Functional Properties of a Phenylalanine Ammonia-Lyase Promoter from *Arabidopsis*

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Phenylalanine ammonia-lyase (PAL) is encoded by a small family of genes in *Arabidopsis*. We cloned and partially characterized one of these genes, PAL1. The deduced amino acid sequence is highly similar to PAL from bean, parsley, and rice. The promoter contains sequence elements homologous to two putative regulatory elements conserved among several phenylpropanoid genes. The regulation of the PAL1 gene was examined by analysis of β-glucuronidase (GUS) activity in transgenic *Arabidopsis* containing PAL1-GUS gene fusions. The PAL1 promoter was activated early in seedling development and in adult plants was strongly expressed in the vascular tissues of roots and leaves, but was not active in the root tip or the shoot apical meristem. In flowers, expression was observed in sepals, anthers, and carpels, but not in petals. Transcripts encoded by the endogenous PAL genes and GUS transcripts from the PAL1-GUS gene fusion were induced by wounding, HgCl₂-stress, and light. Analysis of the regulatory properties of 5' deleted promoters showed that the proximal region of the promoter to −290 was sufficient to establish the full tissue-specific pattern of expression and that the proximal region to −540 was responsive to environmental stimuli. Negative and positive elements were located between −1816 and −823 and between −823 and −290, respectively.

INTRODUCTION

Phenylalanine ammonia-lyase (EC 4.3.1.5, PAL) catalyzes the deamination of L-phenylalanine to trans-cinnamic acid, which is the first step in the biosynthesis of a large class of plant natural products based on the phenylpropane skeleton (Dixon et al., 1983; Jones, 1984). Phenylpropanoids play key roles in plant development and in protection against environmental stresses. Thus, flavonoids are pigments and UV-protectants, and lignin is a major structural polymer in xylem cell walls. Induction of lignin deposition in peripheral tissues and accumulation of furanocoumarin and isoflavonoid-derived phytoalexins help protect against mechanical damage and potential pathogens. In addition, some phenylpropanoids function as regulatory and signal substances. For example, the *Agrobacterium* virulence genes and the *Rhizobium* noduleation genes are induced by compounds such as acetosyringone derived from phenylpropanoid wound metabolites and simple flavonoids such as luteolin, respectively (Downie and Johnston, 1986; Stachel and Zambrisky, 1986).

PAL mRNA and enzyme levels are highly regulated during development and in response to environmental cues, correlated with the accumulation of specific phenylpropanoid products (Lamb et al., 1989). PAL is encoded by a family of three to four genes in bean (Cramer et al., 1989), parsley (Lois et al., 1989), and rice (Minami et al., 1989), and the transcripts of individual PAL genes show highly different patterns of accumulation (Liang et al., 1989a; Lois et al., 1989). Nuclear run-off transcription experiments have shown that elicitor and UV-light induction of PAL mRNA in cell suspension cultures of bean and parsley reflects transient stimulation of PAL gene transcription (Lawton and Lamb, 1987; Lois et al., 1989). Likewise, analysis of the bean PAL2 promoter in transgenic tobacco and potato plants containing PAL2-β-glucuronidase (GUS) gene fusions has demonstrated tissue- and cell-type-specific PAL transcription during development and in response to wounding and light (Bevan et al., 1989; Liang et al., 1989b). Thus, the bean PAL2 promoter is able to integrate a complex set of developmental and environmental cues into a coherent program of expression attuned to the diverse functions of phenylpropanoid natural products.

To help dissect the signal transduction pathways underlying developmental and environmental regulation of PAL promoters, we are examining PAL regulation in *Arabidopsis* and report here on the isolation of an *Arabidopsis* PAL gene and the functional properties of its promoter. *Arabidopsis* is readily transformable (Valvekens et al., 1988), and, hence, we have been able to examine the properties of this promoter in the homologous system. In addition, a number of hormonal and developmental mutants have been characterized in *Arabidopsis* that are likely to be involved in signal pathways for PAL regulation (Meyerow-
Moreover, Arabidopsis provides an excellent system for the creation of transgenic plants containing selectable or screenable marker genes regulated by the PAL promoter, or specific dissected cis-elements, for use in the identification of novel signal pathway mutants that affect PAL promoter activity in trans.

