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4-Coumarate:Coenzyme A Ligase from Loblolly Pine Xylem[1]

Isolation, Characterization, and Complementary DNA Cloning

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4-Coumarate:CoA ligase (4CL, EC 6.2.1.12) was purified from differentiating xylem of loblolly pine (*Pinus taeda* L.). The pine enzyme had an apparent molecular mass of 64 kD and was similar in size and kinetic properties to 4CL isolated from Norway spruce. The pine enzyme used 4-coumaric acid, caffeic acid, ferulic acid, and cinnamic acid as substrates but had no detectable activity using sinapic acid. 4CL was inhibited by naringenin and coniferin, products of phenylpropanoid metabolism. Although the lignin composition in compression wood is higher in *p*-hydroxyphenyl units than lignin from normal wood, there was no evidence for a different form of 4CL enzyme in differentiating xylem that was forming compression wood. cDNA clones for 4CL were obtained from a xylem expression library. The cDNA sequences matched pine xylem 4CL protein sequences and showed 60 to 66% DNA sequence identity with 4CL sequences from herbaceous angiosperms. There were two classes of cDNA obtained from pine xylem, and the genetic analysis showed that they were products of a single gene.

The enzyme 4CL (EC 6.2.1.12) catalyzes the formation of CoA thioesters of cinnamic acids (Lozoya et al., 1988). Aromatic secondary plant metabolites such as benzoic acid, condensed tannins, lignin precursors, flavonoids, and lignans are all derived from the general phenylpropanoid pathway through CoA thioesters (Fig. 1). 4CL has been purified or partially purified from soybean (Knobloch and Hahlbrock, 1975), petunia (Ranjeva et al., 1976), *Forsythia* (Gross and Zenk, 1974), parsley (Knobloch and Hahlbrock, 1977), poplar (Grand et al., 1983), spruce (Lüderitz et al., 1982), pea (Wallis and Rhodes, 1977), and maize (Vincent and Nicholson, 1987). DNA sequences encoding 4CL were obtained from parsley (Lozoya et al., 1988), potato (Becker-André et al., 1991), rice (Zhao et al., 1990), and soybean (Uhlmann and Ebel, 1993).

Lignin in pine shows significant microheterogeneity (Terashima and Fukushima, 1988). The first lignin deposited during the differentiation of xylem is *p*-hydroxyphenyl lignin in the middle lamella and in the cell corners. Guaiacyl lignin is first deposited in the middle lamella and later in the secondary wall. A small amount of syringyl lignin is deposited late in the synthesis of the secondary wall. The formation of different kinds of lignin during xylogenesis could be determined by different isozymes of 4CL. Grand et al. (1983) identified three isozymes of 4CL with different substrate specificities in poplar stems. They suggested that expression of the different 4CL isozymes could regulate the relative abundance of the three monolignols (coumaryl, coniferyl, and sinapyl alcohols) polymerized into different types of lignin. Isozymes of 4CL were also reported in soybean, petunia, pea, and maize (Knobloch and Hahlbrock, 1975; Ranjeva et al., 1976; Wallis and Rhodes, 1977; Vincent and Nicholson, 1987).

Compression wood is formed in gymnosperms in response to mechanical stress and is characterized by higher wood density, increased lignin content, and an increased proportion of *p*-coumaryl alcohol-derived components in the compression wood lignin (Timell, 1986). Differences in lignin composition in normal and compression wood of pine could be due to the presence of different isoforms of 4CL in xylem.

The objective of this study of 4CL in pine was to determine the number and identity of 4CL isozymes involved in lignin biosynthesis in differentiating xylem. Although we have searched carefully for multiple forms of 4CL by protein purification and cDNA cloning, the weight of our evidence argues for a single 4CL isozyme expressed in loblolly pine (*Pinus taeda* L.) xylem. 4CL activity was fractionated using five different chromatographic techniques that all resolved a single peak of 4CL activity. We demonstrated that the purified 4CL protein had the expected substrate specificity and simple enzyme kinetics. In addition, we have partially purified 4CL from differentiating compression wood. The 4CL isozyme active in compression wood formation had the same substrate specificities as the purified enzyme from differentiating normal wood. Protein sequence from the purified 4CL protein confirmed the identity of a 4CL cDNA sequence obtained using anti-

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indoyl phosphate; CAP, cleavable amplified polymorphism; 4CL, 4-coumarate:CoA ligase; coniferin, coniferyl alcohol β-D-glucoside; EG, ethylene glycol; NT, nitroblue tetrazolium; PVDF, polyvinylidene difluoride; V_{max}, maximum rate.

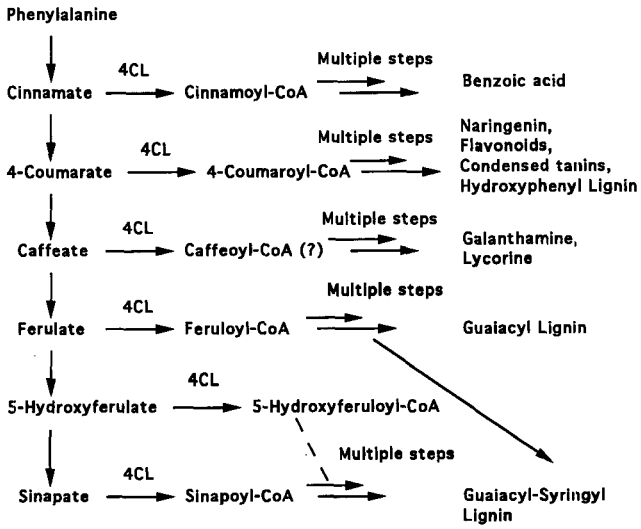

Figure 1. Role of 4CL in the formation of CoA esters of cinnamic acids in phenylpropanoid metabolism. The formation of sinapic acid through 5-hydroxyferulate is presumed to be specific for plants, making guaiacyl-syringyl lignin typical of angiosperms, but an alternative pathway to guaiacyl-syringyl lignin using *O*-methyltransferase is shown.

bodies raised against the purified protein. Genetic analysis showed that the two classes of 4CL cDNA found in pine xylem were encoded by a single gene.

MATERIALS AND METHODS

Plant Material

Differentiating xylem from normal wood was collected during May from fast growing 10- to 13-year-old loblolly pine (*Pinus taeda* L.) trees (O'Malley et al., 1992). Immature xylem was scraped from wood, dropped directly into liquid nitrogen, and stored at −80°C. Compression wood was induced by bending 6- to 7-year-old trees to a 45° angle and tying them to the ground (also in May). Compression wood formation could be detected visibly as early as 3 d after bending. Samples of differentiating xylem from compression wood were harvested into liquid nitrogen by scraping at 7, 15, and 30 d after bending.

Enzyme and Protein Assays and Substrates

The 4CL activity assay was based on that of Lüderitz et al. (1982). The reaction mixture contained 50 mM ferulic acid, 2.5 mM ATP, 7.5 mM $MgCl_2$, and 38 μM CoA in 100 mM Tes (pH 7.6). 4CL activity was monitored by the increase in A_{350}. The molar extinction coefficients of feruloyl-CoA and coumaroyl-CoA in Tris-HCl (pH 7.6) and Tes (pH 7.6) were compared and found to be nearly identical with those given by Gross and Zenk (1966). Enzyme activity is expressed in nanokatals (1 nmol of substrate converted to product per s).

Protein concentration was determined by a modified Bradford dye binding assay (Bio-Rad) using BSA as a protein standard. Protein concentration for purified 4CL was determined by UV *A* (214 nm) and peak integration from the C_4 HPLC reverse-phase column, compared with BSA.

CoA, ATP, $MgCl_2$, *p*-coumaric acid, caffeic acid, ferulic acid, *trans*-cinnamic acid, L-Phe, and naringenin were obtained from Sigma. Sinapic acid was purchased from Fluka (Ronkonkoma, NY). Coniferaldehyde and coniferyl alcohol were synthesized by Synthons, Inc. (Blacksburg, VA). Coniferin was purified from differentiating xylem following the procedure of Savidge (1989). The purity and identity of coniferin were confirmed by reverse-phase HPLC (C_{18}) using an authentic sample provided by R. Savidge (New Brunswick, Canada).

Purification of 4CL from Pine Xylem

Crude extracts were prepared from 200 g of frozen differentiating xylem by homogenization in liquid nitrogen (O'Malley et al., 1992), followed by thawing in extraction buffer (20 mM Tris-HCl [pH 7.5], 10% [v/v] EG, 5 mM DTT, 0.13% [w/v] protamine sulfate, and 5 mM $MgCl_2$) at room temperature. The extracts were filtered and centrifuged and the supernatants were adjusted to pH 7.5 with a few drops of 2 N NaOH.

