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Expression patterns of an *Arabidopsis* phenylalanine ammonia-lyase promoter in transgenic tobacco

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Abstract. We have cloned and characterized one of the genes that encode phenylalanine ammonia-lyase from *Arabidopsis thaliana*. A 1·8-kb fragment containing the upstream region of this gene, *PAL1*, was transcriptionally fused to the β-glucuronidase reporter gene, and the construct was introduced into tobacco by *Agrobacterium*-mediated leaf disc transformation. The distribution of β-glucuronidase activity in the transgenic plants was analysed histochemically. The spatial pattern of activity showed that the *PAL1* promoter is very active in the vascular tissues. No expression was observed in epidermal cells, trichomes and apical meristems. Activation of the *PAL1* promoter in the early stages of seedling development remained confined to the regions adjacent to the root apical meristems; in later stages of seedling development, the pattern of expression was drastically altered. The results suggest that the *PAL1* promoter is under the control of a complex set of signal transduction pathways which lead to its activation in response to tissue-specific, wounding and developmental cues.

Keywords. *Arabidopsis thaliana*; gene structure; gene expression; phenylalanine ammonia-lyase.

1. Introduction

During their growth plants encounter a variety of stresses and, being immobile, have evolved means of overcoming the adverse conditions by switching on and off sets of genes, possibly in a coordinated fashion (Lawton and Lamb 1987; Schmelzer *et al.* 1989; Ward *et al.* 1991). Products of the phenylpropanoid pathway have been shown to play an important role in plant development and in imparting protection against environmental stresses (Cramer *et al.* 1989; Hahlbrock and Scheel 1989). These include a wide array of compounds, such as UV-protectant flavonoid pigments, antimicrobial iso-flavonoid phytoalexins, wound-protectant hydroxy-cinnamic acid esters, and structural components of cell wall like lignin and suberin. Phenylpropanoids or their derivatives are also implicated in the signal transduction mechanism leading to host recognition by *Agrobacterium* (Stachel and Zambryski 1986) and *Rhizobium* (Downie and Johnston 1986). Several genes of the pathway have also been shown to be transcriptionally activated in plant–mycorrhizal symbiotic interaction (Harrison and Dixon 1994).

The enzyme phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) catalyses the first committed reaction of the phenylpropanoid pathway, i.e. conversion of L-phenylalanine

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to *trans*-cinnamic acid. The observation that PAL levels are elevated in response to developmental cues (Jahnen and Hahlbrock 1988) and to various stimuli such as wounding, fungal infection and light (Loschke and Hadwiger 1981; Jones 1984) and the fact that it is responsible for catalysing the first determining step of the pathway emphasize its crucial regulatory role in the synthesis of many important compounds.

Owing to its important role in plant development and defense against pathogen infection, PAL has become one of the most studied enzymes of the phenylpropanoid pathway. Lamb and collaborators (Cramer *et al.* 1985) were the first to isolate a cDNA clone encoding PAL from elicitor-treated bean (*Phaseolus vulgaris*) cells. Following this, *PAL* genes have been isolated and characterized from a number of plant species, including bean (Cramer *et al.* 1989), parsley (Lois *et al.* 1989), rice (Minami *et al.* 1989), pea (Yamada *et al.* 1992), *Arabidopsis* (Ohl *et al.* 1990; Thungapathra 1990; Wanner *et al.* 1995), tomato (Lee *et al.* 1992) and *Trifolium* (Howles *et al.* 1994). Transcriptional activation of *PAL* gene(s) has been shown to be brought about by various biotic and abiotic stress factors, such as fungal infection, elicitors isolated from fungal cell walls, wounding, UV light, glutathione and ethylene, as well as developmental cues (Lawton and Lamb 1987; Dron *et al.* 1988; Liang *et al.* 1989a,b; Choudhary *et al.* 1990; Hashimoto *et al.* 1992; Yamada *et al.* 1992). The inducibility under various conditions indicates existence of multiple sequence elements responsive to different stresses in the upstream region of the *PAL* gene(s). Two sequence elements, one conferring inducibility by UV and the other by elicitor, have been delineated for the parsley *PAL1* gene by footprint analysis of *in-vivo* bound DNA–protein complexes (Lois *et al.* 1989).

At least four isoforms of active PAL with different p*I* and K_m values have been isolated from bean cell cultures (Bolwell *et al.* 1985). In all the cases studied, *PAL* genes are members of a small multigene family. The existence of several members of the *PAL* gene family and multiple isoforms of the enzyme raises the possibility that different members of the gene family encode different isoforms of PAL (Cramer *et al.* 1989; Liang *et al.* 1989a).

Two different bean *PAL* genes, *PAL2* and *PAL3*, show differential activation in response to elicitor treatment (Cramer *et al.* 1989). Shufflebottom *et al.* (1993) extended these results by demonstrating that the promoters of bean *PAL2* and *PAL3* genes direct different spatial and temporal patterns of expression of the reporter gene β-glucuronidase (*GUS*) during development and in response to environmental stimuli in transgenic potato and tobacco plants. This appears to indicate that the members of the bean *PAL* gene family are expressed and regulated differently.

Arabidopsis thaliana, in spite of its small genome size, contains three or four members of the *PAL* gene family (Ohl *et al.* 1990; Thungapathra 1990). As a first step in characterizing all the members of the *Arabidopsis PAL* multigene family, we report here characterization and expression patterns of one of the members, *PAL1* (*AtPAL1*). Inducibility of this gene has been analysed by monitoring expression of a *GUS* reporter gene transcriptionally fused to the *PAL1* promoter in transgenic tobacco plants.

2. Materials and methods

2.1 *Plant material*

Nicotiana tabacum cv. xanthi was the recipient in the transformation experiments.