As a first step in these studies, we show here that PAL is encoded by a small gene family in Arabidopsis. One of the genes, designated PAL1, was cloned and partially characterized. The encoded product is highly related to PAL from other species, and the promoter contains putative regulatory elements conserved in the promoters of several phenylpropanoid biosynthetic genes. We demonstrate that PAL1 exhibits a complex pattern of tissue-specific developmental expression and is also induced by wounding, HgCl$_2$-stress, and light. Functional analysis of 5’ deletions identified regions of the PAL1 promoter involved in determining the quantitative level of expression, tissue specificity, and environmental regulation.

RESULTS

Cloning and Characterization of an Arabidopsis PAL Gene

A 5-kb EcoRI fragment containing an Arabidopsis PAL gene (PAL1) was subcloned from a genomic clone isolated by screening a genomic library from Arabidopsis (Chang and Meyerowitz, 1986) with bean PAL cDNA sequences (Edwards et al., 1985).

To determine the complexity of PAL genes in the Arabidopsis genome, genomic DNA was digested with EcoRI, HindIII, BamHI, and PstI, blotted, and hybridized with a labeled fragment from the Arabidopsis PAL1 gene. The result is shown in Figure 1. Under stringent hybridization and washing conditions, four to five bands of different intensities were observed, indicating that PAL1 is a member of a small family of genes.

We sequenced a 3.1-kb fragment that included 1.8 kb of the promoter and 0.7 kb of the coding region, as shown in Figure 2. The deduced amino acid sequence showed 75%, 77%, and 67% identity to the corresponding PAL sequences from bean (Cramer et al., 1989), parsley (Lois et al., 1989), and rice (Minami et al., 1989), respectively. Sequence comparison indicated that the coding region contained a 448-bp intron with AG/GT and AG/AT consensus boundaries (Breathnach and Chambon, 1981) at the same relative position as in the previously characterized PAL genes from these other species. The transcription start site of PAL1 was mapped by S1 nuclease protection to a position 129 bp upstream of the putative translation initiation codon (data not shown).

The promoter and 5’-untranslated region of the PAL1 gene exhibited no obvious overall sequence homology to

![Figure 1. DNA Gel Blot of Arabidopsis Genomic DNA Probed with a Fragment from the Coding Region of PAL1.](image_url)

Five micrograms of DNA per lane were digested with EcoRI, HindIII, BamHI, or PstI, separated on a 0.8% agarose gel, blotted, and hybridized with a labeled 190-bp StyI-HindIII PAL1 fragment corresponding to the 5’ end of the second exon. The blot was washed in 0.1 x SSC, 0.1% SDS at 65°C and autoradiographed for 3 days. Size markers are given in kilobases.
Expression of PAL 1-GUS Fusion in Transgenic Arabidopsis

To determine the regulatory properties of the Arabidopsis PAL1 promoter during seedling development and in response to different environmental stimuli, 1.8 kb of the PAL1 promoter, subsequently referred to as the "full-length promoter," was cloned into the binary vector pBluescript (Jefferson et al., 1987) as a transcriptional fusion in front of a promoterless p-glucuronidase (GUS) gene. The resulting construct, pS0-I, was transformed into Arabidopsis by the root transformation method (Valvekens et al., 1988), and kanamycin-resistant transgenic plants were analyzed in the T2 generation.

Expression during Early Seedling Development and in Adult Plants

Seeds of transgenic plants transformed with pS0-1 were germinated on medium containing kanamycin and seedlings were stained with 5-bromo-4-chloro-3-indolyl-p-glucuronide (X-Gluc) at various developmental stages, as shown in Figure 3. The strongest GUS staining was found in very young seedlings that had just emerged from the seed coat (48 hr after planting), and staining subsequently declined slowly. GUS staining was found in all tissues of the seedling except the root tip. Staining was most intense in a broad area at the base of the radicle, at the base and the tip of the cotyledons, and associated with the presumptive vascular tissue of the shoot (Figures 3A and 3B). Four to 5 days after germination, GUS staining had decreased in most tissues and become progressively restricted to the vascular tissue (Figures 3C to 3F). The staining intensity became more variable among independent transformants, but remained higher in the root than the shoot.

