Plant Cell Physiol. 47(7): 905–914 (2006)
doi:10.1093/pcp/pcj063, available online at www.pcp.oxfordjournals.org

Distinct Roles of Cinnamate 4-hydroxylase Genes in *Populus*

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Cinnamate 4-hydroxylase (C4H) catalyzes the conversion of cinnamate into 4-hydroxy-cinnamate, a key reaction of the phenylpropanoid pathway which leads to the biosynthesis of several secondary metabolites. *C4H* genes exist as a multigene family in various plant species. In order to understand the roles of individual *C4H* members, four *C4H* cDNAs (*PtreC4H*) were isolated from *Populus tremuloides* and three *C4H* loci (*PtriC4H*) were identified in the *P. trichocarpa* genome. The ability of *Populus* C4H isoforms to convert *trans*-cinnamate into *p*-coumaric acid was verified by the examination of yeast recombinant PtreC4H proteins. *Populus C4H* genes were expressed in various tissues, including developing xylem, phloem and epidermis; however, the expression patterns of individual members were different from each other. Sequential analysis of *C4H* promoters showed that the differential expression of *C4H* genes was associated with *cis*-acting regulatory elements such as box L, box P and H box, suggesting that the divergent C4H isoforms played distinct roles in the production of secondary metabolites. The involvement of specific C4H isoforms in the biosynthesis of guaiacyl and syringyl monolignols is discussed.

Keywords: Cinnamate 4-hydroxylase (C4H) — Lignin — Phenylpropanoid — *Populus tremuloides* — *Populus trichocarpa*.

Abbreviations: C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate coenzyme A:ligase; CPR, NADPH-cytochrome P450 reductase; Ct, threshold cycle; CTAB, cetyltrimethylammonium bromide; DIG, digoxigenin; G, guaiacyl; MSD, mass spectrometer detector; ORF, open reading frame; PAL, phenylalanine ammonia-lyase; RT–PCR, reverse transcription–PCR; S, syringyl; UTR, untranslated region.

The nucleotide sequences reported in this paper have been submitted to the GenBank database under accession numbers DQ522292 (*PtreC4H1-1*), DQ522293 (*PtreC4H1-2*), DQ522294 (*PtreC4H2-1*), DQ522295 (*PtreC4H2-2*), DQ522296 (*PtreC4H1p1*), DQ522297 (*PtreC4H1p2*) and DQ522298 (*PtreC4H2p*).

Introduction

The core reactions of phyenylpropanoid metabolism that produces important secondary metabolites participating in plant development and defense responses (Hahlbrock and Scheel 1989) involve three enzymes, phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), cinnamate 4-hydroxylase (C4H, EC 1.14.13.11) and 4-coumarate coenzyme A:ligase (4CL; EC 6.2.1.12) (Mizutani et al. 1997). PAL catalyzes the deamination of phenylalanine to produce *trans*-cinnamic acid, which is converted to *p*-coumaric acid by an oxidative reaction catalyzed by a cytochrome P450 enzyme, C4H. A thioesterification is then activated by 4CL and the product, *p*-coumaroyl-CoA, is funneled into one of the branched pathways leading to the production of cell wall constituents (lignins), pigments (flavonoids), UV protectants (coumarins) and plant defense compounds (isoflavonoids, furanocoumarins and norlignans) (Whitbred and Schuler 2000, Suzuki et al. 2002, Suzuki et al. 2004).

Genes encoding PAL, C4H and 4CL have been cloned from various plant species (for a recent review, see Li et al. 2006). *PAL* genes present as a small gene family in plants, such as Arabidopsis (Raes et al. 2003) and raspberry (Kumar and Ellis 2001). Divergent PAL genes, exhibiting tissue-specific expression in plants, have been suggested to function in specific metabolic milieus (Kao et al. 2002). For example, one *PAL* gene isolated from *Populus tremuloides* is associated with condensed tannin metabolism, whereas another is involved in monolignol biosynthesis (Kao et al. 2002). Similar to *PAL*, multiple *4CL* gene members have been found in Arabidopsis (Raes et al. 2003), quaking aspen (Hu et al. 1998), tobacco (Lee and Douglas 1996), *P. trichocarpa* (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html), and others. The divergent *4CL* genes have also been found to play a role in the biosynthesis of specific secondary metabolites. In Arabidopsis, *At4CL1* and *At4CL2* function in monolignol biosynthesis (Ehlting et al. 1999, Raes et al. 2003), *At4CL3* activates the biosynthesis of flavonoid (Ehlting et al. 1999), whereas *At4CL4* is most probably related to sinapate activation (Hamberger and Hahlbrock 2004). Additionally, two *4CL* genes isolated from quaking aspen display distinct expression patterns. One presents primarily in developing xylem, while the other

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is expressed in epidermal and leaf tissues (Hu et al. 1998). The results from *PAL* and *4CL* studies indicate that *C4H* genes could also exist as a multigene family in various plant species and the divergent isoforms could play distinct roles in secondary metabolisms.

So far, the *C4H* gene has been studied in several plant species. In Arabidopsis, only one *C4H* gene (*AtC4H*) has been described (Raes et al. 2003). *AtC4H* is expressed in all tissues and responds to light, wounding and fungal infection (Bell-Lelong et al. 1997, Mizutani et al. 1997, Nair et al. 2002, Raes et al. 2003), indicating that it plays diverse functions in phyenylpropanoid metabolism. Conversely, C4Hs are encoded by a small gene family in alfalfa (Fahrendorf and Dixon 1993), maize (Potter et al. 1995), orange (Betz et al. 2001), pea (Whitbred and Schuler 2000), periwinkle (Hotze et al. 1995) and hybrid aspen (Kawai et al. 1996). However, little is known about the roles of individual C4H isoforms in the production of secondary metabolites (Betz et al. 2001). Here we report the characterization of four C4Hs in *P. tremuloides* and three in *P. trichocarpa*. We verified their biochemical functions using yeast recombinant proteins and analyzed their differential expression by in situ hybridization, reverse transcription–PCR (RT–PCR) and real-time PCR. Interestingly, we found that the differential expression of *C4H* genes is associated with *cis*-acting regulatory elements, suggesting that the divergent C4H isoforms play distinct roles in phyenylpropanoid metabolism in *Populus*.