Arabidopsis thaliana, ecotype Columbia-C24, was used as source of *Arabidopsis* genomic DNA. *Arabidopsis* seeds were surface sterilized in 70% ethanol for 15 seconds followed by soaking in 5% (v/v) sodium hypochlorite for 10–15 min. The seeds were then rinsed in sterile water and allowed to germinate on Petri plates containing half-strength MS medium as described (Lindsey 1992).

2.2 *Screening of genomic library*

A genomic library of *Arabidopsis thaliana* (ecotype Columbia) in lambda EMBL3 vector was screened using a cDNA clone from bean (Cramer *et al.* 1985). More than 4×10^4 pfu were screened using the probe radiolabelled either by nick translation (Rigby *et al.* 1977) or by the random primer method (Feinberg and Vogelstein 1983). The genomic library was screened according to the method described (Sambrook *et al.* 1989). Clones containing sequences complementary to *PAL* were identified following four rounds of screening and purification. DNA isolated from the positive lambda clones was mapped with respect to various restriction sites and the *PAL*-complementary DNA sequences were localized by Southern hybridization using the bean cDNA as probe.

2.3 *DNA preparation*

Phage particles were isolated from *E. coli* cell lysate by CsCl gradient centrifugation. Lambda and plasmid DNAs were isolated by procedures described (Sambrook *et al.* 1989). For isolation of plant DNA, the standard protocol (Ausubel *et al.* 1987) was modified. Tobacco leaves and whole *Arabidopsis* seedlings were ground in liquid nitrogen in a mortar. The frozen powder was suspended in an extraction buffer containing 100 mM Tris.Cl (pH 8·0), 100 mM EDTA, 250 mM NaCl, 100 µg/ml proteinase K and 1% (w/v) Sarkosyl. The suspension was incubated at 55°C for 3 h. It was then centrifuged and the supernatant was collected. Nucleic acid was precipitated by addition of 0·6 vol of isopropanol and washed once with 70% ethanol. The pellet was resuspended in TE (10 mM Tris-HCl, 1 mM EDTA) and extracted with equal volume of Tris.Cl (pH 8·0)-saturated phenol very gently, followed by extraction with equal volume of chloroform–isoamyl alcohol (23 : 2). The aqueous phase was collected following centrifugation, and the process of phenol extraction was repeated. DNA was precipitated with ethanol and washed with 70% ethanol. The DNA pellet was finally dissolved in TE.

2.4 *DNA sequencing*

DNA was sequenced by Sanger's dideoxy chain termination method (Sanger *et al.* 1977) using Sequenase (USB) and ^{35}S-labelled dATP. For sequencing using single-stranded DNA templates, various DNA fragments were subcloned into appropriate restriction sites of M13mp18/M13mp19 phage RF DNA. For sequencing with double-stranded DNA templates, the fragments were subcloned into pUC or pBluescript plasmid DNA and the DNA was isolated by the alkali lysis method as described (Sambrook *et al.* 1989). The DNA was further purified by precipitating with 13% PEG in the presence of 2 M NaCl.

2.5 *Blot hybridization analysis*

The *Arabidopsis* and tobacco DNA was digested with various restriction enzymes, fractionated on agarose gels, and blotted onto nylon membrane filters as described (Sambrook *et al.* 1989). Hybridizations to the *Arabidopsis* DNA were performed at 42°C using a labelled probe derived from the *AtPAL1* gene in the presence of formamide. The filters were washed at low (2 × SSC and 0·1% SDS at 60°C for 2 h) or high stringency (0·1 × SSC and 0·1% SDS at 60°C for 2 h). Hybridizations to the transgenic tobacco DNA were performed at 37°C using ^{32}P-labelled GUS-coding sequence (*Eco*RI–*Hin*dIII fragment from the plasmid pGUSN358→S; Clonetech) and the *PAL1* upstream fragment as described above. The filters were washed at a moderate stringency (0·4 × SSC and 0·1% SDS at 50°C for 2 h).

2.6 *Construction of chimaeric genes and transformation*

For generation of chimaeric gene constructs containing the *AtPAL1* promoter upstream of *GUSA* coding sequences, plasmid DNA containing the 4·8-kb *Eco*RI fragment shown in figure 1 was digested with *Eco*RI and end-filled using Klenow enzyme. The DNA was subsequently digested with *Bgl*II and the 1·8-kb fragment, which contains the upstream region, was isolated. The *Bgl*II site is 72 bp upstream of the transcription start site. This fragment was cloned (see right half of figure 1) into pBI101 plasmid (Jefferson *et al.* 1987). The resulting construct contained the *PAL1* promoter upstream of the *GUSA* coding sequence in the correct orientation (figure 1, bottom). The chimaeric gene construct was transferred into *Agrobacterium tumefaciens* strain LBA4404 by the freeze–thaw method (Gelvin *et al.* 1990). Healthy leaves from aseptically grown young tobacco plants were harvested and cut into small squares to provide wounded edges. The leaf pieces were dipped in overnight-grown *Agrobacterium* culture for 5 min and placed upside down on plates containing MS medium supplemented with 1·0 μg/ml 6-benzylaminopurine (BAP), 0·1 μg/ml 1-naphthaleneacetic acid (NAA) and 500 μg/ml carbenicillin for a period of two days. They were then transferred onto plates containing 1·0 μg/ml BAP, 0·1 μg/ml NAA, 500 μg/ml carbenicillin and 150 μg/ml kanamycin, and subsequently to plates containing 1·0 μg/ml BAP, 0·1 μg/ml NAA, 500 μg/ml carbenicillin and 300 μg/ml kanamycin as described (Gelvin *et al.* 1990).

2.7 *Fluorimetric GUS assay*

GUS enzyme activity was determined according to the procedure of Jefferson (1987) by measuring the fluorescence emitted by 4-methylumbelliferone, a GUS cleavage product from methyl-4-umbelliferyl-β-D-glucuronide. Activity is expressed as nanomoles of 4-methylumbelliferone produced per min per mg protein. Protein was estimated by the Bradford (1976) method.

