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RESEARCH PAPER

Transcriptional analysis of differentially expressed genes in response to stem inclination in young seedlings of pine

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Keywords

Conifers; gravitropic response; real-time reverse transcriptase polymerase chain reaction (RT-qPCR); subtractive suppresive hybridisation.

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ABSTRACT

The gravitropic response in trees is a widely studied phenomenon, however understanding of the molecular mechanism involved remains unclear. The purpose of this work was to identify differentially expressed genes in response to inclination using a comparative approach for two conifer species. Young seedlings were subjected to inclination and samples were collected at four different times points. First, suppression subtractive hybridisation (SSH) was used to identify differentially regulated genes in radiata pine (*Pinus radiata* D. Don). cDNA libraries were constructed from the upper and lower part of inclined stems in a time course experiment, ranging from 2.5 h to 1 month. From a total of 3092 sequences obtained, 2203 elements were assembled, displaying homology to a public database. A total of 942 unigene elements were identified using bioinformatic tools after redundancy analysis. Of these, 614 corresponded to known function genes and 328 to unknown function genes, including hypothetical proteins. Comparative analysis between radiata pine and maritime pine (*Pinus pinaster* Ait.) was performed to validate the differential expression of relevant candidate genes using qPCR. Selected genes were involved in several functional categories: hormone regulation, phenylpropanoid pathway and signal transduction. This comparative approach for the two conifer species helped determine the molecular gene pattern generated by inclination, providing a set of *Pinus* gene signatures that may be involved in the gravitropic stress response. These genes may also represent relevant candidate genes involved in the gravitropic response and potentially in wood formation.

INTRODUCTION

Plants have the ability to adjust their growth habit in response to environmental stimuli and this capacity is the key for their survival and perpetuation success. Gravity plays an important role within the environmental stimuli to which plants are exposed. Trees must respond to gravity effects, since in nature stems or trunks can be exposed to snow and heavy wind, generating displacement from normal vertical growth (Timell 1986). The process of gravitropism can be divided into three sequential steps: (i) gravity perception; (ii) signal transduction; and (iii) differential growth response (Fukaki & Tasaka 1999; Haswell 2003). The loss of stem verticality is sensed by an intricate mechanism involving biophysical stimuli perception (organelle movement) and transformation of this perception into physiological signals triggering consecutive molecular events, the synthesis of hormones, transcription factors and, most important, calcium signalling (Muday 2001). The transduction of a gravitropic stimulus triggers differential growth of both upper and lower sides of the stem on inclined plants. This differential growth in trees triggers the formation of reaction wood, which is called compression wood (CW) in gymnosperms. Generally in conifers, eccentric radial growth is associated with CW formation (Timell 1986). This type of wood is formed at the lower side of inclined stems and branches of gymnosperms in response to a non-vertical orientation mainly associated with the initial gravitropic stress (Kozlowski 1971; Larson *et al.* 2001).

The molecular mechanisms that trigger wood formation have been reported in forest trees, and several genomics approaches have been used in poplar, loblolly pine, radiata pine, spruce and eucalyptus (Allona *et al.* 1998; Hertzberg *et al.* 2001; Whetten *et al.* 2001; Kirst *et al.* 2003; Schrader *et al.* 2004; Sterky *et al.* 2004; Andersson-Gunneras *et al.* 2006; Pavy *et al.* 2007; Qiu *et al.* 2008; Li *et al.* 2009). However, the response to gravitropic stimulus at a molecular level in forest trees is still poorly understood (Du & Yamamoto 2007). Some studies have reported genes and proteins regulated in response to inclination or loss of verticality in gymnosperms (Allona *et al.* 1998; Zhang *et al.* 2000; Le Provost *et al.* 2003; Yamashita *et al.* 2008, 2009; Herrera *et al.* 2010).

Considering the large genome size of pine (~25,000 Mbp) (Grotkopp *et al.* 2004) and the consequent difficulties in sequencing the pine genome, expressed sequence tag (EST) sequencing remains an important approach for gene discovery in conifers. Suppression subtractive hybridisation (SSH) is a useful technique to isolate genes that are differentially

expressed between two samples from distinct developmental stages or tissues, allowing the identification of both abundant and rarely expressed transcripts (Diatchenko *et al.* 1996; Vilaine *et al.* 2003). This molecular technology has been successfully applied to the analysis of several tree species transcriptomes during development and in response to stress (Ranjan *et al.* 2004; Derory *et al.* 2006; Foucart *et al.* 2006; Yakovlev *et al.* 2006).

The objective of this study was to gain insight into molecular candidates involved in the gravitropic response using a transcriptomic technique and a comparative approach in two economically important conifer species, *Pinus radiata* D. Don and *Pinus pinaster* Ait. In order to survey genes involved in the gravitropic stress response, SSH libraries were built at different times after inclination in young radiata pine seedlings. Then, candidate genes for radiata and maritime pine were selected from these libraries and validated using quantitative real-time PCR (qPCR). Two questions were addressed: (i) are there molecular differences between these two species in response to gravitropic stimulus; and (ii) do selected genes have the same expression profile in both species?

MATERIAL AND METHODS

Sampling of tissues and differentiating xylem

The experiments were performed in radiata pine (*Pinus radiata* D. Don.) and maritime pine (*Pinus pinaster* Ait.). For maritime pine, 30 half-sib 1-year-old seedlings obtained from the Research Unit of INRA-Pierroton (Cestas, France) were collected per time point after 2, 10 and 24 h and 3 months of leaning by tilting the pots to 45° (Herrera *et al.* 2010). Stems were dissected longitudinally in order to separate the upper from the lower half of the stem to quantify gene expression on either side of the stem. All the samples were used in the qPCR analysis. Thirty upright seedlings were used as control samples and collected after 24 h. The time course considered for maritime pine was the same as reported in Herrera *et al.* (2010), which took into account short- (2 and 10 h) and long-term (24 h and 3 months) responses.

