 bp. A total of 119 TDFs (2.5%) were found to be differentially expressed between EW vs. LW and OW vs. CW. Variation of the expression pattern fell into two classes: (i) those showing quantitative variations (differences in intensity of the cDNA fragments), and (ii) those showing qualitative variations (presence/absence variations). An example of qualitative variation is shown in Fig. 2 for a TDF that is strongly expressed in differentiating LW xylem, independent of wood type (VW, CW or OW). A unique set of 109 TDFs was then obtained following Blast-N analysis, corresponding to a 6.5% rate redundancy. Redundancy among the TDFs could be explained by the use of selective PCR with

**Table 1** Primers pairs used for RT–PCR analysis. $T_a$ Annealing temperature

| Clone ID | Putative function (abbreviation) | Upper primer (5′-3′) | Lower primer (5′-3′) | $T_a$ (°C) |
|---|---|---|---|---|
| PP.PM16 | Glycine-rich protein (GRP) | GTTGGGTGGGGTGAAGATAA | CCGTTCCGAGCAGAGC | 60 |
| PP.PM32 | Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) | CGCTCACTTCCCTCTGTTTTC | CATCTTTTGCGACGGTTTCTC | 61 |
| PP.EM43 | Senescence-associated protein (Sen) | CGTCGTGAGACAGGTTAGTTTTA | AGAGGCGTTCAGTCATAATC | 59 |
| PP.PM66 | Low-molecular-weight heat shock-protein (LMW HSP) | TTCTGCCGTAGCCAATACTC | TGCTCGCCTTTATCTCTTCC | 58 |
| Control | *Trans*-cinnamate 4-hydroxylase (C4H) | GACGGTCTGTTATTGAAATCC | TCTCAACCACTCTCTCATCGC | 58–61 |

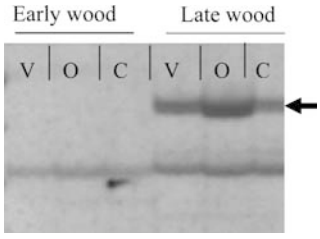


**Fig. 2** Differentially expressed TDF patterns observed on a polyacrylamide silver-stained gel. TDF PP.PM16 (*arrow*) is over-expressed during LW formation in differentiating xylem of maritime pine, whatever the type of wood considered. *V* Vertical, *O* opposite, *C* compression

primer pairs containing common selective nucleotides. Among the 109 unique TDFs, 24 (22%) sequences showed qualitative variations between EW vs. LW, and 85 TDFs (78%) displayed mostly quantitative variations between CW vs. OW, after 8 or 120 days of bending.

## Quantitative analysis of gene expression by reverse Northern and RT–PCR

Expression patterns of the 109 TDFs identified by cDNA–AFLP were analyzed by reverse Northern in order to validate those observed in the cDNA–AFLP assay, and to determine the relative abundance of each transcript in the differentiating xylem of EW and LW (Fig. 3).

For each TDF, ANOVA was performed to detect differentially expressed genes between EW vs. LW. Thirteen TDFs (12%) showed significant ($P < 1\%$) differences in accumulation between EW vs. LW, from which 10 (out of 24) and 3 (out of 85) were previously detected in the cDNA–AFLP screen between EW vs. LW and OW vs. CW, respectively. The higher rate of validation for the TDFs associated with EW vs. LW (42%) is probably due to higher differences in gene expression between these two contrasting types of wood, compared to OW vs. CW, even after 120 days of mechanical bending. It should be noted that only one TDF (PP.EM42, similar to a polyubiquitin) presented a conflicting result between both assays. It is likely that different members of the ubiquitin gene family were revealed by these two techniques.

