Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9284–9288, December 1989
Botany

Developmental and environmental regulation of a phenylalanine ammonia-lyase–β-glucuronidase gene fusion in transgenic tobacco plants

(floral development/lignin/meristem/photoregulation/vascular differentiation)

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Communicated by Eric E. Conn, August 21, 1989 (received for review June 28, 1989)

ABSTRACT **A 1.1-kilobase promoter fragment of the bean (*Phaseolus vulgaris* L.) phenylalanine ammonia-lyase (EC 4.3.1.5) gene PAL2 was translationally fused to the β-glucuronidase reporter gene and transferred to tobacco by *Agrobacterium tumefaciens*-mediated leaf disk transformation. The distribution of β-glucuronidase activity in these transgenic plants is very similar to that of endogenous PAL2 transcripts in bean, with very high levels in petals; marked accumulation in anthers, stigmas, roots, and shoots; and low levels in sepals, ovaries, and leaves. Histochemical analysis of the spatial pattern of β-glucuronidase activity showed that the PAL2 promoter is highly active in the shoot apical meristem, the zone of cell proliferation immediately adjacent to the root apical meristem, and in the early stages of vascular development at the inception of xylem differentiation. Wounding and light evoke specific changes in the spatial pattern of β-glucuronidase activity in stems, including induction in the epidermis. These data indicate that the PAL2 promoter transduces a complex set of developmental and environmental cues into an integrated spatial and temporal program of gene expression to regulate the synthesis of a diverse array of phenylpropanoid natural products.**

Plants elaborate from phenylalanine a wide range of natural products, based on the phenylpropane skeleton, with key functions in development and interactions with the environment (1, 2). Examples include lignin, which is a structural polymer of xylem cell walls and a stress-induced barrier to infection in peripheral tissue, flavonoid pigments and UV protectants, isoflavonoid and furanocoumarin antimicrobial phytoalexins, and wound-protectant cinnamic acid esters. Moreover, phenylpropanoid products function as signals in the rhizosphere for induction of virulence genes in *Agrobacterium tumefaciens* or nodulation genes in *Rhizobium* (3, 4) and internally as modulators of hormone transport or action (5–7) and potential regulators of phenylpropanoid biosynthetic enzyme levels (8, 9).

Phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5) catalyzes the deamination of L-phenylalanine to yield *trans*-cinnamic acid and NH_4^+, which is the first committed step in phenylpropanoid synthesis. PAL activity is highly regulated during development associated with the cell-type specific synthesis of lignin and flavonoid pigments and by an array of environmental cues associated with the synthesis of phenylpropanoid products involved in adaptation or protection (1, 2). In bean (*Phaseolus vulgaris* L.), there is a family of three or four PAL genes (10) that encode distinct polypeptide isoforms (11, 12). The corresponding transcripts exhibit markedly different patterns of accumulation, leading to the selective synthesis of functional variants of the enzyme in different biological situations.

Dissection of the molecular mechanisms underlying the selective expression of PAL genes will reveal how plants incorporate adaptive and protective responses within a flexible developmental program of natural product biosynthesis. As a first step, we have examined the properties of the promoter of the bean PAL2 gene by analysis of the expression of a PAL2–β-glucuronidase (GUS) gene fusion in transgenic tobacco. PAL2 transcripts are found at high levels during floral development in bean and also accumulate in roots and hypocotyls (10, 12). Moreover, PAL2 transcripts are induced by wounding or illumination of hypocotyls and by treatment of cell suspension cultures with fungal elicitor or glutathione.

We show here that GUS activity in transgenic tobacco plants containing the PAL2–GUS gene fusion exhibits a pattern of expression very similar to that of endogenous PAL2 transcripts in bean, indicating that this pattern is established at the transcriptional level by the action of the PAL2 promoter. Histochemical analysis of GUS activity in roots and shoots revealed that the PAL2 promoter is highly active in discrete, localized zones at the apical tips and, in more mature regions, is active specifically in vascular tissue. Wounding and light induce distinct, tissue-type-specific modifications in the spatial pattern of GUS activity, and we conclude that the PAL2 promoter is able to transduce a complex set of developmental and environmental cues into an integrated spatial and temporal program of gene expression attuned to the diverse functions of phenylpropanoid products.