Results

Isolation of PtreC4H cDNAs in aspen developing xylem

In order to isolate multiple *C4H* cDNAs, we screened an aspen developing xylem cDNA library under low stringency using ^{32}P-labeled aspen *C4H* cDNA (Ge and Chiang 1996) as a probe. A total of 30 positive clones were identified. Sequence analysis revealed four different *C4H* cDNA clones, which were named *PtreC4H1-1* (accession No. DQ522292), *PtreC4H1-2* (accession No. DQ522293), *PtreC4H2-1* (accession No. DQ522294) and *PtreC4H2-2* (accession No. DQ522295), respectively. Each of the four *PtreC4H* genes has an open reading frame (ORF) of 1,515 bp coding for a 505 amino acid protein, which contains an N-terminal hydrophobic domain consisting of 21 amino acids, four conserved domains identified in eukaryotic P450s and a heme-binding motif (Kalb and Loper 1988). The putative PtreC4H proteins have a range of 85–99% amino acid sequence identities to the C4Hs in Arabidopsis (Bell-Lelong et al. 1997, Mizutani et al. 1997), *Helianthus tuberosus* (Teutsch et al, 1993), mung bean (Mizutani et al. 1993), alfalfa (Fahrendorf and Dixon 1993), *Catharanthus roseus* (Hotze et al. 1995), parsley (Logemann et al. 1995), hybrid poplar (Ro et al. 2001) and *Populus kitakamiensis* (Kawai et al. 1996).

The four *PtreC4H* cDNAs could be divided into two groups according to sequence comparison. *PtreC4H1-1* and *PtreC4H1-2* showing 99% sequence identity were grouped and named *PtreC4H1* genes. Similarly, *PtreC4H2-1* and *PtreC4H2-2* were grouped and named *PtreC4H2* genes. *PtreC4H1-1* and *PtreC4H1-2* or *PtreC4H2-1* and *PtreC4H2-2* could be allelic variations. The homology between *PtreC4H1* and *PtreC4H2* genes is 91% at the nucleotide level and 97% in their deduced amino acid sequences in the coding region. However, the difference is substantial in the 5′- and 3′-untranslated regions (UTRs). In these regions, approximately 60–70% sequence homology was found between *PtreC4H1* and *PtreC4H2* genes. The divergent sequences in the non-coding regions indicated that the *PtreC4H1* and *PtreC4H2* genes could be regulated differently in various tissues.

Biochemical characterization of PtreC4H proteins

Co-expression of four highly similar *PtreC4H* genes in xylem made us wonder whether they are capable of converting *trans*-cinnamate into *p*-coumaric acid, since the substrate specificity of *PtreC4H*, as a P450, could be changed by a single amino acid substitution (Lindberg and Negishi 1989). To address this question, we analyzed the biochemical function of *PtreC4H* proteins. Protein-coding regions of *PtreC4H1-1* and *PtreC4H2-1* were cloned into a yeast expression vector pYES2.1 under the control of a galactose-inducible GAL1 promoter and expressed in a *Saccharomyces cerevisiae* strain INVSC2(CPR) which carried the *Arabidopsis* NADPH-P450 reductase gene and was previously constructed in our laboratory (Osakabe et al. 1999). A *PtreC4H1-1* and *PtreC4H2-1* activity assay was conducted by a modified in vivo method (Schoendorf et al. 2001, Shimada et al. 2001). Induced culture of the yeast transformants which carried *PtreC4H1-1*, *PtreC4H2-1* or control vector pYES2.1 was incubated with the C4H substrate, *trans*-cinnamate. The ether extracts resulting from these incubations were analyzed by HPLC-UV/mass spectrometer detector (MSD) (Osakabe et al. 1999). As shown in Fig. 1, the substrate *trans*-cinnamate was converted into *p*-coumaric acid in yeast strains carrying *PtreC4H1-1* or *PtreC4H2-1*. There was no such conversion in the yeast strain transformed with control vector, pYES2.1. From this result, we concluded that both *PtreC4H1-1* and *PtreC4H2-1* encode active C4Hs.

In situ expression analysis of PtreC4H genes

To investigate the expression profile of *C4H* genes in *P. tremuloides*, in situ hybridization was performed using digoxigenein (DIG)-labeled sense and antisense RNA probes corresponding to 0.7 kb of the 5′ end of *PtreC4H1-1*.