2.8 *Histochemical staining*

Sections of plant material were cut using a sharp razor blade, and infiltrated with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) staining solution containing

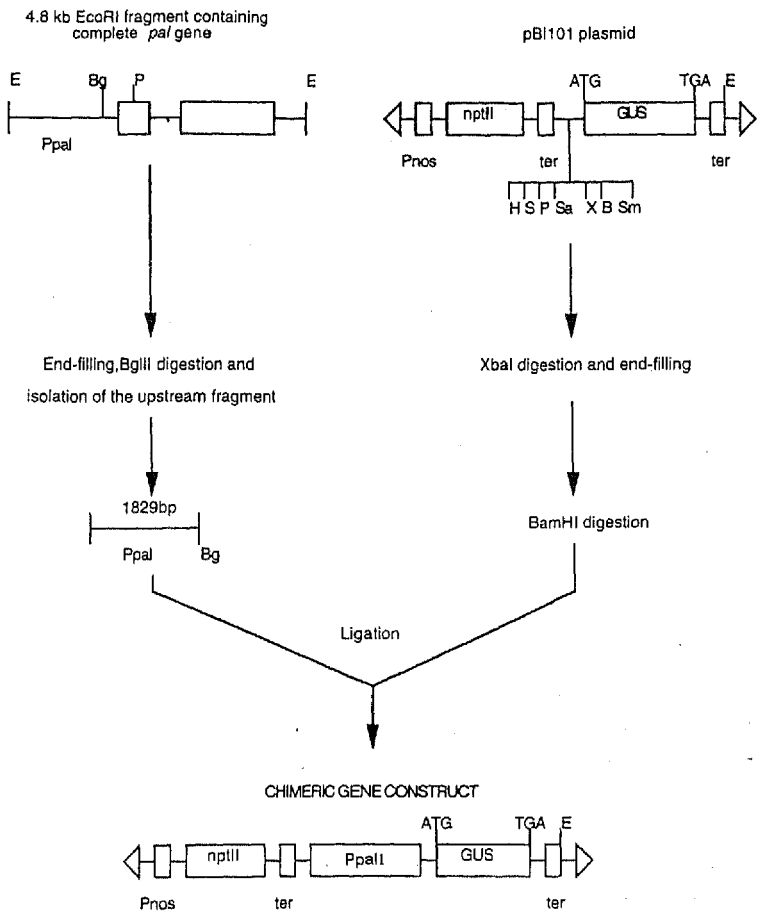

Figure 1. Preparation of the chimaeric gene construct. The *PAL1* promoter of *Arabidopsis thaliana* was cloned into binary vector pBI101 in the right orientation upstream of the *GUS* reporter gene. The abbreviations used are; E, *Eco*RI; Bg, *Bgl*II; P, *Pst*I; H, *Hin*dIII; S, *Sph*I; Sa, *Sal*I; X, *Xba*I; B, *Bam*HI; Sm, *Sma*I; PpalI, *AtPAL1* promoter; Pnos, *nos* promoter; nptII, neomycin phosphotransferase coding region; ter, *nos* terminator; GUS, β-glucuronidase coding region.

1 mg/ml X-gluc in 100 mM phosphate buffer (pH 7·0), and the stained sections were incubated for 12 to 20 h at 37°C (Jefferson 1987). Seedlings were immersed in the X-gluc solution and incubated as described above. Chlorophyll was removed by passing the tissue through several changes of 70% ethanol. After staining the material was transferred to 30% glycerol solution in phosphate buffer (pH 7·0).

3. Results

3.1 *Gene structure and nucleotide sequence*

Positive clones identified after four rounds of screening of the lambda EMBL3

genomic library of *Arabidopsis thaliana* could be grouped into three categories on the basis of restriction pattern. A 4·8-kb *Eco*RI fragment of the lambda clone gAtPAL1 containing the complete *PAL1* gene was subcloned into pUC9 plasmid and was used for this study. The clone was mapped with respect to sites for various restriction enzymes. The 4·8-kb *Eco*RI fragment has a single *Pst*I site that generates 2-kb and 2·8-kb fragments. The identity of the clone containing the *PAL* gene sequence was confirmed by comparing the nucleotide sequence of the clone with that of *PAL* cDNA from bean (Cramer *et al.* 1985) and *PAL* genes of rice (Minami *et al.* 1989). The *Arabidopsis PAL1* gene was sequenced and the DNA sequence has been deposited in the EMBL, GenBank and DDBJ nucleotide databases under accession number X84728. The structure of the gene as deduced from the sequence data reveals two exons and a single intron of 451 bp. The 4·8-kb *Eco*RI fragment also includes 1·8 kb of the upstream region and ~350 bp of the 3′ downstream region.

3.2 *Arabidopsis PAL1 gene is a member of a multigene family*

To assess the number of *PAL* genes in the *Arabidopsis* genome, plant DNA was digested with various restriction enzymes and Southern blots were probed with the 2·8-kb *Pst*I–*Eco*RI fragment which contains most of the *PAL1* coding region. The numbers of bands observed in the Southern hybridization experiments (figure 2) even at high stringency were more than those expected on the basis of the restriction pattern of the gAtPAL1 clone. Lower stringency reveals a few more bands, some strong and others faint. The *Eco*RI digest produces two strong and at least one faint band. The prominent band appears to correspond to the 4·8-kb *Eco*RI fragment subcloned from the lambda clone. The results indicate the presence of at least three members of the *PAL* gene family in the *Arabidopsis* genome. Differences in the intensity of hybridization signals possibly reflect some degree of sequence divergence among various members of the gene family.