For radiata pine, 25 half-sib 1-year-old seedlings per time point, around 30 cm in height, obtained from an open-pollinated orchard (Forestal Mininco, Los Angeles, Chile) were tilted to 45º and harvested after 2.5, 10 and 24 h and 1 month. This sampling time course was established based on a photographic sequence, where the initial response to gravitropic stimulus at the apical zone was observed after 2.5 h of inclination (see Video clip S1). Twenty-five upright trees were sampled as controls. In addition, two different stem sampling procedures were used: (i) a longitudinal cut along the stem to separate the upper from the lower half; and (ii) transverse cuts to divide stems in three different height sections, named Stem 1 for the apical zone, Stem 2 for the medial zone and Stem 3 for the basal zone of inclined seedlings. Tissues were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. In all treatments, radiata and maritime pine seedlings were maintained under long-day conditions of 16-h light/8-h dark and illuminated with halogen lamps (314–494 μmol m^{-2} s^{-1}) at 25 °C.

RNA isolation and SSH library construction

Total RNA from different tissues was extracted according to Le Provost *et al.* (2007) for both species. All libraries were comprised of a unique tissue, *i.e.* whole stem side, collected on several genotypes of radiata pine. Double-stranded cDNAs were obtained using the Smart PCR cDNA Synthesis Kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's procedure. The PCR-Select cDNA Subtraction Kit (Clontech) was used for the generation of SSH libraries according to the manufacturer's instructions. Eight SSH libraries were obtained for radiata pine (Table 1). Subtracted or forward libraries were built considering the control as driver, and cDNA from 2.5 h inclined stems (lower or upper half) as tester. The second library was built at 10 h inclination for both upper and lower half of the stem. In this case, the driver corresponded to an equimolar mix of cDNA from 2.5 h and cDNA from control. The subsequent libraries were built by subtracting the previous times (control, 2.5 and 10 h) in order to obtain genes expressed only at the new times of inclination (Table 1).

Amplified fragments were cloned into StrataClone PCR Cloning Vector pSC-A (Stratagene, CA, USA), transferred into Strataclone SoloPack competent cells, and finally plated onto LB agar containing 100 μg ml^{-1} ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 μg ml^{-1} 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Plates were incubated at 37 °C overnight to obtain a subtracted EST bank.

Table 1. Distribution of ESTs within the eight SSH libraries.

	2.5 h		10 h		24 h		1 month		
	Inf	Sup	Inf	Sup	Inf	Sup	Inf	Sup	Total
ESTs	478	450	323	384	382	384	384	367	3152
Discarded	3	5	10	15	2	15	7	3	60
ESTs useful	475	445	313	369	380	369	377	364	3092
Contigs	78	43	15	30	23	14	13	4	220
Singletons	245	351	205	210	246	221	240	265	1983
Cont + Singl = Informative sequences	323	394	220	240	269	235	253	269	2203
Anot from TIGR	117	122	86	105	107	9	63	96	705
Anot from NCBI	41	37	35	101	29	37	23	22	325
TIGR + NCBI = Anot total	158	159	121	206	136	46	8	118	952
No Hits Seq	165	235	99	34	133	189	245	151	1251

Expressed sequence tag (EST) sequencing and bioinformatic analysis

Differentially expressed ESTs were selected and DNA inserts from the clones were first amplified using M13 universal primers, and products were sent to be sequenced at a commercial DNA sequencing service (Macrogen, Seoul, Korea). ESTs were cleaned of vector and primer sequences using the VecScreen program (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Finally, sequences were assembled into contigs using the CAP3 program included in Vector NTI Advance 9.0 (Invitrogen, Carlsbad, CA, USA). Based on the qualified sequences, the search for sequence similarity was performed using the BLASTN algorithm (Altschul *et al.* 1990) against public databases: TIGR (http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi) and BLASTX against the NCBI database (http://blast.ncbi.nlm.nih.gov). Similarity scores between the cDNA clones and known sequences were represented by the BLASTN and BLASTX probability E-value (E-value$\leq 10^{-4}$). A functional category was then assigned to each gene product according to the FunCat (Functional Catalogue) from MIPS (Munich Information Center for Protein Sequences; Ruepp *et al.* 2004).

Quantitative PCR expression analysis

The mRNA expression level of selected candidate genes was measured using quantitative PCR. Reaction and quantification were performed with the Chromo4™ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) following the procedure described by Paiva *et al.* (2008). Primers for quantitative qPCR (Higuchi *et al.* 1993) were designed using Beacon Designer, v. 2.0 software (PremierBiosoft, Palo Alto, CA, USA). Serial dilutions of amplified PCR products were used as standard templates to assess the PCR efficiency for each primer pair (Table 2). Triplicates of cDNA were used to minimise experimental error, and each PCR run was carried out at least in duplicate for each of the two RNA extraction sets. A melting curve analysis was performed for each set of primers in order to avoid non-specific amplification. Additionally, agarose gels were run in order to discard potential multiband products for each qPCR gene analysed.

Template cDNA for all samples was synthesised using 1 μg of DNase-treated total RNA using the iScript cDNA Synthesis Kit (Bio-Rad), in accordance with the manufacturer's instructions. The first-strand RT reaction product was diluted 10-fold, and 2 μl were used for each qPCR reaction. The cycle threshold (Ct) line was determined manually as the point where the R^2 value for the standard curve reached its highest point. iQ SYBR Green Supermix (Bio-Rad) was used for all qPCR quantification in a final volume of 20 μl, following the manufacturer's protocol. All experiments were run using a Chromo4™ Multicolor Real-Time PCR Detection System (Bio-Rad) with the folloing cycling conditions: a denaturing step at 95 °C for 15 s followed by an annealing/extension step at 60 °C for 45 s, as recommended by the manufacturer. The instrument was set to measure dye florescence at the end of each cycle at the 60 °C annealing/extension step and performed a melting curve at the end of each reaction.

