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Isolation and characterization of a wound inducible phenylalanine ammonia-lyase gene (*LsPAL1*) from Romaine lettuce leaves

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Phenylalanine ammonia-lyase (PAL) catalyses the first step controlling the rate of phenylpropanoid metabolism. Wounding is a ubiquitous stress in nature and in the harvesting and preparation of fruits and vegetables that induces an increase in PAL activity, an accumulation of phenolic compounds and subsequent tissue browning. A wound-inducible PAL gene (*LsPAL1*) was isolated from Romaine lettuce by RT-PCR. The putative protein encoded by *LsPAL1* is similar to predictive polypeptides sequences for other PALs. The kinetics of PAL mRNA accumulation is similar to those of induced PAL enzyme activity, with enzyme activity following mRNA accumulation by 12 h. Wound-induced PAL transcripts accumulated in cells close to the wound sites. Tissue printing showed that PAL mRNA was associated with tissue next to the epidermis and vascular bundles. A heterologous PAL protein was expressed in *E. coli* and was found to show significant PAL activity.

Introduction

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyses the deamination of phenylalanine to produce *trans*-cinnamic acid, the first step controlling the rate of phenylpropanoid metabolism (Koukol and Conn 1961). The production of phenylpropanoid compounds is important in plant development, plant-microbe signalling and plant defence (Hahlbrock and Scheel 1989). Proteins with PAL activity occur in plants and microorganisms (Camm and Towers 1973), and have been isolated from different species of plants and extensively studied at the molecular level. PAL genes in some species belong to a small gene family (Cramer et al. 1989, Lois et al. 1989, Minami et al. 1989, Ohl et al. 1990, Lee et al. 1992, Subramaniam et al. 1993, Zhu et al. 1995, Fukasawa-Akada et al. 1996). Multiple PAL genes have been described in potato (Joos and Hahlbrock 1992), while there is possibly only one PAL gene in loblolly pine (Whetten and Sederoff 1992).

Changes in PAL activity, together with other enzymes involved in phenylpropanoid biosynthesis, and the accumulation of various phenolic compounds is a plant response to stress (Dixon and Paiva 1995). Wounding is one of many stresses that induces a PAL-promoter (Bevan et al. 1989, Shufflebottom et al. 1993, Prasad et al. 1995, Zhu et al. 1995, Sriprasertsak et al. 1999), triggers the accumulation of PAL mRNA (Lawton and Lamb 1987, Ohl et al. 1990, Ishizuka et al. 1991, Joos and Hahlbrock 1992, Diallinas and Kanellis 1994, Fukusawa-Akada et al. 1996, McConn et al. 1997), and increases PAL activity (Minamikawa and Uritani 1965a, Goldstein et al. 1972, Wong et al. 1974, Tanaka and Uritani 1976, Ke and Saltveit 1989a, Kamo et al. 2000, Hisaminato et al. 2001). Inhibitors of gene transcription and protein synthesis reduce the wound- and ethylene-induced rise in PAL activity, suggesting that de novo PAL synthesis is required for these responses (Minamikawa and Uritani 1965b, Hyodo and Yang 1971, Chalutz 1973, Matsushita and Uritani 1975, Ke and Saltveit 1989a).

Wound-induced changes in PAL activity during the preparation and storage of fresh-cut lettuce have been extensively studied (Saltveit 1997). The increase in PAL activity in lettuce correlates with the severity of wounding,

Abbreviations – hsp, heat-shock proteins; hs, heat-shock; PAL, phenylalanine ammonia lyase; POD, peroxidase; PPO, polyphenyl oxidase.

the propagation of putative wound signal(s), and the accumulation of simple phenolic compounds involve in secondary processes such as tissue browning (Ke and Saltveit 1989a, Loaiza-Velarde et al. 1997). There are differences in the responsiveness among cultivars and types of lettuce to wounding (Tomás-Barberán et al. 1997a, Cantos et al. 2001). However, increased PAL activity is common enough to be suggested as a predictor of lettuce storage quality (Couture et al. 1993).

Browning of tissue adjacent to sites of wounding is associated with the oxidation of phenolic compounds (Amiot et al. 1997). In fresh cut lettuce, browning is one of the most important factors reducing quality (Lopez-Galvez et al. 1996a, Saltveit 1997), and many different approaches have been developed to reduce wound-induced tissue browning (Tomás-Barberán et al. 1997b, Saltveit 2000).

Although PAL activity is often analysed during studies of fresh cut lettuce, we are not aware of the isolation of a wound-induced PAL gene or a molecular characterization of the wound response. The objective of this research was to isolate and characterize a PAL gene from leaves of wounded lettuce.

Materials and methods

Plant material

Heads of Romaine lettuce (*Lactuca sativa* L., var. Longifolia) were obtained from commercial sources, transported to the Mann Laboratory at the University of California, Davis and held at 0.5°C until used. Complete leaves or 1 cm pieces of achorophyllous mid-rib tissue were stored at 10°C for 36 h to evaluate the kinetic of PAL activity and gene expression. The fully expanded leaves used in the experiments were chosen from the middle of the head eliminating the damaged outer leaves and immature inner leaves. Pieces of mid-rib tissue (1.5 cm) were excised and kept for 12 h or 24 h at 10°C and then dissected into 0.5 cm sections to evaluate PAL activity and potential wound signal movement. For tissue printing experiments, 1.5 cm pieces were stored under the same condition as described above and dissected in 0.3 cm pieces before analysis. Based on preliminary kinetic studies of wound-induced PAL activity, all tissue was stored at 10°C.

