
Structure, Inheritance, and Expression of Hybrid Poplar (Populus trichocarpa \times Populus deltoides) Phenylalanine Ammonia-Lyase Genes

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A heterologous probe encoding phenylalanine ammonia-lyase (PAL) was used to identify PAL clones in cDNA libraries made with RNA from young leaf tissue of two Populus deltoides \times P. trichocarpa F1 hybrid clones. Sequence analysis of a 2.4-kb cDNA confirmed its identity as a full-length PAL clone. The predicted amino acid sequence is conserved in comparison with that of PAL genes from several other plants. Southern blot analysis of genomic DNA from parental and hybrid individuals, restriction site polymorphism in PAL cDNA clones, and sequence heterogeneity in the 3' ends of several cDNA clones suggested that PAL is encoded by at least two genes that can be distinguished by HindIII restriction site polymorphisms. Clones containing each type of PAL gene were isolated from a poplar genomic library. Analysis of the segregation of PAL-specific HindIII restriction fragment-length polymorphisms demonstrated the existence of two independently segregating PAL loci, one of which was mapped to a linkage group of the poplar genetic map. Developmentally regulated PAL expression in poplar was analyzed using RNA blots. Highest expression was observed in young stems, apical buds, and young leaves. Expression was lower in older stems and undetectable in mature leaves. Cellular localization of PAL expression by in situ hybridization showed very high levels of expression in subepidermal cells of leaves early during leaf development. In stems and petioles, expression was associated with subepidermal cells and vascular tissues.

The enzyme PAL (EC 4.3.1.5) plays a key role in linking primary metabolism to phenylpropanoid metabolism by catalyzing the deamination of L-Phe to produce trans-cinnamic acid. This reaction is considered a key step in phenylpropanoid metabolism (Jones, 1984; Hahlbrock and Scheel, 1989) because it provides an entry point for the biosynthesis of a large number of natural products derived from the phenylpropane skeleton. Consistent with the diverse roles played by these phenylpropanoid-derived compounds, PAL enzyme levels are under both developmental and environmental control (Hahlbrock and Scheel, 1989). The accumulation of PAL mRNA and the activity of PAL promoters varies during the differentiation of certain cells, tissues, and organs, and in response to stresses such as wounding, pathogen infection, and elicitor treatment (Lawton and Lamb, 1987; Bevan et al., 1989; Liang et al., 1989b; Lois et al., 1989; Ohl et al., 1989; Schmelzer et al., 1989; Lois and Hahlbrock, 1992). PAL is encoded by small gene families in several plants (Cramer et al., 1989; Lois et al., 1989; Ohl et al., 1989; Gowri et al., 1991), but recent reports suggest that potato contains 40 to 50 PAL genes (Joos and Hahlbrock, 1992), whereas loblolly pine may contain a single PAL gene (Whetten and Sederoff, 1992). Differential expression of individual PAL genes in response to stress and during development has been documented in parsley and bean (Liang et al., 1989a; Lois et al., 1989; Lois and Hahlbrock, 1992).

A major product of phenylpropanoid metabolism is lignin, and most of the enzymic steps required for the biosynthesis of lignin monomers (cinamyl alcohols) have been defined (Lewis and Yamamoto, 1990). However, the regulation of the biosynthesis and polymerization of lignin monomers is not well understood. Because PAL plays a key role in linking primary metabolism to phenylpropanoid metabolism, it is likely to be important in channeling carbon into the biosynthesis of lignin monomers, and PAL genes are likely to be coordinately regulated with those encoding enzymes specific to lignin biosynthesis. In woody plants, lignin biosynthesis is developmentally regulated during wood formation and a significant proportion of carbon is diverted into its production. In spite of this, there is relatively little information regarding the structure and regulation of PAL genes in trees. Recently, PAL was purified from loblolly pine (Whetten and Sederoff, 1992) and jack pine (Campbell and Ellis, 1992c), and a PAL cDNA clone from loblolly pine was isolated and sequenced (Whetten and Sederoff, 1992). In jack pine cell cultures, the induction of PAL enzyme activity by elicitor treatment occurs coordinately with increases in the activities of lignin-specific enzymes and is followed by the deposition of apparently genuine gymnosperm lignin in cell walls (Campbell and Ellis, 1992a, 1992b).

