Pergamon

PII: S0031-9422(98)00336-7

Phytochemistry Vol. 49, No. 8, pp. 2233–2243, 1998

Printed in Great Britain
0031-9422/98/$ - see front matter

DIFFERENTIAL EXPRESSION AND PROPERTIES OF PHENYLALANINE AMMONIA-LYASE ISOFORMS IN TOMATO LEAVES

ANNAMRAJU D. SARMA, YELLAMRAJU SREELAKSHMI and RAMESHWAR SHARMA*

School of Life Sciences, University of Hyderabad, Hyderabad 500046, India

(*Received* 24 *November* 1997)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; phenylalanine ammonia-lyase; isoforms; differential induction; properties.

Abstract—In tomato leaves, excision and light treatments increase phenylalanine ammonia-lyase activity, which is contributed by three PAL isoforms. These isoforms possessed similar native and subunit Mr, but were different in their p*I*, Km for Phe and optimal pH for activity. Also these were differentially induced and affected by metabolites belonging to particular branches of phenylpropanoid pathway. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The phenylpropanoid biosynthetic pathway synthesizes a variety of phenolics such as flavonoids, phytoalexins, lignins and benzoic acid derivatives [1–3]. These compounds perform a variety of functions in plants; as small Mr compounds, they serve as sunscreens, antioxidants, anti-microbial agents, colored pigments and as polymers, suberins and lignins, they constitute structural components [4]. There is evidence that phenylpropanoid derivatives are also involved in cell division [5] and recognition by *Agrobacterium*, *Rhizobium* of their respective hosts [6]. The phenylpropanoids are derived from *trans*-cinnamic acid, which is produced by deamination of phenylalanine, catalysed by the enzyme L-phenylalanine ammonia-lyase (PAL). Regulation of the activity of this enzyme is important in modulating phenylpropanoid biosynthesis in plants [7], and recently, using transgenic plants, it has been shown that PAL activity is a major control point for regulating the phenylpropanoid pathway [8, 9].

In several plant species, such as *Arabidopsis* [10], bean [11], parsley [12], pea [13], poplar [14], rice [15], tomato [16] PAL is encoded by a small multi-gene family. Being a key enzyme, the expression of the PAL gene is regulated by a diverse array of factors such as injuries, infections, environment and developmental stage of the plant [10, 12, 13, 17–21]. The characterization of individual PAL promotors of French bean, *PAL2-GUS* and *PAL3-GUS* showed that these constructs exhibit different spatial and temporal patterns of expression during development and in response to environmental stimuli [17, 19]. While PAL activity is primarily regulated at the level of gene expression, several reports have indicated additional regulatory mechanisms, such as feed-back activation or inhibition by products from branches of phenylpropanoid pathway [22], enzyme inactivation [23] and phosphorylation [24].

In many species, such as *Oenothera*, potato, alfalfa and *Vicia faba*, PAL consists of multiple isoforms [25–28] and it is expected that in addition to post-translational modifications, some of the isoforms may be products of different members of the PAL gene family [17]. Relatively few studies have examined the contribution of PAL isoforms to different branch pathways of phenylpropanoid metabolism. In oak, three isoforms of PAL were isolated from leaf [22] and each PAL isoform was found to be associated with a specific metabolic pathway and organelle. Moreover, individual PAL isoforms were under negative feedback control by products of their own branch, whereas metabolites of other branch pathways of phenylpropanoid metabolism activated them. In transgenic tobacco, over-expression of bean PAL-2 gene increased the level of chlorogenic acid, but not of the flavonoid rutin [9].

In tomato leaves, PAL is encoded by a family of at least five genes [16] and the sequence analyses indicated the frequent occurrence of premature stop

*Author to whom correspondence should be addressed.

codons within three of the five genes, which causes early termination of these genes, resulting in truncated expression and thereby leading to the formation of inactive variants. Purification of PAL from tomato cell cultures infected with *Verticillium albo-atrum* showed only a single active PAL form [29]. However, studies on photoinduction of PAL and anthocyanin in tomato showed that, though the light-mediated increase in PAL in tomato seedlings is not correlated with the formation of anthocyanin, inhibition of PAL reduced the anthocyanin accumulation, indicating the possible existence of a specific pool of PAL regulating anthocyanin synthesis [30].

In the present study, we examined the number of active PAL forms expressed in intact and excised leaves of tomato. In tomato leaves, at least three active isoforms of PAL were present and excision and light treatment of leaves enhanced the activity of two isoforms. All three isoforms of PAL were purified and their molecular properties were studied. We report that the inducible isoforms of PAL were differentially regulated by metabolites of the phenylpropanoid pathway, which may in turn regulate the flux into different branches of the pathway.

RESULTS

Induction of PAL activity

Figure 1 shows that the PAL activity of 3-week-old tomato leaves was at a steady level. However, the excision of leaves stimulated PAL activity with a peak level at 24 h. In comparison with intact leaves, excision stimulated PAL activity by 7-fold at 24 h in leaves incubated in darkness. The incubation of leaves in light further stimulated PAL increase and it was 10-fold higher than the basal level. However, the profile of increase in PAL activity was similar in both dark- and light-exposed leaves. On incubation of excised leaves longer than 24 h, PAL activity declined. In view of this, for PAL purification, excised leaves incubated in light for 24 h were used.

