Plant Physiol. (1994) 106: 877–886

Phenylalanine Ammonia-Lyase in Tobacco[1]

Molecular Cloning and Gene Expression during the Hypersensitive Reaction to Tobacco Mosaic Virus and the Response to a Fungal Elicitor

Luca Pellegrini[2], Odette Rohfritsch, Bernard Fritig, and Michel Legrand*

Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg Cedex, France

A tobacco (*Nicotiana tabacum* L. cv Samsun NN) cDNA clone coding the enzyme phenylalanine ammonia-lyase (PAL) was isolated from a cDNA library made from polyadenylated RNA purified from tobacco mosaic virus (TMV)-infected leaves. Southern analysis indicated that, in tobacco, PAL is encoded by a small family of two to four unclustered genes. Northern analysis showed that PAL genes are weakly expressed under normal physiological conditions, they are moderately and transiently expressed after wounding, but they are strongly induced during the hypersensitive reaction to TMV or to a fungal elicitor. Ribonuclease protection experiments confirmed this evidence and showed the occurrence of two highly homologous PAL messengers originating from a single gene or from two tightly co-regulated genes. By in situ RNA-RNA hybridization PAL transcripts were shown to accumulate in a narrow zone of leaf tissue surrounding necrotic lesions caused by TMV infection or treatment with the fungal elicitor. In this zone, no cell specificity was observed and there was a decreasing gradient of labeling from the edge of necrosis. Some labeling was also found in various cell types of young, healthy stems and was shown to accumulate in large amounts in the same cell types after the deposition of an elicitor solution at the top of the decapitated plant.

In plants, PAL (EC 4.3.1.5) catalyzes the conversion of L-Phe to *trans*-cinnamic acid in the first step of the phenylpropanoid pathway, which supplies the precursors for flavonoid pigments, lignin, UV protectants, and furanocoumarin phytoalexins (Hahlbrock and Grisebach, 1979; Vance et al., 1980; Grisebach, 1981; Hahlbrock and Scheel, 1989; Lewis and Yamamoto, 1990). The phenylpropanoid metabolism is activated in response to a wide array of developmental and environmental cues, and, consequently, phenylpropanoids are synthesized during the normal process of development and under various stress conditions such as UV irradiation, mechanical wounding, or pathogen attack (Lawton and Lamb, 1987; Dixon and Lamb, 1990). Phenylpropanoids are also known to act as molecular signals in recognition processes between *Agrobacterium* and *Rhizobium* and their host plants (Lynn and Chang, 1990).

Fluctuation in PAL activity has been shown to be a key element controlling the synthesis of phenylpropanoids, and, in many cases, an increase in the amount of PAL mRNA has been shown to underlie the increase of PAL activity (Edwards et al., 1985; Fritzemeier et al., 1987; Lawton and Lamb, 1987; Orr et al., 1993). In elicited cultured pine cells, for instance, the induction of PAL activity occurs concurrently with increases in the activities of lignin-specific enzymes and is followed by the deposition of apparently genuine gymnosperm lignin in the cell walls (Campbell and Ellis, 1992a, 1992b). Activity of PAL and other enzymes of the phenylpropanoid pathway is also highly stimulated in tobacco (*Nicotiana tabacum* L.) leaves reacting hypersensitively to TMV (Fritig et al., 1973; Legrand et al., 1976). Activation of phenylpropanoid metabolism is believed to result in phenolic polymer deposition, which builds up new mechanical barriers against pathogen invasion (Favali et al., 1978; Legrand, 1983).

Among tobacco enzymes involved in the phenylpropanoid metabolism, OMTs of class I and II and cinnamyl alcohol dehydrogenase have been cloned (Jaeck et al., 1992; Knight et al., 1992; Pellegrini et al., 1993). Class I OMT and cinnamyl alcohol dehydrogenase genes have been shown to be constitutively expressed in vascular tissue of healthy plants, whereas class II OMT transcripts were detected only upon infection or elicitation of tobacco tissues. Only a very partial PAL cDNA has been obtained from a tobacco cDNA library (Brederode et al., 1991), but these authors did not detect any PAL gene expression during the hypersensitive reaction of tobacco to TMV.

Here we report the characterization of a near full-length clone isolated from a cDNA library made from 48-h TMV-infected tobacco leaves. By RNA blot analysis and in situ RNA hybridization the pattern of expression of PAL genes has been investigated in healthy or TMV-infected or elicitor-treated tobacco plants. Genomic DNA blot analysis disclosed the presence of a very limited number of bands, indicating the presence of two to four genes in the tobacco genome. RNase protection assay revealed the presence of two highly

[1] This work was supported by a grant from the Commission of the European Communities (E.C.C.; ECLAIR-OPLIGE AGRE-0021). L.P. was the recipient of a Predoctoral Training Mobility Grant from the E.C.C.

[2] Present address: Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, MD 21210.

*Corresponding author; fax 33–88–61–44–42.

Abbreviations: DIG, digoxigenin; OMT, *O*-methyltransferase; PAL, phenylalanine ammonia-lyase; SSC, standard sodium citrate; TMV, tobacco mosaic virus.

identical messengers originating either from differential polyadenylation of the same transcript or from the co-expression of two highly homologous genes.

MATERIALS AND METHODS

Plant Material

Tobacco plants (*Nicotiana tabacum* L. cv Samsun NN) were grown in a greenhouse under controlled conditions. Thirty-day-old plants were infected by rubbing fully expanded tobacco leaves with a TMV suspension (0.2 μg/mL) in water containing carborundum powder as abrasive. An 8.0 μg/mL solution of megaspermin, a fungal elicitor purified from culture medium of *Phytophthora megasperma* (Kauffmann et al., 1993), was sprayed underneath fully expanded tobacco leaves (approximately 0.8 mL/leaf). Leaves rubbed with an aqueous carborundum suspension or sprayed with water were used as wounded samples or unwounded controls, respectively. Samples were harvested at different times after infection or elicitation and frozen in liquid nitrogen. For elicitation of stems, plants were decapitated and 0.1 μg of megaspermin in 20 μL of water was applied on the stem section. For in situ hybridization, 1.5 × 0.3 cm leaf strips including infection sites identified by UV epifluorescence were cut. Each sample consisted of material collected from three plants (one leaf per plant).

RNA Extraction and mRNA Purification

Total RNA was prepared from frozen leaves (−80°C) by several successive extractions with 0.3 M Tris-HCl, pH 8, and with phenolchloroform and precipitation with 2 M LiCl and ethanol. The RNA was dissolved in water and stored at −20°C. By spectrophotometry, the yield was estimated to about 280 μg RNA g^{-1} fresh weight. The integrity of RNA was checked by electrophoresis on 1% agarose gels and ethidium bromide staining. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose (New England Biolabs) chromatography (Aviv and Leder, 1972; Sambrook et al., 1989).

cDNA Library Screening

A λZapII (Stratagene) cDNA library made from 48-h TMV-infected leaves (Pellegrini et al., 1993) was screened using a 597-bp tobacco PAL cDNA fragment (N. Favet, unpublished result). cDNA inserts were rescued in pBluescript SK(+) plasmids by R408 helper phage-mediated in vivo excision, as described by the manufacturer (Stratagene). Plasmidic DNA was purified with Qiagen columns (Qiagen, Hilden, Germany). cDNA inserts were excised by *Not*I digestion and sized on 1% agarose gels.