3.3 *Transgenic tobacco plants with AtPAL1–GUS*

Fifty different transformed plants were selected on the basis of degree of *GUS* expression. Transformants (T_0) showing high *GUS* expression were selected for this study. To determine if more than one copy of the chimaeric gene construct was integrated, plant DNA was isolated and digested with *Eco*RI, *Bam*HI, or *Eco*RI and *Hin*dIII, and hybridized with *GUS* and *PAL* probes. The construct does not contain any internal *Bam*HI site but contains unique *Hin*dIII and *Eco*RI sites (see figure 3). The *Eco*RI site is present near the T-DNA left border. On the basis of the restriction map of the construct, an intact integrated copy of the construct should produce fragment(s) of at least 5·5 kb in the *Eco*RI and *Bam*HI digests, and a 4·1-kb fragment in the *Eco*RI–*Hin*dIII double digest. The autoradiogram (figure 3) shows a number of bands of different sizes, suggesting more than one site of integration in the tobacco genome. Integrity of the internal region (Ppal1–GUS) of at least some of the constructs in the transgenic plants is confirmed by the presence of the expected strong 4·1-kb band in the *Eco*RI–*Hin*dIII double digest. The double digest also reveals a larger and a smaller fragment.

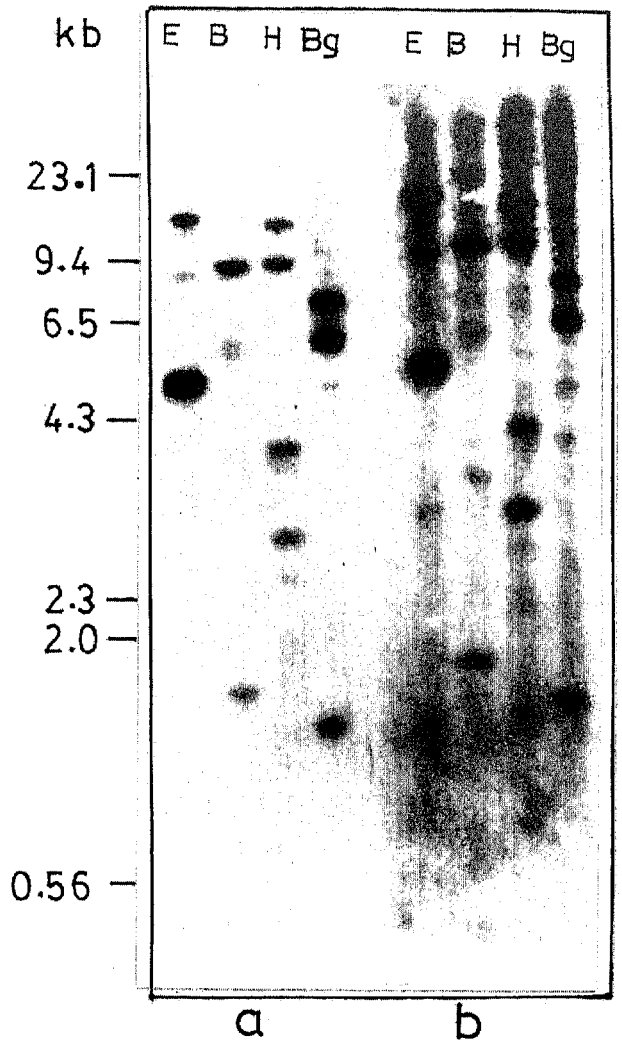

Figure 2. Genomic blots of *Arabidopsis thaliana* DNA digested with *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H) or *Bgl*II (Bg) and probed with the *Pst*I–*Eco*RI fragment containing part of the first exon and the complete second exon of the *PAL1* gene at (a) high and (b) low stringency. The strongly hybridizing band in the *Eco*RI digests probably represents the 4·8-kb *Eco*RI fragment isolated from the lambda clone.

3.4 *Spatial pattern of PAL1 promoter-directed GUS expression*

Fluorimetric assay of GUS activity was done to analyse the pattern of expression of the *AtPAL1–GUS* construct. Tissue from T_1 plants was homogenized in GUS extraction buffer and fluorescence of the extracts was monitored. The experiments revealed highest *GUS* expression in roots (figure 4). Expression in roots was three- to four-fold higher than in shoots and leaves and two-fold higher than in whole