Among the 13 differentially expressed TDFs, a search against the GenBank database showed that 7 TDFs corresponded to known-function genes, 1 to an unknown protein, and 5 did not match any published sequence in public databases and were classified as hypothetical protein (Table 2). Two TDFs were found to be specifically expressed in EW-associated xylem [PP.PM32 was 53% identical to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and PP.PM60bis was of unknown function], 6 were up-regulated in EW [from a 2.12- to a 10-fold ratio: the only gene with a known function, PP.EM39, was 49% identical to 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase], whereas 5 TDFs were down-regulated in EW (from 1.8- to 16.6-fold ratio: among known-function genes, PP.PM16 was 96% identical to a GRP, PP.PM66 was 77% identical to a heat-shock protein (HSP), PP.PM31 was 48% identical to a tubulin α chain, and PP.EM31 was 87% identical to UDP-glucose uridyltransferase). The ACC oxidase and α-tubulin that were identified by cDNA–AFLP presented clear qualitative differences between CW and OW at both day 8 and day 120. Contradictory results were obtained by reverse Northern, since ACC oxidase was mainly expressed in EW (day 8) of a straight tree and α-tubulin in LW (day 120) of a straight tree. These results illustrate the interaction effect between gravity and seasonal effects on gene expression.

RT–PCRs were performed on TDFs showing high [e.g. G3PDH, Sen (a putative senescence-associated protein)] and low (e.g. HSP, GRP) EW/LW ratios in the reverse Northern experiment (Fig. 4a). These four TDFs showed the same expression pattern in both procedures, i.e. up-regulation of G3PDH and Sen in EW and down-regulation of HSP and GRP in EW. For two genes highly expressed in LW (HSP and GRP), the trend of gene expression was also investigated across the whole experiment, i.e. in OW and CW after 8, 35, 66 and 120 days of bending. The GRP showed a progressive accumulation along the season in OW, whereas the transcript was present at a very low level in CW after only 120 days of bending (Fig. 4b), contrasting with the results obtained on VW-associated xylem (Fig. 4a). This expression profile was also observed for the HSP although it showed some basal expression even in early CW.

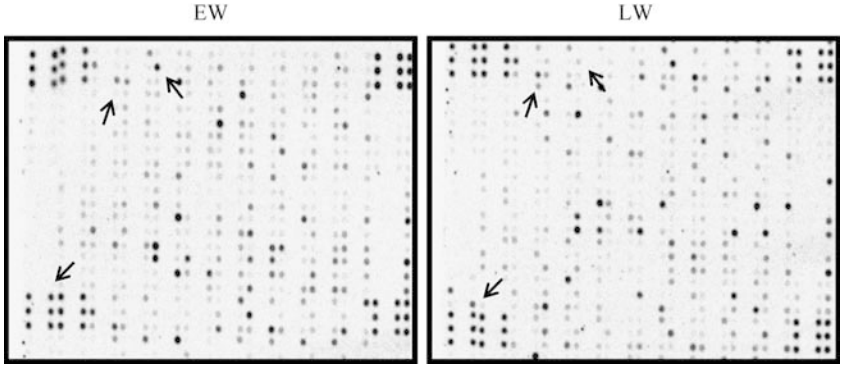


**Fig. 3** Reverse Northern experiment. Differentially expressed ESTs between EW and LW in differentiating xylem of maritime pine are indicated by arrows. *EW* Membrane hybridized with cDNA corresponding to differentiating xylem associated with early wood, *LW* membrane hybridized with cDNA corresponding to differentiating xylem associated with late wood

**Table 2** Homologies of the differentially expressed cDNA–AFLP fragments to sequences in databases (Nov. 2002). All are BLAST-X scores

