Plant Physiol. (1992) 98, 380-386
0032-0889/92/98/0380/07/$01.00/0

Received for publication April 1, 1991
Accepted August 16, 1991

Phenylalanine Ammonia-Lyase from Loblolly Pine[1]

Purification of the Enzyme and Isolation of Complementary DNA Clones

Ross W. Whetten* and Ronald R. Sederoff

Department of Forestry, North Carolina State University, Raleigh, North Carolina 27695-8008

ABSTRACT

Phenylalanine ammonia-lyase (EC 4.3.1.5) has been purified from differentiating secondary xylem of loblolly pine (*Pinus taeda* L.). Native molecular weight of the enzyme was estimated to be 280,000, with a subunit molecular weight of 74,000; isoelectric point, 5.8; and Michaelis constant for L-phenylalanine, 27 micromolar. No evidence was obtained for the existence of isoforms of the enzyme, nor for negative cooperativity of substrate binding. Polyclonal antibodies were raised against the phenylalanine ammonia-lyase subunit and used to identify a *pal* clone in an expression library of xylem complementary DNA (cDNA). Polymerase chain reaction, using oligonucleotide primers made from N-terminal amino acid sequence and from the 5′ end of the clone isolated from the expression library, was also used to isolate cDNA clones. These methods yielded cDNA clones covering the protein coding region of the *pal* messenger RNA. Comparisons of nucleotide sequence of *pal* cDNAs from pine, bean, sweet potato, and rice showed 60 to 62% identity between the pine clone and the angiosperm clones.

The development of secondary xylem in woody perennials is an interesting biological process that has received little research attention at the molecular level. Inquiry into the processes of wood differentiation has been limited to physiological and ultrastructural studies, which have laid a foundation upon which to build a more detailed understanding of intracellular events. We are beginning to apply tools of molecular biology to problems of xylem differentiation and development that are difficult to address at the level of physiology and ultrastructure.

We have chosen to focus on lignification as an important aspect of wood formation. Lignin is a complex polymer of three phenylpropanoid subunits deposited in the cell walls of vascular elements after cell growth has ceased. Sucrose supplied by the phloem is transformed into phenylalanine via the shikimic acid pathway, and lignin monomers are produced from phenylalanine by a branch of phenylpropanoid metabolism. The pathway of lignin precursor biosynthesis is well established (see ref. 7 for review). The first step of the phenylpropanoid pathway is deamination of phenylalanine by PAL[2] (EC 4.3.1.5) to yield *trans*-cinnamic acid. Hydroxylation of cinnamic acid produces *p*-coumaric acid; additional hydroxylation and methylation of *p*-coumarate produce first ferulic acid, then sinapic acid. These three acids are coupled to CoA and reduced to the corresponding alcohols. The end products of this pathway are *p*-coumaryl, coniferyl, and sinapyl alcohols. The relative proportions of the three precursors in lignin varies from species to species and even between cell types within a single individual (14). In general, lignin in angiosperms contains more *p*-coumaryl and sinapyl alcohol residues than does gymnosperm lignin; coniferyl alcohol is the primary lignin monomer in most gymnosperms.

The regulation of synthesis and polymerization of lignin monomers is still poorly understood. Synthesis of lignin monomers could be regulated at multiple points; the entry of sugars into the shikimic acid pathway or the entry of phenylalanine into the phenylpropanoid pathway are obvious possibilities. Feedback regulation of many or all of the enzymes in the pathway is another alternative. None of the intermediates in the phenylpropanoid pathway, from phenylalanine to coniferyl alcohol, accumulates to significant levels in differentiating xylem of lodgepole pine (24). The only lignin-related compound that does accumulate is coniferin, the 4-*O*-β-D-glucoside of coniferyl alcohol (25). This observation supports the suggestion (26) that the enzymes involved in synthesizing lignin monomers may be coordinately regulated and arranged in multi-enzyme complexes, so that intermediates are not allowed to accumulate.

We are developing tools to investigate more intensively the regulation of the lignification process. We have chosen to work with *Pinus taeda* L. (loblolly pine) for several reasons. The wood of pines is anatomically simpler than that of angiosperms: more than 90% of the volume of pine wood is occupied by tracheids (8). The relative abundance of this cell type will simplify biochemical and molecular analysis of intracellular events during tracheid differentiation. Data and materials from an extensive loblolly pine breeding program are also available to us, and plantations of rapidly growing trees are locally accessible.

In this article, we report the purification and characterization of PAL from differentiating xylem of loblolly pine and the isolation of cDNA clones of the protein coding region of

[1] Supported by the North Carolina State University Forest Biotechnology Industrial Associates Group.

[2] Abbreviations: PAL, L-phenylalanine ammonia-lyase (EC 4.3.1.5); SSC, standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0); PCR, polymerase chain reaction; pI, isoelectric point.

the *pal* mRNA. The identity of the cDNA clones was established by comparison of nucleotide sequence to amino acid sequence obtained from purified protein, expression of antigenic material in *Escherichia coli,* and comparison with other cloned *pal* sequences. The isolated PAL protein of loblolly pine is similar in physical characteristics to the enzyme from angiosperms, and the cDNA sequence shows considerable similarity to *pal* genes isolated from herbaceous angiosperms.

