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Secondary xylem-specific expression of caffeoyl-coenzyme A 3-*O*-methyltransferase plays an important role in the methylation pathway associated with lignin biosynthesis in loblolly pine

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Abstract

Two types of structurally distinct *O*-methyltransferases mediate the methylation of hydroxylated monomeric lignin precursors in angiosperms. Caffeate 3-*O*-methyltransferase (COMT; EC 2.1.1.68) methylates the free acids and caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT; EC 2.1.1.104) methylates coenzyme A esters. Recently, we reported a novel hydroxycinnamic acid/hydroxycinnamoyl CoA ester *O*-methyltransferase (AEOMT) from loblolly pine differentiating xylem that was capable of methylating both acid and ester precursors with similar efficiency. In order to determine the possible existence and role of CCoAOMT in lignin biosynthesis in gymnosperms, a 1.3 kb CCoAOMT cDNA was isolated from loblolly pine that showed 79–82% amino acid sequence identity with many angiosperm CCoAOMTs. The recombinant CCoAOMT expressed in *Escherichia coli* exhibited a significant methylating activity with hydroxycinnamoyl CoA esters whereas activity with hydroxycinnamic acids was insignificant. Moreover, 3.2 times higher catalytic efficiency for methylating caffeoyl CoA over 5-hydroxyferuloyl CoA was observed which could serve as a driving force towards synthesis of guaiacyl lignin. The secondary xylem-specific expression of CCoAOMT was demonstrated using RNA blot analysis, western blot analysis, and *O*-methyltransferase enzyme assays. In addition, Southern blot analysis indicated that CCoAOMT may exist as a single-copy gene in loblolly pine genome. The transgenic tobacco plants carrying loblolly pine CCoAOMT promoter-GUS fusion localized the site of GUS activity at the secondary xylem tissues. These data suggest that CCoAOMT, in addition to AEOMT, plays an important role in the methylation pathway associated with lignin biosynthesis in loblolly pine.

Introduction

Gymnosperms and angiosperms are two highly diverged groups of land plants. During evolution, gymnosperms appeared earlier in the Permian through Triassic periods than angiosperms which appeared in the later Cretaceous period (Higuchi *et al.*, 1977). Evolution of lignin is presumed to be closely related to adaptation and evolution of land plants. The xylem from coniferous gymnosperms contains lignin rich in guaiacyl (G) moieties (4-hydroxy-3-monomethoxyphenyl) whereas lignin from angiosperm dicots have, in addition, large fractions of syringyl (S) moieties (3,5-dimethoxy-4-hydroxyphenyl). The main difference between G lignin and S lignin is the degree of methoxylation which is intimately associated with the ease of degrading G-S lignin in angiosperms as compared to degradation of G lignin in gymnosperms during chemical pulping of wood (Chang and Sarkanen, 1973; Chiang and Funaoka, 1990). *O*-methyltransferase (OMT) which catalyzes the methylation of hydroxylated monomeric lignin precursors

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF036095 (loblolly pine CCoAOMT cDNA) and AF098159 (loblolly pine CCoAOMT promoter).

(Figure 1), therefore, occupies a pivotal position in lignin biosynthesis pathway as well as in genetic engineering of conifer trees for better pulping efficiency (Bugos *et al.*, 1991; Whetten *et al.*, 1998).

In angiosperms, two structurally distinct OMTs orchestrate the methylation of two different types of lignin precursors (Figure 1). The enzyme, caffeate 3-*O*-methyltransferase (COMT, EC 2.1.1.68) methylates caffeic acid and 5-hydroxyferulic acid (Bugos *et al.*, 1991), whereas another OMT enzyme, caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT; EC 2.1.1.104) methylates caffeoyl-CoA and 5-hydroxyferuloyl CoA (Ye *et al.*, 1994) (Figure 1). Both COMT and CCoAOMT enzymes in angiosperms are bi-specific; however, substrate specificities of gymnosperm OMTs are much less explored. It was commonly believed until recently that gymnosperm COMTs are monospecific with a preference for only caffeic acid and were not efficient in utilizing 5-hydroxyferulic acid as a substrate (Shimada *et al.*, 1972; Kuroda *et al.*, 1975). It is also not known if CCoAOMT is involved in the process of methylating the lignin precursors in gymnosperms.

Although cDNAs or genes encoding COMTs and CCoAOMTs have been reported from many angiosperm species, no OMT-like cDNA sequence was reported from any gymnosperm species until recently. We described the cloning and characterization of a novel hydroxycinnamic acid/hydroxycinnamoyl CoA ester *O*-methyltransferase (AEOMT) cDNA from loblolly pine xylem (Li *et al.*, 1997). Recombinant AEOMT was capable of methylating both acid and ester precursors with similar efficiency. Thus gymnosperm AEOMT was proposed to perform dual functions of two structurally dissimilar angiosperm enzymes, COMT and CCoAOMT. However, in extracts of loblolly pine secondary xylem, hydroxycinnamoyl CoA esters were methylated more efficiently than their corresponding hydroxycinnamic acids (Li *et al.*, 1997). This difference in activity between recombinant AEOMT and crude enzyme system from loblolly pine xylem in utilizing hydroxycinnamoyl CoA esters and hydroxycinnamic acids led us to investigate the possible existence of CCoAOMT in loblolly pine xylem.

Here, we present the results of cloning and characterization of a CCoAOMT cDNA from loblolly pine differentiating xylem. The CCoAOMT transcripts and proteins are mainly expressed in developing secondary xylem tissues of loblolly pine. The recombinant CCoAOMT preferentially methylated hydroxycinnamoyl CoA esters above hydroxycinnamoyl acids and caffeoyl CoA was methylated 3.2 times more efficiently than 5-hydroxyferuloyl CoA. Loblolly pine CCoAOMT promoter-GUS fusion localized the site of GUS activity at the secondary xylem in transgenic tobacco plants. These data suggest that CCoAOMT, in addition to AEOMT, may play an important role in the methylation of the lignin precursors in differentiating secondary xylem from loblolly pine.