Poplar is emerging as a useful model system for the investigation of the genetics and molecular biology of woody species. In addition to its small genome, ease of vegetative

1 Supported by a grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada to C.J.D. E.K.M. was supported by an NSERC graduate fellowship.
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Abbreviations: 4CL, 4-coumarate:CoA ligase; PAL, phenylalanine ammonia-lyase; pfu, plaque-forming unit; RFLP, restriction fragment-length polymorphism; TD hybrid, Populus trichocarpa \times P. deltoides hybrid.
Plant Material

Clonally propagated poplar genotypes used included TD hybrid H11-11 (parental individuals unknown), Populus deltoides ILL129, P. trichocarpa 93-968, TD hybrids 53-242 and 53-246, derived from a cross between 93-968 and ILL129, F2 individuals derived from a cross between 53-242 and 53-246, and backcross (B1) individuals derived from a cross between 53-246 and ILL129 (H. Bradshaw and R. Stettler, personal communication). Plant material was obtained from greenhouse-grown cuttings or from field-grown trees.

cDNA Library Construction and Screening

cDNA libraries containing 2 × 10^6 recombinants were constructed with young leaf RNA from TD hybrids H11-11 and 53-246 in λZAPII (Stratagene, San Diego, CA), and approximately 1 × 10^5 pfu of the H11-11 library were initially screened with a 1.5-kb potato PAL cDNA clone (encompassing the 3' end of the gene) at reduced stringency as described (Moniz de Sá et al., 1992). To obtain a full-length clone, both libraries were screened a second time with a 550-bp EcoRI-PstI fragment from the 5' end of a poplar PAL cDNA obtained in the first screening. Approximately 1 × 10^5 pfu of each library were plated; 10 plaques from the H11-11 library and 14 plaques from the 53-246 library hybridized, and several of these were purified and further characterized. No potential full-length clones were obtained from the 53-246 library, but one H11-11 clone (PAL H11-7) contained a potentially full-length 2.4-kb insert.

Genomic Library Construction and Screening

A genomic library in AGEM12 (Promega, Madison, WI) was constructed using DNA from TD hybrid 53-242. Genomic DNA (10 μg) was partially digested with MboI, and the ends were partially filled in with A and G using Klenow enzyme to create fragments unable to self-ligate. Aliquots of this DNA (0.5 μg) were ligated with 2.5 μg of AGEM12 arms containing partially filled-in XhoI sites (XhoI half-site arms, Promega). Following packaging (Gigapack Gold packaging extracts, Stratagene), recombinant phage were titered using Escherichia coli KW251 as a host. The final library consisted of approximately 1 × 10^6 recombinants. A total of about 7 × 10^5 phage were screened using the PAL H11-7 cDNA as a hybridization probe. Phage and phage DNA were purified by standard methods (Sambrook et al., 1989).

Nucleic Acid Isolation and Hybridization

DNA was isolated from the leaves of greenhouse-grown poplar cuttings by a modification of the CTAB method as described by Doyle and Doyle (1990). To increase DNA yields, the hexadecyltrimethylammonium bromide concentration was increased from 2 to 4%. RNA was isolated by the method of Parsons et al. (1989) from greenhouse-grown poplar cuttings of TD hybrid clone H11-11, or from tissue isolated from a 4-year-old field-grown H11-11 tree. Southern blots, northern blots, preparation of labeled probes, and hybridizations were performed according to standard methods (Sambrook et al., 1989) or as described (Moniz de Sá et al., 1992). In situ hybridization was performed using sections taken from greenhouse-grown H11-11 plants as described (Reinold et al., 1993). 35S-Labeled anti-sense and control sense RNAs transcribed in vitro from the PAL H11-7 cDNA clone were used as hybridization probes.

DNA Sequence and Linkage Analysis

DNA sequences were determined by the dideoxy chain termination method using double-stranded plasmid templates (Sambrook et al., 1989). Both strands of overlapping clones generated from the PAL H11-7 cDNA clone by restriction enzyme digestion or exonuclease III deletion (Sambrook et al., 1989) were sequenced. Sequence analysis was carried out using the Wisconsin Genetics Computing Group software package. Segregating populations of F2 individuals derived from a cross between TD hybrids 53-242 and 53-246 and backcross individuals derived from a cross between hybrid 53-246 and its male parent P. deltoides clone ILL129 (H. Bradshaw and R. Stettler, personal communication) were used to test linkage between RFLPs. Linkage analysis and calculation of log of odds scores were done using the MAPMAKER program (Lander et al., 1987).
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RESULTS

Poplar PAL cDNA Clones

Measurement of PAL enzyme activities in a variety of poplar tissues and organs indicated that the highest activities were present in developing xylem scraped from woody stems and in young leaves near the shoot apex (data not shown). In mature, fully expanded leaves, PAL enzyme activity was severalfold lower than in young leaves (not shown). Based on these results, we used young leaf tissue as a source of mRNA for the construction of poplar cDNA libraries. We reasoned that this mRNA population would contain transcripts of PAL genes expressed in different tissue types, since the biosynthesis of soluble phenylpropanoid compounds is likely to occur in leaf epidermal and/or mesophyll cells, whereas the biosynthesis of lignin would occur in the developing vascular system of the leaves. Thus, if potential PAL gene family members were differentially expressed in these different tissues, they would be represented in such libraries.

cDNA libraries were constructed in λZAPII with poly(A)+ RNA isolated from young leaves of TD hybrids H11-11 and 53-246 and were screened using a potato PAL cDNA clone (Fritzemeier et al., 1987; Joos and Hahlbrock, 1992) as a hybridization probe.