PAL activity assay

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity was measured as previously described by Ke and Saltveit (1986) with some modifications. Briefly, 4 g of lettuce tissue was homogenized with 16 ml 50 mM borate buffer (pH 8.5) containing 5 mM of 2-mercaptoethanol and 0.4 g insoluble polyvinylpolypyrrolidone. The homogenate was filtrated through 4 layers of cheesecloth and centrifuge at 20 000 *g* at 4°C for 20 min. One-ml aliquots of the supernatant were assayed for PAL activity after addition of 110 µl of 100 mM L-phenylalanine and incubated at 40°C for 30 min. Previous to the absorbance readings; the proteins were precipitated by the addition of trichloroacetic acid at 4% (w/v). The samples with TCA were incubated for 5 min at room temperature and centrifuged at 10 000 *g* for 5 min. Absorbance of the supernatant was read at 290 nm at the beginning and after the incubation time. An aliquot of 1 ml of 1/20 dilution of the fraction was assayed similarly to the crude extract of lettuce tissue when assaying the fractions obtained from purified fusion proteins. PAL activity was calculated as mol of cinnamic acid per g of tissue produced under the specific conditions, or as µmol of cinnamic acid produced per mg of protein per hour for fractions of purified fusion protein.

Reverse transcription polymerase chain reaction (RT-PCR)

Whole leaves and pieces of mid rib tissue were stores at 10°C for 0, 2 and 20 h. Total RNA was obtained by a phenol extraction method as described by Sambrook et al. (1989). The extracted total RNA (1 µg) was used to obtain the first strand cDNA by reverse transcription (RT) using the RETROscript kit (Ambion, TX) following the manufacturer's instructions and amplified by PCR using degenerative primers. Degenerate primers were designed using an alignment of predicted amino acids sequences of published PAL genes. The PAL sequences used in this alignment were: *Helianthus annuus* (GenBank accession number: Y12461), *Arabidopsis thaliana* (L33678), *Petroselinum crispum* (X81159), *Daucus carota* (D85850), *Nicotiana tabacum* (AB008199), *Oryza sativa* ssp. *japonica* (X16099) and *Triticum aestivum* (X99705). The forward degenerate primer (5′-GAYCCNYTNAAYTGGGG-3′; Y = C,T; R = A,G and N = A,C,T,G) and the reverse primer (5′-CCYTGRAARTTNCCNCCRTG-3′) were chosen between the most conserved regions of the predicted protein sequences alignment. The PCR cycle with degenerate primers was conducted at 94°C for 10 min, followed by 7 cycles of touch down PCR consisted in: denaturation, 94°C for 1 min; annealing starting at 56°C and ending at 50°C for 1 min and extension, 72°C for 2 min; after the touch down, 30 cycles of PCR were used with an annealing temperature of 50°C and with a final extension of 72°C for 7 min. The PCR reaction was: 5 µl of RT first strand cDNA product, 0.2 µM of each degenerate primers, 2.5 units of Amplitaq Gold (Applied Biosystems, CA, USA), 0.2 mM dNTP mix (Applied Biosystems), 5 µl of AmpliTaq Buffer (containing $MgCl_2$) and water to reach 50 µl total volume. The tubes were covered with 3 drops of mineral oil (Sigma, St. Louis, MO) and run in a GeneAmp PCR system 2400 Applied Biosystems thermo cycler. The RT-PCR products were separated by electrophoresis in a 1% agarose gel. The bands were stained with ethidium bromide, extracted out of the gel and purified by using QIAquick gel extraction kit (Qiagen, CA). The purified DNA was cloned in pCR-2.1 vector using TOPO TA cloning kit (Invitrogen, CA) following the manufacturer's instructions. The cloned

RT-PCR products were sequenced at the Advanced Plant Genetics Facilities at UC Davis and compared with sequences contained at the National Center for Biotechnology Information (NCBI) by the BLAST program.

Rapid amplification of cDNA ends (RACE)

The SMART RACE cDNA kit (Clontech, CA) was used to obtain the full length (or near full length) PAL gene once the DNA fragment (1.1 Kb) obtained by RT-PCR was sequenced. Total RNA (1 μg) was extracted from wounded lettuce after 12 h at 10°C and was utilized to generate the first strand of cDNA following the manufacturer's instructions. The cDNA obtained was the template of PCR reactions using a reverse gene-specific primer (5′-CCCTTCTTTCGGCTGTAACTCG-3′) and a forward gene specific-primer (5′-TTATGCTCTCCG-TACATCTCCC-3′) in combination with the SMART RACE cDNA kit to obtain the 5′ RACE and 3′ RACE, respectively. The PCR conditions for the 5′ RACE reaction was composed by 94°C for 10 min, followed by 5 cycles of touch down PCR consisting of: denaturation, 94°C for 1 min; annealing starting at 72°C and ending at 68°C for 1 min and extension, 72°C for 2 min; after the touch down, 25 cycles of PCR was used with an annealing temperature of 68°C and with a final extension of 72°C for 7 min. The PCR conditions for the 3′ RACE reaction was composed of 94°C for 10 min, followed by 8 cycles of touch down PCR consisting of: denaturation, 94°C for 1 min; annealing starting at 72°C and ending at 65°C for 1 min and extension, 72°C for 2 min; after the touch down 30 cycles of PCR was used with an annealing temperature of 65°C and with a final extension of 72°C for 7 min. As verification, the full length *LsPAL1* gene was obtained by PCR reactions using a forward specific primer located at the beginning of the 5′RACE fragment (5′-GAG-CAATCTGATCAATACCCATTC-3′) in combination with the primer form SMART RACE cDNA kit. The PCR conditions for the full length PCR reactions were 94°C for 10 min, followed by 15 cycles of touch down PCR consisting of: denaturation, 94°C for 1 min; annealing starting at 72°C and ending at 50°C for 1 min and extension, 72°C for 2 min 30 s; after the touch down 30 cycles of PCR was used with an annealing temperature of 50°C and with a final extension of 72°C for 7 min. The PCR products were cloned using TOPO TA kit, subcloned into pBluescript II KS (Stratagene, CA) and sequenced. The sequences were aligned using Sequencher software (Gene Codes Corp., Ann Arbor, MI). The longest open reading frame (ORF) was found using DNASTAR software (DNASTAR, Madison, WI). The ORF was compared with protein sequences at NCBI using BLAST search.