The protein in the crude supernatant was batch adsorbed to DEAE-Sephacel (packed volume, 90 mL; bead size, 40–160 μm). DEAE beads with adsorbed protein were loaded on a partially filled DEAE column (2.5 cm in diameter; final volume, 100 mL). The remaining supernatant was loaded on the column (2.5 mL/min) to ensure that all 4CL had been bound. The column was eluted for 160 min at a flow rate of 2.5 mL/min with a linear gradient of 75 to 400 mM Tris-HCl (pH 7.5) containing 10% EG and 5 mM DTT. Fractions containing more than half the peak activity of 4CL were pooled and concentrated (Centriprep-30, 30,000 mol wt cutoff), filtered (0.2 μM), and loaded on a Sephacryl-200 gel-filtration column (90 × 2.5 cm). Proteins were eluted with a solution containing 10 mM Tris-HCl (pH 7.5), 5 mM DTT, and 10% EG. All open column separations were carried out at 4°C.

Further Purification of 4CL Using HPLC

All HPLC operations were carried out at room temperature. 4CL-containing protein from gel-filtration column fractions were concentrated using Centriprep-30, diluted 20-fold with ion-exchange column equilibration buffer (10 mM Na/K PO_4, pH 6.4, containing 10% EG and 5 mM DTT), and loaded on a CM 825 ion-exchange chromatographic column (Shodex, Tokyo, Japan; carboxymethyl cation-exchange HPLC, 8 × 75 mm). The column was run for 4 min after the same buffer was loaded (0.7 mL/min). Proteins were eluted in 20 min (1 mL/min) by a linear gradient of 10 to 350 mM Na/KH_2PO_4 (pH 6.4) containing 10% EG and 5 mM DTT.

Concentrated fractions from cation-exchange HPLC were loaded onto a Protein-Pak DEAE-8HR column (Waters, Milford, MA; anion exchange, 10 × 100 mm). Proteins were eluted with a linear gradient of 0 to 0.4 M NaCl in 20 mM Tris-HCl (pH 7.6), 10% EG, and 5 mM DTT (1 mL/min for 50 min).

The pooled fractions from anion-exchange HPLC were loaded onto a Blue-5PW column (TosoHAAS, Philadelphia, PA; reactive dye, 8.0 × 75 mm). After 20 min (0.3 mL/min at 10 mM Tris) proteins were eluted with a linear gradient of 0.01 M to 1.25 M Tris-HCl (pH 7.5), 20% EG, and 5 mM DTT for 120 min. The purified 4CL protein was transferred into 60 mM Tris-HCl (pH 7.5), 1% EG by repeated centrifugal concentration in a Centricon-30, and stored at −80°C. Purified proteins were further characterized by silver-stained SDS-PAGE, C4 HPLC reverse-phase chromatography (O'Malley et al., 1992), and IEF.

One nanokatal of dye HPLC-purified 4CL protein (10 μg) was loaded onto a native IEF gel (Novex, San Diego, CA; pH 3–7) and run by standard methods except that the cathode buffer (pH 8.5) was prepared by mixing 20 mM Tris base and 50 mM Gly. Thin slices (0.6 mm) were cut with a razor blade and assayed for 4CL activity after 2 h of reaction, instead of the standard 2-min assay.

Estimation of Molecular Mass

The native molecular mass of 4CL was estimated by Sephacryl-200 gel-filtration chromatography. The molecular mass of denatured 4CL protein was determined using SDS-PAGE. Reference protein standards used in these two analysis were from Sigma and Pharmacia, respectively.

Estimation of K_m

The estimation of K_m of pine 4CL for the synthesis of cinnamic acid thioesters was carried out in 100 mM Tes buffer (pH 7.6) at 25.5 ± 0.5°C. 4CL enzyme for the K_m determinations was purified through the DEAE column on the HPLC. K_m values were determined for 4CL activity from differentiating xylem of normal and compression wood and in the presence of two chemical inhibitors. Each experimental treatment was replicated three times and was randomized for analysis to eliminate systematic error. K_m values (±SE) were estimated by nonlinear regression (Leatherbarrow, 1987).

cDNA Library Screening

Construction and characterization of the λ ZAP cDNA library from differentiating xylem were described by Whetten and Sederoff (1992). Polyclonal antibodies against highly purified 4CL (C_4 HPLC reverse-phase column) were raised in female New Zealand White rabbits. Immunoscreening of 250,000 plaques of amplified library was carried out using a 1000-fold dilution of rabbit anti-4CL serum as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit serum as the secondary antibody (Sigma). After 4CL cDNA sequences were obtained, a second unamplified cDNA library (λ ZAP II vector) was screened using a hybridization probe made from an internal region of the 4CL cDNA (position 280–942). Approximately 10^5 phages were transferred to Hybond-N membrane (Amersham). The filters were prehybridized overnight and hybridized for 2 d at 60°C in a buffer containing 2× SSPE and 0.5× PE (25 mM Tris-HCl, pH 7.5, 0.05% Na PPi, 0.5% SDS, 0.1% PVP-40, 0.1% Ficoll, 0.1% BSA, 2.5 mM EDTA, 0.25 mg/mL herring sperm DNA). The filters were then washed once with 2× SSPE at 60°C for 15 min and twice with 0.2× SSPE, 0.1% SDS at 60°C for 30 and 45 min.

Classification of 4CL cDNA Clones

4CL cDNA clones from the second library screening were cut with *Eco*RI, *Hin*dIII, *Pst*I, and *Xho*I. The cloning sites for the cDNA-cloning vector were *Eco*RI and *Xho*I. Restriction fragments were analyzed by agarose gel electrophoresis (1%) and reconfirmed by Southern blot hybridization using the 662-bp probe. The untranslated 3′ end of some 4CL cDNA clones was analyzed by DNA sequencing. After the two 4CL full-length cDNA sequences were known, the cDNA clones were again cut with *Sac*II, *Rsa*I, and *Hae*III restriction enzymes to distinguish between these groups.

Segregation Analysis of 4CL CAPs

PCR primers were designed to amplify 4CL sequence containing restriction site polymorphisms that distinguished the two classes of cDNA (CAPs, Konieczny and Ausubel, 1993). 4CL CAP-1 and CAP-2 spanned the cDNA sequence from 134 to 891 and 1057 to 1569 bp, respectively. Pine megagametophyte genomic DNA from loblolly clone 7–56 was prepared following the method of Doyle and Doyle (1990). The DNA was diluted and concentrated three times using a centrifugal filtration device (Centricon-100, Amicon). A modified "hot start" PCR method (D'Aquila et al., 1991; Chou et al., 1992) was carried out in a 25-μL reaction mixture in a 250-μL Eppendorf tube in three steps.

Step 1: Fifty microliters of mineral oil were added to PCR reaction mixture 1 that contained 5 ng of genomic DNA, 25 ng each of primer, 1× PCR buffer, 0.025 mM deoxyribonucleotide triphosphate, 0.5 mM $MgCl_2$, and sterile water to 20 μL. Reaction mixture 1 was spun briefly and heated at 95°C for 25 min. Step 2: Taq polymerase mixture 2 contained 5 μg of purified BSA, 1× PCR buffer, 1 unit Taq DNA polymerase, and sterile water (total volume of 5 μL) and was combined with reaction mixture 1 that was cooled to 85°C. Step 3: The PCR reaction was initiated by heating the combined reaction mixture at 94°C for 50 s, cooling to 63 or 67°C for 1 min, and then extending the products at 72°C for 3 min. Amplification was carried out over 40 or 50 cycles, ending with an extension period at 72°C for 5 min.

Genetic segregation analysis of 4CL CAPs was accomplished by restriction enzyme digestion of the DNA fragments PCR amplified from megagametophyte DNA, followed by electrophoresis on 2% (w/v) NuSieve (FMC, Rockland, ME) agarose gel electrophoresis. A single DNA fragment of 4CL CAP-1 was obtained (50 cycles, annealing temperature of 63°C) from all samples and the fragment could be cut with *Sac*II to distinguish 4CL-A and 4CL-B after the DNA was purified (DNA affinity [Qiagen, Chatsworth, CA] spin columns and two 70% ethanol washes). The amount of DNA synthesized for 4CL CAP-2 (40 cycles, annealing temperature of 67°C) was not sufficient for detection with ethidium bromide and sometimes more than

one DNA band was PCR amplified. Hence, PCR products were separated on a 2% agarose gel and extracted using DNA affinity Spinbind columns (FMC). DNA was then cut with *Hae*III, separated by electrophoresis, blotted from the gel, and probed.