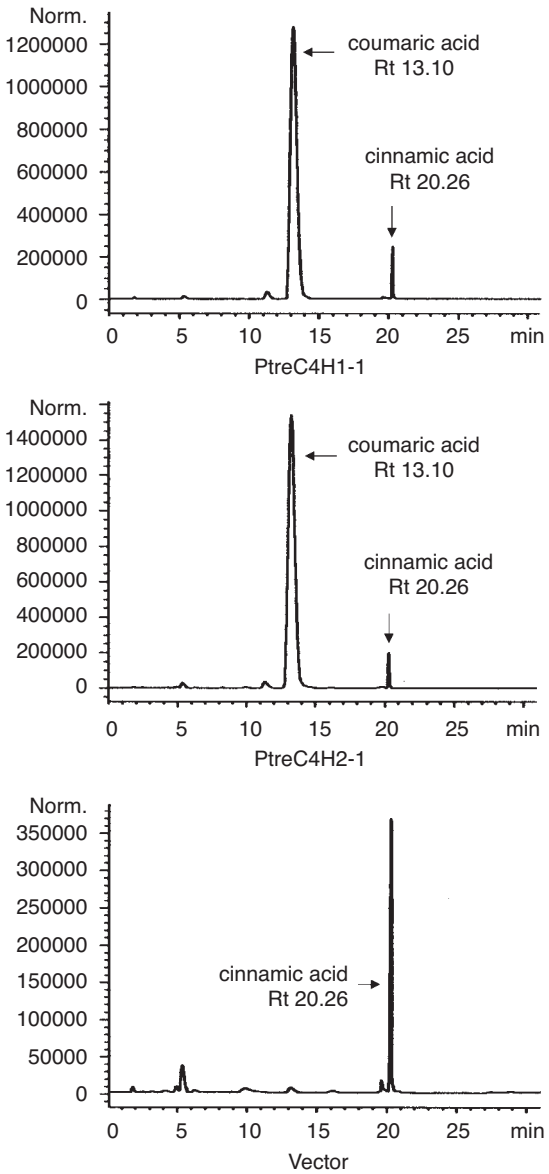

Fig. 1 HPLC-UV/MS determination of the catalytic activities of C4H recombinant proteins. *Saccharomyces cerevisiae* strain INVSC2(CPR) (Osakabe et al. 1999) harboring *PtreC4H1-1*, *PtreC4H2-1* or control vector (pYES2.1/V5-His-TOPO, Invitrogen) was firstly incubated in galactose-containing medium for induction of *CPR* and *PtreC4H* recombinant proteins and then in the medium supplemented with *trans*-cinnamate for determination of C4H activities. The reaction products were analyzed by HPLC-UV/MS. *Trans*-cinnamate was apparently converted to *p*-coumaric acid in yeast cells carrying *PtreC4H1-1* or *PtreC4H2-1*. No conversion was detected in cells transformed with control vector.

As *PtreC4H* genes shared a high level of homology, the hybridization signals detected by the *PtreC4H1-1* probe might reflect the collective expression of the four *PtreC4H* genes. As shown in Fig. 2, *PtreC4H* transcripts were not detected in young stems (the fourth internode, Fig. 2A) and young leaves (the third and sixth leaves, Fig. 2G, H) of *P. tremuloides* plants, suggesting that the expressional levels of *C4H* genes in these tissues are low; however, in the older tissues (Fig. 2B–D, F, I), the transcripts are abundant in developing xylem, phloem and epidermis tissues, suggesting that C4H isoforms have a broad range of physiological functions in plants.

Quantitative real-time PCR analysis of PtreC4H expression

Because of the high level of sequence homology among *PtreC4H* cDNAs, it is difficult to distinguish the transcripts of individual *PtreC4H* genes by in situ hybridization. To solve this problem, gene-specific RT–PCR was conducted using a gene-specific forward primer located in the 5′ UTR and a reverse primer located in the coding region. The results showed that the expression of the *PtreC4H1* genes was the most abundant in developing xylem, followed by phloem and mature leaf tissues; however, *PtreC4H2* genes were expressed strongly in developing xylem, more weakly in phleom, and were undetectable in mature leaf tissues (Fig. 3).

To analyze quantitatively the differential expression of divergent *PtreC4H* genes further, real-time PCR using gene-specific primers located in the coding region was carried out. As shown in Fig. 4, the fold changes of expression of the *PtreC4H2* genes in xylem compared with those in young leaves or phloem were apparently higher than those of *PtreC4H1* genes, although both *PtreC4H1* and *PtreC4H2* genes were highly expressed in xylem, suggesting that *PtreC4H2* genes were more xylem specific.

Identification and real-time PCR analysis of C4H genes in P. trichocarpa

Although we applied a low stringency screening strategy when isolating *PtreC4H* cDNAs, it might not be capable of collecting all the *C4H* genes in *P. tremuloides*. With the availability of the *P. trichocarpa* genome sequence, which has been predicted at this stage to contain approximately 58,036 gene models (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html), we were able to analyze the genomic constitution of *C4H* genes in a tree species.

In the *Populus* genome, we identified three *C4H* homologs as putative *PtriC4H* genes. Their gene models are estExt_Genewise1_v1.C_LG_XIX2612, eugene3.00131281 and fgenesh1_pg.C_scaffold_164000060, respectively (Table 1). Sequence comparison of the three *PtriC4H* gene models revealed that estExt_Genewise1_v1.C_LG_XIX2612 and eugene3.00131281 showed 92% homology at the nucleotide level and 97% identity at the amino acid level, while fgenesh1_pg.C_scaffold_164000060 had low homology (about 66%) at both the nucleotide and amino acid levels to estExt_Genewise1_v1.C_LG_XIX2612

