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Regulation of resin acid synthesis in *Pinus densiflora* by differential transcription of genes encoding multiple 1-deoxy-D-xylulose 5-phosphate synthase and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase genes

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Summary *Pinus densiflora* Siebold et Zucc. is the major green canopy species in the mountainous area of Korea. To assess the response of resin acid biosynthetic genes to mechanical and chemical stimuli, we cloned cDNAs of genes encoding enzymes involved in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (1-deoxy-D-xylulose 5-phosphate synthase (PdDXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (PdDXR) and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (PdHDR)) by the rapid amplification of cDNA ends (RACE) technique. In addition, we cloned the gene encoding abietadiene synthase (PdABS) as a marker for the site of pine resin biosynthesis. *PdHDR* and *PdDXS* occurred as two gene families. In the phylogenetic trees, PdDXSs, PdDXR and PdHDRs each formed a separate clade from their respective angiosperm homologs. *PdDXS*2, *PdHDR*2 and *PdDXR* were most actively transcribed in stem wood, whereas *PdABS* was specifically transcribed. The abundance of *PdDXS*2 transcripts in wood in the resting state was generally 50-fold higher than the abundance of *PdDXS*1 transcripts, and *PdHDR*2 transcripts were more abundant by an order of magnitude in wood than in other tissues, with the ratio of *PdHDR*2 to *PdHDR*1 transcripts in wood being about 1. Application of 1 mM methyl jasmonate (MeJA) selectively enhanced the transcript levels of *PdDXS*2 and *PdHDR*2 in wood. The ratios of *PdDXS*2 to *PdDXS*1 and *PdHDR*2 to *PdHDR*1 reached 900 and 20, respectively, on the second day after MeJA treatment, whereas the transcript level of *PdABS* increased twofold by 3 days after MeJA treatment. Wounding of the stem differentially enhanced the transcript ratios of *PdDXS*2 to *PdDXS*1 and *PdHDR*2 to *PdHDR*1 to 300 and 70, respectively. The increase in the transcript levels of the MEP pathway genes in response to wounding was accompanied by two orders of magnitude increase in *PdABS* transcripts. These observations indicated that resin acid biosynthesis activity, represented by *PdABS* transcription, was correlated with the selective transcriptions of *PdDXS*2 and *PdHDR*2. Introduction of *PdDXS*2, *PdHDR*1 and *PdHDR*2 rescued their respective knockout *Escherichia coli* mutants, confirming that at least these three genes were functionally active. Intracellular targeting of the green fluorescent protein fused to the N-terminal 100 amino acid residues of these genes in the *Arabidopsis* transient expression system showed that the proteins were all targeted to the chloroplasts. Our results suggest that the MEP pathway regulates resin biosynthesis in the wood of *P. densiflora* by differential transcription of the multiple *PdDXS* and *PdHDR* genes.

Keywords: 1-deoxy-D-xylulose 5-phosphate reductoisomerase, resin biosynthesis.

Introduction

Terpenoids are found as or as part of the key molecules participating in respiration, photosynthesis and regulation

of growth and development. One of the roles of terpenoids is the protection of plants from herbivores and pathogens (Croteau et al. 2000, Chappell 2002). In particular, oleoresin, composed of monoterpenes and diterpene resin acids, plays an important defensive role in conifers (Trapp and Croteau 2001*a*).

Until two decades ago, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the building blocks of terpenoids, were thought to be synthesized exclusively by the mevalonate pathway in all the organisms (Goldstein and Brown 1990). However, Rohmer et al. (1993) identified a new pathway, the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Figure 1), also leading to the production of IPP and DMAPP. It is now evident that in plants, the two pathways operate in different subcellular compartments, and hence have different roles. The MEP pathway resides in the plastid, whereas the mevalonate pathway is in the cytosol (Rohmer 1999). The end products of the MEP pathway are generally known to constitute the building blocks of mono-, di- and tetraterpenes, whereas the mevalonate pathway supplies the precursors of sesqui- and triterpenes. Nevertheless, cross talk between the MEP pathway and the mevalonate pathway is known to occur (Laule et al. 2003).

The initial reaction of the MEP pathway is catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS, EC 2.2.1.7) (Sprenger et al. 1997, Lange et al. 1998). Because the product of this reaction, 1-deoxy-D-xylulose 5-phosphate, can serve not only as a precursor of the essential cofactors, pyridoxal and thiamine, but also as the building block of the terpenes, IPP and DMAPP (Julliard and Douce 1991), the first step of the MEP pathway is regulated so that the demands for the various end-products can be met. Most plants have been found to have multicopies of the *DXS* gene. Class 1 *DXS* has a role in household functions, whereas class 2 *DXS* is correlated with mycorrhizal carotenoid and ginkgolide biosyntheses (Walter et al. 2002, Kim et al. 2006*c*). Recently, Seetang-Nun et al. (2008) showed that the gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR, EC 1.1.1.267) in several angiosperms is a multicopy gene with an unknown physiological function. Moreover, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (HDR, EC 1.17.1.2), the enzyme catalyzing the last step of the MEP pathway, is encoded by a multicopy gene in the gymnosperms *Ginkgo biloba* L., *Pinus taeda* L. and *Cycas revoluta* Thunb. with two to three copies in each plant (Kim et al. 2008*a*). Another multicopy gene in the MEP pathway is the gene encoding 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (CMK, EC 2.7.1.148) in *G. biloba* (Kim et al. 2008*b*). In all the MEP pathway, multicopy genes of gymnosperm origin so far identified, at least one copy of each gene is selectively involved in secondary metabolism.

Pinus densiflora Siebold et Zucc., one of the most important forest species in Korea, has been used for thousands of years as timber for buildings and furniture as well as firewood; it is also highly prized as an ornamental plant. Several pine forests have been designated by the government as timber resources to replace or rebuild ancient buildings. Unfortunately, these pine forests have suffered from serial attacks by the insects *Dendrolimus spectabilis* Butler in the 1960s and *Thecodiplosis japonensis* Uchida et Inouye in the 1980s. For the past two decades, these forests have been devastated as a result of the inadvertent introduction of the pine wilt disease nematode, which is transmitted by the Japanese pine sawyer beetle *Monochamus alternatus* Hope (Fielding and Evans 1996, Woo et al. 2007). Little is known about the biochemical or genetic makeup of *P. densiflora*; therefore, a study of the genes related to resin production is necessary to understand the physiological response of trees to invasion by insects and particularly the pine wilt disease nematode.