Purification of 4CL Specific Antibody

4CL fusion protein (LacZ-4CL) was expressed in *Escherichia coli* (strain BB4) from a 1.7-kb cDNA and concentrated from a crude lysate by ammonium sulfate precipitation. Partially purified 4CL fusion protein was run on an SDS-polyacrylamide gel and immobilized by electroblotting onto a PVDF membrane. The region of the filter that strongly bound 4CL antibody corresponded with 45-kD markers. This region was cut out and used to purify 4CL polyclonal antibodies. The anti-4CL polyclonal antibodies were adsorbed to the membrane-bound fusion protein in buffer (0.05% Tween-20, Tris-buffered saline; Bio-Rad) and eluted with 0.2 M Gly (pH 2.8) containing 1 mM EDTA (Sambrook et al., 1989). The pH of the eluted antibodies was adjusted to 8.0 with 1 M Tris base. Purified antibodies were brought to a final concentration of 1% BSA (w/v), 1× PBS and stored at −80°C. Crude xylem extract was used to titer the affinity-purified antibodies by western blotting.

Substrates Used in Western Blots of 4CL

Antibody was detected in plaque lifts and SDS-PAGE-derived blots by staining with BCIP (Bio-Rad) and NT (Bio-Rad) as substrates for secondary antibody-bound alkaline phosphatase. IEF-derived blots were stained with BCIP and NT as well as with chemiluminescent substrate, [3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt, Bio-Rad], as substrate for alkaline phosphatase.

Protein and Nucleic Acid Sequencing and Analysis

Protein sequence determination was carried out by the University of Wisconsin Biotechnology Center, using a pulsed-liquid phase sequencer (Applied Biosystems, Foster City, CA; model 477/120). Peptide sequences were obtained from unfractionated cyanogen bromide cleavage products by *o*-phthaladehyde blocking and sequencing (Wadsworth et al., 1992). This method produces sequences of peptides beginning with Pro residues, because Pro is not blocked by *o*-phthaladehyde. Two internal peptide sequences were determined.

Pine 4CL cDNAs were partially sequenced by making primers from regions of sequence conserved between 4CL of rice (Zhao et al., 1990) and parsley (Lozoya et al., 1988). The remaining sequence was obtained from vector primers and primers designed from pine 4CL sequence. Both strands of the clones were sequenced by Applied Biosystems automated DNA sequencing (Interdisciplinary Centre for Biotechnology Research, University of Florida, Gainesville, FL).

4CL protein and DNA sequences from pine (*P. taeda* L., Fig. 7), parsley (*Petroselinum crispum*, full length), potato (*Solanum tuberosum* L., full length), rice (*Oryza sativa* L., full length), and soybean (*Glycine max* L., partial sequence) were aligned using GAP (sequence analysis software, Genetics Computer Group, University of Wisconsin; Devereaux et al., 1984). Sequences not aligned at the 5′ end of the first four taxa were excluded from analysis, leaving 1600 bp of consensus sequence. The two 4CL cDNA clones from soybean (GM14 and GM16), however, were not full length and consisted of 914 and 1320 bp, respectively.

RESULTS

Purification of 4CL from Loblolly Pine Xylem

Loblolly pine 4CL was purified from differentiating xylem by chromatography using five column systems (Table I; Fig. 2, A–C). All chromatographic separations yielded only a single peak of 4CL activity. A peak of 4CL activity coincident with a protein peak was obtained after anion-exchange HPLC (Fig. 2B) and again after dye HPLC (Fig. 2C). The purified 4CL protein yielded a single peak on a C_4 HPLC reverse-phase column (Fig. 2D). As the purification steps proceeded, a single protein band (64 kD) became prominent on a silver-stained SDS-polyacrylamide gel (Fig. 3). The full purification protocol could be accomplished in 3 d.

Table I. *Purification of 4CL from P. taeda xylem*

Purification Step	Protein	Specific Activity	Purification	Total Activity	Yield
	mg	*nkatal/mg*	*-fold*	*nkatal*	*%*
Crude extract	456	0.3	1	127	100
DEAE column (Anion exchange)	131	0.9	3	111	88
Sephacryl-200 (Gel filtration)	26	3.2	11	83	65
CM825 HPLC (Cation exchange)	2	18.0	65	36	28
DEAE 8HR HPLC (Anion exchange)	0.15	158.0	571	24	19
Blue-5PW TOSOHAAS HPLC (Reactive dye)	0.036	248.1	895	9	7

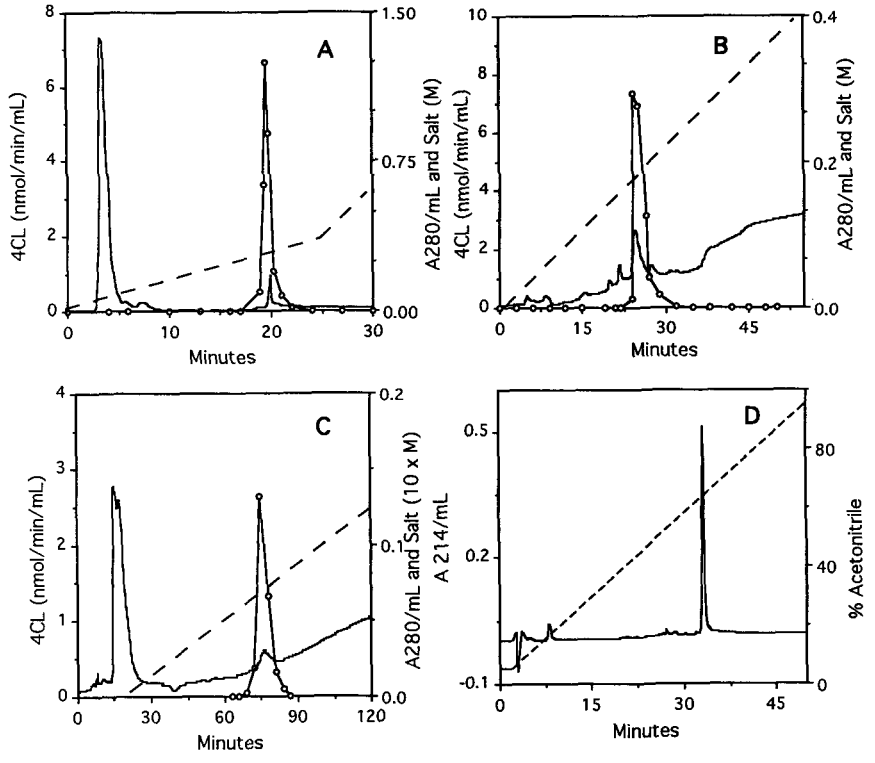

Figure 2. Sequential purification of 4CL from differentiating xylem of normal wood of loblolly pine. A, Cation exchange, CM825, HPLC; B, anion exchange, DEAE, 8HR, HPLC; C, Blue dye, 5PW, HPLC; D, C_4 reverse-phase HPLC. O, 4CL activity; - - - -, salt; —, protein.

Characterization of 4CL

Physical and Biochemical Properties

The apparent molecular mass of native 4CL enzyme was 60 kD, based on Sephacryl-200 gel-filtration chromatography. The similarity of the molecular mass estimates for the denatured protein (SDS-PAGE, Fig. 3) and the native protein confirmed that pine 4CL was a monomer. The pH optimum of 4CL was 7.6 in both Na/K PO_4 and Tes buffers.

Substrate Specificity

The affinity of 4CL for cinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, and sinapic acid was determined and expressed as K_m values (Table II). Purified 4CL enzyme yielded simple Michaelis-Menten kinetics in the presence of *p*-coumaric acid as substrate (Table II; Fig. 4). No 4CL activity was detected using sinapic acid as substrate. The K_m for cinnamic acid was approximately 10-fold higher than for the other substrates, which had K_m values in the range of 4 to 10 μM (Table II). The V_{max}/K_m values indicate that the enzyme has highest activity with coumaric acid, followed by caffeic acid, ferulic acid, and cinnamic acid (Table III).

Inhibition of 4CL Activity

4CL activity was inhibited by CoA, naringenin, coniferaldehyde, coniferyl alcohol, and coniferin. Naringenin is derived from *p*-coumaroyl-CoA and is a precursor of flavonoids. Coniferaldehyde, coniferyl alcohol, and coniferin are all derived from feruloyl-CoA. Pine 4CL activity was reduced one-half in the presence of 13 μM naringenin, 500 μM CoA, 250 μM coniferaldehyde, 250 μM coniferyl alcohol, and 5 mM coniferin. L-Phe and ATP had no inhibitory effect on enzyme activity at 30 and 50 mM concentrations, respectively. Cinnamate, coumarate, ferulate, caffeate, and sinapate did not have any significant inhibitory effect on 4CL activity at 1.0 mM. Coniferin (20 mM) and naringenin (30 μM) had no inhibitory effect on Glc-6-P dehydrogenase (Sigma), an enzyme not involved in phenylpropanoid metabolism.