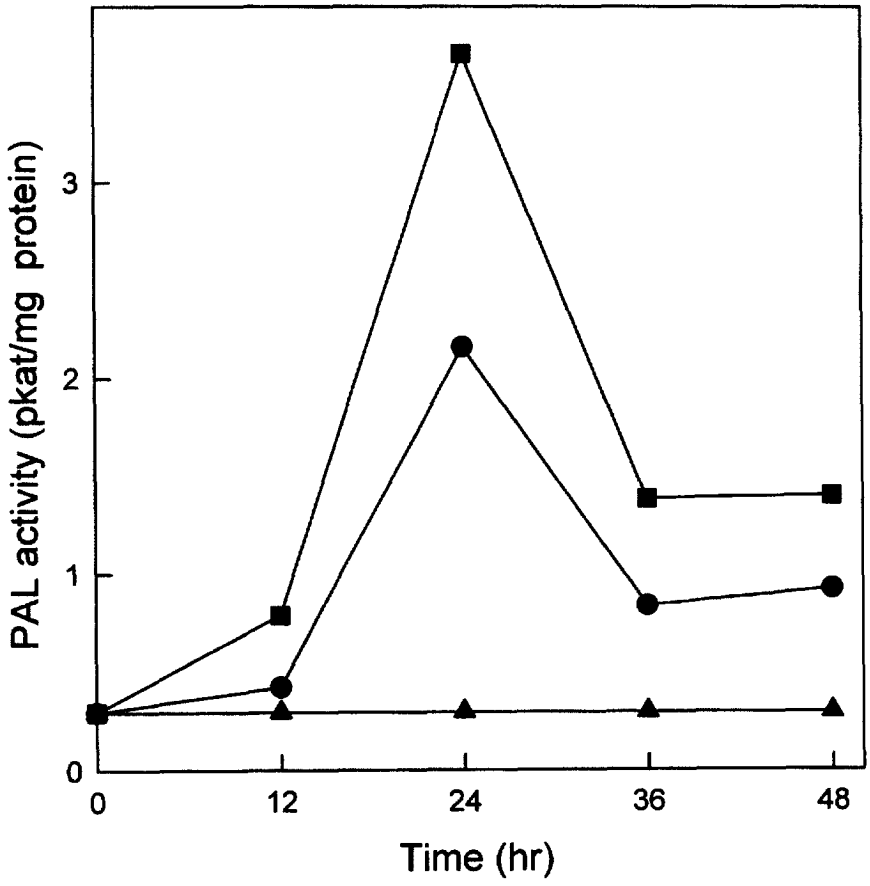

Fig. 1. Effect of excision on PAL activity of tomato leaves. The tomato seedlings were grown under continuous light for three weeks. Three grams (fr. wt) of tomato leaves were excised and were either incubated in dark (●) or in continuous light (■). The control leaves (▲) were obtained from intact seedlings. At the time intervals indicated, leaves were homogenised and the homogenate was processed till protamine sulphate step. PAL activity and protein were determined after dialysing the supernatant obtained after protamine sulphate precipitation.

Table 1. Purification of PAL from excised leaves of tomato

Purification step	Total activity (pkat)	Protein (mg)	Specific activity (pkat/mg)	Purification (fold)	Yield (%)
Protamine sulphate	1460	272	5.36	1	100
Phenyl-Sepharose	1320	8	165	30.7	90
Gel filtration	900	2	450	83.9	61.6
DEAE-Sepharose					
Total	862	1.6	539	101	59
PAL-I	88	0.1	880	164	6.0
PAL-II	515	0.5	1030	192	35.2
PAL-III	259	1.0	259	48.3	17.7

Purification of PAL

PAL was purified from tomato leaves in a 4-step procedure including protamine sulphate precipitation, phenyl-Sepharose, gel filtration and DEAE-Sepharose chromatography (Table 1). Addition of protamine sulphate and acetone wash clarified crude homogenate by removing most of the pigments and phenolic compounds. Thereafter, upon phenyl-Sepharose chromatography, PAL activity eluted as two major peaks, one with Buffer-A wash and another with Buffer-C (data not shown). This step purified PAL by 30–40 fold, with a recovery of 90%. On gel filtration, PAL activity eluted as a single peak with 60% recovery and 84-fold purification. On DEAE-Sepharose chromatography, PAL eluted as three major peaks and these peaks were designated as PAL-I, -II and -III in the order of their elution [Fig. 2(A)]. On chromatofocussing [Fig. 2(B) and (C)] at 7–4 and also at 6–4 pH gradients, the PAL activity resolved into three peaks with the corresponding p*I* values at 5.4, 5.01 and 4.82.

The purity of the PAL isoforms after DEAE-Sepharose step was examined by non-denaturing PAGE. Electrophoresis of individual PAL isoforms showed a single band for PAL-I and PAL-II, however, PAL-III exhibited one major band and two minor bands (data not shown). On SDS-PAGE, each isoform of PAL showed a major band of Mr 80 ± 2 kDa along with two or three minor bands in the range upto 50 ± 5 kDa. It has been observed for several species that the native PAL subunit is inherently unstable *in vitro* and during purification breaks down to yield lower Mr partial degradation products [27, 31, 32]. Western blot analysis of protein bands separated after SDS-PAGE, showed that these bands also cross-reacted with tobacco PAL antibody, indicating that they may share common epitope(s) (Fig. 3, upper inset). Gel chromatography indicated a Mr of 320 kDa (Fig. 3, lower inset), whereas, upon SDS-PAGE, three isoforms of PAL showed a Mr of 80 kDa (Fig. 3), indicating that PAL may be a tetramer with four subunits.

Kinetic properties

The initial velocity of PAL activity was analysed for the PAL isoform mixture and also for individual PAL isoforms. The Lineweaver–Burk plot [Fig. 4(A)] of enzyme eluted from the phenyl-Sepharose step, which contained all the PAL isoforms, gave a typical downward curvilinear graph with a maximum and a minimum apparent K_m values of 1120 μM and 60 μM, respectively [Fig. 4(A)]. However, when the kinetic analysis of individual PAL isoforms was performed, each form exhibited a normal Michaelis–Menten saturation pattern [Fig. 4(B)–(D)], i.e. a straight line on a Lineweaver–Burk plot with individual K_m values of PAL-I = 840 μM, PAL-II = 121 μM and PAL-III = 465 μM. This suggests that the non-linear plot obtained for the mixture is a result of varying K_m values of the individual isoforms. A large difference in K_m values also suggests the structural and functional dissimilarity between the enzymes. Since K_m is a property that is directly related to the pH of the medium, we examined the pH optimum for each PAL isoform. Figure 5 shows that PAL-II and PAL-III have pH optima at 8.8 and 9.5, respectively, whereas, for PAL-I, the pH optimum is at 10.5.

