SUBGROUP 4 R2R3-MYBS IN CONIFER TREES: GENE FAMILY EXPANSION AND CONTRIBUTION TO THE ISOPRENOID- AND FLAVONOID-ORIENTED RESPONSES

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Abstract

Transcription factors play a fundamental role in plants by orchestrating temporal and spatial gene expression in response to environmental stimuli. Several R2R3-MYB genes of the Arabidopsis subgroup 4 (Sg4) share a C-terminal EAR motif signature recently linked to stress response in angiosperm plants. It is reported here that nearly all Sg4 MYB genes in the conifer trees Picea glauca (white spruce) and Pinus taeda (loblolly pine) form a monophyletic clade (Sg4C) that expanded following the split of gymnosperm and angiosperm lineages. Deeper sequencing in P. glauca identified 10 distinct Sg4C sequences, indicating over-representation of Sg4 sequences compared with angiosperms such as Arabidopsis, Oryza, Vitis, and Populus. The Sg4C MYBs share the EAR motif core. Many of them had stress-responsive transcript profiles after wounding, jasmonic acid (JA) treatment, or exposure to cold in P. glauca and P. taeda, with MYB14 transcripts accumulating most strongly and rapidly. Functional characterization was initiated by expressing the PtMYB14 gene in transgenic P. glauca plantlets with a tissue-preferential promoter (cinnamyl alcohol dehydrogenase) and a ubiquitous gene promoter (ubiquitin). Histological, metabolite, and transcript (microarray and targeted quantitative real-time PCR) analyses of PtMYB14 transgenics, coupled with mechanical wounding and JA application experiments on wild-type plantlets, allowed identification of PtMYB14 as a putative regulator of an isoprenoid-oriented response that leads to the accumulation of sesquiterpenes in conifers. Data further suggested that PtMYB14 may contribute to a broad defence response implicating flavonoids. This study also addresses the potential involvement of closely related Sg4C sequences in stress responses and plant evolution.

Key words: Gene family expansion, gymnosperms, isoprenoid metabolism, MYB transcription factors, microarray RNA profiling, Picea glauca, plant evolution, stress response, terpenes, tissue-specific expression.

Introduction

Since their divergence ~300 million years ago (Mya) (Magallón and Sanderson, 2005), gymnosperm and angiosperm plants have diversified their strategies to cope with changing environmental conditions, competing plants, potential pests, and foraging animals (Brooker, 2006; Agrawal, 2007). As a result of their early divergence and adaptations to harsh environments, gymnosperms have acquired unique traits that distinguish them from herbaceous
and woody angiosperms. For example, defence mechanisms have been described in conifer trees such as pines and spruces which can be constitutive or induced in response to biotic and abiotic stresses and lead to the formation of physical barriers (i.e. formation of traumatic resin ducts and calcium oxalate crystals, and cell wall lignification) and/or to the synthesis of phenolic compounds, or volatile and non-volatile terpenoid compounds (for a review, see Keeling and Bohlmann, 2006). Several genes and proteins related to these responses have been identified, but gene regulators that enable plants to cope with environmental challenge remain largely uncharacterized outside of angiosperm plant systems.

Regulation of gene expression plays a fundamental role in plant response to environmental stimuli. Transcription factors (TFs) belonging to the ERF, bZIP, and WRKY families have been linked to a suite of mechanisms leading to defence and stress responses (Singh et al., 2002; Fujita et al., 2006). A few members of the R2R3-MYB family have also been implicated in plant stress responses to cold (Agarwal et al., 2006), UV (Jin et al., 2000), and wounding (Taki et al., 2005), but R2R3-MYB genes have primarily been shown to regulate plant secondary metabolism (Vom Endt et al., 2002). Furthermore, they are well known as positive and negative regulators of biosynthetic enzymes required for the production of phenylpropanoids (Legay et al., 2007), flavonoids (Grotewold, 2005), and benzenoids (Verdonk et al., 2005).

Sequence analyses of available plant genomes have shown that R2R3-MYB genes form one of the largest and most diverse families of TFs in plants. For instance, between 109 and 192 R2R3-MYB genes were identified in Arabidopsis thaliana, Oryza sativa, Populus trichocarpa, and Vitis vinifera genomes (Jia et al., 2004; Yanhui et al., 2006; Matus et al., 2008; Wilkins et al., 2009). Despite large-scale gene discovery in conifer trees (e.g. Kirst et al., 2003; Pavy et al., 2005; Ralph et al., 2007), relatively few R2R3-MYB genes have been described in conifers or gymnosperms. The full-length cDNA sequence for 18 different putative R2R3-MYB genes, previously reported in Pinus taeda (lobbolly pine) and Picea glauca (white spruce), were shown to have diverse transcript profiles (Bedon et al., 2007). Analyses of this relatively limited set of sequences suggested that several R2R3-MYB gene duplications post-date the angiosperm–gymnosperm split. DNA-binding domains (DBDs) were highly conserved between gymnosperm and angiosperm R2R3-MYBs, and many of the C-terminal motifs described in angiosperms (Kranz et al., 1998) could be found in conifers. However, conifer-specific C-terminal amino acid motifs also suggested possible functional divergence between closely related MYBs from the major plant phylla. A more extensive characterization of the conifer R2R3-MYB gene family and the associated putative functional domains may aid in elucidating potential functional conservation and divergences.

Plant R2R3-MYB genes are classified into different subgroups based on DNA binding affinity (Romero et al., 1998) and C-terminal amino acid motifs (Kranz et al., 1998; Matus et al., 2008). Some C-terminal motifs are required for transcriptional activation (Li et al., 2006), repression (Jin et al., 2000), or protein interactions (Zimmermann et al., 2004). For example, the R2R3-MYB genes of subgroup 4 (Sg4) (Kranz et al., 1998) and C2 repressor motif clade (Matus et al., 2008) harbour the core $^{D_{1}}E_{N_{L}}^{N_2}L$ from the ERF-associated amphiphilic repression (EAR) motif proposed to play key roles in defence and stress responses (Ohta et al., 2001; Weigel et al., 2005). For example, the Sg4 gene AtMYB4 was shown to regulate UV stress and wound responses in Arabidopsis (Jin et al., 2000; Taki et al., 2005). It was shown that several conifer sequences including the P. glauca PgMYB5, 10, and 13, and the P. taeda PtMYB14 gene products share the core EAR motif (Bedon et al., 2007). This led to the hypothesis that these conifer MYBs may play a role in defence, although such a role had yet to be supported by functional evidence.

Gain-of-function experiments using constitutive expression constructs in tobacco, Arabidopsis, and spruce have recently shed light on the functions of conifer MYBs. The P. taeda genes PtMYB1 (Patzlaff et al., 2003a; Bomal et al., 2008), PtMYB4 (Patzlaff et al., 2003b), and PtMYB8 (Bomal et al., 2008) have been linked to phenylpropanoid metabolism, and PtMYB1 and PtMYB4 to ammonium assimilation (Gomez-Maldonado et al., 2004). However, non-constitutive expression may be more informative considering unforeseen effects that may arise when investigating closely related sequences. For example, tissue-specific expression has been shown to be an efficient approach to investigate the function of TFs (Zhang, 2003).

The present report describes an experimental approach driven by the discovery and analysis of a relatively large number of closely related Sg4 R2R3-MYB sequences in P. glauca and P. taeda. Transcript accumulation following mechanical wounding, exposure to cold, and jasmonic acid (JA) application was consistent with a role for several of the sequences in responses to environmental stimuli or stresses. The P. taeda PtMYB14 was selected as a candidate gene for overexpression in P. glauca (white spruce) using the tissue-preferential cinnamyl alcohol dehydrogenase promoter (CADpro; Bedon et al., 2009) and the constitutive ubiquitin promoter (UBPpro; Christensen et al., 1992). Comparative analyses of histological, metabolite, and transcriptional phenotypes resulting from these constructs were discussed in relation to the putative involvement of MYB14 in isoprenoid and flavonoid metabolism in conifers.