MATERIALS AND METHODS

Experimental Material

Differentiating secondary xylem was harvested from rapidly growing 10- to 12-year-old loblolly pines (*Pinus taeda* L.) in May, when the rate of diameter growth was at or near maximum. Trees were felled and the logs cut to 30- to 50-cm lengths. The bark was then removed and discarded, and the differentiating xylem scraped from the surface of the peeled log directly into liquid nitrogen. The frozen tissue was subsequently stored at −80°C until needed. This material is similar to the "cambial sap" obtained by Lüderitz and Grisebach (17) from Norway spruce, but is more properly called differentiating xylem (28).

Determination of Enzyme Activity and Protein Concentration

PAL activity was detected using an assay modified from a previously described method (12). Briefly, the reaction mixture contained 50 mM Tris-HCl, pH 8.8, 1 mM L-phenylalanine, and enzyme in a total volume of 1.2 mL. The reaction was allowed to proceed for 30 to 60 min at 30°C and was stopped by the addition of 1 mL of 2 N HCl. The reaction mixture was then extracted with 1.5 mL of toluene by vortexing for 10 s, and the mixture centrifuged at 750 *g* for 5 min to separate the phases. The A_{290} of the cinnamic acid recovered in the toluene phase was measured against a blank of toluene alone. A standard curve of absorbance as a function of cinnamic acid concentration was constructed using assay mixtures that contained known amounts of cinnamic acid, but no enzyme. These mixtures were extracted and examined by spectrophotometry as described above for enzyme assays. The identity of the reaction product was confirmed in the initial stages of method development by comparison of HPLC retention time and UV spectrum with that of authentic cinnamic acid (Sigma). Enzyme activity is expressed in nanokatals; 1 katal represents the conversion of 1 mol of substrate to product per second.

Protein concentration was determined with the Bradford dye-binding assay (Bio-Rad), using BSA as a standard.

Purification

The following buffers were used: buffer A, 0.1 M potassium phosphate, pH 7.5; buffer B, 20 mM Tris-HCl, pH 7.5; buffer C, 400 mM Tris-HCl, pH 7.5; and buffer D, 20 mM Tris-HCl (pH 7.5), 5 mM $NaHSO_3$. All buffers contain 5 mM DTT, and buffers B and C also contain 10% (v/v) ethylene glycol. Buffer pH was determined at 25°C.

All purification steps were carried out in a cold room at 4°C. Approximately 200*g* of frozen xylem were ground to a powder in a Waring Blendor cooled with liquid nitrogen. The powder was then transferred to a glass beaker and a twofold (v/w) excess of buffer A added. The frozen mixture was stirred gently with a spatula until the buffer thawed, then squeezed through 10 layers of cheesecloth (premoistened with buffer A) and filtered through one layer of Miracloth.

The filtrate was centrifuged at 20,000*g* for 30 min. Ammonium sulfate fractionation was carried out on the supernatant, and the fraction precipitating between 40 and 70% of saturation was taken for further purification. The precipitate was resuspended in a minimum volume of buffer B and dialyzed against two changes of buffer B. Insoluble material was removed by centrifugation at 14,000*g* for 20 min. The supernatant was diluted to a protein concentration of less than 15 mg/mL and loaded onto a 2.5 × 20-cm column of DEAE-Sephacel. The column was washed with buffer B until the protein content of the effluent returned to baseline level, determined by an in-line UV monitor. A linear gradient elution was then carried out with 250 mL each of buffer B and buffer C, and 5-mL fractions were collected.

Fractions containing PAL activity were pooled and concentrated to 1 mL using centrifugal ultrafiltration devices (Centriprep 30; Amicon). The concentrated material was then loaded onto a 1.5 × 80-cm Sephacryl S-300 gel filtration column and eluted with buffer D, and 2-mL fractions were collected. Fractions containing PAL activity were pooled and concentrated as before, and the purified enzyme preparation stored at −70°C. The S-300 column was calibrated for native mol wt determination using marker proteins from Sigma: bovine thyroglobulin (M_r 669,000), equine apoferritin (M_r 443,000), sweet potato β-amylase (M_r 200,000), yeast alcohol dehydrogenase (M_r 150,000), and BSA (M_r 66,000).

Protein Electrophoresis and Blotting

Protein separation by SDS-PAGE followed the method of Laemmli (13), using a mini-gel format (Hoefer Mighty Small) or a Phast System (Pharmacia). Size standards for SDS-PAGE were purchased from Pharmacia or Sigma and consisted of *Escherichia coli* β-galactosidase (M_r 115,000), rabbit phosphorylase b (M_r 97,000), BSA (M_r 66,000), chicken ovalbumin (M_r 45,000), and bovine carbonic anhydrase (M_r 29,000). Isoelectric focusing was done on a Phast System. Protein standards were from Pharmacia; the relevant proteins were amyloglucosidase (pI 3.5), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin (pI 5.2), bovine carbonic anhydrase (pI 5.85), and human carbonic anhydrase (pI 6.55). Electrophoretic transfer of proteins to nitrocellulose was done with a semi-dry transfer unit (ABN Poly-Blot) according to the manufacturer's recommendations. Protein gels were silver stained using a silver stain kit (Stratagene) according to the supplier's instructions.