In this study, we cloned the genes encoding DXS, DXR and HDR, the enzymes responsible for catalyzing the

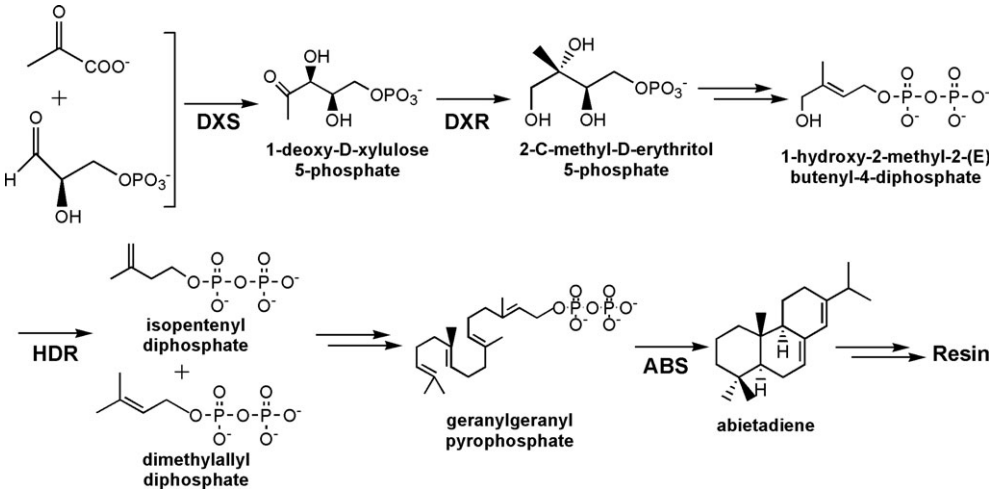

Figure 1. Biosynthesis of resin through MEP pathway in *P. densiflora*. A color version of this figure is available as Supplementary Data at *Tree Physiology* Online.

synthesis of the precursors for resin production, and the gene encoding abietadiene synthase (ABS, EC 4.2.3.18), which catalyzes the first committed step of resin synthesis in *P. densiflora*. To characterize the biological roles of these genes, the trees were wounded or treated with methyl jasmonate (MeJA) to mimic stresses of biological and mechanical origins, and the transcription patterns of the genes were assessed. We demonstrated differential transcription of the *DXS* and *HDR* multigenes in resin biosynthesis.

Materials and methods

Plants

Current-year shoots from grafted 6-year-old saplings of *P. densiflora* forma *multicaulis* Siebold et Zucc., planted in the outdoor nursery at Yongin-Si, Kyunggi-Do Province and strobili from a 15-year-old tree in the same region were collected on May 2006 for the preparation of mRNA. Current-year shoots from the same population were used for MeJA and wounding treatment studies in mid-June and mid-August 2007, respectively. The leaves, bark and wood were separated immediately after harvest and were frozen in liquid nitrogen until processed. Each treatment consisted of three trees that were sampled separately.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from each organ and tissue by the cetyltrimethylammonium bromide method (Chang et al. 1993). Single-strand cDNA was synthesized from total RNA with the GeneRacer Kit (Invitrogen, Carlsbad, CA). The reaction mix for the reverse transcriptase polymerase chain reaction (RT-PCR) contained 11 μl of RNase-free water mixed with 2 μg of total RNA (2 μl), 2 μl of 10× buffer, 1 μl of each 5 mM dNTP, 2 μl of 10 μM oligo (dT) primer, 1 μl of 10 U μl^{-1} RNase inhibitor and 1 μl of 4 U μl^{-1} Omniscript reverse transcriptase (Qiagen, Düsseldorf, Germany), in a final volume of 20 μl. The mixture was incubated for 60 min at 37 °C and for 5 min at 95 °C.

Rapid amplification of cDNA ends

Rapid amplification of cDNA ends (RACE) was performed using the GeneRacer™ Kit (Invitrogen). All the primers for the RACE PCR are listed in Table 1. The oligonucleotide primers for isolating the conserved core fragments were designed from the previously identified conserved amino acid sequences of DXS, DXR, ABS and HDR from other plants. One-twentieth of the RT-PCR product was used as the template for PCR amplification. The PCR program consisted of 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C (*DXS*), 67 °C (*DXR*) or 50 °C (*HDR*) for 1 min and extension at 72 °C for 1 min. The 35 cycles were preceded by a denaturation step at 94 °C for 5 min and ended with a final extension at 72 °C for 10 min. The PCR products were purified with the PCR Purification Kit (Takara, Shiga, Japan), cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and finally sequenced. All primers for RACE were designed based on the core sequences (Table 1). We performed 5′-RACE PCR using the Generacer 5′ primer and the reverse primers, and 3′-RACE PCR using the Generacer 3′ primer and the forward primers of each gene. All PCR assays were run as described for the PCR program.

Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) was carried out using the Rotor-Gene 2000 Real Time Amplification System (Corbett Research, Mortlake, NSW, Australia) and Quantitect SYBR Green PCR system (Qiagen) in a 50 μl reaction mixture containing 25 μl of 2× master mix, 2 μl (10 pmol) of each primer, 1 μl of cDNA (100 ng) and 20 μl of RNase-free water. The thermal cycling profile consisted of: stage 1, 95 °C for 15 min; stage 2, 94 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s for a total of 40 cycles. The reaction was run in triplicate. The standard curves were made by running the QRT-PCR of each gene ranging from 1×10^3 to 1×10^7 copies μl^{-1}; and the copy number was calculated as described by Yin et al. (2001).

Complementation assay

To confirm the activities of the *P. densiflora* MEP pathway enzymes, *Escherichia coli dxs* disruptant DXM3 (Kim et al. 2005), *dxr* disruptant DYM1 (Kuzuyama et al. 1999) and DLYT1, the *lytB* disruptant (Kim et al. 2008*a*), were spread on LB medium containing 25 μg ml^{-1} kanamycin and 0.01% of 2-C-methyl-D-erythritol (ME) for DXM3 and DYM1, and 0.1% mevalonate for DLYT1. We transformed DXM3, DYM1 and DLYT1 with the plasmids pMW118-DXSs, pMW118-DXR and pMW118-HDRs, respectively, prepared by inserting genes corresponding to the mature proteins (Table 1) into the pMW118 vector (Nippon Gene, Arakawa, Japan), and by selecting on LB plates.

MeJA treatment and wounding

The 6-year-old saplings grown in the field were sprayed with 1 mM MeJA solution containing 10% ethyl alcohol in mid-June 2007. One current-year shoot (15–20-cm long) per tree was collected from three trees for 3 days after the treatment. Each shoot was immediately separated into bark, needle and wood, and then frozen in liquid nitrogen before the total RNA was isolated. For the wounding experiments, one current-year shoot per tree was transversely cut with a razor blade every 1 cm along the shoot and the second cut was made after 2 h on the other side (Phillips et al. 2007).

Organelle-targeting analysis

The N-terminal 100 amino acid residues of PdDXSs, PdDXR and PdHDRs were amplified and fused to the pSMGFP vector. Protoplasts were isolated from *Arabidopsis*

Table 1. Primers used in this study.