Materials and methods

Plant material

The loblolly pine seeds germinated for one month in the greenhouse. Pine seedlings of 10 cm height were harvested and roots, needles and stems were collected separately for molecular and biochemical analysis. Differentiating secondary xylem tissue was collected from vegetatively propagated 3-year old loblolly pine tree (genotype 1932 grown in the Forest Experimental Station of International Paper, Bainbridge, GA) and all tissue samples were stored immediately in liquid nitrogen until use.

cDNA cloning of loblolly pine CCoAOMT

Total RNA isolated from secondary xylem of loblolly pine was used for first-strand cDNA synthesis by Superscript II reverse transcriptase (Gibco). This pool of cDNAs and a pair of conserved CCoAOMT primers were used for amplification of loblolly pine CCoAOMT fragment by PCR. Many pairs of degenerate primers were designed on the basis of conserved cDNA sequences of angiosperm CCoAOMTs. Two degenerate primers (5′-ATYGGTGTYTACACYGG-3′, sense primer; 5′-CCATCACCRACMGSAAGG-3′, antisense primer) amplified a cDNA fragment of about 430 bp which was cloned into pCR2.1 vector (Invitrogen), sequenced and confirmed to be a CCoAOMT cDNA fragment. This 430 bp PCR product was used as a probe to screen the loblolly pine differentiating xylem cDNA library (Zhang and Chiang, 1997). Six positive phage plaques were identified in the primary screening of about 12 000 plaques. A cDNA clone of about 1.3 kb was isolated through two more rounds of screening. This cDNA clone was sequenced and the DNA/Protein sequence analysis was performed using the GCG software package Version 9.0.

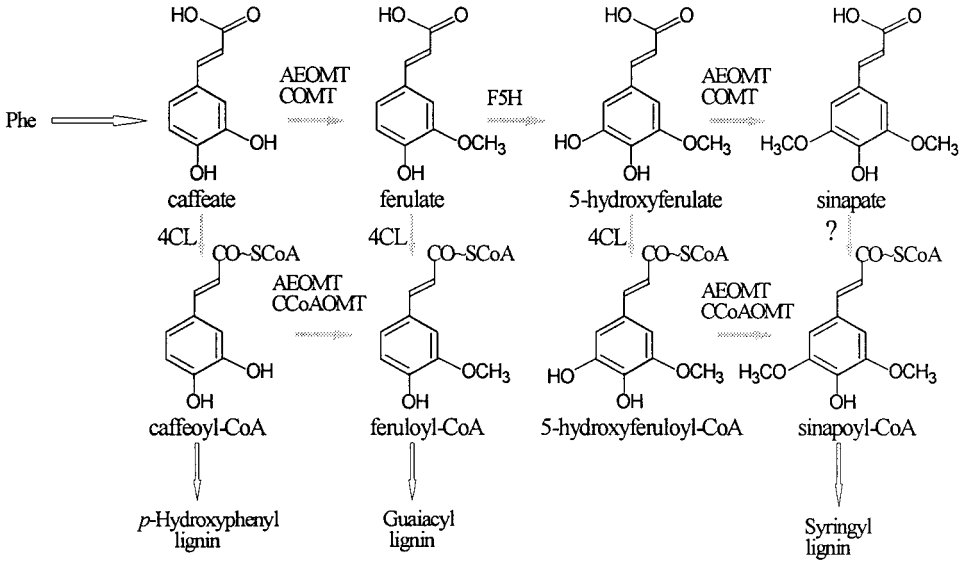

Figure 1. The general lignin biosynthesis pathway. Question mark indicates unconfirmed step. Phe, phenylalanine; COMT, caffeate 3-*O*-methyltransferase; CCoAOMT, caffeoyl-CoA 3-*O*-methyltransferase; AEOMT, hydroxycinnamic acid/hydroxycinnamoyl-CoA ester OMT; F5H, ferulate 5-hydroxylase; 4CL, *p*-coumarate-CoA ligase. Unfilled arrow indicates multiple steps leading to end products.

Expression of recombinant CCoAOMT in Escherichia coli

PCR was employed to introduce a *Sph*I site at the 5′ end and a *Hin*dIII site at the 3′ end of the coding region of loblolly pine CCoAOMT and the PCR product was cloned into pQE-32 vector with a His-tag at the N-terminal (Qiagen). This construct was transferred into *E. coli* M15-[pREP4] strain. The control M15-[pREP4] cell strain contained only pQE-32 vector without insert. The growth and induction of bacterial cells were optimized according to the manufacturer's instructions. The recombinant CCoAOMT proteins were purified using a His•Bind Resin (Novagen) as described by Li *et al.* (1997). Polyclonal antibodies against the purified recombinant pine CCoAOMT protein were raised in rabbits by Alpha Diagnostic International (San Antonio, TX). Protein concentrations were determined by using BioRad Protein Assay and BSA as a standard. SDS/PAGE and western blot analysis were performed as described (Li *et al.*, 1997).

Biochemical and molecular analysis

The crude proteins were extracted from seedling roots, needles, stems and secondary xylem tissues as well as bacterial cells expressing loblolly pine CCoAOMT according to Li *et al.* (1997) and used for COMT enzyme assays with caffeic acid and 5-hydroxyferulic acid (Bugos *et al.*, 1991) and CCoAOMT enzyme assays using caffeoyl-CoA and 5-hydroxyferuloyl-CoA (Li *et al.*, 1997). Genomic DNA and total RNA were isolated from loblolly pine tissues and Southern and northern blot analyses were performed with CCoAOMT cDNA by the procedures described by Zhang and Chiang (1997).