Nucleotide Sequence Analysis

cDNA clones were sequenced on both strands by the dideoxy chain-termination method (Sanger et al., 1977) using double-stranded plasmid templates (Sambrook et al., 1989) and T7 DNA polymerase (Pharmacia). Deleted clones were generated by exonuclease III digestion and sequenced at their extremities. Synthetic oligonucleotide primers (20 mer) were designed from the cDNA sequence previously determined and used to sequence the internal regions. The DNA sequence was analyzed using the Genetics Computer Group software (Devereux et al., 1984).

Subcloning and Probe Synthesis

Plasmid PAL.E-3′ was constructed by subcloning a 387-bp, *Bgl*II-*Not*I digest into the vector pSK(+) (Stratagene). This construct consisted in the last 183 bp of the 3′ translated region and the 204 bp of the 3′ untranslated region of PAL.E cDNA insert, including an 18-base poly(A) tail. According to standard methods (Sambrook et al., 1989) and the manufacturer's recommendation, [α-^{32}P]UTP-labeled (Amersham) or DIG-UTP-labeled (Boehringer) RNA transcripts were generated by in vitro transcription of the PAL.E-3′ plasmid in sense or antisense orientation using T3 or T7 RNA polymerase (Boehringer), respectively.

[α-^{32}P]dCTP-labeled DNA probes were generated by random oligonucleotide-primed synthesis from the gel-purified *Not*I/*Not*I insert of the PAL.E clone. The probe, purified on Sephadex G-50 columns (Pharmacia), was used at a 2 × 10^6 dpm/mL concentration in standard hybridization solution.

Genomic DNA Analysis

Nuclear genomic DNA from *N. tabacum* was purified from 5 g of young leaves according to Saghai-Maroof et al. (1984). DNA digested to completion with one or two restriction enzymes was phenol/chloroform purified, ethanol-precipitated, and resolved on 0.75% agarose (FMC BioProducts, Strand, Denmark). Depurinated gels were blotted on Hybond N membranes (Amersham) and hybridized at 42°C in standard hybridization solution containing 50 or 30% formamide for high- or moderate-stringency conditions, respectively. After 14 h of hybridization the filters were washed twice in 2× SSC, 0.1% SDS at 60°C for 15 min. Two additional washes were performed for 15 min at 60°C in 0.1× SSC, 0.1% SDS or 1× SSC, 0.1% SDS for high or moderate stringency, respectively.

RNA Blot Analysis

Ten micrograms of total RNA was resolved on formaldehyde-agarose gels and blotted on Hybond N membrane (Amersham). Prehybridization, hybridization, and washes were carried out at the most stringent conditions according to standard methods (Sambrook et al., 1989). Hybridization signals were revealed by exposing the membranes to an RX film (Fuji) at −80°C for 15 to 24 h with an intensifying screen (DuPont).

RNase Protection Analysis of PAL.E Transcripts

[α-^{32}P]UTP-labeled antisense PAL.E-3′ transcripts (2 × 10^5 cpm) were ethanol precipitated with 5 μg of total cellular RNA or 5 μg of *Escherichia coli* tRNA (RNase free, Boehringer). The pellet was rinsed with ice-cold 70% ethanol and resuspended in 20 μL of hybridization buffer (10 mM Tris-HCl, pH 7.5, 1.2 M NaCl, 5 mM EDTA). Samples were heated at 85°C for 5 min to denature the RNAs and then incubated

for 2 h at 70°C. RNaseONE digestion buffer for adenine uracil-rich sequences (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) (105 μL) and 5 units of RNaseONE (Promega) were added and samples were incubated at 20°C for 1 h. RNaseONE digestion was stopped by addition of 180 μL of stop solution (300 mM Na acetate, pH 5.5, 0.1% SDS, 100 μg/mL *E. coli* tRNA) followed by vortexing. Samples were ethanol precipitated, washed in ice-cold 70% ethanol, and resuspended in 6 μL of loading dye (80% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 0.1% SDS). Protected fragments were resolved on a polyacrylamide/7 M urea sequencing gel, fixed in a fixing solution (5% methanol, 5% acetic acid), and dried prior to autoradiography.

In Situ RNA-RNA Hybridization

Fresh tissue from TMV-infected leaves, megaspermin-elicited leaves and stems, and healthy leaves and stems was fixed in fixing solution (4% paraformaldehyde, 0.25% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.2) and embedded in Paraplast Plus (Sherwood Medical, Athy, Ireland). Six-micrometer sections were cut using a rotary microtome (Jung, Nussloch, Germany), mounted on gelatin-coated slides, and heated at 50°C overnight. After removal of Paraplast with toluene, sections were treated with proteinase K (0.2 μg/mL, Sigma) in 1 mM $CaCl_2$ solution for 6 min at 20°C and re-fixed for 30 min in fixing solution. Tissue sections were covered with 60 μL of hybridization solution (50% deionized formamide, 10% dextran sulfate, 4× SSC, 5× Denhardt's solution, 0.1% SDS, containing 600 ng of DIG-labeled sense or antisense PAL.E-3′ transcript) under a sealed coverslip. Sealed slides were incubated overnight at 50°C and washed in 0.5× SSC for 4 h at 42°C, then rinsed in the same solution at 37°C for 30 min and treated with RNase A (20 μg/mL, Sigma) for 30 min at 37°C to remove single-stranded RNA. Slides were washed again for 2 h in 0.5× SSC at 42°C and then rinsed in the same solution for 15 min at room temperature.

Immunodetection of DIG-labeled RNA transcripts was performed with alkaline phosphatase-conjugated anti-DIG antigen-binding fragments (Boehringer) following the manufacturer's recommendations. Pictures were taken using a standard bright-field Reichert microscope.

Assay of PAL Activity

PAL activity was assayed by measuring the rate of formation of labeled cinnamic acid from labeled Phe. Soluble proteins were extracted by grinding 10 g of freshly harvested leaf tissues at 4°C in 50 mL of 0.1 M borate buffer, pH 8.8, containing 5 mM mercaptoethanol in the presence of quartz sand. The mixture was filtered through a double layer of cheesecloth. The filtrate was centrifuged at 13,000 rpm for 10 min and the supernatant was used for enzymatic assays. Protein concentration was measured by the Bradford method (Bradford, 1976). PAL activity was assayed with 1 mL of 0.25 M borate buffer, pH 8.8, containing 300 μM ^{14}C-labeled Phe and with 50 or 100 μL of enzyme extracts. After 1 h of incubation at 37°C, the reaction was stopped with two drops of 9 N sulfuric acid. The labeled cinnamic acid was extracted and its radioactivity was measured by scintillation counting as described earlier (Legrand et al., 1976).

