Plant Physiol. (1997) 113: 65–74

Molecular Cloning of 4-Coumarate:Coenzyme A Ligase in Loblolly Pine and the Roles of This Enzyme in the Biosynthesis of Lignin in Compression Wood[1]

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Two genomic sequences encoding 4-coumarate:coenzyme A ligase (4CL; EC 6.2.1.12) in loblolly pine (*Pinus taeda* L.) were cloned. Both sequences contained three introns and four exons with identical coding sequences predicting 537 amino acids. Two of the three introns in these two clones were different both in sequence and in length. Sequences of both 4CL clones were found in all nine megagametophyte DNAs tested, providing genetic evidence that these two 4CL genomic sequences are nonallelic genes. Our analyses suggest that there are at least two distinct, intron-containing 4CL genes, at least one of which is transcribed into 4CL mRNA in developing xylem tissue of loblolly pine. The levels of 4CL gene transcription in xylem were influenced by compressional stress, resulting in an elevated 4CL enzyme activity with 4-coumaric acid. 4CL enzyme activity with ferulic acid remained unchanged, whereas with caffeic acid it was significantly inhibited. Exogenously applied *trans*-cinnamic acid in the protein extracts from normal wood xylem caused inhibition of 4CL activity toward caffeic acid similar to that under compressional stress. The implications of this cinnamic acid-modulated effect on 4CL enzyme activities toward different substrates in regulating monolignol synthesis in xylem under compressional stress are discussed.

Plants adapt to changes and stress in their environment to survive. When a plant is forced out of its natural equilibrium position in space by environmental perturbations, a unique growth pattern is initiated to restore its orientation. In conifers, bending of the stem or branches results in the formation of special woody tissues that have the ability to restore the original orientation. Collectively, these woody tissues are called compression wood, which is characterized by a high quantity of lignin and a low content of cellulose (Timell, 1986). In general, the lignin content in normal wood of conifers is about 30%, whereas compression wood has a lignin content as high as 40% (Timell, 1986). In normal conifer wood tissue, lignin is formed by the polymerization of coniferyl alcohol (Fig. 1, 11) to form guaiacyl lignin, with the incorporation of a small but basal amount (approximately 5% of the total lignin) of 4-hydroxyphenyl lignin derived from 4-coumaryl alcohol (Fig. 1, 5). In compression wood, coniferyl alcohol copolymerizes with a higher amount of 4-coumaryl alcohol that has multiple conjugating sites to form a highly condensed lignin (the so-called "abnormal" lignin) (Timell, 1986; Higuchi, 1990). The condensed lignin in compression wood may also be used by trees to resist pathogen attack (Chen and Chang, 1985). In wound-healing plant tissues, the 4-coumaryl moiety is also incorporated into suberin, forming a barrier to pathogens (Kolattukudy and Köller, 1983). An exceptionally high number of 4-coumaryl units are found in the lignin synthesized by cultured spruce (*Picea abies*) cells under elicitation (Lange et al., 1995). Thus, the occurrence of an abnormal number of 4-coumaryl moieties in conifer plant cells appears to be a common stress-inducible metabolic response that is indispensable to the defense system in plants.

4CL (EC 6.2.1.12), which catalyzes the activation of 4-coumaric acid (Fig. 1, 2), caffeic acid (Fig. 1, 6), or ferulic acid (Fig. 1, 8) in conifers, is considered to be a key stress-response enzyme and has been extensively studied in many plant species. 4CL enzymes have been purified or partially purified from soybean and parsley (Knobloch and Hahlbrock, 1975, 1977), *Forsythia* (Gross and Zenk, 1974), petunia (Ranjeva et al., 1976), pea (Wallis and Rhodes, 1977), spruce (Lüderitz et al., 1982), poplar (Grand et al., 1983), maize (Vincent and Nicholson, 1987), and loblolly pine (*Pinus taeda* L., Voo et al., 1995). The cDNA sequences have been reported for parsley (Lozoya et al., 1988), potato (Becker-André et al., 1991), soybean (Uhlmann and Ebel, 1993), loblolly pine (Voo et al., 1995), Arabidopsis (Lee et al., 1995), aspen (Kawaoka and Chiang, 1996), and tobacco (Lee and Douglas, 1996). 4CL genomic DNAs have been isolated and sequenced for parsley (Douglas et al., 1987; Lozoya et al., 1988), rice (Zhao et al., 1990), and potato (Becker-André et al., 1991). Genomic DNA for loblolly pine 4CL has not, to our knowledge, been previously cloned and characterized.

[1] This work was supported by a grant (no. IBN 9118386) from the National Science Foundation and in part by a grant from McIntire-Stennis Forestry Research Program to V.L.C.

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Abbreviations: CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA/5-hydroxyferuoyl-CoA 3-*O*-methyltransferase; CCR, cinnamoyl-CoA oxidoreductase; C3H, 4-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; COMT, caffeate 3-*O*-methyltransferase; 4CL, 4-coumarate:CoA ligase; nt, nucleotide; PAL, Phe ammonia-lyase.

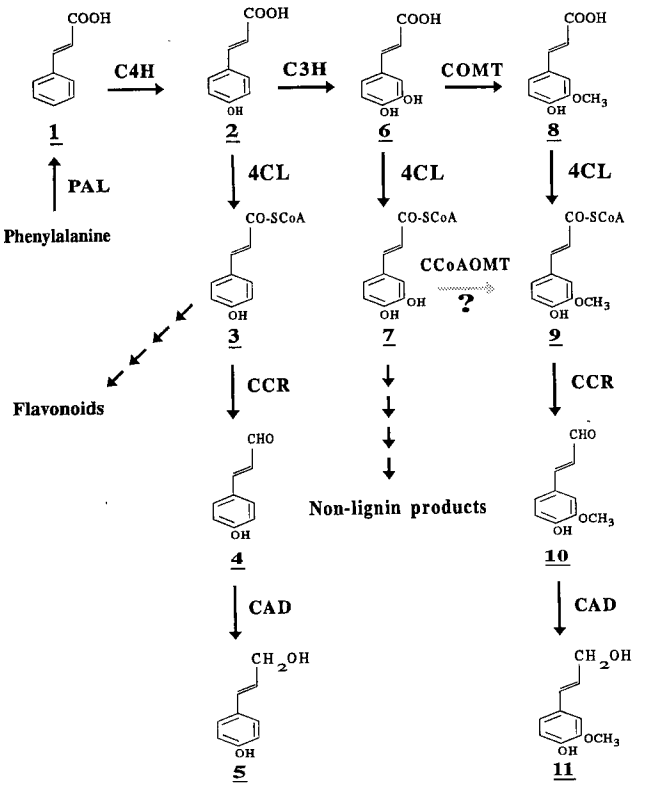

Figure 1. The proposed pathway of lignin biosynthesis in gymnosperms. The pathway intermediates are indicated with underlined numbers. A question mark (?) indicates an unknown step.