The expression levels were normalised with the stable expression level of a 40S ribosomal protein S27 gene, denoted Ge066D02 (accession BX252550). Data were analysed using the Excel (Microsoft) macro GENEX v1.10 (gene expression analysis for iCycle iQ® real-time PCR detection system, v1.10, 2004; Bio-Rad), using methods derived from the algorithm of Vandesompele *et al.* (2002).

Accession numbers

The sequences from radiata pine validated with qPCR have been deposited in the dbESTs database of the NCBI (http://

Table 2. Sequences of primers used for gene expression analysis by qPCR.

target gene	accession no.	primer forward	primer reverse	efficiency (%)	product size (bp)
Ge066D02 Ribosomal protein S27	BX252550	5′-TTTTAGGAAGAAGGGTGATTGACT-3′	5′-ATTAGAAACCAACGAGGCTGTC-3′	97	151
EIN3-like protein (radiata pine)	GO344431	5′-TTGGTCGCAAGTTTGTCTTCT-3′	5′-CGATCAAGCACATTTCCATTT-3′	97	93
EIN3-like protein (maritime pine)		5′-CGCTTCTTTCTGCCCTTATG-3′	5′-GAGTAGTTGGCCACCAAGGA-3′	98	75
Homeobox protein PpHB6	GO344433	5′-CCACCGTAAATAAGCTTTGCT-3′	5′-CCATCCATTGTCTGGCATAG-3′	99	85
Auxin-repressed protein	GO344428	5′-AGCCAGACAGTGGCCTCA-3′	5′-CCCCCACTCCCTTCTTTTAG-3′	100	100
Phenylalanine ammonia-lyase	GO344434	5′-TCAACAAAAAGATCCGAGGAC-3′	5′-CCTGGCCCATTCTGAAATAA-3′	100	86
Chalcone synthase	GO344430	5′-GGAGAAGCCCTGTTTCGAG-3′	5′-TGAGGCCTACCTCTCTCAGG-3′	100	95
Flavanone 3-hydroxylase (radiata pine)	GO344432	5′-CTGCCTGGATGCTGTCTGT-3′	5′-TGTAGACAAGTCGGTGGAAGC-3′	97	92
Flavanone 3-hydroxylase (maritime pine)		5′-CATGGAAGAGCCCATCACTT-3′	5′-CGACTTTGGCCAACTTTTTC-3′	100	76
TRANSPARENT TESTA 12 (radiata pine)	GO344437	5′-CTGTTACCGTAGCCGGAGAG-3′	5′-TTGTCTCCACACTTGCAACC-3′	97	88
TRANSPARENT TESTA 12 (maritime pine)		5′-GGTTTTTGCAGGCTCAGAAC-3′	5′-GCAGCCCAAGTCAACAAAAT-3′	99	85
Porin (radiata pine)	GO344436	5′-CTGCTACCGATACCAAACGA-3′	5′-GGCAAGGTGAACCAGAAAAA-3′	100	86
Porin (maritime pine)		5′-CAGTGCCTTGGTCAGAGGTT-3′	5′-TCTGCAACAGCACTGGATTC-3′	92	95
Profilin-1	GO344438	5′-GTTTGGGCTCAGAGCGATAG-3′	5′-GCTCGGCAAAATCATTCACT-3′	100	76
Protein translation factor SUI1 homologue 1	GO344435	5′-TTCGTGACCTCGCTTAGGAT-3′	5′-GCACAAGAGGATGGACGAAT-3′	99	100

www.ncbi.nlm.nih.gov/dbEST/index.html; accession numbers GO344428, GO344430–GO344438). All ESTs obtained, and non-redundant sequences from SSH libraries were also submitted to EMBL (http://www.ebi.ac.uk/embl/index.html; under accession numbers, FR729927–FR731105), including some low-quality sequences, which were not considered in the 942 unigenes listed in Table S1.

Statistical analysis

The gene expression statistical analyses were performed using Statistica for Windows (version 7.0) (StatSoft, Inc., Tulsa, OK, USA). Analysis of ANOVA/LSD *post hoc* was used, and significant differences were determined at $P \leq 0.05$, or non-parametric ANOVA Scheirer-Ray-Hare, which is an extension of the Kruskal–Wallis test, depending on the normality assumptions.

RESULTS AND DISCUSSION

Phenotypic characterisation of gravitropic stressed pine seedlings

Previous studies on maritime pine have shown that phenotypic characteristics of CW appear after <1 month of inclination (Ba *et al.* 2010). In order to obtain radiata pine samples with the characteristic anatomy of CW, sampling over the course of 1 month was undertaken. Additionally, the reorientation of the apex in maritime pine was filmed, showing that it occurs after about 2 h of inclination (Herrera *et al.* 2010). For this reason, 2 h was considered as the first sampling time in maritime pine. The same process was filmed for the reorientation response of radiata pine (Video clip S1), showing apex visual reorientation to 45° inclination after 2.5 h of leaning. This time (2.5 h) was considered as the first sampling time for radiata pine.

SSH library construction, assembly and functional annotation

Identification of ESTs differentially expressed during gravitropic response was performed based on the construction of subtractive cDNA libraries. A total of 3152 ESTs, ranging from 100 to 1600 bp for the eight libraries, were obtained. A total of 3092 high-quality ESTs were obtained for assembly, generating a gene index of 2203 elements (220 contigs and 1983 singletons), assuming that a singleton EST corresponded to a unique EST. However, the number of unique sequences may be overestimated because some sequences could come from non-overlapping regions within the same gene. An overview of all the libraries showed an average redundancy of 57.2% (Table 1). This parameter was estimated considering the following formula [1 – (#unigenes/#high quality genes)]. Contig size ranged from 596 to 1567 bp, whereas singleton size ranged from 105 to 818 bp. Among the 220 contigs, the number of ESTs per contig ranged from two (36 contigs) to 68 (one contig). In summary, each contig contained an average of six ESTs and most of the contigs (75%) contained five ESTs. The sequencing success rate was 80%, which is comparable to a previous report for pine (Cairney *et al.* 2006).

