

RESEARCH PAPER

# Involvement of *Pinus taeda* MYB1 and MYB8 in phenylpropanoid metabolism and secondary cell wall biogenesis: a comparative *in planta* analysis

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## Abstract

The involvement of two *R2R3-MYB* genes from *Pinus taeda* L., *PtMYB1* and *PtMYB8*, in phenylpropanoid metabolism and secondary cell wall biogenesis was investigated *in planta*. These pine *MYBs* were constitutively overexpressed (OE) in *Picea glauca* (Moench) Voss, used as a heterologous conifer expression system. Morphological, histological, chemical (lignin and soluble phenols), and transcriptional analyses, i.e. microarray and reverse transcription quantitative PCR (RT-qPCR) were used for extensive phenotyping of *MYB*-overexpressing spruce plantlets. Upon germination of somatic embryos, root growth was reduced in both transgenics. Enhanced lignin deposition was also a common feature but ectopic secondary cell wall deposition was more strongly associated with *PtMYB8*-OE. Microarray and RT-qPCR data showed that overexpression of each *MYB* led to an overlapping up-regulation of many genes encoding phenylpropanoid enzymes involved in lignin monomer synthesis, while misregulation of several cell wall-related genes and other *MYB* transcription factors was specifically

associated with *PtMYB8*-OE. Together, the results suggest that *MYB1* and *MYB8* may be part of a conserved transcriptional network involved in secondary cell wall deposition in conifers.

## Introduction

The plant cell wall is a composite assembly of complex polymers that confer structure and provide protection to the cell and to the whole plant. The major polymers of the primary cell wall include cellulose, hemicelluloses, and pectins (Cosgrove, 1999). The secondary cell wall that is formed mostly in vascular cells and fibres also contains a large proportion of lignin. In trees, lignin synthesis is of major importance because of the production of wood which is typically made up of 20–30% lignin on a dry weight basis. Therefore, the formation of secondary xylem (i.e. wood) entails the partitioning of a significant proportion of fixed carbon resources into the synthesis of lignin-building blocks through the phenylpropanoid pathway (Amthor, 2003; Boerjan *et al.*, 2003). Although the

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enzymes and genes of phenylpropanoid and monolignol biosynthetic pathways have been extensively studied (Humphreys and Chapple, 2002; Boerjan *et al.*, 2003), the mechanisms regulating their expression are still a matter of debate. In addition, molecular mechanisms regulating metabolic flux through the phenylpropanoid biosynthetic pathway are very complex and, to date, remain unclear.

Recent experimental evidence indicates that monolignol synthesis genes are under transcriptional control (Rogers and Campbell, 2004). Among the different classes of transcription factors (TFs) directly or indirectly implicated in lignification, *R2R3-MYBs* are strong candidates for the regulation of phenylpropanoid enzymes and monolignol biosynthesis (Rogers and Campbell, 2004; Groover and Robischon, 2006). The *R2R3-MYBs* form one of the largest families of TFs in plants. Numerous studies characterizing their molecular function have assigned diverse roles to these TFs, including cell fate or organ identity (Rogers and Campbell, 2004; Paux *et al.*, 2005; Chen *et al.*, 2006; Groover and Robischon, 2006). *R2R3-MYBs* also govern many aspects of secondary metabolism in *Arabidopsis* (Vom Endt *et al.*, 2002) as well as in angiosperm and gymnosperm trees (Rogers and Campbell, 2004; Groover and Robischon, 2006).

The involvement of *R2R3-MYBs* in the transcriptional control of phenylpropanoid and flavonoid metabolic pathways has been well documented in plant model systems. Different *R2R3-MYBs* have been shown to bind the promoters of most genes in these pathways (Rogers and Campbell, 2004); they may bind either alone (Patzlaff *et al.*, 2003a, b; Gomez-Maldonado *et al.*, 2004; Goicoechea *et al.*, 2005), or in heteroduplex with bHLH proteins (Goff *et al.*, 1992; Debeaujon *et al.*, 2003) or with WD-40 proteins (Koes *et al.*, 2005; Morita *et al.*, 2006). In woody species, however, only a few functional studies have addressed this question for *MYBs*. For example, the poplar gene *PttMYB21a* was proposed to be a negative regulator of *CCoAOMT* expressed in vascular tissues (Karpinska *et al.*, 2004). On the other hand, the genes *PtMYB1* and *PtMYB4* from loblolly pine (Patzlaff *et al.*, 2003a, b; Gomez-Maldonado *et al.*, 2004) and *EgMYB2* from eucalyptus (Goicoechea *et al.*, 2005) appear to be transcriptional activators of genes encoding lignin synthesis enzymes. These three *MYBs* are preferentially expressed in developing xylem tissues, bind AC elements, and activate transcription from lignin biosynthetic gene promoters in transient assays in yeast or plant cells (Patzlaff *et al.*, 2003a, b; Gomez-Maldonado *et al.*, 2004; Goicoechea *et al.*, 2005). Overexpression of *PtMYB4* resulted in ectopic lignification in tobacco (Patzlaff *et al.*, 2003b) and in *Arabidopsis* (Newman *et al.*, 2004). Overexpression of *EgMYB2* in tobacco plants leads to altered lignin structure, to thicker secondary cell walls, and to the up-regulation of lignin-related genes (Goicoechea *et al.*, 2005). Although data from these studies

and others implicated *MYBs* in the lignification of tree woody tissues, knowledge is still limited about the number and the respective roles of *MYB* TFs during wood formation.

This report represents overexpression experiments carried out with two *Pinus taeda R2R3-MYB* genes, namely *PtMYB1* and *PtMYB8*, aiming to develop new insights into the involvement of *MYBs* in gymnosperm trees. *PtMYB1* has been hypothesized to regulate lignin biosynthesis in differentiating xylem because of its ability to bind AC elements and to activate transcription from the *phenylalanine ammonia-lyase (PAL)* promoter (Patzlaff *et al.*, 2003a). This hypothesis remained to be tested *in planta*. *PtMYB8* was investigated because its closest homologue in spruce, *PgMYB8*, had transcript accumulation that was strongly preferential to secondary xylem and that increased during compression wood formation (Bedon *et al.*, 2007). Overexpression was preferred to knockout/knockdown analysis since it is less affected by functional redundancy, which can be especially acute for TFs from large gene families such as *MYBs* (Schwechheimer *et al.*, 1998; Zhang, 2003). This problem has been recently underlined for T-DNA knockout lines of *AtMYB46* where no phenotypes were observed (Zhong *et al.*, 2007). In addition, conifers possess a very large genome with a larger proportion of multigenic families compared with angiosperms (Kinlaw and Neale, 1997), which may add another level of complexity for the design of knockout/knockdown experiments. For this report, the use of spruce [*Picea glauca* (Moench) Voss, a member of the Pinaceae] for transformation provided an expression system that is taxonomically much closer to pine than *Arabidopsis* and tobacco employed in previous reports (Patzlaff *et al.*, 2003a, b). The extent of ectopic secondary cell-wall deposition that is reported in transgenic spruce plantlets overexpressing these *MYBs* as well as their microarray, and reverse transcription quantitative PCR (RT-qPCR) transcript profiles helped to assess and compare the putative involvement of *PtMYB1* and *PtMYB8* in lignification and secondary cell wall biogenesis.

## Materials and methods

### Vector construction and spruce transformation

*PtMYB1* (AY356372; Patzlaff *et al.*, 2003a) and *PtMYB8* (DQ399057; Bedon *et al.*, 2007) cDNA from *Pinus taeda* L. (loblolly pine) were used to conduct gain-of-function experiments in *Picea glauca* (Moench) Voss (white spruce). Gain of function was obtained by inserting the full-length cDNA in front of the maize ubiquitin promoter (Christensen *et al.*, 1992), followed at the 3' end by the 35S terminator. This constitutive expression vector was obtained by modifying the pRT106 (Topfer *et al.*, 1993) plant expression vector. For *Agrobacterium tumefaciens*-mediated stable transformation, cassettes were digested by *Hind*III and cloned into pCAMBIA1305.2 (www.cambia.org). The resulting plasmid was

then transferred into the *A. tumefaciens* strain C58 pMP90 (Koncz and Schell, 1986).

The white spruce embryogenic line Pg653 was used in the present study, and was initiated and maintained as described by Klimaszewska *et al.* (2001). Genetic transformations were carried out also as described in Klimaszewska *et al.* (2004). Once co-cultivated, explants were decontaminated from *A. tumefaciens* with cefotaxim and transferred onto fresh medium containing cefotaxim and kanamycin. Kanamycin-resistant embryonal tissues were screened for positive X-gluc staining (Klimaszewska *et al.*, 2004) and assayed for *PtMYB1* and *PtMYB8* mRNAs accumulation by RT-qPCR (see below). Transgenic lines (each representing an independent transformation event) exhibiting a range of *PtMYB* mRNAs levels were selected for somatic embryo maturation and plantlet production.

#### *Transgenic lines and culture conditions used for somatic plantlet production*

For *in vitro* monitoring, somatic embryos were produced from selected transgenic lines overexpressing *PtMYB1* (lines 4, 12, 14, 24, and 26) and *PtMYB8* (lines 1–13) as well as wild-type and pCAMBIA control lines; 50–100 embryos per transgenic line were germinated according to Klimaszewska *et al.* (2004) and the experiment was repeated twice or three times. When allowed by the phenotype, 10- to 14-week-old transgenic plantlets were transplanted in a mix of moss, vermiculite, and turface (ratio 4:2:1, v/v/v) and grown in a mist environment for 15 d before being transferred to the greenhouse under a photoperiod of 16 h light at 24 °C, and 8 h dark at 20 °C.

For the microarray experiments, somatic embryos were germinated for 3 weeks on MLVG medium supplemented with 58 mM sucrose (Klimaszewska *et al.*, 2004). Two transgenic lines per transgene (lines 4 and 14 for *PtMYB1*, and lines 1 and 2 for *PtMYB8*), as well as four control lines (Pg653), were used in a randomized complete block design, with four biological replicates per line and 50 whole plantlets per replicate.