Isolation of pine CCoAOMT promoter region and transfer of promoter-GUS fusion to tobacco

Five loblolly pine Genome Walker libraries were constructed using Universal GenomeWalker Kit (Clontech). An antisense primer (5′-CTTACACTCCGCTGCAGCAACACTTGT-3′) complimentary to 85–102 (from the 5′ end) of the pine CCoAOMT cDNA sequence (Figure 2) was synthesized and used to PCR-based amplification of 5′ regulatory sequences. After secondary PCR walking, about 1.2 kb fragment was cloned in pCR2.1 vector. The genomic DNA insert was sequenced from both ends using an ABI310 Genetic Analyzer (Perkin Elmer). The sequence analysis indicated that this sequence extends upstream of the cDNA clone. To examine the regulatory activity of CCoAOMT promoter, 829 bp promoter fragment was cloned into the pBI101 vector (Clontech) between *Hin*dIII and *Bam*HI restriction sites. PCR was employed to introduce *Hin*dIII site at the 5′ end and *Bam*HI at the 3′ end of the CCoAOMT promoter. The PCR product was sequenced and confirmed to

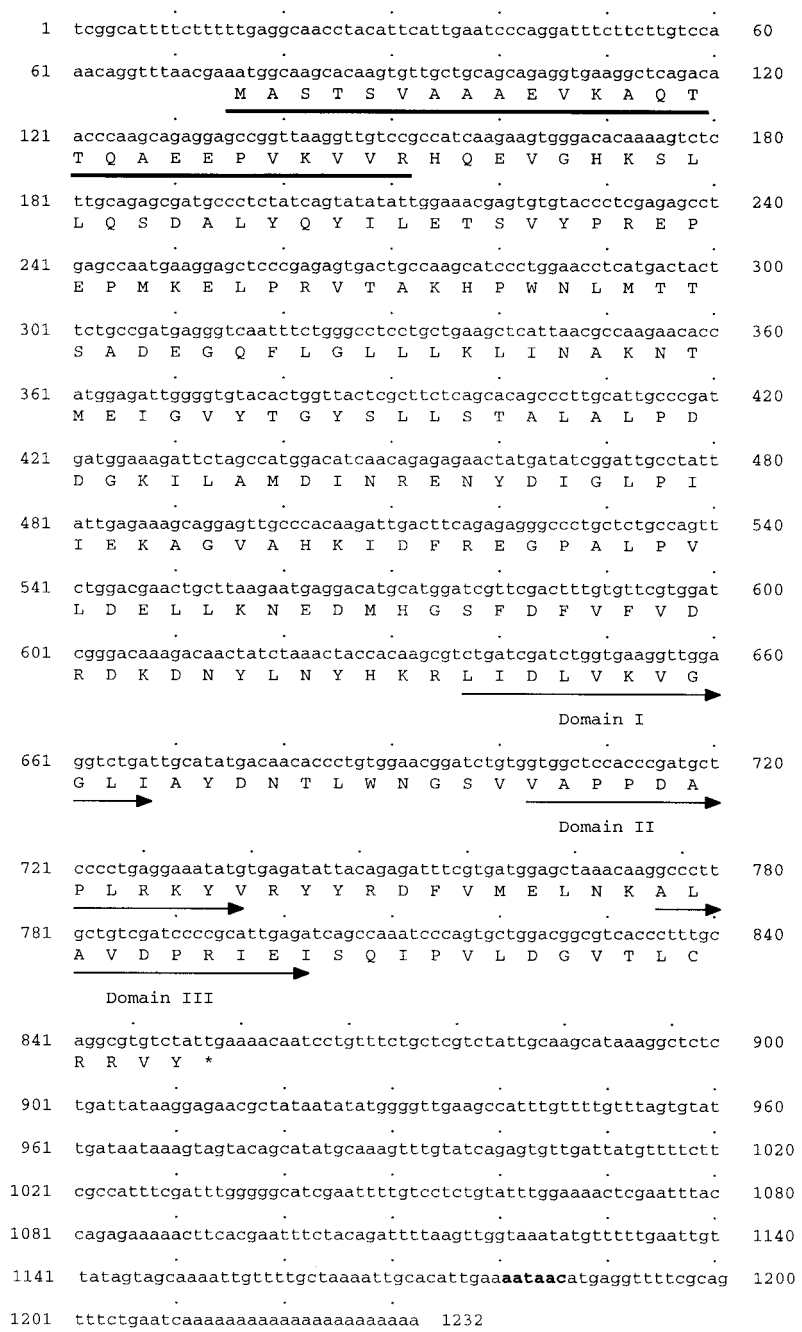

Figure 2. Nucleotide sequence and deduced amino acid sequence of loblolly pine CCoAOMT cDNA. The putative polyadenylation signal is indicated in bold. The suggested SAM binding domains I, II, and III (Joshi and Chiang, 1988; Li *et al.*, 1997) are indicated by arrows. The highly divergent region from angiosperm CCoAOMTs at the N-terminal is bold-underlined.

be identical to the genomic sequence. The plasmid DNA was introduced into *Agrobacterium* strain C58. The promoter-GUS fusion was transferred into *Nicotiana tabacum* leaf disks under selection of kanamycin according to the transformation protocol used in our laboratory (Horsch *et al.*, 1988). Tissue extracts and fluorometric measurements of GUS activity were conducted as described by Jefferson *et al.* (1987). For histochemical staining, hand sections of tobacco stems were incubated in 1 mM X-Gluc (5-bromo-4-choro-3-indole-D-glucuronic acid) as described by Jefferson *et al.* (1987).