To elucidate the potential functions of ESTs from the libraries, a similarity search was performed using the basic local alignment search tool (BLASTN) in TIGR and NCBI databases for identification of homologous/orthologous sequences (Table S1). Using the pine and spruce index from TIGR, only 705 unigenes were assigned as known function genes. A gene discovery rate (defined here as [hypothetical protein (no hits)/informative reads (contigs + singletons)] * 100) of 34.8% was calculated. This was comparable to findings reported in Rengel *et al.* (2009) for differentiating eucalyptus xylem and by Derory *et al.* (2006) for bud burst in oak. The SSH technique is known to equalise the expression level of rare and abundant transcripts (Diatchenko *et al.* 1996; Vilaine *et al.* 2003); therefore genes annotated as 'new' may either represent genes specific to perennial species or to secondary xylem formation. A total of 952 ESTs displayed homology to sequences available in both TIGR and NCBI databases, of those sequences 614 had known functions and 328 ESTs had unknown functions, including hypothetical proteins.

Functional classification of expressed genes in SSH libraries

The distribution of the expressed genes in response to inclination showed differences between the upper and lower halves during both the early (Fig. 1A–D) and late (Fig. 1E–H) response, according to functional category of MIPS (Ruepp *et al.* 2004). At the earliest time point (2.5 h), the most expressed groups of genes were related to cellular communication and signal transduction in both upper and lower sections of the stem, followed by protein synthesis (upper half) and metabolism (lower half) (Fig. 1A,B). At 10 h, the main group of genes expressed in the upper half was also those related to protein synthesis, whereas the main group of genes in the lower half was assigned as encoding proteins with a binding function or cofactor requirement. In addition, two other functional categories of genes were highly expressed in the upper half (cellular communication and signal transduction) and in the lower half (protein synthesis) (Fig. 1C,D). After 24 h of stress, two functional categories corresponding to proteins with a binding function and protein synthesis were overexpressed in the upper and lower part of the stem (Fig. 1E,F).

Genes in the metabolism category were overexpressed on both sides of the stem. *Endoxyloglucan transferase (EXT), xyloglucan endotransglycosylase/hydrolase (XTH)*, *β-1,3-glucanase* and a *glucanase*-like gene (Table S1), which are involved in secondary wall remodelling and degradation, were also identified. *XTH* has been widely studied and is related to normal and reaction wood formation in angiosperms (Mellerowicz *et al.* 2008; Baba *et al.* 2009; Nishikubo *et al.* 2011). Another gene expressed was an *expansin*, which encodes for an enzyme important for cell wall remodelling, breaking hydrogen bonds between cellulose microfibrils and hemicellulose and allowing the hydrolase to access the cell wall (McQueen-Mason & Cosgrove 1995; Rose & Bennett 1999). Regarding cell wall metabolism, one field of investigation is the dynamics of cell wall remodelling in order to elucidate the mechanisms of development of a specific type of wood. In gymnosperms, Zhang *et al.* (2000) reported differential expression of genes that encodes cell wall proteins in differentiating xylem from inclined loblolly pine.

The *C3HC4 ring-finger* type, *BTF3b* type, *zinc-finger* type, *homeobox PpHB6*, *GT*-like trihelix, *MADS-box jointless*