To determine the pattern of PAL1-GUS expression in adult plants transformed with pSO-1, the T2 progeny of seedlings were stained with X-Gluc. The staining was most intense in young tissues, and became progressively reduced to the vascular tissue (Figures 3C to 3F).

Expression during Early Seedling Development and in Adult Plants

Figure 2. Sequence of the PAL1 Promoter, 5'-Untranslated Region, and Part of the Coding Region.

The transcription start site is at +1, a putative TATA homology is other PAL genes, but a computer-aided nucleotide sequence comparison identified several repeats of two small elements, which are conserved among the promoters of several phenylpropanoid biosynthetic genes and which coincide with inducible in vivo footprints in the parsley PAL promoter (Lois et al., 1989). In the 5'-untranslated leader, a 9-bp element is identical to an element in the bean CHSl5 gene at a similar location, which coincides with an elicitor-inducible DNase I hypersensitive site (Lawton et al., 1990).
several independent transformants were grown for 3 to 5 weeks on selective medium and stained with X-Gluc to visualize GUS activity. Figure 3 shows that the pattern found in young seedlings was maintained in the adult plant, with stronger staining in roots than in stems or leaves (Figures 3G and 3H). At the tissue level, staining was most pronounced in the vascular tissue (Figures 3L and 3M), but lower levels of GUS activity were occasionally observed in other tissues.

Young flower buds did not stain initially for GUS activity (Figure 3O), but staining appeared slowly as the flower buds developed. In a fully developed flower, GUS staining was found in the sepals, in the stamen, and at the tip and base of the carpel, but no staining was detected in petals (Figures 3N and 3P). Intense GUS staining was also observed in pollen grains, and analysis of flower buds at different developmental stages showed that GUS staining in pollen became visible before the pollen developed its yellow pigmentation (data not shown).

Staining intensity could be quite variable even among the progeny of individual transformants and within a plant between different organs. Thus, two leaves of the same size and developmental stage sometimes showed a striking difference in staining intensity. However, the basic qualitative features of high vascular expression, lack of expression in the root tip, and a characteristic pattern of floral expression were always maintained.

**Induction by Wounding, HgCl₂, and Light**

Three-week-old transgenic plants were wounded, incubated in an HgCl₂ solution, or transferred to white light after 4-day dark adaptation, and then harvested 6 hr to 12 hr after stimulation. GUS activity was measured using the fluorimetric assay and, to our surprise, no significant induction of GUS activity was observed against a background of significant GUS activity arising from sustained developmental expression of the gene fusion, we subsequently examined changes in the levels of PAL and GUS transcripts in transgenic plants after wounding, HgCl₂ treatment, or transfer to white light after 4-day dark adaptation. Total RNA was extracted from whole plants harvested 1 hr to 4 hr after treatment and subjected to RNA gel blot analysis using labeled PAL1 or GUS probes. The results are shown in Figure 4. Transcripts encoded by the endogenous PAL genes were markedly induced by all three stimuli (Figure 4, top panel). Additional experiments measuring mRNA during a longer time course showed that the induction by wounding and HgCl₂ stress was transient with the highest levels 1 hr to 2 hr after stimulation (data not shown). Light induction resulted in a slower accumulation of PAL transcripts, with a maximum 2 hr to 4 hr after transfer to light.

GUS mRNA was induced by all three stimuli and accumulated with kinetics very similar to those observed for PAL mRNA (Figure 4). In control plants transformed with a cauliflower mosaic virus 35S promoter-GUS gene fusion, the endogenous PAL mRNA was induced by wounding, whereas GUS mRNA remained relatively low (Figure 4, w).

| Table 1. GUS Activity in Plants Transformed with pSO-1 after Wounding or HgCl₂ Treatment |
|-----------------|-----------------|-----------------|-----------------|
|                 | GUS Activity    |                 |                 |
|                 | (pmol MU*/min·mg protein) |
| Control         | 29.2 ± 1.8      | Wounded         | 31.2 ± 2.3      |
| HgCl₂           | 27.3 ± 1.9      |                 |                 |

GUS activity was determined from whole plants 8 hr after wounding or transfer to a 100 µM HgCl₂ solution. The data are mean values and average deviations from the mean calculated from 14 measurements on individual transformants.