RNA gels and northern blots

Total RNA was extracted from achlorophyllous mid rib lettuce tissue of mature leaves. A phenol extraction method was performed as described by Sambrook et al. (1989). The total RNA electrophoreses were performed in 1.3% (w/v) agarose gels with 7% (v/v) formaldehyde. The RNA was transferred overnight onto Hybond N+ membrane (Amersham Pharmacia, NJ, USA) and UV cross-linked. Riboprobes were generated using DIG labelled dNTP (Boehringer Mannheim, Indianapolis, USA) as described in Nonogaki et al. (2000). The probe corresponded to the first 383 bp of the *LsPAL1* gene (accession number AF299330). The membranes were prehybridized for 30 min in a buffer composed by 5× SSC, 50% (v/v) formamide, 4% (w/v) blocking reagent (Boehringer Mannheim), 0.2% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine. The hybridization and washing of the membranes and the detection of the signal by chemiluminescence was done as described by Nonogaki et al. (2000). The quantification of the relative expression of signal was performed by integration of the signal by densitometer scanning (IS-1000 Digital Imaging System, Alpha Innotech Corporation, CA) of the Northern blot normalized by the integration of the ethidium bromide stained ribosomal RNA of the same sample. In some figures the value 1 was arbitrarily assigned to zero time.

Southern blot

DNA was extracted from young lettuce leaves (centre of the head) using a DNeasy plant mini kit (Qiagen) following the manufacturer instructions: 8 μg of genomic DNA was digested with *Eco*RI and *Xba*I (New England Laboratories, MN). The digested DNA was separated in a 0.8% (w/v) agarose gel and overnight alkali transferred in a Hybond N+ membrane (Amersham Pharmacia, NJ, USA). The full-length LsPAL1 probe was prepared by digestion of the vectors containing the clone with *Spe*-I and *Xho* (New England Laboratories, MN), consequently gel purified by QIAquick gel extraction (Qiagen) and labelled using Alk Phos Direct kit (Amersham Pharmacia biotech) following the manufacturer's instructions. The prehybridization and hybridization was done at 55°C in a rotisserie oven for 1 h and overnight, respectively. The primary washings (twice) were performed at 65°C and secondary washing (2) at room temperature. The signal detection was done by chemiluminescence by using CDP-Star (Amersham Life Science, NJ, USA), following the instruction manual, on an X-ray film (Fuji Super RX, Tokyo, Japan).

Tissue printing

Pieces (1.5 cm) of mid rib lettuce tissue were stored at 10°C for 24 h. These pieces were cut into five 0.3-cm thick sections labelled A to E. A thin layer (approximately 1 mm) of each section was cut transversally with a scalpel and dried in paper towels for a few seconds to remove excess liquid. Printing was performed pressing the section against Hybond N+ membrane (Amersham Pharmacia) for 5 s. The membranes were dried on 3MM (Whatman, UK) paper at room temperature, UV cross-linked, and treated as Northern blots as previously

described. The signal was detected colorimetrically as described by Nonogaki et al. (2000).

Expression of the fusion protein

Expression and purification of the fusion protein in *E. coli* was conducted essentially according to Nonogaki et al. (2000) with slight modifications. The *Eco*RI site-linked forward primer (5′-CGGAATTCATGGA-GAACGGTAAT-3′) and *Xba*I site-linked reverse primer (5′-CGTCTAGACTAACATATTGGAAG-3′) were designed for the coding region of *Ls*PAL. The PAL open reading frame was cloned into the *Eco*RI and *Xba*I site in the pMALc vector (New England Laboratories) and used for bacterial transformation. The transformed bacteria were incubated overnight at 37°C. An aliquot of the overnight culture was used to inoculate an incubation broth for 4 h at 37°C and protein synthesis was induced by isopropyl-1-thio-β-D-galactopyranoside (IPTG, 2 mM) for 2 h. The cells were harvested by centrifugation and resuspended in sonication buffer (Nonogaki et al. 2000). After an overnight freezing period, the cells were thawed and sonicated for around 1–5 min to release a higher amount of soluble fusion protein. The soluble protein was purified as Nonogaki et al. (2000) and separated by electrophoresis in a 10% acrylamide gel. The bands were stained with Coomassie Brilliant Blue (Fisher, PA, USA) for approximately 1 h, and de-stained to visualize the major bands. A prestained broad range protein standard (Bio-Rad, CA) was used to estimate the molecular weights.

Protein electrophoresis

Protein separation was performed by SDS-PAGE in a 10% (w/v) acrylamide gels as described by Laemmli (1970). A Mini-Protean II (Bio-Rad) electrophoresis system was used. The gels were loaded with equal volume of protein sample preparation (10 µl), which were previously kept for 5 min at 90–95°C. A prestained broad range protein standard (Bio-Rad) was used to estimate the molecular weights. After electrophoresis the proteins were stained with Coomassie Brilliant Blue (Fisher) for 4 h or overnight and distained with methanol, water and acetic acid mixture (45:45:10).