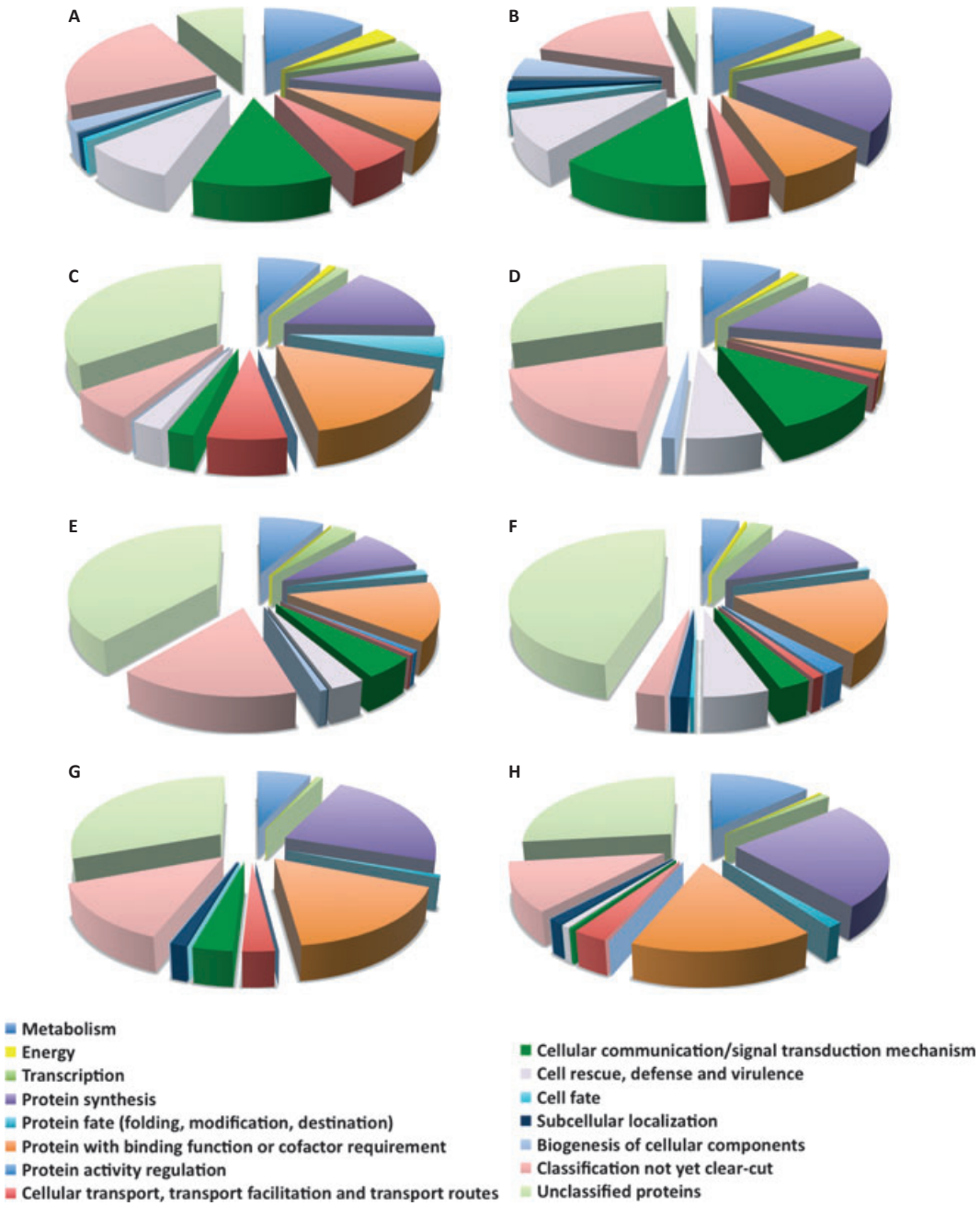

Fig. 1. Functional classification of radiata pine genes differentially expressed in each library of gravitropic response. (A) Library 2.5 h from the upper half of the stem. (B) Library 2.5 h from the lower half of the stem. (C) Library 10 h from the upper half of the stem. (D) Library 10 h from the lower half of the stem. (E) Library 24 h from the upper half of the stem. (F) Library 24 h from the lower half of the stem. (G) Library 1 month from the upper half of the stem. (H) Library 1 month from the lower half of the stem.

(*LeMADS*), *auxin-regulated protein (IAA8)*, *EIN3*-like, *ring-finger 110*, *MADS box* (*lbMADS3*) and *WD-40 repeat* proteins were identified within the group of transcription factors (Table S1). These transcription factors are believed to regulate secondary wall biosynthesis (Oh *et al.* 2003; Rogers *et al.* 2005; Groover & Robischon 2006). Some *Arabidopsis* MADS-box genes have been implicated in the regulation of lignin biosynthesis (Liljegren *et al.* 2000). ESTs libraries generated from six types of wood that form xylem in radiata pine showed that transcription factors are expressed (Li *et al.* 2009). These observations indicate a plausible function of transcription factors in the gravitropic response and consequent wood formation.

Several ESTs showed high identity with genes related to calcium intracellular homeostasis. Different *calmodulins* were expressed at both sides of the stem at 2.5 h, but at 10 h they were only expressed only in the lower half. *Calcium-binding protein* genes were overexpressed and identified at 2.5 h in the upper side. These proteins are capable of fixing calcium ions that regulate the activity of certain enzymes and play a

role as second messengers in plant cells (Sanders *et al.* 1999; McAinsh & Pittman 2009). It has been reported that touch-induced (*TCH*) genes are activated in response to thigmomorphogenesis (Braam & Davis 1990). *TCH1* encodes one of the *Arabidopsis* calmodulins (*CaM2*) (Braam & Davis 1990), *TCH2* and *TCH3* encode CaM-like proteins (Sistrunk *et al.* 1994; Khan *et al.* 1997; McCormack & Braam 2003) and *TCH4* is a xyloglucan endotransglucosylase/hydrolase (XTH; formerly abbreviated XET) (Xu *et al.* 1995; Purugganan *et al.* 1997; Campbell & Braam 1998; Rose *et al.* 2002). In our experiments, ESTs with high homology to these genes were observed, supporting the idea that calcium and dynamic cell wall remodelling are involved in the gravitropic response, consistent with data presented in Lee *et al.* (2005).

After 1 month of inclination, the two most highly expressed functional categories were genes related to protein synthesis and proteins with binding function in both sections of the stem (Fig. 1G,H). It should be noted that for long-term response, the protein-binding functional category is overexpressed in both parts of the stem; therefore, we hypothesise that these proteins could be involved in the formation of compression wood. A large number of genes involved in protein synthesis were identified as differentially expressed during wood development between growing seasons in maritime pine (Paiva *et al.* 2008), indicating that genes involved in these molecular processes probably control wood formation during the gravitropic response.

Gene expression using qPCR analysis

To validate SSH data, a comparative analysis was performed in both radiata and maritime pine. The main goal was to identify candidate genes involved in the gravitropic response in young seedlings. Ten genes were selected and validated with qPCR. The following criteria were applied for the selection of candidate genes: (i) genes highly affected in their expression profile using the SSH approach; and (ii) the effects of these genes in the response to gravity stress in other conifer species. Using this strategy, six primer pairs for both radiata and maritime pine (*auxin-repressed protein*, *chalcone synthetase*, *homeobox protein PpHB6*, *phenylalanine ammonia-lyase*, *profilin1*, *PTFSUI1*) and four specific sets of primers specific to each species (*EIN3-like protein*, *flavanone 3-hydroxylase*, *porin*, *transparent testa 12*) were designed (Table 2). PCR efficiencies ranged from 92% to 100%, which is consistent with correct *Taq* polymerase activity.

Overview of qPCR experiments

Seven genes were differentially expressed on both sides of inclined stems in radiata and maritime pine (*EIN3*, *homeobox protein PpHB6*, *PAL*, *CHS*, *flavanone 3-hydroxylase*, *TT12*, *porin*; Fig. 2). Additionally, gene expression analysis was performed in a time course for different height positions of inclined stems of radiata pine. Samples obtained from the apical zone (Stem 1), medial zone (Stem 2) and basal zone (Stem 3) of inclined stems were assessed. The expression profiles differed depending on position, indicating both spatial and temporal regulation of gene expression.