* MU, methylumbelliferone.

Because there was some variability in the assay, and a transient environmental induction might not be readily observed against a background of significant GUS activity arising from sustained developmental expression of the gene fusion.
Effect of 5’ Deletions on Organ-Specific and Tissue-Specific Expression

The effect of 5’ promoter deletions on the basal level of GUS expression was examined in 3-week-old T2 plants transformed with the constructs pSO-1 to pSO-4 and is shown in Figure 5. Deletion of the promoter from −1832 to −816 resulted in a 30% increase in GUS activity extractable from whole plants. Further deletions of the promoter to −540 and −290 led to stepwise decreases to about 30% and 2%, respectively, of the activity of the full-length promoter.

Figure 6 shows the levels of extractable GUS activity in leaves, stems, and roots of adult T2 plants containing the PAL1-GUS gene fusion and consecutive 5’ promoter deletions (pSO-1 to pSO-4). Progressive deletion of the promoter to −290 (pSO-4) did not cause qualitative changes in organ-specific expression. Irrespective of the extent of the deletion, the relative expression levels in different organs increased in the order leaves < stems < roots.

To investigate the effects of 5’ promoter deletions on the tissue-specific pattern of PAL1 promoter activity, transgenic plants were histochemically stained for GUS activity. Although the staining intensity in different organs was found to be quite variable among the progeny of an individual transformant, no difference was found in the tissue-specific pattern of GUS expression between plants containing the full-length promoter and the various promoter deletions (Figures 3I and 3K). Thus, plants containing the shortest promoter fragment (pSO-4, −290) still expressed GUS in the vascular tissue and in the same flower organs as plants transformed with pSO-1 containing the full promoter. However, consistent with the markedly reduced overall GUS activity extractable from the SO-4 plants, GUS staining in the histochemical assay developed much more slowly and only after prolonged incubation of tissue specimens.

Effect of 5’ Deletions on mRNA Induction by Environmental Stimuli

Transgenic plants SO-1 to SO-4 were wounded, incubated in an HgCl2 solution, or transferred to light after dark adaptation. Plants were harvested 1 hr to 4 hr after stim-
DISCUSSION

Isolation and Structure of an Arabidopsis PAL Gene

In this paper we describe the isolation and partial characterization of an Arabidopsis PAL gene using a heterologous PAL cDNA probe from bean. A genomic DNA gel blot of DNA digested with four restriction enzymes and probed with a fragment homologous to a highly conserved part of the second exon revealed one to two strong bands and two to four weaker bands under stringent hybridization and washing conditions. The PAL probe was only 190 bp and did not contain an internal site for any of the four restriction enzymes used in this experiment. Therefore, the number of bands should reflect the number of PAL-related sequences in the genome. In the experiment shown in Figure 1, genomic DNA was isolated from the Columbia ecotype, whereas PAL1 was cloned from a library of Landsberg ecotype DNA. However, using Landsberg DNA in an equivalent experiment resulted in the same pattern of bands except for the 8.5-kb band in HindIII-cut DNA, which was missing in the Landsberg blot (data not shown). The data indicate that the PAL1 gene is a member of a small family of genes. The prominent band in EcoRI-digested genomic DNA probably corresponds to the 5-kb EcoRI fragment that was initially subcloned from the genomic λ clone.