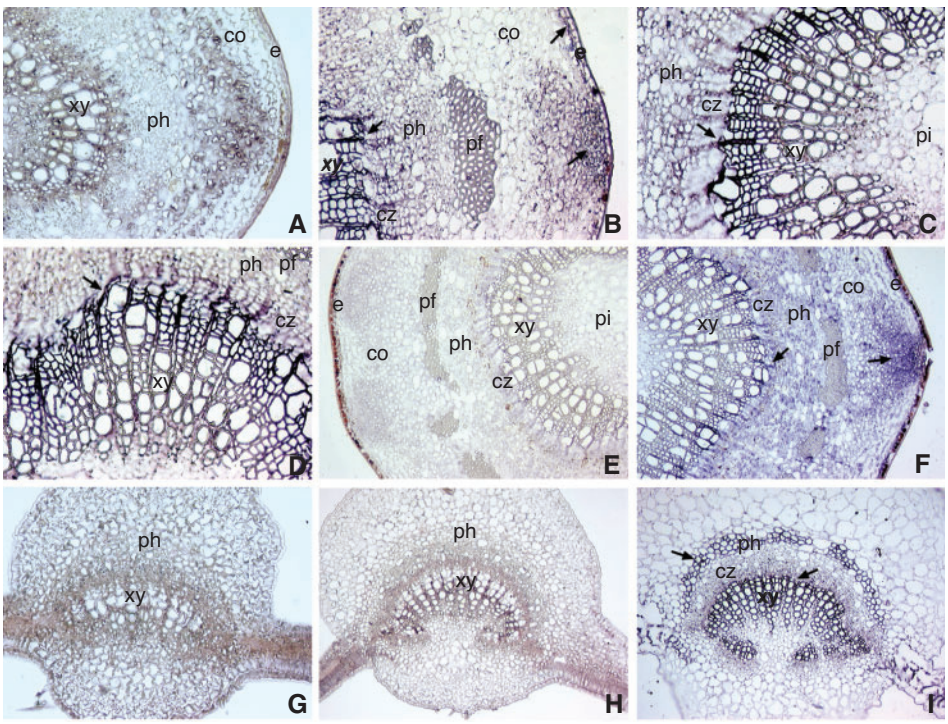

Fig. 2 In situ hybridization of *PtreC4H* genes. In situ localization was performed using DIG-labeled sense (E) and antisense (A–D and F–I) RNA probes (corresponding to 0.7 kb of the 5′ region of *PtreC4H1-1*). The internodes 4 (A), 8 (B and C), 12 (D and E) and 14 (F), and leaves 3 (G), 6 (H) and 14 (I) of several young *Populus tremuloides* plants grown in a greenhouse were examined. The expression of *PtreC4H* genes was detected in developing xylem, phloem and epidermis tissues, and in adaxial cells (arrows) of older tissues. *PtreC4H* genes were also expressed in rapidly dividing cortical cells (B and F, arrows) that would later develop into lenticels. co, cortex; cz, cambial zone; e, epidermis; ph, phloem; pf, phloem fiber; pi, pith; xy, xylem.

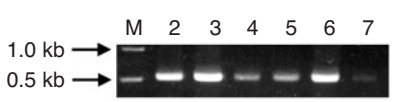

Fig. 3 RT–PCR analysis of *PtreC4H* genes. Total RNA from phloem (lanes 2 and 5), xylem (lanes 3 and 6) or leaf (lanes 4 and 7) tissues was reverse transcribed and amplified using *PtreC4H1* (lanes 2–4) or *PtreC4H2* (lanes 5–7) gene-specific primers. Molecular marker (M) and molecular weight (arrows) are indicated.

or eugene3.00131281. Comparing the *PtriC4H* genes with the isolated *PtreC4H* cDNAs, we found that estExt_Genewise1_v1.C_LG_XIX2612 showed the highest homology (about 98%) with *PtreC4H1-1* and *PtreC4H1-2*, while eugene3.00131281 was more similar to *PtreC4H2-1* and *PtreC4H2-2* showing about 98% homology. The homology between fgenesh1_pg.C_scaffold_164000060 and four *PtreC4H* genes was only about 66%. Therefore, estExt_Genewise1_v1.C_LG_XIX2612, eugene3.00131281 and fgenesh1_pg.C_scaffold_164000060 were designated *PtriC4H1*, *PtriC4H2* and *PtriC4H3*, respectively (Table 1).

To analyze the *PtriC4H* gene expression in *P. trichocarpa*, we extracted total RNAs from eight different tissues, including young leaves, mature leaves, young stems,

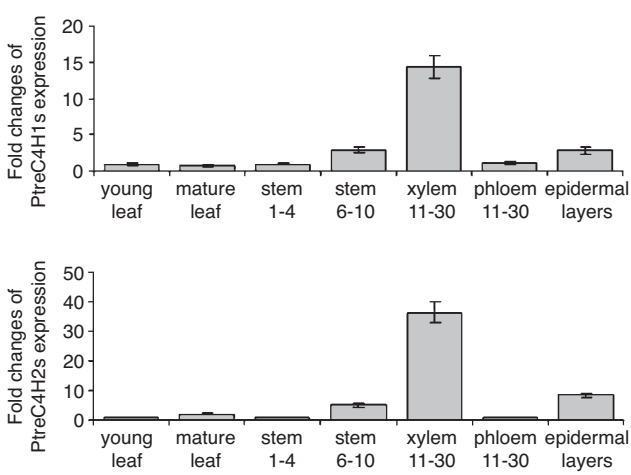

Fig. 4 Real-time PCR analysis of *PtreC4H* genes. Transcripts of *PtreC4H* genes in young leaves (first–fourth from the top), mature leaves (12th and 13th from the top), young stems (first–fourth from the top), maturing stems (sixth–10th from the top), and developing xylem, phloem and epidermal layers (from the 11th to the 30th internode) of *P. tremuloides* were quantified by quantitative real-time PCR and normalized to the level of 18S rRNA in the sample. Error bars represent the standard deviations of three PCR replicates of a single reverse transcription reaction. The normalized transcripts in young leaves were set arbitrarily to 1.

Table 1 *C4H* genes in the genome of *P. trichocarpa*

Genes	Gene models	Gene locations
PtriC4H1	estExt_Genewise1_v1.C_LG_XIX2612	LG_XIX:10989564–10993103
PtriC4H2	eugene3.00131281	LG_XIII:12820991–12825368
PtriC4H3	fgenesh1_pg.C_scaffold_164000060	scaffold_164 : 432544–434026

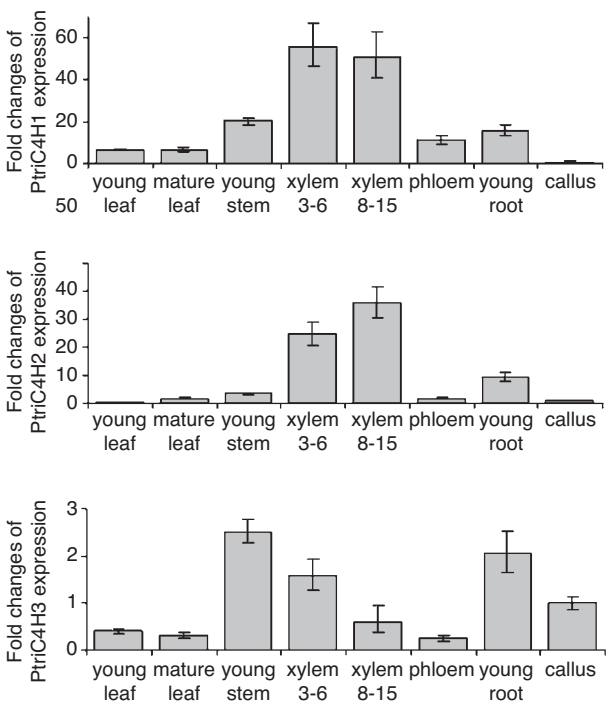

Fig. 5 Real time PCR analysis of *PtriC4H* genes. Transcripts of *PtriC4H* genes in young leaves, mature leaves, young stems, developing xylem from the third to the sixth internode, developing xylem from the eighth to the 15th internode, phloem, young roots (1–3 cm) and callus tissues of *P. trichocarpa* (Nisqually-1) were quantified by quantitative real-time PCR and normalized to the level of 18S rRNA in the sample. Error bars represent the standard deviations of three PCR replicates of a single reverse transcription reaction. The normalized transcripts in calli were set arbitrarily to 1.