Kutsuki and Higuchi (1981) were the first to demonstrate that 4CL was one of the enzymes involved in lignin biosynthesis and that it had increased activity in the xylem of compression wood of *Thuja orientalis* and *Metasequoia glyptostroboides*. Elevated activities of 4CL and CCR were also found in the xylem of compression wood of loblolly pine (Popko, 1993). Recently, Voo et al. (1995) reported a comprehensive study of 4CL enzyme activity, cDNA cloning from developing xylem, and segregation of 4CL gene fragments in megagametophyte DNAs of loblolly pine. They concluded that in the loblolly pine genome there is a single 4CL gene encoding two xylem 4CL mRNAs that are allelic. Also, no evidence was found for a different form of the 4CL enzyme in the xylem of compression wood. In this paper we report the cloning, sequence characterization, and Southern analysis of 4CL genes from genomic DNA and the gene segregation in megagametophyte DNAs of loblolly pine. Changes in 4CL enzyme activities with various substrates during the first 48 h of compression wood formation are also described. Based on our analysis, we propose a possible role for 4CL, in conjunction with pathway intermediates, in the regulation of abnormal lignin synthesis in compression wood of loblolly pine.

MATERIALS AND METHODS

Differentiating secondary xylem was collected from 3-year-old loblolly pine (*Pinus taeda* L.), ground in liquid nitrogen, and used immediately for RNA isolation or stored in liquid nitrogen until use. Vegetatively propagated 3-year-old loblolly pine trees (genotype no. 1932 grown in the Forest Experimental Station of International Paper, Bainbridge, GA) were used. Compression wood was induced by bending the tree stem to a 45° angle for various periods, and differentiating secondary xylem in the compression area was collected directly into liquid nitrogen. Normal wood xylem was collected from the stem of the same genotype without bending.

RNA Isolation and cDNA Library Construction

Total RNA was isolated from the xylem of normal wood and compression wood following the method of Bugos et al. (1995). Poly(A)$^+$ RNA from normal wood xylem was used to construct a cDNA library using the Stratagene cDNA cloning kit (λZAP II vector).

Isolation of 4CL cDNA Clones

λ-phage cDNAs were isolated from the amplified cDNA library (Sambrook et al., 1989). Based on a GenBank entry of the pine 4CL cDNA sequence (accession no. U12012), primers were designed to frame the entire 4CL-coding sequence plus parts of its 5′ and 3′ noncoding regions: primer A, 5′-CATTCAATTCTTCCCACTGCAGGC; primer B, 5′-GCGATCGCAAGAGTGTAGGGCGTT. PCR was used to amplify the 4CL cDNA using a DNA thermal cycler (model 480, Perkin-Elmer). The reaction mixture (50 μL) contained about 50 ng of λ-phage cDNA, 0.2 μM each primer, 200 μM each deoxyribonucleotide triphosphate, 1× PCR reaction buffer, and 2.5 units of *Taq* DNA polymerase. The PCR was initiated by denaturing the reaction mixture at 94°C for 3 min, followed by 35 cycles of 94°C (1 min), 58°C (1 min), 72°C (2.5 min), and a final 10-min extension at 72°C. Ten microliters of the reaction were used for agarose gel electrophoresis. The amplified DNA fragments were cloned into plasmid vector using the TA-cloning kit (Invitrogen, San Diego, CA).

Southern Blot Hybridization

Genomic DNA was isolated from the needles or xylem of loblolly pine according to the method of Doyle and Dickson (1987). DNA (15 μg each) was digested separately with *Eco*RV, *Eco*RI, *Hin*dIII, *Bam*HI, *Dra*I, and *Xba*I, separated by agarose gel electrophoresis, and blotted onto a membrane (Hybond-N+, Amersham). A fragment of loblolly pine 4CL cDNA (0.5 kb) void of any of the restriction sites mentioned above was labeled with [^{32}P]dCTP using the DECA Prime II DNA labeling kit (Ambion, Austin, TX). The blot was hybridized to the probe at 65°C for 1.5 h, using Rapid-hyb buffer (Amersham). The membrane was washed with 2× SSC-0.5% SDS at room temperature for 20 min and 0.5× SSC-1% SDS at 65°C for 15 min twice and exposed to Kodak x-ray film at −80°C.

PCR Amplification, Cloning, and Sequencing of 4CL Genes

Primers A and B (see above) were also used to amplify 4CL genes with genomic DNA as a template. The genomic

DNA was isolated from a single tree, the xylem of which was used for isolating mRNA to construct the cDNA library. PCR was carried out in a 50-μL reaction mixture containing 200 ng of genomic DNA, 0.2 μM each primer, 200 μM each deoxyribonucleotide triphosphate, 1× PCR reaction buffer, and 2.5 units of *Taq* DNA polymerase. The PCR reaction mixture was denatured at 94°C for 3 min, followed by 35 cycles of 94°C (1 min), 58°C (1 min), and 72°C (4 min), and was ended with a 10-min extension at 72°C. Ten microliters of the reaction was used for agarose gel electrophoresis and blotted for hybridization to a probe of [^{32}P]dCTP-labeled whole 4CL cDNA (1.9 kb). Fragments of about 2.7 kb were strongly hybridized. The PCR products were cloned using the TA-cloning kit (Invitrogen). Clones with inserts of 2.7 kb were sequenced and confirmed to encode 4CL. To identify different intron-containing fragments of 4CL genes in the loblolly pine genome, three additional pairs of primers designed from conserved sequences for all plant 4CLs studied so far were used to carry out independent PCR amplifications using genomic DNA as the template. One of the primer pairs, primer C (5′-AGGCTGGCTCCACACAGG) and primer D (5′-TCCTGCTCGCTGATTTCC), was also used to amplify 4CL DNA fragments (approximately 0.7 kb), which included introns and 240 bp of coding sequence, to further verify the existence of introns in 4CL genes from different genotypes of loblolly pine.