An Eadie-Scatchard plot of 4CL enzyme kinetic data in the presence and absence of inhibitors suggested noncompetitive inhibition (Fig. 4). On these plots, parallel lines are indicative of noncompetitive inhibition. Lines fitted to the data on the Eadie-Scatchard plots had slopes that were not significantly different from each other ($P \leq 0.07$). The approximate K_m value of pine xylem 4CL for *p*-coumarate was 12 $\pm$ 1 μM in the absence of inhibitor, 27 $\pm$ 5 μM in the presence of 10 μM naringenin, and 17 $\pm$ 2 μM in the presence of 2 mM coniferin.

Heterogeneity of Purified 4CL

Reactive dye-purified 4CL enzyme yielded two bands on silver-stained IEF gels (Fig. 5A). Affinity-purified anti-4CL (25- to 50-fold dilution) recognized a single 64-kD band on western blots of crude xylem protein and recognized both of the protein bands detected by IEF of purified 4CL (Fig. 5B). Enzyme activity assays on IEF gel slices clearly showed that both protein bands had 4CL activity (Fig. 5D). The second band, however, had a much reduced 4CL activity, although a similar amount of protein was present (Fig. 5A). Both bands had the same expected substrate

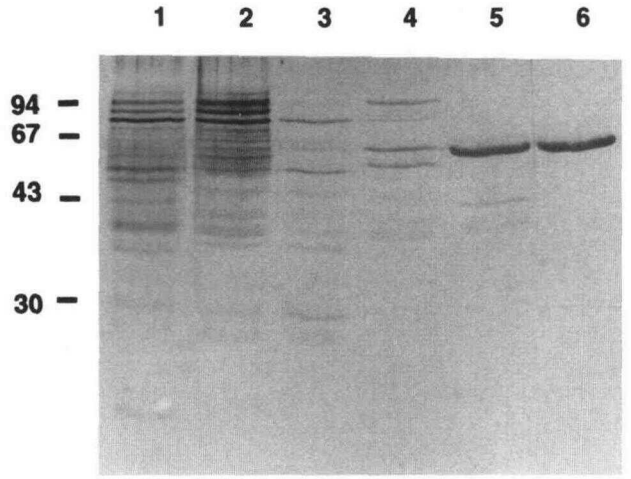

Figure 3. Silver-stained SDS-polyacrylamide gel showing the sequential purification of 4CL from *P. taeda*. Lanes 1 to 6 were loaded with 1 μg of protein. Lane 1, Crude xylem extract; lane 2, anion exchange, DEAE, open column fraction; lane 3, Sephacryl S200 gel-filtration fraction; lane 4, cation exchange, CM825 fraction; lane 5, anion exchange, DEAE, 8HR fraction; lane 6, Blue dye, 5PW, fraction. The position of mol wt standards (not shown) is indicated to the left of lane 1, with mass in kD.

preference for ferulic and *p*-coumaric acids. Further biochemical analysis of 4CL heterogeneity revealed by IEF suggested that the extra band was an artifact of protein purification.

Comparison of 4CL from Compression Wood and Normal Wood

4CL enzyme activity from differentiating xylem of compression wood was characterized and compared with 4CL from differentiating xylem of normal wood. Crude extracts from compression wood (specific activity, 0.85 nkatal/mg) were first partially purified by Sephacryl-200 gel-filtration chromatography, and then the fractions containing 4CL activity were combined and concentrated (specific activity, 1.75 nkatal/mg). The 4CL activity from compression wood was further fractionated using a DEAE-ion-exchange column on HPLC (Fig. 6). The elution profile from the DEAE column resolved many distinct protein peaks using a gradient elution of 0 to 0.5 M salt over 1 h, but the 4CL activity resolved in only a single peak (specific activity, 7.2 nkatal/mg). Substrate specificities of the partially purified 4CL from differentiating compression wood were almost identical with purified 4CL protein from normal wood (Tables II and III). The 4CL antiserum bound to a single 64-kD protein band on western blots for protein samples from compression wood (Fig. 7B), similar to the 4CL from normal wood (Fig. 7A).

Identification of the Xylem 4CL cDNA

An 8000-fold dilution of the rabbit polyclonal antiserum recognized a single band of 64 kD on western blots of crude xylem protein (Fig. 7). This antiserum was used to screen an amplified pine xylem cDNA library. Twelve 4CL cDNA clones that belonged to one class of 4CL cDNA clones were identified that carried inserts of 0.9, 1.2, and 1.7 kb. Identical nucleotide sequences were obtained for the overlapping sequences. All 12 4CL cDNA clones have identical 3′ end nucleotide sequences.

The correspondence of the 4CL cDNA clones with the purified 4CL protein was established from protein sequence. Purified 4CL protein was cleaved with cyanogen bromide at Met residues. Internal peptides for sequencing were identified following the method of Wadsworth et al.

Table II. *Apparent K_m values of 4CL from differentiating xylem of normal wood and compression wood*
Enzyme assays were carried out at 25.5 ± 0.5°C using DEAE-HPLC-purified protein.

Variable Substrate	Fixed Substrate Concentrations	K_m Normal[a] Wood (± SD)	K_m Compression[a] Wood (± SD)
		μM	μM
Coumaric acid	25 μM CoA 0.8 mM ATP 8.0 mM $MgCl_2$	6.8 ± 0.7	6.9 ± 0.5
Ferulic acid	25 μM CoA 0.8 mM ATP 8.0 mM Mg Cl_2	9.1 ± 0.7	10.0 ± 0.7
Caffeic acid	25 μM CoA 0.8 mM ATP 8.0 mM Mg Cl_2	4.6 ± 0.3	4.2 ± 0.3
Cinnamic acid	25 μM CoA 0.8 mM ATP 8.0 mM $MgCl_2$	79.4 ± 12	71.5 ± 14
ATP	25 μM CoA 8.0 mM $MgCl_2$ 200 μM Ferulic acid	76.6 ± 6	
CoA	2.5 mM ATP 8.0 mM $MgCl_2$ 200 μM Ferulic acid	4.1 ± 0.8	

[a] Average of three determinations.

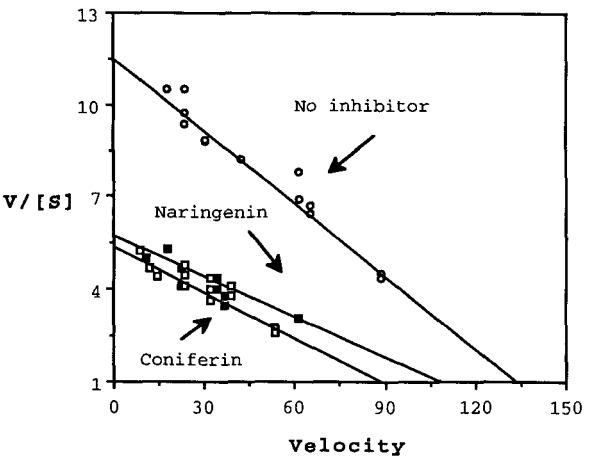

Figure 4. Eadie-Scatchard plot of 4CL kinetics data with *p*-coumaric acid in the absence (○) and presence of two inhibitors: 10 μM naringenin (■) and 2 mM coniferin (□).

(1992). Two different internal peptide sequences (34 and 24 amino acids) were obtained. The inferred peptide sequence from the 4CL cDNA matched 94% of the two internal peptide sequences from purified protein (Fig. 8). The close match was within the expected accuracy for the protein-sequencing procedure that was used (95 ± 5%, Speicher et al., 1990). The percentage match was calculated as the number of positive correct residue assignments over the sum of positive assignments (50/53). Protein-sequencing cycles that were not assigned an amino acid or had tentative amino acid assignments were not scored. The protein sequence data and the inferred protein sequence coded by the cDNA clones differed for three positive assignments. However, re-examination of the protein-sequencing chromatograms revealed that the inferred amino acid was the secondary candidate at all three locations.