Many gene families are smaller in Arabidopsis than in most other plant species (Meyerowitz, 1987). In contrast, the Arabidopsis PAL gene family is comparable in size to the PAL gene families in bean, parsley, and rice. The observation that the small genome of Arabidopsis contains a PAL gene family of similar complexity to those in species with much larger genomes is consistent with the hypothesis that individual PAL genes may encode variant products with distinct functional specializations. This hypothesis was initially proposed to account for the differential regulation of members of the PAL gene family in bean and parsley (Liang et al., 1989a; Lois et al., 1989) and the different biochemical properties of specific bean PAL enzyme subunit isoforms (Bolwell et al., 1985).

Figure 7. RNA Gel Blot of GUS mRNA from Plants Transformed with pSO-1 to pSO-4 or pBI121.

Whole plants were wounded, transferred to a 100-μM HgCl₂ solution, or transferred to white light following 4 days of dark adaptation, and harvested 1 hr to 4 hr after treatment. Total cellular RNA was extracted and fractionated by gel electrophoresis, blotted, and hybridized to labeled probes homologous to GUS. c, control plants harvested immediately after wounding or transfer to light; w, wounding; H, HgCl₂ solution. Size markers are given in kilobases.
Sequencing 0.7 kb of the putative coding region confirmed that this *Arabidopsis* gene encodes PAL. The deduced amino acid sequence showed a high degree of similarity to PAL from bean, parsley, and rice, with most differences representing conservative changes. The amino terminus is quite divergent in both length and sequence. As expected, the sequences from the three dicotyledonous plants are more closely related to each other than to the rice PAL sequence.

**Developmental and Tissue-Specific Regulation**

To facilitate analysis of the tissue-specific developmental regulation and stress induction of the PAL1 gene and to obtain some information on the functional organization of its promoter, we generated four PAL1 promoter-GUS transcriptional fusions and analyzed these constructs in transgenic *Arabidopsis*. As expected, considerable variation was found in the absolute levels of expression in different transformants, probably due to positional effects from insertion of the transgene at different sites in the genome. This problem was overcome by analyzing several independent transformants in each case. Moreover, qualitative properties such as tissue-specific patterns of expression did not vary between independent transformants containing the same construct.

The regulation of a reporter gene by a heterologous promoter has been shown in many systems, including the bean PAL2-GUS gene fusion in transgenic tobacco, to be an accurate reflection of the intrinsic regulatory properties of the promoter (Benfey and Chua, 1989; Bevan et al., 1989; Liang et al., 1989b; Schmid et al., 1990). The *Arabidopsis* PAL1 promoter was very active during the early stages of seedling development. GUS staining was found in all tissues except the root tip and was most pronounced at the base of the radicle. The cotyledons accumulate high levels of anthocyanins at this stage; we propose that during the 1st day or 2nd day after emerging from the seed coat the seedling is vulnerable to UV light and pathogens, and, thus, PAL is induced in the shoot for rapid accumulation of pigments and in the root possibly for defense against microbial attack. In adult plants, PAL was expressed in the vascular tissue of roots, shoots, and leaves. This observation is consistent with the vascular expression of the bean PAL2-GUS gene fusion in transgenic tobacco and potato (Bevan et al., 1989; Liang et al., 1989b) and supports the hypothesis that PAL expression associated with the initiation of lignin synthesis is an early marker for xylem differentiation during vascular development.

In *Arabidopsis* flowers, the PAL1-GUS gene fusion was expressed in sepals, anthers, and the carpel but not in the white unpigmented petals. After the flower bud opened and the stigma became susceptible to fertilization, GUS staining developed at the base of the stigma. The functional significance of PAL1 expression in this tissue is not known, but a possible explanation would be that during outgrowth of the pollen tube the pollen specifically interacts with a defined region of the stigma. A second zone of PAL1-GUS expression of unknown function developed at the base of the ripening silique in the abscission zone of sepals and petals. The strong expression found in pollen correlates with pigment biosynthesis. Staining pollen from different developmental stages showed that the onset of PAL1 expression slightly preceded the accumulation of the yellow pollen pigment, which in petunia was identified as the phenylpropanoid tetrahydroxychalcone (de Vlaming and Kho, 1976).