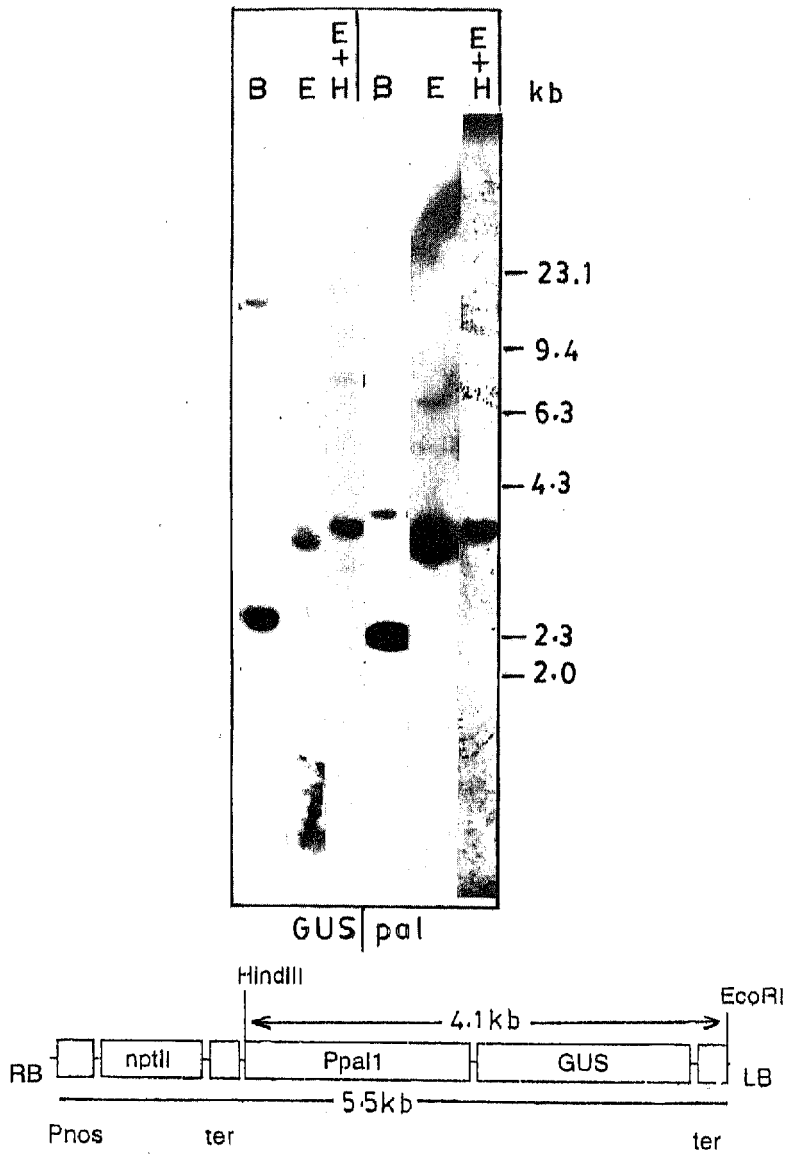

Figure 3. Genomic blots of DNA isolated from transgenic tobacco plants. DNA was digested with *Bam*HI (B), *Eco*RI (E), or *Eco*RI and *Hin*dIII (E + H) and probed with *GUS* coding or *PAL* promoter sequences. The restriction map is shown below the autoradiogram. The gene construct contains *Hin*dIII and *Eco*RI sites but does not contain any internal *Bam*HI site as indicated in the map. Thus the *Bam*HI and *Eco*RI digests are expected to produce a fragment of at least 5·5 kb from the intact gene construct. Similarly the *Eco*RI–*Hin*dIII double digest should produce a fragment of approximately 4·1 kb.

plants. Similar results were obtained by slot blot analysis of *GUS*-specific transcript (data not shown).

Transformants (T_0) showing high *GUS* expression and the kanamycin-resistant progeny of these plants were assayed for *GUS* expression using the chromogenic

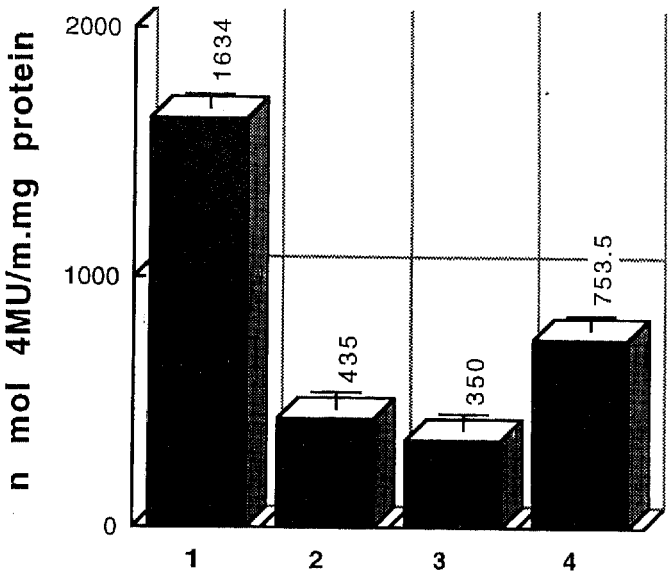

Figure 4. Fluorimetric assay of organ-specific expression of the *PAL1–GUS* construct in organs of 60-day-old plants. Fluorescence was converted to nanomoles of 4-methyl-umbelliferone produced per min per mg protein. The columns represent (1) roots, (2) shoots, (3) leaves and (4) whole plants.

substrate X-gluc (Jefferson *et al.* 1987). Independent transformants exhibited similar patterns of expression though they showed different levels of expression ranging from high to low. Emerging shoots of transformed plants show high degree of expression confined to leaf veins and midribs (figure 5a). A similar pattern of staining is observed in mature leaves (figure 5,c–e). Transverse sections of stems and petioles reveal expression confined to vascular-bundle regions (figure 5,g&j). At higher magnification (figure 5,h–k), the expression appears to be confined to xylem. *GUS* expression appears to be initiated in the parenchymatous cells that act as primordia for the xylem elements, and as the cells differentiate, possibly getting lignified, the intensity of staining increases (figure 5,i&k). The youngest vessels closest to the vascular cambium show mild expression while the older ones towards the central pith region stain intensely. The expression is mainly confined to xylem rays and associated parenchymatous cells. In thicker sections, mild expression in the outer phloem ray cells can also be seen (figure 5i). No *GUS* expression was visible in epidermal cells in transverse sections of stems and petioles and in trichomes (figure 5,j&l) as was seen in the case of expression driven by the bean *PAL2* promoter (Bevan *et al.* 1989; Shufflebottom *et al.* 1993). Thick longitudinal sections of the growing apex of the plants do not show much staining except in the developing vascular bundles at the tip (figure 5,m&n). Expression in roots was also confined to xylem.

3.5 *Activation of PAL1 promoter in response to developmental cues and wounding*

Seeds of transgenic plants were germinated on MS medium containing kanamycin

and seedlings at different stages of development were stained for GUS activity by immersing in X-gluc. Various stages of development exhibiting a changing pattern of *GUS* expression are shown in figure 6,a–f. In germinating seeds, GUS staining is confined to the region surrounding the emerging radicle (figure 6a). As the plumule and radicle elongate and the cotyledonary leaves expand, GUS activity is observed in the root-tip region (figure 6b). This expression near the root tip extends