Characterization of the Xylem 4CL cDNA

Diversity of 4CL cDNA Clones from Xylem

An unamplified pine xylem cDNA library from loblolly pine clone 7–56 was screened using a 700-bp probe made from an internal region of the 1.7-kb cDNA clone, and 38 4CL cDNA clones were obtained. Two full-length 4CL cDNA clones were sequenced, 4CL-A and 4CL-B (GenBank accession Nos. U12012 and U12013). The open reading frame was assigned at position 1 because this Met residue was only four amino acids away from the N-terminal protein sequence. The predicted molecular mass of the 4CL protein, 58,598 D, was consistent with the estimate obtained by gel-filtration chromatography. The two clones differed at 11 nucleotides (99.5% sequence identity). Eight were substitution mutations in the protein-coding region, resulting in one amino acid change from Gly to Glu. Three were insertion mutations in the 3′ untranslated region. There was a microsatellite sequence in the 3′ region that differed between 4CL-A and 4CL-B [$(AG)_5$ versus $(AG)_6$]. A unique restriction endonuclease site for *Hae*III occurred in 4CL-A, and 4CL-B has unique restriction endonuclease sites for *Sac*II and *Rsa*I.

We used the unique restriction sites in 4CL-A and 4CL-B to categorize the 38 4CL cDNA clones. The expected *Xho*I-*Hin*dIII DNA fragments were found for 34 of the 38 4CL cDNA clones. Sequencing of 3′ end of the untranslated cDNA region of 20 cDNA clones showed that nine had $(AG)_5$ and 11 had $(AG)_6$ in the 3′ end region. The cDNAs that had the $(AG)_6$ microsatellite sequence were all cut by *Rsa*I and *Sac*II. One of the cDNA clones had a 100-nucleotide deletion that caused a frameshift but was otherwise identical with 4CL-A. The DNA sequences flanking the deletion were similar to an intron-splicing junction. The remaining four cDNA clones had complex patterns of restriction endonuclease cleavage products (e.g. two *Xho*I sites). Sequence analysis near the *Xho*I sites suggested that these clones contained multiple inserts (i.e. cloning artifacts). Thus, the 4CL cDNA clones belonged to only two types of messages, 4CL-A and 4CL-B, after excluding cloning artifacts and a possible "pseudosplicing" event.

Amplification of Xylem 4CL Sequences from Genomic DNA

Primers were chosen from the 4CL cDNA sequences to amplify two regions that contained restriction site polymorphisms between the two classes of 4CL messenger RNA, 4CL-A and 4CL-B (Fig. 9). If the two classes of 4CL cDNAs were allelic products of a single gene, then these primers could PCR amplify polymorphic genomic 4CL DNA sequences that segregate in Mendelian ratios (CAPs, Konieczny and Ausubel, 1993). A restriction digest of 4CL CAP-1 that was amplified from pine needle diploid DNA yielded the expected three DNA fragments (Fig. 10A, lane

Table III. *Substrate specificities of 4-coumarate:CoA ligase from differentiating xylem of normal wood and compression wood*

V_{max} was calculated for enzyme assays containing 100 nmol of the corresponding cinnamic acids, 8.0 mM $MgCl_2$, 0.8 mM ATP, and 0.025 mM CoA. Values were the average of three reactions. 4CL used in these assays was purified to step 5 for normal wood (reactive dye HPLC) and DEAE-HPLC for compression wood. Substrate specificities (V_{max}/K_m) of cinnamic acids are shown in parentheses.

Substrates	Normal Wood		Compression Wood	
	V_{max}	Relative V_{max}/K_m	V_{max}	Relative V_{max}/K_m
	nkatal/mg		*nkatal/mg*	
4-Coumaric acid	210	100 (31)	7.1	100 (1.0)
Caffeic acid	128	90 (28)	3.8	90 (0.9)
Ferulic acid	183	65 (20)	5.9	59 (0.6)
Cinnamic acid	29	1 (0.38)	0.64	1 (0.01)

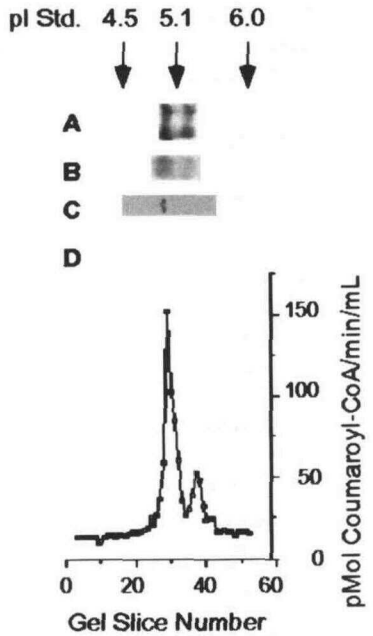

Figure 5. IEF (pH 3–7) of purified 4CL resolved two bands. A, Purified 4CL (2 μg) was subjected to IEF and silver stained. B, Purified 4CL (2 μg) was subjected to IEF, transferred to PVDF membrane, and detected with affinity-purified polyclonal rabbit anti-4CL (50-fold dilution). The detection system was the chemiluminescent substrate, 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl1,2-dioxetane disodium salt. C, Western blot showing the presence of a single 4CL protein band in crude xylem extract. Crude protein (20 μg) was subjected to IEF, transferred to PVDF membrane, and detected with affinity-purified polyclonal rabbit anti-4CL. The blot was stained with BCIP and NT. D, Profile of 4CL activity in slices from an IEF gel loaded with 10 μg of purified 4CL. The horizontal scale of A, B, and C is not identical with that of D. The position of pI standards is shown at the top of A.

1). 4CL CAP-2 did not amplify intensely and could be resolved only by autoradiography. 4CL CAP-2 amplified from pine needle diploid tissue and cut with *Hae*III yielded the expected four DNA fragments (Fig. 10B, lane 1).

Segregation Analysis

4CL CAP-1 was amplified from genomic DNA isolated from 44 megagametophytes from loblolly pine tree 7–56, then cut with *Sac*II, and analyzed on a 2% NuSieve (FMC) agarose gel. The megagametophyte samples yielded two distinct types of PCR-amplified DNA fragments, either uncut (757 bp from 4CL-B) or cut (234 and 523 bp from 4CL-A). The ratio of cut and uncut CAP-1 DNA fragments (24:20) was not significantly different from the 1:1 ratio expected for allelic segregation at a single locus ($\chi^2 = 0.32$, 1 degree of freedom, Fig. 10A).

4CL CAP-2 was amplified from megagametophyte DNA, cut with *Hae*III, and then analyzed by Southern blotting. The megagametophyte samples yielded the two predicted types of PCR-amplified DNA fragments for alleles at a single locus, either two bands (162 and 350 bp from 4CL-A) or three bands (128, 162, and 222 bp from 4CL-B), but also yielded a third type that contained all four DNA fragment sizes (Fig. 10B). The 350-bp fragment segregated in a 1:1 ratio and corresponded exactly with 4CL-A CAP-1. The complex segregation ratio of 3:15:10 suggested that more than one 4CL genomic sequence was amplified. A model that could explain the complex segregation ratio incorporated a second locus, 4CL-* that yielded the three-band pattern from 4CL-B CAP-2 and gave the four-band pattern in combination with 4CL-A CAP-2 (Fig. 10B). The recombination frequency estimate, 0.21, suggested linkage but was not significantly different from 0.50 ($\chi^2 = 3.51$, 1 degree of freedom, $P < 0.10$). 4CL-* must have significantly different DNA sequence from the expressed 4CL locus because only one allele of 4CL-* was amplified and there was no evidence of 4CL-* from 4CL CAP-1. Thus, genetic analysis of 4CL CAPs showed that the 4CL-A and 4CL-B messages expressed in xylem were products of a single xylem 4CL locus in the loblolly pine genome.

Sequence Alignment of 4CL

Alignment and comparison of deduced amino acid and DNA sequences of pine 4CL cDNAs and 4CL cDNAs from four other plant species revealed several regions of similarity (Fig. 11). A high level of sequence similarity exists in internal regions and at the carboxyl end of the proteins. Box I (Fig. 11) was suggested to be the AMP-binding domain (Schröder, 1989; Bairoch, 1991) that is common to a number of prokaryotic and eukaryotic ATP-dependent enzymes. Box II (Fig. 11) was suggested to be associated with catalytic activity (Becker-André et al., 1991). The sequence difference between pine and angiosperms was consistent with an ancient divergence of the angiosperm and gymnosperm lineages (Table IV).

DISCUSSION

We purified and characterized the 4CL enzyme that functions in lignin biosynthesis in the xylem of loblolly pine. Differentiating pine xylem contained only one major form of 4CL. No other protein with similar catalytic activity

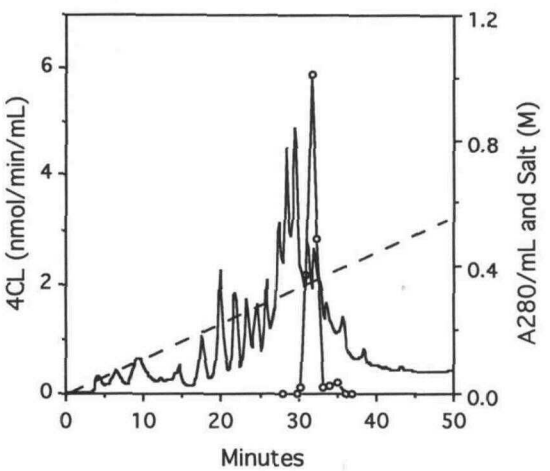

Figure 6. Partial purification of 4CL from differentiating xylem of compression wood of loblolly pine through anion exchange, DEAE, 8HR, HPLC. ○, 4CL activity; – – – –, salt; —, protein.