MATERIALS AND METHODS

PAL2–GUS Gene Fusion. A 5.3-kilobase *Eco*RI fragment containing the complete transcription unit of the bean (*P. vulgaris* L.) cv. Canadian Wonder PAL2 gene and 1170 base pairs (bp) of the nucleotide sequence upstream of the transcription start site (10) was cloned in pSP64. The 1635-bp *Eco*RI–*Nar* I fragment at the 5′ end of the insert was subcloned into the *Sma* I site of pSP64 to give pSPP2. This plasmid was digested with *Ava* II, which cuts once 60 bp 3′ of the PAL2 translation initiation codon (10), and the *Ava* II site was filled in with Klenow fragment of DNA polymerase before digestion with *Hin*dIII. The *Hin*dIII–*Ava* II fragment was cloned into the *Hin*dIII–*Sma* I site of the GUS expres-

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Abbreviations: GUS, β-glucuronidase; PAL, phenylalanine ammonia-lyase.

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sion binary vector pBI101.3 (13) to give the PAL2–GUS gene fusion pBPG1. This construct contained 33 bp of pSP64, followed by the 1170-bp 5′ flanking sequence, the 98-bp leader sequence, and the first 60 bp of the coding sequence of PAL2 fused in-frame with the GUS coding sequence. The translational fusion was confirmed by direct double-stranded sequencing with a GUS-specific primer (14).

Transgenic Tobacco. pBPG1 was mobilized from *Escherichia coli* HB101 into *A. tumefaciens* LBA 4404 (15), and transgenic tobacco plants were generated by the leaf disk method (16). Transformed plants were selected on Murashige and Skoog medium (17) containing kanamycin at 200 μg/ml and carbenicillin or cefatoxim at 500 μg/ml. The same procedure was used to obtain transgenic plants transformed with pBI101.3, which contains a promoterless GUS gene, and pBI121, which contains a cauliflower mosaic virus 35S–GUS gene fusion (13). Tissue from transgenic plants was snap frozen in liquid air and stored at −70°C. Tissue that was wound-induced by chopping into small pieces with a razor blade was incubated in the dark at 25°C under sterile conditions on filter paper moistened with 5 mM sodium phosphate buffer (pH 5.5). In light-induction experiments, explants were removed from light-grown plants and maintained in darkness for 2 weeks. These plants, which had generated young, white, nonchlorophyllous leaves, were then transferred to continuous white light [115 microeinsteins (1 einstein = 1 mol of photons) per sec per m^2] or were maintained in darkness as controls. Transformants were grown to maturity under greenhouse conditions, and, immediately after opening, flowers were dissected into sepals, colored and noncolored parts of petals, anthers, stigmas, and ovaries. In each case, extracts were made of organs collected from five flowers of a single plant, together with samples of leaf tissue.

Nucleic Acid Isolation and Analysis. DNA was isolated from tobacco leaves by the cetyltrimethylammonium bromide procedure (18). RNA was extracted from bean and tobacco plants by phenol extraction and was isolated as described (19). Southern blots of genomic DNA from transgenic plants were probed with the *Hin*dIII–*Eco*RI fragment containing the PAL2–GUS gene fusion by using standard procedures (20).

The transcription start site of the PAL2–GUS gene in transgenic tobacco was compared with that of the endogenous bean PAL2 gene by RNase protection with pSPP21, which was derived from pSPP2 by deletion of the *Ppu*MI–*Nar* I fragment (bp 1330–1365) and religation after filling in with the Klenow fragment of DNA polymerase (12). ^{32}P-labeled RNA probes were transcribed from linearized pSPP21. The probe (10^6 cpm) was mixed with 20 μg of total cellular RNA and 50 μg of *E. coli* tRNA (RNase free) and was lyophilized. Hybridization was performed in 80% (vol/vol) formamide/0.25 M Pipes, pH 6.4/0.4 M NaCl/5 mM EDTA. The hybridization solution was incubated at 80°C for 10 min and then at 45°C for 6 hr prior to RNase digestion and analysis of protected fragments by electrophoresis in 7% polyacrylamide gels (21).

GUS Assays. GUS was assayed in tissue extracts by fluorimetric determination of the production of 4-methylumbelliferone from the corresponding glucuronide (14). Protein was determined by the Bradford method (22), and GUS is expressed as picomoles of product per min per mg of protein. Histochemical localization of GUS activity *in situ* was performed with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (13). Stem sections were cut by hand prior to enzyme incubation; flowers and root squashes were directly incubated in substrate solution. Lignin and wall-bound phenolics in stem sections were monitored by UV fluorescence microscopy, using a Nikon Diaphot TMD microscope.

