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Structure, Inheritance, and Expression of Hybrid Poplar (*Populus trichocarpa* × *Populus deltoides*) Phenylalanine Ammonia-Lyase Genes[1]

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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* × *P. trichocarpa* F_1 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 popular 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 *Hin*dIII restriction site polymorphisms. Clones containing each type of *PAL* gene were isolated from a poplar genomic library. Analysis of the segregation of *PAL*-specific *Hin*dIII 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 (cinnamyl 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

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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* × *P. deltoides* hybrid.

propagation, and transformability (Parsons et al., 1986; Fillatti et al., 1987; de Block, 1991; Leple et al., 1992), genetically defined material is available from a three-generation pedigree originating from a cross between a *Populus trichocarpa* female tree and a *P. deltoides* male tree (Bradshaw and Stettler, 1992; H. Bradshaw and R. Stettler, personal communication). Furthermore, many TD F_1 hybrid individuals from this and similar crosses have fast-growing phenotypes that make them superior for woody biomass production in certain sites (Heilman and Stettler, 1985; Heilman et al., 1990). Apart from its involvement in the biosynthesis of lignin, which accounts for 21 to 23% of *P. trichocarpa* wood dry weight (Swan and Kellogg, 1986), phenylpropanoid metabolism is unusually active in the buds, leaves, and bark of various *Populus* species, where a large number of phenylpropenoic acids and esters, flavones and flavanones, and their glycosides accumulate (see for example, Pearl and Darling, 1968, 1971; Hegnauer, 1973; Wollenweber, 1975; Greenaway et al., 1990; English et al., 1991). Some of these compounds may play defensive roles (Shain and Miller, 1982).

We are interested in the mechanisms regulating both lignin biosynthesis and the biosynthesis of the array of soluble phenylpropanoid-derived compounds in poplar, and we have chosen to focus part of our work on the regulation of the *PAL* gene(s) in this tree. We previously identified and partially sequenced a poplar *PAL* cDNA clone and showed that *PAL* mRNA accumulation and enzyme activity is rapidly and massively induced in elicitor-treated poplar suspension-cultured cells (Moniz de Sá et al., 1992). In this paper, we report the sequence of a full-length poplar *PAL* cDNA clone and show that, in the poplars used in our studies, two *PAL* genes are located at independently segregating genetic loci. We also show that *PAL* expression is developmentally regulated in poplar and that large amounts of *PAL* mRNA accumulate in a cell type-specific manner in developing leaves and shoots.

MATERIALS AND METHODS

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, F_2 individuals derived from a cross between 53-242 and 53-246, and backcross (B_1) 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 500-bp *Eco*RI-*Pst*I 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 λGEM12 (Promega, Madison, WI) was constructed using DNA from TD hybrid 53-242. Genomic DNA (10 μg) was partially digested with *Mbo*I, 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 λGEM12 arms containing partially filled-in *Xho*I sites (*Xho*I 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 hexadecyltriemethylammonium 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). ^{35}S-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 F_2 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 logarithm of odds scores were done using the MAPMAKER program (Lander et al., 1987).