Antibody Production and Protein Sequencing

Polyclonal antibodies to PAL were elicited in female New Zealand White rabbits. Antigen was prepared by SDS-PAGE separation of about 50 μg of purified protein and excision of

the 74-kD PAL band. The gel slices were lyophilized, ground to a powder, and resuspended in distilled water. The initial injection contained rehydrated gel emulsified with Freund's complete adjuvant, whereas booster injections were emulsified with Freund's incomplete adjuvant. Rabbits received booster injections 12 weeks after the initial injection, and blood was drawn 2 weeks after the booster injection.

Protein sequence analysis was carried out by the University of Wisconsin Biotechnology Center on 250 pmol of protein purified through the S-300 step.

Library Construction and Screening

Total RNA was isolated from differentiating xylem using a guanidine thiocyanate extraction buffer and centrifugation through a CsCl pad (23). Polyadenylated RNA was isolated on poly-U paper (Amersham). The library was constructed in the Lambda ZAP II vector (Stratagene), using cDNA synthesized for directional insertion (Stratagene Uni-ZAP XR cDNA synthesis kit). Approximately 20,000 primary plaques were obtained; the library was then amplified as recommended by Stratagene. Immunoscreening of aliquots of the amplified library was carried out using 1:1000 dilutions of rabbit anti-PAL serum as primary antibody and alkaline phosphatase-coupled goat anti-rabbit immunoglobulin serum (Sigma) as the secondary antibody. Colorimetric detection of the bound alkaline phosphatase was done using standard methods (23).

Nucleic Acid Manipulations

Routine manipulations such as plasmid isolation, subcloning, restriction digests, and gel electrophoresis were carried out using standard methods (23). Recovery of plasmid from Lambda ZAP II was done according to a protocol supplied by Stratagene. DNA sequences were determined by the dideoxy method from sets of nested deletion subclones (9). Agarose gel separation of restriction fragments of genomic DNA was done with 10 μg of genomic DNA per lane, and the DNA was transferred to a nylon hybridization membrane (Zetabind; AMF/Cuno) using the alkaline transfer procedure (22). The membrane was prehybridized at 65°C for 2 to 4 h in 6× SSC; 100 mM Tris (pH 7.5); 10 mM EDTA; 0.2% $Na_4P_2O_7$; 2% SDS; 0.4% each BSA, Ficoll, and PVP; and 0.1% degraded herring DNA. Isolated cDNA insert was labeled with two radioactive nucleotides by hexamer-primed synthesis (6). Hybridization was carried out at 65°C for 18 to 36 h in 4× SSC; 25 mM Tris (pH 7.5); 2.5 mM EDTA; 0.5% SDS; 0.05% $Na_4P_2O_7$; 0.1% each BSA, Ficoll, and PVP; and 0.25 mg/ml herring DNA. Final washes of the hybridized membranes were done at 0.5× SSC, 0.1% SDS at 65°C. PCR amplification was carried out using a GeneAmp kit (Perkin Elmer Cetus), using 0.4 μM degenerate N-terminal primers and 0.04 μM unique sequence internal primer. Template for the amplification reactions was a diluted sample of first strand cDNA synthesized from total xylem RNA using an oligo dT primer. PCR products were cloned into pCR1000 (18), then subcloned into Bluescript for sequencing.

Data Analysis

Enzyme kinetic data were analyzed by nonlinear regression using the Enzfitter software package (Elsevier). Equations for estimation of mol wt and pI were fitted to data derived from standard proteins by linear regression using Cricket Graph. DNA sequence analysis was carried out using the Genetics Computing Group software package (5).

RESULTS

Purification

PAL was purified 400-fold after ammonium sulfate fractionation and two chromatographic steps, with a yield of about 11% of the activity present in the original homogenate (Table I). A lower mol wt polypeptide frequently copurifies with PAL; we believe this to be a degradation product of PAL because it cross-reacts with antisera raised against the 74-kD PAL subunit (Fig. 1). A similar lower mol wt band in preparations of PAL from suspension cultures of bean was shown to be a breakdown product by peptide mapping and immunological cross-reactivity (2).

Properties of Pine PAL

The mol wt of the pine PAL subunit, estimated from the mobility on SDS-PAGE, is 74,000. The native mol wt of PAL, estimated by gel filtration chromatography on Sephacryl S-300, is 280,000, in agreement with the tetrameric nature of PAL in all organisms examined to date. Flat bed isoelectric focusing of purified PAL next to a set of standards yielded an estimate of pI 5.8.

The purified enzyme follows Michaelis-Menten kinetics, with a K_m of 27 μM for L-phenylalanine. A double reciprocal (Lineweaver-Burk) plot shows no sign of an inflection point, and the Hill coefficient was approximately unity. This observation contrasts with observations on PAL purified from many other higher plants. The enzyme is commonly described as showing negative cooperativity (10).

Purified PAL protein was submitted to N-terminal amino acid sequence analysis, and 22 amino acids of sequence were obtained. A degenerate nucleotide sequence that could encode the observed amino acids was inferred, and an oligonucleotide primer was synthesized from a portion of the sequence, using inosine in positions where any of three or four nucleotides might occur (Fig. 2).