Results and discussion

Isolation and characterization of pine CCoAOMT cDNA

To date, a large number of highly conserved CCoAOMT cDNAs have been isolated from angiosperm species but there is no report from any gymnosperm species (Joshi and Chiang, 1998). Our initial attempts to clone CCoAOMT cDNA using PCR and reverse transcriptase PCR strategies were unsuccessful (Li *et al.*, 1997). Moreover, no hybridization signal was detected in loblolly pine genomic DNA Southern blot and xylem RNA northern blot when probed with *Stellaria* CCoAOMT cDNA as a representative angiosperm cDNA. This led us to believe that if there is a CCoAOMT in loblolly pine, its cDNA sequence must be diverged from *Stellaria* CCoAOMT cDNA. By carefully designing several new sets of conserved primers on the basis of available CCoAOMT sequences and performing RT-PCR-mediated amplifications using total RNA from loblolly pine xylem as a template, we succeeded in amplification of a cognate CCoAOMT cDNA fragment. The amplified 430 bp cDNA fragment showed 75–80% identity with the corresponding region from angiosperm CCoAOMT cDNAs. Using this cDNA fragment as a probe, we screened the loblolly pine developing xylem cDNA library, and an about 1.3 kb cDNA clone was isolated. DNA sequence analysis indicated that this loblolly pine CCoAOMT cDNA is 1232 bp long with an open reading frame of 777 bp (Figure 2) that encodes a protein of 259 amino acid residues with a calculated molecular weight (M_r) of 29 145. The coding region is flanked at the 5′ and 3′ end by 75 bp and 377 bp of non-coding regions, respectively. The flanking sequences upstream of the first ATG codon match with our recent suggestions for plant-specific ATG context (Joshi *et al.*, 1997) and a putative polyadenylation signal is present 33 bp upstream from the polyadenylation site as observed in many plant genes (Joshi, 1987a).

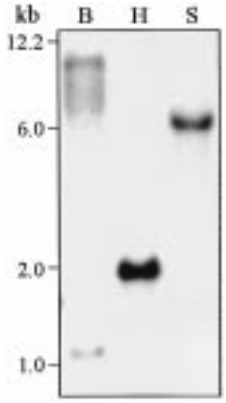

Figure 3. Southern blot hybridization of loblolly pine CCoAOMT gene. Total genomic DNA (50 μg/lane) was digested with *Bam*HI (B), *Hin*dIII (H) and *Sac*I (S). The blot was hybridized with ^{32}P-labeled pine CCoAOMT cDNA probe. The molecular size is shown on the left in kb.

To determine the copy number of CCoAOMT gene, Southern blot analysis was performed. Genomic DNA was digested with restriction enzymes, *Bam*HI, *Hin*dIII and *Sac*I individually. None of these restriction enzymes cut the pine CCoAOMT cDNA. The blot was probed with full-length pine CCoAOMT cDNA under high-stringency conditions. As shown in Figure 3, only one band was detected in *Hin*dIII and *Sac*I digestions. These results suggest that CCoAOMT may exist as a single-copy gene in the loblolly pine genome. One band along with another high-molecular-weight band was detected in *Bam*HI digestion indicating that there may be a *Bam*HI restriction site in the non-coding region of CCoAOMT gene in the loblolly pine genome (1C = ca. 21–22 pg; Wakamiya *et al.*, 1993).

In order to examine the expression patterns of loblolly pine CCoAOMT in the intact plants, various loblolly pine plant organs/tissues such as needles, roots, and stems from seedlings and differentiating secondary xylem from mature trees were collected. Total RNAs isolated from these tissues were blotted onto a nylon membrane and probed with a ^{32}P-labeled full-length pine CCoAOMT cDNA (Figure 4). A strong hybridization signal was detected in differentiating secondary xylem and a weak signal was observed in seedling stems. No hybridization signal was detected in needles and roots from seedlings (Figure 4). These results indicate that loblolly pine

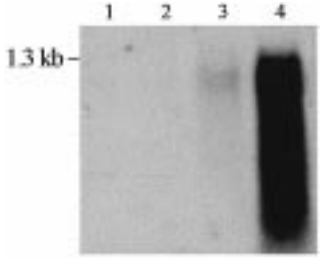

Figure 4. Northern blot analysis of pine CCoAOMT. Total RNAs (25 μg) from seedling needles (lane 1), seedling roots (lane 2), seedling stems (lane 3) and differentiating xylem (lane 4) were fractionated by gel electrophoresis, and blotted onto a nylon membrane. The equal loading of RNA amounts was judged by ethidium bromide staining. The blot was hybridized to ^{32}P-labeled pine CCoAOMT cDNA. The molecular size is shown in kb on the left.

CCoAOMT gene is expressed in the secondary xylem tissue of mature trees with a weak expression in seedling stems that contain primary xylem as the lignifying tissue. However, other tissues from mature trees should be examined in the future to substantiate this conclusion.

DNA and amino acid sequence analysis of CCoAOMT from loblolly pine

The nucleotide and amino acid sequences of most of the angiosperm CCoAOMTs are highly conserved (Table 1). The deduced loblolly pine CCoAOMT shares 79–82% identity with many CCoAOMT sequences available in the GenBank except two sequences from *Stellaria* and *Arabidopsis* where identity is only 51–52% (Table 1). Earlier, we did not detect the hybridization signal in the loblolly pine genomic DNA when using *Stellaria* CCoAOMT as a probe (Li *et al.*, 1997). That observation also indicated that *Stellaria* and loblolly pine CCoAOMT cDNAs are likely to be structurally different. The three domains, proposed to be involved in the SAM-binding of OMTs, as well as other CCoAOMT-specific sequence motifs (Joshi and Chiang, 1998; Li *et al.*, 1997) are also found and located at the respective regions in loblolly pine CCoAOMT. Domain I (LIDLVKVGGLI), domain II (VAPPDAPLRKYV) and domain III (ALAVDPRIEI) were at amino acid residues 188–198, 210–221 and 234–243, respectively (Figure 2). A distinct dissimilarity of N-terminal sequence (26 amino acid residues) was also observed between loblolly pine CCoAOMT and many angiosperm CCoAOMTs. Similar to loblolly pine AEOMT (Li *et al.*, 1997), such structural difference may be related to some functional difference between loblolly pine and angiosperm CCoAOMTs.