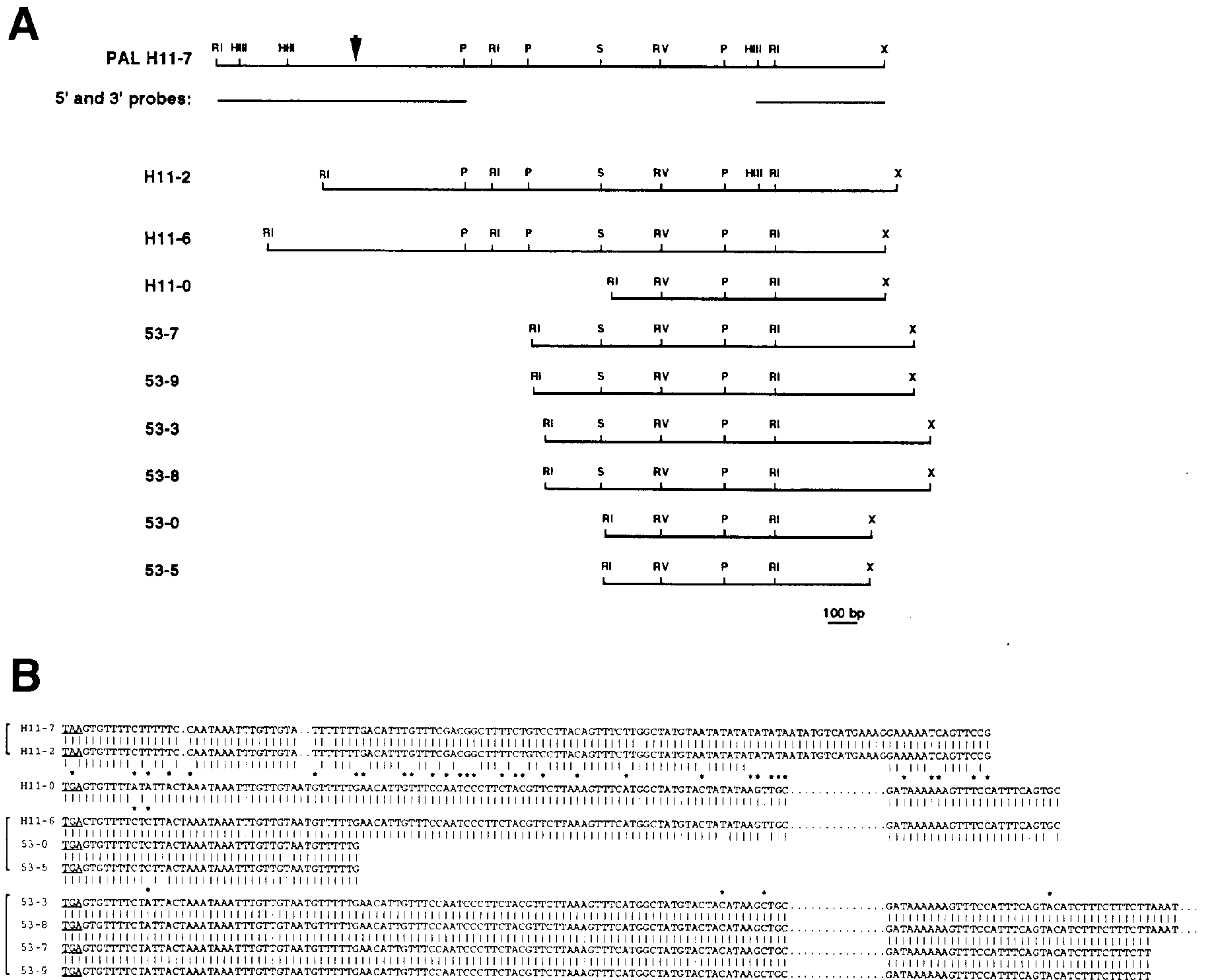

Figure 1. Restriction maps and 3′ sequence comparisons of *PAL* cDNAs. A, Restriction maps of cDNA clones derived from the H11 (H11-7, etc.) and 53-246 (53-7, etc.) libraries. The vertical arrow above the H11-7 map indicates the predicted intron location. Shown below the H11-7 map are the locations of 5′ and 3′ hybridization probes used in Southern blot hybridizations (see Fig. 4). *Eco*RI and *Xho*I 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, *Hin*dIII; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sac*I; X, *Xho*I.

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.

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 et al., 1992) 65%. This amino acid sequence conservation provides unequivocal evidence for the identity of H11-7 as *PAL*. As noted by others (Gowri et al., 1991), the greatest amino acid sequence divergence between *PAL* genes was found at the N-terminal and C-terminal ends of the predicted amino acid sequences. In contrast, even the most diverged amino acid sequences in this comparison (those of poplar *PAL* 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 *Hin*dIII site near their 3′ ends (H11-7 and H11-2), and those lacking this site (all remaining clones examined). A second *Hin*dIII 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 *Hin*dIII 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′ *Hin*dIII site) were identical, but were only about 85% identical to these regions in the remainder of the cDNAs, which lacked *Hin*dIII 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 iden-

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pop   M----ETVTKNGYQNGSLESLC-----------VNQRDPLSWGVAAEAMKGSH   38
pot   M---APSIAQ  HV  EV EVLWKK---------SIH   N EM VDSLR      41
alf   M----  ISAAITK NAN  F LIHAKNNNNMK  EA   N               49
par   MENG-NHA T  HV  NGMDF -----------MKTDE  Y  I  D  T      41
rice  M-------AG  P--------------------I KE   N  A  AE A      26
pine  ?TQAN VQV STGLCTDFG --------------SGS   N VR  K  E      38

pop   LDEVKRMVADYRKPVVKLGGE-TLTIAQVASIA-GHD--TGDVKVELSESARP   87
pot        K  DEF   I   W  -   V       -NA NK SGF         A   92
alf            E      R    -    S   A  -A  --  - Q D      D   97
par            E           E    S   A S-AR --GSG T     A  A   91
rice           QF E L  IQ A-  RVG   AV QAK --AAR A   D E      76
pine  FE   A  DS FGAKEISIE KS   SD  AV -RRS--QVK  LD-AAA KS   87