Figure 1. Restriction maps and 3' sequence comparisons of PAL cDNAs. A, Restriction maps of cDNA clones derived from the H11 (H11-7, etc.) and 53-246 (53-7, etc.) libraries. The vertical arrow above the H11-7 map indicates the predicted intron location. Shown below the H11-7 map are the locations of 5' and 3' hybridization probes used in Southern blot hybridizations (see Fig. 4). EcoRI and XhoI sites at the ends of the cDNAs were derived from cDNA cloning. The 5' ends of the clones are at the left. B, Alignment of the 3' untranslated sequences of the PAL cDNAs shown in A. Stop codons are underlined. Vertical lines indicate sequence identity; locations of base pair differences are indicated by a star (*) beneath the sequences. The 3' terminal ends of clones 53-8 and 53-9 are not shown. HIII, HindIII; P, PstI; RI, EcoRI; RV, EcoRV; S, SacI; X, XhoI.
A number of positive clones were purified and appeared to be authentic PAL clones based on partial sequence analysis (Moniz de Sá et al., 1992). One clone (PAL H11-7) from the H11-11 library was 2.4 kb in size and, thus, long enough to contain a complete copy of PAL. Figure 1A (top) shows a restriction map of this cDNA clone, which was sequenced in its entirety (data not shown; the complete nucleotide sequence has been deposited in GenBank, accession number L11747). An open reading frame encoding 715 amino acids was deduced from the nucleotide sequence and is shown in Figure 2. This length is similar to that reported for other PAL genes (Cramer et al., 1989; Lois et al., 1989; Minami et al., 1989; Gowri et al., 1991; Joos and Hahlbrock, 1992; Whetten and Sederoff, 1992). The 5' and 3' untranslated sequences of the H11-7 clone were 102 and 128 bp in length, respectively, and the overall nucleotide sequence identity to the portion of the potato PAL cDNA clone (Joos and Hahlbrock, 1992) used as a probe was approximately 78%. We conclude that this clone represents a full-length or near full-length poplar PAL cDNA clone.

A comparison of the deduced amino acid sequence of the H11-7 PAL gene to that of several other PAL genes (Fig. 2) indicated a high degree of similarity to these genes. The overall identity to potato PAL (Joos and Hahlbrock, 1992) was 81%, to alfalfa PAL (Gowri et al., 1991) 85%, to parsley PAL (Lois et al., 1989) 81%, to rice PAL (Minami et al., 1989) 70%, and to pine PAL (Whetten and Sederoff, 1992) 65%. This amino acid sequence conservation provides unequivocal evidence that this clone represents a full-length or near full-length 3' end of the predicted amino acid sequences. In contrast, even the most diverged amino acid sequences in this comparison (those of poplar and pine PAL) were nearly identical in several internal regions. All PAL genes studied to date contain a single intron, which splits an Arg codon conserved in most PAL genes (Gowri et al., 1991), located at nucleotide 516 in the PAL H11-7 clone (Figs. 1A and 2). Thus, we predict that an intron is situated at this position in poplar PAL genes.

To assess possible sequence heterogeneity among poplar PAL cDNAs, restriction maps of a number of additional cDNA clones from the H11-11 and 53-246 cDNA libraries (ranging in size from 2.2–1.0 kb) were compared with that of full-length clone H11-7. Figure 1A shows that, whereas most restriction sites were conserved, the clones could be grouped into two classes—those containing a HindIII site near their 3' ends (H11-7 and H11-2), and those lacking this site (all remaining clones examined). A second HindIII site near the 5' end of clone H11-7 was also absent in clone H11-6, the only other cDNA long enough to contain this portion of the gene. The polymorphic HindIII sites occur at the 3' and 5' ends of the clones, where sequence divergence between different genes would be likely to occur (see Fig. 2), and their presence suggests that the cDNAs may have arisen from two separate PAL genes. Further evidence for distinct genes came from sequence differences in the 3' untranslated regions of the cDNAs (Fig. 1B). Comparison of these sequences revealed that the 3' untranslated regions of H11-7 and H11-2 (both of which contain a 3' HindIII site) were identical, but were only about 85% identical to those regions in the remainder of the cDNAs, which lacked HindIII sites. Clones within this second class of cDNAs shared 97% or greater sequence identity in their 3' untranslated regions. The H11-11 cDNAs in this class differed in sequence at two nucleotide positions, whereas two 53-246 cDNAs were identical in these positions.
tential in sequence to one of these H11 cDNAs and the others differed in sequence from the H11-11 clones at four positions (Fig. 1B).