Results and discussion

Wounding lettuce mid-rib tissue induced a 6-fold increase in PAL activity (Fig. 1). PAL activity in 1-cm pieces and comparable tissue excised from non-wounded whole leaves held at 10°C for 36 h did not differ substantially during the first 2 h, with levels of 0.10 ± 0.02 µmol cinnamic acid $g^{-1} h^{-1}$. After 6 h, PAL activity in the wounded tissue started to increase, reaching a maximum activity of 0.60 ± 0.02 µmol cinnamic acid $g^{-1} h^{-1}$ at 24 h, and then declining about 50% to 0.30 ± 0.02 µmol cinnamic acid $g^{-1} h^{-1}$ at 36 h. From 6 to 36 h, the non-wounded control exhibited fairly stable background levels of PAL activity (0.08 µmol cinnamic acid $g^{-1} h^{-1}$). These kinetics of wound-induced PAL activity are similar to those previously reported for fresh cut Iceberg and Romaine lettuce (Ke and Saltveit 1989a, Lopez-Galvez et al. 1996b, Loaiza-Velarde and Saltveit 2001).

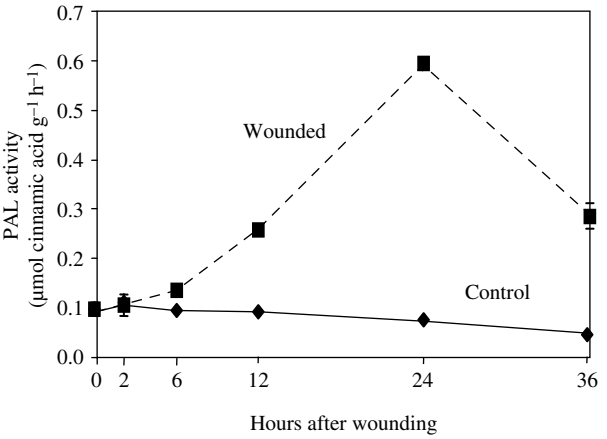

Fig. 1. PAL activity of wounded mid rib lettuce tissue (1 cm^2) stored at 10°C for 36 h. As a control, whole lettuce leaves were stored under the same conditions and tissue was excised immediately before the assay. The PAL activity was measured as accumulation of cinnamic acid.

If wound-induced PAL activity in lettuce is the result of de novo protein synthesis (Ke and Saltveit 1989a), then wound-induced PAL mRNA must accumulate prior to the rise in PAL activity (Fig. 1). Total RNA was extracted from non-wounded and wounded lettuce tissue held at 10°C for 0, 2 and 20 h. Extracted RNA was used to obtain cDNA by reverse transcription, which was then used as a template in a PCR reaction with degenerate primers designed for the highly conserved regions of the predicted PAL amino acid sequences in published databases. A PCR product (1.1 Kb) from the reaction using total RNA extracted from 20 h wounded samples was cloned, sequenced and compared by BLAST search at NCBI. The product was not observed in unwounded samples (data not shown). The results of the analyses indicated that there was a high probability that the clone was a fragment of a PAL gene.

The full length, or near full-length gene, was obtained by RACE. The PAL lettuce gene was named *LsPAL1* (accession number AF299330). It is important to mention that the *LsPAL1* cDNA differed from the 1.1 Kb obtained by RT-PCR, although both of them shared similar motifs: 87% and 93% similarity in cDNA and predictive amino acid sequence, respectively. We subsequently cloned the full length or near to full length gene corresponding to a RT-PCR product named *LsPAL2* (AF411134), which will be characterized in future work.

The longest open reading frame of LsPAL1 encoded a predicted protein of 711 amino acids (Fig. 2) with a putative molecular weight of 77.4 kDa. This size is