                                                    ▼
pop   GVKASSDWVMDSMDKGTDSYGVTTGF-GATSHRRTKQGGALQKELIRFLNAGI  139
pot                S            -C        N         K     V  144
alf         E   E  N            -     S                    149
par         E      N            A     S                    144
rice  R     E ILTCIAH G I       - G       D P   V  L Y      126
pine  R EE   N  LTQ T    T       -        N  AE    P       V  137

pop   FGNGTETCHTLPHSATRAAMLVRINTLLQGYSGIRFEILEAITRLLNNNITPC  192
pot         ST                                   K I S      197
alf         SN    KT                             KP  KTV    202
par       SD---N  KT  HS                D   R    KP  KFV QN  194
rice    T SDG-    SETV                           K   TGV    180
pine  L KCP --NV SEDT        T           WD   TVEK   AGL  K  190

pop   LPLRGTITASGDLVPLSYIAGLLTGRPNSKATGPTGEVLDAAEAFKAAGIESG  245
pot         V                        V  S SK   D   RV AVSG   250
alf                                  H  S    N K   NL   NAE  255
par   I                                   VI SPE  KNL VEGAE  247
rice                        I     AQ IS D RKV       L    G   233
pine                             RVRSRD IEMSG   L KV L KP  243

pop   FFELQPKEGLALVNGTAVGSGLASMVLFETNVLAVLSELLSAIFAEVMNGKPE  298
pot                        M  I  YDS I   MF V               303
alf                           I     A I      V         Q    308
par                        M        A I    A VM      F Q    300
rice    T N      I    S   A  AT M DA I       V   V C        286
pine  -          I    SM AA   I C DA    L    VI   M C       295

pop   FTDHLTHKLKHHPGQIEAAAIMEHILDGSAYMKAAKKLHETDPLQKPKQDRYA  352
pot      Y                            S V   Q    M           356
alf                                   S V        I           361
par                                     V   Q    M           353
rice              S D         A  SF SH   VN M                339
pine     P           M       YV    S   H A    MN          G  346

pop   LRTSPQWLGPQIEVIRFSTKSIEREINSVNDNPLIDVSRNKAIHGGNFQGTPI  404
pot                  AA   M                                  409
alf             LV                                  L        414
par             L     S                             L        406
rice             Q    AA       V       V    H G     L        392
pine            V I   SA HM            V    A D     L        401

pop   GVSMDNVRLAIASIGKLLFAQFSELVNDFYNNGLPSNLTASRNPSLDYGFKGA  457
pot        T    L     M          Y          G                462
alf        T    L     M                   S                  467
par        T      A   M  -                SGG                448
rice       A      N   M        E      T   AG               T 445
pine       L   S SA   M          Y  G     SGGP         L     454

pop   EIAMASYCSELQYLANPVTTHVQSAEQHNQDVNSLGLISSRKTAEAVDILKLM  510
pot               F     N                   A                515
alf                                         A   N   IE   Q   520
par               F     N                       S   E        511
rice         S        I N                 V A   L            498
pine         T   L      S                 V A   S   I        507

pop   STTFLVALCQAIDLRHLEENLKSAVKNTVSQVSKRVLTTGANGELHPSRFCEK  563
pot    S Y                    V       A  T  I  I     A       568
alf    S   I                 NS       A KT   M V             573
par       G                  T      S A      M V             564
rice  TS YI      V        I  S    C T A K   MNPT D SSA   S   551
pine  LS Y T     V        MLATN QI    A KT S  L GL E G        560

pop   ELLKVVDREYVFAYVDDPCSATYPLMQKLRQVFVDHALENGENEKNFSTSVFQ  616
pot      R      L T A     S             L    MK   S    INS I 621
alf   D        H    I          S        L    V    S    N   I 626
par   D  R    G I   I                 TL E    K  D  R L   I  617
rice  N TAID    V  Y D               A.L E   TS DR-RARGLR L  603
pine  D QV   E V   Y D    N S   T     NIL ER FK A G  DPN   I N 613

pop   KIEAFEEELKALLPKEVESARAAYDSGNSAIDNKIKECRSYPLYKFVREELGT  669
pot     G   D  N V            LLE   PS P R T        RL  Q    674
alf     AT      T          T  E   PT P   NG                  679
par     AT  D                 LE   PT P R E            K      670
rice  DHQVRGGAPLCAA GDRGRP RRRQRT-APVA R V S  F   R      C    655
pine    PV  A    Q EPQ SL  ES  K T PLP R Q         E  NQ       666