#### *Plantlet growth quantification, tissue sampling, and histology*

Image capturing was used for plantlet growth quantification. The length of hypocotyl and root was determined by using ImageJ 1.32 Software (W Rasband, National Institute of Health, USA; <http://rsb.info.nih.gov/ij/>). Elongations were calculated from five replicates, with 6–10 plantlets per replicate, and the experiment was repeated twice. Afterwards, tissues of *in vitro* plantlets were sampled as follows: roots and hypocotyls were rapidly separated on the medium by using a scalpel and were immediately fixed as described below when used for histology, or frozen into liquid nitrogen and stored at –80 °C for further molecular analysis.

For histology, the tissue samples (three plantlets per transgenic line per replicate, with five replicates) were fixed for 24 h under low vacuum in 2% (v/v) paraformaldehyde, 3% (v/v) glutaraldehyde, 0.1 M cacodylate buffer (pH 7.2) supplemented with 1 mM CaCl<sub>2</sub>, and 1% (w/v) sucrose. Samples were dehydrated in graded ethanol series and toluene, before infiltration with paraffin for 7 d. Thin sections (5 µm) were prepared by using a microtome. Paraffin-free sections were transferred to water and, after incubation in the mordant 2% (w/v) ZnCl<sub>2</sub>, were stained in Sharman's safranin O–orange G–tannic acid (Sharman, 1943). All sections were observed under a Zeiss Axioskop microscope (Jena, Germany) fitted with a digital camera.

#### *RNA extraction, cDNA synthesis, and real-time RT-qPCR analysis*

RNA extraction, cDNA synthesis and real-time RT-qPCR analyses were performed as described in Bedon *et al.* (2007) with minor

modifications. Briefly, total RNA samples were obtained by using a Mixer Mill MM300 engine (Retsch, Germany) to grind tissue (from spruce and pine) in 1.5 ml Ependorf microtubes, and were extracted following the procedure of Chang *et al.* (1993). Total RNA samples were treated with amplification grade DNase I (Invitrogen, Carlsbad, CA, USA) and purified using RNeasy columns (Qiagen). cDNAs were synthesized from 1 µm of purified RNA using SuperScript II (Invitrogen) according to the manufacturer's instructions. For RT-qPCR quantification of each target RNA, a 5-fold dilution of cDNA mixture was used as template. Thermocycling, conducted using an Opticon2 DNA Engine (MJ Research Inc.), was initiated by a 2 min incubation at 95 °C followed by 35 cycles (95 °C for 10 s; 55 °C for 40 s; 72 °C for 6 s) with a single fluorescent reading taken at the end of each cycle. A standard dilution series covering five orders of magnitude were prepared for the target and reference genes from 1 ng µl<sup>-1</sup> PCR amplicon of each cDNA to produce solutions covering 10<sup>-1</sup> to 10<sup>-6</sup> ng µl<sup>-1</sup>. All mRNA levels were calculated from threshold cycle values, normalized with respect to the transcript level of *ELONGATION FACTOR 1-alpha (EF1a)* with 100% identity to *Picea abies* AJ132534) and expressed as a ratio relative to the control. For endogenous spruce *MYB* genes, absolute quantity of transcripts was calculated using standard curves as described by Rutledge and Côté (2003). Technical duplicates were performed for each sample amplification to assess variation in RT-qPCR data; a mean variation in the threshold cycle of 0.31±0.10 and 0.36±0.07 (corresponding to 23.6% and 28.3% of the fold change, respectively) was obtained between technical duplicates for sequences related to cell wall and secondary metabolism, and for *MYB* sequences, respectively. The means of these duplicates were used to calculate fold change ratios for each biological replicate, the mean ratio being obtained from three or four biological replicates. The specific primer pairs of target and reference genes, designed using Primer3 software (Rozen and Skaletsky, 2000), are given in Table S1 (see Supplementary data available at *JXB* online).

#### *Microarray experiment*

All the information regarding microarray manufacture and quality control are detailed in Appendix S1 (see Supplementary data available at *JXB* online). The microarray experiment was designed in total compliance with MIAME guidelines. Briefly, total RNA was extracted as described above. For each sample, 1.0 µg of total RNA was used for indirect RNA amplification performed with the Superscript™ Indirect RNA Amplification System kit (Invitrogen) according to the manufacturer's instructions. Dye coupling was performed on 5 µg of amplified RNA with either Alexa Fluor® 555 or Alexa Fluor® 647 reactive dyes (Invitrogen).

Pre-hybridization and hybridization protocols are described in Appendix S1 (see Supplementary data available at *JXB* online). Eight hybridizations (including four dye swaps) were carried out for each of the *PtMYB1* and *PtMYB8* versus wild-type (WT) comparisons. Array images were scanned using ScanArray Express (Perkin Elmer) and image analysis was performed with Quantarray software (Packard BioSciences, version 3.0, 2001).

#### *Statistical analysis of microarray data*

A complete list of microarray data from *PtMYB1*-OE and *PtMYB8*-OE in spruce is presented in Table S2 (see Supplementary data available at *JXB* online). Data were analysed in R (Ihaka and Gentleman, 1996), mainly using the BioConductor suite of packages (Gentleman *et al.*, 2004). Spot intensities were analysed with the LIMMA package from Bioconductor (Smyth, 2005). Data normalization was performed using the composite method based on Lowess curves (Yang *et al.*, 2002). Normalized data were then

statistically analysed using the linear model and empirical Bayes analyses in LIMMA. The results were corrected using the Benjamini and Hochberg (1995) method of false discovery rate (1%). In this study, the focus was placed on differentially expressed genes that gave a *P* value <0.01 (from the LIMMA), and met a  $\log_2$  ratio threshold of 0.8 (1.75-fold change) between WT and *PtMYB*-OE transgenics. For each transgenic construct, data from the two different lines were analysed separately and only the differentially expressed genes identified in both lines for a given transgene were considered (i) to reduce the impact of positional effects of the transgene, and (ii) to ensure that the differential expression was not the effect of only one transgenic line. Therefore, genes showing differential expression for only one transgenic line were not retained.

#### Soluble phenolic metabolite analysis

Spruce hypocotyl tissue from 7-week-old plantlets (~20 mg) was suspended in 1.5 ml of methanol:water:HCl (48.5:48.5:1, v/v/v), pulverized in a cell disrupter (FastPrep) at high power for 20 s, and then extracted for 4 h at 50 °C in a heating block. The homogenate was centrifuged for 10 min at 18900 *g*, and the supernatant equally divided into two 600  $\mu$ l aliquots. Subsequently, 1 ml of distilled water was added to methanolic extracts followed by an equal volume of ethyl-ether. After mixing thoroughly, the ether phase was retained. This extraction was performed twice and the ether phases were pooled. The ether phase was concentrated to dryness in a speedvac and resuspended in 100  $\mu$ l methanol before injection in a Summit HPLC system (Dionex) fitted with a 0.2 $\times$ 150 mm Pursuit column (Waters; 5  $\mu$ m particle size), an autosampler, and a photodiode array detector. The elution at a rate of 1 ml min<sup>-1</sup> was performed at 45 °C using a linear gradient from 100% eluant A (CH<sub>3</sub>COOH, 5%) to 80% eluant B [CH<sub>3</sub>COOH (20%):CH<sub>3</sub>CN, 75:25, v/v] over 60 min, followed by a 10 min wash with 100% B, and 10 min reaclimatizing with 100% A.

#### Acetyl bromide (AcBr) lignin determination

Samples were first produced to generate a standard curve for lignin determinations via the AcBr method as follows. Oven-dried 2-year-old spruce stems (Pg653) were ground in a Wiley mill to pass through a 40-mesh screen, and then soxhlet-extracted with acetone for 24 h. Holocellulose was purified from the extractive free wood by reacting 200 mg of ground wood with 1 ml of NaClO<sub>2</sub> solution (400 mg 80% sodium chlorite, 4 ml distilled water, 0.4 ml glacial acetic acid) in a 25 ml round-bottom flask maintained at 90 °C in an oil bath. An additional millilitre of NaClO<sub>2</sub> solution was added every 30 min and the samples removed to a cold water bath after 2 h. Samples were then filtered through a coarse crucible and dried overnight. Holocellulose composition was determined gravimetrically and characterized for residual lignin content by Klason lignin determination. For lignin isolation, ground, extract-free 2-year-old spruce wood was ball milled as per Stewart *et al.* (2006) and lignin was isolated according to Björkmann (1956). Isolated holocellulose and lignin samples were then recombined in varying ratios, ranging from 100:0 to 0:100, and validated by Klason analysis, to produce a standard curve for the AcBr technique.

Seven-week-old hypocotyls from transgenic and wild-type spruce were ground to a powder in a mortar and pestle with liquid nitrogen; they were subsequently pre-extracted overnight in hot acetone to remove free phenolics prior to lignin determination using the AcBr method. Acetone-extracted samples, as well as the standard curve mixtures (~5 mg), were accurately weighed into Teflon-capped glass vials, in which 0.5 ml of AcBr solution was added [25% (v/v) AcBr in glacial acetic acid]. Samples were incubated in a heating block at 50 °C for 2 h with occasional mixing. After 2 h, the solution was transferred to a 10 ml volumetric flask with ~5 ml of glacial acetic acid and 2 ml of 2 M

NaOH. Then, 0.35 ml of 0.5 M hydroxylamine was added. The volume was made up to exactly 10 ml with glacial acetic acid and the solution thoroughly mixed. The hypocotyls and standard curve solution were then read at 280 nm and lignin content interpreted.

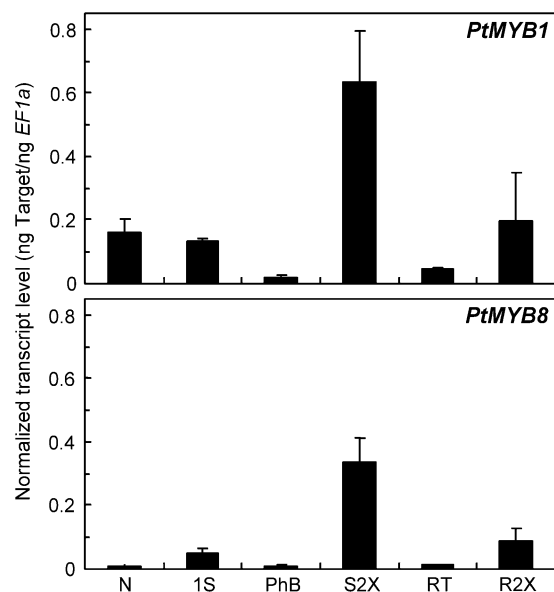
## Results

### *PtMYB1* and *PtMYB8* display overlapping transcript profiles preferential to differentiating secondary xylem

Transcript abundance of *PtMYB1* and *PtMYB8* was surveyed in several organs or tissues from 2-year-old *Pinus taeda* trees by using real time RT-qPCR analysis. As shown in Fig. 1, *PtMYB1* and *PtMYB8* transcripts preferentially accumulated in S2X and R2X, congruent with a role in xylem differentiation. The results also showed that *PtMYB1* transcript levels in those tissues are higher than the ones of *PtMYB8*. Interestingly, the expression profiles of these two *MYB* genes were similar to those reported for their closest homologues from spruce, *PgMYB1* and *PgMYB8* (Bedon *et al.*, 2007). For these pine and spruce pairs of *MYB* sequences, pair-wise optimal alignments gave amino acid identities of 99.1% and 98.3% for the DBD and of 87.1% and 93.1% for the complete coding sequence (Bedon *et al.*, 2007).