Materials and methods

DNA cloning

Conifer Sg4 R2R3-MYB sequences were isolated by using 3’-rapid amplification of cDNA ends (RACE; SMART RACE cDNA Amplification Kit, BD Biosciences Clontech, CA, USA) from P. glauca (Pg) (needles, mechanically wounded seedling stems) and P. taeda (Pt) (seedlings, non-wounded stems and bark). Total RNAs were isolated as in Chang et al. (1993). An Sg4-specific and partially degenerate primer 5’-TGGCGYTCTTCC-CAAGGCC-3’ where Y=C, T, or U, and S=C or G) was designed based on the WRSLPKAA amino acid sequence from PtMYB14,
PgMYB5, PgMYB10, and PgMYB13 (Bedon et al., 2007), PtMYB1 and PtMYB4 (Patzlaff et al., 2003a and b), and PmMBF1 (Xue et al., 2003). Touchdown PCR for the nested 3′-RACE reaction used a DNA engine PTC-225 thermal cycler (Biorad, Hercules, CA, USA) with five cycles of two steps at 94 °C for 30 s and 72 °C for 3 min, five cycles of three steps at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min, and 25 cycles of three steps at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. Amplification products were separated on a 1% agarose gel, gel extracted (Gel Extraction Kit, Qiagen, Mississauga, CA, USA), ligated to pCR2.1, and transformed into TOPO™ competent cells (TA cloning Kit, Invitrogen, Carlsbad, CA, USA) prior to sequencing.

Sequence comparisons and phylogenetic tree construction

Phylogenetic tree construction used 45 different R2R3-MYB cDNA sequences from *P. taeda*, *P. glauca*, and several angiosperms (Kranz et al., 1998; Karpinska et al., 2004; Bedon et al., 2007; Matus et al., 2008; Wilkins et al., 2009), and two R1R2R3-MYBs (AtMYB3R4 and PtMYB3R3) as an outgroup. Several alignment and phylogenetic construction methods were tested (see methods and results in Supplementary Table S1 available at JXB online). Clustal W sequence alignments (Thompson et al., 1994) were obtained with the MEGA 4.0.2 software (Tamura et al., 2007), with parameters set as follows: gap opening penalty/gap extension penalty of 25/1 for pairwise alignment and 5/1 for multiple alignment by using the blosum protein weight matrix for amino acid sequences, and 15/6 for both pairwise and multiple alignment with IUB (International Union of Biochemistry) DNA weight matrix for nucleotide sequences (transition weight of 0.5). Two Neighbor–Joining trees were constructed by MEGA 4.0.2 software using (i) amino acid sequences with the Jones–Taylor–Thornton substitution model (Gamma parameter of 1.0 and pairwise gap deletion); and (ii) nucleotide sequences with the p-distance model including transitions and transversion (all codon positions selected and complete deletion). Both bootstrap consensus trees were inferred from 1000 replicates. The MEME analysis software (Bailey and Elkan, 1994) was used to identify C-terminal conserved motifs of 4–6 amino acids in 42 Sg4 sequences with the occurrence parameter for a single motif set at zero or one motif. Amino acid sequence similarities (Fig. 1C) were obtained with Clustal W (blosum matrix, gap open, 25; gap extend, 1) in the Bioedit software (Version 6.0.7). Pairwise sequence similarities between *Pt/PgMYB14* and *PgMYB15* were obtained with the Smith–Waterman algorithm in the Emboss package (Matrix: EBLOSUM62, gap penalty, 3.0; extend penalty, 0.1).

**Plasmid constructs and stable transformation of white spruce**

Constructs were generated for constitutive and tissue-preferential expression of the complete coding sequence of the *P. taeda* PtMYB14 and of the uidA *β-glucuronidase (GUS)* reporter gene. The maize UBpro (constitutive overexpression) (Christensen et al., 1992) and the *P. glauca* CADpro (tissue-preferential overexpression) (Bedon et al., 2009) were cloned into the pMJM expression vector (Bomal et al., 2008). The CADpro is a 1.163 bp genomic DNA fragment of upstream flanking sequence of the *CAD* gene (Bedon et al., 2009). Stable *A. tumefaciens* tumefaciens transformed by the four constructs into *P. glauca* embryos/tissues (genotype Pg653), selection of the transgenic lines (independent transformation event), and production of transgenic spruce plantlets were described (Bomal et al., 2008).

**Histology, and histochemical and fluorometric GUS assays**

Tissue samples (hypocotyls and roots) were fixed and paraffin embedded as described in Bomal et al. (2008). Semi-thin paraffin-free sections were stained in Sharman’s safranin O/orange G/tannic acid as per Sharman (1943). For X-gluc staining, samples were prepared essentially as described in Hawkins et al. (1997). Briefly, samples were pre-treated for 30 min in cold 90% acetone to facilitate substrate penetration and prevent transgene induction. Samples were then rinsed twice in 100 mM potassium phosphate buffer (pH 7.0) and incubated for 4 h in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in the dark at 37 °C under blue light illumination. All observations were performed with an Axioskop microscope (Zeiss, Jena, Germany) fitted with a digital camera. MUG (4-methylumbelliferyl glucuronide) fluorometric enzymatic assays were as described in Côté and Rutledge (2003) and used from four biological replicates of 25 hypocotyls (with cotyledons) per line.

**Terpene extraction and analysis**

Terpene extractions were based on procedures of Martin et al. (2002). All steps were carried out using 2 ml vials with a teflon-coated screw cap (Hewlett-Packard, Palo Alto, CA, USA). Samples (~200 mg) were submerged in 1.5 ml of methanol:HCl (95:5, v/v), pulverized at high power for 20 s in a cell disputer (FastPrep), and incubated for 4 h at 40 °C in a constant temperature heating block. Following extraction, the homogenate was centrifuged for 10 min at 13 000 rpm; the supernatant was dried on a Speedvac and the pellet was resuspended in 200 µl of HPLC grade methanol. Detection and identification of anthocyanins was achieved by liquid chromatography–mass spectrometry (LC-MS) on an HP 1100 LC-MS-Trap XCT plus. The methanolic extracts were separated on an SB C-18 Zorbax Rapid Resolution 4.6×150 mm 3.5 µm column at 40 °C at a flow rate of 1 ml min⁻¹, using a linear gradient from 95% solvent A and 5% solvent B to 75% solvent A and 30% solvent B over 37 min. Solvent A was water with 0.2% formic acid and solvent B was acetonitrile with 0.2% formic acid. Detection was monitored with an HP 1100 photodiode array detector with the reference wavelength set to 520 nm, while mass determination was achieved by electrospray ionization (ESI) in negative ion polarity.

**Determination of starch content, and analysis of anthocyanins**

For starch content determination, soluble metabolites were extracted by overnight incubation at ~20 °C with methanol:chloroform:water (12:5:3, v/v/v). The sample was centrifuged, the supernatant removed, and the remaining pellet washed twice with fresh methanol:chloroform:water (12:5:3). The dried residual pellet was weighed, and then hydrolysed using 4% sulphuric acid at 121 °C for 4 min. The liberation of glucose, representing starch, was quantified directly by anion exchange high-performance liquid chromatography (HPLC; Dionex, Sunnyvale, CA, USA) on a DX-600 equipped with a Carbopac PA1 column and an electrochemical detector (Coleman et al., 2006).

For anthocyanin analysis, ~20 mg of tissues were suspended in 1.5 ml of methanol:HCl (95:5, v/v), pulverized at high power for 20 s in a cell disputer (FastPrep), and incubated for 4 h at 40 °C in a constant temperature heating block. Following extraction, the homogenate was centrifuged for 10 min at 13 000 rpm; the supernatant was dried on a Speedvac and the pellet was resuspended in 200 µl of HPLC grade methanol. Detection and identification of anthocyanins was achieved by liquid chromatography–mass spectrometry (LC-MS) on an HP 1100 LC-MS-Trap XCT plus. The methanolic extracts were separated on an SB C-18 Zorbax Rapid Resolution 4.6×150 mm 3.5 µm column at 40 °C at a flow rate of 1 ml min⁻¹, using a linear gradient from 95% solvent A and 5% solvent B to 75% solvent A and 30% solvent B over 37 min. Solvent A was water with 0.2% formic acid and solvent B was acetonitrile with 0.2% formic acid. Detection was monitored with an HP 1100 photodiode array detector with the reference wavelength set to 520 nm, while mass determination was achieved by electrospray ionization (ESI) in negative ion polarity.