Nt sequencing of the cloned 4CL cDNA and genes was performed for both strands by the dideoxynucleotide chain termination method of Sanger et al. (1977) using the Sequenase kit from United States Biochemical and synthetic oligonucleotides.

Protein Extraction and Enzyme Assays

Differentiating xylem from normal wood and compression wood of trees (genotype no. 1932) bent for various periods was ground in liquid nitrogen and homogenized in extraction buffer (0.2 M Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 5 mM DTT, 30% glycerol, and 1 μg/mL leupeptin). The extracts were centrifuged at 18,000*g* at 4°C for 15 min, and the supernatants were used for enzyme assays.

The 4CL activity assay was based on the direct spectrophotometric method of Knobloch and Hahlbrock (1977). The reaction mixture contained 0.2 M Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 0.8 mM ATP (Sigma), 0.1 mM substrate (4-coumaric acid, ferulic acid, caffeic acid, or *trans*-cinnamic acid; Sigma), and 25 to 30 μL of crude extract in 1 mL. The reaction was initiated by the addition of CoA (Sigma) to a final concentration of 25 μM and carried out at 22 to 24°C. The increase in A_{333} (4-coumaroyl-CoA), A_{346} (feruloyl-CoA), or A_{350} (caffeoyl-CoA and cinnamoyl-CoA) over 12 min was monitored. The specific enzyme activity is expressed as picokatal (1 picomol of substrate converted to product per second) per milligram of total extractable protein. At least three replicate samples were assayed for each treatment.

COMT activity was assayed with caffeic acid as the substrate according to the method of Kuroda et al. (1975). The reaction mixture contained 0.1 M Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM caffeic acid, and 50 μL of crude extracts in a total volume of 200 μL. After preincubation at 30°C for 5 min, the reaction was initiated by the addition of 100 nmol of diluted *S*-adenosyl-L-[*methyl*-^{14}C]Met (Amersham) and incubated at 30°C for 20 min. The reaction was terminated by the addition of 10 μL of 4 N HCl. Radioactivity in diethyl ether extraction was determined by liquid scintillation counting. Protein concentrations in crude extracts were determined by the Bradford dye-binding assay (Bio-Rad) using BSA (Sigma) as a standard.

Northern Blot Hybridization

Total RNA (20 μg per lane) was denatured by 50% deionized formamide and 2.2 M formaldehyde at 65°C for 15 min, separated by 2.2 M formaldehyde and 1.2% agarose gel electrophoresis with 40 mM Mops, and blotted onto Hybond-N+ membranes (Amersham). ^{32}P-labeled loblolly pine 4CL cDNA (1.9 kb) was hybridized to the blots at 65°C for 1.5 h using Rapid-hyb buffer (Amersham). The membranes were washed with 2× SSC-0.5% SDS at room temperature for 20 min and twice with 0.5× SSC-1% SDS at 65°C for 15 min, and then they were exposed to Kodak x-ray film at −80°C. The RNA blots were then stripped and hybridized separately to ^{32}P-labeled loblolly pine cDNAs of PAL, COMT, or CAD.

RESULTS

The Loblolly Pine Genome Contains Two 4CL Genes with Introns

Based on the 4CL cDNA sequences available in the GenBank database, primers A and B were designed to PCR amplify 4CL genes using genomic DNA as a template. The PCR products were cloned, and 10 clones with inserts of 2.7 kb were sequenced and confirmed to code for 4CL. All 10 4CL clones contained an identical coding sequence (four exons) and three introns with different sequences that segregate these 10 clones in two 4CL gene groups, i.e. *lp4cl-1* and *lp4cl-2*. Of the three introns, intron II (90 bp) is identical for both 4CL genes, whereas introns I and III are different both in sequence and in length. Intron I in both genes has four *Dra*I sites (Fig. 2); however intron I (425 bp) of *lp4cl-1* has an internal *Eco*RI site that is absent in intron I (431 bp) of *lp4cl-2*. Overall, the sequences of intron I in both genes are highly similar, with a greater than 90% identity.

Sequences in intron III are distinctly different between *lp4cl-1* and *lp4cl-2*. Intron III in *lp4cl-2* contains one *Dra*I site and is 30 bp longer than intron III in *lp4cl-1*, which has two *Dra*I sites (Fig. 2). Within intron III, two regions show high sequence similarity between these two 4CL genes. One region of 169 bp, located at the 5′ splice junction of intron III in *lp4cl-1* (nt 1921–nt 2090) shows 96% sequence identity with the corresponding region of intron III in *lp4cl-2*. The other is a 47-bp fragment near the 3′ splice junction of intron III in these genes (nt 2223–nt 2269), with an identity of 98% between these two regions. However, the sequences in the middle portion of intron III (124 bp for *lp4cl-1* and 150 bp for *lp4cl-2*) are highly divergent, with no obvious sequence similarity.