Usage	Primer name	Sequence (5′→3′)
Conserved core sequence	DXS-F	ATNGGNGATGGWGTNATGAC
	DXS-R	TCAAACTTTSACAACWCCATG
	DXR-F	AAGCCHACDGTKGCWGCMATWGAA
	DXR-R	TCHACVGCYTTCTCATTDGCDGCR
	ABS-F	CATATGAYACWGCTTGGGTRGCAAG
	ABS-R	CTTCTDGCCTCCARMCTTGGCATAC
	HDR-F	CAAATTGCVTATGAAGCVAG
	HDR-R	ATRAGATCVAGCTTHTCCTT
RACE	Generacer 5′	CGACTGGAGCACGAGGACACTGA
	Generacer 3′ nested	CGCTACGTAACGGCATGACAGTG
	DXS1-F	AGGTTGCCAAGGGTGTTACCAAACAACTCG
	DXS1-R	CCAGCTCCTCAAACAGCGTGGAACGG
	DXS2-F	CATTGGACCTGTGGATGGGCACAACATT
	DXS2-R	CCTTGGCGGCTTCTCTGAGTTTGCGAAG
	DXR-F	ATCACCGTTGATTCTGCCACCTTG
	DXR-R	GGCCAATCCCTGAATGCCCCTCCAGAAG
	ABS-F	TTGCATTTCCGGGAGAAACCATCCTGG
	ABS-R	ACTGAGGCTTCTCAGAGCCATCAACTGCTG
	HDR1-F	GGACACTGACATGGACTGAAGGAGTA
	HDR1-R	AAGCTCATAAAATGTTCATTCACA
	HDR2-F	GGAAAAGACAATGATGCGTAGATTTGGTGT
	HDR2-R	ACCCACGGACAGGTTGTGTCCACAATCTGT
ORF-PCR	DXS1-F	ATGGCATCGTCTGCTGTAATCC
	DXS1-R	TCAAGACATTACTTGCAGTGCTTCTC
	DXS2-F	ATGGCAATTGCAAGCAGGGCAG
	DXS2-R	TTATCGGTGCTTCAGAAGAGCATCA
	DXR-F	ATGGCAGTGACAATTCCGCTGG
	DXR-R	TCAAACTGTGGCAGGCTCCAAGC
	ABS-F	ATGGCCTTGCCTTCCTCTTCATTGTC
	ABS-R	CTAAGCAACCGGGGGGAAAAGGGAG
	HDR1-F	ATGCATTCGAGCCTCAGCTTTGCA
	HDR1-R	TTATACCATCTGCAACGCCTCCTCATCC
	HDR2-F	ATGGCTCAAGCGTGCGCGGT
	HDR2-R	CTATGCTGCTTGCAGAACCTCTTCTCGTTT
Mature protein	DXS1-F	CGGGATCCTGCCAGTGCTTTATCTGACC
	DXS1-R	CGGGATCCTCAAGACATTACTTGCAGTGC
	DXS2-F	CGGAATTCTGTGGAGGTTGCTTCAAAGAG
	DXS2-R	CGGAATTCTTATCGGTGCTTCAGAAGAGC
	DXR-F	CGGGATCCTGCCTTTGAAAGAACTACACA
	DXR-R	CGGGATCCTCAAACTGTGGCAGGCT
	HDR1-F	CGGGATCCTAGAAGTGATGCTGCTCCTAG
	HDR1-R	CGGGATCCTTATACCATCTGCAACGCCT
	HDR2-F	CGGGATCCTCGATGCCACGGAGGA
	HDR2-R	CGGGATCCCTATGCTGCTTGCAGAACCT
QRT-PCR	DXS1-F	CCATTCTTCGGGACGTCAAAGCTACACACA
	DXS1-R	AACCAGACGATGAACAACCAGGAACAGCTGT
	DXS2-F	CAATTTTAGAGAAGATTAAAAGCATGCCGG
	DXS2-R	CGTTCAAAGCCAGGAAATGGGAGACATG
	DXR-F	ATGGCAGTGACAATTCCGCTGG
	DXR-R	TCAAACTGTGGCAGGCTCCAAGC
	ABS-F	AGCTCGCCAGCATCTACAGCGGCTG
	ABS-R	GAAATGGTGATCGATCCCTAGCCTCTCAAC
	HDR1-F	GTAGGACCAAGAACTTTAAGCAA
	HDR1-R	CCTGTATGTAACCTACAGCGCT
	HDR2-F	CAGAGTGCCCAAGTGAAGATGT
	HDR2-R	GACTCGGACGCCTCCACA
Targeting	DXS1-TA-F	CGGGATCCTATGGCATCGTCTGC
	DXS1-TA-R	CGGGATCCACCTCAAAAATTATGTCAGA
	DXS2-TA-F	CGGGATCCTATGGCAATTGCAAGC
	DXS2-TA-R	CGGGATCCTAATTAACGGTGTCAAGC

(continued on next page)

Table 1. Continued.

Usage	Primer name	Sequence (5′→3′)
	DXR-TA-F	CGGGATCCTATGGCAGTGACAATTCC
	DXR-TA-R	CGGGATCCCAATGTCTGAGTTCCG
	HDR1-TA-F	GCGGATCCAATGCATTCGAGCCTCAG
	HDR1-TA-R	GCGGATCCTCCTTGTGACCAAATCCCT
	HDR2-TA-F	GCGGATCCAATGGCTCAAGCGTGC
	HDR2-TA-R	GCGGATCCTACCCAAATCCTTTTCGTT

thaliana and transfected by a modified polyethylene glycol method (Abel and Theologis 1994). Each plasmid containing 20 μg DNA (1 μg μl^{-1}) was transformed into 300 μl of protoplast suspension (10^6 ml^{-1}), and the transformed protoplasts were incubated at 22 °C overnight in the dark. Expression of the fusion protein was observed with an MRC-1024 confocal laser scanning microscope system (Bio-Rad).

Bioinformatics analysis

Amino acid sequences of all genes were aligned with the ClustalW program (http://clustalw.genome.jp/) and viewed by the BioEdit program. Phylogenetic trees were constructed with the TreeTop phylogenetic tree prediction program (http://www.genebee.msu.su/services/phtree_reduced.html). In the bootstrap, the multiple alignments were resampled 100 times. Theoretical molecular masses and p*I* values were calculated with the Compute pI/M_w tool (http://ca.expasy.org/tools/pi_tool.html). The putative target location of the plant was predicted online with the ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), TargetP (http://www.cbs.dtu.dk/services/Tar getP) and PSORT (http://psort.ims.u-tokyo.ac.jp/form.html) programs. The full-length cDNA sequences of *PtDXS*s from the partial EST data (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine) were assembled with TIGR Gene Indices Clustering Tools (http://compbio.dfci.harvard.edu/tgi/software/).

Results

Cloning and sequence analysis of PdDXSs, PdDXR, PdABS and PdHDRs

Based on the conserved region of the plant enzymes, each primer pair (Table 1) was designed, and the conserved fragment of each gene was obtained. The fragment sizes were as follows: 517 bp for *PdDXS*, 561 bp for *PdDXR*, 1144 bp for *PdABS* and 705 bp for *PdHDR*. *PdDXS* and *PdHDR* appeared as two different sequences. A full-length cDNA including the open reading frame (ORF) of each gene was obtained by 3′- and 5′-RACE (Table 1). The ORF size, number of amino acid residues, theoretical M_r and p*I* of each protein are shown in Table 2. The proteins encoded by *PdDXS*1 (GenBank Accession No. EU439293), *PdDXS*2 (EU438118) and *PdDXR* (EU439294) showed homologies higher than 75% compared with other DXSs and about 80% identity with the other plant DXRs (data not shown). The PdHDRs (*PdHDR*1, EU439296; *PdHDR*2, EU439297) had a 75% homology with other plant HDRs (data not shown).

All *P. densiflora* proteins that were examined showed high homologies ranging between 93% and 97% with their corresponding *P. taeda* proteins. The primary structures of *P. densiflora* and *P. taeda* proteins differed by only 33, 46, 12 and 3 amino acid residues for DXS1, DXS2, HDR1 and HDR2, respectively (Figures 2 and 3). The protein encoded by *PdABS* (EU439295) had seven extra residues (DNQAKKL), stretching from positions 47 to 53, compared with the PtABS sequence (AY779541) (Ro and Bohlmann 2006).

Phylogenetic analysis

To determine the relationships between the cloned genes and their homologs, phylogenetic trees were constructed (Figure 4). Each copy of PdDXS and PtDXS paired with the class 1 and class 2 DXSs (Walter et al. 2002), respectively, formed a distinctive gymnosperm group within each class of DXS (Kim et al. 2006*c*). We labeled the genes as *PdDXS*1 and *PdDXS*2 according to their affinity toward class 1 and class 2 *DXS*s, and *PdHDR*s were similarly numbered based on their clustering with the *Ginkgo HDR*1 and *HDR*2 (Kim et al. 2008*a*). PdDXR belonged to the gymnosperm subclade, and PdHDRs also formed a gymnosperm

Table 2. Sequence sizes of genes and gene products obtained from *P. densiflora* needles.