Differential induction of PAL isoforms

To monitor the specific induction in levels of individual PAL isoforms on excision of leaves, the relative levels of each PAL isoform were analysed by ion-exchange chromatography at different time intervals after excision. In the intact leaves, all three PAL isoforms were at nearly similar levels. Figure 6 shows that the excision-mediated increase in PAL activity in leaves incubated in light is mainly contributed by increase in the levels of PAL-II and PAL-III. Both isoforms showed a maximal level at 24 h, which is 6-fold higher for PAL-II and 3-fold higher for PAL-III, compared to their basal levels. For leaves incubated in dark, the magnitude of PAL induction was less than that in light-incubated leaves and PAL-III was stimulated more than PAL-II. By contrast, the excision and light treatment had no substantial effect on the activity of PAL-I which

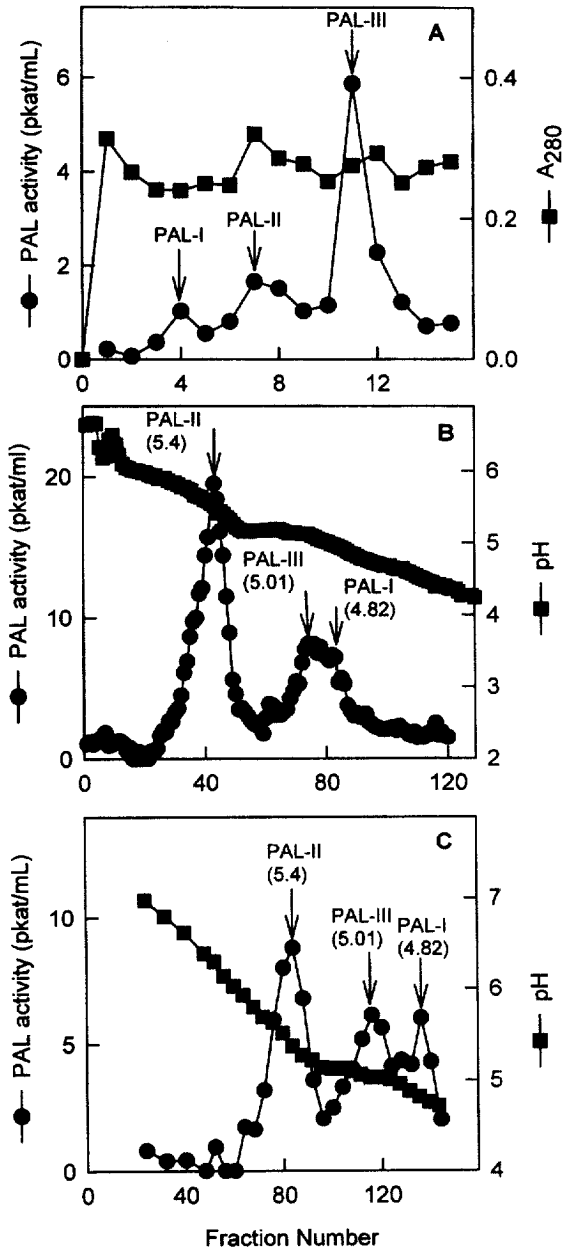

Fig. 2. Analysis of PAL activity by ion-exchange and chromatofocussing chromatography. A, Elution profile of tomato PAL extracted from the un-induced leaves on DEAE-Sepharose CL 6B. PAL fraction obtained from the gel filtration step was loaded onto DEAE-Sepharose column and the enzyme was eluted with a step gradient of NaCl (0.0–0.4 M). PAL-I was eluted at 0.0 mM, PAL-II at 100 mM, and PAL-III at 300 mM salt concentration. B, Elution profile of PAL activity extracted from induced (excision-light exposed) leaves on PBE-94 chromatofocussing column. PAL fraction obtained from phenyl-Sepharose step was loaded onto PBE-94 column. PAL was eluted with polybuffer-74 over a pH range of 6–4. C, Elution profile of PAL activity from induced (excision-light exposed) leaves on PBE-94 chromatofocussing column over a pH range of 7–4. The values in parenthesis indicate p*I* of respective PAL isoforms.

remained at steady level during the time period analysed.

Effect of phenylpropanoid metabolites on PAL isoforms

The possibility of individual PAL isoforms regulating different branch pathways of phenylpropanoid metabolism was examined by incubating several phenolic compounds with inducible PAL isoforms i.e., PAL-II and PAL-III. Table 2 shows that PAL-II and PAL-III were inhibited by these compounds in a differential fashion. While PAL-III was sensitive to metabolites of phenolic acid pathway, only high concentrations of these were inhibitory to PAL-II. However, both PAL-II and PAL-III, were insensitive towards *p*-hydroxybenzoic acid, excluding the possibility of the involvement of these isoforms in the biosynthesis of benzoic acid derivatives. Quercetin, a flavanol, has an opposite effect on these forms, i.e. it was inhibitory to PAL-III with increasing concentrations, whereas, at the same concentrations, it activated PAL-II. In contrast, naringenin had no effect on PAL-II, but inhibited PAL-III with increasing concentrations. These results indicate that PAL-III isoform is probably associated with the phenolic acid biosynthetic pathway, whereas PAL-II may be the isoform contributing towards flavanol and anthocyanin biosynthetic pathways.

DISCUSSION

The PAL activity of tomato leaves was contributed by three isoforms of which the levels of two isoforms (PAL-II and PAL-III) were stimulated by excision and light. Such selective induction of individual PAL isoforms may be regulated at the level of expression of PAL genes. Several reports have shown that the injury to plants caused by wounding or excision initiates a set of responses such as production of hormones like systemin and jasmonic acid, which in turn stimulate the expression of genes regulating pathogenesis related proteins, enzymes involved in lignification and protective metabolites encoded by the phenylpropanoid pathway [3]. It is likely that the observed stimulation of PAL isoforms is a part of overall defense strategy of plants against injury. The mechanism underlying excision-mediated induction of PAL isoforms is not known and can only be speculated. In tomato cotyledons, the light-mediated PAL induction was suppressed by inhibitors of the transcription and translation indicating an involvement of gene expression and translation [30]. In addition, the expression of PAL appears to be regulated at multiple loci in a stimulus specific fashion, the wounding and infection of tomato stimulated the transcription of PAL5 gene [33]. However in tomato isolines resistant to infection by *Verticillium*