pop   VLLTGEKVQSPGEEFDKVFTAMCQGKIIDPMLECLGEWNG------------SPLPIC  715
pot   E       R     IE        N Q N  L    KS   ------------A       720
alf   G     N I     C  L S           L         ------------A       716
par   EY      T      E   I  SK       L    ES   ------------A       716
rice   F     LK     CN   LGIS    L    D   K    ------------E     N 701
pine  K  S TRTI    VIEV YD ISEDVK V LFK  DG K TLAHSEINNLPR    YND 739

pine  CHDLSPRILLL   747
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Figure 2. Comparison of the predicted poplar *PAL* amino acid sequence with that of other *PAL* genes. The deduced amino acid sequence of poplar *PAL* H11-7 (pop) is aligned with deduced amino acid sequences of potato (pot, Joos and Hahlbrock, 1992) alfalfa (alf, Gowri et al., 1991), parsley (par, Lois et al., 1989), rice (Minami et al., 1989), and pine (Whetten and Sederoff, 1992) *PAL* genes. Amino acids are shown when they differ from the poplar sequence. Dashes indicate gaps introduced to maximize alignment. The N-terminal sequence of pine PAL has not been published.

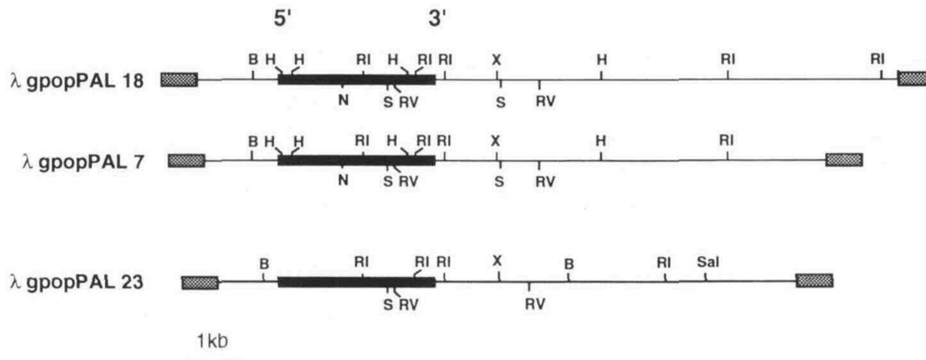

Figure 3. Restriction maps of poplar *PAL* genomic clones. Clones were derived from a genomic library constructed with DNA from TD hybrid 53-242. Stippled boxes indicate λGEM12 vector DNA. Closed boxes indicate the approximate locations of *PAL* genes as determined by hybridization with H11-7 cDNA probes. B, *Bam*HI; H, *Hin*dIII; RI, *Eco*RI; RV, *Eco*RV, N, *Not*I; S, *Sac*I; Sal, *Sal*I; X, *Xba*I.

tical 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 F_1 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 *Hin*dIII sites. These and other restriction sites within the gene are colinear with those in cDNA clone H11-7 except that a 0.8-kb *Hin*dIII-*Eco*RI 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 *Not*I 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 *Hin*dIII sites and a *Not*I 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 *Hin*dIII restriction site polymorphisms within the transcribed and flanking genomic regions, and a *Not*I 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 to the H11-7 cDNA. Of the restriction enzymes used, two (*Xba*I and *Bam*HI) do not cut within either class of *PAL* cDNAs.