### *PtMYB1*- and *PtMYB8*-OE in spruce leads to ectopic secondary cell wall deposition

Transgenic spruces overexpressing (OE) *PtMYB1* and *PtMYB8* were generated to gain insight into their role as



**Fig. 1.** Transcript profiles of *PtMYB1* and *PtMYB8* in pine. Total RNA was isolated from various tissues of 2-year-old *Pinus taeda* trees. Transcript levels were determined by RT-qPCR from three biological replicates and were normalized relative to *EF1-alpha* expression level. Error bars represent standard deviation. N, Needle; 1S, primary shoot, corresponding to elongating shoot (no apical bud) with no sign of secondary growth; PhB, phloem-bark; S2X, shoot secondary xylem; RT, root tip; R2X, root secondary xylem.

potential regulators of lignification. Five *PtMYB1*-OE lines and 13 *PtMYB8*-OE lines with strong transgene expression (determined by RT-qPCR analysis of transgene RNA levels, not shown) were selected for *in vitro* growth monitoring and phenotypic analyses.

The *PtMYB1* transgenic spruce plantlets produced roots that were 30–80% shorter (mean decrease of 40%) depending on the transgenic line compared with WT and pCAMBIA controls (Fig. 2A, B). *PtMYB1*-OE plantlets survived subsequent transfer to soil but their growth was moderately to severely delayed, particularly for line 4 (Fig. 2C). Histological observations of *PtMYB1* transgenic plantlets provided evidence of ectopic secondary cell wall deposition in a few cells displaying the pink colour that is diagnostic of lignified cell walls following Sharman's staining (Sharman, 1943). Significant cell wall thickening characteristic of sclerenchyma-like elements was observed among the parenchyma cells, located close to the shoot apical meristem (Fig. 2E) and peripheral to vascular tissues (Fig. 2G); this phenomenon was never observed in WT control plantlets (Fig. 2D, F). The hypocotyls of *PtMYB1* transgenic plantlets also had altered vascular radial patterning as the phloem zone was expanded and contained longer files of phloem cells (Fig. 2I) compared with the WT control (Fig. 2H). No unusual secondary cell wall deposition was noticed in the root or needles of *PtMYB1* overexpressors (Fig. 2K, M) compared with the WT control (Fig. 2J, L).

In *PtMYB8* transgenic plantlets, the root growth was strongly reduced compared with the untransformed control (Fig. 2N), with a mean decrease in length of 68% (Fig. 2O). Two *PtMYB8* transgenic lines also developed shorter hypocotyls (L1 and L2, Fig. 2N, O). *In vitro* development of *PtMYB8*-OE plantlets was poor, and none of them survived the subsequent transfer to soil. Histological analyses using Sharman's staining provided evidence of extensive ectopic secondary cell wall thickening (pink staining) in *PtMYB8* transgenics after 7 weeks in germination. Sclerenchyma-like elements were observed among the parenchyma cells, located in the periphery of vascular tissues (Fig. 2S). The pink staining also revealed cell wall thickening in root cortical cells (Fig. 2T), as well as the presence of sclerenchyma-like elements in the needle (Fig. 2U). In WT control plantlets, the staining was restricted to vascular elements in the hypocotyl, root, and needle (Fig. 2P–R).

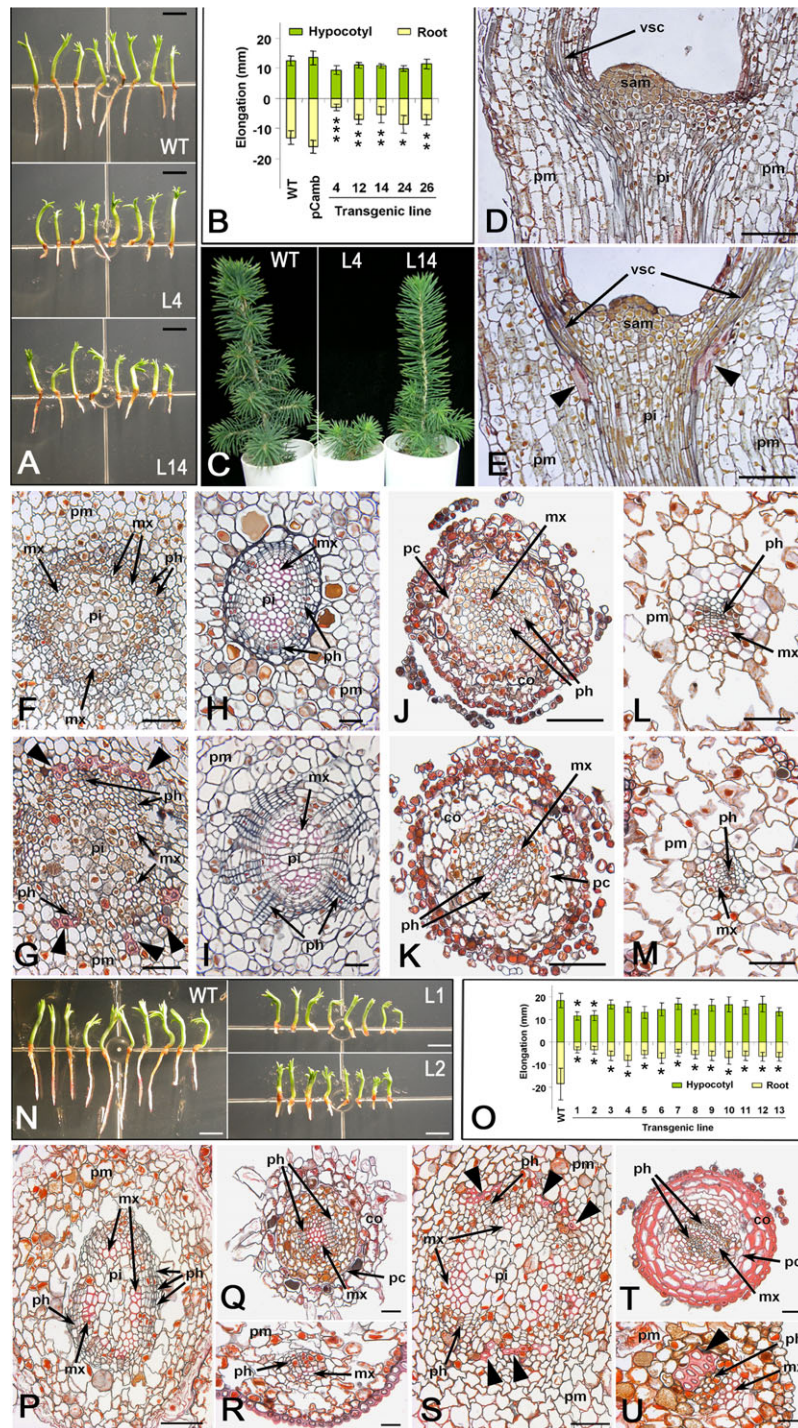
#### *Lignin and phenolics in PtMYB1 and PtMYB8 overexpressing spruce*

Secondary cell walls are normally composed of a large proportion of lignin (Campbell and Sederoff, 1996; Amthor, 2003). In light of the observed altered secondary cell wall deposition in *PtMYB1*- and *PtMYB8*-OE transgenic spruces (Fig. 2), lignin content and phenolic compound profiles were quantified. As a consequence of

the small sample size, lignin determinations used the AcBr technique on 7-week-old hypocotyls. Both the *PtMYB1* and *PtMYB8* overexpressors had significant increases in lignin content, which ranged from 10.1% to 11.2% increase in *PtMYB1*-OE, and 5.3% to 12.1% in *PtMYB8*-OE, compared with controls (Fig. 3A). The amount of soluble phenolic compounds that could be recovered by methanol extraction from *PtMYB1* and *PtMYB8* transgenic and wild-type plantlet tissues was also examined. Visual inspection of the HPLC chromatograms clearly indicates that there is an overall decrease in the amount of low molecular weight phenolics in both transgenics compared with WT (Fig. 3B).