Regulation of hormonal control

Three genes (*EIN3*, *homeobox protein PpHB6*, *auxin-repressed protein*) were validated using the qPCR approach. A comparison of these genes showed differential expression in the two species (Fig. 2). *EIN3* transcript expression increased about twofold in comparison to control stems and the opposite side. *Homeobox* transcripts showed a strong induction peak of four-fold in the upper half compared to controls (non-inclined) and twofold compared to the lower half after 10 h of inclination in radiata pine. In maritime pine, the incre-

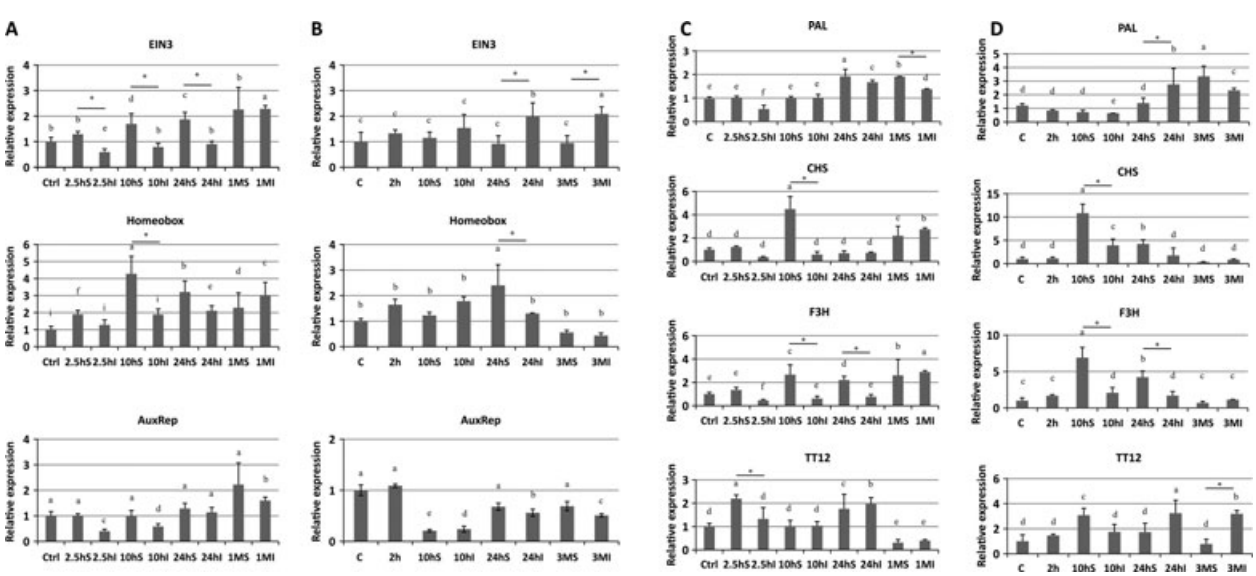

Fig. 2. qPCR analysis of genes related to hormonal signal transduction and transcription factors in (A) radiata pine and (B) maritime pine, in superior (upper) and inferior (lower) halves of stems. Genes involved in phenylpropanoid metabolism and vacuolar membrane flavonoid transport in (C) radiata pine and (D) maritime pine in superior (upper) and inferior (lower) halves of stems. Asterisks indicate differences in expression level between sides of inclined stems and different letters indicate significant differences with respect to the control of non-inclined stem ($P \leq 0.05$).

ment was about twofold after 24 h compared to controls and opposite side of the stem (Fig. 2). Moreover, when gene expression was analysed at different height postions on the stem, three-fold increased expression of *EIN3* and *homeobox protein PpHB6* genes was observed at 2.5 and 10 h, respectively, in the basal part of the inclined stem (Fig. S1A,B). These results indicate that expression of these genes occurs mainly in the basal zone of stems and on a specific side, suggesting that they could coordinate a molecular mechanism to promote recovery of vertical growth. In addition, the *EIN3* expression pattern is different in the two species after 24 h of inclination, suggesting the involvement of this gene in several metabolic and developmental processes.

EIN3-like protein is a transcription factor dependent on the ethylene signal (Alonso & Stepanova 2004). This gene showed differential expression in stems and induction only in the upper half in inclined seedlings of radiata pine. These results may indicate possible participation of ethylene signalling in the gravitropic response at early time points. Ethylene regulates many and diverse metabolic and developmental processes in plants, from seed germination to organ senescence, and plays a major role as signal molecule at low concentrations in response to stress and tolerance to infection in several species (Abeles *et al.* 1992; Morgan & Drew 1997). Recently, ethylene signalling has been related to control of expression of flavonol biosynthesis genes (Lewis *et al.* 2011), suggesting a possible relation between this signal transduction, flavonol accumulation and auxin distribution in inclined stems.

PpHB6 belongs to a transcription factor family characterised by having a homeodomain (McGinnis *et al.* 1984). This family is present in several development processes in plants, including apical meristem development, response to light and to hydric stress (Henriksson *et al.* 2005; Ariel *et al.* 2007), and was recently reported to regulate expression of the limiting enzyme ACC oxidase in ethylene biosynthesis during fruit ripening (Lin *et al.* 2008). The differential expression of *EIN3-like protein* and *homeobox PpHB6* in the upper half is consistent with a time course suggesting the involvement of ethylene in response to inclination, similar to the response in maritime pine, where *ACC oxidase* is differentially expressed in xylem from compression wood in adult trees (Plomion *et al.* 2000). Moreover, Le Provost *et al.* (2003) reported the differential expression of *ACC oxidase* in compression wood, which could support the involvement of ethylene as a modulator of the response. The expression of this ethylene-related gene *EIN3* was induced in the upper half of inclined stems. This differential expression probably occurs either because (i) ethylene is produced directly in this part of the stem in radiata pine seedlings, or (ii) *EIN3* could be activated by crosstalk with another signal in the ethylene signal transduction pathway.