RESULTS

Isolation and Sequence Analysis of PAL cDNA Clones

A 597-bp cDNA that shared over 74% identity with the PAL gene sequences available in the EMBL data bank (N. Favet, unpublished results) was used as probe to screen a λZapII (Stratagene) cDNA library made from 48-h TMV-infected tobacco leaves (Pellegrini et al., 1993). Approximately 1.0×10^5 plaque-forming units were plated. Four rounds of screening allowed the isolation of nine positive clones. Their cDNA inserts were sized on agarose gels after excision by *Not*I digestion and were found to have different lengths, ranging from 1.7 to 2.4 kb. Only one clone, named PAL.E, harbored a cDNA insert corresponding to the expected full-length size. Therefore, PAL.E was sequenced on both strands by sequencing deleted clones obtained by exonuclease III digestion. Figure 1 shows the nucleotidic sequence of PAL.E, which is 2431 bp long with an open reading frame of 2136 bp (positions 91–2226), a 90-bp leader sequence, and a 3′ untranslated region of 204 bp, including an 18-nucleotide poly(A) tail. A putative polyadenylation signal was found 20 nucleotides before the poly(A) tail.

The analysis of the PAL.E cDNA clone predicted a polypeptide of 712 amino acids whose calculated molecular mass was 76,515 D. This value is about 5,000 D larger than the molecular mass estimated by SDS-PAGE for PAL partially purified from TMV-infected tobacco leaves (P. Geoffroy, personal communication). This result may indicate that tobacco PAL protein is rapidly proteolyzed, as it is in bean and alfalfa, where a native PAL subunit of 77 kD is inherently unstable and gives rise to shorter degradation products (Bolwell et al., 1985; Jorrin and Dixon, 1990). The predicted isoelectric point of PAL.E is 6.65.

The deduced tobacco PAL.E protein shares, at the amino acid level, a degree of similarity higher than 80% with those deduced from cDNA clones isolated from dicotyledonous species such as bean (Edwards et al., 1985), parsley (Lois et al., 1989), alfalfa (Gowri et al., 1991), potato (Joos and Hahlbrock, 1992), pea (Kawamata et al., 1992), loblolly pine (Wetten and Sederoff, 1992), and poplar (Subramaniam et al., 1993). When this paper was in process a very similar cDNA clone was isolated from tobacco cell culture (Nagai et al., 1994). Also, six potential *N*-glycosylation sites (Asn-X-Thr/Ser, underlined in Fig. 1) are conserved in PAL.E, thus suggesting the glycosylation of tobacco PAL, as has been demonstrated for potato PAL (Shaw et al., 1990).

All nine cDNA clones isolated were shown to have the same restriction map as the PAL.E clone (data not shown). These clones were sequenced for approximately 350 bp at each extremity and in some internal regions using primers designed for PAL.E cDNA sequencing. All of these nucleotide sequences matched perfectly those of PAL.E, suggesting that all of the cDNAs isolated were synthesized from the same PAL mRNA species.

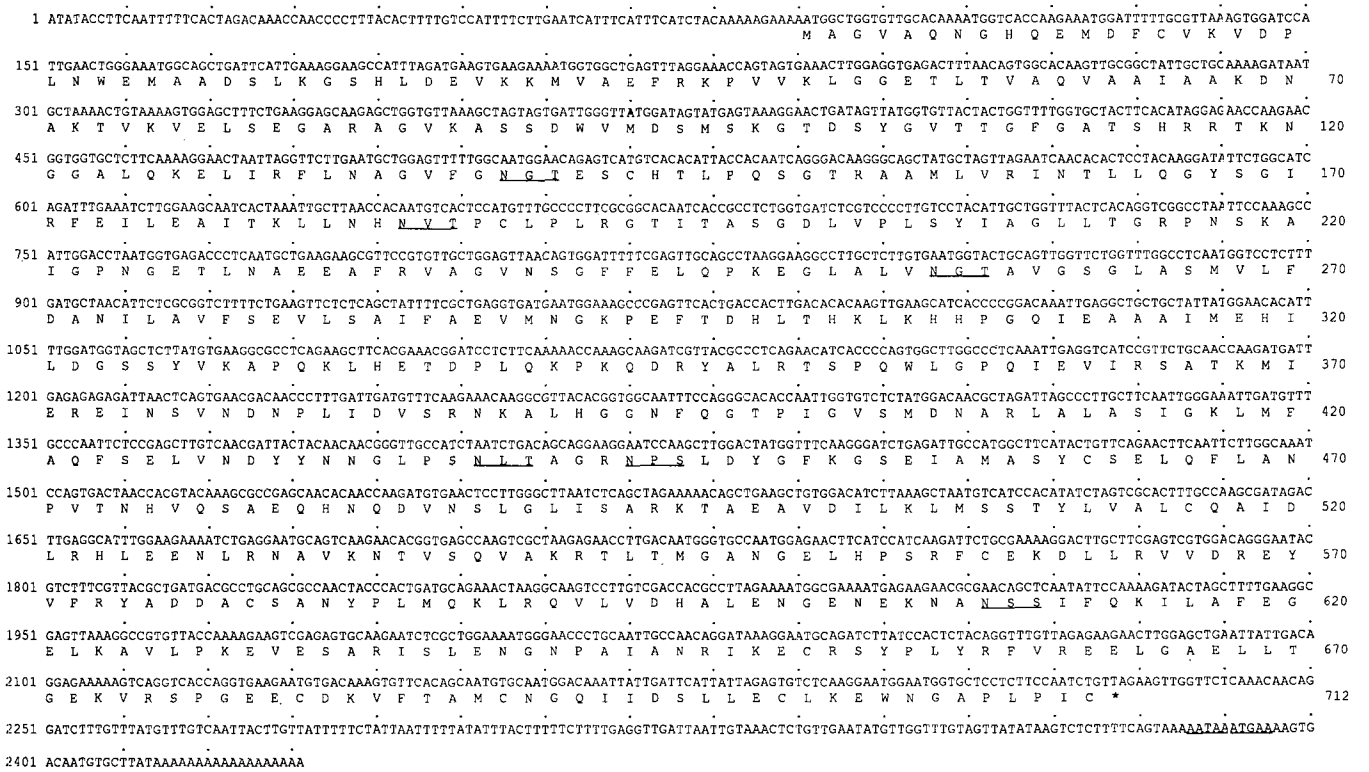

Figure 1. Nucleotide and deduced amino acid sequences of the tobacco PAL cDNA. Nucleotides are numbered from the first base of the cDNA insert. A potential polyadenylation signal and glycosylation sites are underlined. The deduced amino acid sequence is indicated below the nucleotide sequence in the single-letter code. The first Met of the open reading frame is designated as the first amino acid of the putative PAL protein. The termination codon is indicated by an asterisk.

PAL Gene Organization

Southern analysis performed at the most stringent hybridization conditions using PAL.E cDNA as a probe lighted up three to four fragments (Fig. 2). Identical results were obtained using moderate-stringency conditions (data not shown), thus making unlikely the presence of other PAL genes with high similarity to PAL.E. The number of the hybridized fragments obtained with restriction enzymes that have (*Bam*HI and *Hin*dIII) or do not have (*Eco*RI and *Eco*RV) a recognition sequence within the PAL.E cDNA indicates that the tobacco PAL gene family is composed of two to four genes.

All PAL genes studied so far contained at the same position a single intron that splits an Arg codon conserved in most PAL genes (Gowri et al., 1991). A codon for an Arg residue (Arg^{130}) is also found in PAL.E cDNA and may indicate the same type of gene organization in tobacco. The small number of hybridizing restriction fragments revealed by Southern analysis is also in accordance with this hypothesis, since it makes unlikely the occurrence of large intervening sequences.