| Clone ID | Size (bp) | Accession No. | Expression pattern cDNA | | Function (abbreviation, EC, accession) | BLASTX score, E-value |
|---|---|---|---|---|---|---|
| | | | AFLP[a] | Reverse Northern[b] | | |
| PP.PM32 | 433 | BX000609 | EW | E | Glyceraldehyde-3-phosphate-dehydrogenase (G3PDH, EC. 1.2.1.13, P12859) | 145, 3e-34 |
| PP.PM60bis | 241 | BX000978 | EW | E | Hypothetical protein | – |
| PP.PM48 | 261 | BX000732 | EW | 10 | Hypothetical protein | – |
| PP.PM14 | 435 | BX000729 | OW8 | 5 | Hypothetical protein | – |
| PP.EM24 | 307 | BX000713 | EW | 4.34 | Hypothetical protein | – |
| PP.EM39 | 364 | BX000581 | CW8 and CW120 | 3.44 | ACC oxidase (ACC oxidase, T09145) | 112, 2e-24 |
| PP.EM43 | 409 | BX000584 | EW | 2.85 | Putative senescence-associated protein (Sen, BAB33421) | 98, 2e-20 |
| PP.PM33 | 723 | BX000610 | EW | 2.12 | Unknown protein (NP_565167) | 108, 9e-23 |
| PP.EM31 | 260 | BX000576 | LW | 0.56 | UDP-glucose pyrophosphorylase (UDPGP, EC.2.7.7.9, BAB69069) | 148, 1e-35 |
| PP.EM42 | 223 | BX000715 | EW | 0.52 | *Pinus taeda* EST[c] | 151, 2e-36 |
| PP.PM31 | 370 | BX000608 | CW8 and CW120 | 0.36 | Tubulin α chain (Tub, CAB66336) | 80, 3e-23 |
| PP.PM66 | 260 | BX000656 | LW | 0.16 | Class-I LMW heat-shock protein (LMW HSP, S71768) | 110, 3e-24 |
| PP.PM16 | 193 | BX000600 | LW | 0.06 | Glycine-rich protein (GRP, AAF75823) | 101, 2e-21 |

[a]cDNA–AFLP fragments were isolated from xylem associated with compression wood (CW), opposite wood (OW) after 8 or 120 days of bending, and early wood (EW) or late wood (LW)
[b]Ratio of the expression levels (EW/LW).*E* denotes EW-specific expression after reverse Northern analysis
[c]Match to a *Pinus taeda*(BG275672) EST similar to polyubiquitin-like protein (S25164)

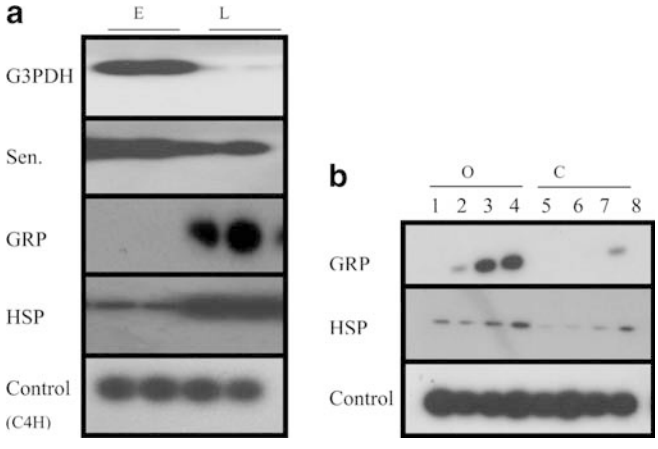


**Fig. 4a, b** RT–PCR analysis of four cDNAs associated with different types of wood in differentiating xylem of maritime pine. *G3PDH* Glyceraldehyde 3-phosphate dehydrogenase, *Sen.* a putative senescence-associated protein, *HSP* low-molecular-weight heat-shock protein, *GRP* glycine-rich protein, *C4H trans*-cinnamate-4-hydroxylase. **a** Expression level in xylem collected on a straight tree and associated with early wood (*E*, day 8), and late wood (*L*, day 120). Two replicates of the RT–PCR were performed. **b** Expression level in xylem associated with opposite wood (*O*) after 8 (*lane 1*), 35 (*lane 2*), 66 (*lane 3*) and 120 (*lane 4*) days of bending and with compression wood (*C*) after 8 (*lane 5*), 35 (*lane 6*), 66 (*lane 7*) and 120 (*lane 8*) days of bending. PCR products were sampled after 15 cycles of PCR

The putative roles of the proteins encoded by these TDFs are consistent with biological processes known to occur during xylogenesis.