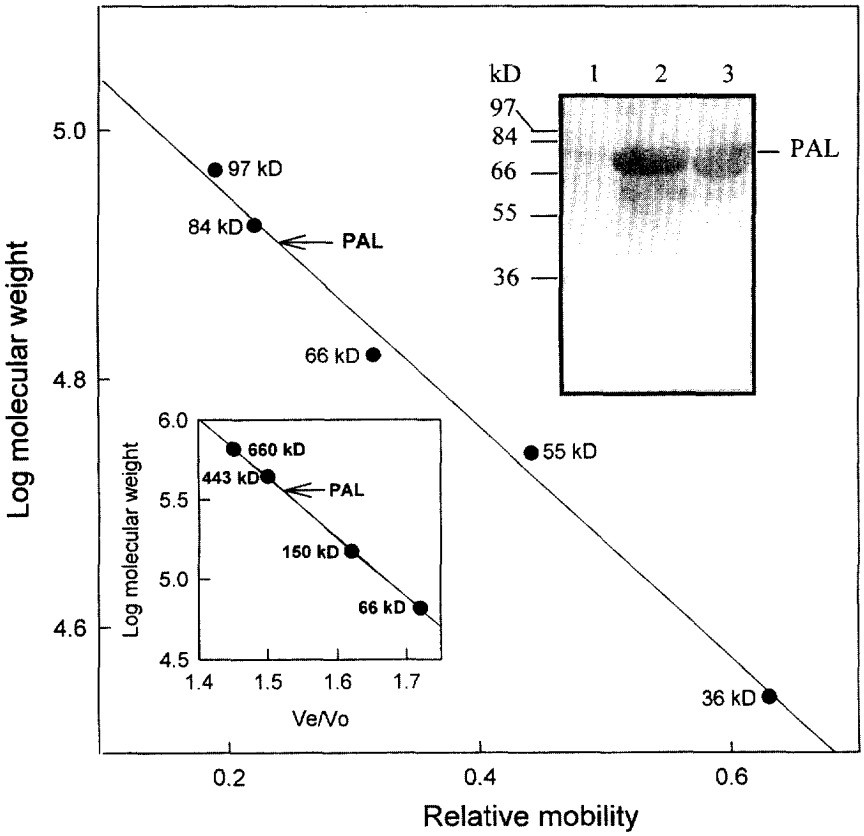

Fig. 3. Determination of Mr of PAL. The PAL subunit Mr was estimated by SDS-PAGE, using a calibration curve obtained for relative mobilities of markers run on a 12% acrylamide gel (see, materials and methods). The upper inset shows the Western blot of PAL after SDS-PAGE; Lane 1, crude-extract; Lane 2, pooled fraction from phenyl-Sepharose and Lane 3, pooled fraction from gel-filtration. The Mr of standard proteins are indicated on the left margin of the inset. The lower inset shows calibration curve used to determine the native Mr of PAL by gel-filtration chromatography on a Sepharose CL-6B column.

albo-atrum, infection-induced increase in PAL activity was not accompanied by a corresponding increase in PAL mRNA levels, indicating an additional regulatory step at post-transcriptional level [34].

The phenylpropanoid pathway, of which PAL is a key enzyme, synthesizes several molecules, of which compounds such as coumarins, chlorogenic acid, wall bound phenolics and lignin, etc. are specifically produced on injury [3]. It is likely that the induced PAL isoforms may contribute specifically to the formation of these compounds. This possibility was examined by purifying PAL isoforms and comparing properties of the inducible isoforms. PAL was purified from tomato leaves using a four step procedure with a final separation of isoforms by DEAE-Sepharose column. The procedure adapted in our study has higher yields and specific activity of PAL than the one used earlier for tomato cell suspension cultures [29]. Moreover, in tomato cell suspension cultures, only a single isoform of PAL was observed, whereas, in leaves, at least three isoforms of PAL were present [Fig. 2(A)]. The chromatofocussing of PAL confirmed the existence of three isoforms of PAL and observed p*I* values for isoforms were in a range reported for PAL isoforms from other species [31, 27].

The native Mr of PAL (320 kDa) obtained on gel chromatography and subunit Mr (80 ± 2 kDa) on SDS-PAGE indicated that, in native form, PAL exists as a tetramer. The observed Mr value of 80 kDa for a PAL subunit corresponds with the predicted Mr from tomato PAL gene sequence that encodes a 721 amino acid polypeptide [16], but is at variance with the observed 74000 Mr for PAL purified from elicitor-treated tomato cell cultures [29]. While reasons for difference in Mr of PAL purified from tomato leaf and cell culture is not known, in the *Phaseolus vulgaris* suspension cultured cells, stationary phase had a 83 kDa PAL isoform, whereas elicitor treated cells had a 77 kDa PAL isoform [35].

PAL is also regarded as a kinetically complex enzyme that exhibits non-Michaelis–Menten behavior exhibiting negative cooperativity with respect

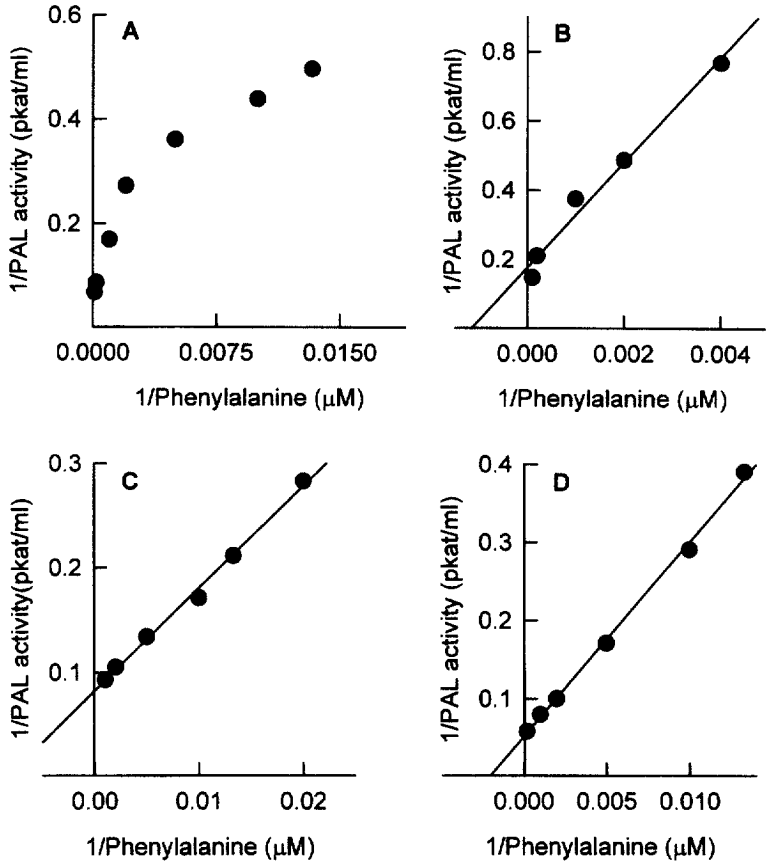

Fig. 4. Lineweaver–Burk plots of initial rate data for tomato PAL. A, The PAL fraction obtained from phenyl-Sepharose step containing all three isoforms. B, PAL-I, C, PAL-II, D, PAL-III obtained after ion-exchange step. Initial rate is the value obtained as A_{290} hr^{-1} at varying concentrations of phenylalanine, against a blank without phenylalanine.