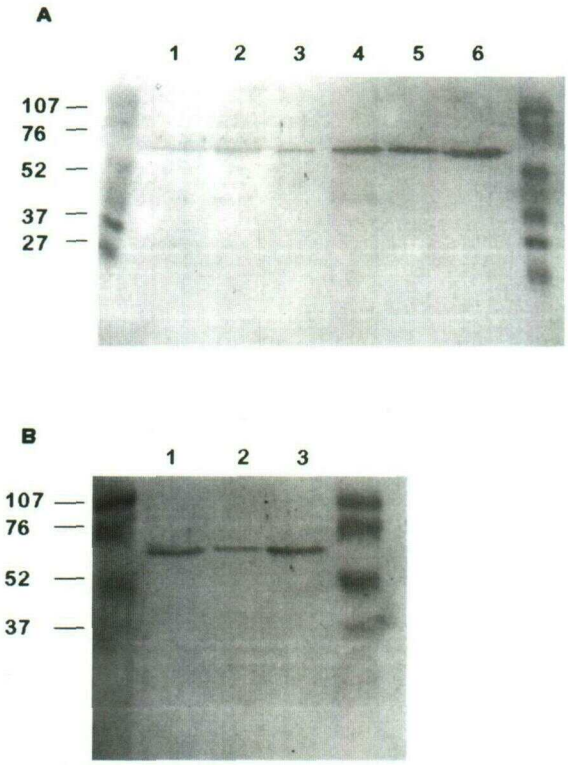

Figure 7. Western blots that show a single band of antigen in all purification steps from normal and compression wood samples. Both blots were stained with BCIP and NT. A, Western blot demonstrating the cross-reactivity of an 8000-fold dilution of polyclonal antibody with a single band at all purification steps of 4CL from normal wood. Lane 1, Crude xylem extract; lane 2, anion exchange, DEAE, open column; lane 3, Sephacryl S200 gel filtration; lane 4, cation exchange, CM825, HPLC; lane 5, anion exchange, DEAE, 8HR HPLC; lane 6, Blue dye, 5PW, HPLC. The amount of protein per lane decreased from lane 1 to lane 6 so that a faint band could be detected in the crude sample and the purified sample did not overdevelop. The position of mol wt standards is indicated to the left of lane 1. Protein standards (in kD) from top to bottom are 107, 76, 52, 36.8, and 27.2. B, Western blot with anti-4CL serum (a 10,000-fold dilution of antibody) and protein from compression wood in three purification steps. Lane 1, Crude xylem extract; lane 2, Sephacryl S200 gel filtration; lane 3, anion exchange, DEAE, 8HR HPLC. The amount of protein in lane 1 was twice as much as in the other two lanes so that a faint band could be detected in the crude sample. The position of mol wt standards is indicated to the left of lane 1. Protein standards (in kD) from top to bottom are 107, 76, 52, and 36.8.