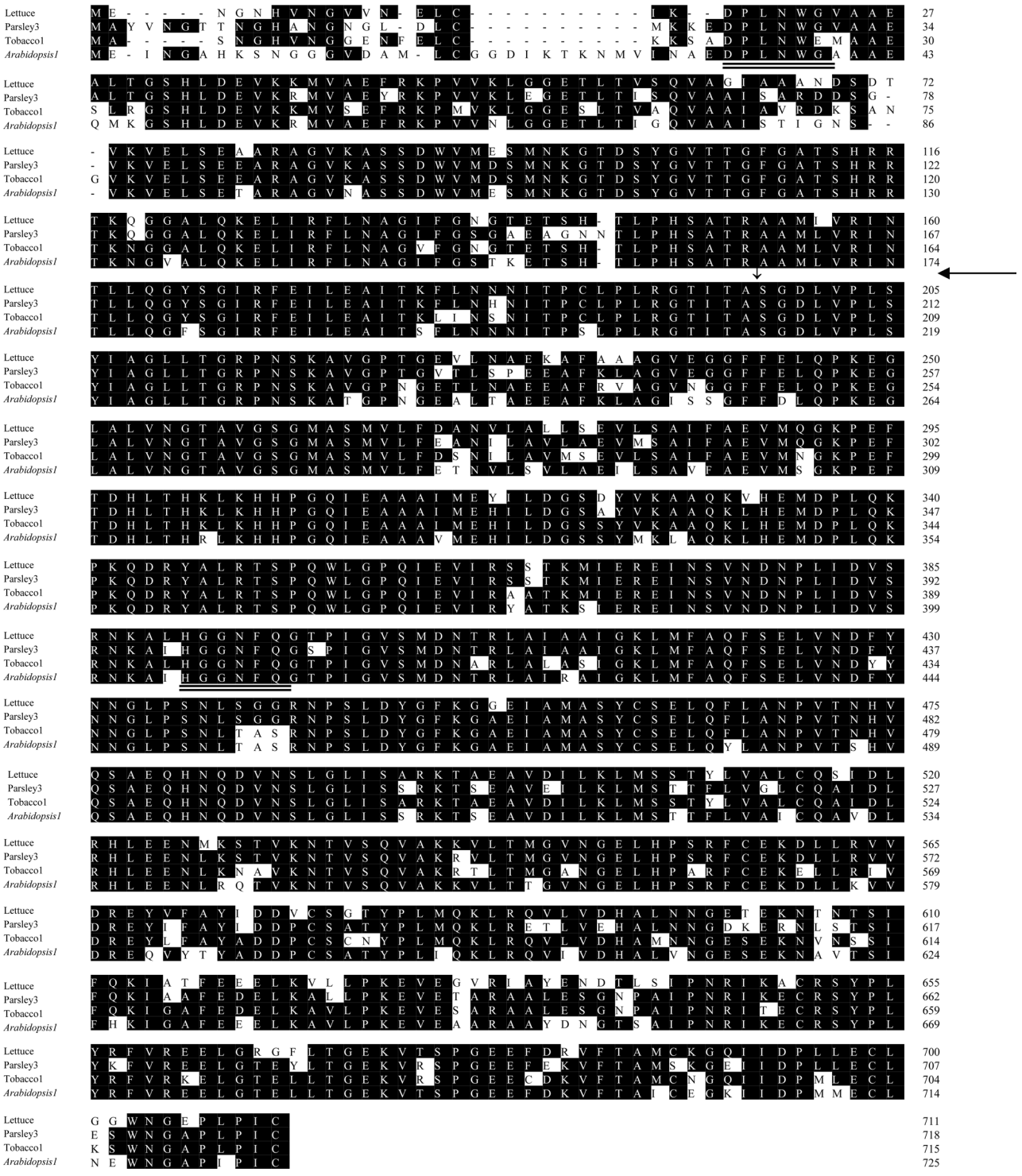

Fig. 2. Multiple clustal alignment of the predicted amino acid sequences of PAL protein sequences from lettuce (LsPAL1, GeneBank accession number AF299330), parsley (PAL3, P45729), tobacco (M84466) and *Arabidopsis* (L33677). The amino acid sequences used to design degenerate primers are double underlined. The putative active site is indicated with ↓ (Schuster and Retey 1994). Amino acids identical to the consensus are reversed shading.

similar to other PAL proteins from different species. Using parsley PAL as a model, Schuster and Retey (1994) predicted that the active site was associated with a serine residue that is converted to dehydroalanine. This serine residue is conserved among species and is located at position 198 in the LsPAL1 predictive peptide (Fig. 2). Since the lettuce PAL was obtained from RNA extracted from wounded leaves, the predicted amino acid

sequences was compared by cluster analyses with three other deduced PAL polypeptides associated with wounded leaves in parsley (parsley3; Lois et al. 1989, Appert et al. 1994), tobacco (tobacco1; EMBL M84466, Fukusawa-Akada et al. 1996) and *Arabidopsis* (Arabidopsis1; Wanner et al. 1995, McConn et al. 1997). The similarity between wound-induced lettuce PAL and PAL from parsley, tobacco or *Arabidopsis* PAL was 85.7%, 84.5% and 81.4%, respectively.

Southern blot analyses were performed with a full-length probe of LsPAL1 (2.4 Kb). The results suggest that lettuce PAL belongs to a small gene family (data not shown), which is in agreement with reports of PAL genes in other herbaceous and woody species (see Introduction).

The accumulation of PAL transcripts was studied in wounded lettuce pieces. Total RNA was extracted from 1 cm pieces of lettuce mid-rib that had been stored at 10°C for 0–36 h (Fig. 3A). A weak signal was observed from the 0 time sample and arbitrarily assigned a value of 1 (Fig. 3B). In some experiments PAL transcript accumulation at time 0 was considerably higher, but the subsequent kinetics of induction were similar to those reported here. The initially higher background level of PAL transcripts in some lettuce tissue was most likely due to pre- or post-harvest stresses (e.g. temperature, injury, drought, light, etc.) that have been shown to increase the background level of PAL transcript genes (Lois et al. 1989) and PAL activity (Couture et al. 1993).

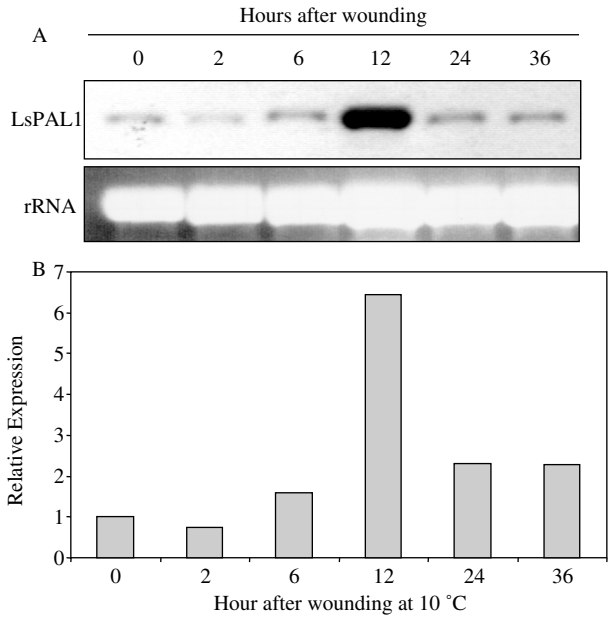

Fig. 3. Northern blot of total RNA (4 μg) from 1 cm mid-rib lettuce tissue similar to use in Fig. 1A were blotted and hybridized with the LsPAL1 riboprobe. The ethidium bromide stained ribosomal RNA prior to blotting is shown to confirm equal loading. B, the relative expression of the LsPAL1 obtained by densitometer scanning analysis of LsPAL1 Northern blot signals normalized by ribosomal RNA ethidium bromide staining. The value 1 was arbitrarily assigned to the sample at 0 h.