developing xylem from the third to the sixth internode, developing xylem from the eighth to the 15th internode, phloem, young roots (1–3 cm) and callus tissues. We then detected transcripts of the individual *PtriC4H* genes in those total RNAs by quantitative real-time PCR (Li et al. 2005). The expression patterns of *PtriC4H* genes were found to be distinct from each other (Fig. 5). *PtriC4H1* was strongly expressed in the developing xylem from the third to the sixth internode and the expression was at lower levels in the developing xylem from the eighth to the 15th internode, young stems, young roots, phloem, young leaves and mature leaves. The expression of *PtriC4H1* in calli was undetectable. In contrast, the transcripts of *PtriC4H2* in the xylem from the eighth to the 15th internode were significantly more abundant than that in the xylem from the third to the sixth internode, although both *PtriC4H1* and *PtriC4H2* were expressed strongly in the xylem tissues. However, the expression pattern of *PtriC4H3* was very different; it was strongly expressed in the young stems and young roots.

Analysis of Populus C4H gene promoters

In order to understand the regulatory mechanisms underlying different expression patterns of *Populus C4H* genes, the promoter sequences from the *PtreC4H* genes were isolated through genome walking. The promoter sequences of *PtreC4H1-1* and *PtreC4H1-2* were highly similar to each other. However, the homology between group *PtreC4H1* and *PtreC4H2* promoters was in the range of 51–55%. The divergent upstream sequences among the two group *PtreC4H* genes suggested that the mechanisms of gene regulation might be different.

We also analyzed the promoter sequences of the *PtriC4H* genes in the *P. trichocarpa* genome database at http://genome.jgi-psf.org/Poptr1/Poptr1.home.html. A 1.1 kb sequence upstream of the coding sequence was extracted from each of the *PtriC4H* genes, named *PtriC4H1p*, *PtriC4H2p* and *PtriC4H3p*, respectively. Comparison of the promoter sequences indicated that *PtriC4H3p* has 44–45% homology with both *PtriC4H1p* and *PtriC4H2p*; and *PtriC4H1p* has 55% homology with *PtriC4H2p*. Meanwhile it was found that *PtriC4H1p* is highly homologous to *PtreC4H1p* (about 83% sequence homology) and *PtriC4H2p* is similar to *PtreC4H2p* (about 60% sequence homology); whereas, the homologies between *PtriC4H3p* and the promoters of *PtreC4H*s are about 46%.

Further sequence analysis indicated that the potential *cis*-regulatory elements in the promoters were different (Table 2). The box L, one of the three sequence motifs (box P, YTYYMMCMAMCMMC; box A, CCGTCC; and box L, YCYYACCWACC) that have previously been shown to be present in plant *PAL* and *4CL* gene promoters (Logemann et al. 1995), is identified in both *PtreC4H1p*s and *PtriC4H1p* but not in either *PtreC4H2p*, *PtriC4H2p* or *PtriC4H3p*. Box P is present

Table 2 Number of *cis*-acting regulatory elements in the promoters of C4H genes

cis-elements	PtreC4H1p1	PtreC4H1p2	PtreC4H2p	PtriC4H1p	PtriC4H2p	PtriC4H3p
Box P (YTYYMMCMAMCMMC)	–	–	–	–	–	1
Box A (CCGTCC)	–	–	–	–	–	–
Box L (YCYYACCWACC)	2	2	–	1	–	–
AC element (CYCACCWACC)	2	2	–	1	–	–
Box H (CCTACCNNNNNNNCT)	–	–	–	–	–	1

only in *PtriC4H3p* but not in the promoters of the other *C4H* genes. Moreover, *PtriC4H3p* contains an H box (CCTACCNNNNNNNCT), which is essential for both light regulation and elicitor induction (Loake et al. 1992).

Discussion

Although only one *C4H* gene was described in Arabidopsis (Raes et al. 2003), four *C4H* homologs in *P. tremuloides* and three in *P. trichocarpa* were identified in this study. Using a yeast expression system (Osakabe et al. 1999), we engineered two quaking aspen cDNAs, *PtreC4H1-1* and *PtreC4H2-1*, into *S. cerevisiae* and found that the recombinant proteins were able to convert *trans*-cinnamate into *p*-coumaric acid (Fig. 1), confirming that the isolated *PtreC4Hs* encode active C4Hs in *P. tremuloides*. Thus, *Populus* C4Hs are encoded by a small gene family with at least four genes in *P. tremuloides* and three in *P. trichocarpa*.

Based on sequence similarity, C4H proteins have been classified into two classes, class I and class II (Nedelkina et al. 1999, Betz et al. 2001, Raes et al. 2003). Except for the C4Hs from *Mesembryanthemum crystallinum* (AAD11427), *Citrus sinensis* (*CsC4H1*) (AF255013), *Phaseolus vulgaris* (CAA70595), *Nicotiana tabacum* (AAK62344 and AAK62345) and *Zea mays* (CYP73A8), which belong to class II, most of the others including *CsC4H2* (AF255014) are class I C4Hs (Nedelkina et al. 1999, Betz et al. 2001, Raes et al. 2003). Phylogenetic analysis suggested that the identified *PtreC4H* genes, *PtriC4H1* and *PtriC4H2*, which show about 90% identity at the amino acid level to *CsC4H2*, belong to class I; while *PtriC4H3*, having 86% identity at the amino acid level to *CsC4H1*, is a member of class II. Although the class I *C4H* genes are widely identified in plant species, the class II *C4H* genes so far are found only in a few species, including *P. trichocarpa*, *Zea mays* (Potter et al. 1995) and *Citrus* (Betz et al. 2001).