Table I. *Purification of PAL from P. taeda*

Purification Stage	Total Protein	Specific Activity	Purification	Yield
	mg	*nanokatal/g*	*factor*	*%*
Crude extract	470	130	1	100
$(NH_4)_2SO_4$ fractionation	260	200	1.5	85
DEAE column	4.5	3,800	29	28
S-300 column	0.13	51,500	400	11

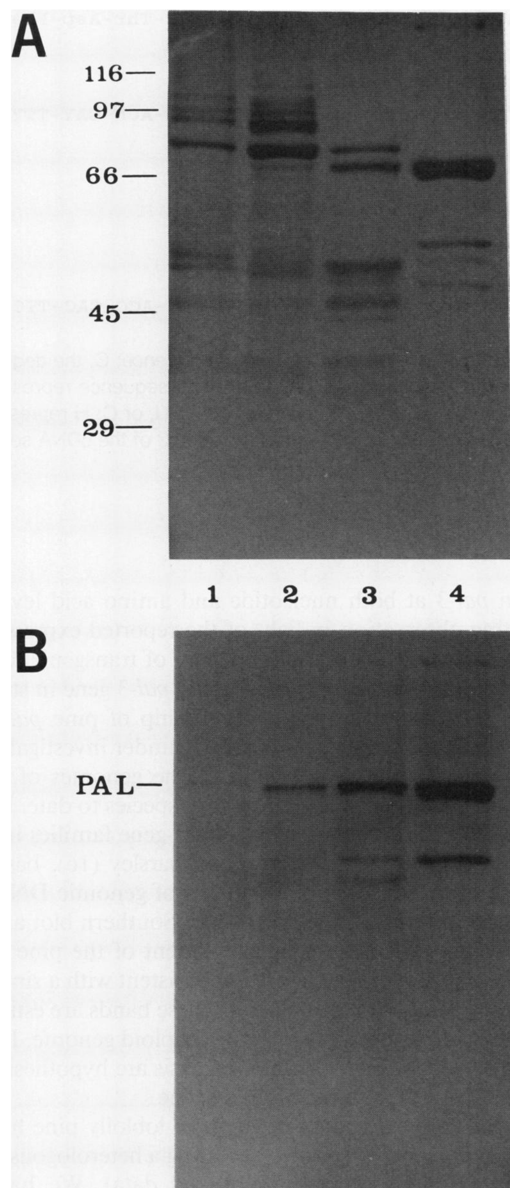

Figure 1. Samples from the purification of pine xylem PAL. A, Silver-stained SDS gel; B, Western blot with anti-PAL serum: lane 1, crude xylem extract; lane 2, ammonium sulfate fraction; lane 3, DEAE fraction; lane 4, Sephacryl fraction. Numbers in the margin of A are the molecular masses (in kD) of the size standards described in "Materials and Methods."

Cloning and Analysis of Pine PAL cDNA

The anti-PAL serum was used to screen a cDNA expression library, and five putative PAL clones were picked. Successive rounds of screening were carried out to plaque-purify the recombinant phage, and a clone was isolated that was reproducibly immunopositive, both on plaque screens and on Western blots of crude *E. coli* lysates (data not shown). The nucleotide sequence of the cDNA insert of this clone was determined, and the insert was labeled and used to probe the library for clones with longer inserts. An oligonucleotide primer was also synthesized from sequence at the 5′ end of the partial cDNA, and used in conjunction with the N-terminal degenerate primer to amplify a segment of *pal* cDNA corresponding to the 5′ half of the protein coding region. The longest clone isolated from the expression library overlaps the clones isolated using PCR by 213 nucleotides; the sequence of this region is identical in all the clones, with the exception of a single base deletion in one clone isolated from the expression library. The nucleotide sequence of the PCR-derived clone matches the coding sequence inferred from the N-terminal amino acid sequence (Fig. 2), with the exception of a single base insertion. We believe this insertion to be an artifact, because it occurs in the region specified by the N-terminal primer.

Comparison of the pine cDNA sequences with nucleotide sequences of bean (4), sweet potato (27), and rice (19) *pal* clones showed high similarity between all sequences. The combined pine *pal* sequence is 62% identical to bean *pal*-2, 60% identical to bean *pal*-3, 62% identical to sweet potato *pal,* and 61% identical to rice *pal* at the nucleotide level in the protein coding region. A display of the alignment of the pine *pal* sequence to the bean *pal*-2 and rice *pal* sequences (as representative dicot and monocot) shows that the observed sequence similarity is highest through the open reading frame in both cases (Fig. 3). Translation of the combined pine cDNA sequences in the reading frame established by the N-terminal amino acid sequence yields a polypeptide of 750 amino acids. The predicted pine protein has 78% similarity and 65% identity at the amino acid level to bean PAL-2, 73% similarity and 60% identity to bean PAL-3, 76% similarity and 64% identity to sweet potato PAL, and 76% similarity and 61% identity to rice PAL.

Organization of PAL Genes in the Pine Genome

Southern blot analysis of loblolly pine genomic DNA, using as a probe a fragment containing the region of overlap between the library and PCR cDNA clones, suggests that the corresponding PAL gene is present as a single copy in the genome. After washing at 0.5× SSC at 65°C, a single dark band is observed in DNA digested with *Eco*RI, *Hind*III, *Bam*HI, or *Kpn*I (Fig. 4). Reconstruction experiments using cloned DNA indicate that the dark bands are present in the pine genome at the single copy level (data not shown). Lighter bands are also visible in overexposures of Southern autoradiograms; these may represent diverged sequences that only weakly hybridize to the probe.