The accumulation of PAL transcripts (Fig. 3A), showed an increase in the first 6 h, reaching a 6-fold maximum after 12 h at 10°C, and then declining at 24 and 36 h (Fig. 3A,B). A comparison between the kinetics of PAL mRNA accumulation and PAL enzyme activity (Fig. 1) showed that the maximum in PAL transcript accumulation precedes the maximum in enzyme activity by 12 h. Although the probe contained the 119 bp of the 5′ untranslated region and the 264 bp of the amino terminal coding region, the possibility exists that it could have hybridized with more than one PAL gene mRNA. Researchers have used specific probes to discriminate between different PAL genes (Liang et al. 1989, Lois et al. 1989, Joos and Hahlbrock 1992). However, the number of PAL genes or polymorphic forms of the gene in the lettuce genome is unknown. Since the maximum accumulation of PAL transcripts occurred after 12 h at 10°C, while the highest level of PAL activity occurred at 24 h, these times were used in future experiments.

PAL activity was initially similar in the three 0.5 cm sections excised from the same region of whole leaves that is excised to make the 1.5 cm mid-rib pieces. The whole leaves were stored at 10°C for 12 or 24 h (Fig. 4). Activity in the sections increased slightly in leaves held for 24 h. This increase may have been due to slight injury when detaching the whole leaves from the head, or from a slight temperature stress during handling. In contrast, PAL activity in excised 1.5 cm pieces increased substantially during the 12 and 24 h of storage. The 0.5 cm pieces from the edges of the 1.5 cm piece had significantly higher PAL activity than did the tissue excised from the centre of the piece. This difference was more pronounced at 24 h than at 12 h (Fig. 4).

Northern blots run with the 12 h tissue (Fig. 5) showed a similar pattern of PAL mRNA accumulation to that shown with PAL activity (Fig. 4). PAL mRNA

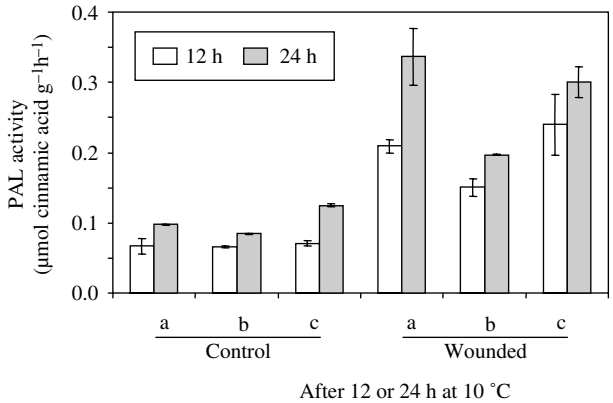

Fig. 4. PAL activity of 0.5 cm lettuce mid-rib fragments extracted of pieces of lettuce (1.5 cm) or whole leaves, stored at 10°C for 12 or 24 h. The 1.5 cm mid-rib pieces were divided in three 0.5 cm sections in order to obtain two ends (a and c) and a centre piece (b) to evaluate the differences in PAL activity as accumulation of cinnamic acid. Vertical atop bars mean standard error.

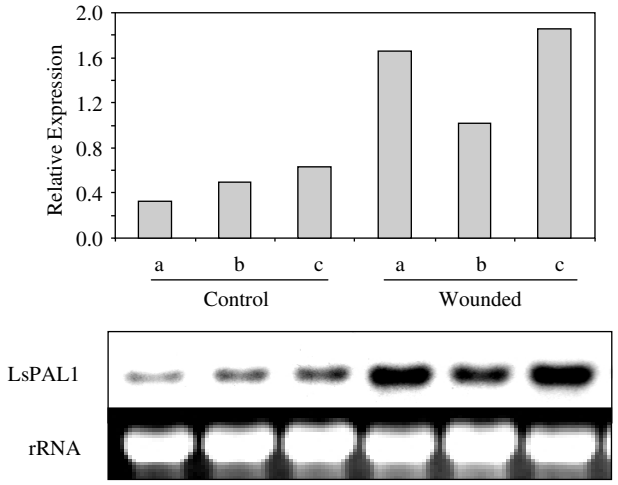

Fig. 5. Northern blot of total RNA (3 μg) from 0.5 cm sections from control (unwounded) and wounded mid-rib lettuce tissue after 12 h of stored at 10°C. The RNAs were blotted and hybridized with LsPAL1 riboprobe. An ethidium bromide stained ribosomal RNA prior to blotting is shown to confirm equal loading. At the bottom the relative expression of the LsPAL1 mRNA obtained by densitometer scanning analysis of LsPAL1 Northern blot signals normalized by ribosomal RNA ethidium bromide staining.

accumulation was stimulated by wounding and was greatest at the edges of the excised 1.5 cm pieces. Tissue in the centre 0.5 cm had 60% of the PAL mRNA accumulated in the edges.

The differential level of activity and accumulation of PAL transcript as a function of the distance from the wound site has been described in other species. Minamikawa and Uritani (1965b) reported that PAL activity in slices of wounded sweet potato tubers, was highest in tissue closest to the wound site. Wong et al. (1974) showed that PAL activity was stimulated up to 0.8 cm from the site of wounding in strawberries leaves. Borchert (1978) described differential PAL activity in slices of wounded potato tuber, with the highest activity in tissue closest to the wounded surface.

The progressive induction of PAL activity from the site of injury into the tissue could be the result of a transmitted or propagated wound signal(s). A putative wound signal appears to be responsible for the induction of PAL activity in Iceberg lettuce leaf tissue as far as 2.5 cm from the site of injury (Ke and Saltveit 1989a, Saltveit 1997). However, the nature of the putative wound signal is elusive. The application of exogenous ethylene can increase PAL transcript (Ecker and Davis 1987) and activity (Hyodo and Yang 1971, Hyodo et al. 1978, Ke and Saltveit 1989a, 1989b, Lopez-Galvez et al. 1996b, Kamo et al. 2000). Wounding can also increase ethylene production from lettuce tissue, and it is tempting to hypothesize a connection between wound-induced ethylene and ethylene-induced PAL activity. However, a kinetic analysis of the induction of PAL activity following wounding or ethylene exposure showed that ethylene is not the primary wound signal (Ke and Saltveit 1989a).