Fifteen poplar PAL genomic clones were identified in a genomic library made from F1 hybrid 53-242, a member of the three-generation pedigree and a sibling of clone 53-246. Figure 3 shows the restriction maps of representative clones. The location and orientation of PAL genes were determined by hybridization with probes taken from cDNA clone H11-7 (not shown). Based on these analyses, the clones shown all contain complete PAL genes. Clones gpopPAL 7 and 18 contain overlapping regions of genomic DNA, and the PAL gene within these clones contains three HindIII sites. These and other restriction sites within the gene are colinear with those in cDNA clone H11-7 except that a 0.8-kb HindIII-EcoRI fragment from the 5' end of the cDNA is replaced with a 1.4-kb fragment in the genomic clones. This is consistent with the predicted location of a single intron in the gene. Additionally, a NotI restriction site was present in the genomic copy of the PAL gene, but not in the cDNA copy. The location of this site suggests that it is present in the intron. A second class of PAL genomic clones (represented by gpopPAL 23, Fig. 3) lacked HindIII sites and a NotI site within the gene and within the genomic clones. All other restriction sites within the PAL genes were conserved in relation to those in gpopPAL 7 and 18 and the PAL cDNA clones. Thus, there appear to be at least two distinct PAL genes in poplar, distinguishable by HindIII restriction site polymorphisms within the transcribed and flanking genomic regions, and a NotI site polymorphism within the intron.

**PAL Gene Family Number and Inheritance**

To obtain a better estimate of the number of PAL genes in poplar, we performed genomic Southern blots using DNA from the P. trichocarpa (clone 93-968) and P. deltoides (clone ILL129) parents of hybrid 53-246, as well as DNA from hybrids 53-246 and H11-11. Figure 4A shows a Southern blot of the DNA from these individuals hybridized with the complete PAL cDNA (see Fig. 1). Ten-microgram samples of genomic DNA from poplar clones 93-968 (P. trichocarpa female parent), ILL129 (P. deltoides male parent), 53-246 (an F1 hybrid from the 53-968 x ILL129 cross), and H11-11 (an F1 hybrid from a different P. trichocarpa x P. deltoides cross), were digested with the restriction enzymes shown, separated on 0.8% agarose gels, and blotted to nylon membranes. B, BamHI; RV, EcoRV; H, HindIII; X, XbaI.

![Figure 4](https://www.plantphysiol.org)
**EcoRV** cuts within all of the cDNA clones and, as discussed above, HindIII cuts within some but not others (Fig. 1A). The small number of hybridizing XbaI and BamHI restriction fragments in the two parental clones confirms that PAL is encoded by a small gene family in each of these plants (shorter exposure of the autoradiogram revealed that the upper 11-kb XbaI fragment in 93-968 was a doublet, and one of the larger BamHI fragments in 93-968 was not consistently observed and appeared to be a partial digestion product). EcoRI-PstI and HindIII-XhoI restriction fragments taken from the 5' and 3' ends of the H11-7 cDNA (Fig. 1A), respectively, hybridized to the same XbaI and BamHI restriction fragments (Fig. 4, B and C), suggesting that these fragments contain complete copies of PAL genes. The 6.5-kb BamHI restriction fragment in ILL129 was identical in size to the BamHI fragment containing the PAL gene in gpopPAL 23 (Fig. 3), indicating that the gene contained on this clone was inherited from this parental individual.

An approximately 2.5-kb HindIII fragment that hybridized to the entire H11-7 cDNA was present in all individuals (Fig. 4A), but hybridization to this fragment was not observed using the 3' probe (Fig. 4C). Because the fragment is about 0.6 kb larger than the 1.8-kb internal HindIII restriction fragment in PAL H11-7, it is likely to represent an intron-containing gene-internal fragment specific to PAL genes containing HindIII sites. Three additional HindIII fragments were observed in each parental clone. Although the 3' probe hybridized to each of these fragments (Fig. 4C), a probe taken from the 5' end of H11-7 (Fig. 1A) hybridized only to the large (estimated sizes 16-23 kb) HindIII fragments (Fig. 4B). This suggests that the large HindIII fragments contain complete PAL genes, whereas the three smaller HindIII fragments (about 6.5 and 7.5 kb in parental clone 93-968 and 4.5 kb in parental clone ILL129, Fig. 4A), which did not hybridize to the 5' probe, contain only the 3' ends of PAL genes, as predicted from cDNA and genomic clone restriction maps. The 4.5-kb HindIII fragment from ILL129 is identical in size to the HindIII fragment at the 3' end of the PAL gene in genomic clones gpopPAL 7 and 18 (Fig. 3), indicating that the gene contained on these clones was inherited from this parental individual.

The probe taken from the 3' end of H11-7 hybridized with differential efficiency to different genomic restriction fragments (Fig. 4C). In particular, the probe hybridized strongly to the smaller HindIII fragments containing PAL 3' ends and poorly to the larger HindIII fragments containing complete genes. This could be explained by sequence divergence between PAL genes in their 3' ends, such that the 3' probe hybridized preferentially to restriction fragments from homologous H11-7-type PAL genes (i.e. those containing internal HindIII sites), and weakly to fragments containing different PAL gene family members (i.e. those lacking internal HindIII sites).