to its substrate, L-phenylalanine [7, 28], and the same was observed for tomato PAL, when kinetic properties of a mixture of PAL isoforms were analysed. However, the above kinetic behaviour was a consequence of the presence of PAL isoforms, each exhibiting different K_m values for phenylalanine, leading to nonlinear kinetics as a function of its concentration. Individually, each PAL isoform showed a normal Michaelis–Menten kinetics, but they differed with respect to their affinity towards L-phenylalanine. Overall, our results indicate that similar to species such as alfalfa and *Vicia faba*, which have multiple isoforms of PAL [27, 28], tomato leaf also possesses PAL isoforms bearing distinct kinetic properties and isoelectric points, but having identical native and subunit Mr.

PAL is a tetrameric enzyme and as reported in several species, is encoded by a multigene family. It is possible that the expression of multiple genes in combination with post-translational modifications may give rise to hetero-tetrameric enzymes, which may differentially regulate a pathway. There is significant divergence in amino acid sequence of three bean PAL genes, which may signify the potential for functional differences in properties of encoded proteins [11]. However, Appert *et al.* [36], found that individual cDNA clones of parsley PAL when expressed in *E. coli* assembled into the corresponding homotetramer, exhibiting identical kinetic properties with similar K_m (15–24.5 μM), temperature and pH optima, which was in contrast to the differential expression of these PAL isoforms in various parts of parsley plants. They suggested that the occurrence of multiple gene copies may also have other functions in addition to encoding multiple isozymes.

In tomato, PAL gene family consists of at least five genes, but DNA sequence analysis showed that among these, PAL1, PAL3 and PAL4 harbor premature stop codons, which may result in the generation of truncated inactive polypeptides [16, 37], hence these genes may be either inactive or pseudogenes. Assuming the remaining two PAL genes to be actively transcribed and translated, the observed three isoforms of PAL may be encoded by these genes. Alternatively, the PAL isoforms may arise as a consequence of post-translational modifications of products of one or more genes.

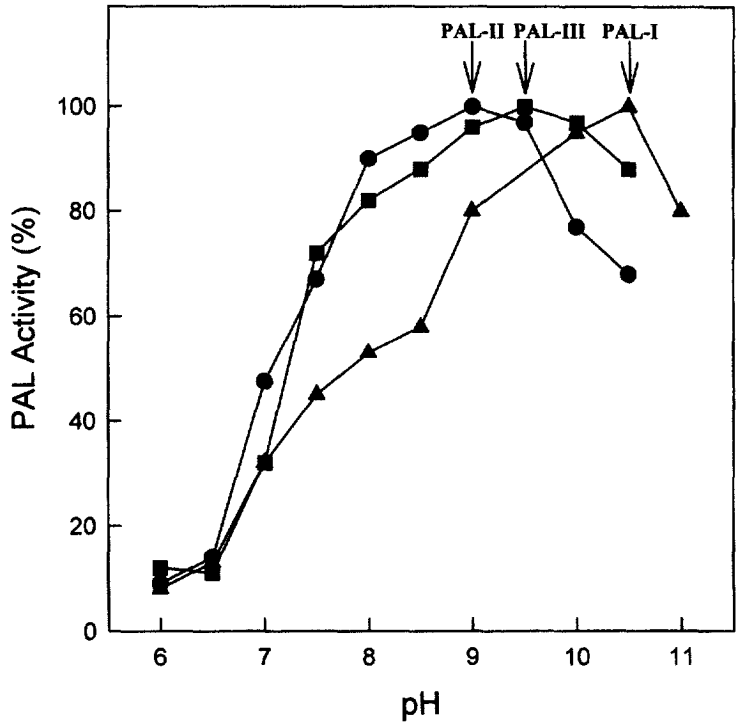

Fig. 5. Activity–pH profiles of different PAL isoforms. Individual PAL isoforms obtained after DEAE-Sepharose were assayed for their optimal pH for activity using different buffers ranging from pH 5-11 (for details, see text). For each isoform, activity is represented as percentage of maximum activity obtained at optimal pH.

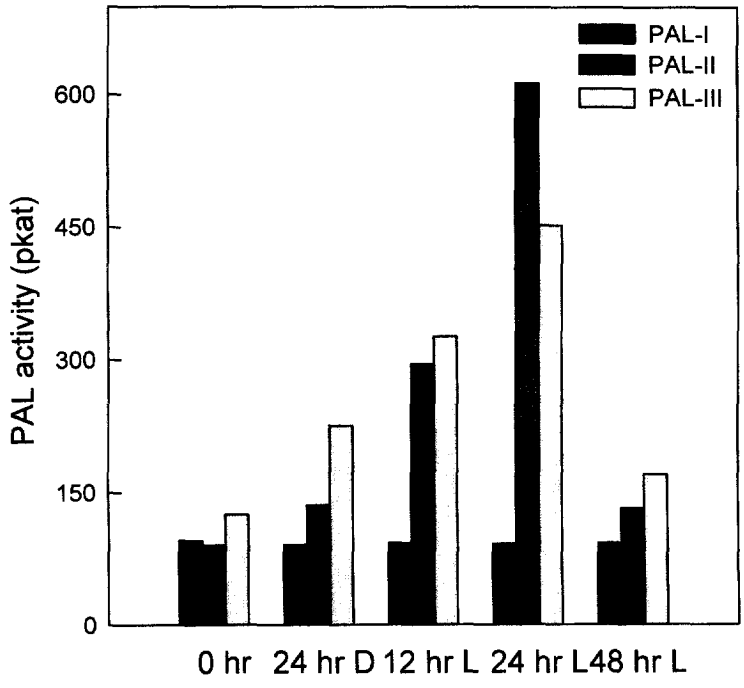

Fig. 6. Effect of excision and light treatment on induction of different PAL isoforms. Three-week-old tomato leaves were excised and were either incubated in light (L) or dark (D). At time points indicated, PAL was extracted, purified till DEAE-Sepharose step. Relative amounts of the three isoforms were compared against their individual basal levels at zero time.