Gas chromatography (GC)–MS analysis of monoterpenes and sesquiterpenes was carried out with a Hewlett-Packard 6890 GC-MS system, using a DB-WAX column (0.25 mm×0.25 µm×30 m,
Fig. 1. Sequence analyses of angiosperm and conifer Sg4 R2R3-MYBs define the conifer specific subclade Sg4C (grey boxes) and identify associated amino acid motifs. (A and B) Rooted Neighbor–Joining trees were obtained with MEGA 4 software (Tamura et al., 2007) and Clustal W alignments of (A) the amino acid sequence (WRSLPKAAG in R2 to the predicted stop codon) or (B) the MYB DBD nucleotides (see Materials and methods). Bootstrap values >50% are shown. Other alignment methods and tree construction algorithms gave consistent results and are detailed in Supplementary Table S1 at JXB online. *Picea glauca (filled lozenges) and Pinus taeda (open lozenges) sequences reported here; known repressors of phenylpropanoid/lignin pathways are in bold. AtMYB3R4 and PttMYB3 are the outgroup, and AtMYB13, 20, and 123 (not of Sg4) are landmarks. Bars indicate the evolutionary distance as a percentage. (C) Amino acid motifs in the Sg4C C-termini ([1]–[6]) (MEME software). Top: MEME motif logos; bit scores indicate the information content for each position. Bottom: Clustal W alignment of predicted amino acid sequences and conserved motifs (shaded): G1 (EIPAFQ) and G2 (DF^F/Q/xx)
Tissue sample preparation, total RNA extraction, and cDNA synthesis were as described in Bomal et al. (2008). The real-time quantitative PCRs (RT-qPCRs) were assembled in a LightCycler® 480 Multiwell plate 384 (Roche, Basel, Switzerland) using a pipetting robot (EpMotion 5075, Eppendorf, Hamburg, Germany). The thermal cycling used a LightCycler® 480 (Roche), a 15 min activation period at 95 °C followed by 40 cycles (95 °C for 10 s, 55 °C for 60 s, and 72 °C for 30 s), and fluorescence readings taken at the end of each cycle. Melting curves were used to verify amplicon purity. Crossing point (Cp) values were determined with the LC480 software, and standard curves were used to transform Cp values into numbers of transcript molecules. Standard curves were based on dilution series covering five orders of magnitude into numbers of transcript molecules. Standard curves were determined with a mean number of 10 0.146 ng µl−1 prepared for each cDNA linearized with EcoRI or BamHI, purified on Qiaquick columns (Qiagen), and verified on a BioAnalyser (model 2100, DNA 1000 LabChip kit, Agilent Technologies). Transcript levels were normalized against the transcript level of ELONGATION FACTOR 1-z (EF-1-z) or CELL DIVISION CYCLE 2 (CDC2).

Most of the DNA sequences used in gene expression experiments came from expressed sequence tag (EST) databases (Supplementary Table S5 at JXB online). In addition, cDNA fragments for nine different enzymes of the mevalonate (MVA) and the meytherythiol phosphate (MEP) pathways were isolated from P. taeda by PCR and used to produce standard curves for RT-qPCR. Primers were designed with sequences of the TIGR pine gene index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gmain.pl?gdb=–pine) with the Primer 3 software (Supplementary Table S4). PCR (50 µl reaction volume) used 0.2 µM of forward and reverse primers, 2 mM MgSO4, 0.2 mM dNTPs at 10 mM each, 1 µl of pine cDNA, and 1 U of Platinum® Taq DNA polymerase High Fidelity (Invitrogen, CARlife). The cycling conditions were 94 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 68 °C for 2 min, and 68 °C for 5 min.

Microarray experiment and data analysis

The microarray experiment was designed in compliance with MIAME guidelines and the data are deposited in ArrayExpress. Tissues were isolated from 4-week-old somatic plantlets from two transgenic embryogenic lines per construct (lines 16 and 18 for UBlPproPMYB14, lines 10 and 14 for CADproPMYB14) and an untransformed control line (wild type). Total RNAs were extracted from four biological replicates of 25 plantlets (hypocotyls and cotyledons) per line. Transcript profiling used a 9K spruce microarray, RNA indirect amplification, microarray hybridization, image analysis, and statistical analyses methods (Bomal et al., 2008).

Eight hybridizations (two replicates per line in each dye) were used for each transgene construct versus wild-type comparison. Differentially expressed transcripts were identified in each comparison based on a F-value <0.01 (from the LIMMA), a log2 ratio ≥0.5 (1.41-fold change), and a false discovery rate (FDR) of 1% (Benjamini and Hochberg, 1995). A common set of genes was identified based on the overlap between the lists from each construct. The statistical analysis was enhanced by using a permutation test to evaluate the number of genes that would pass, under the null hypothesis, the conditions to be part of the overlapping set by chance alone. For this, a set of permuted data files was obtained by assigning the original data set at random to one of the two possible treatments (i.e. wild type or transgenic) without replacement (including the correct assignment). Independent data analyses were performed with the 70 permuted data files for each construct type. All the 4900 possible pairwise combinations between the two comparisons were examined in R to identify potential overlaps occurring by chance alone. A maximum of five genes in the overlap of two sets was obtained with a mean number of 0.153 genes over the 4900 pairwise combinations, while the correct assignment in both comparison gave an overlap of 53 sequences.

Wounding, JA treatment, and exposure to cold in pine and spruce in vitro plantlets

Pinus taeda seeds were sterilized by soaking in ethanol (95%) for 5 min, sodium hypohlorite (2%) with Tween-80 (0.1%) for 10 min, and H2O2 (10%) for 5 min, then ethanol (70%) for 5 min, and rinsed three times for 10 min in sterile distilled water. Seeds were stratified by soaking for 24 h in sterile MilliQ water at room temperature in the dark and then scarified by cutting the radicular tegument, set to germinate on half-strength MS medium supplemented with 0.5% activated charcoal, and incubated at 25 °C under continuous light (40 µmol m−2 s−1). Picea glauca plantlets were obtained from mature somatic embryos (P653) and germinated as described (Bomal et al., 2008).

Treatments were applied to 4-week-old plantlets as follows. Mechanical wounding was achieved by pinching the hypocotyl with forceps and by cutting one-third of the cotyledons and needles with scissors. Wounded tissues were sampled for analysis at 0, 1.5, 6, and 24 h after wounding. JA (Sigma-Aldrich, Buchs, Switzerland) was dissolved in methanol and 100 µM solution was injected as described (Bomal et al., 2008) with the null hypothesis, the conditions to be part of the overlapping set by chance alone. A maximum of five genes in the overlap of two sets was obtained with a mean number of 0.153 genes over the 4900 pairwise combinations, while the correct assignment in both comparison gave an overlap of 53 sequences.

Wounding, JA treatment, and exposure to cold in pine and spruce in vitro plantlets
sprayed onto the aerial portion of unwounded plantlets; controls were sprayed with methanol alone. JA-treated hypocotyls and cotyledons were harvested for analysis at 0 h and 24 h after treatment. Exposure to cold consisted of incubating 4-week-old plantlets for 24 h in a growth cabinet at 4 °C, while the control plants were maintained at 23 °C. Three biological replicates for each of the above treatments were used in a randomized complete block design, with each replicate being comprised of three plantlets (pine seedlings in magenta boxes) or 10 plantlets (spruce somatic seedlings in Petri dishes).

**Results**

PtMYB14 and related sequences define a large conifer subclade within Sg4 of R2R3-MYBs

The isolation of several new cDNA sequences closely related to *PtMYB14* (Bedon et al., 2007) brings the number of *Sg* gene sequences to 10 in *P. glauca* and seven in *P. taeda* (Fig. 1). Six distinct partial cDNAs were obtained for each of these species (see Materials and methods), with predicted coding sequences ranging from 159 to 230 amino acids, from the WRSLPKAA motif in the DBD to the putative stop codon. The *PgMYB17* full-length cDNA sequence of 236 predicted amino acids was identified by EST mining (Pavy et al., 2005) by full-length sequencing. The new sequences are numbered in continuation with a previous report (Bedon et al., 2007).