As expected, the F1 TD hybrid 53-246 contained restriction fragments derived from both parents (Fig. 4A). However, some fragments in the parental plants appeared to be allelic because not all were inherited. For example, 53-246 inherited only one of the two smaller HindIII fragments from the *P. trichocarpa* parent and one of two large HindIII fragments from the *P. deltoides* parent. Although the *P. trichocarpa* and *P. deltoides* parental individuals of hybrid clone H11-11 are unknown, most of the PAL-specific restriction fragments in this clone were identical in size to fragments from 93-968 and ILL129. This suggests conservation of restriction sites flanking the PAL genes in the parents of 53-246 and the parents of H11-11.

To define genetically the number of poplar PAL loci, we used advanced generations of the poplar pedigree to analyze the segregation of PAL-specific HindIII RFLPs observed in Figure 4. DNA extracted from 30 F2 and 30 B1 individuals (obtained from crosses between F1 hybrid 53-246 and its male sibling 53-242 and between 53-246 and its male parent ILL129, respectively; H. Bradshaw and R. Stettler, personal communication), as well as DNA from parental and F1 individuals, was cut with HindIII and Southern blots were hybridized with the H11-7 cDNA. Representative lanes of the blots are shown in Figure 5. The 2.5-kb PAL gene-internal fragment was observed in all lanes, whereas the other fragments corresponded to those shown in Figure 4. Clone 53-242 showed a pattern of HindIII restriction fragments very similar to its sibling 53-246, except that two different parental HindIII fragments were inherited (the larger of the two smaller fragments from 93-968, labeled T2a, and the smaller of the two large fragments from ILL129, labeled D1b, Fig. 5). Analysis of the inheritance of the polymorphic HindIII fragments in F2 individuals showed that the T1, D1a, and D1b fragments, corresponding to the class of large HindIII fragments containing complete PAL genes, were allelic (for example, the genotypes of F2 individuals 2, 3, 4, and 6 are D1a/D1b, D1a/D1a, T1/T1, and T1/D1b, respectively; Fig. 5). Similarly, fragments T2a, T2b, and D2 (corresponding to the class of HindIII fragments containing 3' ends of PAL genes) were also allelic (for example, individuals 1, 2, 3, and 5 had the genotypes T2a/T2b, D2/D2, T2b/D2, and T2a/D2, respectively; Fig. 5). Segregation of these RFLPs in the B1 generation (derived from a cross between 53-246 and ILL129) confirmed this allelism (for example, individuals 3, 7, and 9 had the genotypes T1/D1b, T1/D1a, and D1a/D1b, whereas individuals 3 and 4 had the genotypes T2b/D2 and D2/D2; Fig. 5). The two groups of allelic RFLPs appeared to segregate independently, suggesting the presence of two separate, unlinked PAL loci in these poplar individuals. Accordingly, the loci were designated PAL T1/D1 and PAL T2/D2 (T and D indicate the parental origin of the particular allele), respectively.

Linkage of the two PAL loci to random RFLP markers segregating in the same population of F2 and B1 individuals (H. Bradshaw and R. Stettler, personal communication) was tested. Whereas no linkage of the two poplar PAL loci to each other was observed, PAL was mapped to a linkage group in the emerging poplar genetic map (Fig. 6) and PAL2 was not linked to any identified poplar RFLP. We conclude that in *P. deltoides*, *P. trichocarpa*, and their hybrids, PAL genes are located at two unlinked loci.

**Developmental Regulation of PAL Expression**

We previously showed that poplar PAL mRNA accumulation is rapidly and massively induced in elicitor-treated poplar suspension-cultured cells (Moniz de Sá et al., 1992). In this...
study, we used northern blots to examine organ- and tissue-specific accumulation of PAL transcripts in H11-11 poplar plants. The left side of Figure 7 shows the results of a northern blot in which the poplar PAL H11-7 cDNA was hybridized to total RNA extracted from young stems (green and non-woody, at the plant apex), buds (dormant apical buds), young leaves (leaves just below the apex up to 1 cm in length), and mature (fully expanded) leaves. Poly(A)+ RNA isolated from young leaves was included as a control. High levels of expression were observed in young stems, buds, and young leaves, but no detectable expression was observed in mature leaves (visual inspection of stained gels and hybridization membranes indicated approximately equal loading and transfer). In a second blot (right side of Fig. 7), PAL RNA accumulation in young stem tissue was compared with that in tissues of mature stems. To obtain xylem and phloem tissue from mature woody stems, bark was peeled from 4-year-old
stem sections, exposing developing xylem tissue on the remaining mature stem and cambium, phloem, and cork cambium on the peeled bark. Cells (mature stem and bark, Fig. 7) were scraped from these respective sources for RNA isolation. Although PAL RNA was clearly detectable in mature stem and bark tissues, the amount of PAL RNA appeared to be lower in these tissues than in young stem tissue (Fig. 7). Thus, PAL gene(s) appear to be most highly expressed in immature tissues near the shoot apex and are expressed at moderate levels in stems undergoing secondary growth. We used in situ hybridization to define the cellular localization of PAL mRNA accumulation in poplar leaves and stems. Figure 8 shows the results of hybridizations of an antisense poplar PAL probe to sections of leaves taken at various stages of development. The youngest leaves, taken from the shoot apex before significant expansion had taken place, showed high levels of PAL expression, much of which was localized in cells near both leaf surfaces (Fig. 8, A and B). Closer examination of these sections showed that high levels of PAL RNA accumulated in a single cell layer just under the epidermis (Fig. 8, C and D). At the upper surface of the leaf, these cells later differentiated into the palisade parenchyma, and those at the lower surface became spongy parenchyma (Fig. 8, H and I; Esau, 1977). There was little or no RNA accumulation in the epidermal cell layer itself, and the amount of PAL mRNA that accumulated in the subepidermal cell layer was markedly lower in cells surrounding developing midveins, which do not differentiate into palisade and spongy parenchyma later in leaf development (Fig. 8, C and D). Within the midvein, expression was associated with cells surrounding a group of differentiated tracheary elements (Fig. 8, C and D). Older leaves retained this basic pattern of expression. Figure 8, E through G, shows hybridization to an expanding young leaf approximately 1.5 cm in length. High levels of PAL expression in these sections were found in two subepidermal cell layers at the upper leaf surface and in a single cell layer at the lower surface. Again, subepidermal expression was much lower in cells immediately surrounding the leaf midvein (Fig. 8, F and G). Within the rest of the leaf, expression was restricted to cells in the xylem and phloem of the midvein and to cells adjacent to tracheary elements of developing lateral veins (Fig. 8, E–G).