Gene name	Full length (bp)	Open reading frame (bp)	Amino acid sequence (bp)	M_r (kDa)	p*I*
*PdDXS*1	2605	2124	707	76.0	6.40
*PdDXS*2	2406	2223	740	79.2	8.54
PdDXR	1765	1440	479	51.9	6.08
PdABS	2898	2577	858	97.9	5.57
*PdHDR*1	1917	1458	485	54.4	6.27
*PdHDR*2	1610	1464	487	54.7	5.85

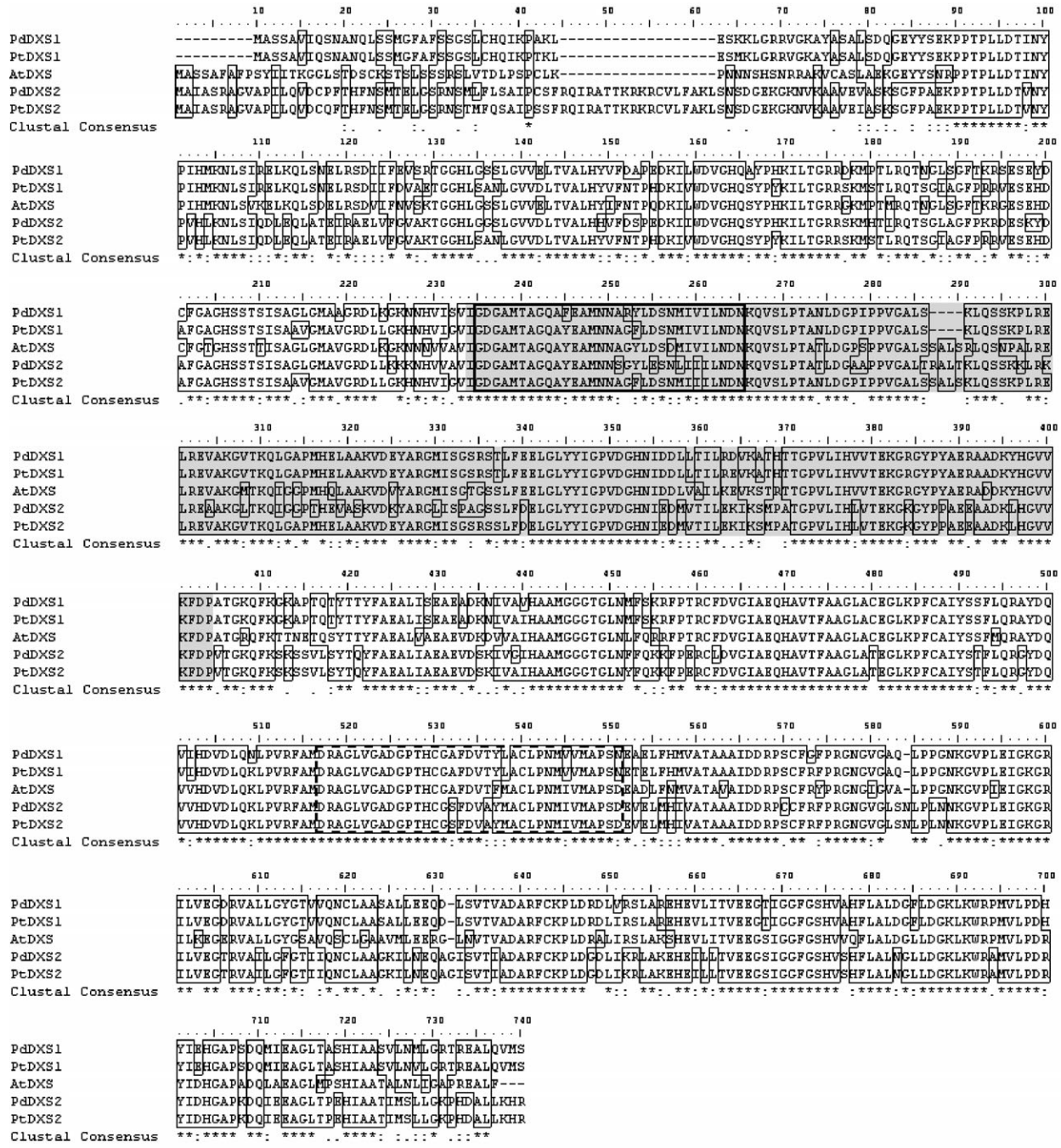

Figure 2. Comparison of the amino acid sequences of PdDXS1 and PdDXS2 with those of PtDXS1 (*P. taeda*, EU862298) and PtDXS2 (EU862297). Solid and dashed boxes represent the consensus TPP-binding and TK motif for the functional activities, respectively. The gray box is the conserved core region used in RACE.

subclade, which was again separated into two sub-subclades: type 1 HDR (HDR1) and type 2 HDR (HDR2).

Complementation assay

The *E. coli dxs* disruptant transformed with *PdDXS*2 (Figure 5) was able to grow in the absence of ME, whereas *PdDXS*1 and *PdDXR* did not complement the mutants (data not shown); however, this failure does not mean that the proteins encoded by these genes were not functional in planta. *PdHDR*1 and *PdHDR*2 successfully complemented the *E. coli lytB* disruptant (Figure 6). These results indicate that *PdDXS*2, *PdHDR*1 and *PdHDR*2 encoded functionally active enzymes.