Phylogenetic trees constructed with sequences from *Picea, Pinus,* and several angiosperms (*Arabidopsis, Oryza, Populus,* and *Vitis*) group the 17 conifer sequences with the *PgMYB* genes (Fig. 1A, B). They show that conifers have many *Sg* sequences compared with most angiosperms. *Picea* has 10 distinct sequences compared with 3–8 in the *Arabidopsis, Oryza, Populus,* and *Vitis* genomes (Matus et al., 2008; Wilkins et al., 2009). Nearly all of the *Sg* conifer sequences formed a monophyletic subclade, referred to as *Sg4C* (Fig. 1A, B). Different alignment methods and tree construction algorithms were compared to validate the robustness of the *Sg4C* subclade (see Supplementary Table S1 at *JXB* online). The DBD sequences alone gave well-conserved tree topologies, with the *Sg4C* subclade supported by high bootstrap values (without including *PtMYB22*) or moderate bootstrap values (with *PtMYB22*) (Fig. 1B; Supplementary Table S1 at *JXB* online). Analyses encompassing the DBD and the C-terminus clearly excluded *PtMYB22* from *Sg4C* and suggested C-terminal conservation among *Sg4C* sequences.

The degree of C-terminal conservation was shown by amino acid motif analysis (MEME software). Despite the highly variable C-terminus in MYBs, six conserved amino acids motifs were identified ([1]–[6]), two of which were uniquely conserved in sequence and location in *Sg4C* ([3] and [4], Fig. 1A, C). The *Sg4* sequences from all taxa harboured the C1 motif of unknown function and the C2 repressor domain containing the core from the EAR motif (motif C2-1, Fig. 1A, C) that is characteristic of *Sg4* (Kranz et al., 1998) also known as the C2 repressor motif clade (Matus et al., 2008). The G1 and G2 motifs located between C1 and C2 appear to be a signature of the *Sg4C* MYBs (Fig. 1A). The G1 motif was unique to *Sg4C* members, while the G2 motif occurs in some *Sg* angiosperms but at a different location.

*Sg4C* transcript levels are modulated in response to stresses

The accumulation of *Sg4C* transcript was monitored with gene-specific RT-qPCR plantlets subjected to mechanical wounding, JA application, and exposure to cold (Fig. 2). It was hypothesized that *Sg4C MYB* expression may be modulated by stresses because several sequences containing the EAR repressor motif have been implicated in responses to stresses, including drought, cold, salt, and oxidative stress (Ohta et al., 2001; Weigel et al., 2005; Kazan, 2006, and references therein). In *Arabidopsis*, the *Sg* gene *AtMYB4* negatively regulated the formation of UV-protectant sinapate esters and its expression was modulated by UV-B (Jin et al., 2000).

A time course evaluation of the response to mechanical wounding indicated differential accumulation among *Sg4C* transcripts (Fig. 2A). The transcripts of *PtMYB14* in pine, and *PgMYB14* and *PgMYB15* in spruce accumulated strongly and rapidly (after 90 min), and decreased after a few hours. The spruce *PgMYB16* and *PgMYB18* also accumulated transiently, but after 6 h and less strongly than the *PgMYB14* and *PgMYB15* transcripts. Many of the other *Sg4C* transcripts were transiently down-regulated after wounding (after 90 min) and some were unaffected. In JA-treated plantlets, *PtMYB14* and *PtMYB13* transcripts increased 14-fold and 2-fold, respectively, while *PgMYB14*...
and \textit{PgMYB15} transcripts increased 4-fold and 2-fold (Fig. 2B). Transcripts of \textit{allene oxide cyclase} (\textit{AOC}), a reporter gene for the JA pathway (Wasternack, 2007), accumulated in JA-treated plantlets. Following an exposure to cold, the level of many \textit{Sg4C} transcripts decreased slightly, and only \textit{PgMYB18} transcripts increased marginally (Fig. 2C).

The most strongly induced sequences, \textit{PtMYB14} and the closest spruce homologues \textit{PgMYB14} and \textit{PgMYB15}, were analysed in more detail. The predicted amino acid sequence of \textit{PtMYB14} shares 82.4\% and 81.1\% identity with \textit{PgMYB14} and \textit{PgMYB15}, respectively. The spruce \textit{MYB14} and \textit{MYB15} sequences share 90.2\% amino acid identity and 95\% nucleotide identity. In 2-year-old untreated trees (Fig. 2D), \textit{PtMYB14} RNA transcripts accumulated preferentially in the bark and were detected at low levels in all tissues tested. The transcripts \textit{PgMYB14} and \textit{PgMYB15} accumulated preferentially in the bark, as well as in the root tip and the apical portion of the stem. Based on these data, \textit{PtMYB14} was selected for functional analysis.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Stress-responsive transcript profiles of \textit{Sg4C} sequences in \textit{P. taeda} and \textit{P. glauca} determined by RT-qPCR. (A–C) Transcript accumulation (fold change relative to controls) in 4-week-old plantlets in response to (A) mechanical wounding (after 0, 1.5, 6, and 24 h), (B) jasmonic acid (after 24 h) and (C) exposure to cold (24 h at 4 °C); \textit{AOC}, \textit{allene oxide cyclase}. (D) Transcript accumulation of \textit{PtMYB14}, \textit{PgMYB14}, and \textit{PgMYB15} in 2-year-old trees. N, needle; AS, apical stem (young elongating shoot); S2X, stem differentiating secondary xylem; WX: whole stem xylem; B, bark; RT, root tip. Data were from three biological replicates and were normalized relative to transcript levels of \textit{EF1-a} (A–C) or both \textit{EF1-a} and \textit{cdc2} (D). Significant Student’s test at 0.05 (*), 0.01 (**), or 0.001 (***)..}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Constitutive and tissue-preferential overexpression of \textit{PtMYB14} alters development}
\end{figure}

Two different promoters were employed to express \textit{PtMYB14} in stably transformed \textit{P. glauca}, as a heterologous conifer expression system. The \textit{P. glauca CADpro} was used for tissue-preferential expression (Bedon et al., 2009) and the maize \textit{UBIpro} drove constitutive expression (Christensen et al., 1992). The \textit{CADpro} was shown to give xylem and bark preferential expression in young trees, and \textit{CAD} transcripts also accumulate in response to mechanical wounding in these tissues (Bedon et al., 2009). The \textit{CAD} transcript profile overlaps with those of \textit{PtMYB14} and \textit{PgMYB14}, but \textit{CAD} transcripts accumulate at higher levels.

The cellular and tissue activity of each promoter was shown in stably transformed \textit{P. glauca} plantlets harbouring promoter–GUS constructs (Fig. 3). Histochemical GUS staining indicated strong enzyme activity in all cells with the \textit{UBI} promoter (Fig. 3A) and activity localized to differentiating tracheids and xylem ray cells with the \textit{CAD} promoter (Fig. 3B). Quantitative enzyme assays comparing the two constructs were consistent with the large differences in transgene transcript accumulation, but showed that each construct gave GUS activity levels that varied little between the different organs (Fig. 3C).

Constitutive overexpression of \textit{PtMYB14} (\textit{UBIproPtMYB14-OE}) produced a strong phenotype affecting plantlet morphology (Fig. 3D, E). A few weeks after germination, the hypocotyls and cotyledons were hypertrophic and red pigmentation had accumulated compared with controls, but root development was normal (Fig. 3E). Approximately 10 weeks after germination, plantlets withered and were unsuitable for transfer to soil. Histological observation of \textit{UBIproPtMYB14-OE} hypocotyls showed that the vasculature was disorganized and had relatively few tracheids with more variable diameters (Fig. 3H, asterisk), and the parenchyma was expanded and contained inner cells accumulating numerous starch grains (Fig. 3H, black arrows) and outer cells accumulating phenolic compounds (Fig. 3K, white arrows). Polyphenolic parenchyma cells (white arrowheads), seen in the \textit{UBIproPtMYB14-OE} plantlets (Fig. 3H, K) but not in controls (Figure 3G, I).
are part of constitutive and inducible defences in conifers (Franceschi et al., 2005).

The CADproPtMYB14-OE plantlets were morphologically more similar to the controls (Fig. 3F); they developed nearly normal hypocotyls, although a slight increase in pigmentation was noted. Histological observations clearly revealed altered cellular organization and development (Fig. 3G): the inner parenchyma cells accumulated starch grains (Fig. 3I) but fewer than in the UBIproPtMYB14-OE plantlets (Fig. 3H). Within the vasculature, the CADproPtMYB14-OE tracheids were narrower and had thinner cell walls than the wild type; and polyphenolic parenchyma cells were observed within the vasculature, the pith, and the outer parenchyma (Fig. 3I, L).