Tissues within leaves of intermediate age, approximately 5 cm in length (about one-third full size), had acquired characteristics resembling their final differentiated forms. Thus, the subepidermal cells at the upper side of the leaf blade had acquired the characteristic shape of palisade parenchyma cells, they contained differentiated chloroplasts, and they were clearly different in morphology from those subepidermal parenchyma cells in the midrib. Spongy parenchyma cells had begun to lose their compactness, the epidermal cells had enlarged, and differentiated xylem was prominent in the leaf midvein (Fig. 8H). PAL mRNA appeared to be less abundant in sections of these leaves (Fig. 8I). Expression was most prominent in the subepidermal palisade parenchyma cells, but was not apparent in the subepidermal parenchyma cells surrounding the midrib (distinguishable from palisade parenchyma cells by their shape and lack of chloroplasts). Some expression was also observed in spongy parenchyma cells near the lower leaf surface. In vascular tissues of the midrib, PAL expression was localized in both xylem and phloem (Fig. 8I). Phloem expression was likely to be associated with the differentiation of phloem fiber cells, a prominent feature of the phloem in mature leaves (Fig. 8I). Finally, examination of sections from mature, fully expanded leaves showed that mesophyll, epidermal, and vascular tissues were fully differentiated (Fig. 8I). No PAL RNA was detected in these sections by in situ hybridization (Fig. 8K).

Sections through petioles and stems were also used for in situ hybridization experiments (Fig. 9). Bright- and dark-field images of PAL probe hybridization to the petiole of an expanding leaf are shown in Figure 9, A and B. Here, expression was highest in two to three cell layers near the epidermis and in the developing vascular tissues in the center of the organ. Closer examination of these sections showed that, as in leaf blades, PAL mRNA accumulated in parenchyma cells immediately below the epidermis (Fig. 9, C and D). Bright- and dark-field images of PAL probe hybridization to a longitudinal section through the shoot apex are shown in Figure 9, E and F. PAL expression was high in subepidermal cell layers and in files of cells throughout the section. Differentially tracheary elements were evident near some of these files of hybridizing cells (Fig. 9F), suggesting that the PAL expression in these cells is associated with the differentiation of lignified cells of the vascular system. Finally, the PAL probe was hybridized to stem cross-sections several nodes below the apex, in which secondary growth was apparent (Fig. 9, G and H). Highly lignified tracheary elements in the xylem as well as phloem fibers were prominent (Fig. 9G), but relatively weak hybridization of PAL probe to these sections was observed. Nevertheless, silver grain accumulation above background levels was evident over lignified tracheary elements of the xylem and fiber cells of the phloem, as well as over phloem and parenchyma cells near the phloem fiber cells (Fig. 9H). In contrast, few silver grains were observed over epidermal or cortex parenchyma cells in the same sections.