RESULTS

Transformation of Tobacco with PAL2–GUS. The PAL2–GUS gene fusion was transferred to tobacco by leaf disk transformation. Plantlets were regenerated under kanamycin selection and then analyzed for GUS expression. Of 30 kanamycin-resistant transformants, 20 showed GUS activity in extracts of wounded leaf tissue. Genomic DNA from four plants selected for further study contained two to five copies of the neomycin phosphotransferase II gene for kanamycin resistance per haploid genome.

Transcription of the PAL2–GUS gene fusion was monitored in an RNase protection experiment with a labeled probe transcribed from pSPP21. This probe contains 1170 bp immediately 5′ of the transcription start site of PAL2 in bean, the 98-bp leader sequence, and 60 bp of the open reading frame. This region of the PAL2 gene is precisely the region that is retained in the PAL2–GUS translational fusion. RNA isolated from leaves of transgenic tobacco plant BPG1(6) protected the same size fragment as RNA from wound-induced bean hypocotyls (Fig. 1), indicating that the expression of the PAL2–GUS gene fusion in transgenic tobacco involves the same transcription start site as that used by the endogenous PAL2 gene in bean. Moreover, severalfold higher levels of the PAL2–GUS transcript were observed in tobacco leaf tissue that had been induced by wounding compared to unwounded leaves (Fig. 1), and there was a

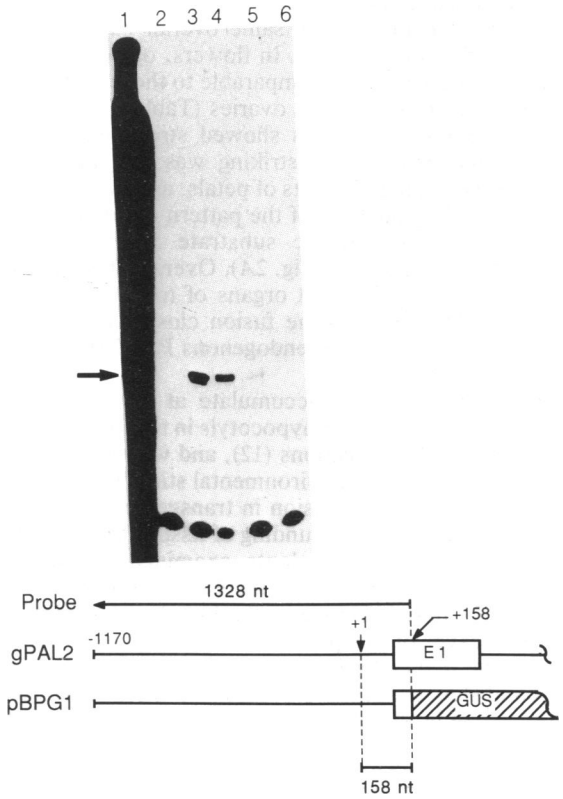

FIG. 1. RNase protection analysis of PAL2–GUS transcripts. ^{32}P-labeled RNA transcribed *in vitro* from the linearized plasmid pSPP21 was analyzed by polyacrylamide gel electrophoresis after the treatments indicated. Lane 1, no treatment; lane 2, RNase treatment in the absence of hybridizable RNA; lanes 3–6, RNase treatment following hybridization to total cellular RNA from bean hypocotyls 12 hr after excision wounding (lane 3), from leaf tissue of transgenic tobacco plant BPG(6) 12 hr after excision wounding (lane 4), from unwounded leaves of BPG(6) (lane 5), and from leaf tissue of an untransformed tobacco plant (lane 6). E1 denotes exon I of the PAL2 gene (gPAL2). The graphic depicts the region of PAL2 and pBPG1 spanned by the probe. Nucleotides (nt) are numbered relative to the *in vitro* transcription start site.