The two constructs gave contrasting PtMYB14 transgene transcript levels and differentially impacted upon transcript accumulation of other Sg4C sequences (Fig. 4). Relative to the endogenous PgMYB14, the UBIpro construct resulted...
in a 300-fold increase in expression and the CADpro construct gave a 3-fold increase (Fig. 4A). The other Sg4C sequences tested were all down-regulated with the UBIpro construct (4- to 14-fold), but not significantly with the CADpro construct (Fig. 4B).

Constitutive and tissue-preferential overexpression of PtMYB14 has overlapping impacts on the spruce transcriptome

As described above, partly overlapping phenotypes affecting hypocotyl development were observed with constitutive and tissue-preferential overexpression of PtMYB14. Microarray transcript profiles (9K custom cDNA microarray) were obtained for UBIproPtMYB14 and CADproPtMYB14 plantlets and compared in order to assess the impact of both types of overexpression upon gene expression relative to the controls.

The constructs resulted in very different numbers of gene sequences being statistically different from the controls (Supplementary Fig. S1A at JXB online). A total of 517 and 78 of these sequences had a fold change of ≥1.4, for UBIpro and CADpro, respectively. The proportion of up- and down-regulated sequences also varied with the construct, i.e. 59–41% for the UBIpro lines and 94–6% for the CADpro lines. Based on GO and KEGG annotations, 8.9% and 13.5% of these misregulated sequences were assigned to the ‘biotic/abiotic stress’ category for UBIpro and CADpro constructs, respectively (Supplementary Fig. S1B). BlastX results against Uniref100, AGI10.0, PGI5.0, and SGI1.0 indicated that several other sequences appear to be related to stress and defence responses, thus increasing the latter proportions to 34% and 41% for the UBIpro and CADpro construct, respectively (Supplementary Table S2 at JXB online).

Despite these apparent differences, there was a significant overlap among the differentially expressed genes between the UBIproPtMYB14 and CADproPtMYB14 lines which was comprised of a total of 53 common sequences (Table 1). The probability of such an overlap occurring by chance was estimated by permutation analysis to be <2.04×10⁻³. These include 49 sequences with increased transcript abundance compared with the control, and only three genes that were down-regulated, namely a heat shock protein (BT101895), an expansin, and an extensin-like protein. Approximately two-thirds of the genes misregulated with CADproPtMYB14 were also misregulated in a similar manner with the UBIproPtMYB14 construct, although the fold change ratios were generally higher in the UBIpro-overexpressers (Table 1). The genes in common and their putative roles in metabolism or in stress and defence response are described below.

RT-qPCR analyses were performed to validate the results of the microarray analyses. A set of 24 genes related to cell wall biogenesis, defence response, as well as primary and secondary metabolism was tested and confirmed for both types of transgenics plantlets (Table 1, and Supplementary Table S2 at JXB online).

Fig. 4. Transcript accumulation of the PtMYB14 transgene and other Sg4C sequences in hypocotyls and roots of 4-week-old wild-type and transgenic plantlets. Mean (±SE) of RT-qPCR determinations in control (wt), UBIproPtMYB14-OE (lines 16 and 18), and CADproPtMYB14-OE (lines 10 and 14) plantlets of four biological replicates, normalized relative to EF1-α transcripts. na, not amplified. Significant Student’s test at 0.05 (*), 0.01 (**), or 0.001 (***)

Table 1. Two-thirds of the genes misregulated with CADproPtMYB14 were also misregulated in a similar manner with the UBIproPtMYB14 construct, although the fold change ratios were generally higher in the UBIpro-overexpressers. The genes in common and their putative roles in metabolism or in stress and defence response are described below.

RT-qPCR analyses were performed to validate the results of the microarray analyses. A set of 24 genes related to cell wall biogenesis, defence response, as well as primary and secondary metabolism was tested and confirmed for both types of transgenics plantlets (Table 1, and Supplementary Table S2 at JXB online).
Table 1. Overlapping set of differentially expressed genes in UBIProPtMYB14 and CADproPtMYB14 spruce plantlets

| Sequence b (cDNA model) | GenBank accession no. b | Annotation c | Accession no. | E-value | AP d | FC e | P-value f | FC e | P-value f |
|------------------------|------------------------|-------------|---------------|--------|------|------|-----------|------|-----------|
| GO:0008629 Lipid metabolic process; GO:0016042 Lipid catabolic process | | | | | | | | | |
| GQ03221_G05 | BT110413 | Acyl-CoA thioesterase | Q8GYW7 | 3E-17 | JA | 1.5 | 1.2E-03 | 1.5 | 9.7E-03 |
| GQ04104_D12 | BT119330 | Acyl-CoA synthetase | Q94622 | 5E-46 | JA | 1.8 | 6.9E-05 | 1.5 | 9.0E-03 |
| GQ02802_D14 | BT103799 | Acyl-CoA synthetase (4CL-like) | Q9M0X9 | 5E-49 | JA | 2.0 | 1.9E-07 | 1.7 | 1.8E-04 |
| GQ00407_D22 | BT118519 | EDS1, lipase class 3 | Q6SGA1 | 0E+00 | SA | 2.3 | 4.8E-07 | 2.3 | 1.4E-05 |
| GQ01307_L15 | CK441554.2* | EDS1, lipase class 3 | Q8L12 | 3E-20 | SA | 1.5 | 2.5E-06 | 1.3 | 2.8E-03 |
| GQ02802_I24 | BT103811 | Fructokinase, putative | Q9C524 | 4E-41 | SA | 2.2 | 3.4E-06 | 1.8 | 1.7E-03 |
| GO:0008152 Metabolic process | | | | | | | | | |
| GQ03005_P01 | BT106595 | ATP:cytoplasmic dehydrogenase | Q93YH3 | 4E-121 | TCA | 1.4 | 3.8E-03 | 1.4 | 9.2E-03 |
| GQ03230_I01 | BT102565 | Cystathionine γ-synthase | P55217 | 9E-69 | TCA | 1.3 | 5.8E-04 | 1.4 | 9.7E-04 |
| GQ0086910 Transport | | | | | | | | | |
| GQ03108_L15 | BT118163 | ABC transporter, PRD-like | Q6SU87 | 1E-35 | FV | 2.2 | 2.6E-05 | 1.6 | 8.0E-04 |
| GQ0061018 Electron transport | | | | | | | | | |
| GQ03709_B14 | BT115936 | UDP-glucose dehydrogenase | Q6RK07 | 0E+00 | FV | 1.5 | 1.2E-05 | 1.3 | 5.1E-03 |
| GQ03508_P12 | BT114142 | Flavonoid 3′,5′-hydroxylase | Q04773 | 7E-40 | FV | 1.9 | 2.5E-06 | 2.0 | 2.3E-05 |
| GO:0009664 Cell wall organization and biogenesis | | | | | | | | | |
| GQ02806_E03 | BT110611 | Expansin | Q84UT0 | 1E-69 | TCA | 1.3 | 1.4E-04 | –1.8 | 9.0E-03 |
| GQ03902_B01 | BT117517 | Chitinase class II | Q5EM9 | 3E-94 | TCA | 3.9 | 1.4E-08 | 2.2 | 2.2E-04 |
| GQ00833_E11 | CK438292.2* | Receptor-like protein kinase | Q8LP72 | 1E-27 | FV | 2.9 | 9.5E-08 | 2.3 | 4.6E-05 |