DISCUSSION

In this paper, we describe the characterization, inheritance, and tissue-specific expression of PAL genes from poplar, an angiosperm tree of commercial importance in temperate regions worldwide. To our knowledge, this is the first report of the genomic organization and developmentally regulated expression of PAL genes from a woody species; the only other reported cloning of a PAL gene from trees comes from work in loblolly pine (Whetten and Sederoff, 1992). The deduced amino acid sequence of the poplar PAL gene represented by PAL H11-7 is clearly related to that of other PAL genes and is more closely related to dicotyledonous angiosperm PAL genes than to those of rice or pine (Fig. 2). Others have noted that the amino and carboxy termini of PAL genes are most highly diverged (Gowri et al., 1991; Joos and Hahlbrock, 1992). Comparison of the poplar PAL H11-7 sequence to that of other PAL genes (Fig. 2) showed a similar pattern and indicated that large stretches of amino acid identity between poplar PAL and other PAL genes are mainly restricted to the predicted second exon. The deduced size of the poplar PAL
Figure 8. Localization of PAL expression in poplar leaves by in situ hybridization. 35S-Labeled PAL H11-7 antisense transcripts were hybridized to leaves of different ages. A–D, Unexpanded leaves at the shoot apex. A, Bright-field illumination; B, dark-field illumination. C and D, Higher magnification under phase contrast (C) and bright field (D). E–G, Expanding leaf 1.5 cm in length. E, Bright-field illumination. F and G, Higher magnification of midrib region under phase contrast (F) and bright field (G). H and I, Expanding leaf 5 cm in length. H, Phase contrast; I, bright field. J and K, Fully expanded leaf under phase contrast (J) and bright field (K). e, Epidermal cell layer. Bars = 25 μm.

protein is very close to that of other dicotyledonous PAL proteins.

Several lines of molecular evidence suggest the presence of two PAL genes in poplar. A collection of PAL cDNA clones from two TD hybrids fell into two groups: those containing HindIII sites and those lacking them. Although most cDNA clones were not long enough to detect the presence or absence of HindIII sites at their 5' ends, the structure of genomic clones indicated the existence of PAL genes completely lacking HindIII sites and genes containing sites at their 3' and 5' ends (Fig. 3). Thus, the two types of PAL genes appear to have arisen from two distinct classes of
Figure 9. Localization of PAL expression in petioles and stems by in situ hybridization. \(^{35}\text{S}\)-Labeled \textit{PAL H11-7} antisense transcripts were hybridized to stem and petiole sections. A–D, Cross-sections through the petiole of an expanding leaf. A, Bright-field illumination; B, dark-field illumination. C and D, Higher magnification under phase contrast (C) and bright field (D). E and F, Longitudinal section through a stem apex under bright-field (E) and dark-field (F) illumination. G and H, Cross-section through a green stem several nodes below the apex under phase contrast (G) and bright field (H). G and H are composites of two photographic images taken from the same tissue section. a, Shoot apex; e, epidermal cell layer; p, phloem; pf, phloem fibers; t, tracheary element; x, xylem. Bars = 25 \(\mu\text{m}.\)
PAL genes, distinguishable by HindIII restriction site polymorphism. PAL gene polymorphism was also reflected in sequence heterogeneity within the 3' untranslated regions of the cDNAs, which fell into the same two groups based on sequence similarity (Fig. 1B). Southern blots of poplar genomic DNA provided further evidence for the existence of a small PAL gene family in poplar. A small number of genomic restriction fragments hybridized to the cDNA probe, and these PAL-specific RFLPs were largely conserved between TD hybrid 53-246 and the unrelated TD hybrid H11-11.

Genetic analysis of HindIII RFLP inheritance in advanced generations of the poplar pedigree indicated that the large genomic HindIII fragments that appeared to contain complete PAL genes were allelic, as were the smaller HindIII fragments that contained 3' ends of the other class of PAL genes. These two groups of PAL-specific RFLPs segregated independently, providing strong evidence for the existence of two unlinked PAL loci in poplar. If each PAL locus contains a single PAL gene, this analysis provides formal genetic proof for the existence of just two PAL genes in these poplar genotypes. Our analysis of genomic clones to date has provided no evidence for clustering of poplar PAL genes. The structures of cDNA and genomic clones clearly indicate that the HindIII restriction fragments specific to the PAL D2/T2 locus arise from single genes (Figs. 3 and 4). However, we have not yet isolated overlapping genomic clones that would span the length of the large HindIII fragments containing the PAL D1/T1 locus. Thus, we cannot exclude the possibility that more than one PAL gene is located on the HindIII fragments at this locus.

In angiosperms for which molecular data are available, PAL is encoded by at least three to four gene family members, and sometimes more (Cramer et al., 1989; Lois et al., 1989; Ohl et al., 1989; Gowri et al., 1991; Joos and Hahlbrock, 1992). In contrast, PAL appears to be encoded by a single gene in pine, as the only other tree for which there is molecular information (Whetten and Sederoff, 1992). Whereas the biological significance of the limited number of PAL genes in both poplar and pine is unclear, it is evident that large PAL gene families are not required in trees for the biosynthesis of large amounts of lignin and other phenylpropanoid compounds.

HindIII-digested poplar DNA sometimes showed variable and faint hybridization to additional HindIII fragments on Southern blots hybridized to the full-length PAL H11-7 probe (particularly evident in Fig. 5). These fragments were not observed using a 3' hybridization probe, and similar fragments were less prominent in other restriction digests (Fig. 4, A and C) but were seen using the H11-7 5' probe (Fig. 4B). Thus, rather than representing potentially divergent PAL genes, the fragments may be due to weak hybridization of the full-length or 5' probe to genomic restriction fragments consisting of the extreme 5' end of PAL genes with internal HindIII sites and flanking genomic DNA. Such 5' fragments would contain only about 80 bp in common with the probe, and thus would be expected to hybridize weakly.