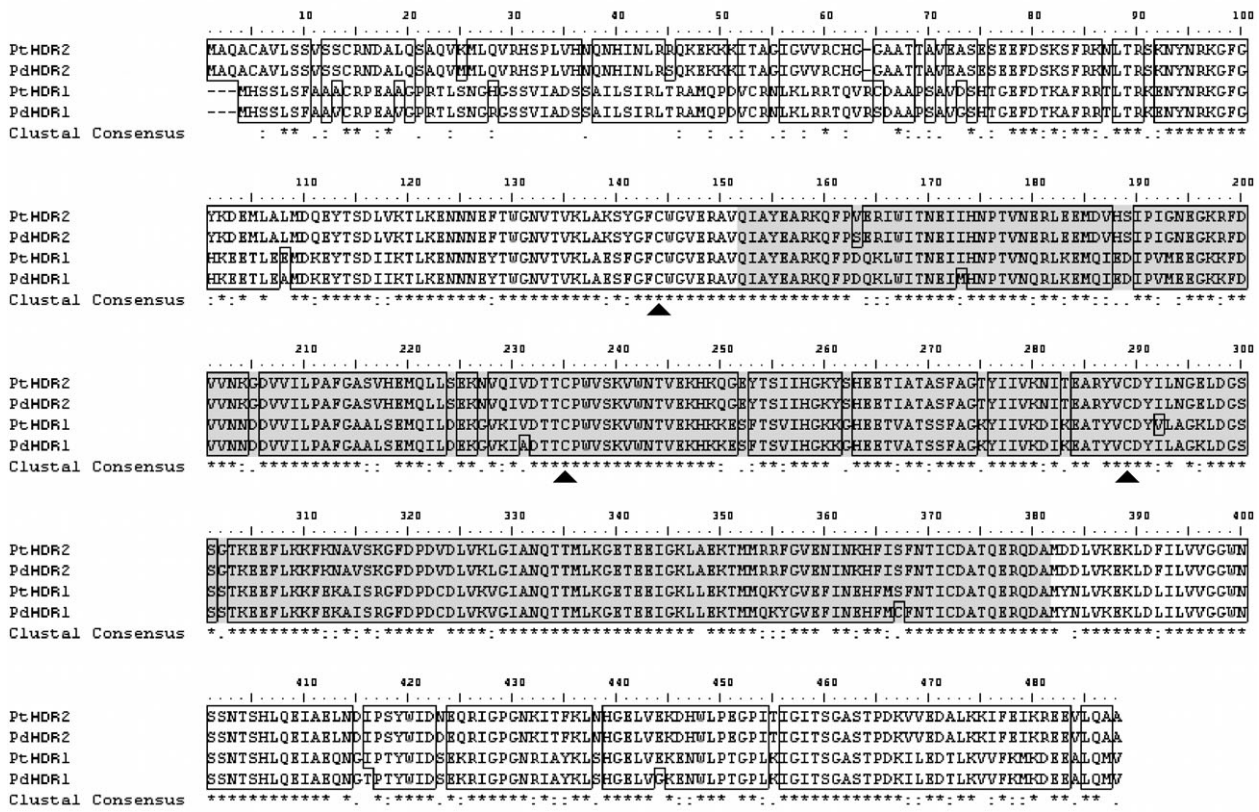

Figure 3. Amino acid sequences of *P. densiflora* HDRs aligned with those of *P. taeda* HDRs. The HDRs analyzed were: PdHDR1 (*P. densiflora*, EU439296), PdHDR2 (EU439297), PtHDR1 (*P. taeda*, EF095154) and PtHDR2 (EF095155). Conserved cysteine residues for functional activity are marked with dark triangles. The gray box is the HDR-conserved core region used for RACE.

Transcript levels among tissues and organs

Transcript abundances of *PdDXS*2, *PdHDR*2 and *PdABS* were two- to threefold higher in wood than in other tissues and organs (Figure 7). The level of *PdDXS*1 transcripts was also two- to sixfold higher in wood than in other tissues and organs; however, the resting level of *PdDXS*2 transcripts was about 50 times higher than that of the *PdDXS*1 transcripts in wood. The resting transcript levels of *PdHDR*1 in the leaves and in the bark were not substantially different from that in wood. Although the resting transcript levels of *PdHDR*1 and *PdHDR*2 in wood were comparable, the *PdHDR*2 transcript level was an order of magnitude higher in wood than in other organs. These results were consistent with the transcript level patterns of *PtHDR*s in the tissues of *P. taeda* reported by Kim et al. (2008*a*). The *PdABS* transcript occurred mostly in wood.

Induction of transcription by MeJA and wounding

Transcript levels of *PdDXS*s, *PdDXR*, *PdHDR*s and *PdABS* in wood were measured after treatment of trees with 1 mM MeJA (Figure 8). The treatment not only enhanced transcript levels of *PdDXS*2 and *PdHDR*2, but also those of *PdDXS*1, *PdHDR*1 and *PdDXR*. By the second day of the MeJA treatment, transcription of *PdHDR*2 in wood gradually increased by a factor of 10, and then decreased to the normal level on the third day. In contrast, on the second day of the MeJA treatment, the *PdHDR*1 transcript level showed only a twofold increase, whereas the *PdDXS*2 transcript level showed a 1300-fold increase, and the *PdDXS*1 transcript level increased by only 10-fold. Transcription of *PdABS* was initially suppressed, but then increased to surpass the normal level by the third day after MeJA treatment.

Changes in the transcript levels of the genes induced by mechanical wounding occurred earlier than those induced by MeJA (Figure 9). Transcript levels of *PdDXS*2, *PdDXR*, *PdHDR*1, *PdHDR*2 and *PdABS* reached maxima on the first day after wounding with increases of about two orders of magnitude, except for *PdIDR*1, which showed only a threefold increase before gradually decreasing to the normal level after the fifth day following wounding.

Although both the MeJA and wounding treatments enhanced the transcript levels of both isogenes of *PdDXS*s and *PdHDR*s, the relative degree of increases favored *DXS*2 and *HRD*2. Under the resting condition, the ratio of the transcript level of *PdDXS*2 to *PdDXS*1 was about 6, whereas that of *PdHDR*2 to *PdHDR*1 was close to 3.5 (Figure 7). The MeJA treatment increased the *PdDXS*2 to *PdDXS*1 ratio to 900 and the *PdHDR*2 to *PdHDR*1 ratio