was detected, even in samples from compression wood, which produces lignin with a different composition in response to mechanical stress. Some heterogeneity of 4CL enzyme was detected by IEF, but it was likely due to either artifacts of purification or minor sequence variation between allelic forms of 4CL. Two classes of 4CL cDNA clones were isolated from a xylem cDNA library and shown to be 99.5% identical in sequence. Mendelian analysis of restriction site polymorphisms in 4CL sequence tag sites PCR amplified from genomic DNA demonstrated that the two classes of cDNA were alleles at one locus in the loblolly pine genome.

```
-207 .....CTCA
-138 TTCAATTCTTCCCACTGCAGGCTACATTTGTCAGACACGTTTTCCGCCATTTTTCGCCTGTTTCTGCGG
-69  AGAATTTGATCAGGTTCGGATTGGGATTGAATCAATTGAAAGGTTTTTATTTTCAGTATTTCGATCGCC
1    ATGGCCAACGGAATCAAGAAGGTCGAGCATCTGTACAGATCGAAGCTTCCCGATATCGAGATCTCCGAC
     M  A  N  G  I  K  K  V  E  H  L  Y  R  S  K  L  P  D  I  E  I  S  D
70   CATCTGCCTCTTCATTCGTATTGCTTTGAGAGAGTAGCCGAATTCGCAGACAGACCCTGTCTGATCGAT
     H  L  P  L  H  S  Y  C  F  E  R  V  A  E  F  A  D  R  P  C  L  I  D
138  GGGGCGACAGACAGAACTTATTGCTTTTCAGAGGTGGAACTAATTTCTCGCAAGGTCGCTGCCGGTCTG
     G  A  T  D  R  T  Y  C  F  S  E  V  E  L  I  S  R  K  V  A  A  G  L
207  GCGAAGCTCGGGTTGCAGCAGGGGCAGGTTGTCATGCTTCTCCTTCCGAATTGCATCGAATTTGCGTTT
     A  K  L  G  L  Q  Q  G  Q  V  V  M  L  L  L  P  N  C  I  E  F  A  F
276  GTGTTCATGGGGGCCTCTGTCCGGGGCGCCATTGTGACCACGGCCAATCCTTTCTACAAGCCGGGCGAG
     V  F  M  G  A  S  V  R  G  A  I  V  T  T  A  N  P  F  Y  K  P  G  E
                                                    :  :  :  :  :
                                                    P  x  Y  I  P  G  E
345  ATCGCCAAACAGGCCAAGGCCGCAGGCGCGCGCCATCATAGTTACCCTGGCAGCTTATGTTGAGAAACTG
     I  A  K  Q  A  K  A  A  G  A  R  I  I  V  T  L  A  A  Y  V  E  K  L
     :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :
     I  A  K  Q  A  K  A  A  G  A  R  I  I  V  T  L  A  a  Y  V  e/r K  L
414  GCCGATCTGCAGAGCCACGATGTGCTCGTCATCACAATCGATGATGCTCCCAAGGAAGGTTGCCAACAT
     A  D  L  Q  S  H  D  V  L  V  I  T  I  D  D  A  P  K  E  G  C  Q  H
     :  :  :  :
     A  D  L  Q
483  ATTTCCGTTCTGACCGAAGCCGACGAAACCCAATGCCCGGCCGTGAAAATCCACCCGGACGATGTCGTG
     I  S  V  L  T  E  A  D  E  T  Q  C  P  A  V  K  I  H  P  D  D  V  V
552  GCGTTGCCCTATTCTTCCGGAACCACGGGGCTCCCCAAGGGCGTGATGTTAACGCACAAAGGCCTGGTG
     A  L  P  Y  S  S  G  T  T  G  L  P  K  G  V  M  L  T  H  K  G  L  V
621  TCCAGCGTTGCCCAGCAGGTCGATGGTGAAAATCCCAATCTGTATTTCCATTCCGATGACGTGATACTC
     S  S  V  A  Q  Q  V  D  G  E  N  P  N  L  Y  F  H  S  D  D  V  I  L
690  TGTGTCTTGCCTCTTTTCCACATCTATTCTCTCAATTCGGTTCTCCTCTGCGCGCTCAGAGCCGGGGCT
     C  V  L  P  L  F  H  I  Y  S  L  N  S  V  L  L  C  A  L  R  A  G  A
759  GCGACCCTGATTATGCAGAAATTCAACCTCACGACCTGTCTGGAGCTGATTCAGAAATACAAGGTTACC
     A  T  L  I  M  Q  K  F  N  L  T  T  C  L  E  L  I  Q  K  Y  K  V  T
828  GTTGCCCCAATTGTGCCTCCAATTGTCCTGGACATCACAAAGAGCCCCATCGTTTCCCAGTACGATGTC
     V  A  P  I  V  P  P  I  V  L  D  I  T  K  S  P  I  V  S  Q  Y  D  V
897  TCGTCCGTCCGGATAATCATGTCCGGCGCTGCGCCTCTCGGGAAGGAACTCGAAGATGCCCTCAGAGAG
     S  S  V  R  I  I  M  S  G  A  A  P  L  G  K  E  L  E  D  A  L  R  E
                                 :  :  :     :  :  :  :  :  :  :  :  :
                                 P  L  G  A  E  L  E  D  A  L  R  E
966  CGTTTTCCCAAGGCCATTTTCGGGCAGGGCTACGGCATGACAGAAGCAGGCCCGGTGCTGGCAATGAAC
     R  F  P  K  A  I  F  G  Q  G  Y  G  M  T  E  A  G  P  V  L  A  M  N
        :  :  :  :  :  :  :  :  :
     X  F  P  K  A  I  F g/d Q
1035 CTAGCCTTCGCAAAGAATCCTTTCCCCGTCAAATCTGGCTCCTGCGGAACAGTCGTCCGGAACGCTCAA
     L  A  F  A  K  N  P  F  P  V  K  S  G  S  C  G  T  V  V  R  N  A  Q
1104 ATAAAGATCCTCGATACAGAAACTGGCGAATCTCTCCCCGCAACAATCAAGCCGGCGAAATCTGCATCCGC
     I  K  I  L  D  T  E  T  G  E  S  L  P  H  N  Q  A  G  E  I  C  I  R
1173 GGACCCGAAATAATGAAAGGATATATTAACGACCCGGAATCCACGGCCGCTACAATCGATGAAGAAGGC
     G  P  E  I  M  K  G  Y  I  N  D  P  E  S  T  A  A  T  I  D  E  E  G
1242 TGGCTCCACACAGGCGACGTCGGATACATTGACGATGACGAAGAAATCTTCATAGTCGACAGAGTAAAG
     W  L  H  T  G  D  V  G  Y  I  D  D  D  E  E  I  F  I  V  D  R  V  K
1311 GAGATTATCAAATATAAGGGCTTCCAGGTGGCTCCTGCTGAGCTGGAAGCTTTACTTGTGGCTCATCCG
     E  I  I  K  Y  K  G  F  Q  V  A  P  A  E  L  E  A  L  L  V  A  H  P
1380 TCAATCTCTGACGCAGCAGTCGTTCCTCAAAAGCACGAGGAGGCGGGCGAGGTTCCGGTGGCCTTCGTG
     S  I  S  D  A  A  V  V  P  Q  K  H  E  E  A  G  E  V  P  V  A  F  V
1449 GTGAAGTCGTCGGAAATCAGCGAGCAGGAAATCAAGGAGTTCGTGGCAAAGCAGGTGATTTTCTACAAG
     V  K  S  S  E  I  S  E  Q  E  I  K  E  F  V  A  K  Q  V  I  F  Y  K
1518 AAAATACACAGAGTTTACTTTGTGGATGCGATTCCTAAGTCGCCGTCCGGCAAGATTCTGAGAAAGGAT
     K  I  H  R  V  Y  F  V  D  A  I  P  K  S  P  S  G  K  I  L  R  K  D
1587 TTGAGAAGCAGACTGGCAGCAAAATGAAAATGAATTTCCATATGATTCTAAGATTCCTTTGCCGATAAT
     L  R  S  R  L  A  A  K  >
1656 TATAGGATTCCTTTCTGTTCACTTCTATTTATATAATAAAGTGGTGCAGAGTAAGCGCCCTATAAGGAG
1725 AGAGAGAGCTTATCAATTGTATCATATGGATTGTCAACGCCCTACACTCTTGCGATCGCTTCAATATGC
1794 ATATTACTATAAACGATATATGTTTTTTTTTTT
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Figure 8. Comparison of 4CL-purified protein sequences with the protein sequence coded by the pine 4CL cDNA. The full-length 4CL cDNA sequence was obtained from a second cDNA library screening using a probe made from internal region of the 1.7-kb cDNA clone obtained from first cDNA library screen. The predicted amino acid sequence is shown directly below the DNA sequence. Confident calls of amino acids are shown in uppercase letters. Tentative identifications are indicated by lowercase letters. :, Sequence identity.

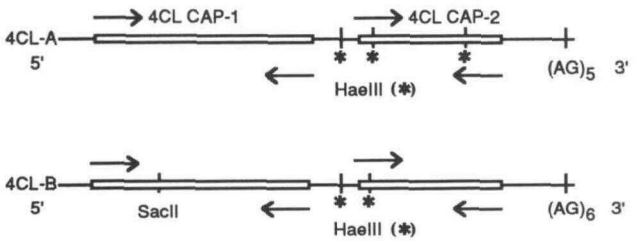

Figure 9. Diagram showing the locations of the CAPs for 4CL-A (GenBank accession No. U12012) and 4CL-B (GenBank accession No. U12013). The positions of the polymorphic *Sac*II and *Hae*III restriction sites are indicated, as well as the $(AG)_n$ microsatellite sequence. The boxes and arrows indicate the PCR fragments that were amplified. The length of the aligned 4CL cDNA sequences was 1829 nucleotides.

The result of our analysis is strong evidence that only one 4CL gene is expressed at a significant level in pine xylem. For 38 independent 4CL cDNAs, a third cDNA class with a frequency ≥ 0.078 would be detected with $P \geq 0.95$. (The probability of sampling at least one of a third class of cDNA in n samples is $P = 1 - (1 - f)^n$, where f is the frequency of the third class.) Thus, the products of a second 4CL gene would have low abundance and probably little significance in coding for 4CL protein that could affect the pool of CoA thioesters in pine xylem. A Southern analysis using 1700 bp of the 4CL cDNA sequence as the hybridization probe was consistent with a single copy of 4CL in the pine genome (not shown). PCR amplification of a related 4CL genomic DNA sequence for CAP-2 suggested a pseudogene.