Table 1. Comparison of loblolly pine CCoAOMT cDNA with angiosperm CCoAOMTs.

	1	2	3	4	5	6	7	8	9	10	11	12
1		82	80	82	79	82	80	81	81	79	51	52
2	67		83	84	83	85	83	84	85	83	51	53
3	64	69		83	88	86	85	85	88	86	53	53
4	62	69	71		87	86	84	85	86	86	52	53
5	63	69	73	69		90	90	88	92	89	54	53
6	66	70	70	71	69		97	97	91	87	54	53
7	66	70	71	71	70	92		97	91	85	53	51
8	64	71	70	73	70	90	93		90	86	53	52
9	66	69	70	72	74	71	71	71		89	54	55
10	62	69	73	71	70	71	71	71	71		52	53
11	54	53	55	55	56	55	55	56	53	56		51
12	54	57	58	57	55	56	57	57	59	55	53	

The percentages of nucleotide sequence identity are shown at the lower left diagonal half, whereas the percentages of amino acid sequence identity are shown at the upper right diagonal half. CCoAOMT cDNAs (GenBank accession numbers in parenthesis) from: 1, loblolly pine (AF036095); 2, *Zinnia* (U13151); 3, alfalfa (U20736); 4, parsley (M69184); 5, aspen (U27116); 6, 7, 8, 9, tobacco (U62734, U62735, U62736, and Z56286, respectively); 10, grape (Z54233); 11, *Stellaria* (L22203); 12, *Arabidopsis* (L40031). The sequences were analyzed with the GCG sequence analysis package.

Heterologous expression of pine CCoAOMT cDNA in E. coli, *western blot analysis and comparison of enzyme activities between recombinant proteins and CCoAOMT activity in the loblolly pine plants*

To determine the substrate specificities of loblolly pine CCoAOMT, its coding region was cloned into pQE-32 expression vector (Qiagen) and purified protein of about 30 kDa was used to prepare polyclonal antibodies in rabbits. Loblolly pine CCoAOMT and AEOMT antibodies (Li *et al.*, 1997) were tested if they reacted to each other's antigen in the western blot of crude protein extracts. The results showed that pine CCoAOMT or AEOMT antibodies reacted only with their cognate proteins (Figure 5). No signal was detected in the seedling needles when either AEOMT or CCoAOMT antibodies were applied (Figure 5). This may be correlated with the very small amount of lignification that occurs in seedling leaves. In seedling roots, a signal was only detected with AEOMT antibodies (Figure 5). Seedling stem showed a weaker signal than secondary xylem tissue with CCoAOMT antibody but AEOMT showed almost same intensity

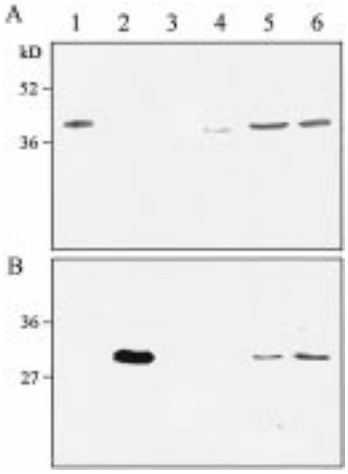

Figure 5. Western blot analysis using loblolly pine AEOMT(A) and CCoAOMT(B) antibodies. Lane 1, total proteins (5 μg) from *E. coli* expressing AEOMT cDNA; lane 2, total proteins (5 μg) from *E. coli* expressing pine CCoAOMT cDNA; lane 3, total proteins (15 μg) from pine seedling needle extract; lane 4, total proteins (15 μg) from seedling root extract; lane 5, total proteins (15 μg) from seedling stem extract; and lane 6, total proteins (15 μg) from differentiating xylem extract. The protein molecular weight is shown at the left in kDa.

of signal in both seedling stem and secondary xylem tissue. The CCoAOMT activity, therefore, appears to be closely associated with the lignification in the secondary xylem tissues of loblolly pine. It has not escaped our attention that there is some discrepancy between the strength of CCoAOMT mRNA signal and the amount of CCoAOMT protein in seedling stem and secondary xylem tissues in our northern and western blots, respectively. However, amounts of CCoAOMT mRNA and proteins can not be directly compared in such analyses since post-transcriptional regulatory events in loblolly pine plants could affect the outcome.

Table 2 shows the enzyme activities of pine CCoAOMT recombinant protein with four lignin precursor substrates, namely caffeoyl CoA, 5-hydroxyferuloyl CoA, caffeic acid, and 5-hydroxyferulic acid. The recombinant pine CCoAOMT exhibited a significant methylating activity with hydroxycinnamoyl CoA esters whereas the activities with hydroxycinnamic acids were insignificant. Moreover, 3.2 times higher catalytic efficiency for methylating caffeoyl CoA than for 5-hydroxyferuloyl CoA was observed. Similar results have also been reported for recombinant CCoAOMTs from many angiosperm species (e.g. Inoue *et al.*, 1998; Martz *et al.*, 1998). The high efficiency of converting caffeoyl CoA to feruloyl CoA by CCoAOMT is likely to drive the metabolic flow of lignin precursors towards coniferyl alcohol-mediated G lignin biosynthesis in loblolly pine. The relative activities of crude proteins from secondary xylem of loblolly pine (Table 2) also indicate that in this tissue hydroxycinnamoyl CoA esters are methylated more efficiently than hydroxycinnamic acids. However, kinetic characterization of the CCoAOMT enzyme should be done to confirm these conclusions.