Other compounds could serve as the wound signal. Methyl jasmonate, another proposed wound signal (Farmer and Ryan 1990), increased PAL mRNA and PAL activity in soybean cell cultures and induced the accumulation of secondary metabolites in plant cell cultures; e.g. lettuce (Gundlach et al. 1992). ABA, another putative wound signal (Peña-Cortes et al. 1989), increased PAL activity in potato cell cultures during suberization (Cottle and Kolattukudy 1982). Other reports link ABA with the induction of PAL during fruit development, but the increase in PAL activity occurred days after application, not rapidly as seen with wounding (Revilla and Gonzalez-San Jose 1997, Kondo et al. 1998). Salicylic acid (SA), another signal molecule associate with plant–pathogen interactions, increased the PAL transcript when applied in significant concentration on young kiwifruit leaves (Reglinski et al. 1997). However, these examples, and others that were reviewed by Jones (1984), are not directly related with wounding. The possibility of cross-talk signalling pathways, involving these signal molecules with others, cannot be ruled out.

Exposure of Romaine lettuce leaves to ABA, JA, MeJA, or SA did not induce changes in PAL activity, the concentration of phenolic compounds or browning in mature leaf tissue similar to the level induced by wounding (Campos-Vargas and Saltveit 2002). In contrast to mature leaves, JA, MeJA and SA did induce elevated levels of PAL activity, the accumulation of phenolic compounds and tissue browning in younger leaves; however, the levels induced were far lower than those induced by wounding. Wound induced phenolic metabolism in mature leaves appears to be induced by signals different from those functioning in young leaves.

Tissue printing was used to identify the specific parts of the mid-rib tissue that are associated with the wound-induced accumulation of PAL transcript and to get more information about the propagation of the putative wound signal(s). Excised 1.5 cm mid-rib tissue pieces were stored at 10°C for 24 h and then sliced into 0.3-cm sections (a–e) for tissue-printing (Fig. 6). An antisense

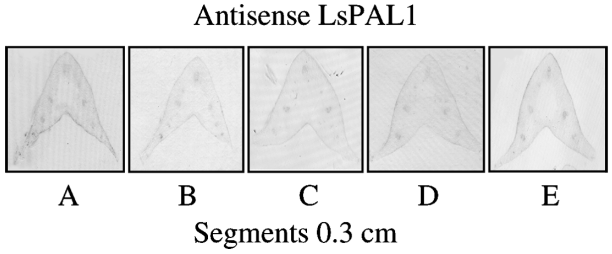

Fig. 6. Pieces of lettuce mid-rib (1.5 cm) were stored at 10°C for 24 h. After this period the pieces were divided in 0.3 cm segments (A–E). A, Tissue printing of a layer (around 1 mm) of the outer face of the 0.3 cm segments of tissue mid rib. The blots were hybridized with antisense LsPAL1 riboprobe for localization of PAL transcript after wounding.

probe and colorimetric signal detection were used to visualize the accumulated PAL mRNA. The greatest intensity was detected close to the epidermis and vascular bundles in the end sections (a,e), with lower intensity signals near the same tissues in the inner (b,d) and centre (c) sections. Ke and Saltveit (1989a) described an increase in lignification (which requires the products of PAL metabolism) in wounded cells and cells a few layer beneath the wound in Iceberg lettuce. A similar situation could occur in the Romaine leaf tissue.

The pattern observed with tissue printing is similar to that observed with the Northern blot of total RNA extracted from the similar sections (Fig. 7). The end sections accumulated the highest levels of the PAL mRNA transcript. Based on relative expression values, the levels of PAL mRNA were almost double those detected in inner sections and similar to those shown in Fig. 4B. Distribution of PAL transcripts observed with tissue printing and Northern blot analysis are in agreement with the descriptions of PAL localization after wounding in other species. Zhu et al. (1995) used a PAL-GUS reporter construct in rice and tobacco to show increased activity around the wound site in leaves of transgenic plants. A similar situation was observed in wounded leaves of transgenic tobacco transformed with an *Arabidopsis* (Prasad et al. 1995) or pea (Sriprasertsak et al. 1999) PAL promoter fused to GUS. Shufflebottom et al. (1993) transformed potato, tobacco and *Arabidopsis* plants with two bean PAL promoters fused with GUS. The GUS activity of one of the constructs (PAL2 GUS) was localized near the immediate wound site, while the other (PAL3 GUS) exhibited activity in a wider area.

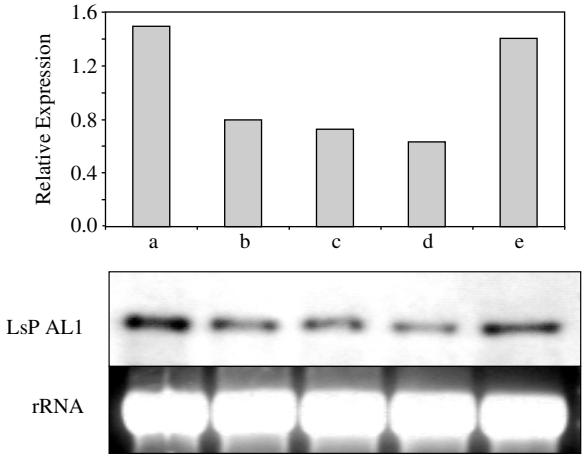

Fig. 7. Northern blot with total RNA from 0.3 cm mid rib segments obtained under the conditions described above, hybridized with LsPAL1 riboprobe. An ethidium bromide staining ribosomal RNA prior to blotting is shown to confirm equal loading. At the bottom the relative expression of the LsPAL1 obtained by densitometer scanning analysis of LsPAL1 Northern blot signals normalized by ribosomal RNA ethidium bromide staining.