DISCUSSION

This article describes the purification and characterization of PAL from differentiating xylem of loblolly pine and the isolation of corresponding cDNA clones. The enzyme is similar in its physical properties to PAL purified from angiosperm sources, but differs from PAL preparations from bean and alfalfa in its apparent lack of isozymes. Extensive purification of PAL preparations from elicitor-treated bean and alfalfa cell suspension cultures yielded multiple PAL isozymes of differing pI and K_m (2, 11). Individual isozymes assayed separately

A: Val-Ala-Ala-Ala-Glu-Ile-Thr-Gln-Ala-Asn-Glu-Val-Gln-Val-Lys-Ser-Thr-Xxx-Leu-Xxx-Thr-Asp-Phe-Gly

B: GTN-GCN-GCN-GCN-GAR-ATH-ACN-CAR-GCN-AAY-GAR-GTN-CAR-GTN-AAR-WSN-ACN-NNN-YTN-NNN-ACN-GAY-TTY-GGN

C: GAR-ATI-ACI-CAR-GCI-AAY-GAR-G

D: AGCG-CAG-GCG-AAC-GAA-GTT-CAA-GTT-AAA-AGC-ACT-GGG-CTG-TGC-ACG-GAC-TTC-GGC

Figure 2. A, The N-terminal amino acid sequence determined from purified PAL protein; B, the inferred nucleotide sequence; C, the degenerate oligonucleotide primer; D, the nucleotide sequence of the 5′ end of the PCR-derived cDNA clone. Xxx in the peptide sequence represents an uninterpretable residue. In the nucleotide sequences, I represents deoxyinosine; R represents A or G; Y represents T, U, or C; H represents A, T, or C; W represents A or T; S represents C or G; and N represents any base. The extra G residue present at position 2 of the cDNA sequence is attributed to an artifact and was not included in further sequence analysis.

display Michaelis-Menten kinetics, but mixtures of the isozymes show the apparent negative cooperativity characteristic of PAL preparations from many angiosperm sources (10). PAL isolated from loblolly pine xylem is homogeneous with respect to pI and shows Michaelis-Menten kinetics. The same is true of PAL isolated from elicitor-treated suspension cultures of *Pinus banksiana* (3). These findings, taken together, suggest that gymnosperms may be different from angiosperms in the organization and regulation of PAL activity.

The identity of the cDNA clones was established by several independent methods. The first clones were isolated from an expression library using polyclonal antiserum raised against gel-purified antigen, and confirmed by Western blot analysis of crude *E. coli* lysates (data not shown). The nucleotide sequence inferred from the N-terminal amino acid sequence matches the nucleotide sequence of the cDNA clones isolated by PCR (Fig. 2). The combined nucleotide sequence of the cDNA clones shows substantial similarity to *pal* genes from other plant species (Fig. 3).

The pine *pal* cDNA clones were isolated in a two step process involving isolation of partial cDNAs from an expression library, then PCR amplification with primers based on N-terminal amino acid sequence and nucleotide sequence from a partial cDNA. A formal possibility exists that mRNAs encoded by different genes could have served as templates for the library and the PCR reaction, yielding a hybrid cDNA. We do not believe this to be likely, based on the similarity of the two independent clones and on the single-copy representation of these sequences in the genome. The sequences of the clones isolated by PCR agree completely with the sequence of clones from the library within the 213 nucleotide region of overlap, with the exception of a single-base deletion in one library-derived clone. Southern analysis of the pine genome using a probe including this segment indicates that this region occurs in a single copy in the genome (Fig. 4), although diverged related sequences may be present.

Comparison of *pal* cDNA sequences from bean, rice, sweet potato, and pine shows significant similarity in the open reading frame and in the predicted polypeptide products. The pine *pal* sequence is slightly more similar to bean *pal*-2 than to bean *pal*-3 at both nucleotide and amino acid levels, an interesting observation in light of the reported expression of the *pal*-2 gene in differentiating xylem of transgenic tobacco plants (1) and lack of expression of the *pal*-3 gene in stems of bean (15). The evolutionary relationship of pine *pal* genes with those of angiosperms is currently under investigation.

The organization of *pal* genes in the genomes of higher plants has been described in only four species to date. PAL is reported to be encoded by small multi-gene families in bean (4), rice (19), *Arabidopsis* (21), and parsley (16), based on evidence from Southern blot analysis of genomic DNA and isolation of genomic or cDNA clones. Southern blot analysis of pine genomic DNA, using a fragment of the pine xylem *pal* cDNA as probe, reveals bands consistent with a single *pal* gene corresponding to this cDNA. These bands are estimated to occur at the level of one copy per haploid genome. Lighter bands detected by Southern blot analysis are hypothesized to represent diverged copies of the *pal* gene.

Another group investigating PAL in loblolly pine has isolated a putative *pal* genomic clone using a heterologous probe (S Smith, K Hutchison, unpublished data). We have sequenced portions of this clone, in collaboration with Smith and Hutchison, and find segments of the sequence to be about 70% identical to our clones, whereas other segments are unrelated (data not shown). This finding indicates that the pine genome contains other sequences related to the *pal* gene corresponding to the cDNAs described here. It is not yet clear whether these related sequences are functional *pal* genes; experiments are now in progress to search more rigorously for other transcriptionally active *pal* genes in loblolly pine.