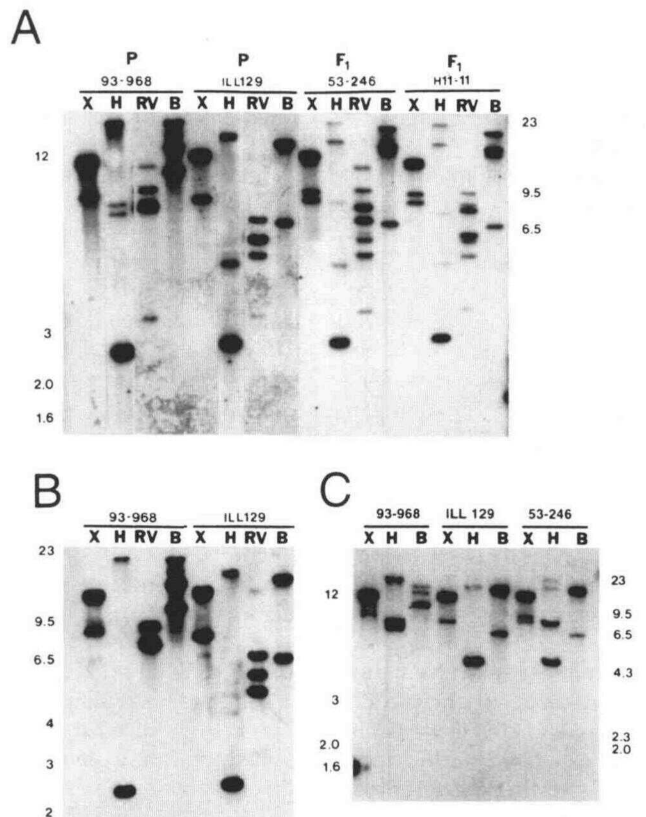

Figure 4. Southern blot analysis of *PAL* genes in parental and hybrid poplar clones. A, Southern blot probed with the complete *PAL* H11-7 cDNA. B, The same blot as in A was stripped and rehybridized to a probe derived from the 5′ end of the *PAL* H11-7 cDNA (see Fig. 1). C, Southern blot probed with a 3′ fragment of the *PAL* H11-7 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 F_1 hybrid from the 53-968 × ILL129 cross), and H11-11 (an F_1 hybrid from a different *P. trichocarpa* × *P. deltoides* cross), were digested with the restriction enzymes shown, separated on 0.8% agarose gels, and blotted to nylon membranes. B, *Bam*HI; RV, *Eco*RV; H, *Hin*dIII; X, *Xba*I. Migration of size standards in kb is shown at the sides of the blots.

*Eco*RV cuts within all of the cDNA clones and, as discussed above, *Hin*dIII cuts within some but not others (Fig. 1A). The small number of hybridizing *Xba*I and *Bam*HI 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 *Xba*I fragment in 93-968 was a doublet, and one of the larger *Bam*HI fragments in 93-968 was not consistently observed and appeared to be a partial digestion product). *Eco*RI-*Pst*I and *Hin*dIII-*Xho*I restriction fragments taken from the 5′ and 3′ ends of the H11-7 cDNA (Fig. 1A), respectively, hybridized to the same *Xba*I and *Bam*HI restriction fragments (Fig. 4, B and C), suggesting that these fragments contain complete copies of *PAL* genes. The 6.5-kb *Bam*HI restriction fragment in ILL129 was identical in size to the *Bam*HI 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 *Hin*dIII 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 *Hin*dIII restriction fragment in *PAL* H11-7, it is likely to represent an intron-containing gene-internal fragment specific to *PAL* genes containing *Hin*dIII sites. Three additional *Hin*dIII 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) *Hin*dIII fragments (Fig. 4B). This suggests that the large *Hin*dIII fragments contain complete *PAL* genes, whereas the three smaller *Hin*dIII 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 *Hin*dIII fragment from ILL129 is identical in size to the *Hin*dIII 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 *Hin*dIII fragments containing *PAL* 3′ ends and poorly to the larger *Hin*dIII 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 *Hin*dIII sites), and weakly to fragments containing different *PAL* gene family members (i.e. those lacking internal *Hin*dIII sites).

As expected, the F_1 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 *Hin*dIII fragments from the *P. trichocarpa* parent and one of two large *Hin*dIII 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 *Hin*dIII RFLPs observed in Figure 4. DNA extracted from 30 F_2 and 30 B_1 individuals (obtained from crosses between F_1 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 F_1 individuals, was cut with *Hin*dIII 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 *Hin*dIII restriction fragments very similar to its sibling 53-246, except that two different parental *Hin*dIII 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 *Hin*dIII fragments in F_2 individuals showed that the T1, D1a, and D1b fragments, corresponding to the class of large *Hin*dIII fragments containing complete *PAL* genes, were allelic (for example, the genotypes of F_2 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 *Hin*dIII 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 B_1 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 F_2 and B_1 individuals (H. Bradshaw and R. Stettler, personal communication) was tested. Whereas no linkage of the two poplar *PAL* loci to each other was observed, *PAL1* 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