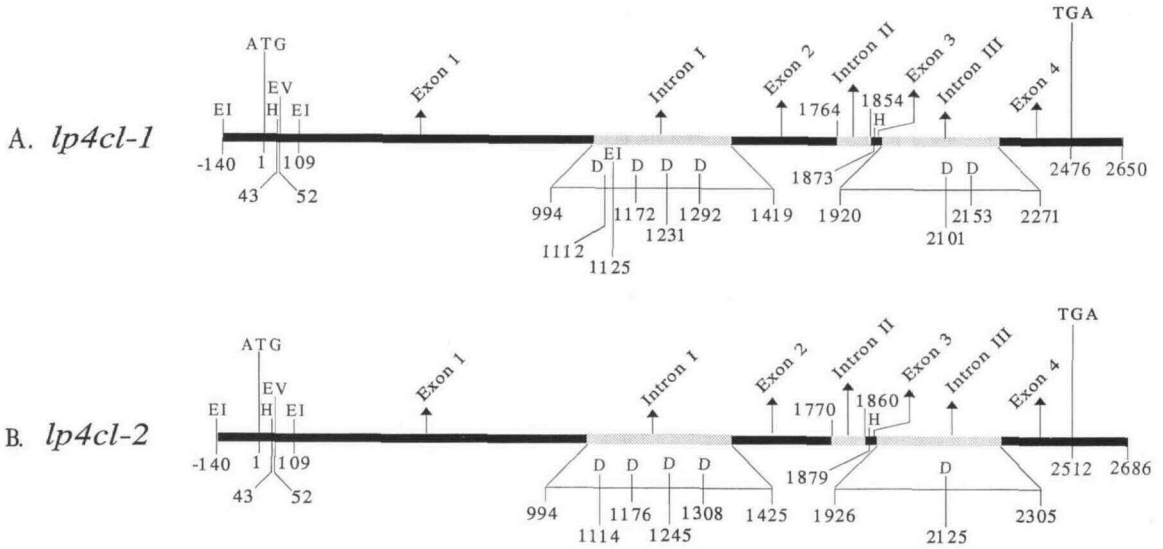

Figure 2. Restriction map of 4CL genes from loblolly pine. The start codon (ATG) of translation is indicated by the number 1. The 5′ noncoding region is numbered as minus. Restriction enzymes used in the Southern blot analysis are shown as: EV, *Eco*RV; EI, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; D, *Dra*I; and X, *Xba*I. The termination codon (TGA) is also presented. A, Gene *lp4cl-1*. B, Gene *lp4cl-2*. GenBank accession numbers for *lp4cl-1* and *lp4cl-2* are U39404 and U39405, respectively.

It has been reported that there was only a single-copy gene of 4CL in the loblolly pine genome (Voo et al., 1995). To verify the size of the 4CL gene family in loblolly pine, a fragment of the 4CL-coding region (nt 109–nt 595; Fig. 2) was used to probe a Southern blot of genomic DNA digested by six restriction enzymes (*Eco*RV, *Eco*RI, *Hin*dIII, *Bam*HI, *Dra*I, and *Xba*I). Based on the DNA sequences, both 4CL gene clones contain one site each for *Eco*RV, two sites for *Hin*dIII, and no restriction site for *Bam*HI and *Xba*I. Also, two *Eco*RI and six *Dra*I sites occur in *lp4cl-1*, whereas one *Eco*RI and five *Dra*I sites are in *lp4cl-2* (Fig. 2). The probe used for hybridization corresponds to a fragment in exon 1. This fragment does not contain any site that would be restricted by any of the six enzymes used for Southern analysis, allowing each hybridizing band to represent a distinct 4CL-coding gene.

Two hybridizing bands were detected for genomic DNA digested with five of six restriction enzymes used (Fig. 3). The *Bam*HI blot showed only one band of 4.6 kb, which could be two DNA fragments of a similar size from two different 4CL genes. Since there are two *Eco*RI sites in *lp4cl-1* (Fig. 2), an expected hybridizing band of about 1.0 kb (nt 109–nt 1125) was observed for this gene. Another band of 2.6 kb is assumed to represent *lp4cl-2*. For *Eco*RV-digested DNA, two hybridizing bands of 6.5 and 3.2 kb were observed, whereas 7.0- and 4.5-kb bands were detected after *Xba*I digestion. For the *Hin*dIII-digested DNA blot, an expected hybridizing band of about 1.8 kb (nt 43–nt 1873 for *lp4cl-1* in Fig. 2A and nt 43–nt 1879 for *lp4cl-2* in Fig. 2B) should appear for both 4CL genes since there are two *Hin*dIII restriction sites within the coding region in each of the 4CL genes. However, an extra band of 3.4 kb was not expected (Fig. 3, lane H), and may have been the result of incomplete *Hin*dIII digestion. For *Dra*I restriction, each 4CL gene should include only one digested fragment that hybridizes to the probe segment. Hence, two hybridizing bands are expected, each of which represents a fragment from a distinct 4CL gene. Furthermore, the size of these hybridizing bands should be larger than 1.1 kb, since the first *Dra*I site in either of the 4CL genes is located around nt 1112 (Fig. 2). As expected, the hybridization showed two fragments of 1.6 and 2.4 kb (Fig. 3, lane D). Overall, the results of gene cloning, sequencing, and Southern blot hybridization all indicate the existence of two distinct, although similar, intron-containing 4CL genes in the loblolly pine genome.

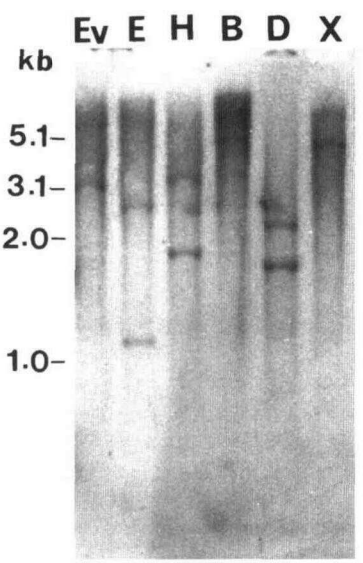

Figure 3. Southern blot hybridization of 4CL genes. Genomic DNA (15 μg each sample) of loblolly pine was digested with *Eco*RV (Ev), *Eco*RI (E), *Hin*dIII (H), *Bam*HI (B), *Dra*I (D), and *Xba*I (X), blotted onto a nylon membrane, and hybridized to P-labeled 4CL cDNA probe A (0.5 kb) corresponding to a portion of exon 1.

Voo et al. (1995) PCR-amplified 28 megagametophyte (haploids; loblolly pine genotype no. 7–56) and one diploid genomic DNA. One of the primer sets they used was apparently located around nt 1000 to nt 1613 in their 4CL cDNA. They showed the segregation of DNA fragments that matched the size of a cDNA fragment in all 29 genomic DNA samples, implying no introns within the regions they studied, in contrast to our data that this region contains two introns (introns II and III). To examine whether our cloned 4CL genes also exist in the megagametophyte DNAs of genotype 7–56, we PCR-analyzed the megagametophyte DNAs provided by one of the authors (R. Whetten) of Voo et al. (1995). A set of primers (primers C and D) was used to amplify a portion of the 4CL genes from the megagametophyte DNAs. Primers C and D are located in nt 1238 to nt 1255 and nt 1477 to nt 1460, respectively, according to the cDNA sequence by Voo et al. (1995).