Table 2. Effect of various phenylpropanoid compounds on activity of PAL-II and PAL-III isoforms. PAL-II and PAL-III isoforms obtained after DEAE-Sepharose chromatography were assayed for 1 h at 37°C in the presence of 1 mM phenylalanine and varying concentration of individual phenolic metabolites. The PAL activity is expressed as percent residual PAL activity with respect to control enzyme sample without phenolic compounds. Each value is a mean of three experiments

Inhibitor type	Inhibitor concentration (μM)	Residual PAL activity (%)	
		PAL-II	PAL-III
Cinnamic acid	50	70.9	0.00
	100	47.0	0.00
	250	0.66	0.00
p-Coumaric acid	50	76.1	0.00
	100	31.1	0.00
	250	0.0	0.00
Ferulic acid	50	73.1	0.00
	100	48.1	0.00
	250	0.0	0.00
Caffeic acid	50	86.8	91.3
	100	96.0	68.5
	250	204	0.0
p-Hydroxy-benzoic acid	50	120	119
	100	111	98.3
	250	112	76.0
Naringenin	50	108	125
	100	105	94.0
	250	73.7	50.5
Quercetin	50	84.0	95.0
	100	109	87.0
	250	126	45.4

While this possibility has not been examined *in vivo*, McKegney *et al.* [38] observed that a single PAL gene over-expressed in insect cell cultures produced differently charged subunits on two dimensional-IEF-PAGE, indicating that post-translational modifications can generate small charge differences. It is possible that such modifications of the PAL subunits may cause the formation of multiple PAL isoforms. Equally plausible is the possibility that a single gene may possess alternate initiation sites which may induce a specific isoform. In fact, in tomato, the promotor of PAL5 gene possesses two initiation sites, encoding a shorter and longer transcript of which the longer transcript was expressed constitutively. The environmental signals such as light and wounding caused a stimulation in accumulation of shorter transcript of PAL [33]. Though our study has shown three isoforms of PAL, whether these are encoded by a single or different PAL gene can be answered only after the amino acid sequencing of individual PAL isoforms and comparison with the available gene sequences.

The stimulus-specific induction of PAL isoforms may have an *in vivo* function to channel the products into a specific branch of the phenylpropanoid pathway. In tomato leaf, all three isoforms are constitutively expressed and excision and light specifically induced only PAL-II and PAL-III isoforms. Thus the induction of these isoforms may accelerate the flux into the general phenylpropanoid pathway is indicated by the observation that both the inducible isoforms have lower K_m value and thus have a high affinity for phenylalanine. Similarly, in bean and alfalfa, exposure to a fungal elicitor leads to preferential induction of PAL isoforms with lower K_m values [27, 31], indicating that these isoforms may regulate the production of specific metabolites related to stress responses.

The results on incubation of individual isoforms with the metabolites of phenylpropanoid pathway indicate such a possibility, that in addition to differential expression, the inducible PAL isoforms may modulate the biosynthesis of specific metabolites. It has been suggested that there may be channeling of substrate during the initial steps of phenylpropanoid pathway [39] and PAL activity may be a key locus in the regulation of overall flux into the pathway [8, 9]. The feedback inhibition of PAL-III activity by cinnamic acid, coumaric acid, caffeic acid and ferulic acid, intermediates of lignin and also chlorogenic acid biosynthesis pathway, suggests that PAL-III may regulate the flux into this pathway. In transgenic tobacco, the over-expression of bean *PAL-2* increased the flux into the chlorogenic acid [9]. Both PAL isoforms appear to cross regulate each other as caffeic acid, which inhibits the PAL-III activity, activates PAL-II at higher concentration. Among the two isoforms, PAL-II may increase the flux into anthocyanin biosynthesis, as higher concentrations of quercetin stimulated PAL-II activity. In contrast, the single PAL isoform purified from tomato cell cultures, showed no inhibition with cinnamate, caffeate, quinate and chlorogenate, indicating that this isoform may regulate a different phenylpropanoid branch pathway [29]. The information on feedback inhibition of PAL isoforms in other species is limited. In *Oenothera*, among the two PAL isoforms present, there was some evidence that only PAL-2 was involved in flavonoid biosynthesis [25]. In oak, benzoic acid and its derivatives inhibited one PAL isoform; caffeic and ferulic acids were inhibitory to the other PAL isoform, whereas gallic acid activated both PAL isoforms [22]. Thus, it is reasonable to speculate that the individual tomato PAL isoforms may be associated with different pathways that are differentially regulated and might even be localized in distinct tissues.

EXPERIMENTAL

Plant growth conditions

Tomato (*Lycopersicon esculentum* cv pusa early dwarf)) seeds were surface sterilised for 5 min with 0.01% (w/v) NaOCl solution, followed by washing with H_2O. The seeds were sown in soilrite (vermicu-

lite and peat mixture) and grown at $25 \pm 2°$ under continuous white light (100 μmol m^{-2} s^{-1}) obtained by using three white (Phillips BA 85)) and one (Phillips BA 82) tubelights. Seedlings were irrigated with H_2O for 3 weeks and the leaves were then harvested. The excised leaves were incubated on a filter paper moistened with H_2O either under continuous light or in darkness. For PAL purification, the excised leaves were incubated in light for 24 h, frozen in liquid N_2 and processed as described below.

Buffers

The following buffers were used: Buffer-A: 0.1 M K–Pi, pH 7 containing 1 mM PMSF and 10 mM β-mercaptoethanol; Buffer-B: Buffer-A containing ammonium sulphate (25% w/v); Buffer-C: Buffer-A containing 10% (v/v) ethylene glycol; Buffer-D: 50 mM Tris–HCl, pH 8 containing 1 mM PMSF and 10 mM β-mercaptoethanol and Buffer-E: 20 mM Tris–HCl, pH 8 containing 1 mM PMSF and 10 mM β-mercaptoethanol.

Data analysis

All the experiments were repeated a minimum of 3–5 times and the representative figures were presented here.