The PAL1 promoter apparently confers a different pattern of expression in *Arabidopsis* flowers than the bean PAL2 promoter in tobacco flowers (Liang et al., 1989b). Thus, PAL2 expression, measured fluorimetrically, was high in petals but was very low in sepals. Another striking difference in tissue-specific expression is that the bean PAL2 promoter confers strong expression in tobacco root tips and shoot apical meristems (Liang et al., 1989b), whereas the *Arabidopsis* PAL1 promoter is silent in these tissues. The differences in floral expression may reflect fundamental differences in the underlying signal pathways between *Arabidopsis* and tobacco, rather than functional differences between the *Arabidopsis* PAL1 and bean PAL2 promoters, because the bean CHS15 promoter, which is strongly expressed in bean and transgenic tobacco flowers, is not active in petals of transgenic *Arabidopsis* (J. Kooter, unpublished observation). However, the differences between bean PAL2 and *Arabidopsis* PAL1 expression in apical meristems may reflect intrinsic properties of these promoters underlying the differential regulation of members of the PAL gene family.

**Induction by Wounding, HgCl	extsubscript{2}, and Light**

PAL is induced in many plant species by various stress-related stimuli including wounding, heavy-metal stress, and light. In bean and parsley, this induction has been shown to be due to transcriptional activation of the respective genes (Lawton and Lamb, 1987; Lois et al., 1989). Surprisingly, we were unable to detect a significant increase in GUS activity in *Arabidopsis* plants transformed with the PAL1-GUS gene fusion after various treatments that are known to induce PAL in other systems. However, measurement of PAL and GUS mRNA in transgenic plants showed that both endogenous PAL genes and the gene fusion were rapidly induced in response to wounding, HgCl	extsubscript{2} stress, and light. The coordinate induction of the gene fusion and the endogenous PAL genes indicated that the PAL1 promoter contained the regulatory elements necessary for environmental induction. The RNA gel blot data (Figure 4) showed that environmental induction of PAL and GUS transcripts was transient, and, hence, in-
creases in GUS activity from transient induction of GUS mRNA might well not be detected against the basal GUS activity accumulated from continuing developmental expression of the gene fusion. The rapid induction of PAL transcripts by wounding and HgCl₂ stress is consistent with the rapid induction of PAL transcripts by treatment of Arabidopsis cell suspension cultures with the microbial elicitor α-1,4-endopolygalacturonase acid lyase (Davis and Ausubel, 1989).

Effect of 5' Deletions on Promoter Activity

To gain some information on the functional organization of the PAL1 promoter, we compared the expression of three different 5'-deleted promoter-GUS fusions with the full-length promoter construct and determined whether specific regulatory properties were changed by the deletions. Deleting the promoter from −1616 to −832 gave a 30% increase in GUS activity, suggesting that a negative cis-element might be located in this region. Although some variation in GUS activity was found among individual plants transformed with the same construct, the values in this experiment were obtained from a large number of primary transformants. Negative regulatory elements several hundred nucleotides upstream from the transcription start site have been found in a number of plant promoters including the bean CHS15 promoter (Dron et al., 1988; J. Kooter, unpublished results) and a tobacco chlorophyll a/b-binding protein promoter (Castresana et al., 1988), and may be a common feature.

By deleting the promoter from −832 to −540 and further to −290, we observed stepwise reductions in promoter activity, suggesting that at least two positive cis-elements were located in this region. Although both GUS activity and mRNA levels decreased upon deleting from −832 to −540, inducibility by wounding, stress, and light, as measured by the ratio of induced to basal level, was not significantly altered. Therefore, we conclude that elements required for PAL induction by exogenous stimuli are located downstream of position −540. Although the proximal 290 nucleotides of the promoter still retained all aspects of tissue-specific regulation, wound, stress, and light inducibility could not be demonstrated for this construct, possibly because of its very low overall level of expression. Thus, at present we do not know whether this construct has lost inducibility or whether induced GUS mRNA levels were still below the detection limit.