*Cell wall GRP* A *Pinus taeda* GRP (AF101787) belonging to the cell wall-associated protein class has recently been found to be highly expressed in the differentiating xylem of OW compared to CW (Allona et al. 1998; Zhang et al. 2000). In addition, this transcript was not detected in xylem collected from straight trees (VW). In the present study using cDNA–AFLP and reverse Northern assays, a *Pinus pinaster* cDNA fragment (PP.PM16:193pb), similar to the *P. taeda* GRP, was found to be differentially expressed between EW vs. LW (Figs. 2, 4a). A full-length cDNA (AL750579) of 583 bp was retrieved from the *P. pinaster* xylem EST database. Blast-X analysis showed that the full coding sequence of this GRP was 96% identical to the *P. taeda* GRP. This gene has a putative open reading frame of 393 bp encoding a 131-amino-acid protein. The protein had a predicted molecular mass of 12.7 kDa and an isoelectric point of 10.1. We examined the expression pattern of PP.PM16 in differentiating xylem associated with different wood types (EW vs. LW, OW vs. CW) using an RT–PCR assay. As observed for the ortholog in *P. taeda* (Zhang et al. 2000), the transcript accumulated over time in xylem associated with OW, and to a much lesser extent in CW (Fig. 4b). Interestingly, PP.PM16 also accumulated in straight stems but only during LW formation (Fig. 4a). To our knowledge, such molecular heterochrony (defined here as the prolongation of the EW formation period due to CW formation) has never been reported in a tree species.

The parallel that can be drawn between (i) the phenotypic proximity of LW and OW or EW and CW in terms of chemical, anatomical and ultrastructural

properties (reviewed by Plomion et al. 2001), and (ii) the similar profiles in transcript accumulation of this GRP in these four tissues, constitutes a strong case to propose this gene as an "expressional candidate" for wood properties. Cell wall GRPs have been described as existing in $\beta$-pleated sheets composed of varied anti-parallel strands and they may be linked to aromatic residues of lignin through tyrosine residues. They are localized in vascular tissues and are thought to provide elasticity as well as tensile strength during vascular development (Cassab 1998).

*Low-molecular-weight heat-shock protein (LMW HSP)* Clone PP.PM66, identical to a Douglas fir class-I LMW HSP, showed an expression pattern similar to the GRP, supporting the result found at the protein level by Plomion et al. (2000). This gene was also up-regulated in LW (Fig. 4a) although it was basally expressed in EW, it accumulated over time in xylem associated with OW, and to a lesser extent in CW (Fig. 4b), indicating a molecular heterochrony as reported for the GRP. In plants, it is well known that LMW HSPs are produced in response to various stresses (Vierling 1991; Waters et al. 1996; Costa et al. 1998). Synthesis also occurs during developmental processes such as pollen or seed maturation (Puigderrajols et al. 2002) and early seedling growth (Kaukinen et al. 1996). In Douglas fir, expression of a class-I LMW HSP was shown to be under the control of several plant growth regulators (Kaukinen et al. 1996). The pattern of expression of class-I LMW HSPs and their regulation by phytohormones suggests specific roles for this HSP in wood formation rather than in the water-deficit response.

*Glyceraldehyde-3-phosphate dehydrogenase* Clone PP.PM32 was very similar to a G3PDH and the transcript of this gene was specifically detected in EW-associated xylem by the three transcriptional techniques: cDNA–AFLP, reverse Northern (Table 2) and RT–PCR (Fig. 4a). In *P. taeda*, this transcript also over-accumulated in CW (Whetten et al. 2001), a result confirmed here by RT–PCR on differentiating xylem collected on the lower and upper sides of a 14-year-old tree that was bent for 2 years (Fig. 5). This enzyme catalyzes the only oxidative reaction that occurs during glycolysis, a central pathway for energy production in cells (Higuchi 1997). In young trees, Zobel and Van Buijtenen (1989) reported that the proportion of EW was greater than LW, corresponding certainly to an increase in cambial activity. CW is also characterized by an important cambial activity resulting in an eccentric radial growth (Timell 1986). Given the similarity between EW and CW in terms of prominent cambial activity, huge energy demand exists in these two tissues compared to LW and OW. We could therefore hypothesize that the increase in cambial activity in CW and EW requires a large amount of energy through ATP and glycolysis leading to an over-expression of G3PDH.