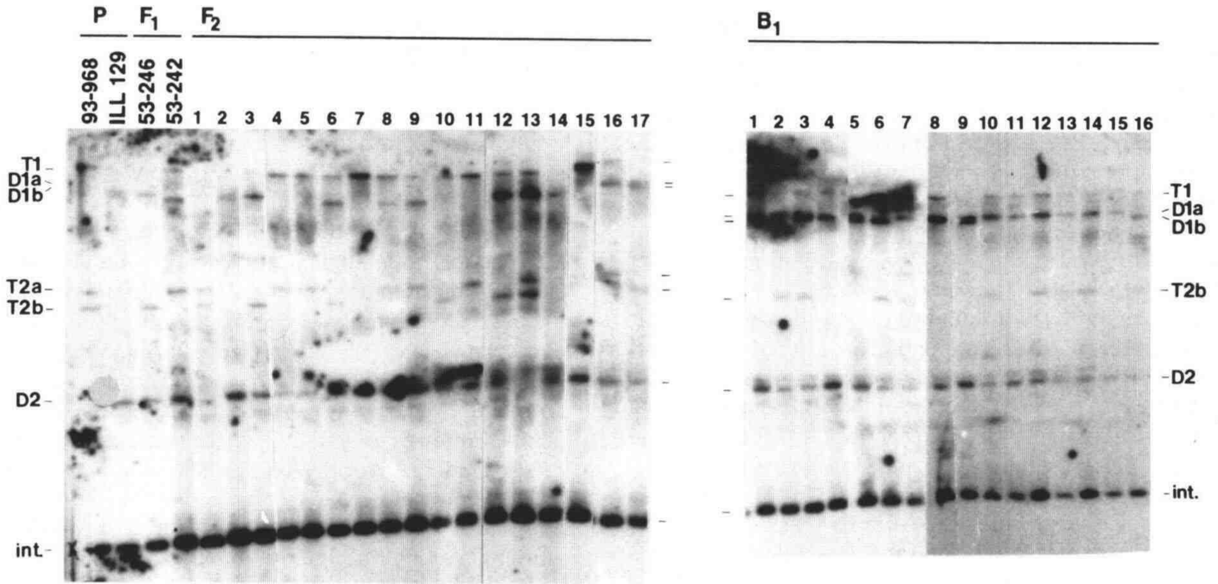

Figure 5. Segregation of *PAL*-specific RFLPs in F_2 and backcross generations of a three-generation poplar pedigree. Genomic DNA (3 μg) from parental clones 93-968 and ILL129, F_1 hybrid sibling clones 53-242 and 53-246, F_2 progeny derived from a cross between clones 53-242 and 53-246, and B_1 progeny derived from a backcross between parental clone 93-968 and F_1 hybrid clone 53-242 was digested with *Hin*dIII, separated on 0.8% agarose gels, blotted to nylon membranes, and hybridized to a ^{32}P-labeled *PAL* H11-7 cDNA probe. The prominently hybridizing *Hin*dIII restriction fragments are indicated to the sides of the blots. Letters (D or T) refer to the origin (*P. deltoides* or *P. trichocarpa*) of segregating fragments. Fragments that appear to segregate as separate genes are indicated by numbers (1 and 2); apparent alleles are indicated by lowercase letters (a and b). The prominent small fragment (int.) is a gene-internal *Hin*dIII restriction fragment.

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

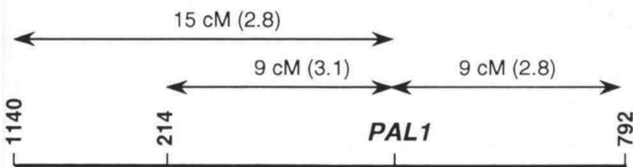

Figure 6. Linkage of *PAL1* to RFLP markers. Linkage of *PAL1*-specific polymorphisms to RFLP markers segregating in F_2 and backcross generations of a three-generation pedigree was established using the MAPMAKER II program. Distances are given in centimorgans (cM) with logarithms of odds scores in brackets.

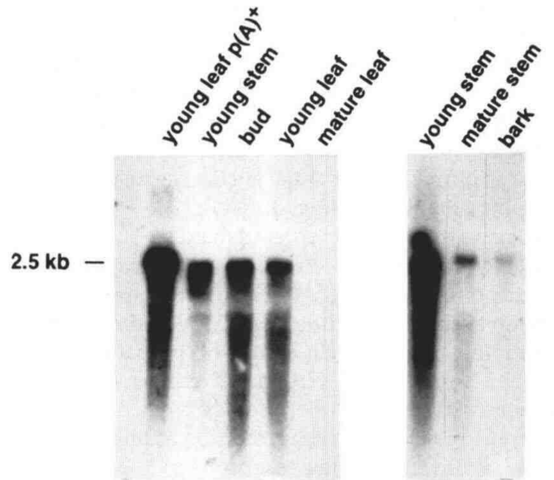

Figure 7. Northern blot analysis of RNA from various poplar tissues. Ten migrograms of total RNA from the organs indicated, or 3 μg of poly(A)$^+$ RNA from young leaves, was fractionated on two separate gels (left and right), blotted, and hybridized to a ^{32}P-labeled *PAL* H11-7 cDNA probe. The size of the *PAL*-specific band, shown at left, was estimated from the migration of size standards and by comigration with transcripts from parsley cells that hybridized to *PAL* (not shown).