In loblolly pine, different organs of a seedling or mature tree synthesize different amounts of lignin. We examined if OMT activities from different tissues/organs also differ significantly. Table 2 shows the specific activities of various crude protein extracts for four lignin precursor substrates. Both seedling stem extract and differentiating secondary xylem extracts efficiently catalyzed the methylation of all four substrates tested, although much higher activities were observed in the differentiating secondary xylem than seedling stems. In agreement with this observation, western blots showed stronger AEOMT and CCoAOMT signal in secondary xylem than in seedling stems containing mainly primary xylem (Figure 5). No methylating activity was observed in the seedling needle extracts. In agreement with this, no western blot signal was detected in the seedling needles when either AEOMT or CCoAOMT antibodies were applied (Figure 5). In seedling roots, enzyme activities were detected only with hydroxycinnamic acids but not with hydroxycinnamoyl CoA esters. Consistently, western blot signal was only detected by AEOMT antibodies in seedling roots (Figure 5). Thus, enzyme activities and western blot analysis provided a similar picture of two OMTs involved in methylation of lignin precursors in various loblolly pine tissues. Both AEOMT and CCoAOMT enzymes appear to be closely associated with the lignification in the primary and secondary xylem tissues of loblolly pine stem and AEOMT seems to be mainly involved in root lignification. However, more detailed study is required to confirm the exclusive role of AEOMT in root xylem lignification.

COMT in angiosperms mainly catalyzes methylation at the hydroxycinnamic acid level and favors 5-hydroxyferulic acid as a substrate. It has long been believed that the preference of COMT with 5-hydroxyferulic acid as a substrate may be responsible for syringyl lignin synthesis (Bugos *et al.*, 1991; Higuchi, 1985). Thus, it is likely that CCoAOMT's preference for caffeoyl CoA in gymnosperms and angiosperms might serve as a kinetic force to drive

Table 2. Substrate specificities of loblolly pine CCoAOMT in *E. coli* and crude extracts of various loblolly pine organs/tissues.

	E. coli recombinant CCoAOMT		Loblolly pine secondary xylem extracts		Loblolly pine seedling stem extracts		Loblolly pine seedling root extracts		Loblolly pine seedling needle extracts	
	sp. act.	rel. act	sp. act.	rel. act.	sp. act.	rel. act.	sp. act.	rel. act.	sp. act.	rel. act
C-CoA	6568	100	18250	100	1561	100	ND	–	ND	–
5HF-CoA	2073	32	16008	88	619	40	ND	–	ND	–
CA	361	5	10907	60	620	40	388	–	ND	–
5HFA	542	8	9839	54	540	35	513	–	ND	–

C-CoA, caffeoyl-CoA; 5HF-CoA, 5-hydroxyferuloyl-CoA; CA, caffeic acid; 5HFA, 5-hydroxyferulic acid; ND, not detected; sp. act., specific activity expressed in pmol min^{-1} g^{-1}; rel. act, relative activity (in %). The specific activities shown were mean of three independent assays. Specific activity with caffeoyl-CoA was taken as 100 to calculate relative activities with other substrates.

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   1  AAACTACGTA ATTTACTTAA TTCAAATATT ATAAACTTTT CAAAAATAAT
  51  CGCGGGAAAT GAAATAAAAT AAAATAAAAT AAAATAAAAT TAAATTAAAT
 101  TAAATCAAAT CACCTAAAAT CACAACCTCG CTGTAATAAA ATATAAATAA
 151  ATAAAAAACT AACTAACTAA AACAAACTAA AACTTATAAC TTAGATGTGG
 201  CCCCAAAAAA GAAAGAAAGA AGGAAGCACA GTTCCCTTCT CTGGTGAAAT
 251  AAATGCAGCA TGTTGATAAT ATGCGCAGAT AAGCCTTGTT GGTTCCAAAA
 301  TATTATATTC CGTAGCTTGA GGTTATGAAT CGTGATAAAA CGTGAATTCA
 351  ACTTTGGCAT AAACCAAACT CAACTACGGT AATGCGGTTG GTGTCGGAAT
 401  CCAACAACTT CCAGGAAAAT GCGTGACACC ATGATTGATT GAGCCGACAC
 451  CGTCCACCCT GGTCACTTTC CGGGGTTGGT AAAGAGTAGG TGGCTTAGAT
 501  CGTTAACACT AGCGATTAGG TTTAGCGGGA CCTGTTCAAA AGGAAATTGG
 551  GTAAGAGGTC GCGAGTTCGA GCTCGATTGG ATGGAGTCCC GTGGTCAAAC
 601  TGACAGCGAC TGACACACAG CACATGAAAT TAGCAAGGGA TTGGGATGAT
 651  TTCGATAGGA CTGTCGATCA GAACGAGAAG TTCAAGCAAA GTTTTGATGG
 701  TCAAAACAGT AAGGCAACGC CAACCTACCA ACTCCCAATG GGCCCTCCCA
 751  GGAGCCCTAG TCATCCCCAC TATCTCATCT TCGTATAAAG ATCTGCCCAC
 801  CTTCTGCTAT TCCTCCACAC AATTCGTCTT CGGCATTTTT CTTTTTGAGG
 851  CAACCTACAT TCATTGAATC CCAGGATTTC TTCTTGTCCA AGCAGGTAAT
 901  TACTCACACA ATGGCATTTT GAGATTCAGG GCTTTGGTTT ACCTTAGATC
 951  GTTGGATAAT TTGGGTAATT GGACCTTTAA TGCAAGATCT GGGTTTCAGA
1001  CCCGTTGGGG AATGCCTGGT AATATTGGGG CTTTTCTGTT TTTTTTCCC
1051  AGCTGGTATT TAGATCTGTT TTCAACTGTT TTGCAGGTTT AACGAAATGG
1101  CAAGCACAAG TGTTGCTGCA GCAGAGGTGA AG
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Figure 6. DNA sequence of the loblolly pine CCoAOMT promoter region. AC-rich elements are italicized and underlined, CCAAT box is bold and underlined, TATA box is bold, putative transcription start site is indicated by a vertical line and an arrow, 5′ intron is underlined and first ATG is indicated with shadowed outline.