Northern Blot Analysis

It has been reported that PAL gene expression was not stimulated in tobacco leaves by TMV infection, wounding, or UV light (Brederode et al., 1991). These data were at odds with PAL gene inducibility by various stimuli shown in many plant species and with the increased PAL activity measured in tobacco leaves reacting hypersensitively to TMV (Fritig et al., 1973; Legrand et al., 1976). Therefore, we reinvestigated PAL expression at the level of both gene expression and enzyme activity in tobacco leaves reacting hypersensitively to TMV or in leaf and stem tissues treated with a fungal elicitor or in wounded leaf tissues.

Northern analysis using the full-length PAL.E cDNA to synthesize the labeled probe revealed a single band of approximately 2.5 kb. A high level of accumulation of PAL transcripts was observed in tobacco leaves reacting hypersensitively to TMV (Fig. 3, lanes E and F). The most intense signal was detected 38 h after infection, as soon as the first lesions appeared. A strong stimulation of PAL activity was measured in the same samples. The maximum of enzyme activity was found 45 h postinoculation (Table I), in agreement with detailed kinetic studies described previously for several enzymes of the phenylpropanoid pathway (Legrand et al., 1976). Tobacco leaves and stems were also elicited with megaspermin, a fungal elicitor inducing necrosis of tobacco tissues within a few hours after treatment (Kauffmann et al., 1993). A transient, rapid, and sharp induction of PAL transcript accumulation was observed in megaspermin-treated

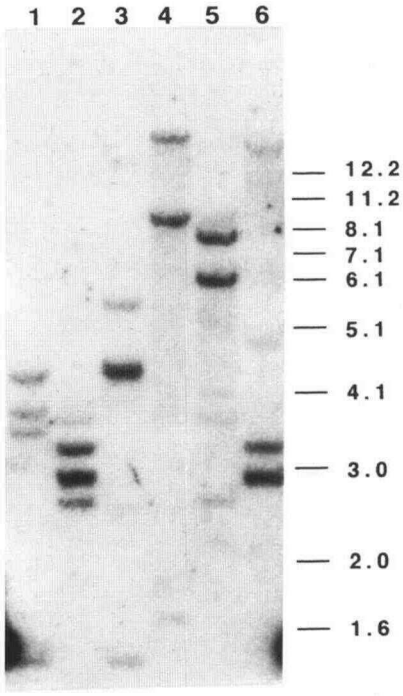

Figure 2. DNA blot analysis of tobacco genomic DNA. The full-length tobacco PAL.E cDNA was used as probe. Ten micrograms of DNA digested with *Eco*RV + *Hin*dIII (lane 1), *Bam*HI + *Eco*RI (lane 2), *Hin*dIII (lane 3), *Eco*RV (lane 4), *Eco*RI (lane 5), or *Bam*HI (lane 6) was analyzed. Molecular marker sizes are in kb.

leaves (Fig. 3, lanes L, M, N) and stems (data not shown). Signal intensity was maximum as soon as 8 h posttreatment and then decreased slowly (Fig. 3, lanes L, M, N). An increase in enzymatic activity was measured over the same period (Table I), in agreement with the fact that PAL messenger amount remained above the control level (compare lanes L, M, N to lanes G, H, I).

Wounding by carborundum treatment was found to induce a rapid and transient accumulation of PAL transcripts. However, in wounded samples (Fig. 3, lanes B, C, D) PAL transcripts accumulated much less than in TMV-infected or elicited tissues, and no significant increase in PAL activity was detected (Table I). Moreover, 45 h after wounding PAL mRNAs (Fig. 3, lane D) dropped to values close to those measured in untreated leaves.

Tobacco plants were grown for 6 weeks in a 16:8 light:dark photoperiod. Healthy, water-sprayed tobacco leaves were harvested at 8 AM, 1 PM, 6 PM, and 10 PM, corresponding to 2, 7, 12, and 16 h of light, respectively. In northern analysis, very similar amounts of PAL messenger were detected in the different samples (Fig. 3, lanes A, G, H, I). However, PAL activity measured in these samples showed a 2.5-fold higher enzymatic activity in the samples collected in the morning than in those harvested later (Table I), thus suggesting a posttranscriptional control of PAL activity during the day, but this was not investigated further. At any rate, these fluctuations in PAL activity were limited compared to the PAL levels reached after TMV infection or elicitation.

RNase Protection Experiments

RNase protection analysis was performed to investigate whether several PAL genes were expressed in response to stress. From the PAL.E-3′ construct, a labeled antisense transcript of 456 bp was generated (Fig. 4A) and used to protect PAL mRNAs from RNase degradation. The RNase protection experiments are presented in Figure 4B. A radioactive band was revealed in all samples (in the presence or absence of cellular RNAs) and corresponded to a small portion of undegraded probe that probably hybridized with the DNA template [Fig. 4, band P(456)]. Two other strong bands appeared only after hybridization of the radioactive probe with cellular RNAs extracted from TMV-infected leaves (Fig. 4, lane 4) or megaspermin-elicited leaves (Fig. 4, lane 5). In the presence of RNAs from healthy leaves faint bands were detected at the same positions, thus demonstrating that the same transcripts were present in healthy leaves but in much smaller amounts. The two induced species clearly visible in samples from tissues bearing TMV lesions or treated with the elicitor were 387 and 329 bp long, respectively.

The longest protected fragment corresponded to the 183-bp downstream region of the PAL.E open reading frame and to the 204-bp 3′ untranslated sequence of the PAL.E transcript as shown in Figure 4A. The other protected fragment is 58 bp smaller and is probably derived from a transcript homologous to PAL.E in the 3′ coding region but with a shorter or slightly divergent untranslated sequence. This situation may arise from alternate polyadenylation of the same transcript or from the expression of two highly homologous genes. In the latter case the two genes should be tightly co-regulated, as indicated by the similar intensities of the two protected bands (Fig. 4B). Another possibility would be that the small fragment arose from a partially degraded PAL.E mRNA, but this appears unlikely since only two definite species were present when the RNase protection assay was performed with RNAs from TMV-infected or elicitor-treated leaves.

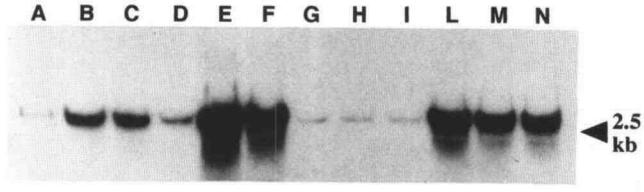

Figure 3. Northern blot analysis of PAL transcripts from various tissues. Blots were probed with the full-length PAL.E cDNA insert. Each lane was loaded with 10 μg of total RNA from untreated healthy leaves harvested at 8 AM (A); leaves rubbed with water and carborundum and harvested 8 h (B), 38 h (C), or 45 h (D) after treatment; TMV-infected leaves harvested 38 h (E) or 45 h (F) after inoculation; leaves sprayed with water and harvested at 1 PM (G), 8 PM (H), or 10 PM (I); or leaves sprayed with megaspermin solution and extracted 8 h (L), 18 h (M), or 23 h (N) after treatment. Tobacco plants from the same batch were used. The position of a 2.5-kb RNA marker is indicated.