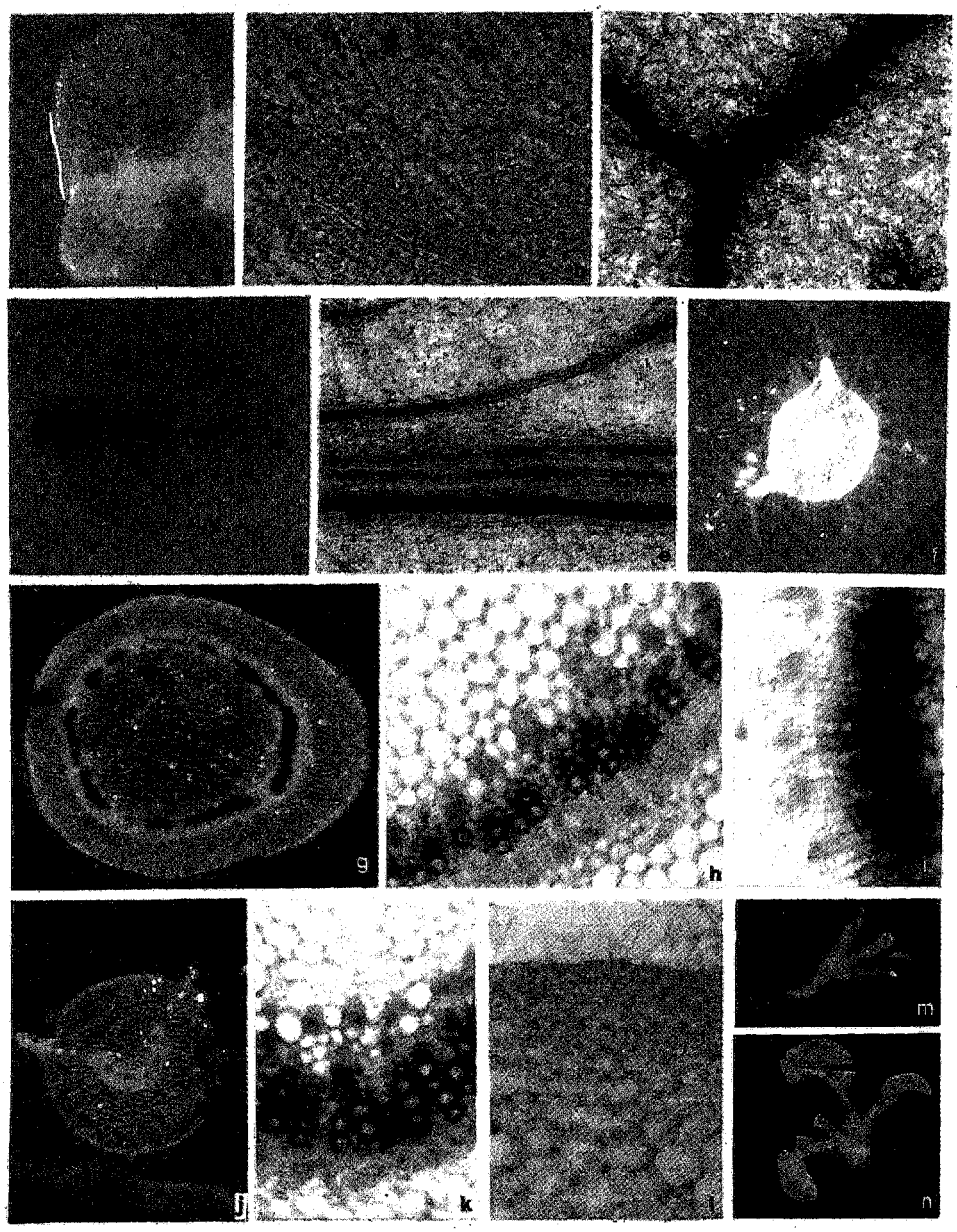

Figure 5.

proximally as the seedlings develop (figure 6,c&d). The pattern of *GUS* expression shows considerable change as the third leaf expands around the 10th day after germination. At this stage intense GUS staining is present in leaves and the lower regions of root, and is confined to the vascular region in the remaining portion of the root (figure 6e). As the seedlings grow further, the GUS staining appears to move further up along the root and only a small section in the middle of the root is not stained (figure 6f). In 20- and 35-day-old seedlings, intense staining is observed in the lower part of the root, vascular region of stem, and leaves, and no staining is observed in the root-tip region (figure 6,g&h). Thus the pattern of *GUS* expression driven by the *PAL1* promoter in transgenic tobacco seedlings undergoes a dramatic shift from very localized expression up to 8–10 days to extensive expression in the vascular tissues of root, stem and leaves of older seedlings. Although the degree of expression among different transformants was variable, the general spatial pattern of expression was similar.

To study the effect of wounding on *PAL1* promoter, leaves of a 20-day-old seedling were excised and wounded with a paper punch. The leaves were immediately immersed in X-gluc solution. Figure 6i shows an injured leaf with intense staining around the wounded regions. Incubation of the excised leaf in X-gluc solution also revealed intense staining in the vascular bundles surrounding the point at which the leaf was excised, while the mesophyll region did not stain. In both cases, the effect on expression of *GUS* is highly localized. Similar staining of a leaf not subjected to wounding produced a picture like the one in figure 5d.

4. Discussion

We have described cloning and characterization of the *Arabidopsis PAL1* gene using a heterologous *PAL* cDNA probe from bean and pattern of activation of the *Arabidopsis PAL1* promoter in transgenic tobacco. The Southern hybridization experiments with *Arabidopsis* DNA digested with different restriction enzymes and a probe containing the major part of *PAL1* coding sequence produced several bands;

Figure 5. Histochemical localization of GUS activity in various tissues. (a) Emerging shoot initial of transformed tobacco showing intense *GUS* expression confined to leaf midribs and veins. (b) Leaf of untransformed plant (16 × magnification). (c) Leaf of transformed plant (16 ×) showing intense GUS staining in vascular bundles. (d) A complete leaf of a seedling (6 ×) showing intense GUS staining in the midrib, veins and veinlets. (e) Higher magnification (16 ×) of the leaf shown in (d). (f) Transverse section of petiole of untransformed plant. (g) Transverse section of stem of transformed plant showing intense GUS staining in the vascular bundles and no expression in the epidermis. (h) Higher magnification (25 ×) of the transverse section in (g) showing *GUS* expression confined to xylem elements in the vascular tissue. (i) Thick transverse section of stem at higher magnification (25 ×) showing mild *GUS* expression in the outer phloem ray cells. (j) Transverse section of petiole of transformed plant showing intense *GUS* expression in vascular bundles; no expression is seen either in the epidermal cells or in trichomes. (k) Higher magnification (16 ×) of (j) showing intense expression associated with the xylem elements. (l) Transverse section of stem showing cells of epidermis and trichomes at higher magnification (25 ×) suggesting no activation of the *AtPAL1* promoter in these cells. (m & n) Thick section of stem apex and axillary bud regions showing no expression either at the growing apex or in axillary bud.