Moreover, *auxin-repressed protein* displayed little expression profile variation in both species in the lower half of the stem, about twofold less than in controls and the opposite side of the stem (Fig. 2). When the expression profile of this gene across the whole stem was analysed, repression at all stem height positions was observed in radiata pine seedlings (Fig. S1C). *Auxin-repressed protein* has been described as repressed in the presence of auxin and has been related to dormancy (Stafstrom *et al.* 1998). In this context, the complex signal transduction cascade that regulates differential cell elongation and causes gravitropic responses still remains unclear. A role for auxin in tropic bending was proposed in the Cholodny–Went hypotheses, which argues that unequal distribution of auxin between the opposite sides of an organ causes differential cell elongation (Went 1974).

Phenylpropanoids and cellular transport of flavonoids

Four genes from this functional category (*PAL* (EC 4.3.1.24), *CHS* (EC 2.3.1.74), *F3H* (EC 1.14.11.9), *TT12*; Fig. 2) were analysed by qPCR. Interestingly, two (*CHS* and *F3H*) showed a comparable expression profile in both species. *CHS* increased expression about four-fold in radiata and ten-fold in maritime pine compared to both controls and the lower stem side. It should be noted that these genes are repressed in the lower half of the stem, and for *CHS* the main variation was observed at 10 h and for *F3H* the main variation was observed at 10 and 24 h of inclination, with induction of about three-fold and five-fold over the control stem in radiata and maritime pine, respectively. In contrast, the two remaining genes (*PAL* and *TT12*) displayed a different expression profile for the two species. *PAL* was differentially expressed in both species in the long time course experiment (24 h, 1 and 3 months) about twofold over the control stem, and *TT12* was induced after short times of inclination only in the upper half and was repressed after 1 month of stress in radiata pine, but displayed a more variable profile in maritime pine. Concerning maritime pine, in the lower half of the stem a down-regulation was observed at 10 h, whereas up-regulation was observed at 24 h and 3 months of inclination. Finally, *PAL* tended to increase only in the basal stem portion at 10 and 24 h in radiata pine when the transcript profile was analysed in different stem sections (Fig. 3A). On the other hand, *CHS* was induced twofold at 2.5 h only in the basal part of the stem, but the transcripts were abundant in medial and basal portions in radiata pine at 10 h (Fig. 3B). For *F3H*, transcript analysis showed a statistically significant increase of four-fold at 10 h in the basal portion of the stem (Fig. 3C). For *TT12*, an induction of eight-fold was observed in apical and medial sections of the stem at early times of inclination (Fig. 3D).

The phenylpropanoid pathway can be described as having one general reaction, where *PAL* is involved, and downstream the CHS enzyme drives the synthesis of lignins or flavonoids (Beritognolo *et al.* 2002). The expression of *CHS* and *F3H* was strongly induced in both pine species in the upper half of the stem; this could indicate that the response includes draining the route for the biosynthesis of flavonoids and anthocyanins. F3H is the key enzyme in flavonoid and anthocyanin biosynthesis (Kim *et al.* 2008), and the CHS enzyme takes part upstream of F3H in plant secondary metabolism. CHS catalyses the condensation of three molecules of malonyl-CoA and one molecule of p-coumaryl-CoA to yield chalcone, a precursor in the biosynthesis of flavonoids (Abe 2008). The reorientation toward flavonoid biosynthesis could be related to auxin accumulation in the tissues involved. This supports the idea that flavonoids inhibit auxin transport *in vivo* (Brown *et al.* 2001; Besseau *et al.* 2007), and also correlates with the observation of *AuxRep* gene down-regulation only in the lower half of inclined stems. Moreover, the

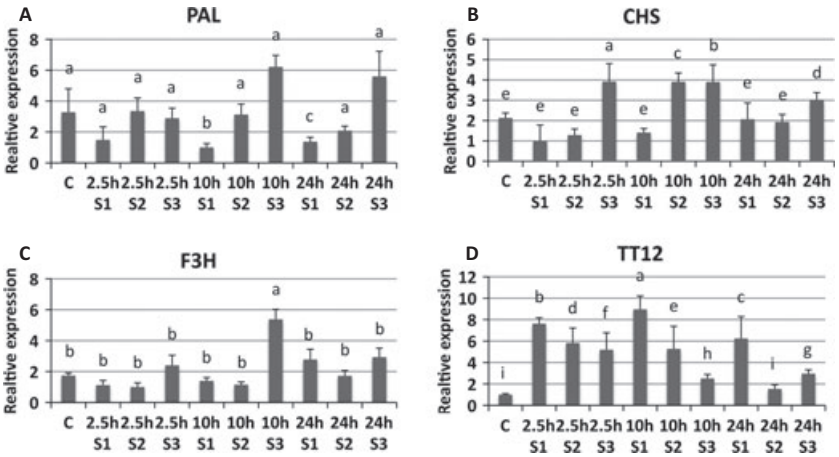

Fig. 3. qPCR analysis of genes involved in phenylpropanoid metabolism and vacuolar membrane flavonoid transport at different height positions of stems of radiata pine. Different letters indicate significant differences with respect to the control of non-inclined stems ($P \leq 0.05$).

expression of *transparent testa 12 (TT12)*, a gene directly related to flavonoid availability at cellular level, which encodes a vacuolar membrane transporter capable of sequestering excess flavonoids in the vacuole (Marinova *et al.* 2007), is an indication of a compensatory mechanism to regulate the quantity of free flavonoids in the cytoplasm. The results observed for *PAL* show an increment in transcripts on both sides of inclined stems, indicating that initial phenylpropanoid substrates for flavonoid biosynthesis in the upper half and lignin biosynthesis in the lower half could be supplied. *CHS* could initially be draining the route to biosynthesis of flavonols in order to avoid auxin accumulation in the upper half, nevertheless at longer times of inclination, this gene is down-regulated and most probably, switches to promote lignin biosynthesis. These observations correlate with the idea that xylem cells of the lower side are round in shape, and their cell walls are thicker, typical phenotypic characteristics of CW formation due to lignin accumulation (Timell 1986; Ba *et al.* 2010; Donaldson *et al.* 2010).