Table I. *PAL activity in healthy, TMV-infected, and elicited tobacco leaves*

Tobacco leaves were either infected by rubbing with a 0.2 μg/mL TMV solution (controls were mock-inoculated with water) or elicited by spraying with an 8.0 μg/mL megaspermin solution (controls were sprayed with water). PAL activity of the extracts was assayed with 1 mL of 0.25 M borate buffer containing 300 μM ^{14}C-labeled Phe as substrate. Time after treatments is indicated in parentheses. Each activity value, expressed as pkat/μg protein, represents the mean value of three measurements.

Time of Sampling	Control		Sample	
	Treatment	Activity	Treatment	Activity
	Water-sprayed:		Megaspermin-sprayed:	
1 PM	(8 h)	2.7	(8 h)	3.2
6 PM	(18 h)	1.1	(18 h)	5.3
10 PM	(23 h)	1.1	(23 h)	8.0
	Mock-inoculated:		TMV-infected:	
8 AM	(38 h)	2.5	(38 h)	10.1
4 PM	(45 h)	1.6	(45 h)	12.6

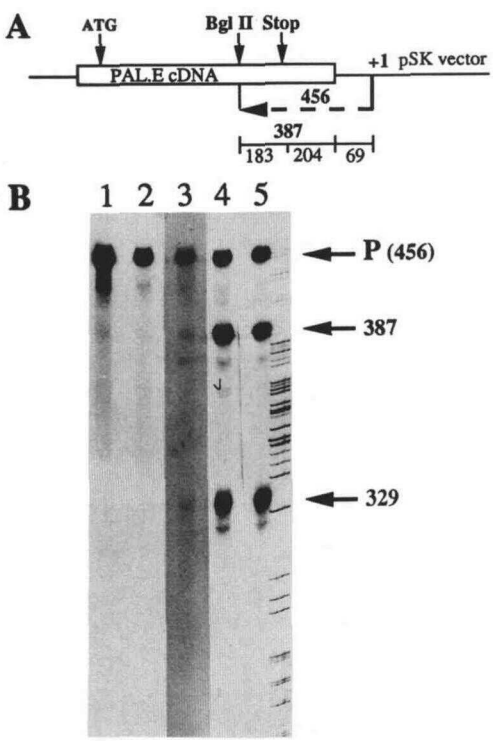

Figure 4. RNase protection analysis of tobacco PAL transcripts. The PAL.E-3′ clone was linearized by *Bgl*II digestion and ^{32}P-labeled RNA was transcribed in vitro by T7 RNA polymerase. A, The region of PAL.E and pSK(+) spanned by the probe. B, The radioactive probe was analyzed on a polyacrylamide gel without treatment (lane 1) or after RNaseONE treatment following hybridization with 5 μg of *E. coli* tRNA (lane 2) or hybridization with 10 μg of total cellular RNA from healthy tobacco leaves (lane 3), from 38-h TMV-infected leaves (lane 4), or from megaspermin-sprayed leaves harvested 8 h after treatment (lane 5). To detect the radioactive bands in lane 3 the gel was exposed about 5-fold longer than in other cases. Arrows denote protected PAL fragments, P denotes the undigested probe, and numbers refer to the size in bp.

In Situ RNA-RNA Hybridization

To define the spatial pattern of expression of PAL transcripts in tobacco leaves and stems reacting hypersensitively, serial sections of TMV-infected leaves and megaspermin-treated leaves and stems were analyzed by in situ RNA hybridization. When observed under UV light, 6-d-old lesions appear surrounded by a sharp, fluorescent ring of reacting cells about 1 to 1.5 mm in width (Fig. 5A). The fluorescence is due to the accumulation of phenylpropanoids, primarily coumarins (Fritig et al., 1972). TMV-infected leaf samples consisted of leaf strips, each containing a single 48-h TMV lesion of less than 1 mm diameter. Collected samples were quickly observed under UV light to verify the presence of only one lesion per sample.

When observed at low magnification, in situ-hybridized serial sections of TMV-infected leaves revealed different intensities of labeling. The staining intensity increases from the right to the left side of the section shown in Figure 5B, i.e. when one gets closer to the necrotic area. This appears more clearly at higher magnification: the cells that accumulate fluorescent compounds also exhibit a strong accumulation of PAL transcripts (Fig. 5F); the tissue of the intermediate area covering a few hundred microns displayed a slightly weaker signal (Fig. 5E); beyond this latter region (Fig. 5D) PAL signal intensity was low and equal to that observed in the healthy control (data not shown). However, the signal measured in these nonelicited tissues (Fig. 5D) was significantly higher than the background revealed by hybridizing the sections with a sense probe (Fig. 5C). This demonstrates a constitutive level of PAL gene expression in tobacco leaves, which is in accordance with the results of northern and RNase protection experiments and also with the enzyme activity measured in healthy leaves. It is also noteworthy that no cell-specific or tissue-specific expression of the PAL genes was observed in either the healthy or the infected tobacco leaves.

Megaspermin spray treatment led to elicitation of the hypersensitive response in smaller areas than those observed 48 h after TMV inoculation, but the same distribution of PAL transcripts was observed (data not shown).