We are interested in PAL because of its role in lignification, an important developmental process in xylem. PAL catalyzes the first step in phenylpropanoid biosynthesis, and has been suggested as a possible control point to regulate the flow of carbon into lignin (20). Although the biochemical pathway of lignin precursor biosynthesis is well known, there is still much to be learned about how it is controlled. Questions also remain regarding which cells are making lignin precursors, where the precursors are stored, and how they are transported into the cell wall, where lignification occurs. The availability of anti-

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pine    1                                                                                           ACGCAGGCGAA
bean    1 ATTCACCACATTACGCACTACTTTTCCTCTCTCCGTCTCCTCATTCCTTCATTTAAATCTGCTTCCCCACTCACCTACCCAAACGCGCC  TA CT AC
rice    1                                           CAAGAAGAGCTCATCACCTTCTCCTCCGGCTCTTCTTCAGTATCGAAT CTCTCTTCT

pine   12 TGAAGTTCAAGTTAAAAGCACTGGGCTGTGCACGGACTTCGGCTCGTCTG....GCAGCGATCCACTGAACTGGGTTCGAGCAGCCAAGGCCATGGAAGG
bean  101   G CGCA C CCC  T G AAA A GC TTCGTCGTCA T  CGCCAATGCCGCGG G  C  G  C      CCGCT  G  GG    GC CTCG
rice   59 CCTC  G CCACC CC C   C TAGCAATGG   G AA   GC  ATCAACAA G  .  C  G  C      GCGCG  G  GGCC AG    CC

pine  108 AAGTCACTTTGAGGAAGTGAAAGCGATGGTGGATTCGTATTTCGGAGCCAAGGAGATTTCCATTGAAGGGAAATCTCTGACAATCTCAGACGTTGCTGCC
bean  201 C      C C  C  G  C  GCGC     C CCGA   CCG AAGC  ... C G GCG C C GC  TC GA G  C  C   G TC G  A  G
rice  158 C  C   C C  C  G     GCGC      ...  C G   C C AGCC CT G CAAG  CC G  CGCCA C  CCGCG  GGCC G  G  C

pine  208 GTTGCTCGAAGATCGCAAG......TGAAAGTGAAATTGGATGCTGCGGCTGCCAAATCTAGGGTCGAGGAGAGTTCAAACTGGGTTCTCACCCAGATGA
bean  298 AC    ......GG    C ACCAGGGTCTCAA GTGGA CTG  G A T C    GGG CT C   A   CC TCAGTG       GA GGAGAGC   G
rice  255   C  C AGGCCAA G C CCGCGCGCGTC CCGTCGA CTC AC A  AG   CGCC CC C   A   CC  CAGCG G   A C      TGC  CG

pine  302 CCAAGGGGACGGATACCTATGGTGTCACTACTGGTTTCGGAGCCACTTCTCACAGGAGAACAAACCAGGGAGCCGAGCTTCAGAAGGAGCCGATCCGCTT
bean  392 A         T  C GT  C   A    C  C  G     T  A  C  C   C CC T  C  A  A  T G  CCT G           TT   A G
rice  355   C C  CGGC  C T   C  C     C  C  C     C G   C  C   C CC C  C  GG C  CC     CC  C  AGTC     TCC  A GCA

pine  402 CTTGAATGCCGGAGTTCTTGGCAAATGCCCAGAAAATGTTT......TGTCCGAAGATACTACCCGGGCTGCCATGCTGGTTCGGACGAATACTCTGCTG
bean  492 T        T  GA AT        TG TA   GTCCAAC GCACCC AC  C CAC G A  TA A  A  T     T  GA AGT   C     T  C
rice  455 TC C  C      A CT C    CTG  T C ...   GCCACACGC  C GTCG  G  GGTG     G        C  G  C TC  C  C  C  C

pine  496 CAGGGCTACTCGGGCATAAGGTGGGACATTCTTGAAACTGTGGAGAAGCTGTTGAATGCGGGGCTGACCCCAAAGCTGCCCTTAAGAGGAACCATAACTG
bean  592   A  G      A  A  T  A TT  A   T G  GG CA CACC      T     CAACAACA T  T   TGTT    AC    G  T  A  T  A
rice  552               C     CC   TC  G  C  C  GG CA CACC      C C  CA C  CG C  G  GTGC     GC CC T  C     C  C

pine  596 CTTCTGGTGATCTGGTTCCCCTGTCTTATATTGCTGGGCTCTTGACCGGGAGGCCTAATTCCAGAGTCAGATCCAGAGATGGAATTGAAATGAGCGGAGC
bean  692  A          T  A  TT   A  C     C  TT GC A  T  T     A  C    AG CTGTTGGTCCCTC    GAGATTC    AT CTAA
rice  652  G  C     C              C  C     C  C   A C     CC C  C  CG GCAG C  TC  GCCC  C  C GGA GG  GA  CC

pine  696 CGAAGCGCTCAAGAAAGTGGGCCTGG...AAAAGCCCTTTGAATTGCAGCCTAAAGAAGGTCTGGCCATTGTCAATGGCACTTCAATGGGAGCAGCACTG
bean  792 G     CT TG ATTG CCAA A T GTTCTG  TT      G     A     G           T   C    G     A  GG TG T  CT T GCT
rice  852    G   T     CTC CC   A C AGGGTGGCTT   CACGC  A C  C  G           C     C             G  CG     GT G  G  C