#### *PtMYB1- and PtMYB8-OE have overlapping impact on spruce transcriptome*

As described above, similarities were observed in the *PtMYB1* and *PtMYB8* overexpression phenotypes, including ectopic secondary cell wall deposition. However, the phenotypes conferred by the two transgenes were clearly distinct, implying that the functions of these two genes may not be completely redundant. Transcript profiling was carried out with custom 9K cDNA microarrays in order to further characterize similarities and differences in the effects of the two *MYB* genes. Two independent transgenic lines per construct were used for comparison with wild-type background. The focus was placed on statistically significant genes that gave a 1.75-fold difference or greater ( $\log_2$  ratio of 0.8) between the transgenic and controls ( $P$  value <0.01; false discovery rate, 1%). Each transgenic line was analysed separately: for *PtMYB1*, the number of up- and down-regulated sequences were, respectively, 52 and 35 for line 4, and 37 and 16 for line 14; for *PtMYB8*, the number of up- and down-regulated sequences were, respectively, 107 and 187 for line 1, and 89 and 159 for line 2. However, only those genes that had differential transcript levels in both lines for each *MYB* transgenic were considered, so as to reduce the potential influence of position effects of the transgene. Based on these criteria, the overexpression of *PtMYB1* had a moderate impact on the spruce transcriptome with a total of 34 up-regulated and 12 down-regulated genes. A stronger effect on spruce transcriptome was observed in *PtMYB8*-OE, with a total of 79 up-regulated and 138 down-regulated genes. Functional annotations obtained from the blastx procedure against Uniref100 and a search against PGI5.0 and PFAM databases indicate that a large proportion of differentially expressed genes had a predicted function that could be linked to cell wall biogenesis, secondary metabolism (phenylpropanoid, flavonoid, and terpenoid) and related pathways (shikimate and *S*-adenosyl methionine), as well as stress and detoxification (Table 1; see Table S2 in Supplementary data available at *JXB* online). Many genes with unknown function were also misregulated in *PtMYB1* (19%) and *PtMYB8* (31%)



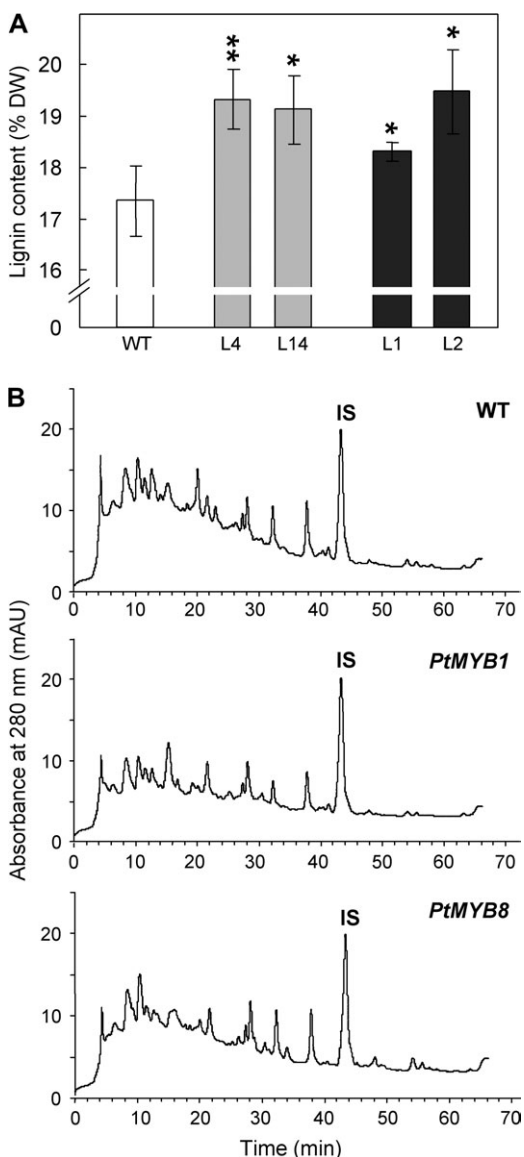
**Fig. 2.** Phenotypes induced by *PtMYB1* and *PtMYB8* overexpression in spruce. (A–M) *PtMYB1*-OE phenotypes: (A) 7-week-old *in vitro* plantlets in wild type (WT) and two independent *PtMYB1*-OE transgenic lines (L4, L14); (B) root and hypocotyl growth in 7-week-old plantlets produced from five independent *PtMYB1*-OE lines [significant differences in elongation between *PtMYB1*-OE lines and control one are indicated by asterisk according to Student's *t*-test at a level of 0.05 (\*), 0.01 (\*\*), or 0.001 (\*\*\*)]; (C) plantlet morphology in wild type (WT) and two independent transgenic lines (L4, L14) of *PtMYB1*-OE transgenic spruce after transfer to soil; (D–M) 5  $\mu$ m sections in hypocotyl, root, and needle of WT (D, F, H, J, L) and *PtMYB1*-OE (E, G, I, K, M) *in vitro* plantlets. Longitudinal (D, E) and cross- (F, G) sections in hypocotyl of 16-d-old *in vitro* plantlets. Sclerenchyma-like elements around the vascular cylinder are shown (black arrowheads) by pink staining characteristic of lignified cell walls (Shaman, 1943). Cross-sections in hypocotyl (H, I), root (J, K), and needle (L, M) are of 7-week-old plantlets. Supernumerary phloem cells can be observed in *PtMYB1*-OE section (I). No ectopic lignification was observed in root and needle of *PtMYB1*-OE transgenic spruce (K, M) compared with the control (J, L). (N–U) *PtMYB8*-OE phenotypes: (N) 7-week-old *in vitro* plantlets in WT and *PtMYB8*-OE independent transgenic lines (L1, L2); (O) root and hypocotyl growth in 7-week-old plantlets from 13 independent transgenic lines; no plantlet survived transfer to soil. Significant differences in elongation between *PtMYB8*-OE lines and the control one are indicated by an asterisk according to Student's *t*-test at a level of

overexpressors (see Table S2 in Supplementary data available at *JXB* online). Overlapping and distinct responses in both transgenics are described below.

Overlapping response in terms of the pattern of differential expression was observed when comparing *PtMYB1* and *PtMYB8* transgenics (Table 1, genes listed in bold). All the up-regulated genes that overlapped in these transgenics were also preferentially expressed in differentiating secondary xylem (Table 1). They included: four genes that were associated with the phenylpropanoid pathway, i.e. *phenylalanine ammonia-lyase* (*PAL*), *4 coumarate coA ligase* (*4CL*), *hydroxycinnamoyl transferase* (*HCT*), and *caffeic acid O-methyl transferase* (*COMT*) (Table 1); two *pinoresinol-lariciresinol reductase* (*PLR1* and *PLR2*) genes, that are involved in lignan synthesis primarily derived from the monolignols, *E-coniferyl*, and *E-p-coumaryl* alcohols (Kwon *et al.*, 2001); and transcripts encoding 3-deoxy-D-arabinoheptulosonate 7-phosphate synthetase (*DAHP*), an important enzyme of the shikimate pathway, which provides precursors for phenylpropanoid metabolism leading to monolignols and flavonoids (Herrmann, 1995; Amthor, 2003). Both transgenics also displayed a strong up-regulation for an uclacyanin, a plant-specific blue copper protein that might participate in redox processes occurring during the primary defence response and/or lignin formation in plants (Nersissian *et al.*, 1998). Conversely, the overlapping transcripts that had reduced expression in both transgenics were preferential to phloem-bark or needle tissue or did not show any tissue preference (Table 1). Reduced transcript levels were observed in both transgenics for a xyloglucan endo-transglycosylase (*XTH*) that could be associated with cell wall biogenesis and reassembly, as well as for a canonical class III peroxidase (MN5232900). Three other genes, that could be associated with stress and detoxification, such as *glyoxalase*, *LEA EMB7* (MN5251934), and r4g30 protein coding gene, were strongly down-regulated, particularly in *PtMYB8*-OE. A *beta-primeverosidase* (MN5233446), which could be linked to geraniol hydrolysis, was also strongly down-regulated in both transgenics, as well as two genes of unknown function. Only a single transcript, a carbonic anhydrase, was strongly up-regulated in *PtMYB1* transgenics and down-regulated in *PtMYB8* transgenics. Carbonic anhydrases have been shown to facilitate the transport of inorganic carbon and to catalyse carboxylation–decarboxylation reactions essential to photosynthesis and/or respiration in plants (Shiraiwa and Miyachi, 1985).

Distinct transcriptional responses were also observed between the transgenics. Transcripts distinctly misregulated in either *PtMYB1* or *PtMYB8*-OE were generally preferential to secondary xylem when up-regulated, whereas they were assigned to needles, phloem-bark, or did not show preference to any particular tissue when down-regulated (Table 1). Large parts of the sequences which were only differentially expressed in *PtMYB8*-OE were associated with cell-wall organization and biogenesis (Table 1). Indeed, several genes related to cellulose (*CesA*), hemicellulose (*CLS*, *GT2*), and pectin (*GT8*) metabolism were up-regulated, together with an epimerase (*NSI*) and an arabinogalactan protein (*AGP1*). In parallel, several genes related to cell wall expansion and reassembly were down-regulated in *PtMYB8*-OE, such as *GH17*, *GH28*, *UDPGT*, *XTH*, and *PAE*, in addition to needle preferential transcripts encoding a *CesA* and a *CSL*-like. Upstream of the phenylpropanoid pathway, additional transcripts representing genes from the shikimate pathway were either up-regulated (*chorismate mutase*) or down-regulated (*prephenate dehydratase*). Other down-regulated sequences were similar to phenylpropanoid pathway genes, like *coumaroyl CoA O-methyltransferase*-like (*CCoAOMT*-like) and *cinnamoyl CoA reductase*-like (*CCR*-like) genes, as well as an *N-hydroxycinnamoyl benzoyltransferase* (*HCBT*), and were preferential to needle and/or phloem/bark tissues (Table 1). Genes linked to *S*-adenosyl-L-methionine, a potential methyl group donor for methoxylation reactions in the monolignol biosynthetic pathway (Amthor, 2003), were also misregulated in *PtMYB8*-OE (Table 1). Transcripts corresponding to enzymes involved in the early steps of the flavonoid pathway, such as *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *dihydroflavonol 4-reductase* (*DFR*), and *flavonol synthase* (*FS*), also showed clear down-regulation in both *PtMYB8* transgenic lines (Table 1). A clear decrease in transcript abundance was observed for several peroxidase genes, some of which could be associated with lignin polymerization (MN5235950; Koutaniemi *et al.*, 2005) or linked to other oxidative processes (MN5233396). Genes associated with stress and detoxification were generally down-regulated, as shown for *glutathione S-transferase*, as well as those related to the terpenoid pathway. In *PtMYB1*-OE, additional up-regulated sequences were related to the phenylpropanoid pathway such as *trans-cinnamate 4-hydroxylase* (*C4H*), and to the cell wall as an *alpha-expansin* which was up-regulated 2-fold (Table 1). In turn, a peroxidase, as well as

0.01 (\*). (P–U) Sections (5 µm) of paraffin-embedded hypocotyl (P, S), root (Q, T), and needle (R, U) in wild-type (P–R) and *PtMYB8*-OE (S–U) *in vitro* plantlets. Sclerenchyma-like elements around the vascular cylinder can be seen in the *PtMYB8*-OE transgenic (black arrowheads) in hypocotyl (S) and needle (U). Lignified cell wall staining (pink) was observed in parenchyma (S) and cortical (T) cells only in the transgenics. All histological sections were stained in safranin O–orange G–tannic acid after mordanting in 2% ZnCl<sub>2</sub> (Sharman, 1943). Scale bars correspond to 5 mm (A, N), 50 µm (D, E, L, M, P, Q, S, T), 25 µm (F, G, R, U), 20 µm (H, I), and 100 µm (J, K). mx, metaxylem; ph, phloem; pm, parenchyma; pi, pith; co, cortex; pc, pericycle; sam, shoot apical meristem; vsc, vascular cells.