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Table 1. Continued

| Sequence^b (cDNA model) | GenBank accession no.^b | Annotation^c | Predicted function | Accession no. | E-value | AP^d | FC^e | P-value^e | FC | P-value |
|-------------------------|-------------------------|--------------|--------------------|---------------|---------|-------|-------|-----------|-----|---------|
| GO:0045449 Regulation of transcription | | | | | | | | | | |
| GQ03111_L15 BT112195 | Zinc finger CCCH-type protein | Q10EL1 | 7E-05 | | 1.5 | 5.7E-03 | 1.6 | 8.0E-03 |
| GQ02902_P24 BT106103 | Parathymosin-like | Q6Z35 | 2E-21 | | 1.5 | 1.3E-04 | 1.4 | 7.1E-03 |
| GO:0006950 Response to stress; GO:0006952 Defense response; GO:0006911 Response to wounding | | | | | | | | | |
| GQ02905_A21 BT106211 | Heat shock protein (HSP70) | Q03883 | 5E-08 | | 1.4 | 3.3E-04 | 1.4 | 5.3E-03 |
| GQ0132_K16 BT101895 | Heat shock protein (HSP71) | Q9ZSU3 | 3E-03 | | -1.5 | 1.4E-03 | -1.5 | 6.6E-03 |
| GQ04105_K15 BT119437 | Glycine-rich protein (Grp94) | Q8H66B6 | 8E-96 | | 1.5 | 7.4E-04 | 1.5 | 4.3E-03 |
| GQ04110_H09 BT119814 | Glutathione S-transferase, Tau class | Q4PNY9 | 5E-57 | | 2.2 | 2.7E-06 | 1.7 | 1.3E-03 |
| GQ0204_N24 BT103013 | Glutathione S-transferase, Tau class | Q65056 | 3E-73 | | 2.1 | 8.6E-07 | 1.6 | 2.3E-03 |
| GQ02818_G19 BT105094 | Dehydrin | Q3ZDL1 | 0E+00 | | 1.4 | 1.4E-04 | 1.5 | 3.8E-04 |
| GQ03702_K04 BT115610 | ADR1-like 1, CC-NBS-LRR prot. | Q1L6F3 | 3E-77 | | 1.4 | 2.2E-03 | 1.6 | 9.7E-04 |
| GQ03308_B18 BT111949 | ADR1-like 1, NBS-LRR protein | Q2VWL9 | 9E-18 | | 2.7 | 1.3E-06 | 1.7 | 6.2E-03 |
| GO:0006508 Proteolysis | | | | | | | | | |
| GQ02810_F11 BT104435 | Serine carboxypeptidase-like 46 | Q1EP77 | 3E-117 | | 1.5 | 2.0E-05 | 1.5 | 3.3E-04 |
| GQ03811_C08 BT117017 | Serine carboxypeptidase-like 28 | Q0SFBS5 | 3E-47 | | 1.3 | 6.2E-03 | 1.5 | 1.2E-03 |

Miscellaneous

| Sequence^b | GenBank accession no.| Annotation^c | Predicted function | Accession no. | E-value | AP^d | FC^e | P-value^e | FC | P-value |
|-------------|---------------------|--------------|--------------------|---------------|---------|-------|-------|-----------|-----|---------|
| GO:003519_E01 BT114678 | Plasma membrane- associated protein | Q1ECE0 | 1E-34 | | 1.5 | 7.4E-04 | 1.6 | 5.0E-04 |
| GQ04104_H01 BT113347 | Alternative oxidase 1b | Q84V46 | 0E+00 | | 1.3 | 6.2E-04 | 1.5 | 3.4E-04 |
| GQ03310_J01 BT11230 | Expressed protein | Q82812 | 1E-19 | | 1.5 | 4.8E-04 | 1.4 | 1.0E-02 |
| GQ03102_D11 BT107053 | DNAJ heat shock family protein | Q05977 | 7E-21 | | -1.4 | 2.6E-03 | 1.5 | 4.1E-03 |
| GQ03237_M12 BT11544 | No hit | N/A | N/A | | 1.7 | 5.5E-06 | 1.4 | 4.3E-03 |
| PMYB14 DQ399056 | Transgene | ABD60279 | 0E+00 | | 9.1 | 7.3E-08 | 2.0 | 3.2E-02 |

^a The 53 shared genes were found in each of the constructs by analysing four replicates from two independent lines in each construct (P-value <0.01, and log2 ratio ≥0.5, FDR of 1%). A permutation analysis using random assignment gave an average number of 0.153 overlapping genes occurring by chance and a probability of obtaining 53 genes <2.04×10^-10 (1/4900).

^b Representative cDNA clone from Arborea EST and full-length insert cDNA database (www.Arborea.ulaval.ca); accession numbers for cDNA clones GenBank, except where marked *; these are for ESTs (dbEST). IDs in bold indicate that the microarray result was validated by RT-qPCR.

^c Functional annotation methods are as described in Pavy et al. (2003); accession numbers are for UniProt (www.uniprot.org/uniprot)

^d AP, associated pathway: FV, flavonoid; TCA, tricarboxylic acid cycle; TP, terpenoid.

^e Fold change (FC) relative to the wild type. (+) are over-represented and (–) are under-represented in transgenics.

^f Student’s t-test level of confidence.

Misregulation of transcripts related to metabolic processes, to defence, and to stress responses in PtMYB14-OE spruce

The overlapping transcript profile data indicate that PtMYB14 overexpression most clearly impacted gene sequences linked to isoprenoid/terpenoid, flavonoid, pyruvate, and citrate [tricarboxylic acid (TCA)] metabolism, as well as the lipid catabolic process leading to JA biosynthesis (Table 1). The common set of up-regulated transcripts included several sequences that were most similar to genes of the cytosolic MVA pathway leading to isoprenoid formation, such as acetoacetyl-CoA thiolase (AACT), 3-hydroxy-3-methylglutaryl-CoA-synthase (HMGS), and mevalonate decarboxylase (MVD1), as well as an α-farnesene synthase (AFS) from the downstream pathway leading to sesquiterpenes, and a chalcone synthase (CHS) involved in flavonoid biosynthesis (Table 1). Congruent with these data, several transcripts associated with electron transport were misregulated, including a cytochrome p450 monoxygenase, a flavonoid 3',5'-hydroxylase, and an FAD-binding domain-containing protein. Transcripts accumulated for ATP-binding cassette (ABC) family transporters (PDR-like), defined as important partners in processes such as pigment accumulation, detoxification, and oxidation damage (Theodoulou, 2000), for NAD-dependent malate oxidoreductase and ATP: citrate lyase that could be linked to pyruvate and the citrate cycle (TCA), and for lipoxygenase (LOX), acyl-CoA
synthase, and acyl-CoA thioesterase linked to \( \alpha \)-linolenic acid metabolism leading to JA biosynthesis.

A large proportion of the remaining misregulated sequences common to both constructs could be linked to defence and stress responses. These included sequences coding for activated disease resistance (ADR) 1 proteins (Table 1), defined by functional domains such as leucine-rich repeat (LRR), Toll- and interleukin-1 receptor-like (TIR), coiled-coil (CC), receptor-like kinase, and NBD (nucleotide binding) which have been implicated in defence response and plant resistance (Fluirh, 2001). Other misregulated stress-responsive sequences, included those coding for dehydrin as well as glycine-rich (GRP) and heat-shock (HSP) proteins, in addition to glutathione S-transferases (Table 1). The strong up-regulation of wound-responsive class II and IV chitinases (Hietala et al., 2004) was common to both constructs, together with the accumulation of transcripts encoding serine carboxypeptidase hydrolytic enzymes shown to function as acyltransferase in secondary metabolism (Lehfeldt et al., 2000). Transcripts of reporter genes from the salicylic acid (SA) response such as EDS1-like and phytoalexin deficient 4 (PAD4) (Wiermer et al., 2005), as well as transcripts of MAP kinase 6 associated with transcriptional regulation and signal transduction in the JA, SA, and ethylene response in Arabidopsis (Fujita et al., 2006), also accumulated with both constructs (Table 1).

PtMYB14 overexpression stimulated terpene and anthocyanin accumulation in spruce plantlets

In light of the overlapping transcript profiles, the increased pigmentation, the presence of polyphenolic cells, and the accumulation of starch grains in parenchyma cells observed with PtMYB14-OE constructs, the aim was to gather further information on the metabolite content of transgenic plantlets. Extensive identification and quantification of monoterpenoids, sesquiterpenoids, and diterpenoids, known plant defence compounds (Martin et al., 2002; Keeling and Bohman, 2006; Phillips et al., 2007), was performed by GC-MS (Fig. 5, Supplementary Table S3 at JXB online). The diversity of sesquiterpenes was clearly increased following both tissue-preferential and constitutive expression of PtMYB14 in spruce plantlets (Fig. 5). Longifolene, \( \alpha \)-bergamotene, germacrene D, and two unidentified sesquiterpenes were detected uniquely in the transgenics (Fig. 5). In addition, the amount of most of the detected sesquiterpenes increased significantly in UBIproPtMYB14-OE plantlets relative to controls (Supplementary Table S3 at JXB online). The total amount of monoterpenes also significantly increased 3-fold in UBIproPtMYB14-OE but not in the CADproPtMYB14-OE plantlets compared with controls (Fig. 5, Supplementary Table S3). Diterpene accumulation was not substantially affected in the transgenics plantlets.