Comparison of the Arabidopsis PAL1 promoter with promoters from other phenylpropanoid genes identified sequence elements homologous to two "boxes" conserved among many phenylpropanoid genes (Cramer et al., 1989; Lois et al., 1989) that had been shown to display elicitor-inducible and light-inducible footprints in vivo (Lois et al., 1989). The sequence comparison in Figure 8 shows that the PAL1 promoter contains three elements (box 1) homologous to the proximal element from the parsley PAL promoter and two elements (box 2) homologous to the distal element. The sequence comparison includes homologous elements from the bean PAL2 promoter. In addition, we found a 9-bp AC-rich element in the 5'-untranslated region of PAL1 (box 3) that is also present in the bean CHS15 gene at a very similar location and coincides with an elicitor-inducible hypersensitive site in this gene (Lawton et al., 1990). The −290 promoter fragment still contains one homolog of each of the boxes 1, 2, and 3. Our data do not prove that these elements are involved in regulation of the Arabidopsis PAL1 promoter but are consistent with this hypothesis, and further functional analysis of the promoter will be necessary to establish their regulatory functions.

The isolation of the Arabidopsis PAL1 gene and characterization of the functional properties of its promoter open up two new approaches for dissection of the underlying signal pathways in addition to the direct characterization of cis-elements and cognate transcription factors. First, the effects of well-characterized Arabidopsis mutants on PAL1 promoter activity can be analyzed. For example, auxin is thought to be a major factor determining the spatial organization of the vascular system (Sachs, 1986), and, thus, expression of PAL1 in the initial stages of xylem differentiation may be affected in the axr1 mutant, which

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**Figure 8.** Conserved Sequence Elements in the Promoters of Several Phenylpropanoid Biosynthetic Genes.

Matches to the consensus sequences noted by Lois et al. (1989) (boxed areas) and matches of at least 4/6 (box 1) or 3/4 (box 2) are shown in capital letters. The positions of the sequences are given relative to the transcription start sites (see text for details). The corresponding bean sequences are from the PAL2 gene (Cramer et al., 1989).
is insensitive to auxin (Estelle and Somerville, 1987). Likewise, det1 affects many aspects of photomorphogenesis, including accumulation of flavonoid pigments, and, hence, may modulate PAL1 expression, as is the case with several other light-regulated genes (Chory et al., 1989). Finally, the ethylene-insensitive mutant ein (Bleecker et al., 1988) may impact stress activation of the PAL1 promoter. Analysis of PAL1 promoter activity in these genetic backgrounds will help delineate the terminal stages of the specific signal pathways disrupted by these mutations. In addition, transgenic Arabidopsis containing marker genes fused to the PAL1 promoter could be used to screen for novel mutants with defects in the signal pathways involved in PAL regulation. This approach is currently being tested with transgenic plants containing either the Arabidopsis alcohol dehydrogenase gene or bacterial genes for resistance to hygromycin and streptomycin under control of the full-length PAL1 promoter and, in principle, could be extended to synthetic promoters containing specific cis-elements dissected from PAL1.

METHODS

Plant Material

Arabidopsis thaliana ecotype Columbia was used in all gene transfer experiments because Columbia regenerates more efficiently than Landsberg during the transformation procedure. Seeds were surface sterilized by soaking for 1 min in 70% ethanol, then for 10 min in a 1:3:0.01 solution of bleach:H2O:Tween 20 followed by five or six rinses in sterile water. Sterilized seeds were then for 10 min in a 1:3:0.01 solution of bleach:H2O:Tween 20 supplemented with 1% sucrose. To select for transformed plants, kanamycin (50 μg/mL) was added to the medium. Sterile, grown plants were maintained in a 16-hr light/8-hr dark cycle at 22°C.

DNA and RNA Blots

DNA isolation and DNA and RNA transfer and hybridization were performed according to standard protocols (Ausubel et al., 1987). RNA was isolated according to Chomczynski and Sacchi (1987) except that the plant material was homogenized with a Brinkmann homogenizer (Brinkmann, Westbury, NY) for 30 sec at setting 5, rather than with a glass-Teflon homogenizer. Hybridization probes were labeled by random priming to a specific activity of 1 to 5 x 106 cpm/μg DNA. The genomic DNA blot was probed with a 190-bp StyI/HindIII fragment from a highly conserved part of the Arabidopsis PAL1 second exon. For RNA blot hybridization of PAL1 transcripts, a 1.2-kb BglII fragment containing part of the PAL1 coding region was used as probe, and for GUS transcripts, a 2.2-kb SmaI/EcoRI fragment from pBI101 was used as a probe.