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 trachery 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. 8J). Finally, examination of sections from mature, fully expanded leaves showed that mesophyll, epidermal, and vascular tissues were fully differentiated (Fig. 8J). 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. Differentiated 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

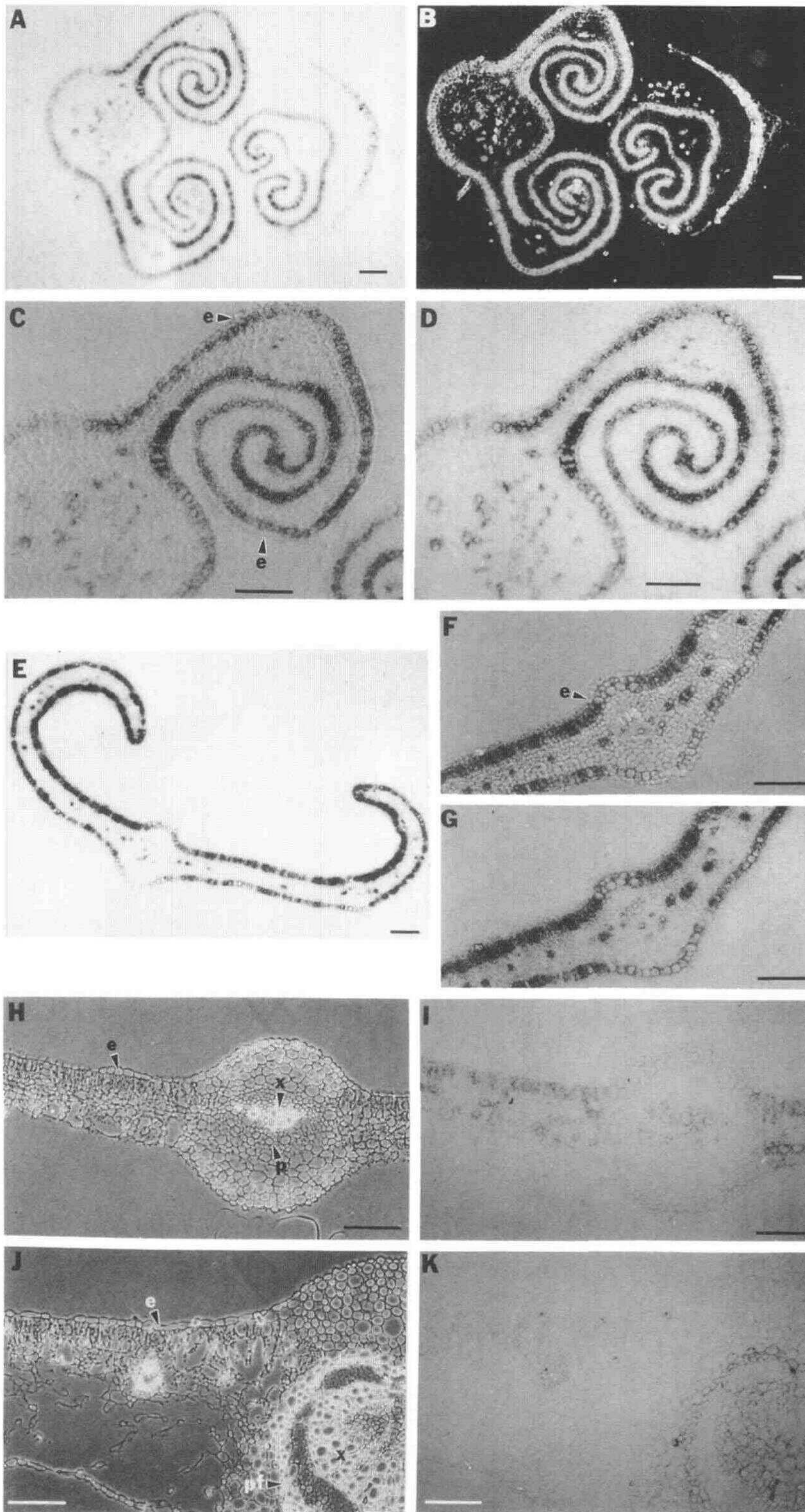

Figure 8. Localization of *PAL* expression in poplar leaves by in situ hybridization. ^{35}S-Labeled *PAL* H11-7 antisense transcripts were hybridized to leaves of different ages. A–D, Unexpanded leaves at the shoot apex. A, Bright-field illumination; B, dark-field illumination. C and D, Higher magnification 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 *Hin*dIII sites and those lacking such sites (Fig. 1). Although most cDNA clones were not long enough to detect the presence or absence of *Hin*dIII sites at their 5′ ends, the structure of genomic clones indicated the existence of *PAL* genes completely lacking *Hin*dIII sites and genes containing sites at their 3′ and 5′ ends (Fig. 3). Thus, the two types of cDNAs appear to have arisen from two distinct classes of