However, based on our 4CL gene sequences, two introns (introns II and III) should be encompassed by these primers. Therefore, using this primer set we expected two PCR fragments (709 and 679 bp), with a 30-bp difference in length, to be generated from the genomic DNA if there are two distinct intron-containing 4CL genes, as shown in Figure 4A. Furthermore, if there is a single 4CL gene per haploid pine genome, there should be a single PCR fragment for each of the megagametophytes (from the same diploid megaspore) with a 1:1 ratio of segregation. Also, if there is no intron within the region amplified, the PCR fragment enclosed by primers C and D would be the same size as a cDNA segment of 240 bp. As shown in Figure 4B, two major PCR bands were observed from each of the megagametophytes, with sizes corresponding to our 4CL gene fragments, and a similar amplification efficiency in each of the haploid genomes, indicating two nonallelic intron-containing genes. Two patterns (Fig. 4B, lanes 1, 2, 8, and 9 versus lanes 3–7) were observed among these nine megagametophytes, with an approximately 1:1 ratio of segregation, which demonstrates the existence of two nonallelic and intron-containing 4CL genes that exhibit allele variations among megagametophytes. A third product with a size matching a 4CL cDNA fragment (240 bp; Fig. 4, lane 10) was also amplified from each megagametophyte with less efficiency, suggesting the absence of introns. Similar patterns were observed in the xylem genomic DNA (diploid) from another genotype (no. 1932) of loblolly pine. Southern blot hybridization of the PCR products to a full-length 4CL cDNA probe indicated that the amplified DNA fragments were indeed from 4CL-coding genes. The presence of a third, intronless 4CL gene in loblolly pine may suggest either a nonfunctional 4CL "processed pseudogene" or a functional gene structurally different from the two intron-bearing genes.

Two Pine 4CL Genes Encode Identical Amino Acid Sequence

Two reported 4CL cDNA clones from loblolly pine (GenBank accession nos. U12012 and U12013; Voo et al., 1995) share 99.5% nt sequence identity and differ at one amino acid, i.e. Glu (GAA) in U12012 versus Gly (GGG) in U12013. The two 4CL genes (*lp4cl-1* and *lp4cl-2*) cloned in this study encode an identical amino acid sequence with Gly (GGG) at nt 1689 to nt 1691, the same as in 4CL U12013. To isolate the 4CL genes with a Glu (GAA) substitution in the coding region and to test the possibility of two different 4CL mRNAs in the pine genotype used in this study, we designed primers to amplify and clone the coding region encompassing nt 1689 to nt 1691. Extracts of a cDNA library and genomic DNA both obtained from a single loblolly pine tree were used as the templates. Fifteen clones from independent experiments were sequenced and all showed GGG (Gly) at the expected position. Since both *lp4cl-1* and *lp4cl-2* predict an identical 4CL amino acid sequence, it remains to be determined whether they are both functional and exhibit differential expressions. At the present stage, we can only speculate that the 4CL mRNAs that Voo et al. (1995) reported and that we report here could be transcribed from at least one of the 4CL genes in loblolly pine.

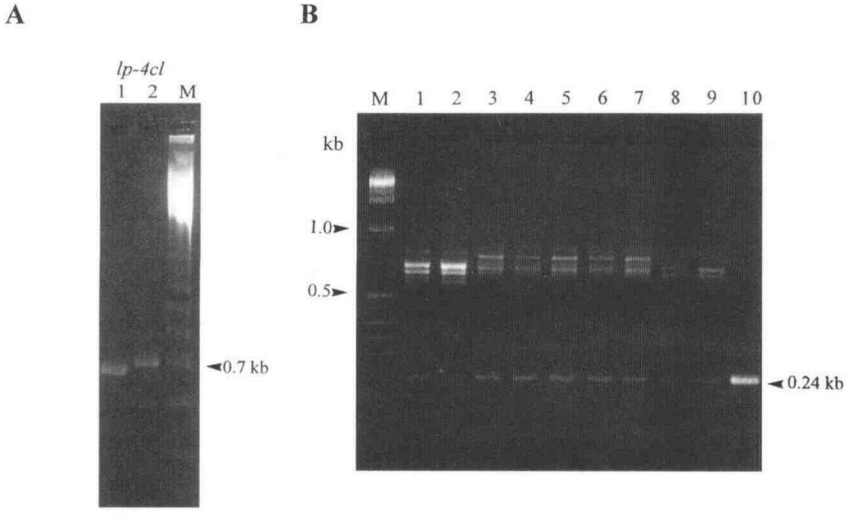

Figure 4. PCR analysis of 4CL genes in the loblolly pine genome. PCR products were separated by 0.5× Tris-borate EDTA buffer 2.5% agarose gel electrophoresis and stained with ethidium bromide. A, PCR amplification of genomic DNA clones of *lp4cl-1* (lane 1) and *lp4cl-2* (lane 2) with primers C and D. Lane M, one hundred base-pair DNA ladder (GIBCO-BRL). B, PCR amplification of loblolly pine genomic DNA preparations with primers C and D. Lane M, one-kilobase DNA ladder (GIBCO-BRL); lanes 1 to 9, genomic DNAs isolated from megagametophyte (haploid) tissue of loblolly pine (genotype 7–56) were used as templates; lane 10, full-length 4CL cDNA was used as a template.

Comparative Analysis of Intron Sequences in Plant 4CL Genes

To our knowledge, nt sequences for 4CL genes have been previously reported for only three plant species: parsley (Lozoya et al., 1988), rice (Zhao et al., 1990), and potato (Becker-André et al., 1991). It is interesting to note that all of these 4CL genes contain four introns in the coding region, whereas the pine 4CL genes possess three introns. Comparison of the 4CL introns in loblolly pine, rice, potato, and parsley revealed that all introns contain conserved sequences essential for proper splicing. Overall, the splice junctions of introns in these four plant 4CL genes are consistent with the conserved sequences found in introns of eukaryotic nuclear genes, suggesting the typical characteristics of intron-containing nuclear 4CL genes (not pseudogenes). All three introns in the pine 4CL genes are highly AT-rich, which is also essential for proper processing of plant introns (Goodall and Filipowicz, 1989).