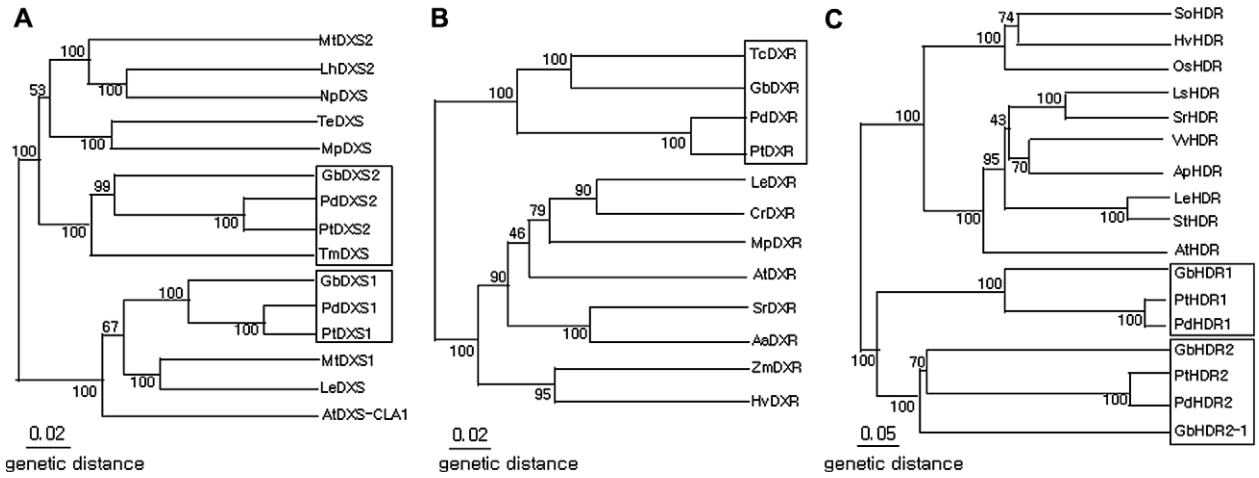

Figure 4. Phylogenetic trees of DXSs, DXRs and HDRs constructed with the Tree Top program. Boxed proteins are of gymnosperm origin. (A) DXS: MtDXS2 (*M. truncatula*, AJ430048), LhDXS2 (*L. hirsutum* Dunal, AY687353), GbDXS2 (AY494185), TeDXS (*T. erecta* L., AF251020), MpDXS (*M. piperita* L., AF019383), NpDXS (*N. pseudonarcissus* L., AJ279019), GbDXS1 (AY505128), TmDXS (*T. media* Rehder, AY505129), MtDXS1 (*M. truncatula*, AJ430047), LeDXS (*L. esculentum* Mill., AF143812), AtDXS-CLA1 (*A. thaliana*, U27099), PdDXS1 (EU439293), PdDXS2 (EU438118), PtDXS1 (*P. taeda*, EU862298) and PtDXS2 (EU862297). (B) DXR: TcDXR (*T. cuspidata* Siebold et Zucc., AY575140), GbDXR (AY494186), LeDXR (*L. esculentum*, AF331705), AtDXR (*A. thaliana*, NM_125674), ZmDXR (*Z. mays* L., AJ297566), HvDXR (*H. vulgare* L., AJ583446), MpDXR (*M. piperita*, AF116825), SrDXR (*S. rebaudiana* Bertoni, AJ429233), CrDXR (*C. roseus* (L.) G. Don, AF250235), AaDXR (*A. annua* L., AF182287), PdDXR (EU439294) and PtDXR (*P. taeda*, EU862299). (C) HDR: OsHDR (*O. sativa* L., AAT77894), ApHDR (*A. palaestina* Boiss., AAG21984), StHDR (*S. tuberosum* L., ABB55395), SrHDR (*S. rebaudiana*, ABB88836), LeHDR (*L. esculentum*, TC124188), VvHDR (*V. vinifera* L., TC32365), LsHDR (*L. sativa* L., TC12240), HvHDR (*H. vulgare* L., TC109922), SoHDR (*S. officinarum* L., TC14752), GbHDR1 (DQ251631), GbHDR2-1 (DQ251632), GbHDR2 (DQ252633), PtHDR1 (*P. taeda*, EF095154), PtHDR2 (*P. taeda*, EF095155), PdHDR1 (EU439296) and PdHDR2 (EU439297). The TC numbers were obtained from the Institute for Genomic Research (TIGR) through the TIGR Gene Indices Clustering Tools (http://www.tigr.org./tdb/tgi/software/). Solid box indicates gymnosperm.

to 20 on the second day (Figure 8). A similar effect was observed after the wounding treatment; the ratios of *PdDXS*2 to *PdDXS*1 and *PdHDR*2 to *PdHDR*1 increased to almost 300 and 70, respectively, on the first day (Figure 9). These results indicate that the transcript levels of *PdDXS*2 and *PdHDR*2 were selectively increased over those of *PdDXS*1 and *PdHDR*1 by both the MeJA and wounding treatments.

Intracellular-targeting analysis

All plant MEP pathway genes possess the N-terminal chloroplast transit peptide sequence that enables the transportation of proteins from the cytosol into the chloroplast (Rodríguez-Concepción and Boronat 2002). The smGFPs fused to the putative transit peptides of PdDXSs, PdDXR and PdHDRs appeared in the chloroplast of the *Arabidopsis* transient expression system, consistent with predictions made by the four transit sequence-predicting programs (data not shown).

Discussion

To study the regulation of diterpene resin biosynthesis in *P. densiflora* at the transcription level, we focused on the multicopy MEP pathway genes *DXS* and *HDR* as possible regulation points. *DXR* and *ABS* were used as the reference genes. The single-copy *DXR* serves in both primary and secondary metabolism, thus its transcription level should reflect the combined flux of the MEP pathway metabolites. The *ABS* gene product catalyzes the cyclization of geranylgeranyl diphosphate to abietadiene as the first committed step of resin biosynthesis. Abietadiene is the precursor of the major pine resin acids, abietic, neoabietic and dehydroabietic acids (Joye and Lawrence 1967). Therefore, the enzyme could serve as a site marker for resin biosynthesis.

Before the transcriptional regulation study, we characterized these genes from *P. densiflora* (Figures 2 and 3) and found that *PdDXS* and *PdHDR* occurred as multicopy genes. The presence of multicopy genes in the MEP pathway and their roles in primary and secondary metabolism have been the focus of several studies. For example, Walter et al. (2002) showed that there are two classes of *DXS* in plants: they reported that the class 2 *DXS* of *Medicago truncatula* Gaertn. is responsible for mycorrhizal carotenoid biosynthesis, whereas the class 1 *DXS* is involved in household functions. Kim et al. (2006*c*) and Phillips et al. (2007) demonstrated that the class 2 *DXS*s in *Ginkgo* and Norway spruce are specifically responsible for diterpene ginkgolide biosynthesis and monoterpene biosynthesis, respectively. The multicopy *HDR* may participate in the metabolic regulation of ginkgolide biosynthesis in the last step of the

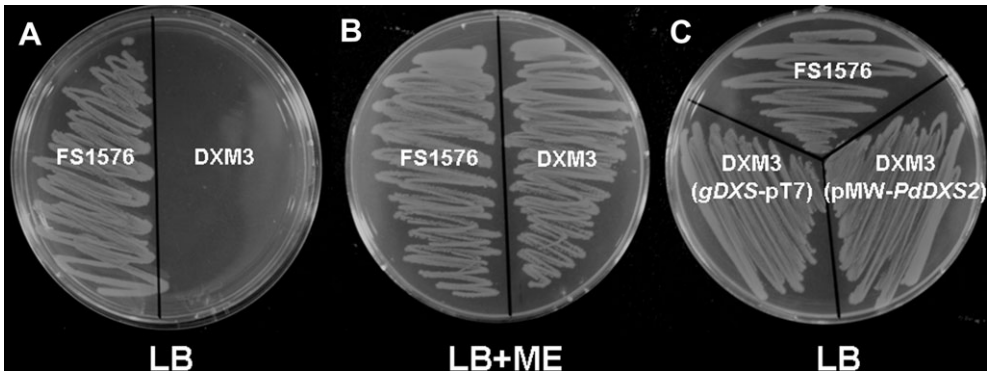

Figure 5. Complementation assay of *PdDXS*. (A) The wild-type *E. coli* strain FS1576 was grown on LB plates without ME. (B) DXM3, the *E. coli* DXS disruptant, was grown on LB plates with ME. (C) The transformants g*dxs*-pT7 and pMW-*PdDXS*2 were grown without ME. g*DXS*-pT7 served as the positive control. A color version of this figure is available as Supplementary Data at *Tree Physiology* Online.

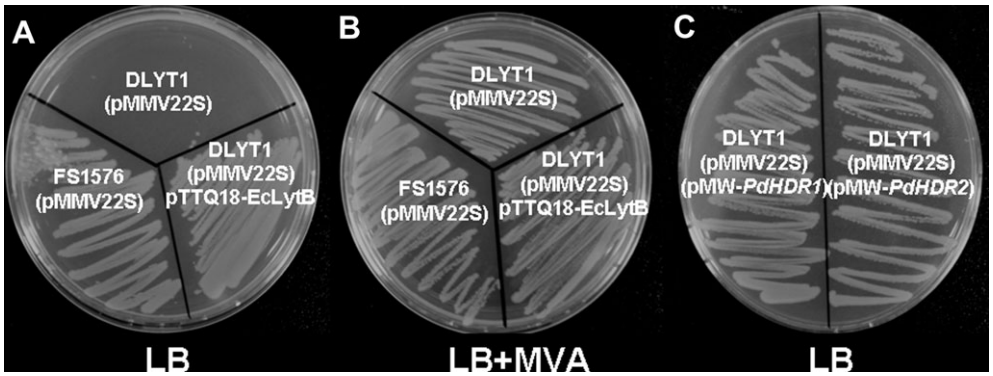

Figure 6. Complementation assay of *PdHDR*. (A) Wild-type *E. coli* strain FS1576 harboring the pMMV22S plasmid was grown on LB medium without mevalonate. DLYT1 (pMMV22S) was rescued by the pTTQ18-EcLytB plasmid when grown on medium without mevalonate. (B) DLYT1 (pMMV22S) was grown on LB medium containing 0.1% mevalonate. (C) DLYT1 (pMMV22S) was rescued by *PdHDR*1 and *PdHDR*2. A color version of this figure is available as Supplementary Data at *Tree Physiology* Online.