**Fig. 3.** Lignin content and free phenolic profiles resulting from *PtMYB1* and *PtMYB8* overexpression in spruce. (A) Lignin content ( $\pm$  SD) of 7-week-old hypocotyls in wild type (WT) and transgenic spruce overexpressing *PtMYB1* (L4, L14), and *PtMYB8* (L1, L2). Lignin content was quantified by the AcBr method, expressed as a percentage of dry weight, acetone-extracted whole hypocotyls, and was calculated from three biological replicates per line and 10 plantlets per replicate. Asterisks indicate that transgenic means were significantly different from wild type, according to Student's *t*-test at  $P \leq 0.05$  (\*) and  $P \leq 0.01$  (\*\*). (B) HPLC profiles of low molecular weight phenolic compounds in 7-week-old wild-type (WT), and *PtMYB1* and *PtMYB8* overexpressing spruce (L4 and L1, respectively). IS, 3,4,5-Trimethoxycinnamic acid as internal standard.

several genes involved in the terpenoid pathway, and stress response and detoxification were down-regulated.

#### Targeted RT-qPCR analysis of phenylpropanoid metabolism, cell-wall, and R2R3-MYB-related genes

RT-qPCR analyses were performed on all the genes involved in the phenylpropanoid pathway to complement

microarray transcript profiles, and were extended to a subset of genes that were related to cell wall biogenesis and to shikimate and flavonoid pathways in order to validate the results from microarray analysis (Fig. 4; see Fig. S1 in Supplementary data available at *JXB* online). Compared with controls, the significant up-regulation of genes involved in phenylpropanoid metabolism was confirmed by Student's *t*-test (Fig. 4). In both transgenics, transcript abundance was increased by  $\sim 2$ -fold for pathway genes going from *C4H* to *CAD*, except for *COMT* and *CCoAOMT* in *PtMYB8*-OE for which transcript abundance was similar to control (Fig. 4, shaded box I). In both transgenics, *PAL* gave smaller and less consistent increases. In addition, the strong up-regulation of the two *PLR* transcripts (around 3-fold for *PLR1*, and  $>10$ -fold for *PLR2*) linked to phenylpropanoid metabolism was confirmed in both transgenics compared with the control (Fig. 4). The down-regulation of flavonoid- (*DFR* and *CHS*) and up-regulation of shikimate (*DAHPh*)-related genes was also confirmed in both transgenics, as shown in Fig. 4. The differential effects on cell wall-related genes in *PtMYB1*-OE compared with *PtMYB8*-OE were also validated (Fig. 4, shaded box II). Compared with control and *PtMYB1*-OE, *GT8*, *CesA*, and *AGP1* transcripts were all increased in *PtMYB8*-OE, while *XTH* was strongly down-regulated in both transgenics.

The RNA levels of *PgMYB1* and *PgMYB8*, previously identified as putative spruce orthologues of *PtMYB1* and *PtMYB8* (Bedon *et al.*, 2007), were examined together with those of the transgenes. In addition, transcript levels of three other spruce *MYBs*, *PgMYB2*, *PgMYB3*, and *PgMYB4*, that were shown to be preferentially expressed in differentiating xylem from 33-year-old white spruce trees (Bedon *et al.*, 2007), were also evaluated. Transgene transcript levels in *PtMYB1* and *PtMYB8* overexpressors were quite similar when normalized relative to *EF1-alpha*, representing a 33-fold and a 40-fold increase in transcript abundance compared with their endogenous counterparts *PgMYB1* and *PgMYB8*, respectively (Fig. 5). *PtMYB1* overexpression led to a small but significant decrease ( $P \leq 0.05$ ) in steady-state RNA of its putative homologue *PgMYB1* (Fig. 5) and of *PgMYB2* and *PgMYB4*, but *PgMYB3* and *PgMYB8* were unaffected. In *PtMYB8*-OE plantlets, transcript levels of *PgMYB1* and *PgMYB3* were significantly up-regulated compared with the control according to Student's *t*-test at  $P \leq 0.05$  and  $P \leq 0.01$ , respectively (Fig. 5), whereas transcript abundance was reduced for *PgMYB4* ( $P \leq 0.01$ ), as well as for *PgMYB8* ( $P \leq 0.05$ ).

#### Discussion

Previous studies have inferred that lignin biosynthesis and deposition in wood are regulated by R2R3-MYBs (see



**Table 1.** Selected genes differentially expressed in *PtMYB1*- and *PtMYB8*-OE transgenic spruce

Microarray experiments were designed with four control (WT) lines, two independent lines per transgenic construct (L4 and L14 for *PtMYB1*; L1 and L2 for *PtMYB8*), and four biological replicates.

Putative gene function <sup>a</sup>	PT <sup>b</sup>	EST ID	<i>MYB1</i> -L4		<i>MYB1</i> -L14		<i>MYB8</i> -L1		<i>MYB8</i> -L2	
			Fold <sup>c</sup>	P <sup>d</sup>	Fold	P	Fold	P	Fold	P
Phenylpropanoid, monolignol related										
<b>Phenylalanine ammonia-lyase (PAL)<sup>e</sup></b>	X,X	MN5159896	<b>+1.9</b>	8E-05	<b>+1.9</b>	1E-04	<b>+2.2</b>	1E-04	<b>+2.0</b>	4E-04
<b>4-Coumarate-CoA ligase (4CL)</b>	X,X	MN5255003	<b>+2.3</b>	9E-06	<b>+2.0</b>	5E-05	<b>+3.1</b>	9E-02	<b>+2.6</b>	8E-02
<b>Hydroxycinnamoyl transferase (HCT)</b>	X,X	MN5171597	<b>+2.0</b>	9E-06	<b>+1.8</b>	6E-05	<b>+2.0</b>	1E-04	<b>+2.1</b>	1E-04
<b>Caffeic acid 3-O-methyltransferase (COMT)</b>	X,X	MN5194345	<b>+2.3</b>	4E-05	<b>+2.1</b>	2E-04	<b>+2.3</b>	2E-04	<b>+3.4</b>	2E-05
<b>Pinoresinol-lariciresinol reductase (PLR1)</b>	X,X	MN5177685	<b>+3.2</b>	6E-07	<b>+2.7</b>	4E-06	<b>+2.2</b>	4E-05	<b>+2.3</b>	2E-05
<b>Pinoresinol-lariciresinol reductase (PLR2)</b>	X,X	MN5194637	<b>+2.4</b>	4E-03	<b>+2.6</b>	4E-03	<b>+3.6</b>	1E-03	<b>+2.8</b>	1E-03
<i>trans</i> -Cinnamate 4-hydroxylase (C4H)	-,X	MN5158256	+2.1	3E-06	+2.0	1E-05	ns	ns	ns	ns
<i>N</i> -Hydroxycinnamoyl,benzoyltransferase (HCBT)	-,P	MN5238027	ns	ns	ns	ns	-2.4	3E-06	-2.1	1E-05
Caffeoyl-CoA 3-OMT-like, putative	N,-	MN5164253	ns	ns	ns	ns	-6.3	6E-07	-6.8	4E-07
Cinnamoyl CoA reductase-like, putative	N,P	MN5235414	ns	ns	ns	ns	-2.4	5E-06	-2.3	1E-07
Cell wall related <sup>f</sup>										
<b>Xyloglucan endo-transglycosylase (XTH)</b>	-,	MN5255573	<b>-3.6</b>	1E-05	<b>-2.9</b>	1E-04	<b>-8.2</b>	5E-07	<b>-8.3</b>	4E-07
Xyloglucanase inhibitor	-,X	MN5163020	ns	ns	ns	ns	-2.5	2E-05	-1.8	7E-04
Cellulose synthase catalytic subunit (CesA)	X,X	MN5175268	ns	ns	ns	ns	+2.2	2E-05	+1.9	2E-04
Cellulose synthase (CesA)	X,X	MN5195335	ns	ns	ns	ns	+3.6	4E-06	+3.5	6E-06
Cellulose synthase (CesA)	N,P	MN5234677	ns	ns	ns	ns	-2.1	5E-05	-2.4	2E-05
Cellulose synthase-like (CSL)	N,-	MN5236143	ns	ns	ns	ns	-3.3	1E-06	-3.1	2E-06
Glycosyl transferase 2 (CSL)	X,X	MN5194187	ns	ns	ns	ns	+2.2	1E-05	+2.2	8E-06
Glycosyl transferase 2 (CSL)	X,X	MN5159543	ns	ns	ns	ns	+2.1	8E-05	+2.3	4E-05
Epimerase (NSI)	X,X	MN5177923	ns	ns	ns	ns	+2.3	5E-06	+2.6	2E-06
Expansin (EXP)	X,P	MN5194355	+2.1	9E-06	+2.1	2E-05	ns	ns	ns	ns
Glycohydrolase (GH17)	-,	MN5251191	ns	ns	ns	ns	-3.9	4E-06	-2.6	7E-05
Glycohydrolase (GH17)	-,X	MN5256029	ns	ns	ns	ns	-2.9	4E-06	-2.2	6E-05
Glycosyl transferase 8 (GT8)	X,X	MN5170784	ns	ns	ns	ns	+3.8	8E-07	+3.0	3E-06
Hydroxyproline-rich glycoprotein-like (HRGP)	X,P	MN5173049	ns	ns	ns	ns	-1.9	2E-04	-1.8	6E-04
Arabinogalactan-like protein (AGP)	X,X	MN5191579	ns	ns	ns	ns	+3.8	8E-07	+4.1	4E-07
Pectin acetyltransferase (PAE)	-,P	MN5240041	ns	ns	ns	ns	-2.1	3E-05	-1.9	1E-04
Glycoside hydrolase (AGAL)	-,	MN5236822	ns	ns	ns	ns	-2.3	1E-06	-2.0	5E-06
Polygalacturonase (GH28)	-,X	MN5233983	ns	ns	ns	ns	-2.9	7E-06	-1.9	5E-04
UDP-glycosyltransferase 85A8 (UDPGT)	-,X	MN5181672	ns	ns	ns	ns	-2.4	6E-05	-2.4	9E-05
Glucosyltransferase-like protein (UDPGT)	N,-	MN5232972	ns	ns	ns	ns	-2.1	1E-06	-1.8	8E-04
Chitinase	-,	MN5181335	ns	ns	ns	ns	-3.2	3E-05	-2.2	6E-04
Chitinase	-,	MN5182703	ns	ns	ns	ns	-2.6	4E-06	-2.5	7E-06
Peroxidases										
<b>Peroxidase (class III)</b>	-,	MN5232900	<b>-1.8</b>	2E-04	<b>-1.8</b>	3E-04	<b>-4.0</b>	4E-05	<b>-3.6</b>	9E-05
Peroxidase	-,P	MN5173202	ns	ns	ns	ns	-2.9	5E-05	-2.3	4E-04
Peroxidase precursor	-,	MN5181972	ns	ns	ns	ns	-2.2	6E-05	-2.0	2E-04
Peroxidase ATP2a	-,	MN5233396	ns	ns	ns	ns	-2.3	3E-06	-1.8	4E-05
Cationic peroxidase 1 precursor (class III)	N,P	MN5235950	ns	ns	ns	ns	-3.2	9E-06	-2.5	8E-05
Shikimate related										
<b>3-Deoxy-DAHP synthase</b>	X,X	MN5251829	<b>+2.9</b>	2E-05	<b>+2.6</b>	7E-05	<b>+3.6</b>	4E-06	<b>+4.1</b>	2E-06
Chorismate mutase	-,X	MN5236775	ns	ns	ns	ns	+2.1	2E-05	+1.8	2E-04
Prephenate dehydratase	N,X	MN5171113	ns	ns	ns	ns	-2.2	1E-05	-1.8	1E-04
Prephenate dehydratase	N,-	MN5240131	ns	ns	ns	ns	-2.3	8E-06	-1.8	2E-04
SAM, SAH metabolism										
<b>Adenosylhomocysteinase</b>	X,X	MN5251787	<b>+1.9</b>	1E-05	<b>+1.9</b>	2E-05	<b>+1.8</b>	3E-04	<b>+1.8</b>	4E-04
Nicotianamine synthase	N,-	MN5182064	ns	ns	ns	ns	+2.3	2E-04	+2.7	8E-05
CBS-domain-containing protein	X,X	MN5174213	ns	ns	ns	ns	+2.4	5E-05	+2.0	3E-04
Homocysteine methyltransferase	X,X	MN5252997	+1.9	4E-06	+1.9	1E-05	ns	ns	ns	ns
Flavonoid related										
Flavonol synthase (FS)	-,	MN5172805	ns	ns	ns	ns	-2.1	1E-04	-2.1	1E-04
Chalcone-flavonone isomerase (CHI)	N,P	MN5163731	ns	ns	ns	ns	-2.1	5E-06	-2.3	2E-06
Flavanone 3-hydroxylase	N,-	MN5192796	ns	ns	ns	ns	-2.0	5E-06	-1.8	2E-05
Dihydroflavonol 4-reductase (DFR)	N,-	MN5235944	ns	ns	ns	ns	-3.0	5E-07	-2.8	7E-07
Chalcone synthase (CHS)	N,-	MN5252787	ns	ns	ns	ns	-3.3	2E-06	-2.4	2E-05
Anthocyanidin synthase	N,-	MN5255315	ns	ns	ns	ns	-3.7	1E-06	-2.8	2E-06
Flavonoid 3',5'-hydroxylase	N,-	MN5255946	ns	ns	ns	ns	-2.7	2E-04	-3.5	3E-05
Chalcone O-methyltransferase (ChOMT)	N,-	MN5255308	ns	ns	ns	ns	-3.3	1E-05	-3.7	8E-06