Determination of PAL activity

PAL activity was assayed using a method described in Ref. [40], in a reaction mixture containing 0.1 M borate buffer, pH 8.8, 10 mM L-phenylalanine and an aliquot of enzyme in a total volume of 1.5 ml. The reaction was carried out for 60 min at 37° and the increase in A_{290} nm was recorded at every 15 min interval. The rate of formation of *trans*-cinnamic acid was taken as a measure of enzyme activity using an increase in 0.01 *A* at A_{290} nm as 3.09 nmol of *trans*-cinnamic acid formed [40]. The PAL activity was expressed in pkat (pmol *trans*-cinnamic acid formed per second). Protein levels were determined by the Bradford dye-binding assay [41] using BSA as a standard.

The effect of phenylpropanoid metabolites on PAL activity was examined by adding respective metabolites to PAL assay mixture. The phenolic compounds were dissolved in 10–50% (v/v) ethanol to make stock solutions (10 mM). The final concentration of phenolics in the PAL assay mixture was 50–250 μM and that of phenylalanine 1 mM. In the control assay mixture an equivalent amount of EtOH was added. The PAL assay was carried out as described above.

Purification of PAL

All purification steps were carried out at 4° unless otherwise specified. 30 g (fr. wt) of excised tomato leaves were ground in liquid N_2 and the powder was washed with 500 ml of chilled acetone (−20°) on a Buchner funnel. The residual acetone was removed by vacuum aspiration. The powder was then homogenised in 50 ml of Buffer-A in the presence of 10–15 g of acid-washed sea sand and 4 g of polyvinylpolypyrrolidone. The homogenate was then diluted with a further addition of 50 ml of Buffer-A and was filtered through a muslin cloth and centrifuged (20000*g*, 30 min). To the resulting supernatant, 10% (w/v) protamine sulphate was added with constant stirring to reach a final concentration of 1.0% and the suspension was centrifuged at 20000*g* for 30 min. Solid ammonium sulphate was then added to the supernatant to reach 25% saturation and the solution was applied at a flow rate of 2 ml min^{-1} to a phenyl-Sepharose column (1.6 × 20 cm) pre-equilibrated with Buffer-B at 25°. The column was washed with 100 ml of Buffer-B and subsequently eluted with 100 ml of Buffer-A, followed by 100 ml of Buffer-C. Fractions (10 ml) exhibiting PAL activity were pooled and concentrated by ultrafiltration to about 1.0–1.5 ml.

The concentrate was then applied to a gel-filtration column (Sepharose CL-6B, 1.0 × 100 cm) pre-equilibrated with Buffer-D and 1 ml fractions were collected. Fractions exhibiting PAL activity were combined and concentrated by ultrafiltration to about 1–2 ml. The concentrate was applied to a DEAE-Sepharose column (1 × 12 cm) pre-equilibrated with Buffer-E. Protein was eluted with a step-gradient of NaCl (0.0–0.4 M, 0.1 M step), each step elution was carried out with 100 ml. The amount of protein in fractions was monitored at A_{280} nm and the PAL activity by the method described above. For the determination of pH optima and K_m for phenylalanine for the 3 isoforms, enzyme fractions were concentrated by ultrafiltration to normalise the salt concentration.

For the time course of PAL induction, 3 g (fr. wt) of excised leaves were harvested at different time intervals and, after freezing in liquid N_2, the samples were processed till protamine sulphate step of purification procedure. The supernatant obtained was dialysed for 8–10 h against 2–3 changes of Buffer-A and the PAL activity and total protein were measured as described in Section 4.4.

Determination of Mr

The Mr of PAL was estimated by gel-filtration chromatography on a Sepharose CL-6B column (1.0 × 100 cm). The column was pre-equilibrated with Buffer-D and was calibrated with the following protein standards: thyroglobulin (660 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa) and BSA (66 kDa). A standard plot was made by plotting the elution volume of standard proteins against logarithm of their Mr and by comparing the elution peak of PAL activity, its Mr was determined.

The Mr of PAL subunits was determined by SDS-PAGE [42] using a 12% separation gel. A standard plot was made using the following standard proteins: rabbit muscle phosphorylase-b (97.4 kDa), rabbit muscle fructose-6-phosphate kinase (84 kDa), BSA (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa) and glyceraldehyde-3-phosphate dehydrogenase (36 kDa).

Electrophoresis and Western analysis

Non-denaturing electrophoresis was performed on 7% (w/v) polyacrylamide gels according to the method described in Ref. [43]. SDS-PAGE was performed with 12% (w/v) acrylamide in the resolving gel. Following electrophoresis, proteins were stained with Coomassie brilliant blue R-250.

Western blotting of purified and partially purified PAL preparations were carried out using the method described previously [44]. Anti-(tobacco PAL) serum (1:400 diluted) as primary antibody and anti-rabbit IgG coupled with alkaline-phosphatase as secondary antibody were used.

Determination of pI

The p*I* values of individual isoforms of tomato PAL were determined by chromatofocussing performed on a PBE-94 column (1.3 × 18 cm) pre-equilibrated with 25 mM imidazole–HCl buffer, pH either 7.4 (for pH 7.0–4.0 gradient) or 6.2 (for pH 6.0–4.0 gradient). The pooled PAL fraction obtained from phenyl-Sepharose chromatography was concentrated by ultrafiltration and was equilibrated with polybuffer-74. The concentrate was applied to the chromatofocussing column and elution was carried out at a flow rate of 30 ml h^{-1} with polybuffer-74 which was diluted 1:8 (v/v) with H_2O and adjusted to pH 4.0. Fractions of 1.5 ml were collected, protein, PAL activity and pH were monitored.

Determination of pH optima

The pH optima for individual PAL isoforms obtained after DEAE-Sepharose chromatography were determined by using the following buffers at 100 mM in the assay mixture: Na citrate or NaOAc buffer (pH 4.0–5.0), MES (pH 6.0), MOPS (pH 6.5), Na–Pi (pH 7.0–7.5), Tris–HCl (pH 8.0–8.5), K borate (pH 9.0–9.5), Na_2CO_3/$NaHCO_3$ (pH 10–11). The activities of PAL isoforms were normalized to 100% at peak value to facilitate comparison.

Acknowledgements—This work was supported by the Department of Science and Technology, New Delhi grant SP/SO/A-30/93 to R. S. Y. S. L. is a recipient of senior research fellowship from the Council of scientific and Industrial Research, New Delhi. We also gratefully acknowledge the gift of tobacco PAL antibody from Professor M. Okazaki, Shinshu University, Ueda, Japan.