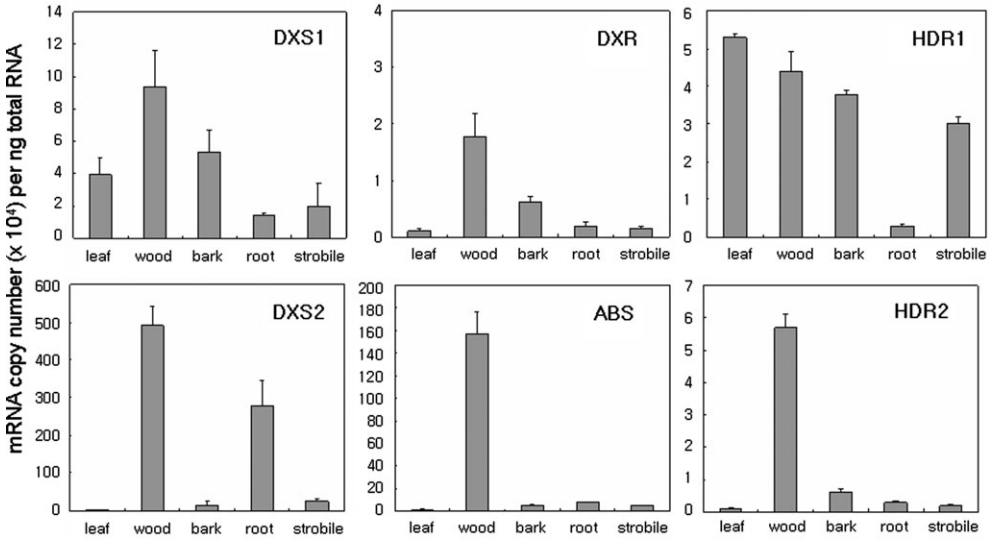

Figure 7. Transcript levels of *DXSs, DXR, ABS* and *HDRs* in *P. densiflora* organs and tissues.

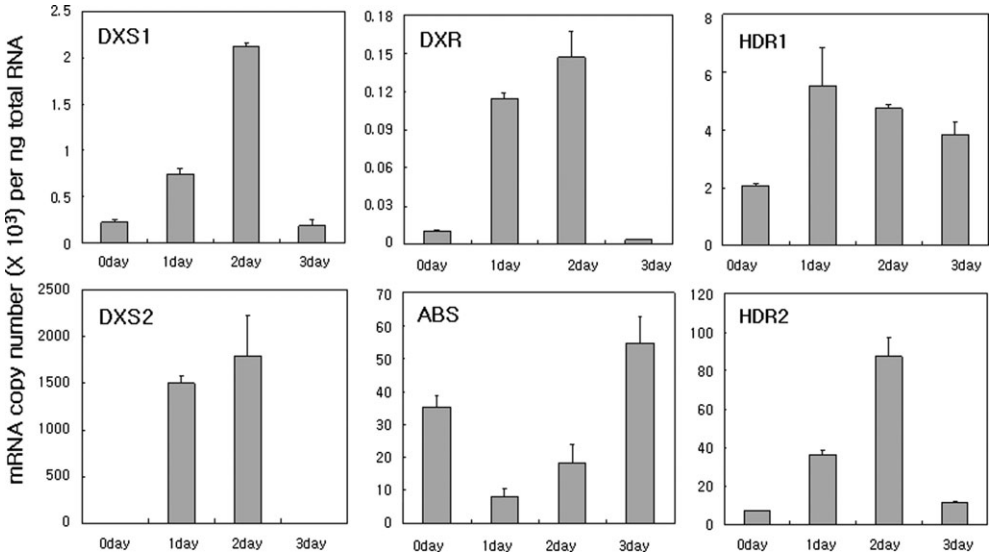

Figure 8. Transcript levels of *P. densiflora* genes after MeJA treatment.

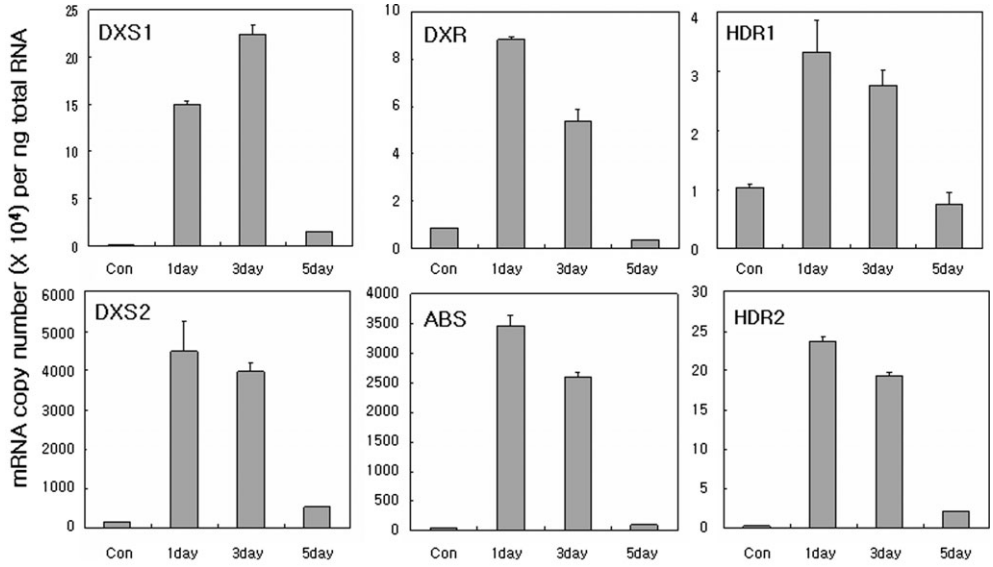

Figure 9. Transcript levels of *P. densiflora* genes after wounding treatment.

MEP pathway through differential transcription of its isogenes (Kim et al. 2008*a*). Differential control of *HDR* isogene transcript levels may provide a more precise manipulation of the metabolite flux to primary and secondary metabolism than the simple, single-point regulation at the DXS-catalyzed step. In *Ginkgo*, an additional regulation point in the MEP pathway, involving the multicopy CMK, has been suggested (Kim et al. 2008*b*). The presence of two copies each of *DXS* and *HDR* in *P. densiflora* suggests the coupling of resin biosynthesis with the specific transcription of one of the isogenes of both *DXS* and *HDR*, in this case *PdDXS*2 and *PdHDR*2. The wood, where the resin-forming cells are located (Trapp and Croteau 2001*a*), was the site of accumulation of transcripts of *PdABS* as well as *PdDXS*2 and *PdHDR*2 (Figure 7), further supporting the involvement of *PdDXS*2 and *PdHDR*2 in resin synthesis.

The phylogenetic trees of the MEP pathway enzymes showed that they belonged to a separate gymnosperm clade that was distinguishable from the angiosperm clade (Figure 4). Their positions in the phylogenetic trees and the order of the gene duplication events of the *P. densiflora* enzymes were consistent with those of the previous studies on *Ginkgo* enzymes (Kim et al. 2006*c*, 2008*a*, 2008*b*)

showing that the divergence of isoprenoid primary and secondary metabolism occurred before the separation of angiosperms and gymnosperms. Thus, the duplication of *PdDXS* into the primary-metabolism-specific class 1 *PdDXS* and the secondary-metabolism-specific class 2 *PdDXS* reflects the timing of this divergence. The differentiation of isoprenoid metabolism into primary and secondary metabolism in the common ancestor of angiosperms and gymnosperms is further supported by the findings of Trapp and Croteau (2001*b*), who concluded that the bifurcation of the terpene synthases in primary and secondary metabolism occurred before the separation of angiosperms and gymnosperms. In contrast, Kim et al. (2008*a*) reported that the multiplication of *GbHDR* occurred after the separation of angiosperms from gymnosperms, and this was corroborated in our study of *PdHDR*.