Protein synthesis and signal transduction

Three genes belonging to this group were assessed (*porin*, *profilin1* and *PTFSUI1*). Only one gene (*porin*) showed variation in expression, while the other two (*profilin1* and *PTFSUI*) displayed very small variation, and were considered as non-differentially expressed between upper and lower stem halves (Fig. 4). In the case of *porin* transcripts, a similar expression pattern was observed at 10 h (about twofold up-regulation in the upper half compared to controls) for both maritime and radiata pine, while a different profile was observed for the long-term response. In radiata pine, qPCR analyses for the different height positions of the stem showed

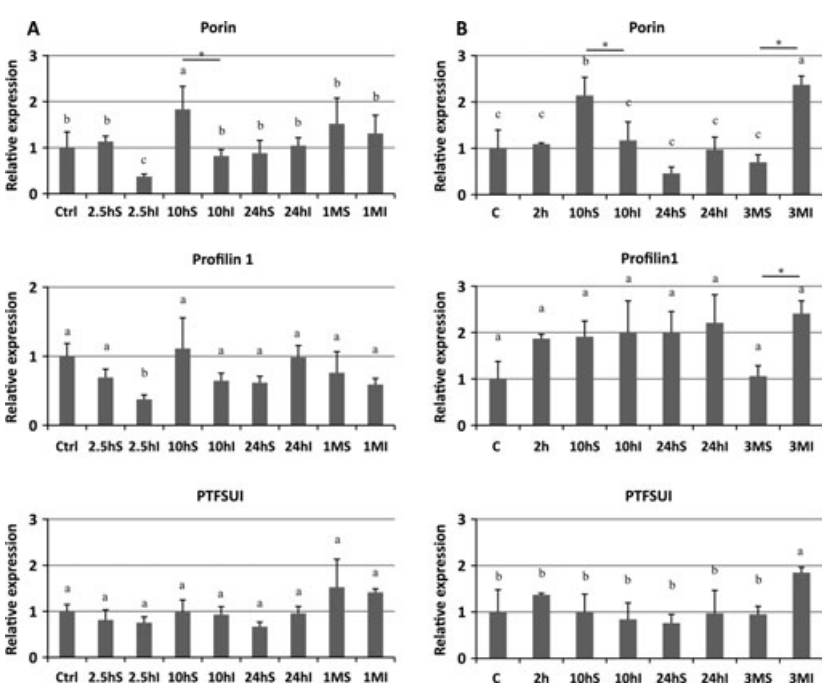

Fig. 4. qPCR analysis of genes related to stress response and signal transduction for protein synthesis in (A) radiata pine and (B) maritime pine in superior (upper) and inferior (lower) halves of stems. Asterisks indicate differences in expression level between sides of inclined stems and different letters indicate significant differences with respect to the control of non-inclined stems ($P \leq 0.05$).

a three-fold induction in the medial part at 10 h of inclination (Fig. S2A). Along the stem, only *profilin1* showed induction of twofold and five-fold in medial and basal parts of inclined whole radiata pine stems, respectively (Fig. S2B). *PTFSUI* showed no statistical differences in expression along the transversal sections, correlating with results obtained in the longitudinal analyses (Fig. S2C).

Hypothetical proteins

A high number of transcripts labelled as 'unclassified proteins' or 'no hits' were observed on both sides and at different times of inclination. This group is probably related to the small length of the SSH fragments that are difficult to identify, or because some fragments correspond to 3′ untranslated regions (UTRs), which are usually the least conserved. These two factors reduce the probability of detecting sequence homologies in the databases. Both BLASTN and BLASTX against a non-redundant database of NCBI were carried out in order over a wide range of species, but no differences were found with either strategy. Some of the 'no hits' may well correspond to unidentified genes, as the SSH technique is reputed to identify new genes that are not easily recovered from total cDNA libraries because their transcripts are not very abundant (Diatchenko *et al.* 1996; Vilaine *et al.* 2003).

CONCLUSIONS

In conclusion, eight SSH libraries were generated to identify molecular players involved in the gravitropic response in two economically important conifer species widely used for reforestation, paper and in the timber industry in the South Hemisphere (radiata pine) and Europe (maritime pine). Additionally, the expression of candidate genes in the upper and lower sides of stems was analysed, as well as along the stem length, thus generating information to help understand the molecular mechanism of bending in terms of spatiotemporal expression of these genes. This information provides a starting point for further studies on the dynamic remodelling of wood formation. These studies not only increase our knowledge of the mechanism underlying the process of gravitropic response, but also provide a powerful genetic tool to increase the value of pine wood for industrial uses.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Figure S1. qPCR analysis of genes involved in hormone signal transduction and transcription factors in different height positions of stems of radiata pine. Different letters indicate significant differences with respect to the control of non-inclined stems ($P \leq 0.05$).

Figure S2. qPCR analysis for stress response genes related to protein synthesis in different height positions of stems of radiata pine. Different letters indicate significant differences with respect to the control of non-inclined stems ($P \leq 0.05$).

Table S1. List of ESTs from SSH libraries.

Video clip S1. Radiata pine apex reorientation on an inclined stem. Movie showing apex reorientation during the first 24 h after plant inclination. Light was supplied laterally.

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