Table 1. Developmental and wound-induced expression of PAL2–GUS

Organ	Condition	Bean PAL2 mRNA, pg/μg of total RNA	GUS activity, pmol of 4-methylumbelliferone per min per mg of protein					
			PAL2–GUS				35S–GUS (pBI121)	Promoterless GUS (pBI101.3)
			pBPG1(1)	pBPG1(4)	pBPG1(5)	pBPG1(6)		
Leaf	Unwounded	0	60	60	340	70	950	0
	Wounded		660	410	450	280	890	0
Stem	Unwounded	40	1090	—	800	660	910	0
	Wounded		2300	—	—	1830	895	0
Root	Unwounded	55	490	—	900	920	960	0
	Wounded		2840	—	—	2510	920	0

corresponding wound induction of extractable GUS activity (Table 1). These data indicate that initiation of transcription in transgenic plants is due to the specific activity of the PAL2 promoter and that measurement of extractable GUS activity provides a marker for expression of the PAL2–GUS gene fusion.

Regulation of PAL2–GUS. Assay of extractable GUS activity was used to monitor the activity of the PAL2 promoter in different organs during development and in response to environmental cues. In transgenic plants BPG1(1), BPG1(4), and BPG1(6), only low levels of GUS activity were observed in leaf extracts, whereas root and stem extracts contained 7- to 18-fold higher levels of GUS activity (Table 1). Leaves of plant BPG1(5) contained somewhat higher levels of GUS activity than leaves of the other transformants, but nonetheless this plant exhibited the same overall pattern of GUS activity: leaf < stem ≈ root. In flowers, only low levels of extractable GUS activity, comparable to those in leaf tissue, were observed in sepals and ovaries (Table 2). In contrast, anthers, stigmas, and petals showed strong expression of GUS activity. Particularly striking was the high level of activity in the pigmented parts of petals, and this distribution was confirmed by analysis of the pattern of GUS activity *in situ* with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (Fig. 2*A*). Overall, the distribution of GUS activity in different organs of tobacco plants containing the PAL2–GUS gene fusion closely resembles the pattern of accumulation of endogenous PAL2 transcripts in bean (Table 1; ref. 12).

PAL2 transcripts also accumulate at levels severalfold higher than normal in bean hypocotyls in response to wounding or dark-to-light transitions (12), and we therefore examined the effects of these environmental stimuli on expression of the PAL2–GUS gene fusion in transgenic tobacco. GUS activity was induced by wounding of tissues from vegetative organs of all transgenic plants examined (Table 1). In BPG1(5) there was a 1.3-fold increase in GUS activity in wounded leaf tissue relative to the high basal level of GUS activity in leaves of this plant. In the other plants, wounding of leaf tissue induced GUS activity 5- to 11-fold. Wounding of root and stem tissue further increased GUS activity above the high basal levels in these organs. Illumination of plants that had been maintained in darkness for 3 days likewise caused a marked increase in extractable GUS activity in both leaf and stem tissue (Table 3).

Plants transformed with a construct containing a promoterless GUS gene showed negligible levels of extractable GUS activity (Table 1). Moreover, plants transformed with plasmid pBI121, which contains a cauliflower mosaic virus 35S–GUS gene fusion, showed comparable high levels of GUS activity in roots, shoots, and leaves, and in these plants GUS activity was not induced by either wounding or illumination. Thus the PAL2–GUS gene fusion exhibits a distinct and specific pattern of developmental and environmental regulation in transgenic tobacco that closely matches the pattern of accumulation of endogenous PAL2 transcripts in bean.

Spatial Pattern of PAL2–GUS Expression. The cell and tissue specificity of PAL2–GUS expression was examined by histochemical analysis of the spatial pattern of GUS activity *in situ*. Roots showed pronounced GUS activity at the apical tip primarily in a band of tissue corresponding to the region of cell proliferation immediately behind the meristem (Fig. 2*B*). GUS activity was also observed further from the root tip, but, in these more mature regions, it was restricted to the vascular tissue.

A close association of PAL2–GUS expression with the vascular system was also observed in stems. Thus in longitudinal sections of stems, GUS activity was confined to prexylem cells, and no activity was observed in other tissues such as the epidermis, cortex, or phloem (Fig. 2 *C* and *D*). Fig. 2*K* depicts vascular organization at the shoot apex in relation to the cross sections examined for GUS activity. Lignin deposition as revealed by UV fluorescence was restricted to the mature xylem tracheary elements immediately adjacent to the prexylem cells exhibiting high levels of GUS activity (Fig. 2*E*). Expression of the gene fusion in this specific lineage could be traced back to the initial stages of vascular development at the shoot tip (Fig. 2*F*). Moreover, in this region PAL2 expression was also associated with the development of vascular connections between leaf primordia and the stem. High levels of GUS activity were also found in the actual apical meristem (Fig. 2*F*). GUS activity did not appear to be confined to a specific cell layer in this region but was distributed throughout the meristem.