lowering the stringency revealed a couple of more bands. The results suggest that the *PAL1* gene belongs to a small multigene family. Isolation of two other different *PAL* genomic clones (data not shown) reinforces this conclusion. Such a conclusion has also been reached by Ohl *et al.* (1990) and recently by Wanner *et al.* (1995). The *PAL1* of this study corresponds to the *PAL1* isolated by Ohl *et al.* (1990) and Wanner *et al.* (1995). Variation in the intensity of hybridization signal may reflect a degree of sequence divergence among various members of the gene family.

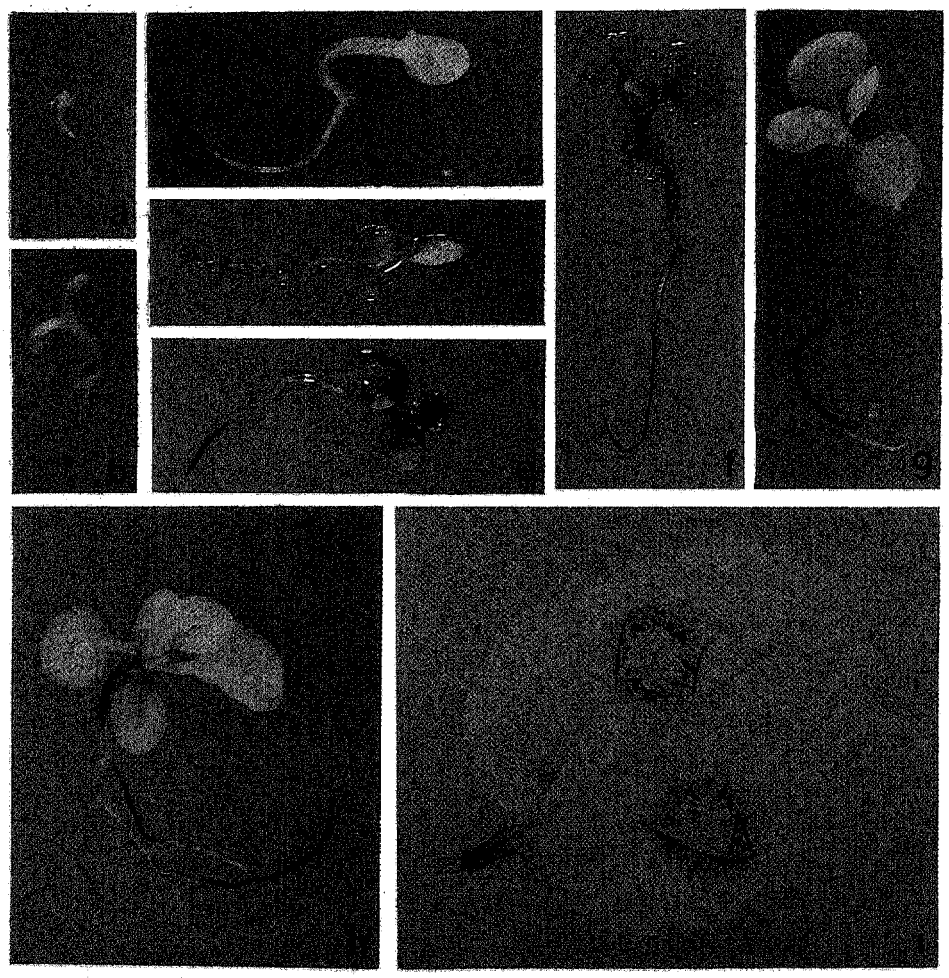

Figure 6. Histochemical localization of developmental and wound-induced *GUS* expression. (a) Germinating seed showing *GUS* expression surrounding the region through which the radicle comes out of the seed coat, possibly induced in response to wound. (b) Three-day-old seedling showing intense *GUS* expression in the root-tip region. (c) Six-day-old seedling showing *GUS* expression at the root-tip region and also in the upper part of the root. (d) Eight-day-old seedling. (e) Twelve-day-old seedling showing a dramatically altered pattern of expression. (f–h) 15, 20- and 35-day-old plants respectively with intense staining in the root and shoot and lower level of *GUS* expression in leaves. (i) Localized *GUS* expression in response to wounding.

In various other plant species, i.e. bean (Cramer *et al.* 1989), parsley (Lois *et al.* 1989), rice (Minami *et al.* 1989), tomato (Lee *et al.* 1992) and pea (Yamada *et al.* 1992), PAL enzyme has been shown to be encoded by a small multigene family. The presence of a *PAL* multigene family in *Arabidopsis* in spite of its smaller genome size probably reflects a different functional role for each member of the gene family. Four isoforms of PAL, with identical molecular weights but different p*I* and K_m values, have been isolated from bean cell cultures exposed to elicitors released from fungal cell walls (Bolwell *et al.* 1985). This suggests the possibility of various members being differentially regulated either in a tissue-specific manner or in response to stress conditions. Such a picture emerges in the case of bean *PAL2* and *PAL3* genes which have been shown to be differentially regulated (Shufflebottom *et al.* 1993). In the light of these results the basic objective of this study was to monitor the pattern of expression of the *Arabidopsis PAL1* promoter as a first step towards assigning a precise role to each member of the *PAL* gene family in terms of response to developmental and environmental cues.