4CL Gene Expression during Compression Wood Formation in Loblolly Pine

The xylem of the loblolly pine tree responds rapidly to compressional stress induced by, for instance, bending of the stem. The brownish coloration of the xylem at the bend site, an indication of compression wood formation, can be visually detected as early as 30 min after bending. Because of the rapid development of compression wood, we examined the 4CL enzyme activities in xylem under compressional stress within 48 h of tree bending. The enzyme activities were assayed with four substrates, *trans*-cinnamic acid (Fig. 1, 1), 4-coumaric acid (Fig. 1, 2), caffeic acid (Fig. 1, 6), and ferulic acid (Fig. 1, 8), which are likely to be involved in pathways of both guaiacyl and 4-coumaryl monolignol synthesis. As expected, essentially no 4CL activity with *trans*-cinnamic acid was found in either the xylem of normal wood or that of compression wood. Compared with normal wood, the 4CL activity with 4-coumaric acid was increased by about 30 to 40% in compression wood xylem during the 2 to 48 h of bending (Fig. 5). However, the activity with ferulic acid remained unchanged throughout the 48 h of compressional stress (Fig. 5). The 4CL activity with caffeic acid in compression wood xylem was reduced to 50 to 83% of the activity in normal wood (Fig. 5). Repeated assays, including the use of independently extracted protein samples, showed a similar trend, i.e. compressional stress induced the 4CL enzyme activity toward 4-coumaric acid and had no effect on the activity with ferulic acid but inhibited the activity with caffeic acid in loblolly pine xylem.

It has been suggested that augmented concentrations of various intermediates in the general phenylpropanoid pathway and monolignols in lignifying tissues are the result of compressional stress (Timell, 1986). Therefore, we investigated the effect of increased levels of intermediates such as cinnamic acid on 4CL activity. It was found, surprisingly, that 4CL activity with caffeic acid in the protein extracts of normal wood xylem was significantly reduced in the presence of exogenously supplied cinnamic acid at concentrations as low as 15 μM (Table I). Cinnamic acid did not affect the 4CL enzyme activities toward 4-coumaric acid or ferulic acid. The cinnamic acid-induced inhibition of 4CL activity toward caffeic acid was not affected by the increased concentrations of caffeic acid, as shown in Table I. The levels of inhibition of 4CL activity toward caffeic acid by various concentrations of supplied cinnamic acid were within the range of the reduction of such activity in compression wood xylem. Hence, the observed reduction of 4CL activity on caffeic acid in compression wood xylem could be attributed at least in part to increased in vivo levels of cinnamic acid.

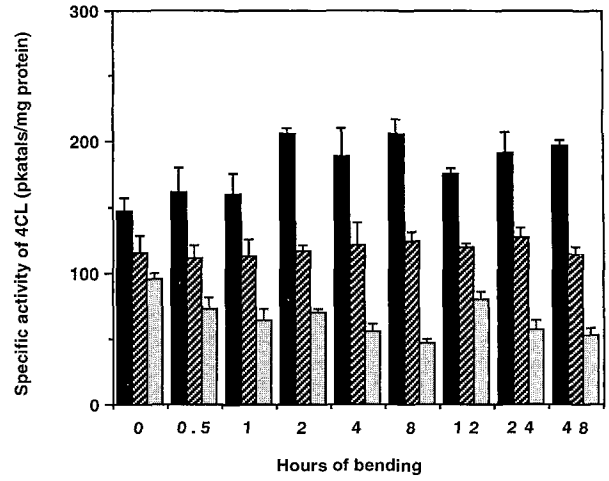

Figure 5. In vitro activities of xylem 4CL enzyme in normal wood (0 h) and compression woods (bending for 0.5 to approximately 48 h) of loblolly pine. Tested substrates shown are 4-coumaric acid (■), ferulic acid (▨), and caffeic acid (▤). Each bar is the mean of measurements of at least three duplicate samples; the error bar is ±SE.

To examine the expression pattern of 4CL genes at the transcription level during compression wood formation, we carried out northern blot analysis using loblolly pine 4CL cDNA as a probe. A single major message of approximately 2.1 kb was detected in RNA extracted from developing xylem tissue of both normal and compression wood. Based on the intensity of the hybridizing bands (Fig. 6A), and with equal loading of RNA samples (Fig. 6B), it appears that the level of 4CL gene expression is higher, although not markedly, in compression wood than in normal wood. Repeated tests of northern blot hybridization to the 4CL DNA probe showed similar results. As a reference, we also used homologous DNA probes to examine the transcription levels of genes involved in lignin biosynthesis pathways and found that the levels of xylem mRNAs for PAL, COMT, and CAD were higher in compression wood than in normal wood of loblolly pine (Fig. 6A). Like 4CL, COMT activities in compression wood xylem within 48 h of bending were 20 to 50% higher than the activities in xylem of normal wood. However, unlike the 4CL enzyme, exogenously added cinnamic acid did not affect the COMT activity (data not shown).

Table I. *Inhibitory effect of cinnamic acid on 4CL activity toward caffeic acid in xylem tissue of loblolly pine*

The 4CL enzyme of normal wood xylem was assayed using 0.1 mM caffeic acid, 4-coumaric acid, or ferulic acid as a substrate, with or without the addition of cinnamic acid. The value was the average of the measurements for three replicate samples, shown with SE. The relative activity is shown in parentheses, and the activity without cinnamic acid is taken as 100.0%.

Cinnamic Acid	Specific Activity		
	Caffeic acid	4-Coumaric acid	Ferulic acid
μM		*pkatal/mg protein*	
0	86.0 ± 0.8 (100.0)	176.0 ± 2.0 (100.0)	110.0 ± 1.5 (100.0)
15	55.3 ± 0.9 (64.3)	nd[a]	nd
30	54.7 ± 0.9 (63.6)	nd	nd
45	56.5 ± 0.8 (65.7)	176.8 ± 3.7 (100.5)	110.6 ± 0.8 (100.5)
75	60.1 ± 2.2 (69.9)	nd	nd
100	58.3 ± 4.5 (67.8)	176.6 ± 1.0 (100.3)	113.6 ± 1.5 (103.3)

[a] nd, Not determined.

DISCUSSION

Overall, our results from gene cloning and sequencing (Fig. 2), Southern blot analysis of diploid DNA (Fig. 3), and PCR analysis of megagametophyte (haploid) and diploid DNAs (Fig. 4) all support the existence of at least two distinct 4CL genes in the loblolly pine genome. Multiple 4CL genes are found in several other plants. Two 4CL genes were found in parsley (Lozoya et al., 1988) and potato (Becker-André et al., 1991), whereas soybean (Uhlmann and Ebel, 1993) and tobacco (Lee and Douglas, 1996) contain a small multigene family. Studies of parsley 4CL showed that two distinct 4CL genes share 99.5% identity in coding region at both nt and amino acid levels and more than 96% identity in both the 3′ untranslated region (within 300 bp) and the 5′ noncoding region (within 500 bp immediately upstream from the translation start codon) (Douglas et al., 1987; Lozoya et al., 1988). Also, these two genes share at least 97% identity for all four introns except the second, which contains two insertions in one of the two 4CL genes (Lozoya et al., 1988). These observations for the two distinct 4CL genes in parsley are very similar to our results with *lp4cl-1* and *lp4cl-2*.