Table 1. Continued

Putative gene function <sup>a</sup>	PT <sup>b</sup>	EST ID	MYB1-L4		MYB1-L14		MYB8-L1		MYB8-L2	
			Fold <sup>c</sup>	P <sup>d</sup>	Fold	P	Fold	P	Fold	P
Terpenoid related										
<b>Beta-primeverosidase</b>	–,–	MN5233446	<b>–3.4</b>	2E-06	<b>–2.1</b>	1E-04	<b>–8.3</b>	1E-06	<b>–9.2</b>	9E-07
Beta-primeverosidase	–,–	MN5251221	ns		ns		–2.9	8E-07	–3.1	4E-07
Beta-primeverosidase	–,–	MN5182631	ns		ns		–6.0	1E-06	–6.0	1E-06
Geraniol 10-hydroxylase	–,–	MN5233989	ns		ns		–4.3	3E-05	–4.5	3E-05
Acetyl Co-A acetyltransferase	–,–	MN5260082	ns		ns		–1.8	8E-06	–2.0	4E-06
Hydroxymethylglutaryl-CoA synthase	–,–	MN5236845	ns		ns		–2.2	2E-06	–2.2	2E-06
Alpha-bisabolene synthase	–,–	MN5251845	–2.6	9E-04	–2.1	8E-03	ns		ns	
Cytochrome P450 (CYP720B1)	N,P	MN5181371	ns		ns		–2.1	1E-06	–2.1	1E-06
CPRD12 protein	N,P	MN5255892	ns		ns		–2.2	2E-06	–2.3	1E-06
Serine carboxypeptidase II	X,X	MN5246267	ns		ns		–2.1	1E-05	–2.0	3E-05
Gamma-tocopherol methyltransferase	–,P	MN5254253	ns		ns		–7.3	1E-06	–7.1	1E-06
Stress and detoxification										
<b>Glyoxalase</b>	–,–	MN5257610	<b>–2.1</b>	1E-03	<b>–2.4</b>	6E-04	<b>–4.3</b>	2E-06	<b>–4.9</b>	1E-06
<b>r40g3 protein</b>	N,P	MN5256759	<b>–2.2</b>	1E-04	<b>–2.3</b>	2E-04	<b>–10.1</b>	3E-06	<b>–9.8</b>	3E-06
<b>Lipase, thioesterase</b>	X,X	MN5176766	<b>+3.0</b>	2E-06	<b>+2.4</b>	1E-05	<b>+2.3</b>	8E-05	<b>+2.4</b>	9E-05
<b>LEA EMB7</b>	–,–	MN5251934	<b>–2.6</b>	1E-03	<b>–2.6</b>	2E-03	<b>–5.9</b>	3E-06	<b>–7.6</b>	1E-06
LEA EMB35	–,–	MN5239233	–2.0	6E-04	–2.2	3E-04	ns		ns	
LEA-EMB7	–,–	MN5181285	ns		ns		–3.2	7E-05	–2.7	2E-04
Glutathione S-transferase	–,–	MN5238832	ns		ns		–2.3	8E-05	–2.3	7E-05
Glutathione S-transferase	–,–	MN5255937	ns		ns		–2.3	2E-05	–2.5	9E-06
Glutathione S-transferase	N,–	MN5256468	ns		ns		–1.9	1E-04	–2.1	5E-05
Beta-cyanoalanine synthase	N,X	MN5258469	ns		ns		+3.2	7E-06	+3.3	7E-06
Vignain precursor	X,–	MN5160240	+2.1	1E-03	+1.8	1E-02	ns		ns	
Vignain precursor	X,–	MN5170536	+2.3	5E-04	+1.9	5E-03	ns		ns	
Phytochelatin synthetase	X,–	MN5207547	ns		ns		+2.8	4E-05	+2.3	2E-04
Phytochelatin synthetase-like	X,–	MN5259467	ns		ns		+2.7	5E-05	+2.1	5E-04
Dehydrin	–,–	MN5161489	ns		ns		–1.8	4E-06	–1.8	3E-06
Dehydrin	–,–	MN5163790	ns		ns		–4.1	7E-06	–3.8	1E-05
BURP domain-containing protein	–,P	MN5163000	ns		ns		–4.8	5E-05	–4.3	1E-04
Arm repeat-containing protein	–,–	MN5195907	ns		ns		–2.0	2E-06	–1.9	4E-06
Lanatoside 15'-O-acetyltransferase	–,P	MN5256118	ns		ns		–2.8	5E-05	–2.1	6E-04
EF-hand Ca <sup>2+</sup> -binding protein	N,–	MN5244381	ns		ns		–2.4	1E-04	–2.4	1E-04
Delta-1-pyrroline-5-carboxylate dehydrogenase	X,X	MN5259351	ns		ns		+2.0	3E-06	+1.8	8E-06
Disease resistance gene	–,X	MN5192231	ns		ns		+2.0	2E-05	+2.0	1E-05
Rapid alkalization factor 3	–,P	MN5243656	ns		ns		–2.3	3E-05	–2.0	2E-04
Fatty acid hydroxylase	–,–	MN5196748	ns		ns		–1.8	2E-05	–1.8	3E-05
Allene oxide synthase precursor	N,–	MN5241220	ns		ns		–2.9	2E-05	–2.7	3E-05
Acyl-CoA synthase (4CL-like)	–,–	MN5255491	ns		ns		–2.3	3E-06	–2.0	1E-05
Metallothionein-like protein	N,–	MN5182650	ns		ns		–3.9	8E-06	–3.4	2E-05
Metallothionein-like protein	–,P	MN5241564	ns		ns		–2.9	7E-06	–2.3	5E-05
Lipid transfer protein	X,X	MN5239177	ns		ns		–2.6	7E-06	–5.7	3E-07
Thioredoxin H-type	–,–	MN5254070	ns		ns		–2.1	9E-05	–1.8	1E-04
Miscellaneous										
<b>Glycine decarboxylase</b>	X,X	MN5241924	<b>+2.0</b>	9E-06	<b>+1.8</b>	1E-04	<b>+1.8</b>	2E-04	<b>+2.2</b>	2E-05
<b>Uclacyanin</b>	N,X	MN5177006	<b>+3.6</b>	2E-06	<b>+3.2</b>	6E-06	<b>+4.6</b>	1E-05	<b>+5.2</b>	8E-06
<b>Carbonic anhydrase</b>	–,–	MN5182742	<b>+3.3</b>	1E-07	<b>+2.6</b>	2E-06	<b>–3.4</b>	5E-07	<b>–3.5</b>	3E-07
<b>Unknown</b>	N,P	MN5161611	<b>–1.9</b>	1E-03	<b>–1.8</b>	4E-03	<b>–2.6</b>	2E-06	<b>–2.4</b>	3E-06
<b>Unknown</b>	–,P	MN5250897	<b>–4.3</b>	8E-06	<b>–3.1</b>	1E-04	<b>–6.0</b>	1E-06	<b>–9.0</b>	4E-07
<b>Transgene</b>	–	MN5173128	<b>+3.1</b>	2E-04	<b>+2.7</b>	7E-04	<b>+5.7</b>	3E-06	<b>+4.7</b>	7E-06

<sup>a</sup> Functional annotations were obtained based on blastx analysis against Uniref100 database, on search against PGI5.0 database, and HMM search against PFAM protein family database.