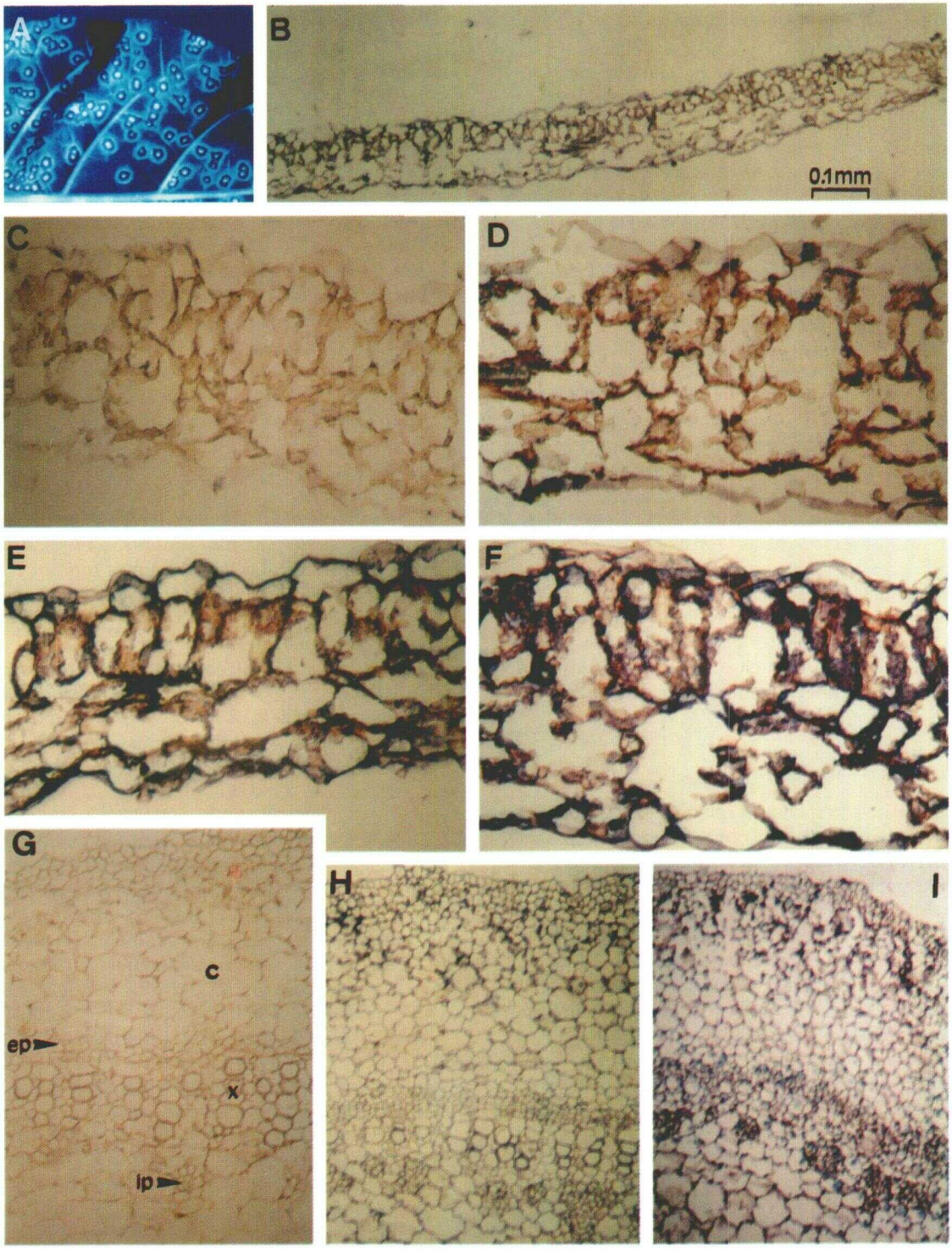

Figure 5. In situ hybridization of TMV-infected leaves and megaspermin-elicited tobacco stems. A, Six-day-old lesions observed under UV light. B, Section through a tobacco leaf 2 d post-TMV infection corresponding to the fluorescent area of A and hybridized with antisense PAL RNA. D, E, and F, Close-up photographs that correspond to the right, middle, and left parts of the section presented in B, respectively. C, Control leaf section hybridized with PAL sense RNA. G, H, and I, Sections through the stem 3 cm from the decapitation site. G, Section from elicitor-treated stem hybridized to sense probe. H, Section from nontreated stem hybridized to antisense probe. I, Section from elicitor-treated stem hybridized to antisense probe. c, Cortex; ep, external phloem; ip, inner phloem; pi, pith; x, xylem.

Tobacco stems also reacted hypersensitively upon megaspermin treatment. Visual inspection under UV light showed a fast appearance of fluorescence along the stem within a few hours after treatment. Thin cross-sections of tobacco stems, 2 to 3 cm from the top of the decapitated megaspermin-treated plant (the site of elicitor deposition), were collected 6 h after elicitation. Compared to equivalent untreated healthy stems (Fig. 5H), a general increase in the amount of PAL transcript was clearly observed in the different tissues of the elicited stem (Fig. 5I): PAL transcript accumulation appeared particularly intense in the internal and external phloem tissues of the stem but was also detected in cortical cells and pith tissues. It is noteworthy that, because proteinaceous elicitor is known to migrate downward in the tobacco stem (Devergne et al., 1992), the intensity of the labeling detected in phloem cells may merely reflect the presence of a high concentration of eliciting molecules in these conductive tissues. No labeling at all was found in control sections hybridized with the sense probe (Fig. 5G), demonstrating the meaningfulness of the staining observed in nontreated stem (Fig. 5H). In fact, in this latter tissue a significant signal was observed in the same cell types that accumulate PAL transcripts in higher amounts upon elicitation. In particular, a clear signal was observed in some subepidermal cells. A similar localization has been reported for PAL of hybrid poplar (Subramaniam et al., 1993). The presence of PAL transcripts in various cell types of tobacco stems was confirmed by observation at higher magnification (not shown) and may arise from an intense metabolic activity in these young tissues. Older lignified tissues have not been examined.

DISCUSSION

The hypersensitive response of plants to pathogens has often been reported to result in cell wall thickening, which impairs the pathogen's spread through plant tissues. Lignin and lignin-like polymers deriving from the phenylpropanoid pathway are thought to play a major role in the building up of these mechanical barriers, which confine the virus to a limited number of cells. As the first enzyme of the phenylpropanoid pathway, PAL plays a key role by controlling the metabolic flux entering the pathway. A strong accumulation of phenylpropanoids and a sharp increase in activity of many biosynthetic enzymes (including PAL) have been reported to occur in Samsun NN tobacco reacting hypersensitively to TMV (Fritig et al., 1973; Legrand et al., 1976). Surprisingly, in a recent work Brederode et al. (1991), using a partial PAL cDNA clone, were unable to detect the accumulation of PAL transcript in TMV-infected Samsun NN leaves. The present study was aimed at clarifying these discrepancies.

A near-full-length cDNA encoding tobacco PAL has been isolated. This cDNA clone was used to synthesize DNA and RNA probes that were used in northern blots and RNase protection assays, respectively, to investigate the pattern of expression of PAL genes under various situations. Two different transcripts were detected in low amounts in healthy material. Upon infection or elicitor treatment the two transcripts accumulated, as indicated by the high level of protected fragments found after RNase action. The two tobacco genes thus appeared to be tightly co-regulated. In the same tissues PAL activity was found to be sharply increased, reaching its maximum a few hours after the maximum mRNA level. Thus, enzyme activity appears to be controlled by messenger amount. Analysis of nuclear run-off transcripts in isolated nuclei has shown that elicitor treatment or UV illumination of cultured parsley cells (Chappell and Hahlbrock, 1984) and wounding or infection of bean hypocotyl tissues (Lawton and Lamb, 1987) stimulate PAL gene transcription. In tobacco a transcriptional control might also underlie PAL mRNA accumulation.

Southern analysis disclosed the presence of a small family of two to four PAL genes in tobacco. PAL genes from other plants, namely parsley, bean, and *Arabidopsis thaliana*, have already been shown to be organized in small families of three to four genes that are expressed differentially during development and in response to different types of environmental stimuli such as UV irradiation, treatment with fungal elicitor, or wounding (Liang et al., 1989; Lois et al., 1989; Ohl et al., 1990; Lois and Hahlbrock, 1992). In potato, the genomic organization of PAL is particularly complex, since as many as 40 genes per haploid genome have been detected, but similar patterns of PAL expression have been reported (Rumeau et al., 1990; Joos and Hahlbrock, 1992). In strawberry, sunflower, and bamboo, only a single form of PAL enzyme has been characterized (Chen et al., 1988; Given et al., 1988; Jorrin et al., 1988), but no information is available about PAL genes in these plants.

Three classes of PAL genes were characterized in bean (Cramer et al., 1989), and distinct patterns of regulation of these genes result in a selective expression of functional variants of PAL enzyme possessing different K_m values (Bolwell et al., 1985). A β-glucuronidase-based reporter system has allowed the dissection of the spatial and temporal patterns of the bean PAL2 and PAL3 promoter activities in transgenic *Arabidopsis*, tomato, and tobacco plants (Shufflebottom et al., 1993). It has been proposed that the differential expression of PAL isoforms accounts for a mechanism by which the plant exerts a metabolic priority for phenylpropanoid biosynthesis under stress conditions (Liang et al., 1989). In tobacco we found a limited number of genes (one or two) whose expression is enhanced by wounding, elicitation, or TMV infection and is likely to account for the increase of PAL activity under these stress conditions. A distinct tobacco cDNA was cloned by Brederode et al. (1991) that shared 70% similarity with the sequence presented in Figure 1 (nucleotides 589–960). These authors discovered a small gene family whose pattern of expression differs clearly from that reported here, since no cognate messenger was detected under various stress conditions, including TMV infection.