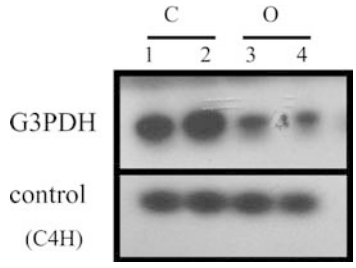


**Fig. 5** RT–PCR analysis of G3PDH. Expression levels in differentiating xylem associated with compression wood (*C*) and opposite wood (*O*) of maritime pine after 2 years of mechanical bending. Two replicates of the RT–PCR were performed. PCR products were sampled after 15 cycles of PCR. *C4H*, *trans*-cinnamate-4-hydroxylase

*Other known-function genes* Besides these three markers for EW (G3PDH) or LW (GRP, LMW HSP) formation, a number of other known-function TDFs were found to be differentially expressed in developing xylem associated with these two tissues, suggesting their specific involvement in wood formation. These included:

– PP.EM31, similar to UDP-glucose pyrophosphorylase. This enzyme was over-expressed in LW. This enzyme plays a central role in the metabolic pathway producing UDP-glucose from glucose-1-phosphate. In plants, the majority of glycosyl units used in the synthesis of cell wall polysaccharides pass through pools of either UDP-glucose and GDP-mannose (Gibeaut 2000). While sucrose synthase (SuSy) is generally regarded as the major enzyme responsible for the supply of UDP-glucose for cellulose biosynthesis (Winter and Huber 2000; Haigler and Ivanova-Datcheva 2001; Uggla et al. 2001; Kutschera and Heiderich 2002) and for biosynthesis of other cell wall polysaccharides (Konishi and Ohmiya 2002) and also possibly for lignin biosynthesis (Savidge et al. 2000), there is increasing evidence that other avenues for the production of specific pools of UDP-glucose within the developing xylem cell may be of critical importance for particular developmental scenari (Hertzberg et al. 2001; Mellerowicz et al. 2001). For example, Hertzberg et al. (2001) found increased expression levels of UTP-glucose-1-phosphate uridinyltransferase in the secondary wall development zone in poplars and suggested that this may be an alternative route for glucose supply for secondary wall biosynthesis. In *P. pinaster*, LW is characterized by a higher cellulose content and thicker wall (G. Chantre, AFOCEL, France, personal communication) and the development of this characteristic will require more glycosyl units to synthesize the polysaccharide matrix. It may be that the specific pools of UDP-glucose from enzymes other than SuSy play an important role in the determination of final cell wall composition. This is yet to be confirmed and awaits further investigation.

- PP.PM31, corresponded to an α-tubulin. Reverse Northern showed that its transcript was over-expressed during LW formation. Whetten et al. (2001) reported on the up-regulation of an α-tubulin during CW formation, supporting our primary finding in the cDNA–AFLP screen (Table 2). Cortical microtubules are mainly composed of α- and β-tubulin. Cortical microtubules could determine the cell wall pattern by defining the position and the orientation of cellulose microfibrils during the differentiation of tracheid elements (Chaffey 2000), probably by guiding the movement of the cellulose-synthesizing complex in the plasma membrane. However, recent studies of *Arabidopsis* mutants indicate that loss of microtubule orientation does not necessarily affect microfibril orientation (Wiedemeier et al. 2002). Cortical microtubules are also involved in different processes such as cell division, cell expansion, cell differentiation and organogenesis (Kost et al. 1999).
- PP.EM43, showed similarity to a senescence-associated protein from *Pisum sativum*. This TDF exhibited over-expression during EW formation (Fig. 4a). Interestingly, Blast-N analysis showed that this protein has strong similarity to 25-S RNA in both species. Senescence-associated proteins are known to be up-regulated during plant senescence, a mechanism under the control of ethylene, an important gaseous hormone also involved in CW formation (Little and Eklund 1999). Although ethylene was shown to induce the expression of several senescence-associated genes (Wang et al. 2000), the role of this TDF in xylogenesis remains unclear.
- PP.PM36, similar to ACC oxidase, an ethylene-forming enzyme, was over-expressed during EW formation. Plomion et al. (2000) reported the up-regulation of this protein during CW formation, supporting our primary finding in the cDNA–AFLP screen (Table 2).