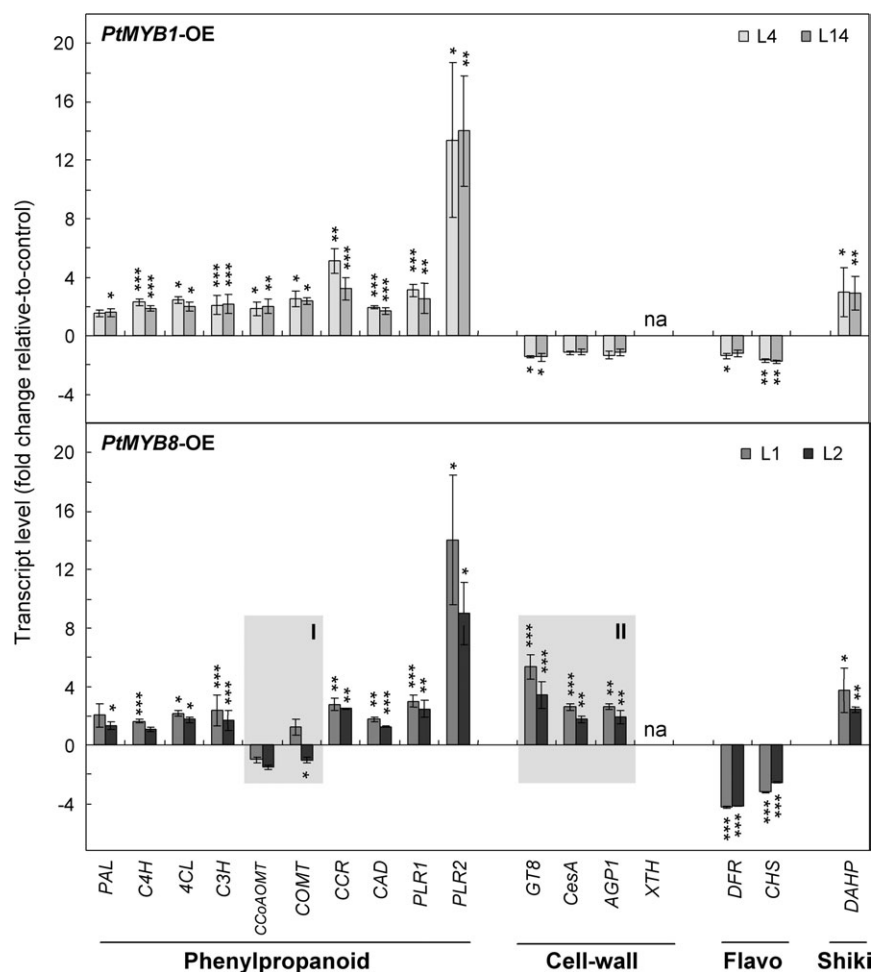
<sup>b</sup> PT, Preferential tissue. Independent microarray experiments performed on a tissue panel of spruce tree (N Pavy *et al.*, unpublished results) showed that some ESTs were preferentially expressed in either differentiating secondary xylem (X), phloem-bark (P), or needles (N), or did not show any preference to particular tissue (–). The first and second letters represent results from two tissue profiling experiments: (1) differentiating secondary xylem versus needle; (2) differentiating secondary xylem versus phloem-bark, respectively.

<sup>c</sup> Fold change relative to the wild-type plantlets. ns, Non-significant.

<sup>d</sup> Student's *t*-test level of confidence.

<sup>e</sup> Genes that are either up-regulated in both *PtMYB1*- and *PtMYB8*-OE, or down-regulated in both *PtMYB1*- and *PtMYB8*-OE, or up-regulated in *PtMYB1*-OE and down-regulated in *PtMYB8*-OE, are shown in bold.

<sup>f</sup> According to Cell Wall Navigator annotations (<http://bioinfo.ucr.edu/projects/Cellwall/index.pl>).



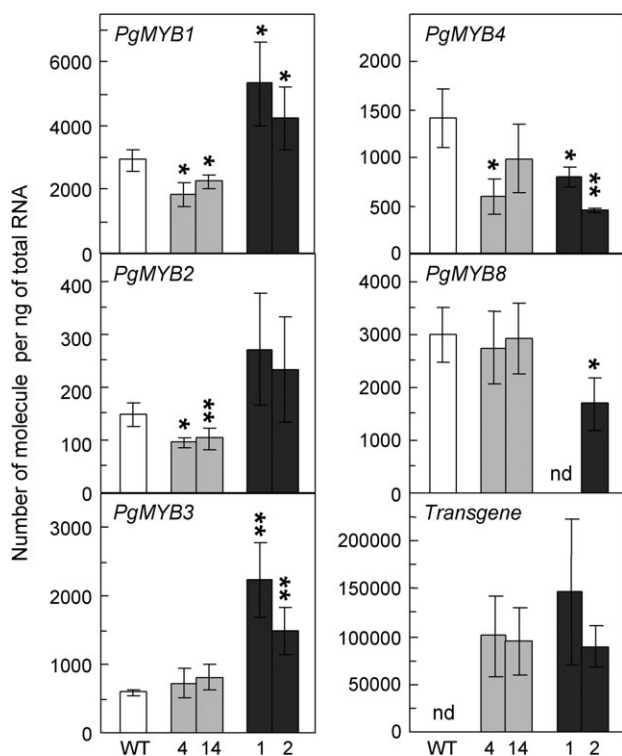
**Fig. 4.** Targeted RT-qPCR analysis in *PtMYB1* and *PtMYB8* transgenic plantlets: validation of microarray and expression data from genes related to secondary metabolism and cell wall assembly. Phenylpropanoid-related genes are *phenylalanine ammonia-lyase* (*PAL*), *cinnamic acid 4-hydroxylase* (*C4H*), *4-coumarate-CoA ligase* (*4CL*), *coumarate 3-hydroxylase* (*C3H*), *caffeate O-methyltransferase* (*COMT*), *caffeoyl-CoA 3-O-methyltransferase* (*CCoAOMT*), *cinnamoyl-CoA reductase* (*CCR*), *cinnamyl-alcohol dehydrogenase* (*CAD*), and *pinoresinol-lariciresinol reductase 1 and 2* (*PLR1* and *PLR2*); cell wall-related genes are *cellulose synthase* (*CesA*), *glycosyltransferase family 8* (*GT8*), *arabinogalactan* (*AGP1*), and *xyloglucan endotransglycosylase/hydrolase* (*XTH*). Flavonoid related genes (Flavo) are *chalcone synthase* (*CHS*), *dihydroflavonol 4-reductase* (*DFR*); the shikimate-related gene (Shiki) is *3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase* (*DAHP*). For both transgenics, transcript accumulation was assessed by RT-qPCR on 3-week-old plantlets produced from two independent transgenic lines for each gene construct (L4 and L14 for *PtMYB1*; L1 and L2 for *PtMYB8*). Transcript levels were determined from four biological replicates (25 plantlets per replicate) and were normalized relative to *EF1-alpha* expression level. Transcript levels were then expressed relative to control plants, and the significance of differential transcript accumulation was evaluated with Student's *t*-test (two-sample, unpaired, one-sided) at  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*), and  $P \leq 0.001$  (\*\*\*). na, Not amplified in *MYB* transgenics.

reviews: Rogers and Campbell, 2004; Groover and Robischon, 2006; Demura and Fukuda, 2007). In gymnosperm trees, however, only *Pinus taeda* *PtMYB4* has been shown to induce ectopic lignification when heterologously expressed in tobacco and *Arabidopsis* (Patzlaff *et al.*, 2003b; Newman *et al.*, 2004). Based on transcriptional activation assays in yeast, *PtMYB1* was also proposed to act as a transcriptional regulator of lignification (Patzlaff *et al.*, 2003a) but this hypothesis remained to be tested *in planta*. The present gain-of-function experiments using a conifer expression system provide experimental evidence that is highly consistent with the involvement of *PtMYB1* as well as *PtMYB8* in lignification. Furthermore, *PtMYB8*-OE additionally led to the misregulation of cell

wall-related transcripts concomitantly with the ectopic deposition of secondary cell wall in different cell types. The putative effects of these *MYBs* on phenylpropanoid metabolism and related pathways, as well as secondary cell wall biogenesis, in conifer trees are interpreted in more detail below.

#### *PtMYB1 and PtMYB8 impact upon phenylpropanoid and shikimate pathways*

The transcript profiles of phenylpropanoid-related genes clearly overlapped in *PtMYB1*- and *PtMYB8*-OE spruce. The overexpression of each *MYB* led to a clear up-regulation of most of the genes evaluated in the phenylpropanoid pathway (from *C4H* to *CAD*), except that



**Fig. 5.** Transcript accumulation of endogenous spruce *MYBs* and pine transgenes in wild-type and *PtMYB1* and *PtMYB8* transgenic plantlets. For both transgenics, transcript accumulation was assessed by RT-qPCR on 3-week-old plantlets produced from two independent transgenic lines (L4 and L14 for *PtMYB1*, grey columns; L1 and L2 for *PtMYB8*, black columns). Transcript levels were normalized relative to *EF1a* expression level and were calculated using standard curves as described by Rutledge and Côté (2003). Bars represent means ( $\pm$ SD) from three biological replicates (15 plantlets per replicate). nd, Not detected. The significance of differential transcript accumulation (up or down) between control and transgenic plantlets was evaluated with Student's *t*-test (two-sample, unpaired, one-sided) at  $P \leq 0.05$  (\*), or  $P \leq 0.01$  (\*\*).