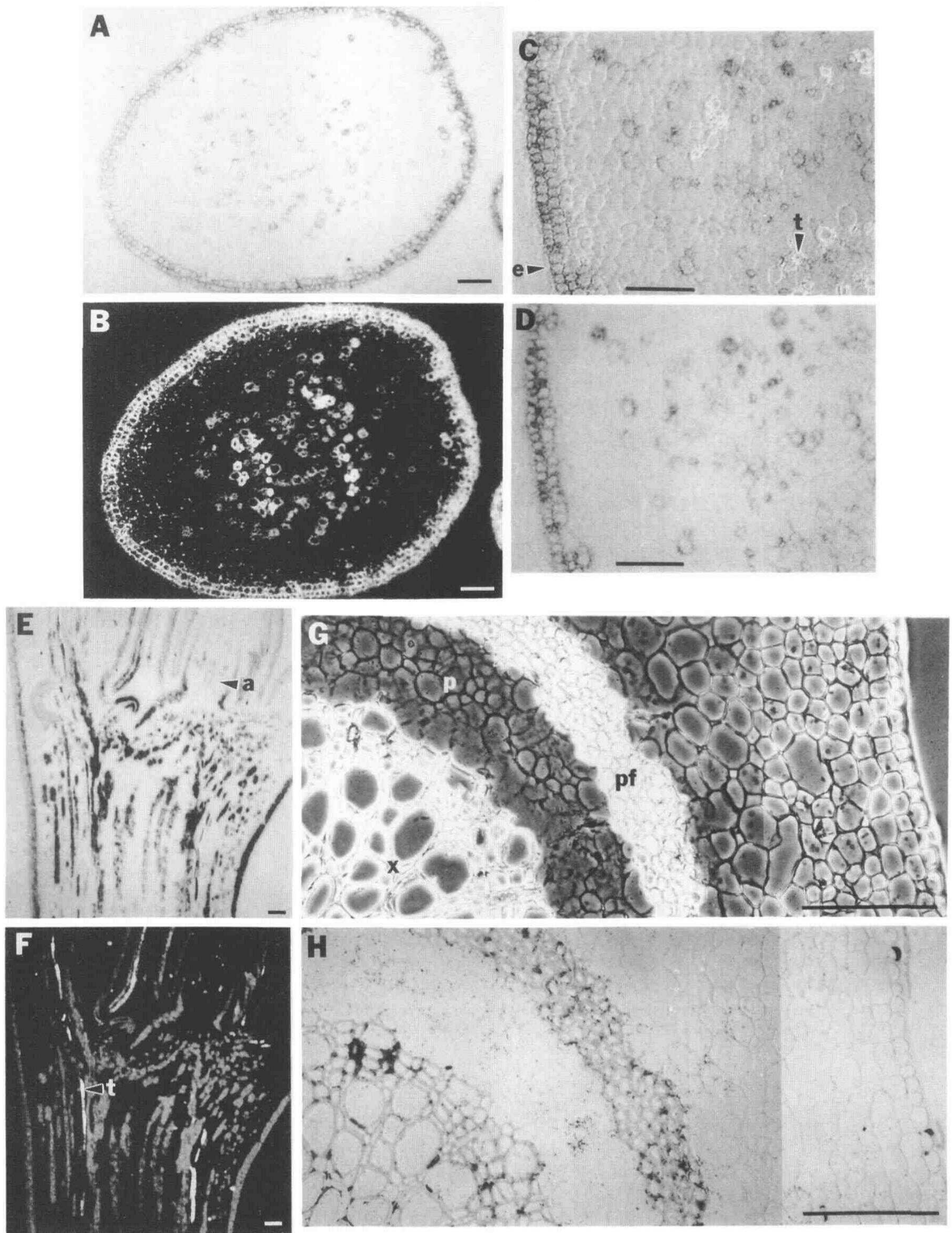

Figure 9. Localization of *PAL* expression in petioles and stems by in situ hybridization. ^{35}S-Labeled *PAL* H11-7 antisense transcripts were hybridized to stem and petiole sections. A–D, Cross-sections through the petiole of an expanding leaf. A, Bright-field illumination; B, dark-field illumination. 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 μm.

PAL genes, distinguishable by *Hin*dIII 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 *Hin*dIII RFLP inheritance in advanced generations of the poplar pedigree indicated that the large genomic *Hin*dIII fragments that appeared to contain complete *PAL* genes were allelic, as were the smaller *Hin*dIII 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 *Hin*dIII 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 *Hin*dIII fragments containing the *PAL D1/T1* locus. Thus, we cannot exclude the possibility that more than one *PAL* gene is located on the *Hin*dIII 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, 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.

*Hin*dIII-digested poplar DNA sometimes showed variable and faint hybridization to additional *Hin*dIII 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 *Hin*dIII 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.

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