pine  793 GCTTCCATTGTGTGTTTCGATGCCAATGTTCTTGCTCTGCTCTCTGAAGTAATCTCTGCCATGTTCTGCGAGGTTATGAATGGGAAGCCTGAGTTTACAG
bean  892   C  T     TCTC  T  A  A  CA C     CG CT G          T  T  A  A  T  TGCT  A  G  C A        C     C  T
rice  852   GG   CC   ATG     C     CA C  C  CG C  G  C  G  GC    G  GG                G     C  C     G    AC  C

pine  893 ATCCATTAACTCACAAGCTGAAGCACCATCCTGGGCAAATGGAAGCTGCAGCGATCATGGAGTATGTCTTGGACGGGAGTTCTTATATGAAACACGCTGC
bean  992    AT  G     T     A          C     C  G  T  G     T  T  T     C CA T     T  A  C          C   GCT   AA
rice  952  C ACC G  C                 C      TCG  C  C  C  C  C      C CA  C C C      C  G TC     GC      CAA

pine  993 TAAGCTCCATGAGATGAATCCTCTGCAGAAGCCAAAGCAGGATCGCTATGGCCTTCGCACTTCGCCTCAGTGGCTCGGCCCTCAGGTGGAGATTATCAGA
bean 1092 G   T G        AG      T        A  C  A  A           C   AA     A  C  A     T  T     AA T  AG G     G
rice 1052 G   G GA C      G C  G      T      G         CA G  C CG   C      G     G               G    A CC   G C   C C

pine 1093 TCTGCAACTCACATGATTGAGCGGGAAATCAATTCTGTGAATGACAATCCAGTAATTGATGTTGCCAGAGACAAAGCTCTACATGGAGGGAATTTCCAGG
bean 1192  T T T  CA GTCA      A     G     C  A  C        C  TT G   AG   GT T  GA     G  CT G      T  T  C     A
rice 1152 G C  C  CA GTCC  C     C  GG     C  C     C        C  G  G  C  C   CCA C C G   G  G  C  C  C  C  C

pine 1193 GCACACCTATTGGTGTTTCCATGGATAATCTTCGTCTGTCAATTTCAGCAATTGGGAAATTGATGTTCGCTCAATTCTCAGAGCTTGTGAATGATTACTA
bean 1292  A  T  A    A  C            CACC    T  G  T   G TT      A   C C      T           T  T     C     C  T
rice 1252     C  C  C     G         C  CGCC      CG C   CG CAAC  C  C  GC C          G  G     C     C     C  G T

pine 1293 CAATGGAGGCTTGCCTTCGAATCTAAGTGGCGGGCCTAATCCCAGCCTGGATTATGGACTGAAAGGGGCCGAGATCGCTATGGCTTCTTACACTTCTGAG
bean 1392     AAT   G         T     C C  C A CAGA  C        T          TT C  G  A  T  A  T  C     A       TGC     A
rice 1352    CAAC  GC  A C  C  C  GGCC   A C GC  C  G    T     C  C  GT C  G  CA           C     C  C    GC

pine 1393 CTTCTTTACCTGGCAAATCCTGTCACCAGCCATGTACAGAGCGCCGAACAGCATAACCAGGATGTCAATTCTCTGGGTCTCGTTTCAGCTAGAAAATCTG
bean 1492   C AA  TT     T  C  C  A           C  A       G  A  C      A     G  C    T      AT GA     T    G   A CA
rice 1452   C AG      C  C  C  CA      A      C          G  G      C        C  G  C  G              C  G  C  G  GA CC

pine 1493 CCGAGGCCATCGATATTCTGAAGCTGATGCTCTCCACATACCTGACAGCTCTGTGCCAGGCTGTGGATTTAAGGCATCTGGAGGAAAACATGCTGGCCAC
bean 1592 AT       TC T  G  C  C    T A    TCT  A  T  T   CGTT  A  T        CA T  C  G      T         G   TT  AA AA
rice 1552 TG       GG G  C  C  C       C    AC        C  A CGTC  C           C  C  CC TC C  C  C        G      CAA AG T

pine 1593 TGTGAAGCAGATTGTTTCTCAGGTAGCCAAGAAAACCCTGAGCACAGGGCTCAACGGGGAGCTTTTGCCAGGCCGTTTCTGCGAAAAGGATTTGCTCCAG
bean 1692      A TG   GAGC       T          GG  T  C C     TG      T  A  A  CCAC  TTCAA A  T  T  G  A C C  T GA A
rice 1652  C  C  A CTGC  CA C       G        GGTG  C C   TGAAC C  C   C  C  C CCAGC CG C   A     G  A CC C    ACC

pine 1693 GTAGTGGATAACGAGCATGTTTTCTCTTACATTGACGATCCGTGCAATGCCAGCTACCCATTGACTCAGAAACTGAGAAACATCCTGGTGGAACGTGCCT
bean 1791    T  T  A GG  T CACA  TG C             C  T     G  G  CA     C    TG  A     A  GC AG G  T       CTA   A
rice 1752   CCA C  CCG     GCC  G   AGC  TGCC     C          GC    A       GC C TG     G  CC CGC G G  C  C  G AC   C

pine 1793 TCAAGAACGCAGAAGGTGAGAAGGATCCCAACACTTCCATTTTCAATAAGATTCCTGTGTTTGAAGCCGAGCTGAAGGCACAGCTTGAACCGCAAGTTAG
bean 1891   GGCC  T G   GAAC     AA CTTG      G  A  C    C A       G ATCA    G AG    T      A C TCT GCCTAA G     GGA
rice 1852     CC G  GC  CC .....CC  G     G GG C    G G    TCC     CA CAA    C  G AG     CCGCT TGC  .  CC     GG  A C