PAL, 4CL and C4H are three enzymes catalyzing the core reactions of phyenylpropanoid metabolism. Together with the results from *PAL* and *4CL* gene studies (Hu et al. 1998, Kao et al. 2002), all the three genes have multiple isoforms in *Populus*. In *P. tremuloides* and *P. trichocarpa*, the *PtreC4H* (Figs. 3, 4) and *PtriC4H* genes (Fig. 5) are differentially expressed, indicating that the divergent *Populus* C4H isoforms play different physiological roles. *PtreC4H2* genes were found to be more xylem specific than *PtreC4H1* genes in *P. tremuloides*. Similar to *PtreC4H* genes, *PtriC4H1* and *PtriC4H2* display distinct expression patterns. *PtriC4H1* is strongly expressed in the developing xylem from the third to the sixth internode and less strongly expressed in the developing xylem from the eighth to the 15th internode; whereas transcripts of *PtriC4H2* in the xylem from the eighth to the 15th internode are significantly more abundant than that in the xylem from the third to the sixth internode. Thus, the physiological functions of *PtreC4H1* and *PtreC4H2* genes in *P. tremuloides* and *PtriC4H1* and *PtriC4H2* in *P. trichocarpa* could be distinct. In addition, *PtriC4H3* shows an expression pattern distinct from that of the other two *C4H* genes; it may play a different role in *Populus* development. The *PtriC4H3* promoter contained a light- and elicitor-responsive *cis*-acting regulatory element, box H (Table 2, Loake et al. 1992), indicating that *PtriC4H3* could be involved in stress responses. However, the involvement of other poplar *C4H* genes in stresses remains to be clarified. Differential expression of *C4H* genes has been reported for *CsC4H1* and *CsC4H2* in *C. sinensis* (Betz et al. 2001). *CsC4H2* that was constitutively expressed in orange flavedo was suggested to play a role as a 'housekeeping' gene in the phenylpropanoid pathway, whereas *CsC4H1* is induced only after wounding treatment (Betz et al. 2001). Taken together, these results indicated that the biosynthesis of specific metabolites might require different C4H isoforms, as in the case of PAL and 4CL isoforms in phenylpropanoid metabolism (Hu et al. 1998, Ehlting et al. 1999, Kao et al. 2002, Raes et al. 2003, Hamberger and Hahlbrock 2004).

On the other hand, the abundant transcripts of *PtreC4H* genes, *PtriC4H1* and *PtriC4H2* in developing xylem suggested that all of them could be involved in the biosynthesis of lignin, a major phenolic polymer that deposits in cell walls. In angiosperm tree development, guaiacyl (G) lignin is preferentially deposited in the xylem of young stems in its predominant cell type, the tracheary vessels; whereas, during maturation of stem, the content of syringyl (S) lignin increases with the development of fiber

and ray cells (Fergus et al. 1970a, Fergus et al. 1970b, Musha and Goring 1975, Saka and Goring 1985). In this study, the accumulation of *PtriC4H1* transcripts was more abundant in tissues (such as the developing xylem from the third to the sixth internode) where G lignin was preferred, and less in tissues (such as the developing xylem from the eighth to the 15th internode) where S lignin was mainly deposited (Fig. 5), showing a close relationship between the expression of *PtriC4H1* and the deposition of G lignin. Furthermore, examining the *cis*-acting regulatory elements in the promoters of *Populus C4H* genes, we found that both *PtreC4H1p*s and *PtriC4H1p* contained the box L element (Table 2) (Logemann et al. 1995). The box L, originally identified in the promoter of the parsley *PAL1* gene by footprint (Lois et al. 1989), was also known to be an AC element (Hatton et al. 1995) and has been found to enhance the xylem-specific expression of monolignol biosynthesis genes (Leyva et al. 1992, Hauffe et al. 1993, Hatton et al. 1995) via interacting with a number of MYB proteins (Tamagnone et al. 1998, Borevitz et al. 2000, Jin et al. 2000, Patzlaff et al. 2003a, Patzlaff et al. 2003b). Examining the promoters of Arabidopsis lignin-related genes, Raes et al. (2003) found that all of the promoters of G rather than S lignin biosynthesis genes had the box L/AC element consensus sequencs, CYCACCWACC. Thus, *PtreC4H1* genes and *PtriC4H1*, showing G lignin-related expression patterns and being regulated by the box L/AC elements, seem to act in the biosynthesis of G lignin. Conversely, the expression level of *PtriC4H2* (Fig. 5) was in line with the biosynthesis of S lignin, showing higher levels in tissues (such as the developing xylem from the eighth to the 15th internode) where S lignin was accumulated and lower levels in tissues (such as the developing xylem from the third to the sixth internode) where G lignin was mainly deposited. Furthermore, both *PtreC4H2p* and *PtriC4H2p* lack the box L/AC element (Table 2), like other genes associated with the biosynthesis of S lignin (Raes et al. 2003). Therefore, we proposed that *PtreC4H2s* and *PtriC4H2* could be involved in the biosynthesis of S lignin.

Materials and Methods

cDNA isolation

To construct a cDNA library, total RNA was extracted from the developing xylem of *P. tremuloides* by the cetyltrimethylammonium bromide (CTAB) method (Chang et al. 1993) and was used for isolation of mRNA by the Poly (A) Quik mRNA Isolation Kit (Stratagene, La Jolla, CA, USA). cDNA was synthesized and ligated into Uni-ZAP XR vector according to the instruction manual of the ZAP-cDNA Synthesis Kit (Stratagene). Ligated DNA was then packaged by Gigapack III Gold Packaging Extract (Stratagene). The constructed library was amplified and screened with ^{32}P-labeled aspen *C4H* cDNA (Ge and Chiang 1996) at 48°C. Thirty positive plaques were isolated after screening a total of 15,000 p.f.u. Ten of the 30 positive plaques were picked up and the pBluescript phagemids were excised from the Uni-ZAP XR vector in vivo by using ExAssist helper phage with *Escherichia coli* host strain SOLR. The rescued clones were analyzed by end sequencing using M13 reverse or forward primer. Four clones with a full-length ORF and showing different end sequences from each other were designated *PtreC4H1-1*, *PtreC4H1-2*, *PtreC4H2-1* and *PtreC4H2-1*, respectively. Another six clones were identical to one of the above four clones. Complete sequencing of the four PtreC4H clones revealed that *PtreC4H1-1* and *PtreC4H1-2*, and *PtreC4H2-1* and *PtreC4H2-1* showed high homology, indicating that they were allelic genes.