Expression of the bean PAL2 gene in tobacco resulted in a few transgenic tobacco plants with unexpectedly reduced levels of PAL activity and abnormal phenotypes (Elkind et al., 1990). It will now be possible to constitutively express the tobacco PAL cDNA in the sense or antisense direction (antisense RNA strategy) and to analyze the effects on PAL activity and phenylpropanoid synthesis. The study of such transgenic plants should enable us to dissect the multiple functions of phenylpropanoid compounds themselves or their derivatives. Indeed, *trans*-cinnamic acid has recently

been shown to be a precursor of salicylic acid, a plant signal implicated in the resistance of tobacco to pathogens (Malamy and Klessig, 1992; Raskin, 1992; Gaffney et al., 1993; Yalpani et al., 1993).

In situ hybridization has shown that many leaf cell types react to TMV infection or elicitor treatment by accumulating PAL transcripts. A similar distribution of PAL transcript throughout the leaf tissue was also found in the case of potato infected with compatible or incompatible races of *Phytophthora infestans* (Cuypers et al., 1988). In parsley leaves reacting hypersensitively to *Phytophthora megasperma* f. sp. *glycinea* a rapid and transient increase of PAL mRNA level was observed very close to the infection sites (Schmelzer et al., 1989; Kombrink et al., 1993). In tobacco leaves bearing necrotic lesions the same localization as that of PAL has been demonstrated for class II OMT, a typical defense gene (Pellegrini, 1994). PAL expression was also examined in stem tissues, and PAL mRNA was detected in various cell types. Sharp increases in mRNA level were shown to occur in the same cell types after elicitation. This contrasts with the data obtained in bean with probes specific for each class of PAL genes (Liang et al., 1989) or in transgenic tobacco expressing a reporter gene under the control of the PAL2 or PAL3 promoter (Shufflebottom et al., 1993). These studies showed that the three bean genes are expressed differentially during development and in response to light, wounding, and infection. In the PAL2 promoter, elements necessary for xylem expression and a negative element that suppresses expression in phloem and perivascular parenchyma have been localized (Leyva et al., 1992). Since the expression pattern of the endogenous PAL gene of tobacco is different, it seems that PAL promoter structure and specificity may vary among plants. A differential regulation of two members of the PAL gene family of potato has also been reported (Joos and Hahlbrock, 1992). In the case of tobacco a very limited number of PAL genes appear to respond in a similar fashion to developmental and environmental cues. The characterization of the promoters of these genes and the study of their specificity by expressing chimeric constructs made of a PAL promoter fused to a reporter gene will provide an opportunity to delineate the spatial and temporal program of expression of each individual gene.

ACKNOWLEDGMENTS

We are indebted to Noëlle Favet for provision of the tobacco PAL probe and to Dr. Serge Kauffmann for megaspermin. Pierrette Geoffroy is gratefully acknowledged for assistance in PAL activity measurements.

Received February 25, 1994; accepted July 10, 1994.
Copyright Clearance Center: 0032-0889/94/106/0877/10.
The GenBank/EMBL accession number for the sequence reported in this article is X78269.

LITERATURE CITED

Aviv H, Leder P (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA **69:** 1408–1412

Bolwell GP, Bell JN, Cramer CL, Schuch W, Lamb CJ, Dixon RA (1985) L-Phenylalanine ammonia-lyase from *Phaseolus vulgaris*. Characterization and differential induction of multiple forms from elicitor-treated cell suspension cultures. Eur J Biochem **149:** 411–419

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brederode FT, Linthorst HJM, Bol JF (1991) Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol Biol **17:** 1117–1125

Campbell MM, Ellis BE (1992a) Fungal elicitor-mediated responses in pine cell cultures. I. Induction of phenylpropanoid metabolism. Planta **186:** 409–417

Campbell MM, Ellis BE (1992b) Fungal elicitor-mediated responses in pine cell cultures. II. Cell wall-bound phenolics. Phytochemistry **31:** 737–742

Chappell J, Hahlbrock K (1984) Transcription of plant defence genes in response to UV light or elicitor. Nature **311:** 76–78

Chen R-Y, Chang T-C, Liu M-S (1988) Phenylalanine ammonia-lyase of bamboo shoots. Biochim Biophys Acta **881:** 210–221

Cramer CL, Edwards K, Dron M, Lang X, Dildine SL, Bolwell GP, Dixon RA, Lamb CJ, Schuch W (1989) Phenylalanine ammonia-lyase gene organization and structure. Plant Mol Biol **12:** 367–383

Cuypers B, Schmelzer E, Hahlbrock K (1988) In situ localization of rapidly accumulated phenylalanine ammonia-lyase mRNA around penetration sites of *Phytophthora infestans* in potato leaves. Mol Plant Microbe Interact **1:** 157–160

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12:** 387–395

Devergne JC, Bonnet P, Panabières F, Blein JP, Ricci P (1992) Migration of the fungal protein cryptogein within tobacco plants. Plant Physiol **99:** 843–847

Dixon RA, Lamb CJ (1990) Molecular communication in interactions between plants and microbial pathogens. Annu Rev Plant Physiol Plant Mol Biol **41:** 339–367

Edwards K, Cramer CL, Bolwell GP, Dixon RA, Schuch W, Lamb CJ (1985) Rapid transient induction of phenylalanine ammonia-lyase mRNA in elicitor-treated bean cells. Proc Natl Acad Sci USA **82:** 6731–6735

Elkind Y, Edwards R, Mavandad M, Hedrick SA, Ribak O, Dixon RA, Lamb CJ (1990) Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc Natl Acad Sci USA **87:** 9057–9061

Favali MA, Conti GG, Bassi M (1978) Modifications of the vascular bundle ultrastructure in the "resistant zone" around necrotic lesions induced by tobacco mosaic virus. Physiol Mol Plant Pathol **13:** 247–251

Fritig B, Gosse J, Legrand M, Hirth L (1973) Changes in phenylalanine ammonia-lyase during the hypersensitive reaction of tobacco to TMV. Virology **55:** 371–379

Fritig B, Legrand M, Hirth L (1972) Changes in the metabolism of phenolic compounds during the hypersensitive reaction of tobacco to TMV. Virology **47:** 845–848

Fritzemeier KH, Cretin C, Kombrink E, Rohwer F, Taylor J, Scheel D, Hahlbrock K (1987) Transient induction of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase mRNAs in potato leaves infected with virulent and avirulent races of *Phytophthora infestans*. Plant Physiol **85:** 34–41

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261:** 754–756

Given NK, Venis MA, Grierson D (1988) Purification and properties of phenylalanine ammonia-lyase from strawberry fruit and its synthesis during ripening. J Plant Physiol **133:** 31–37

Gowri G, Paiva NL, Dixon RA (1991) Stress responses in alfalfa (*Medicago sativa* L.). Sequence analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor-treated cell cultures and developing plants. Plant Mol Biol **17:** 415–429