The highest levels of PAL gene expression in TD hybrid H11-11 were found in buds and in developing stem and leaf tissue near the shoot apex (Fig. 7). This suggests that phenylpropanoid metabolism is very active in these organs and is consistent with the accumulation of large amounts of phenylpropanoid-derived compounds in buds and leaves (Pearl and Darling, 1968; Hegnauer, 1973; Wollenweber, 1975; Greenaway et al., 1990; English et al., 1991). In contrast with the high expression in young leaves, PAL RNA was undetectable in fully expanded leaves, indicating that PAL gene(s) are developmentally regulated during leaf maturation. In situ hybridization to leaves of different ages confirmed the developmental regulation of PAL gene expression during leaf maturation (Fig. 8). Expression was localized in developing vascular tissue, but the highest expression was in a specific cell type that, later in leaf development, differentiated into the palisade parenchyma at the upper leaf surface and into part of the spongy parenchyma at the lower surface. Large amounts of PAL mRNA were present in the subepidermal cells of very young leaves, indicating that PAL expression is activated in these cells early during leaf development. Morphological differentiation of these cells coincided with a decrease in PAL expression (see Fig. 8, H–K). This pattern of PAL gene expression suggests that prior to morphological differentiation as mature, photosynthetically active cells, this particular cell type in poplar becomes biochemically specialized for the biosynthesis of the rich array of phenylpropanoid compounds characteristic of the genus (Hegnauer, 1973; Wollenweber, 1975). In situ hybridization of a poplar cDNA probe encoding 4CL (the last step in the central phenylpropanoid pathway) to similar leaf sections revealed comparable patterns of expression (S. Reinold and C. Douglas, unpublished data), suggesting the coordinate expression of phenylpropanoid genes in these cells.

High levels of PAL expression were evident in very young stem tissue (Fig. 7). In situ hybridization showed that PAL expression in petioles and young stems is associated with cells just below the epidermis, as well as with vascular system differentiation (Fig. 9, A–F). In a manner analogous to PAL expression in leaves, expression in the nonvascular tissues disappears as the stem matures (Fig. 9, G and H), suggesting that, similar to the process in leaves, this expression may be related to the biosynthesis of soluble phenylpropanoid compounds. High levels of gene expression comparable to the subepidermal PAL expression in poplar leaves and stems has not been reported in such cells in other plants. Whereas expression of genes encoding PAL and 4CL was observed in the epidermis of parsley leaves (Schmelzer et al., 1989), this expression was of much lower magnitude than that reported here. Thus, high levels of phenylpropanoid gene expression in specialized subepidermal cells of developing poplar shoots may be a distinctive feature of these plants. However, the specialization of certain cells for natural product biosynthesis is common in plants. For example, oil duct cells in parsley leaves are specialized for furanocoumarin biosynthesis, and PAL and 4CL expression has been specifically localized to these cells by in situ hybridization (Schmelzer et al., 1989).

Despite the requirement for lignin biosynthesis in the tissues of stems undergoing secondary growth, in situ hybridization suggests that PAL expression is much lower in these tissues than in young leaves and stems (Fig. 9, G and H). Northern blot analysis showed that PAL expression is clearly detectable in the developing xylem of mature stems and in the phloem and other tissues scraped from the inner surface
of the bark, but this expression was much lower than that in green, nonwoody stem tissue near the shoot apex (Fig. 7). Thus, moderate levels of PAL expression appear to be sufficient to support the lignin biosynthesis associated with secondary growth.

We are currently engaged in the further characterization of genomic copies of the poplar PAL genes and in developing gene-specific probes to determine if there is differential expression of the PAL genes in TD hybrids and parental trees. The poplar PAL genes will provide useful tools for examining mechanisms regulating gene expression at the poplar shoot apex, during differentiation of secondary xylem, and following perception of environmental stresses. The ability to transform and regenerate transgenic poplar trees (Fillatti et al., 1986; de Block, 1990; Leple et al., 1992) will allow the analysis of cis-acting elements responsible for the developmentally and environmentally regulated expression of poplar PAL genes.

ACKNOWLEDGMENTS

We thank Peter McAuliffe (Scott Paper, Ltd., New Westminster, B.C.) for providing field-grown poplar trees; Toby Bradshaw, Brian Watson, and Reinhard Stettler (University of Washington) for supplying plant material and DNA from the three-generation poplar pedigrees; and Ross Whetten for providing the deduced pine PAL amino acid sequence. We are further indebted to T. Bradshaw and B. Watson for help with RFLP and linkage analysis and for assistance and advice in preparation of the genomic library, and to T. Bradshaw for comments on the manuscript.

Received November 20, 1992; accepted February 10, 1993.

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