Yeast expression vector constructs

To construct the yeast expression vector, forward primer H1-ATG (5′-GTATAATGGATCTCCTCCTCTTG-3′) and reverse primer H1-TAG (5′-GAAACTAAAAGGACCTTGGCT TTG-3′) together with template *PtreC4H1-1* cDNA were used for PCR amplification of the *PtreC4H1-1* ORF. Forward primer H2-ATG (5′-GATCATGGATCTCCTCCTCCTGG-3′) and reverse primer H2-TAA (5′-GAAATTAAAAGGACCTTGGCT TTG-3′) together with template *PtreC4H2-1* cDNA were used for PCR amplification of the *PtreC4H2-1* full-length coding sequences. After 5 min heating at 95°C, 15 cycles of amplification were performed as follows: 1 min denaturation at 94°C, 1 min annealing at 58°C, and 3 min extension at 72°C. The reaction was completed by 25 min extension at 72°C to ensure that the PCR product contained sufficient 3′ A-overhangs. After gel purification, the amplicons were ligated into an autonomously replicating TA expression vector pYES2.1/V5-His-TOPO (Invitrogen, Carlsbad, CA, USA). Two sequence-verified clones pYESC4H1-70 and pYESC4H2-9 contained the identical sequences of *PtreC4H1-1* and *PtreC4H2-1*, respectively, and were used for yeast transformation to express *PtreC4H1-1* and *PtreC4H2-1* driven by the GAL1 promoter.

In vivo analysis of PtreC4H1-1 and PtreC4H2-1 activity in yeast cells

The host *S. cerevisiae* strain INVSC2(CPR) used for expression of *PtreC4H1-1* and *PtreC4H2-1* was previously constructed in our laboratory (Osakabe et al. 1999). *PtreC4H1-1* and *PtreC4H2-1* expression vectors (pYESC4H1-70 and pYESC4H2-9) were introduced into INVSc2(CPR) to create the yeast strains pYESC4H1-70-1 and pYESC4H2-9-2 for co-expression of *Arabidopsis thaliana CPR* and *PtreC4H1-1* or *PtreC4H2-1* cDNAs by using the lithium acetate method as described in the instruction manual of the pYES2.1 TOPO TA Cloning Kit (Invitrogen) and selected on YND/HTA agar plates (6.7 g l^{-1} yeast-nitrogen base without amino acids, 5 g l^{-1} ammonium sulfate, 20 g l^{-1} glucose, 40 mg l^{-1} L-tryptophan, 20 mg l^{-1} L-histidine, 40 mg l^{-1} adenine hemisulfate salt and 20 g l^{-1} agar). Control yeast strain pYES2.1 was also created by transferring the control vector (pYES2.1/V5-His-TOPO) into INVSc2(CPR) cells. To eliminate the uncertainty during microsome preparation, a *PtreC4H1-1* and *PtreC4H2-1* activity assay was conducted by modifying the in vivo method described previously (Schoendorf et al. 2001, Shimada et al. 2001). The pYESC4H1-70-1, pYESC4H2-9-2 and pYES2.1 clones were grown to stationary phase in 3 ml of YND/HTA medium at 30°C. Cells were harvested by centrifuging at 3,000 r.p.m. for 5 min. After washing twice with sterile distilled water, the pellet was then used as an inoculum for 10 ml of expression culture in galactose-supplemented induction medium (20 g l^{-1} bactopeptone, 20 g l^{-1} galactose, 10 g l^{-1} yeast extract) and incubated at 28°C for 7–8 h. After inducing the co-expression of *CPR* and *PtreC4H* genes, the medium was replaced with 5 ml

of fresh induction medium supplemented with 100 μM of the C4H substrate cinnamate and incubated at 28°C for 16 h. The supernatant was collected after centrifugation at 3,000 r.p.m. for 5 min and extracted with 5 ml of ethyl acetate. The solvent phase was washed with 5 ml of sterile distilled water and then dried by a vacuum. The ethyl acetate-extracted and dried reaction mixtures were analyzed by HPLC-UV/MSD as described (Osakabe et al. 1999).

In situ mRNA localization

In situ hybridization was performed by following the procedure previously described (Harding et al. 2002). To generate the probe, *PtreC4H1-1* cDNA in pBluescript SK$^-$ (Stratagene) obtained by screening the cDNA library was digested by *Hin*dIII and *Xho*I. Following blunting of the ends by T4 DNA polymerase and gel recovery, the 3.6 kb DNA fragment was self-ligated by T4 DNA ligase. The resulting plasmid contained 0.7 kb of the 5′ region of *PtreC4H1-1*. This region was then amplified by using T3 as a forward primer and T7 as a reverse primer. The PCR product was used as an in vitro transcription template. DIG-labeled sense and antisense RNA probes of *PtreC4H1-1* were prepared by using a DIG RNA labeling Kit from Roche Applied Science (Indianapolis, IN, USA). The internodes 4, 8, 12 and 14, and leaves 3, 6 and 14 of several young *P. tremuloides* plants grown in a greenhouse were used for in situ hybridization. Sections were photographed using a microscope and digital imaging system (both Nikon).

RT–PCR

Phloem, developing xylem and mature leaf tissues were collected from 1-year-old, greenhouse-grown quaking aspen. Tissues were immediately frozen and stored in liquid nitrogen until used for RNA isolation. Total RNA was isolated by the CTAB method (Chang et al. 1993). cDNA was synthesized by a GeneAmp Gold RNA PCR Core Kit (ABI, Foster City, CA, USA) using 2 μg of total RNA as a template in a 25 μl volume. To amplify a 0.5 kb product, PCR was performed by using the gene-specific primer C4H1F (5′-CTAGAAATCTCTTTCAGTAC TCC-3′, for *PtreC4H1* genes) or C4H2F (5′-TACCTAAAAA GTCCCCACCTCT-3′, for *PtreC4H2* genes) as a forward primer and C4HR (5′-TTAACATCCTC GACAACTTGAG-3′) as a reverse primer, and 1 μL of cDNA as a template. The temperature profile included annealing at 54°C for 60 s and elongation at 72°C for 90 s. Reactions were stopped after 30 cycles.