lignin biosynthesis pathway towards G lignin deposition. AEOMT is likely to provide lignin intermediates through both ester and acid pathways in loblolly pine (Figure 1). Although recombinant AEOMT is capable of efficient methylation of 5-hydroxyferulic acid and 5-hydroxyferuloyl CoA ester and recombinant CCoAOMT is capable of efficient methylation of 5-hydroxyferuloyl CoA ester, these substrates are not likely to be present in gymnosperms due to the absence of F5H enzyme (Higuchi, 1985). Thus both AEOMT and CCoAOMT are capable of methylating the syringyl lignin-specific substrates and there is no need to transfer these genes from angiosperms to gymnosperms to activate the S lignin biosynthesis pathway in gymnosperms as proposed earlier (Bugos *et al.*, 1991).

Isolation of the promoter region of pine CCoAOMT gene and expression of promoter-GUS fusion in transgenic tobacco

About 1.1 kb 5′-flanking region upstream of the first ATG of CCoAOMT gene from loblolly pine was isolated using the Universal GenomeWalker (ClonTech) procedure (Figure 6; GenBank accession number AF098159). We compared the DNA sequence (75 bp) of the 5′-untranslated region from the CCoAOMT cDNA with the corresponding region immediately upstream of the ATG from the genomic CCoAOMT clone. To our surprise, the presence of a 191 bp intron situated 10 bp upstream of ATG was identified (Figure 6). The remaining 65 bp from the 5′-untranslated region of the CCoAOMT cDNA were identical to the genomic region upstream of the putative 5′ intron except for one extra base in the genomic clone. Although the presence of a 5′ intron is also observed in a few other plants (Kloesgen *et al.*, 1986; Christensen *et al.*, 1992), and such introns are important in gene regulation of some monocot genes (McElroy *et al.*, 1990), this is the first observation of a 5′ intron in any phenylpropanoid pathway-related genes in plants. Processing of loblolly pine CCoAOMT pre-mRNA might involve an additional step of intron splicing and this may be important in the regulation of CCoAOMT gene expression in gymnosperms. The 829 bp region of the genomic clone upstream of the 5′ end of the cDNA region appears to be the promoter region of this single-copy CCoAOMT gene. It showed the presence of a typical TATA box (Joshi, 1987b) at the −40 position, CCAAT box-like sequence at the −90 position from the 5′ end of the cDNA region and some AC-rich regulatory elements that occur in several phenylpropanoid genes of plants (Seguin *et al.*, 1997).

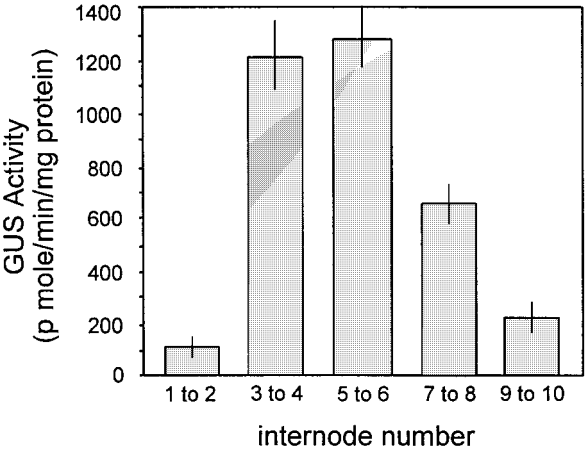

Figure 7. GUS enzyme activity in various internodes of transgenic tobacco plants that were transformed with loblolly pine CCoAOMT promoter-GUS construct. Each experiment was repeated at least three times. The GUS activity was determined with 4-methylumbelliferone glucuronide (MUG) as a substrate.

To examine if this CCoAOMT promoter region does confer the tissue-specific expression, a total of 895 bp upstream of the 5′ intron (829 bp promoter and 66 bp from 5′-untranslated leader) was fused to GUS gene in pBI101 plasmid (ClonTech). This construct was transferred into tobacco plants by *Agrobacterium*-mediated transformation. The GUS activity measurements from various internodes of transgenic tobacco plants with 10 internodes are shown in Figure 7. The highest GUS activity was observed in internodes 3–6 from the top. A minimum activity was noted in the top two and bottom two internodes of transgenic tobacco plants. Histochemical staining for GUS activity also showed intense blue color localized specifically to the secondary xylem of internodes numbers 3–6 (Figure 8A). No staining was observed in internodes 2 and 10 and a weak expression was observed in internode 8. These histochemical observations are in agreement with the GUS activity measurements which indicate that this CCoAOMT promoter is mainly active in developing secondary xylem tissues (Figure 7). Moreover, the presence of GUS was observed to be localized to the ray parenchyma cells of secondary xylem tissues in transgenic tobacco plants (Figure 8B). The importance of ray parenchyma cells as lignin precursor provider to adjacent lignifying xylem elements has been discussed earlier by Feuillet *et al.* (1995).