We isolated a 4CL cDNA clone from pine xylem that contained the full-length coding sequence and that shared extensive inferred protein sequence similarity to 4CL in other plants. The pine 4CL cDNA coded a protein sequence that matched two internal peptide sequences from the purified 4CL protein with an accuracy of 94%. The close match confirmed that the 4CL cDNA clone corresponded with the protein purified from xylem. Extensive regions of the pine 4CL protein sequence were conserved relative to the known angiosperm 4CL sequences, especially for the AMP-binding and enzyme catalytic domains (Schröder, 1989; Bairoch, 1991; Becker-André et al., 1991). Unfortunately, little is known about the substrate specificities of the proteins encoded by some of these 4CL genes.

The pine 4CL was similar in size and substrate specificity to the 4CL enzyme that was purified from spruce (Lüderitz et al., 1982) and parsley (Knobloch and Hahlbrock, 1977). In contrast with angiosperms, sinapate rarely occurs in the gymnosperms (Lewis and Yamamoto, 1990). Loblolly pine 4CL was not detectably active with sinapic acid as the substrate. This result was consistent with earlier observations of 4CL from spruce and several taxa from the Cupressaceae and Taxodiaceae in which sinapate was not acti-

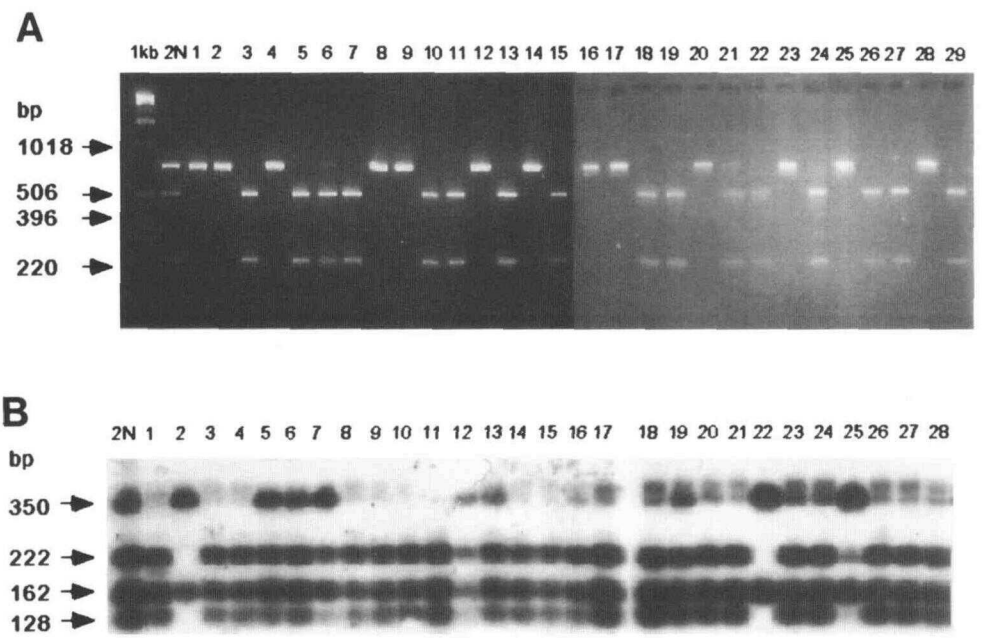

Figure 10. Genetic segregation analysis of 4CL CAPs from pine megagametophyte (haploid) of loblolly pine tree 7–56. A, Segregation 4CL CAP-1 showing uncut DNA fragments corresponding to 4CL-A and *Sac*II restriction fragments that are expected for 4CL-B. DNA standards are shown to the left. The second lane from the left contained DNA fragments PCR amplified from diploid tissue and has all three expected bands; lanes 1 to 29 contained fragments that were PCR amplified from haploid segregants. B, Segregation of 4CL CAP-2 showing two-band patterns expected for 4CL-B when cut with *Hae*III, three-band patterns expected for 4CL-A that has two *Hae*III restriction sites, and four-band patterns expected for diploid tissue but found for some megagametophyte samples. DNA standards are shown to the left. The second lane from the left contained DNA fragments PCR amplified from diploid tissue; lanes 1 to 28 contained DNA fragments that were PCR amplified from haploid tissue. The samples in this figure do not correspond directly with those in A.

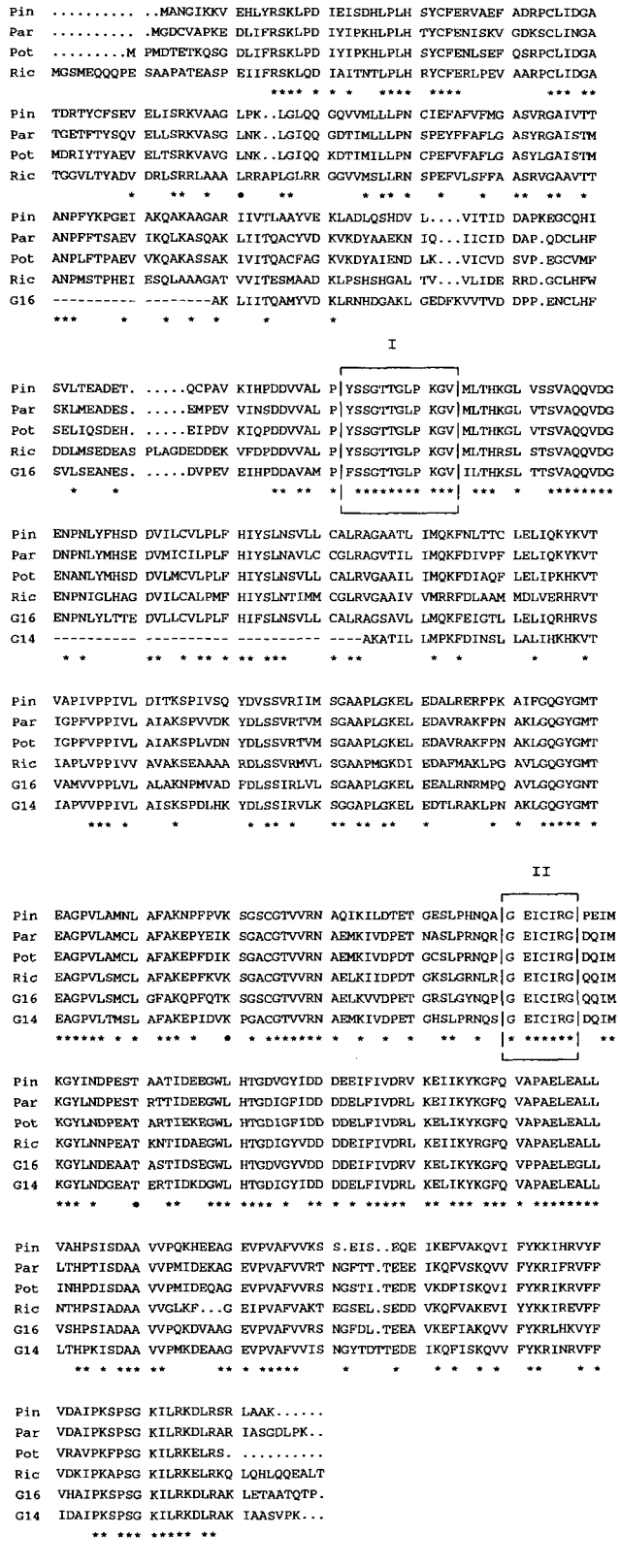

Figure 11. Comparison of the deduced amino acid sequences of pine (Pin), parsley (Par; Lozoya et al., 1988), potato (Pot; Becker-André et al., 1991), rice (Ric; Zhao et al., 1990), and soybean (G14 and G16; Uhlmann and Ebel, 1993) 4CL. The asterisk (*) denotes amino acid identity, and boxes I and II show regions of extended sequence identity for the putative AMP-binding domain (box I) and the putative catalytic site domain (box II). Missing data and gaps were identified as dashes and dots, respectively.

vated by gymnosperm 4CL (Kutsuki et al., 1982; Lüderitz et al., 1982).

Some angiosperm 4CL isozymes have substrate specificities and chemical inhibition properties similar to those of pine 4CL. 4CL isozymes that had no detectable activity with sinapic acid were found in soybean (Knobloch and Hahlbrock, 1975), petunia (Ranjeva et al., 1976), parsley (Knobloch and Hahlbrock, 1977), poplar (Grand et al., 1983), *Forsythia* (Gross and Zenk, 1974), and pea (Wallis and Rhodes, 1977). Ranjeva et al. (1976) suggested that the petunia ferulate:CoA ligase isozyme was involved in lignin biosynthesis. This petunia isozyme was specifically inhibited by naringenin (400 μM), as was the pine 4CL.

The role of 4CL in regulation of phenylpropanoid metabolism and lignin biosynthesis could be different for gymnosperms and angiosperms. 4CL is encoded by at least two genes in poplar (Allina et al., 1993), parsley (Lozoya et al., 1988), and soybean (Uhlmann and Ebel, 1993). The soybean isozymes had different substrate specificities and were differentially regulated. The different angiosperm isozymes could play a regulatory role in lignin biosynthesis or in directing the synthesis of other secondary metabolites derived from activated CoA substrates of 4CL. Preliminary observations from Arabidopsis, however, suggested that 4CL could be encoded by a single gene in this species (Allina et al., 1993). A single isoform of 4CL appeared to be expressed in the xylem of pine. If pine xylem contains only one form of 4CL, then the microheterogeneity of pine lignin observed by Terashima and Fukushima (1988) and the increased synthesis of hydroxyphenyl units in compression wood lignin of pine (Timell, 1986) could not be regulated by differential expression of 4CL isozymes with different substrate specificities.

The regulation of general phenylpropanoid metabolism from Phe ammonia-lyase to 4CL occurs at both the metabolic level and at the level of gene expression (Sato et al., 1982; Bolwell et al., 1986; Jorrin and Dixon, 1990). Less is known about regulation in the lignin biosynthetic pathway (4CL to CAD). Pine xylem 4CL was inhibited by coniferin, a storage or transport form of coniferyl alcohol. Coniferin accumulates to a high concentration (4.5% of tissue wet weight) in the differentiating xylem of pine during the spring (Savidge, 1989). 4CL, like cinnamyl alcohol dehydrogenase (O'Malley et al., 1992), shows possible feedback inhibition by coniferin. Our result suggests coordinate metabolic regulation of the general phenylpropanoid and lignin biosynthetic pathways by a feedback mechanism. Knowledge of the substrate specificity of enzymes and the metabolic regulation of phenylpropanoid metabolism will be important for efforts to genetically engineer lignin biosynthesis.

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Table IV. *Comparison of DNA homology and protein sequence similarity of pine to other plants based on the pine 4CL cDNA coding or inferred protein sequence*

The percentages of DNA homology are shown at the upper right diagonal half, whereas the percentages of protein sequence similarity are shown at the lower left diagonal half. Sequences were aligned using BESTFIT (sequence analysis software, Genetics Computer Group, University of Wisconsin; Devereaux et al., 1984). The gap weight and gap length weight used for the sequence analysis were the default options (DNA: 5.0, 0.3; protein: 3.0, 0.1). The EMBL accession Nos. of plant 4CLs are: rice 4CL, X52623; soybean 4CLs, X69955 (GM16) and X69954 (GM14); pine 4CL, U12012; potato 4CL, M62755; and parsley 4CL, X13324.

	Pine	Potato	Parsley	Rice	Soybean	
					GM16	GM14
Pine (*P. taeda*)		65	64	62	63	66
Potato (*S. tuberosum* L.)	82		74	59	64	73
Parsley (*P. crispum*)	83	89		60	64	71
Rice (*O. sativa*)	78	80	79		66	65
Soybean (*G. max* L.)						
GM16	84	87	84	83		65
GM14	85	88	89	83	84	

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