*PtMYB8*-OE had no effect on the transcript levels of *COMT* and *CCoAOMT* (Table 1; Fig. 4, shaded box I). It appears that different *MYB* genes may act differentially on genes within the lignin biosynthetic pathway in spruce, as it was shown in other plant systems (Patzlaff *et al.*, 2003b; Goicoechea *et al.*, 2005). Other authors have also proposed that more than one *MYB* may act on the same target gene to regulate its expression (Moyano *et al.*, 1996; Tamagnone *et al.*, 1998; Borevitz *et al.*, 2000) and thus provide multiple levels of control for lignin biosynthesis in plants (Tamagnone *et al.*, 1998; Borevitz *et al.*, 2000). The present results provide evidence that several lignin biosynthetic genes can be recruited in spruce as a result of *PtMYB1* or *PtMYB8* overexpression, congruent with the increased lignin deposition (Fig. 3A) and the decrease of low molecular weight phenolic compounds (Fig. 3B) observed in both transgenics.

Despite these observations, restricting the impact of *PtMYB1* and *PtMYB8* to the sole phenylpropanoid biosynthetic pathway may lead to a biased interpretation or to a restricted view of the putative metabolic pathways

affected by *R2R3-MYB* overexpression in spruce. Microarray profiling was useful to assess the variations of transcripts of upstream, and/or downstream biosynthetic pathways linked to phenylpropanoid metabolism, and also provided a potential overview of metabolic routing in *PtMYB1* and *PtMYB8* overexpressors (Table 1). The clear up-regulation of *DAHPh* observed in both *PtMYB1*-OE and *PtMYB8*-OE (Table 1; Fig. 4) may indicate a routing of metabolic flow into the upstream shikimate pathway, which provides precursors required for lignin biosynthesis (Herrmann, 1995; Amthor, 2003). This situation is similar to that observed in petunia where the *R2R3-MYB* gene *ODO1* was shown to be a key regulator of benzenoid compounds responsible for floral scent through the activation of genes in the upstream shikimate pathway (Verdonk *et al.*, 2005). It would thus appear that *PtMYB1* and *PtMYB8* are potential regulators of phenylpropanoid metabolism in conifers, probably by directing metabolic flux into shikimate and monolignol pathways. However, the different transcriptional responses observed for *COMT* and *CCoAOMT* (Fig. 4, shaded box I) may indicate that the two *MYBs* have different impacts in terms of methoxylation in the monolignol biosynthetic pathway. Different responses were also observed for transcripts linked to SAM/SAH metabolism (Table 1), which forms the methyl group donor for lignin monomers (Amthor, 2003).

Comparative analysis of *MYB* overexpressors also suggested an effect on lignan biosynthesis as indicated by a strong up-regulation of two putative pinoresinol/lariciresinol reductase (*PLR*) coding sequences (Fig. 4). Lignans constitute an abundant class of phenylpropanoid compounds which have been supposed to act in defence against pathogens (Gang *et al.*, 1999; Schmitt and Petersen, 2002) but also as important components of heartwood formation (Swan *et al.*, 1969). The general routing for lignan biosynthesis has been strongly suggested to be the result of the conversion from coniferyl alcohol to secoisolariciresinol, and then to matairesinol (Susuki and Umezawa, 2007). This routing starts with stereoselective coupling of coniferyl alcohol monomers to form pinoresinol, the first substrate of the *PLR* enzyme (Davin *et al.*, 1997). A possible explanation for the accumulation of *PLR* transcripts may involve a feed-forward response to the up-regulated monolignol pathway in *MYB*-OE spruces. If such was the case, it would be an indirect effect of *MYB* overexpression. Another hypothesis would be that *PLR* genes are in fact direct targets for *PtMYB1* and/or *PtMYB8*. The >10-fold up-regulation of *PLR2* in both types of *MYB* overexpressors is not inconsistent with this possibility (Fig. 4). As far as is known, no *MYB* has been associated with transcriptional control of genes coding for lignan synthesis enzyme. A gel shift experiment with *PtMYB1*/*PgMYB1* and *PtMYB8*/*PgMYB8* recombinant proteins and *PLR1* and *PLR2* promoter regions should be further considered to test this hypothesis.

### *PtMYB8* as a putative actor in secondary cell wall biosynthesis

The involvement of *R2R3-MYB* TFs in the regulation of secondary cell wall biosynthesis has only been recently reported for *AtMYB26* (Yang *et al.*, 2007) and *AtMYB46* (Zhong *et al.*, 2007) in the model plant *Arabidopsis thaliana*. Overexpression of the *Eucalyptus EgMYB2* in tobacco has been associated with secondary cell wall thickening but not to the regulation of genes associated with secondary cell wall biogenesis, other than those involved in monolignol biosynthesis (Goicoechea *et al.*, 2005). The data presented here show that *PtMYB8* clearly impacted secondary cell wall biogenesis as reflected by the distribution of ectopic sclerenchyma-like elements, and by the deposition of secondary cell walls in parenchyma, and cortical cells (Fig. 2). Congruent with these observations, overexpression of *PtMYB8* led to the misregulation of several genes associated with biosynthetic pathways of cellulose and hemicellulose (Table 1; Fig. 4, shaded box II). In this context, *PtMYB8/PgMYB8* may be considered as putative regulators of secondary cell wall biosynthesis, in agreement with their expression being restricted to secondary xylem in pine and spruce (Bedon *et al.*, 2007; Fig. 1). Consistent with such a role, *PgMYB8*, the closest homologue of *PtMYB8* in spruce, displayed increased transcript accumulation upon the induction of compression wood (Bedon *et al.*, 2007). Here, *PtMYB8*-OE led to the up-regulation of *AGPI* arabinogalactan transcripts (Table 1; Fig. 4) whose up-regulation has been associated with compression wood in spruce (Bedon *et al.*, 2007) and pine (Zhang *et al.*, 2000) and tension wood in poplar (Lafarguette *et al.*, 2004). In conifers, compression wood is produced in bent or leaning trees, and is characterized by an enhanced deposition of secondary cell wall compounds (Timell, 1986), by increased lignin content, and an enrichment in H lignin units in spruce (Lange *et al.*, 1995) and pine (Yeh *et al.*, 2006).

Constitutive overexpression of *PtMYB8* in spruce was also associated with strong decreases in transcript levels of flavonoid, terpenoid, and benzenoid biosynthesis enzymes (Table 1; Fig. 4). Most of these down-regulated sequences were identified as needle or phloem-bark preferential (Table 1), a tissue where *PtMYB8* transcripts normally accumulated at very low level (Fig. 1). Due to the highly conserved DNA binding domain observed among conifer *MYBs* (Bedon *et al.*, 2007), ectopic overexpressing *PtMYB8* in such tissues may have led to the indirect effects linked to non-specific binding to gene promoters caused by *MYB* overexpression. Such a dosage-linked effect would influence most strongly the expression of genes with similar *cis* regulatory motifs, as suggested in *Arabidopsis* (Jin *et al.*, 2000). *MYB* overexpression may also repress promoter activity by competing with cognate

TFs (sequestration) and thus sequestering components of the transcriptional machinery away from *cis* regulatory DNA elements (Gill and Ptashne, 1988).

A parallel may be drawn between the present study and the transcriptional network regulating secondary cell wall biosynthesis recently proposed by Zhong *et al.* (2006, 2007). In this model, the *A. thaliana* NAC domain protein *SND1* activates a transcriptional cascade involving *R2R3-MYB* and *KNOX* TFs, which themselves are strong candidates for the regulation of genes encoding enzymes required for secondary cell wall deposition. These authors further suggested that the control of secondary cell wall biosynthesis by *SND1*-like genes may be a common mechanism in plants (Zhong *et al.*, 2006). The results presented here could indeed indicate that secondary cell wall deposition in conifer trees is controlled by a transcriptional network similar to the *SND1* cascade, in which *PtMYB8/PgMYB8* and *PtMYB1/PgMYB1* may represent putative members. Most of the spruce *MYB* sequences which are misregulated following *PtMYB8* overexpression (Fig. 5) are close homologues of the *MYB* partners described in the *SND1* cascade in *Arabidopsis* (Zhong *et al.*, 2007). These spruce sequences were shown to be highly similar to the *Arabidopsis R2R3-MYB* genes *AtMYB20* (At1g66230) and *AtMYB85* (At4g22680) for *Pg/PtMYB1*, *AtMYB103* (At1g63910) for *Pg/PtMYB2*, and *AtMYB46* (At5g12870) for *Pg/PtMYB4* and *Pg/PtMYB8* (Bedon *et al.*, 2007). These later sequences (*AtMYB46*, *Pg/PtMYB4* and -8) are also highly similar to *EgMYB2* from *Eucalyptus*, shown to control secondary cell wall thickening (Goicoechea *et al.*, 2005). All these spruce *MYBs* are also the most clearly preferential to differentiating secondary xylem tissue (Bedon *et al.*, 2007) supporting their putative involvement in secondary cell wall deposition. In addition, both of the xylem preferential *CesA* coding sequences from spruce (MN5175268 and MN5193335), which were up-regulated in *PtMYB8*-OE (Fig. 4; Table 1), were also most similar to *AtCesA7* and *AtCesA8* associated with *SND1* transcriptional cascade in *Arabidopsis* (Zhong *et al.*, 2007).

In conclusion, *Pg/PtMYB1* and *Pg/PtMYB8* appear to be potentially important players in conifers in the regulation of secondary cell wall biosynthesis, including lignin deposition. Their potential roles were examined in comparison with the transcriptional cascade controlling the formation of xylem secondary cell wall in the model plant *Arabidopsis thaliana*. Together these two systems may provide a comparative framework to assess further hierarchy between TFs but also—and perhaps most interestingly—gain insights into the evolution of regulatory processes of wood formation and related biosynthetic pathways. If *Pg/PtMYB1* and/or *Pg/PtMYB8* are part of a transcriptional network that is conserved between gymnosperm and angiosperm species, it would be of great

interest to identify upstream actors such as NAC-type master genes similar to *SND1* (Zhong *et al.*, 2007), *VND*, and *NTS* (Demura and Fukuda, 2007; Mitsuda *et al.*, 2007), which could be pivotal TFs in secondary cell wall biogenesis in trees.

### Supplementary data

The following supplementary material is available at *JXB* online.

**Table S1.** Primer sequences used for RT-qPCR.

**Table S2.** Complete list of expression data relative to *PtMYB1* and *PtMYB8* overexpression in spruce.

**Fig. S1.** RT-qPCR analysis of transcript accumulation in wild type, *PtMYB1* and *PtMYB8* transgenic plantlets: validation of microarray and expression data from genes related to secondary metabolism and cell wall assembly.

**Appendix S1.** Procedure used for microarray manufacturing.

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