Pine species contain a network of resin ducts located throughout the wood and the bark, and the secretory epithelial cells are associated with the xylem (Trapp and Croteau 2001*a*). Methyl jasmonate and wounding treatments, which mimic pathogen invasion and insect herbivory, respectively, are known to induce the accumulation of transcripts of the gene coding for terpene synthase (Fäldt et al. 2003, Miller et al. 2005) and to enhance terpene biosynthetic enzyme biosynthesis (Steele et al. 1998, Martin et al. 2002) in conifers. In *Pinus*, the mechanical wounding significantly increases the total amount of resin acid and causes swelling of the resin-forming epithelial cells within 2 days after wounding, accompanied by an increased number of plastids (Walter et al. 1989). In *Pinus sylvestris* L., MeJA treatment not only increases the concentration of resin acids but also increases the number of resin ducts in the newly developing xylem (Heijari et al. 2008). Therefore, if the class 2 *DXS* and the type 2 *HDR* are both operating in the resin-specific MEP pathway of *P. densiflora*, the MeJA and the wounding treatments should induce the preferential transcription of these genes in wood. For this reason, we examined the changes in gene transcription levels in response to the MeJA and wounding treatments only in wood. The MeJA treatment caused a gradual accumulation of MEP pathway gene transcripts, whereas actual resin biosynthesis, as represented by the *PdABS* transcript level, was suppressed at the early stage of the response (Figure 8). The delayed increase in the accumulation of *PdABS* transcripts coincided with the return of the MEP pathway gene transcript levels to their resting values. This pattern suggests that the MeJA treatment induced the accumulation of the building blocks required for the de novo biosynthesis of resin. In contrast, wounding exerted an immediate effect on all transcript levels that were analyzed such that the maximal levels of *PdDXS*2, *PdHDR* and *PdABS* transcripts were attained on the first day after treatment (Figure 9), consistent with the physiological need for immediate resin production to prevent the entry of the pathogens and attacking insects through the wound. These results are also consistent with those of the previous studies on Norway spruce (*Picea abies* L.) where both MeJA treatment and mechanical wounding increased the transcript levels of the terpene synthase gene family and the CYP720-type *CYP450* genes that encode the enzymes catalyzing the late steps of terpenoid biosynthesis (Fäldt et al. 2003).

The differential accumulation of *PdDXS*2 and *PdHDR*2 transcripts over *PdDXS*1 and *PdHDR*1 transcripts in wood in response to the MeJA and the wounding treatments again indicated the involvement of *PdDXS*2 and *PdHDR*2 in resin production. Moreover, the concomitant increase in *PdABS* transcripts, a marker of resin acid biosynthesis, and the well-documented increase in resin concentration (Walter et al. 1989, Heijari et al. 2008) by the MeJA and wounding treatments provide strong support for the interpretation that diterpene resin biosynthesis in *P. densiflora* is regulated by the differential transcription of class 2 *DXS* and type 2 *HDR* in wood. This finding is reminiscent of the specific involvement of *DXS*2 and *HDR*2 in ginkgolide biosynthesis in *Ginkgo* (Kim et al. 2006*c*, Kim et al. 2008*a*) and *DXS*2 in monoterpene biosynthesis in *P. abies* (Phillips et al. 2007).

Monoterpenoids and diterpenoids are mostly produced in plastids from isoprene building blocks synthesized by the MEP pathway. All MEP pathway genes of plant origin encode enzymes that have the N-terminal chloroplast transit peptide sequence, which enables the transportation of these proteins from the cytoplasm to the chloroplast (Croteau et al. 2000). We used smGFP as the reporter protein fused to the putative transit peptides of each *P. densiflora* MEP pathway enzyme in the transient expression assays. The transit peptides of PdDXSs, PdDXR and PdHDRs delivered GFP to the chloroplasts (Figure 10), and the unfused smGFP appeared in the cytoplasm (data not shown). ABS is also known to occur in the chloroplast (Ro and Bohlmann 2006), which means that the initial cyclization of GGPP in resin acid synthesis and biosynthesis of the building blocks take place in the same organelle. In *Ginkgo*, *GbMECT*, *GbMECS*, *GbHDR*2, *GbCMK*2 and *GbHDR*2-1 are expressed in chloroplasts (Kim et al. 2006*a*, 2006*b*, 2008*a*, 2008*b*); however, *GbHDR*1 (Kim et al. 2008*a*) and *GbCMK*1 (Kim et al. 2008*b*) were also detected in the cytoplasm and the nucleus. Neither *PdHDR*1 nor *PdHDR*2 displayed such unexpected behavior.

In conclusion, we cloned the *P. densiflora* genes that encode DXS, DXR and HDR, the enzymes catalyzing the first, second and the last step, respectively, of the MEP pathway. Among the examined genes, *DXS* and *HDR* were found to be two-copy genes. Transcript levels of *PdDXS*2 and *PdHDR*2 were highest in wood, where resin production occurs. The transcript levels of *PdDXS*2 and *PdHDR*2 showed preferential increases over those of *PdDXS*1 and *PdHDR*1 in response to the MeJA and wounding treatments. In the protein-targeting experiment, PdDXSs, PdDXR and PdHDRs all appeared in the chloroplast. These observations indicate that PdDXS2 and PdHDR2 are closely correlated with diterpenoid resin production in *P. densiflora*.

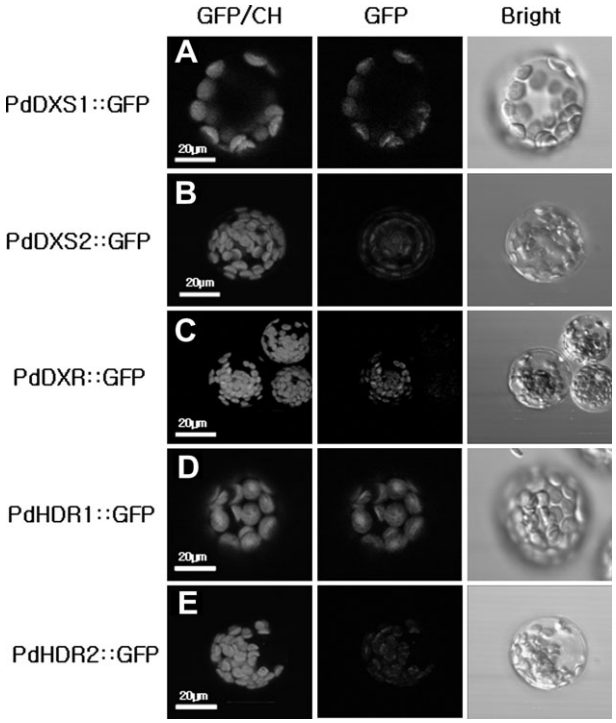

Figure 10. Intracellular localization of the fusion constructs of N-terminal residues of DXSs, DXR and HDRs with smGFP. One hundred N-terminal residues of DXSs, DXR and HDRs from *P. densiflora* were fused in-frame to smGFP and introduced into *Arabidopsis* protoplasts. Abbreviations: GFP/CH, merged images of GFP and chlorophyll auto-fluorescence; GFP, green fluorescence protein images; bright, bright field images. A color version of this figure is available as Supplementary Data at *Tree Physiology* Online.

Supplementary Data

Supplementary data for this article are available at *Tree Physiology* Online.

Acknowledgments

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