*Hypothetical proteins* Among the hypothetical proteins, four were either specific or highly up-regulated during early wood formation (Table 2). Given the huge amount of pine xylem ESTs in public databases (59,859 *P. taeda* and 18,000 *P. pinaster* ESTs), these genes might correspond to very rare transcripts that have not been sequenced in EST projects to date. It is interesting to note that the cDNA–AFLP approach was capable of identifying rare and unusual transcripts not easily found through other methods.

## Toward the validation of expressional candidate genes

Although we are beginning to understand the genetic architecture of quantitative traits [see the growing literature on detection of quantitative trait loci (QTLs)], we still know very little of the actual loci responsible for quantitative variations. As exemplified in this study, functional genomics is discovering remarkable variations in gene expression, thus identifying many good candidate genes. Now the challenge is to discover if such variations really matter and whether they affect phenotypic variation in natural populations. One step forward to complete this objective is to study nucleotide variations in the expressional candidate genes, evaluate if these variations are under selection processes and test their contribution to the genetic variability of the quantitative traits.

While changes in mRNA expression levels do not always lead to changes in protein accumulation, it is clear that cell wall proteins, such as the GRP on which we reported here, play an important role in determining the chemical composition and morphology of the cell wall and that changes in the level of such proteins may lead to different cell wall properties.

The distribution of nucleotide polymorphism found in *P. pinaster* Ait. (27 individuals) for PP.PM16 (GRP) is summarized in Table 3. A total of 11 polymorphic sites (9 SNPs and 2 INDELS) were detected. Nine of them were found in more than one individual. For the two remaining ones only one variant was detected in the sample. Six of these polymorphisms were located in the 3′ untranslated (UTR) region of the exon and those remaining (five) had no predicted consequences for the protein sequence.

Estimates of the nucleotide diversity for both the whole region and its different functional parts (non-coding, coding synonymous and coding non-synonymous) are shown in Table 4. Six haplotypes were identified. Three of

**Table 3** Nucleotide polymorphism of PP.PM16: intraspecific variability in *Pinus pinaster* (Pp) and interspecific variability between *P. pinaster* (*Pp*) and *P. taeda* (*Pt*). *SNP* Single nucleotide polymorphism, *INDEL* insertion/deletion

| | Number of polymorphisms | |
|---|---|---|
| | Pp | *Pp* vs. *Pt* |
| SNP non-coding | 6 | 12 |
| SNP coding | 3 | 6 |
| Synonymous | 3 | 5 |
| Non synonymous | 0 | 1 |
| Transition | 6 | 9 |
| Transversion | 3 | 8 |
| INDEL | 2 | 1 |
| Total | 11 | 18 |

**Table 4** Number of polymorphic sites (S) and nucleotide diversity ($\pi$) in the GRP region sequenced in *Pinus pinaster* (only SNPs were considered in this analysis)

| | Non-coding region | Coding region | | | Total |
|---|---|---|---|---|---|
| | | Synonymous | Non-synonymous | Total | |
| S | 6 | 3 | 0 | 3 | 9 |
| $\pi$ | 0.0055 | 0.00145 | 0 | 0.00145 | 0.00695 |

them exhibited very low frequency, being represented by a single individual.