The UBIproPtMYB14-OE plantlets also accumulated large amounts of anthocyanin \( O \)-glucosides, including delphinidin 3-\( O \)-glucoside, cyanidin 3-\( O \)-glucoside, petunidin 3-\( O \)-glucoside, peonidin 3-\( O \)-glucoside, and malvidin 3-\( O \)-glucoside in UBIpro (Supplementary Fig. S2A at JXB online). The CADproPtMYB14-OE plantlets only gave increased accumulation of cyanidin 3-O-glucoside. Starch content determinations in UBIproPtMYB14-OE plantlets showed a 2-fold increase compared with controls, but no increase was recorded in any of the CADproPtMYB14-OE lines (Supplementary Fig. S2B).

MYB14 transcripts accumulated coordinately with those of isoprenoid/terpenoid-related genes following mechanical wounding

In light of microarray and metabolite determination results (PtMYB14-OE plantlets), it was postulated that PtMYB14 could contribute to the regulation of the isoprenoid and terpenoid pathways, and JA metabolism during defence responses (Table 1, Fig. 6, Supplementary Table S3 at JXB online). Mechanically wounded seedlings (Fig. 2A) were analysed for accumulation of isoprenoid and terpenoid transcripts encoding all of the enzymes from the cytosolic MVA, and plastidial MEP pathways, together with those coding for prenyltransferases and terpene synthases. Transcript accumulation was observed for two reporter genes, a defensin (DEF) for wounding (Pervieux et al., 2004) and an AOC for the JA pathway (Wasternack, 2007) (Fig. 6).

A clear wound response was observed for several isoprenoid and terpenoid pathway transcripts in both pine and spruce when considering cytosolic and plastidial routes. Transcripts for MVA pathway enzymes (AACT, HMGS, and MVD1) increased in both species; the HMGS RNA transcripts rapidly increased 15-fold and 5-fold in pine and spruce, respectively (Fig. 6). Only transcripts of the first MEP pathway enzyme, DXS, accumulated, giving 3-fold and 6-fold increases, respectively. Transcripts also accumulated for prenyltransferases (FPS and GGPPS) and AFS from downstream pathways leading to sesquiterpenes. These transcript accumulation profiles were slightly delayed or simultaneous with PgMYB14 and PtMYB14.

Discussion

In response to developmental and environmental cues, plants activate specific parts of their metabolism to produce a variety of primary and secondary metabolites that contribute to adaptive traits. TFs orchestrate the temporal and spatial expression of the numerous genes related to these complex metabolic routes. Several transcriptional regulators were shown to modulate phenylpropanoid, flavonoid, and benzoid accumulation (Grotewold, 2005), but only a few have been linked to the regulation of isoprenoid metabolism (Xu et al., 2004; Broun et al., 2006; Memelink and Gantet, 2007). This report identified the pine R2R3-MYB gene PtMYB14 as a putative partner in an isoprenoid-oriented response that leads to sesquiterpene accumulation in conifers. It also broadly illustrates the potential involvement of closely related Sg4C sequences in stress responses and plant evolution.
Overlapping transcription, histological, and metabolite phenotypes identified by tissue-specific and constitutive PtMYB14 overexpression approaches

Combining both constitutive and tissue-preferential overexpression strategies was an efficient way to define traits potentially related to PtMYB14 activity. The comparative microarray analyses provided a robust subset of 53 sequences that were mainly up-regulated (49 out of 53) in both types of PtMYB14 overexpressors (Table 1). It is proposed that this set of shared misregulated sequences most probably includes genes and pathways directly linked to the PtMYB14 activity, because their transcripts were modulated similarly (Table 1) despite the very different levels and spatial distributions of PtMYB14 transgene expression observed with the two strategies (Fig. 3A–C). Although the CADproPtMYB14-OE plantlets had fewer misregulated sequences, the majority of them (around two-thirds) were part of the common set. The two types of transgenics also shared common histological and metabolite traits that were considered to be a consequence of PtMYB14 activity. They included (i) polyphenolic parenchyma cells, which are components of constitutive and inducible defence response in conifers (Franceschi et al., 2005), observed within the vasculature, the pith, and the outer parenchyma (Fig. 3H, I); (ii) altered vascular organization and tracheid development compared with wild-type plantlets (Fig. 3); and (iii) an overlapping modulation in the tracheid (Fig. 5, Supplementary Table S3 at JXB online) and anthocyanin profiles (Supplementary Fig. S2). These shared or overlapping phenotypes, i.e. transcript, histological, and metabolite profiles, are proposed to indicate candidate genes and metabolic processes that are influenced by PtMYB14 activity, as opposed to non-specific, or random effects leading to pleiotropic changes.

The phenotypes uniquely observed in the UBIproPtMYB14 overexpressors were considered as potential pleiotropic effects (Fig. 3, Supplementary Fig. S2 at JXB online). They include additional effects on transcript profiles of genes belonging to other secondary metabolism pathways (Supplementary Table S1). For instance, many of the sequences that were down-regulated with the UBIproPtMYB14 construct were linked to shikimate, monolignol, and benzenoid biosynthetic pathways (Supplementary Table S1). Other R2R3-MYBs have been implicated in the transcriptional control of phenylpropanoid (Zhong and Ye, 2010) and benzenoid metabolism through the upstream shikimate pathway (Verdonk et al., 2005). This transcript profile was consistent with the additional modifications in the metabolite profile (i.e. monoterpenes, anthocyanins, and starch) that were observed when PtMYB14 was driven by UBIpro (Fig. 5, Supplementary Fig. S2). Due to the highly

Fig. 5. Mono- and sesquiterpenoids in wild-type and PtMYB14-OE transgenic spruce (raw data). Monoterpenes (A, C, E) and (B, D, F) sesquiterpenes in wild-type (A, B), CADproPtMYB14 (C, D), and UBIproPtMYB14 (E, F) plantlets in three lines per transgenic and three replicates per line. Each value is a mean of a technical duplicate.
conserved DBD observed among conifer MYBs (Bedon et al., 2007), the genes that were uniquely down-regulated with UBIlpro may reflect indirect effects caused by the high level of MYB expression, such as non-specific binding to gene promoters. The PtMYB14 transcript level was ~100-fold higher for the UBIlproPtMYB14 than for the CADproPtMYB14 construct when normalized to CDC2 gene transcripts. 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). Differences in the effects of the two overexpression strategies may also be due to spatial expression of the transgene. A second mechanism by which MYB over-expression may repress gene expression is by competing with cognate TFs (squelching) and thus sequestrating components of the transcriptional machinery away from cis-regulatory DNA elements (Gill and Ptashne, 1988). All the Sg4C sequences described herein share a signature motif similar to the basic helix–loop–helix (bHLH) interaction site identified by Zimmermann et al. (2004) in Arabidopsis. Thus, non-specific effects may entail interactions with other proteins, such as bHLH and WD40 which have been identified as important MYB partners in flavonoid pathways (Vom Endt et al., 2002; Ramsay and Glover, 2005).

Finally, based on the differential expression of other Sg4C transcript levels observed between the UBIlpro- and CADpro-driven overexpression of PtMYB14 (Fig. 4B), one can argue that strong and constitutive PtMYB14 overexpression had a co-suppressing effect on other Sg4C members while it was not the case for moderate and tissue-preferential PtMYB14 overexpression. If other Sg4C members also play a role in regulating defence and stress responses in conifers, as suggested by expression profiles (Fig. 2A–C), it might be expected that their down-regulation would affect gene expression and, in part, explain the large difference in misregulated sequences observed between UBIlpro- and CADpro-driven overexpression of PtMYB14.

Overexpression phenotypes are consistent with a role for PtMYB14 in the regulation of isoprenoid metabolism

Isoprenoids are involved in numerous cellular processes and serve as hormones, defensive agents, membrane constituents, components of signal transduction networks, mating pheromones, and photoprotective agents (Sacchettini and Poulter, 1997). Among the isoprenic-derived compounds (Lichtenthaler, 1999; Lange et al., 2000), sesquiterpenes and sterols are predominantly generated from the cytosolic MVA pathway, while monoterpenes, diterpenes and caroteneoids are synthesized via the plastidic MEP pathway (Wu et al., 2006). A few transcription factors have been linked to the regulation of isoprenoid metabolism in angiosperm plants (Broun et al., 2006; Memelink and Gaetet, 2007). To our knowledge, no MYB has been directly linked to the regulation of isoprenoid pathways.