REFERENCES

1. Hahlbrock, K. and Scheel, D., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1989, **40,** 347.
2. Harborne, J. B., *The Flavonoids: Advances in Research since 1980*. Chapman and Hall, New York, 1988.
3. Dixon, R. A. and Paiva, N. L., *The Plant Cell*, 1995, **7,** 1085.
4. McClure, J. W., Physiology and functions of flavonoids. In *The Flavonoids*, eds. J. B. Harborne, T. J. Mabry and H. Mabry. Chapman and Hall, London, 1975, p. 970.
5. Binns, A. N., Chen, R. H., Wood, H. N. and Lynn, D. G., *Proceedings of National Academy Science U.S.A.*, 1987, **84,** 989.
6. Lynn, D. G. and Chang, M., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1990, **41,** 497.
7. Jones, H. D., *Phytochemistry*, 1984, **23,** 1349.
8. Bate, N. J., Orr, J., Ni, W., Meromi, A., Nadler-Hassar, T., Doerner, P. W., Dixon, R. A., Lamb, C. J. and Elkind, Y., *Proceedings of National Academy of Science U.S.A.*, 1994, **91,** 7608.
9. Howles, P. A., Sewalt, V. J. H., Paiva, N. L., Elkind, Y., Bate, N. J., Lamb, C. and Dixon, R. A., *Plant Physiology*, 1996, **112,** 1617.
10. Ohl, S., Hedrick, S. A., Chory, J. and Lamb, C. J., *The Plant Cell*, 1990, **2,** 837.
11. Cramer, C. L., Edwards, K., Dron, M., Liang, X., Dildine, S. L., Bolwell, G. P., Dixon, R. A., Lamb, C. J. and Schuch, W., *Plant Molecular Biology*, 1989, **12,** 367.
12. Lois, R., Dietrich, A., Hahlbrock, K. and Schulz, W., *EMBO Journal*, 1989, **8,** 1641.
13. Yamada, T., Tanaka, Y., Spriprasertsak, P., Kato, H., Hashimoto, T., Kawamata, S., Ichinose, Y., Kato, H., Shiraishi, T. and Oku, H., *Plant Cell Physiology*, 1992, **33,** 715.
14. Subramaniam, R., Reinold, S., Molitor, E. K. and Douglas, C. J., *Plant Physiology*, 1993, **102,** 71.
15. Minami, E.-I., Ozeki, Y., Matsuoka, M., Koizuka, N. and Tanaka, Y., *European Journal of Biochemistry*, 1989, **185,** 19.
16. Lee, S. W., Robb, J. E. and Nazar, R. N., *Journal of Biological Chemistry*, 1992, **267,** 11824.
17. Liang, X., Dron, M., Cramer, C. L., Dixon, R. A. and Lamb, C. J., *Journal of Biological Chemistry*, 1989, **264,** 14486.
18. Liang, X., Dron, M., Schmid, J., Dixon, R. A. and Lamb, C. J., *Proceedings of National Academy of Science U.S.A.*, 1989, **86,** 9284.
19. Shufflebottom, D., Edwards, K., Schuch, W. and Bevan, M., *Plant Journal*, 1993, **3,** 835.
20. Diallinas, G. and Kanellis, A. K., *Plant Molecular Biology*, 1994, **26,** 473.

21. Hatton, D., Sablowski, R., Yung, M. H., Smith, C., Schuch, W. and Bevan, M., *Plant Journal*, 1995, **7,** 859.
22. Alibert, G., Ranjeva, R. and Boudet, A., *Biochimica Biophysica Acta*, 1972, **279,** 282.
23. Creasy, L. L., *Physiologia Plantarum*, 1987, **71,** 389.
24. Bolwell, G. P., *Phytochemistry*, 1992, **31,** 4081.
25. Neumann, G. and Schwemmle, B., *Journal of Plant Physiology*, 1993, **142,** 135.
26. Ishizuka, M., Yamada, F., Tanaka, Y., Takeuchi, Y. and Imaseki, H., *Plant Cell Physiology*, 1991, **32,** 57.
27. Jorrin, J. and Dixon, R. A., *Plant Physiology*, 1990, **92,** 445.
28. Lopez-Valbuena, R., Obrero, R., Jorrin, J. and Tena, M., *Plant Physiology and Biochemistry*, 1991, **29,** 159.
29. Bernards, M. A. and Ellis, B. E., *Plant Physiology*, 1991, **97,** 1494.
30. Goud, K. V., Sharma, R., Kendrick, R. E. and Furuya, M., *Plant Cell Physiology*, 1991, **32,** 1251.
31. Bolwell, G. P., Bell, J. N., Cramer, C. L., Schuch, W., Lamb, C. J. and Dixon, R. A., *European Journal of Biochemistry*, 1985, **149,** 411.
32. Bolwell, G. P., Sap, J., Cramer, C. L., Lamb, C. J., Schuch, W. and Dixon, R. A., *Biochimia Biophysica Acta*, 1986, **881,** 210.
33. Lee, S. W., Heinz, R., Robb, J. E. and Nazar, R. N., *European Journal of Biochemistry*, 1994, **226,** 109.
34. Lee, S. W., Nazar, R. N., Powell, D. A. and Robb, J. E., *Plant Molecular Biology*, 1992, **18,** 345.
35. Bolwell, G. P. and Rodgers, M. W., *Biochemical Journal*, 1991, **279,** 231.
36. Appert, C., Logemann, E., Hahlbrock, K., Schmid, J. and Amhrein, N., *European Journal Biochemistry*, 1994, **225,** 491.
37. Yeo, Y. S., Lee, S. W. and Chang, Y. D., *Journal of Korean Society of Horticultural Sciences*, 1996, **37,** 537.
38. McKegney, G. R., Butland, S. L., Theilmann, D. and Ellis, B. E., *Phytochemistry*, 1996, **41,** 1259.
39. Hradzina, G. and Jensen, R. A., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1992, **43,** 241.
40. Saunders, J. A. and McClure, J. W., *Phytochemistry*, 1975, **14,** 1285.
41. Bradford, M. M., *Analytical Biochemistry*, 1976, **72,** 1151.
42. Laemmli, U. K., *Nature*, 1970, **227,** 680.
43. Davies, B., *Annals of New York Academy of Sciences*, 1964, **121,** 404.
44. Towbin, H., Stahekin, T. and Gordon, J., *Proceedings of National Academy of Science U.S.A.*, 1979, **76,** 4350.