In order to perform a neutrality test using the dN/dS ratio-based test, the consensus sequence was aligned with the consensus derived from *P. taeda* ESTs. This interspecific analysis detected 18 fixed differences (Table 3), mainly (12 polymorphic sites) located in the non-coding 3′ UTR region. Of the ''coding'' polymorphism only one induced a modification at the amino acid level: a serine (AGC) in *P. pinaster* was replaced by a threonine (ACC) in *P. taeda*. Finally, a significant departure from neutrality ($P$ value = 0.028), which may be explained by purifying selection ($P = 0.013$), was detected for this gene.

The validity of this gene as a good candidate for wood quality is also emphasized by the results obtained from sequence analysis. Strong evidence of departure from neutrality for polymorphism within this gene indicates the importance of this gene for fitness. This result suggests that PP.PM16 is involved in the genetic control of traits related to survival, which is not surprising when considering that GRPs are thought to be involved in vascular development (Cassab 1998) and in the composition and structure of the cell wall.

Given (i) that most of the wood quality-related traits are linked to the properties of the cell wall (wood chemical composition, density...) and that these properties exhibit significant genetic control (Pot et al. 2002), and (ii) that several polymorphisms were detected on this gene, the next step is to test their contribution to the genetic variability of these traits. A first study of this type was performed using 12*12 half-diallele (described by Pot et al. 2002). The parents of the diallele were sequenced for the GRP. We have recently detected significant associations between SNPs in the GRP for lignin, cellulose content and total growth. Although the polymorphisms observed in the studied region of the gene have no effect on the protein sequence, a few studies have shown the importance of such polymorphisms on gene expression patterns. Indeed, there is some evidence that suggests that at the coding level, codon usage bias can affect the translation process (Powell and Moriyama 1997), and that mutation in introns can affect the expression pattern of the gene by inducing abnormal splicing (Brown 1996; Cai et al. 1998). This type of differential expression pattern between genotypes would probably affect the variability of the resulting phenotypic traits.

In any case, it would be interesting to extend the sequenced region to the remaining 5′ end of the gene (171 bp corresponding to 57 amino acids) in order to check if the protein sequence is fully conserved. If such a result were obtained, this would probably mean that this protein is so important for the fitness of the tree that no divergence from the initial sequence is allowed. Sequencing of the promoter region would be another interesting target. Indeed polymorphism in this region would not imply protein sequence alteration, but probably the transcription level for the different alleles of the gene. These differences could be at the origin of phenotypic variations of the ''resulting'' traits.

## Conclusions

Wood properties are functions of the size, shape and arrangement of the cells in the wood. Wood formation is under the coordinated expression of thousands of genes involved in cell division, cell elongation, cell wall deposition and programmed cell death. These genes need to be identified because they are potential targets for the direct modification of wood properties. The use of the cDNA–AFLP technique was an efficient method for isolating expressional candidate genes. The high percentage of transcripts regulated in xylem associated with EW and LW demonstrates the effectiveness of cDNA–AFLP in the determination of the changes involved during this transition. Except for one case, reverse Northern and RT–PCR confirm the results obtained by cDNA–AFLP.

The cDNA–AFLP technique in maritime pine appears to be a useful approach for identifying pine homologues of known genes, as well as discovering novel genes not previously identified in other organisms. TDFs isolated using this technique belong essentially to four functional classes: (i) cell wall formation (e.g. GRP and UDP-glucose pyrophosphorylase), (ii) cytoskeleton (tubulin α chain), stress protein (LMW HSP), (iii) general metabolism (G3PDH), (iv) hormone biosynthesis (ACC oxidase). Among the 13 TDFs isolated, 4 of them were not identified after a search against public databases. The ratio of the expression of these 4 hypothetical proteins in EW and LW is greater than 2.5. Transgenic plants containing over-expression or antisense constructs could be generated in pine, poplar or the model plant species *Arabidopsis* to gain more functional information.

These isolated expressional candidate genes could also serve as molecular markers for identifying QTLs. We have recently localized several QTLs for growth, wood chemical composition, wood anatomy, fiber morphology, kraft pulp production parameters and timber quality in the genetic map of maritime pine (data not shown). Comparison between the map location of the candidate genes and the QTLs will provide us with new insights into the involvement of these genes in the determination of wood properties.

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