Wounding could have caused the accumulation of signals associated with the vascular bundles, but the possibility exists that there is constitutive expression of the PAL gene in this type of tissue. Ohl et al. (1990) observed significant GUS activity in vascular tissue of transformed *Arabidopsis* plants with PAL-GUS construct. Similarly, Prasad et al. (1995) described GUS activity associated with vascular bundle tissue in transgenic tobacco plants transformed with an *Arabidopsis* PAL promoter GUS reporter gene.

A heterologous protein expression system was designed to confirm the identity of the lettuce PAL gene. The strategy described by Nonogaki et al. (2000) was followed to express PAL protein in bacteria. PAL protein has been expressed in *E. coli* (Schulz et al. 1989, Appert et al. 1994, Faulkner et al. 1994, Schuster and Retey 1994, Baedeker and Schulz 1999). The longest open reading frame of the PAL gene was cloned in the pMALc vector, and used to transform *E. coli*. Protein expression was confirmed using the soluble or insoluble (pellet) protein fraction from induced bacteria harbouring an empty vector, or the lettuce PAL putative peptide sequence (Fig. 8A). In induced bacteria with an empty vector the soluble or insoluble PAGE protein profile showed a major band below the 52 kDa marker, that is close to the molecular weight of the maltose binding protein (MBP, 42 kDa). However, in transformed bacteria carrying the PAL insert, the insoluble protein fraction showed a major band around 120 kDa, which is close to the predicted fusion proteins (77 kDa PAL + 42 MBP). This band did not appear when soluble protein was assayed from the same transformed bacteria. Protein synthesized in a bacterial expression system can accumulate as insoluble material in inclusion bodies (Schein 1989). Although most fusion proteins were detected in the inclusion bodies, small amounts of the fusion proteins were present in the soluble fraction as well (barely visible by Coomassie staining in Fig. 8).

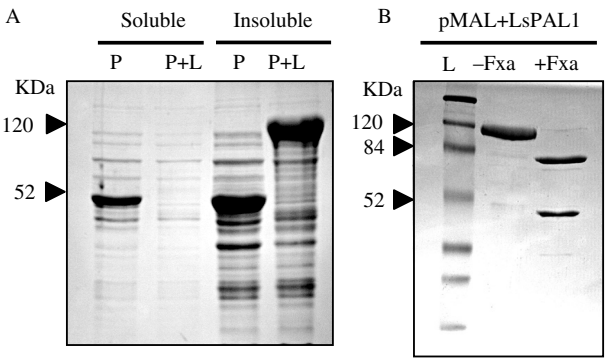

Fig. 8. The LsPAL1 encoded protein was over expressed in *E. coli*. (A) Coomassie Blue stained gel protein electrophoresis of soluble and insoluble fractions of induced bacteria cells containing an empty pMAL vector (P) or with the LsPAL1 insert ($P + l$). Arrowheads indicate molecular markers. (B) Coomassie Brilliant Blue staining gel of affinity purified fusion protein without cleavage with FactorXa (– Fxa) or after to be treated (+ Fxa). The ladder (L) is shown and arrowheads denote selected markers.

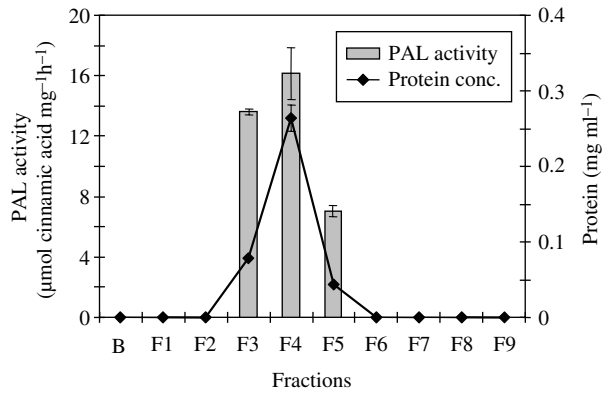

Fig. 9. Affinity purified fusion protein from *E. coli* assayed for PAL activity The graphic shows the activity and protein concentration of the buffer (B) and fractions (F1–9). Vertical line atop bars represents the standard error.

We were able to purify and collect the soluble fusion proteins by affinity purification with amylose resin. The soluble fusion protein was digested with Factor Xa protease to determine the relative molecular weight of the fusion proteins components (Fig. 8B). The fusion protein had molecular weight around 120 kDa (Fig. 8A). After digestion, two major bands are visible; one below the 52 kDa marker (presumably MBP), and the other close to the 84 kDa marker (which could be the 77 kDa predicted PAL protein).

The activity of the PAL fusion protein was assayed to confirm that the cloned gene was a PAL gene (Fig. 9). Affinity purification was used to fractionate the expressed protein. The buffer did not show PAL activity or the presence of soluble protein. Fractions with some level of protein were the only ones that showed PAL activity. No activity was observed when the same fractions were assayed for PAL activity without the addition of substrate (L-phenylalanine). The ability of the cloned gene to produce protein with PAL activity confirms that the gene is a PAL gene.

Although our research was orientated to the study of wound-induced PAL gene expression, the isolated *LsPAL1* could also be expressed during plant development or exposure to biotic or abiotic stresses, as has been described for other PAL genes. The isolation of wound-induced PAL genes from lettuce will aid in our understanding of the processes involved in wounding and phenylpropanoid metabolism dealing with secondary events, such as oxidation and browning, and in the elaboration of new approaches to reduce inherent problems with the postharvest handling and processing of fresh fruit and vegetables.

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