Table 2. PAL2–GUS expression in floral organs

Organ	GUS activity, pmol of 4-methylumbelliferone per min per mg of protein
Sepals	40
Petals (colored part)	4500
Petals (noncolored part)	2000
Stigma	1200
Anthers	1800
Ovaries	120
Leaves	150

GUS activity was measured in extracts of organs collected from five flowers of a single BPG1(6) plant.

Table 3. Light induction of PAL2–GUS expression

Time after illumination, hr	GUS activity, pmol of 4-methylumbelliferone per min per mg of protein			
	PAL2–GUS*		35S–GUS†	
	Leaf	Stem	Leaf	Stem
0	95	410	600	415
6	535	815	—	—
12	1255	900	555	330

*Measured in plant BPG1(6).
†Measured in transgenic plants transformed with pBI121.

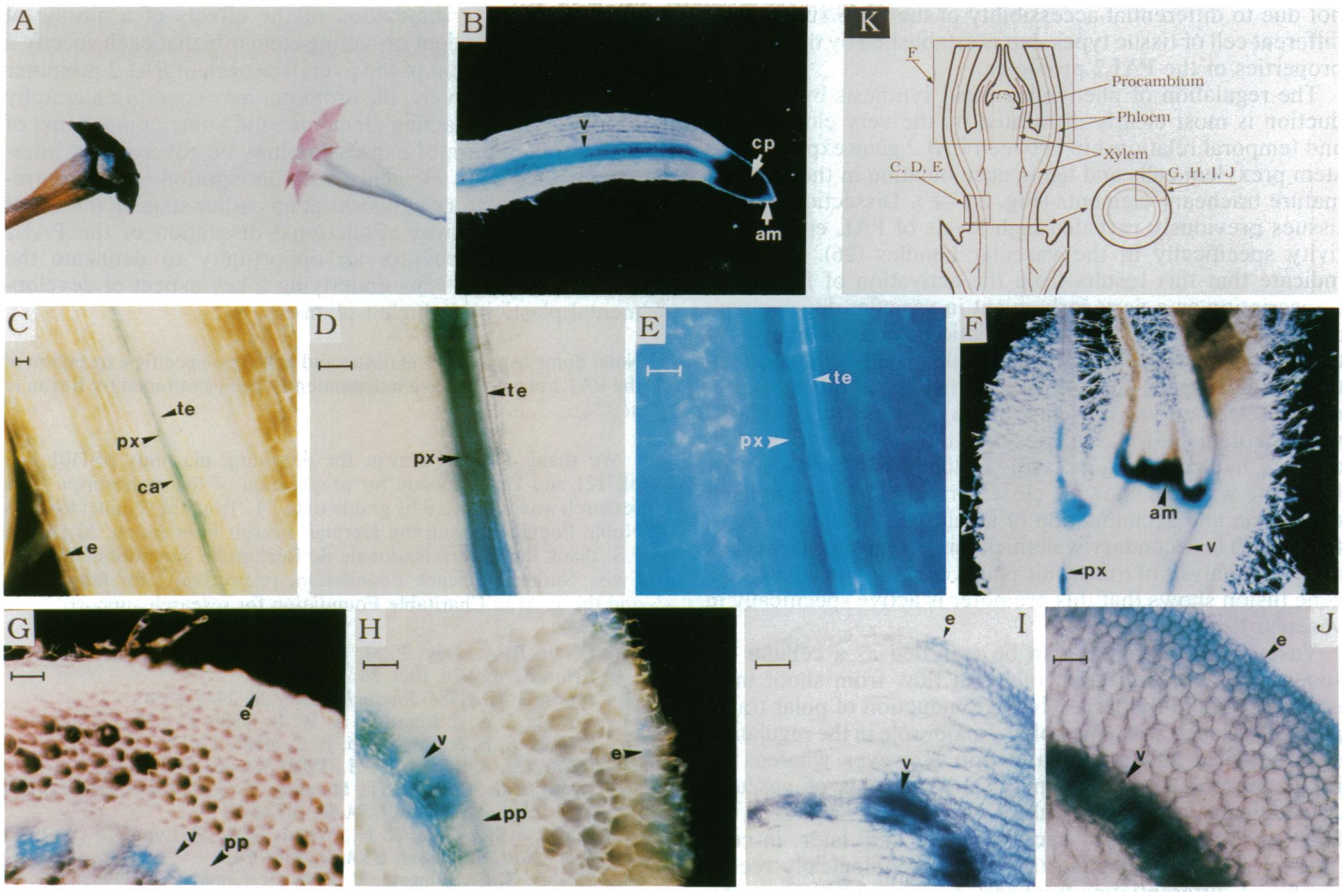

FIG. 2. *In situ* analysis of PAL2–GUS expression. Unless otherwise noted, GUS activity in transgenic plant BPG(6) material was histochemically localized with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide. (*A*) Flowers. (*Left*) Flower stained for GUS activity. (*Right*) Unstained flower from the same plant. (*B*) Root tip. (*C* and *D*) Stem longitudinal sections in the region 20 mm from the apex. (*E*) UV fluorescence of same section as in *D*. (*F*) Shoot apex. (*G*) Stem lateral section 20 mm from the apex. (*H*) Stem lateral section from same region as in *G* but 2.5 mm from the wound surface 16 hr after excision wounding. (*I*) Stem lateral section from etiolated shoot. (*J*) Same as *I* but 16 hr after illumination. (*K*) Vascular organization at the shoot apex in relation to stem cross sections for histochemical analysis. am, Apical meristem; ca, cambium; cp, cell proliferation zone; e, epidermis; pp, perivascular parenchyma; px, prexylem; te, tracheary element; v, vascular tissue. (Bars = 100 μm.)

We examined the effects of environmental stimuli on the spatial pattern of expression of the PAL2–GUS gene fusion by *in situ* analysis of changes in the distribution of GUS activity in stems following wounding or dark-to-light transitions. Wounding further increased GUS activity in prexylem cells above the level observed during normal development (Fig. 2 *G* and *H*). In addition, wounding induced GUS activity in the perivascular parenchyma and the epidermis. Irradiation with either white light (Fig. 2 *I* and *J*) or near-UV light (data not shown) induced GUS activity in the epidermal and subepidermal layers of etiolated shoots. Both forms of irradiation also further increased GUS activity in the vascular region but, unlike wounding, did not induce GUS activity specifically in the perivascular parenchyma, although a weak induction was also observed in the cortical region.