UV autofluorescence for lignin showed the presence of lignin deposition in phloem fibers as well as

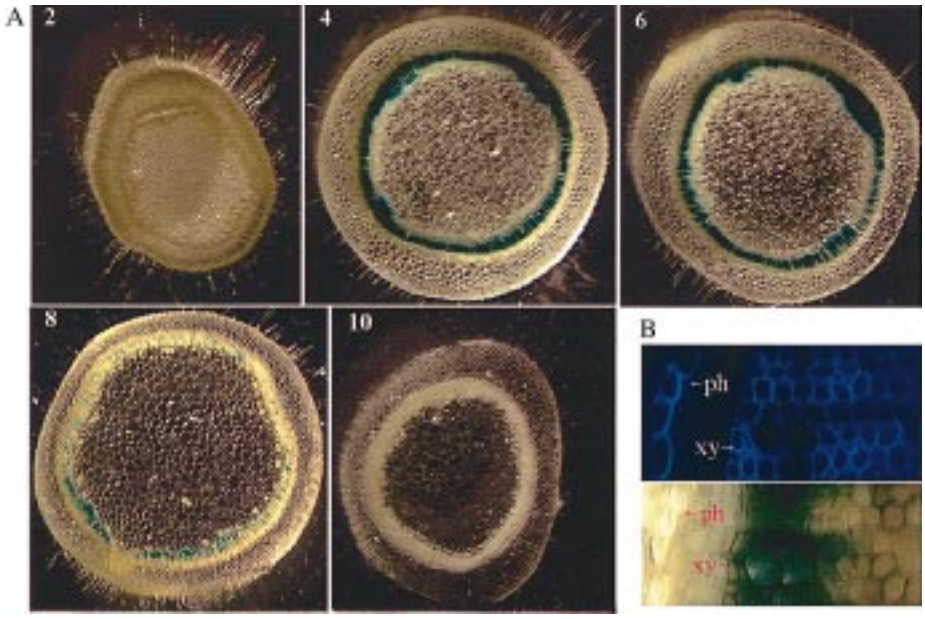

Figure 8. Loblolly pine CCoAOMT promoter-GUS fusion analysis. A. Histochemical localization of GUS activity in various internodes (2–10 as indicated by number) of transgenic tobacco plants that were transformed with loblolly pine CCoAOMT promoter-GUS construct. Note the intense GUS staining in the secondary xylem of internodes 4 and 6. B. Comparison of the CCoAOMT promoter-GUS fusion expression in different lignifying tissues (phloem fibers and xylem) at the 4th internode in a transgenic tobacco plant that was transformed with loblolly pine CCoAOMT promoter-GUS construct. Phloem fibers exhibiting lignin autofluorescence by the UV excitation at 365 nm in the top panel do not show any GUS staining in the bottom panel. Also note the presence of GUS staining in the secondary xylem ray parenchyma in the bottom panel that shows lignin autofluorescence in the top panel.

xylem tissue of tobacco plants but no GUS staining was observed in phloem fibers in any of the transgenic tobacco plants (Figure 8B). Previously, tissue-print hybridization studies localized the predominant presence of CCoAOMT mRNA signal in young differentiating xylem as well as in phloem fibers and xylem cells of old internodes (Ye and Varner, 1995). Using the same technique, alfalfa CCoAOMT transcripts were recently localized to xylem tissue from second internode but not the phloem tissues (Inoue *et al.*, 1998). In contrast, in the third and fifth internodes from alfalfa plants CCoAOMT transcripts were detected in both xylem and phloem (Inoue *et al.*, 1998). Our results with loblolly pine CCoAOMT promoter-GUS fusion indicate the secondary xylem-specific expression of loblolly pine CCoAOMT gene. This is the first demonstration of CCoAOMT promoter activities in any plant species. Moreover, no GUS staining was observed in transgenic tobacco leaf or petiole (Figure 8) and flower tissues transformed with loblolly pine CCoAOMT promoter-GUS fusion (data not shown). Taken all these results together, it can be concluded that the loblolly pine CCoAOMT promoter is mainly expressed in the secondary xylem tissue. It must be, however, noted that, although our results concerning secondary xylem-specific expression in transgenic tobacco are in good agreement with the possible role of CCoAOMT in lignin biosynthesis of loblolly pine, it is difficult to compare the wood anatomy of a perennial gymnosperm with the herbaceous angiosperm and it is possible that the pattern of expression of a promoter from a gymnosperm in an angiosperm species does not fully reflect the pattern of expression of the corresponding gene in homologous system. Further *in situ* hybridization studies are required to confirm these results.

In loblolly pine, CCoAOMT is most probably a single-copy gene. The northern blot analysis indicated that massive amounts of CCoAOMT transcripts in loblolly pine are present in the mature stem tissue containing differentiating secondary xylem as compared to seedling stem tissue containing mainly the primary xylem. Moreover, western blot analysis with CCoAOMT antibody and CCoAOMT enzyme assays from different plant organs/tissue also corroborated the same observation. The CCoAOMT promoter-GUS fusion in transgenic tobacco plants further supports the same conclusion. These data suggest

that CCoAOMT gene is specifically expressed in the secondary xylem tissue in loblolly pine. Availability of CCoAOMT promoter from loblolly pine could be utilized in future for secondary xylem-specific expression of angiosperm genes for genetic engineering of S lignin in gymnosperms. In addition, antisense inhibition of CCoAOMT under the control of loblolly pine CCoAOMT promoter in transgenic loblolly pine plants may help in delignification during the pulping process. Recently, Zhong *et al.* (1998) demonstrated the important role of CCoAOMT in regulating the quality and quantity of lignin by antisense approach in tobacco. Our present study with loblolly pine CCoAOMT opens the door to attempt similar experiments in conifers which are preferred tree species for wood pulp production due to superior fiber properties.

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