Nucleotide Sequence Analysis

A 3.1-kb EcoRI/HindIII fragment of the genomic PAL1 clone was subcloned blunt in both orientations into the EcoRV site of pBluescript (International Biotechnologies, New Haven, CT). A series of nested deletions was generated from these clones by unidirectional exonuclease III/S1 nuclease digestion. Single-stranded DNA was sequenced by the dideoxy chain-termination method (Sanger et al., 1977).

S1 Nuclease Protection

The S1 nuclease protection assay was performed as described by Ryder et al. (1987).

PAL1-GUS Gene Fusions

The EcoRI/HindIII fragment of the genomic PAL1 clone was subcloned into the polylinker of pUC19 to yield pAP-4. Recombinant plasmids containing the chimeric PAL1 promoter-GUS fusions were generated by ligating a 1816-bp EcoRI/BglII fragment (pSO-1), an 832-bp SnaBI/BglII fragment (pSO-2), a 540-bp EcoRV/BglII fragment (pSO-3), and a 290-bp HindIII/BglII fragment (pSO-4) of pAP-4 between the SalI and the BamHI site of pBluescript (Jefferson, 1987). The EcoRI site of pAP-4 and the SalI site of pBluescript were polished with the Klenow fragment of DNA polymerase I before ligation. The binary vectors were transferred from Escherichia coli strain DH5α into Agrobacterium tumefaciens strain LBA4404 by direct DNA transfer (An, 1987).

Transformation

Arabidopsis was transformed with A. tumefaciens strain LBA4404 using the root transformation method (Valvekens et al., 1988) with one modification. Root explants were taken from 10-day-old to 15-day-old seedlings, which were grown in liquid MS medium supplemented with sucrose on a shaker (110 min−1).

Histochemical Staining

Whole plants or detached organs were fixed in 0.3% formaldehyde, 10 mM [2-morpholino]ethanesulfonic acid, pH 5.6, 0.3 M
mannitol for 45 min at room temperature with a brief initial vacuum infiltration, washed several times in 50 mM Na phosphate buffer, pH 7.0, and incubated in phosphate buffer containing 0.2 mg to 1.0 mg of X-Gluc/mL at 37°C for 3 hr to 12 hr (Jefferson, 1987).

Tissue Sectioning

Plant tissue stained for GUS activity was dehydrated in an ethanol series (10%, 30%, 50%, 70%, 90%, 100%) within 24 hr. Ethanol was then replaced by incubation in 25%, 50%, 75%, and 100% xylene in ethanol each for 2 hr. Within the next 2 days to 3 days, xylene was gradually replaced by Paraplast tissue embedding medium (Monoject Scientific, St. Louis, MO). Fifty percent to 100% (v/v) Paraplast pellets were added to the xylene submerged tissue and incubated overnight. The tissue was then heated to 45°C and Paraplast was added until no more dissolved. The tissue was subsequently brought to 57°C and the Paraplast:xylene mixture was replaced by 100% molten Paraplast. Paraplast was changed five to six times until the xylene smell was gone. The tissue Paraplast mixture was cast in molds and allowed to solidify at 4°C. Sections were cut on a microtome (Minotome Cryostat, International Equipment Co., Needham Heights, MA) and the Paraplast was removed by incubating the mounted sections in ethanol. The sections were photographed on a microscope (Diaphot-TMD, Nikon, Tokyo, Japan) using phase contrast.

Fluorimetric GUS Assay

GUS enzyme activity was determined according to Jefferson (1987) by measuring the fluorescence of methylumbelliferyl-β-D-glucuronide produced by GUS cleavage of methylumbelliferyl-β-D-glucuronide. GUS activity is expressed as nanomoles of methylumbelliferyl-β-D-glucuronide per minute per milligram of protein. Protein was determined by the method of Bradford (1976).

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