Until now, plant 4CL genomic sequences were only available for one gymnosperm (loblolly pine) and three angiosperms (a monocot and two dicots). All of these 4CL genes contain introns in their coding regions. Except for their AT-rich nature, no similarity in intron sequences could be found in these known 4CL genes; however, the intron insertion into the coding sequences is highly conserved. All four introns in rice, potato, and parsley 4CL genes are located in a conserved domain of the 4CL amino acid sequence. For the pine 4CL gene, the insert positions for introns I and III are in the same protein domains as those in the 4CL genes of the other three plants. However, intron II in the pine genes is not inserted in the domain where the intron II in other plant 4CL genes is located. Instead, the insertion point is shifted 47 amino acids downstream. Furthermore, intron II in pine 4CL genes splits two amino acids (Q ↓ V), whereas intron II in other plant 4CL genes splits the first and second bases of the same codon for Gly. Since gymnosperms are considered to be more primitive than angiosperms (e.g. rice, potato, and parsley) (Cronquist, 1981), the insertion of intron II in pine 4CL genes could be a more ancient event during 4CL gene evolution. Also, intron IV, which is found in other plant 4CL genes, is absent in the pine 4CL genes. It can be speculated that either angiosperms introduced this intron into the 4CL genes or gymnosperms such as pine lost intron IV from their 4CL genes after the divergence of angiosperms from gymnosperms.

In parsley, two forms of 4CL isoenzymes, differing in three amino acids, exhibited virtually identical catalytic properties (Lozoya et al., 1988). It was believed that these two 4CL isoenzymes were unlikely to play a differential role in parsley phenylpropanoid pathways (Hahlbrock and Scheel, 1989). For loblolly pine, there is no difference between normal and compression wood in partially purified 4CL proteins (Voo et al., 1995). Our studies show that two

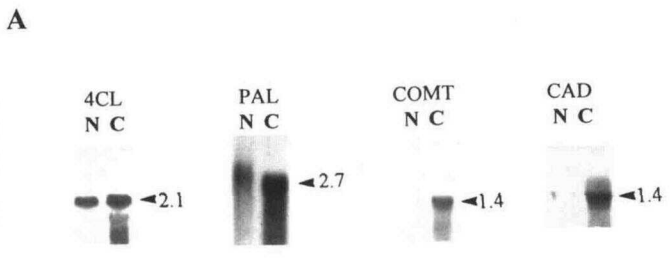

Figure 6. Transcription of genes involved in lignin biosynthesis in normal wood and compression wood of loblolly pine. Total RNAs (20 μg) from developing xylem tissue of normal wood (N) and compression wood (C) were fractionated by gel electrophoresis and blotted to a nylon membrane. A, The blot was hybridized to P-labeled 4CL cDNA. After being stripped, the same blot was separately hybridized to cDNA clones of PAL, COMT, or CAD from loblolly pine. RNA sizes were shown in kilobases. B, Ethidium bromide-staining RNA gel.

distinct 4CL genes predict an identical 4CL protein. Furthermore, the in vitro assay of 4CL enzyme activity of various conifer species indicated that the 4CL enzyme is typical of enzymes with broad substrate specificity and is capable of mediating effectively the CoA ligation of various cinnamic acid derivatives for the formation of different phenolic compounds (Hahlbrock and Grisebach, 1979; Kutsuki et al., 1982; Hahlbrock and Scheel, 1989; Voo et al., 1995). Therefore, it is possible that in loblolly pine only one type of 4CL enzyme, produced by either of the two 4CL genes, is sufficient to initiate the synthesis of various CoA-thioesters of cinnamic acid derivatives at different developmental stages and in various tissues.

Compression wood in conifers is known for its abnormally high content of lignin with the incorporation of unique 4-coumaryl moieties (Timell, 1986). Kutsuki and Higuchi (1981) demonstrated that the enzyme activities of 4CL, PAL, COMT, and CAD are 1- to 3-fold higher in compression wood xylem than in normal wood xylem of *T. orientalis* and *M. glyptostroboides*. These results are similar to our observations of increased 4CL and COMT enzyme activities and higher levels of mRNAs for 4CL, PAL, COMT, and CAD in xylem of compression wood than in normal wood (Figs. 5 and 6). Although the transcript level of the gene encoding CCR is unknown, both the CCR enzyme activity and the content of total xylem proteins in compression wood xylem of loblolly pine were found to be twice as high as that of normal wood xylem (Popko, 1993). Therefore, it is likely that compressional stress induces, to various degrees, the elevated expression of most genes involved in the biosynthesis of monolignols, resulting in an overall augmented synthesis of lignin in compression wood tissues. However, this could not explain the preferential increase in 4-coumaryl alcohol to generate 4-coumaryl-enriched (abnormal) lignin in compression wood (Adler, 1977; Nimz et al., 1981).

At this time, the precise mechanism for a preferential increase in 4-coumaryl moieties in compression wood lignin is still unclear. So far, there is no evidence of novel enzymes specifically responsible for the preferential synthesis of 4-coumaryl lignin in compression wood. However, our finding of the altered substrate specificity of 4CL in loblolly pine due to compressional stress could be a significant step toward an understanding of the mechanism underlying the enhanced synthesis of 4-coumaryl lignin. We found that the 4CL activity toward 4-coumaric acid was increased in xylem of compression wood. The 4CL activity with ferulic acid was essentially the same in compression wood as in normal wood. However, the 4CL activity toward caffeic acid was significantly lower in compression wood xylem (Fig. 5). This implies that since there is only one type of monomeric 4CL enzyme in either normal or compression wood xylem of loblolly pine, the specific catalytic functions of 4CL toward different substrates might be modulated by factors induced under compressional stress. Thus, in compression wood, whereas the overall elevated expression of lignin-related genes might contribute to the increase in total lignin quantity, the induced expression of 4CL mRNA gives rise to an augmented level of 4CL enzyme with an altered functionality that could preferentially convert 4-coumaric acid into 4-coumaroyl-CoA thioester (Fig. 1, 3) for the enhanced synthesis of phenylpropanoids such as 4-coumaryl lignin and flavonoids (Gross, 1985; Dixon and Paiva, 1995). Thus, it appears that the increased 4CL activity toward 4-coumaric acid during compressional stress may contribute mainly to the enhanced synthesis of 4-coumaryl lignin and perhaps flavonoids (Fig. 1).