DISCUSSION

The 1.1-kilobase PAL2 promoter confers on the GUS reporter gene a specific and distinctive pattern of expression during plant development and in response to environmental stimuli. The distribution of extractable GUS activity in transgenic tobacco plants containing the PAL2–GUS gene fusion is very similar to the pattern of accumulation of endogenous PAL2 transcripts in bean (12), indicating that this pattern is established at the transcriptional level by the action of the PAL2 promoter. Nuclear run-off transcription experiments have shown that elicitor induction of PAL mRNA at the onset of the phytoalexin defense response in bean cell suspension cultures is initiated by rapid stimulation of PAL transcription (23). The present data indicate that the transcriptional activity of the PAL2 promoter is likewise a major factor in determining the pattern of accumulation of the corresponding transcript in intact plants during development and in response to wounding or light.

Demonstration of appropriate regulation of the gene fusion by measurement of extractable GUS activity then allowed us to use the histochemical stain for GUS activity *in situ* to examine the tissue and cell-type specificity of the PAL2 promoter. These experiments showed that the PAL2 promoter exhibits a complex, precise spatial pattern of activity (Fig. 2), many facets of which can be directly related to the accumulation of specific phenylpropanoid products—e.g., vascular expression associated with lignin deposition (Fig. 2 *C–E*), light induction in epidermal cells and developmental expression in the colored region of petals associated with flavonoid synthesis (Fig. 2 *A*, *I*, and *J*), and wound induction in epidermal and perivascular parenchymal cells for the synthesis of isoflavonoid phytoalexins and lignin (Fig. 2*H*) (1, 2, 24, 25). This pattern is very different from that observed with a 35S–GUS gene fusion, which expresses GUS activity in many tissues and to particularly high levels in phloem (ref. 13; data not shown). Thus the spatial pattern of GUS activity in transgenic plants containing the PAL2–GUS gene fusion is

not due to differential accessibility of the GUS substrate in different cell or tissue types, but is established by the specific properties of the PAL2 promoter.