pine 1893 TCTGGCCAGAGAAAGTTATGACAAAGGGACCAGCCCTCTGCCCAACAGGATCCAGGAATGCAGGTCTTATCCTCTCTATGAATTTGTGAGAAACCAGCTC
bean 1991 AGGT  A    CTTGCA    G  C ACCAAT TG AA T       A     A     T  T  A       C  CT G   CA G          G GG   T A
rice 1946 AGGCCG  CCGCGTC  CCG CG C  C C    GC    CG CG      C      GTC  GA  C     G  TC  G        CCGC  C  CC CG GG

pine 1993 GGTACCAAGCTTCTGTCTGGAACTCGTACCATTTCCCCTGGTGAAGTGATTGAAGTGGTTTACGACGCTATCAGTGAGGACAAGGTCATAGTCCCTCTCT
bean 2091    G  ATC T G    A    TGAAAAGGTG  C  A  G     A TG   CAAA   G T AGT    GT CC A GA  AA     T AT
rice 2046   CTG GTAT C  CA C   CGAGAAGCT  AG      C  C  G A TGCA CAA    G T CT  GC        CC    G     C     C  A    CA GC

pine 2093 TCAAATGCTTGGATGGGTGGAAAGGAACTCTGGCCCATTCTGAAATAAATAATCTTCCAAGATCGCCTTTATACAACGACTGCCATGATTTGAGTCCTCG
bean 2191   GG       C T GA A        T   TG    .....................C         TT GTTAG   CGT   ..... TTT    T  ATT GAGAGT
rice 2146    G C     C CA G A        C   CGAG CCCTT CCATCA CTA GCC   GA C    TCGC ATTGCC   TGCCAT  AC     C  CGAG AGGAGA

pine 2193 GATCCTTTTGTTGATGCAGTTGTTTACCGATCTGGAATTTGATTGGTCATAAAGCTTGATTTTGTTTTTCTTT......CTCTTGTTTTATACTGCTGGA
bean 2265   G TTC     C AT   A GTA T G GTT    AA A  T   G T     TCTG     C    GTGGCAAA CAA   CACACATACAA     T CAGGCGTGC   T
rice 2246   GAGAC AAAAAT AAAGAAAACGA      C TC TGTA CTTCA ACA A      ACCCTG A T   C     G  CGTTGC GT     G          G  A CTC

pine 2287 TTTGCATCCCATTGGATTTGCCAGTGCCAGAAATATGTAAGGGTGGCAGATCATTTGGGTGATCTGAAACATGTAAAAGTGGCGGATCATTTGGGTAGCA
bean 2365   G CTTGGTTC  poly (A)
rice 2346 G GTTC TTGTG TAGC GCT T CT AGCTTGCCA CC T   C      A G   AGC   C TT    A G T ........ T TC C A AC   TA TTTG

pine 2387 TGCAGATCAGTTGGGTGATCGTGTACTGCTTTCACTATTACTTATATATTTAAAGATCGAACTTTGGGGAATAAAAACATGATTTTAATTGpoly(A)

rice 2438 G GT   AAGTA T C AC    AT C AT C   A AA G TG GG   ATAGA   T T TTG TCC poly (A)
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Figure 3. Nucleotide sequence of the combined pine *pal* cDNA clones compared to full-length cDNAs of bean (*Phaseolus vulgaris*) *pal*-2 and rice (*Oryza sativa*). Positions in the angiosperm sequences having the same nucleotide as the pine sequence are left blank, differences are indicated by the appropriate nucleotide, and gaps inserted to optimize the alignment are indicated by dots. The bean and rice sequences were inferred from published information on genomic DNA sequences, transcription start sites, splice sites, and polyadenylation sites. The PCR-derived pine cDNA clones extend from position 1 to position 1392, and the longest library-derived clone extends from position 1180 to the poly (A) tail.

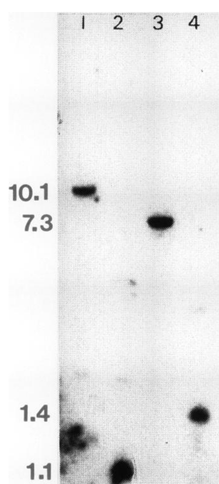

Figure 4. Results of Southern hybridization to genomic DNA of loblolly pine, using a fragment of the pine *pal* cDNA (nucleotides 1180–1820) as a probe. A single dark band is visible in pine DNA digested by *Eco*RI (lane 1), *Bam*HI (lane 2), *Hind*III (lane 3), and *Kpn*I (lane 4). The estimated sizes of the restriction fragments, in thousands of base pairs, are shown on the left.

PAL antibodies and a pine *pal* cDNA clone will make possible studies of the lignin biosynthetic pathway in pine at the molecular level, to address these unanswered questions.

ACKNOWLEDGMENTS

We thank Teresa Zuber, Debbie Fantz, and Stephanie Porter for their able technical assistance; Joe Hughes and Weyerhaeuser Corporation for access to their plantations; Audrey Goodell for advice and assistance with elicitation of antibodies; Hank Bass for advice on screening expression libraries; and Dr. David O'Malley for advice on protein purification.

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