The overlapping transcription and metabolite phenotypes observed upon PtMYB14 overexpression in spruce are clearly consistent with a role in the regulation of isoprenoid metabolism. Several of the up-regulated transcripts were related to early steps in isoprenoid biosynthesis in the cytosolic MVA pathway, such as AACT, HMG-S, and MVDI (Table 1). An AFS in the downstream sesquiterpene pathway was also up-regulated (Table 1; Supplementary Table S2 at JXB online). Furthermore, the metabolite profiles clearly point to the accumulation of more diverse volatile oleoresins such as sesquiterpenes (Fig. 5 and Supplementary Table S3). Similarly, mechanical wounding in wild-type spruce and pine plantlets led to the coordinated accumulation of PtPgMYB14 transcripts with those of AACT and HMGS from the MVA pathway, and prenyltransferase (FPSS) and terpene synthase (AFS) of the sesquiterpene pathway (Fig. 6). In Norway spruce, the induction of genes encoding key steps in the MEP pathway (i.e. DXS) was stimulated by mechanical wounding and by methyl jasmonate (MeJA) (Philipps et al., 2007). Here, the wounding experiment impacted both MVA and MEP pathway transcripts (Fig. 6), but the microarray analyses of PtMYB14-overexpressors did not detect any differentially expressed MEP pathway transcripts (Table 1; Supplementary Table S2). Therefore, it was postulated that PtPgMYB14 may be preferentially involved in isoprenoid metabolism through the cytosolic (MVA) rather than the plastidic (MEP) route, although it may be part of a complex signalling network that controls the wound response in conifers.

The accumulation of transcripts encoding flavonoid pathway enzymes (Table 1) and of cyanidin-3-glucoside (Supplementary Fig. S2 at JXB online) in both transgenics also suggested that PtMYB14 may have a role in the regulation of flavonoid metabolism. Anthocyanins are reported to act as UV-B protectants in addition to taking part in antioxidative defence (Steyn et al., 2002), but their accumulation at particular developmental stages can be deleterious or interfere with normal development (Chawla et al., 1999). Bresseau et al. (2007) reported that flavonoids including anthocyanins can also interfere with plant growth and the development of tracheary elements in Arabidopsis. In spruce, both of the PtMYB14 overexpression approaches clearly altered vascular tissue organization (Fig. 3) and the common set of misregulated transcripts included sequences related to cell wall organization and biogenesis (i.e. expansin, extensin, and chitinases) (Table 1). This experimental evidence suggests two possible explanations. The PtMYB14 acts on genes associated with the anthocyanin pathway in specific tissues during development. Alternatively, PtMYB14 may be part of a complex defensive programme, including terpenoid and anthocyanin production. Further experiments are needed to test these hypotheses.

The differential gene family expansion of Sg4C R2R3-MYBs highlights the distinct evolutionary trajectories of angiosperm and gymnosperm plants

Several conifer sequences form a monophyletic subclade (Sg4C) within Sg4 of plant R2R3-MYBs previously defined by Kranz et al. (1998). Evidence was also presented that conifers have accumulated more Sg4 sequences than angiosperm genomes sequenced to date. Ten Sg4C members were
identified in *P. glauca*, whereas the angiosperms have between three and eight sequences. Considering that cDNA isolation is non-exhaustive, the Sg4C inventory probably remains incomplete. Interestingly, the grapevine (*V. vinifera*) genome has a total of eight Sg4 MYBs, six of which form a monophyletic clade (Matus *et al.*, 2008), representing an angiosperm Sg4 gene family structure that is analogous to the Sg4C in conifers.

Several analyses of expressed gene sequences provide evidence of monophyletic gene family amplifications that occurred after the angiosperm–gymnosperm split, as reported here for Sg4C MYBs. Specific examples include the *P. taeda* arabino-galactan proteins (Zhang *et al.*, 2000) and cellulose synthases (CesA) (Nairn and Haselkorn, 2005), and the *Picea* spp. KNOX-1 gene family (Guillet-Claude *et al.*, 2004) and dirigent proteins (Ralph *et al.*, 2007). This latter protein family and the expansin superfamly (Sampedro and Cosgrove, 2005) provide striking examples of entire subfamilies or subclades that are specific to conifers. Some of these studies have also pointed to reduced complexity of gene family structure in conifers. For example, conifer sequences have been found in only one of the three subbranches in the KNOX-1 gene family, although all of the conifers examined had more gene duplications within this branch (Guillet-Claude *et al.*, 2004). Taken together, these data clearly illustrate the evolutionary trajectories of conifer gene families as being distinct from angiosperms.

The gymnosperms account for four of the five divisions/phyla of seed plants (spermatophytes). A very deep split exists between the gymnosperms which appeared ~300 Mya and the more recent angiosperms estimated to be ~190 million years old (Magallon and Sanderson, 2005). While conifers (coniferales) appeared relatively early in the evolution of gymnosperms, the pinaceae family and its major genera, including those of *Pinus* and *Picea*, are estimated to have radiated ~90–100 Mya. The phylogenetic trees of plant Sg4 sequences include multiple sequences within both the *Picea* and *Pinus* subfamilies (Fig. 1, Supplementary Table S1 at JXB online). This enables it to be postulated that many of the gene duplication events that gave rise to Sg4C actually occurred between 100 and nearly 300 Mya. The relatively short branches in the Sg4C subclade may not suggest such an ancient time frame for these gene duplications; however, conifers are very long lived and notorious for their slow rate of evolution (Savolainen and Pyhäsälä, 2007). Further investigations such as the estimation of rates of substitution and molecular clock analyses are needed to shed light on this question.

The maintenance of duplicated genes within Sg4C over tens of millions of years suggests that selective advantages
have been derived over time from the additional gene copies. Such advantages may arise in the shorter term from enhanced expression, or with time from sequence divergence resulting in modified biochemical functions or biological roles. Expression studies in *Arabidopsis* indicated that highly homologous sequences may have different expression profiles under particular conditions or treatments (Yanhui et al., 2006). Not surprisingly, widespread duplication has been previously invoked as an important mechanism for *MYB* gene evolution leading to the acquisition of new functions, as well as enabling plant adaptation to new environments (Grotewold, 2005). For example, duplication of the *MYB* regulator *p1* and *p2* genes in maize from the ancestral *p* gene resulted in two different expression profiles related to flavonoid regulation. The *p1* gene is expressed in the kernel pericarp, and *p2* is expressed in the developing anther and silk (Zhang et al., 2000).

Transcript expression data suggest that the Sg4C sequences have diverged functionally to some extent. A survey of transcript profiles across different tissues in spruce (Bedon et al., 2007; Fig. 2D), as well as changes in transcript accumulation levels upon wounding, JA application, and exposure to cold (Fig. 2A–C), clearly indicate differential regulation. The majority (but not all) of Sg4C were shown to respond to at least one of the stress treatments tested. Their stress responsiveness and the evidence supporting the involvement of Pt/PgMYB14 in the regulation of isoprenoid metabolism (Fig. 6) lead us to postulate that Sg4C sequences have contributed to the adaptation of gymnosperms and conifers to changing environments throughout evolution.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Figure S1.** Number and functional grouping of genes differentially expressed in *UBLpro* and *CADproPtMYB14-OE* spruce plantlets.

**Figure S2.** Accumulation of anthocyanins and starch in wild-type and Pt*MYB14-OE* transgenic spruce.

**Table S1.** Phylogenetic analysis of the conifer, and angiosperm subgroup 4 R2R3-MYB sequences with different methods of alignments and phylogeny.

**Table S2.** Functional grouping of genes differentially expressed in *UBLpro* and *CADproPtMYB14-OE* spruces.

**Table S3.** Mono-, di-, and sesquiterpenoids in Pt*MYB14-OE* transgenic spruce (raw data).

**Table S4.** Primers sequences used to generate DNA matrices for RT-qPCR analyses in pine.

**Table S5.** Primer sequences used for RT-qPCR in pine and spruce

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