Real-time PCR

Total RNA was isolated from young leaves (first–fourth from the top), mature leaves (12th and 13th from the top), young stems (first–fourth from the top), maturing stems (sixth–10th from the top), developing xylem (from the 11th to the 30th internode), phloem (from the 11th to the 30th internode) and epidermal layers (from the 11th to the 30th internode) of *P. tremuloides* and from young leaves, mature leaves, young stems, developing xylem from the third to the sixth internode, developing xylem from the eighth to the 15th internode, phloem, young roots (1–3 cm) and callus tissues of *P. trichocarpa* (Nisqually-1) by the CTAB method (Chang et al. 1993). Reverse transcription was performed using Taqman Reverse Transcription Reagents (ABI, Foster City, CA, USA). Total RNA (200 ng) was reverse transcribed in a 10 μl reaction mixture containing 1× Taqman RT buffer, 55 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM of random hexamers, 4 U of RNase inhibitor and 31.25 U of reverse transcriptase by incubating at 25°C for 10 min, 37°C for 60 min and 95°C for 5 min. The resulting cDNA was diluted to 1,000 μl with sterile water. Quantitative real-time PCR was carried out in triplicate reactions using an ABI PRISM 7900HT Sequence Detection System (ABI). Three pairs of gene-specific primers (5′-GGCTTGAAATGTGCAATTGATCAT ATTT-3′ and 5′-CCAAGCACGGTATCGAGCTCATCA-3′ for the *PtreC4H1* genes and *PtriC4H1*; 5′-GTTGAAGTGCGCAATA GACCATATCC-3′ and 5′-CCAAGCAAGGTATCGAGCTCAT GG-3′ for the *PtreC4H2* genes and *PtriC4H2*; and 5′-GGTAAGC TGTGCCATGGATCACATAA-3′ and 5′-CCTTTAGGACGGC TCTGATTTCATCT-3′ for *PtriC4H3*) were used to amplify individual *C4H* transcripts in *P. tremuloides* or *P. trichocarpa* tissues. As an endogenous control that was used to normalize template amounts, *P. tremuloides* (AF206999) or *P. trichocarpa* (AY652861) 18S rRNA fragments were amplified using a forward primer (5′-CGAAGACGATCAGATACCGTCCTA-3′) and a reverse primer (5′-TTTCTCATAAGGTGCTGGCGGAGT-3′). PCRs were carried out in a 25 μl volume containing 5.5 μl of diluted cDNA, 200 nM of each primer and 1× SYBR Green PCR Master Mix (ABI) using the following conditions: 50°C for 2 min, 95°C for 10 min, 60 cycles of 95°C for 15 s and 60°C for 1 min. A fter amplification, a thermal denaturing cycle at 95°C for 15 s, 60°C for 15 s and 95°C for 15 s for determining the dissociation curves was applied. The dissociation curves were used for verifying the specificity of PCR amplification. The results from gene-specific amplification were analyzed using a comparative Ct method which uses an arithmetic formula, $2^{-\Delta\Delta Ct}$, to achieve results for relative quantification as suggested by the manufacturer. Ct represents the threshold cycle.

Genome walking

The upstream *PtreC4H* genes were amplified by using a Universal Genome Walker^{TM} Kit (Clontech, Mountain View, CA, USA). Eight enzymes, namely *Puv*II, *Hpa*I, *Pme*I, *Rsa*I, *Dra*I, *Eco*RV, *Ssp*I and *Stu*I, were used to prepare aspen genomic DNA libraries according to the ClonTech protocol. Two *PtreC4H1* gene-specific primers, H1W1 (5′-GGAATATGATCTTCTTTATCC A AAGG-3′) and H1W2 (5′-GTACTGAAAGAGATTTCTAGGG AGAAG-3′), and two *PtreC4H2* gene-specific primers, H2W1 (5′-CAGGAGGAGGAGATCCATGATC-3′) and H2W2 (5′-GA GGTGGGGACTTTTTAGGTAAGAAG-3′), were designed according to the sequence of the 5′ end of *PtreC4H1* and *PtreC 4H2* cDNAs and used for genome walking of *PtreC4H* promoters. Three overlapping fragments were obtained from *Dra*I, *Puv*II and *Hpa*I libraries using *PtreC4H1* gene-specific primers. The longest one was designated *PtreC4H1p1* (accession No. DQ522296). Two PCR walking fragments were obtained from *Stu*I and *Rsa*I libraries by using *PtreC4H2* gene-specific primers. The longer one is about 1.1 kb.

In order to eliminate the mutation due to the two rounds of amplification, forward primer H1-5′ (5′-GAAGCTTATCATTTT GGATTCTTGAATGTG-3′) and reverse primer H1-3′ (5′-CGGA TCCTTTCTAGGGAGAAGAGGGAG-3′) for *PtreC4H1p* and forward primer H2-5′ (5′-ACAATAATGCTAACAAAAGAAAA TAATC-3′) and reverse primer H2-3′ (5′-CGGATCCGATCTTG GAACTGGTTTCTTTGT-3′) for *PtreC4H2p* were designed according to the sequences obtained by genome walking and used to amplify the promoter fragment from the aspen genome directly by PCR. Four PCR clones of *PtreC4H1* gene promoters were picked up and completely sequenced. Sequencing analysis revealed that the four PCR clones (designated *PtreC4H1p2*, accession No. DQ522297) were identical and showed 90% homology to *PtreC4H1p1*, indicating that one of them is the promoter of *PtreC4H1-1* and another is the promoter of

PtreC4H1-2. Meanwhile, three PCR clones of the *PtreC4H2* promoter were picked up and completely sequenced. Sequencing analysis revealed that the two PCR clones (designated *PtreC4H2p*, accession No. DQ522298) were identical and showed a two nucleotide difference from the sequence cloned by genome walking.

Acknowledgments

The project was supported by the US Department of Energy, Office of Basic Energy Sciences, grant number DE-FG02-03-ER15442 to V.L.C.

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(Received March 8, 2006; Accepted May 13, 2006)