To examine the spatial pattern of expression and stress inducibility of *AtPAL1* promoter, the upstream region was transcriptionally fused to the *GUS* coding sequence and these constructs were studied in transgenic tobacco. The Southern blots of DNA isolated from transgenic plants that showed high level of *GUS* expression indicate more than one site of integration of the construct, besides integration of full-length construct. Some small fragments hybridizing to both *PAL* and *GUS* probes are also present. The copy number of the smaller fragments appears to be several-fold higher than that of the larger ones. The presence of fragments smaller than expected in the *Eco*RI and *Bam*HI digests may be due either to integration of a truncated copy of the construct or to sequence rearrangements following integration. The higher copy number of small fragments may result either from amplification of the sequences or from integration of a truncated copy of the construct in tandem. Amplification of sequences is known to occur in plant cell suspension cultures grown progressively in higher concentrations of kanamycin (Jones *et al.* 1994). However, if the higher copy number in the present study is a consequence of gene amplification, then this would be an example of amplification in a growing plant rather than in cell suspension culture.

The observations on organ-specific expression of *GUS* reveal highest activity of *AtPAL1* in roots. Similar results have been obtained by Ohl *et al.* (1990), although the ratios of expression between root and shoot, and root and leaves are higher in the present study. These differences may be due to differences in plant background. The basic patterns of organ-specific expression of *PAL* or *PAL* promoter-directed *GUS* transcript, i.e. highest in root and lowest in leaves, in various plant systems (Liang *et al.* 1989a; Lois *et al.* 1989; Ohl *et al.* 1990; Yamada *et al.* 1992) are similar. This uniformity suggests that the *PAL* promoter is regulated by organ-specific factors that are similar across plant genera.

The GUS reporter system has also permitted analysis of spatial and developmental patterns of *AtPAL1* activation at cell and tissue levels. The *AtPAL1* promoter in transgenic tobacco is active in xylem elements. This is consistent with the role of PAL in catalysing the first step in the pathway leading to the synthesis of lignins. Activity of *PAL1* promoter in epidermal cells of leaves and stem and in trichomes is normally associated with transcription of *PAL1* in response to light (Bevan *et al.* 1989; Liang *et al.* 1989b;. Shufflebottom *et al.* 1993). Surprisingly, our results

show that the *AtPAL1* promoter is not active in epidermal cells of leaves and stem and in trichomes of transgenic tobacco, whereas the bean *PAL2* and *PAL3* promoters have been shown to be active in epidermal cells of stem and in leaf trichomes in tobacco and potato plants. These subtle differences in the expression pattern between bean and *Arabidopsis PAL* promoters in transgenic tobacco could be due to presence/absence of specific sequence elements or because the transcription factors present in tobacco are not able to recognize the *Arabidopsis PAL* promoter sequences. The latter possibility may be ruled out as the generalized tissue-specific expression pattern in transgenic plants is conserved between bean and *Arabidopsis PAL* promoters. This implies that specific sequence elements may be absent in the *Arabidopsis PAL1* promoter. Thus silencing of *Arabidopsis PAL* promoter in epidermis and trichomes might reflect functional differences between *AtPAL1* and bean *PAL* promoters rather than differences in the plant background in terms of *trans*-acting proteins.

We did not observe activity of *AtPAL1* in shoot apical meristems of tobacco. This was also the case in a homologous transgenic system in *Arabidopsis* (Ohl *et al.* 1990). The bean *PAL2* gene promoter was shown to be highly active in apical meristems (Bevan *et al.* 1989; Liang *et al.* 1989b; Shufflebottom *et al.* 1993). The bean *PAL3* promoter was also shown to be active in these tissues though less than bean *PAL2* (Shufflebottom *et al.* 1993). This observation raises the possibility that *PAL1* gene of *Arabidopsis* may not be directly involved in initiation of development and differentiation, in contrast to bean *PAL2* and *PAL3* (Leyva *et al.* 1992; Shufflebottom *et al.* 1993). This proposal derives support from the pattern of expression of the *AtPAL1* promoter in developing seedlings. *GUS* expression surrounding the radicle emerging out of the seed coat may be due to a wound response of *AtPAL1*. Activity of the *AtPAL1* promoter in young tobacco seedlings is confined to certain parts of the root and the pattern changes only after 10 days of seedling growth. This is in contrast to the activity of the promoter in transgenic *Arabidopsis* (Ohl *et al.* 1990). It is unclear whether these differences in pattern of expression are due to fundamental differences in the underlying signal transduction pathways or due to germination and growth conditions. However, *AtPAL1* activity after 10 days is similar in both *Arabidopsis* and tobacco backgrounds.

Our observation of strong activity of *AtPAL1* in a localized fashion in response to wounding is in agreement with the wound inducibility of *PAL* in parsley and bean (Lois *et al.* 1989; Liang *et al.* 1989b; Shufflebottom *et al.* 1993), and contrasts with the observation in *Arabidopsis* (Ohl *et al.* 1990) where no GUS activity could be detected in spite of the presence of *GUS* mRNA in response to wounding. In fact, *AtPAL1* in tobacco exhibits immediate would response. The absence of GUS activity in *Arabidopsis* transgenic plants carrying *PAL–GUS* constructs (Ohl *et al.* 1990) and the strong activity in tobacco (this study) might imply basic differences in underlying signal transduction pathways leading to the wound response. Activation of the *AtPAL1* promoter in vascular tissue appears to be dependent on light since dark-grown plants show negligible histochemically detectable GUS activity (data not shown). This observation is consistent with results of the wounding experiments of this study which were carried out in the dark to exclude the light-inducible response: no GUS activity in vascular tissues away from wounded site was observed.

The differences from results of earlier studies in relation to tissue-specific and developmental pattern of expression and wound stress lead us to hypothesize that

the *AtPALI* used in our study may have subtly different function in the *PAL* gene family of *Arabidopsis thaliana* in comparison with bean genes (Leyva *et al.* 1992; Shufflebottom *et al.* 1993).

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