Grisebach H (1981) Lignins. *In* PK Stumpf, EE Conn, eds, The

Biochemistry of Plants, Vol 7. Academic Press, New York, pp 457–478

Hahlbrock K, Grisebach H (1979) Enzymic controls in the biosynthesis of lignin and flavonoids. Annu Rev Plant Physiol **30:** 105–130

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol **40:** 347–369

Jaeck E, Dumas B, Geoffroy P, Favet N, Inzé D, Van Montagu M, Fritig B, Legrand M (1992) Regulation of enzymes involved in lignin biosynthesis: induction of O-methyltransferases mRNAs during the hypersensitive reaction of tobacco to tobacco mosaic virus. Mol Plant Microbe Interact **5:** 294–300

Joos H-J, Hahlbrock K (1992) Phenylalanine ammonia-lyase in potato (*Solanum tuberosum* L.). Genomic complexity, structural comparison of two selected genes and modes of expression. Eur J Biochem **204:** 621–629

Jorrin J, Dixon RA (1990) Stress responses in alfalfa (*Medicago sativa* L.). II. Purification, characterization, and induction of phenylalanine ammonia-lyase isoforms from elicitor-treated cell suspension cultures. Plant Physiol **92:** 447–455

Jorrin J, Lopez-Valbuena R, Tena M (1988) Purification and properties of phenylalanine ammonia-lyase from sunflower (*Helianthus annuus* L.) hypocotyls. Biochim Biophys Acta **964:** 73–82

Kauffmann S, Baillieul F, Genetet I, Kopp M, Fritig B (1993) Two proteins secreted by *Phytophthora megasperma* elicit necrosis and defence-related responses in tobacco. *In* B Fritig, M Legrand, eds, Mechanisms of Plant Defense Responses. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 140–143

Kawamata S, Yamada T, Tanaka Y, Sriprasertsak P, Kato H, Ichinose Y, Kato H, Oku H (1992) Molecular cloning of phenylalanine ammonia-lyase from *Pisun sativum*. Plant Mol Biol **20:** 167–170

Knight ME, Halpin C, Schuch W (1992) Identification and characterization of cDNA clones encoding cinnamyl alcohol dehydrogenase from tobacco. Plant Mol Biol **19:** 793–801

Kombrink E, Beerhues L, Garcia-Garcia F, Hahlbrock K, Muller M, Schroder M, Witte B, Schmelzer E (1993) Expression patterns of defense-related genes in infected and uninfected plants. *In* B Fritig, M Legrand, eds, Mechanisms of Plant Defense Responses. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 236–249

Lawton MA, Lamb CJ (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. Mol Cell Biol **7:** 335–341

Legrand M (1983) Phenylpropanoid metabolism and its regulation in disease. *In* JA Callow, ed, Biochemical Plant Pathology. John Wiley & Sons, Chichester, UK, pp 367–384

Legrand M, Fritig B, Hirth L (1976) Enzymes of the phenylpropanoid pathway and the necrotic reaction of hypersensitive tobacco to tobacco mosaic virus. Phytochemistry **15:** 1353–1359

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol **41:** 455–496

Leyva A, Liang X, Pintor-Toro JA, Dixon RA, Lamb CJ (1992) Cis-element combinations determine phenylalanine ammonia-lyase gene tissue-specific expression patterns. Plant Cell **4:** 263–271

Liang X, Dron M, Cramer C, Dixon R, Lamb CJ (1989) Differential regulation of phenylalanine ammonia lyase genes during plant development and by environmental cues. J Biol Chem **24:** 14486–14492

Lois R, Dietrich A, Hahlbrock K, Schulz W (1989) A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive cis-acting elements. EMBO J **8:** 1899–1906

Lois R, Hahlbrock K (1992) Differential wound activation of members of the phenylalanine ammonia-lyase and 4-coumarate:CoA ligase gene families in various organs of parsley. Z Naturforsch **47c:** 90–94

Lynn DG, Chang M (1990) Phenolic signals in cohabitation: implications for plant development. Annu Rev Plant Physiol Plant Mol Biol **41:** 497–526

Malamy J, Klessig DF (1992) Salicylic acid and plant disease resistance. Plant J **2:** 643–654

Nagai N, Kitauchi F, Shimosaka M, Okasaki M (1994) Cloning and sequencing of a full-length cDNA coding for phenylalanine ammonia-lyase from tobacco cell culture. Plant Physiol **104:** 1091–1092

Ohl S, Hedrick SA, Chory J, Lamb CJ (1990) Functional properties of a phenylalanine ammonia lyase promoter from *Arabidopsis*. Plant Cell **2:** 837–848

Orr JD, Edwards R, Dixon RA (1993) Stress responses in alfalfa (*Medicago sativa* L.). XIV. Changes in the level of phenylpropanoid pathway intermediates in relation to regulation of L-phenylalanine ammonia-lyase in elicitor-treated cell-suspension cultures. Plant Physiol **101:** 847–856

Pellegrini L (1994) La phénylalanine ammoniac-lyase (PAL) et l'O-méthyltransférase (OMT) de classe II du Tabac, des enzymes du métabolisme phénolique impliquées dans la défense antimicrobienne: clonage des cDNA et étude de la régulation spatio-temporelle des gènes. Thèse de l'Université Louis Pasteur, Stasbourg, France

Pellegrini L, Geoffroy P, Fritig B, Legrand M (1993) Molecular cloning and expression of a new class of ortho-diphenol-*O*-methyltransferases induced in tobacco (*Nicotiana tabacum* L.) leaves by infection or elicitor treatment. Plant Physiol **103:** 509–517

Raskin I (1992) Salicylate, a new plant hormone. Plant Physiol **99:** 799–803

Rumeau D, Maher E, Kelman A, Showalter AM (1990) Extensin and phenylalanine ammonia-lyase gene expression altered in potato tubers in response to wounding, hypoxia and *Erwinia carotovora* infection. Plant Physiol **93:** 1134–1139

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA **81:** 8014–8018

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Schmelzer E, Krüger-Lebus S, Hahlbrock K (1989) Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. Plant Cell **1:** 993–1001

Shaw NN, Bolwell GP, Smith C (1990) Wound induced phenylalanine ammonia-lyase in potato (*Solanum tuberosum* L.) tuber disc. Significance of glycosylation and immunolocalization of enzyme subunits. Biochem J **267:** 163–170

Shufflebottom D, Edwards K, Schuch W, Bevan M (1993) Transcription of two members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell specificities and induction patterns. Plant J **3:** 835–845

Subramaniam R, Reinold S, Molitor EK, Douglas CJ (1993) Structure, inheritance, and expression of hybrid poplar (*Populus trichocarpa* × *Populus deltoides*) phenylalanine ammonia-lyase genes. Plant Physiol **102:** 71–83

Vance CP, Kirk TK, Sherwood RT (1980) Lignification as a mechanism of disease resistance. Annu Rev Phytopathol **18:** 259–298

Wetten RW, Sedoroff RR (1992) Phenylalanine ammonia-lyase from loblolly pine. Purification of the enzyme and isolation of complementary DNA clones. Plant Physiol **98:** 380–386

Yalpani N, Leon J, Lawton MA, Raskin I (1993) Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. Plant Physiol **103:** 315–321