The regulation of phenylpropanoid synthesis by PAL induction is most clearly illustrated by the very close spatial and temporal relationship between PAL2 gene expression in stem prexylem cells and lignin accumulation in the adjacent mature tracheary elements (Fig. 2 *C–E*). Dissection of stem tissues previously revealed high levels of PAL enzyme activity specifically in the vascular bundles (26). Our data indicate that this results from the activation of PAL gene transcription as a very early event in vascular development at the inception of xylem differentiation. A recent analysis of transgenic plants containing an *S*-adenosylmethionine synthetase–GUS gene fusion showed that the synthetase promoter is very active in vascular tissue, presumably correlated with the demand for *S*-adenosylmethionine for the methylation of hydroxycinnamic acids in lignin biosynthesis (27). Likewise we have shown a close correlation between lignin deposition and accumulation of a cell wall glycine-rich protein (GRP) in secondary wall thickenings of protoxylem cells (28), and analysis of transgenic plants containing a GRP–GUS gene fusion shows that this promoter is active specifically in vascular tissue (29).

Vascular development can be regarded as a cellular response to the canalization of auxin flow from shoot meristems mediated by processive autoinduction of polar transport (30). Hence auxin may play a major role in the regulation of these genes during vascularization. However, whereas the PAL2 promoter is active in the initial stages of vascular development in cells very close to the apex (Fig. 2*F*), the GRP promoter does not become active until later, in cells further from the meristem that are morphologically recognizable as differentiating tracheary elements (29). Thus, the PAL2 and GRP promoters may be induced by different components of a putative signal cascade governing lignin deposition during xylogenesis.

A surprising feature of the PAL2 promoter is the high level of activity in the zone of cell proliferation immediately adjacent to the root apical meristem and in the actual shoot apical meristem (Fig. 2*B*). The latter might reflect PAL gene expression associated with the synthesis of flavonoids for protection of meristematic cells against UV damage, but this would not account for the strong expression in root tips. Recent data indicate that flavonoids may be natural regulators of polar auxin transport (5) and that phenylpropanoid derivatives such as dehydrodiconiferyl glucosides have cytokinin-like activities (6, 7). Hence, PAL2 expression in juvenile cells may be related to the generation of morphogenetic signals, and in line with this hypothesis we have recently observed that inappropriate expression of the PAL2 gene in transgenic tobacco causes abnormal plant development (Y. Elkind, M. Mavandad, R.A.D., and C.J.L., unpublished observations).

PAL2 induction by environmental stimuli involves marked changes in the spatial pattern of promoter activity established during development. Moreover, within the same organ, wounding and light evoke distinct, tissue-type-specific changes in PAL2 expression (Fig. 2 *G–J*), which can be directly related to the corresponding protective functions: (*i*) strong induction in the epidermis and subepidermis by light and weak induction in the cortex for the generation of an effective UV barrier, and (*ii*) wound induction in the epidermis and perivascular parenchyma for the prevention of microbial ingress and systemic spread through the vasculature. Thus the PAL2 promoter is able to transduce a complex set of developmental and environmental stimuli into an integrated program of gene expression for the flexible regulation of the first committed step in the synthesis of an array of natural products with diverse functions. This integration may represent a summation of the effects of a mosaic of discrete, independent cis-acting elements that each specify a separate component of the overall pattern of PAL2 promoter activity. Alternatively, the promoter may contain a hierarchy of interactive cis-acting elements, and some components of the overall pattern of expression may be governed by interrelated cis-acting elements with integration of the corresponding regulatory networks at an earlier stage in the signal transduction pathway. Functional dissection of the PAL2 promoter now provides an opportunity to delineate the molecular mechanisms underlying a key aspect of developmental plasticity in higher plants.

Note. Some aspects of the tissue and cell-type-specific expression of the PAL2 promoter have independently been examined by Bevan *et al.* (31).

We thank Michael Bevan for providing plasmids pBI101 and pBI121 and Cindy Doane for preparation of the manuscript. This research was supported by grants to C.J.L. from the Samuel Roberts Noble Foundation and the Herman Frasch Foundation. M.D. and J.S. thank the Centre Nationale de Recherche Scientifique and the Swiss National Science Foundation, respectively, for fellowships and the J. Aron Charitable Foundation for research support.

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