The inhibited 4CL activity toward caffeic acid under compressional stress would reduce the conversion of caffeic acid into caffeoyl-CoA thioester (Fig. 1, 7), which often acts as a precursor to nonlignin products such as chlorogenic acid (Neish, 1964; Lamb, 1979). Thus, the lowered 4CL activity toward caffeic acid in compression wood xylem would not reduce lignin biosynthesis. On the contrary, the reduced 4CL activity for caffeic acid would channel less phenylpropanoid to nonlignin products, allowing a preferential distribution of phenylpropanoids for lignin (4-coumaryl lignin in particular) biosynthesis in compression wood. Therefore, the 4CL enzyme, with altered activities toward different substrates, could act as a regulator for the distribution of cinnamic acid derivatives into various phenylpropanoid pathways, favoring a characteristic flux of precursors for the formation of 4-coumaryl-enriched lignin required in compression wood tissue.

An alternative methylation step has been proposed for lignin biosynthesis in the angiosperm *Zinnia*, in which the CoA-ligated product of caffeic acid, caffeoyl-CoA thioester (Fig. 1, 7), is a necessary intermediate for the biosynthesis of guaiacyl lignin through the mediation of CCoAOMT (Ye et al., 1994). It is not clear at the present time whether the CCoAOMT activity toward caffeoyl-CoA thioester exists in loblolly pine xylem and what roles (if any) this enzyme might play in the biosynthesis of guaiacyl lignin and in the formation of compression wood in conifers.

The changes in catalytic function of a single type of 4CL enzyme toward different substrates in compression wood of loblolly pine (Fig. 5) have not, to our knowledge, been observed before. Our study strongly indicates that 4CL enzyme activity toward different substrates is likely modulated by pathway intermediates. Exogenously applied cinnamic acid at concentrations of 15 to 100 μM is shown to be able to interfere with the 4CL enzyme activity in normal xylem, resulting in changes in its catalytic specificity toward caffeic acid in a manner similar to that in compression wood xylem (Table I). It has been estimated that the endogenous concentrations of free cinnamic acid in bean and alfalfa cell cultures are 4 to 25 and 5 to 30 μM, respectively (Mavandad et al., 1990; Loake et al., 1991; Orr et al., 1993). Although the actual pool sizes of cinnamic acid in normal and compression wood of loblolly pine are currently unknown, the levels (15–100 μM) of cinnamic acid used in the enzyme assays (Table I) are likely within the physiological concentrations that could be induced in compression wood xylem.

It has been hypothesized that phenylpropanoid pathway intermediates such as cinnamic acid and 4-coumaric acid may act as both negative and positive regulators of gene

expression within the phenylpropanoid pathway (Bolwell et al., 1988; Mavandad et al., 1990; Loake et al., 1991, 1992; Orr et al., 1993; Ni et al., 1996). Our studies support this hypothesis. Furthermore, we present here the first observation, to our knowledge, that at the enzyme level cinnamic acid, a poor substrate itself for 4CL, can specifically inhibit 4CL activity toward caffeic acid while having no observed effect on 4CL activities for other substrates. It is interesting to note that cinnamic acid is an intermediate representing the entry to various phenylpropanoid biosynthetic pathways and has been shown to have effects on gene expression of phenylpropanoid pathway enzymes such as PAL, C4H, and chalcone synthase (Bolwell et al., 1988; Mavandad et al., 1990; Loake et al., 1991; Orr et al., 1993). It has been reported that the activity of recombinant 4CL proteins toward cinnamic acid could be inhibited by proteinaceous components from tobacco stem extracts, suggesting posttranslational interactions of 4CL enzyme with cellular protein factors (Lee and Douglas, 1996). Taken together, it is possible that the activity of the 4CL enzyme toward different substrates could be modulated by factors such as pathway intermediates and other components induced by compressional stress of the pine xylem. Also, it could be envisaged that overproduction of cinnamic acid to exceed a threshold level maintained under normal growth may signal an altered status for phenylpropanoid biosynthesis in cells as a response to external stimuli such as compressional stress.

In this study, we focused on cloning loblolly pine 4CL genes, confirming the existence of two distinct 4CL genes, and analyzing the roles of 4CL substrate specificity in relation to the formation of abnormal 4-coumaryl lignin in loblolly pine compression wood. We have demonstrated, for the first time to our knowledge, the effect of cinnamic acid, a pathway intermediate, on the substrate specificity of the 4CL enzyme and proposed a possible role of cinnamic acid in regulating 4CL enzyme activities, probably at the posttranslational level, during compression wood formation. However, it is unclear how cinnamic acid interacts with the 4CL protein to affect the enzyme activity toward caffeic acid. Also, cinnamic acid does not appear to affect the 4CL activity toward 4-coumaric acid, at least in vitro (Table I). Therefore, cinnamic acid could not be attributed to the increased levels of 4-coumaric acid-4CL activity during compressional stress (Fig. 5). Other factors induced under compressional stress may also be involved in the modulation of 4CL enzyme activity and mRNA transcription. The underlying mechanisms for either up- or down-regulating the 4CL activities toward different substrates in response to mechanical stress remain to be elucidated. Nevertheless, our studies propose the possibility that pathway intermediates may play an important role, at the enzyme level, in regulating the microheterogeneity of lignins in response to developmental or stress signals.

ACKNOWLEDGMENTS

The authors thank Drs. Ronald Sederoff, Ross Whetten, and David O'Malley (North Carolina State University, Raleigh) for their comments and discussions about the manuscript and for providing genomic DNAs isolated from megagametophyte and diploid tissues of loblolly pine clone 7–56; Drs. Richard Smeltzer and Daniel Carraway (International Paper) and Laigeng Li for helping collect compression xylem tissues of loblolly pine clone 1932; Xiao-Lu Jin for assistance with the enzyme assay; and Dr. Shekhar Joshi for critically reading the manuscript.

Received June 28, 1996; accepted October 10, 1996.

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