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Purification and characterization of UV-B induced phenylalanine ammonia-lyase from rice seedlings

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Abstract

On exposure of rice seedlings to UV-B radiations, a four fold induction of PAL activity was observed. Purification of induced PAL revealed the presence of a single isoform with a molecular mass 320 kD and a subunit Mr of 84 kD. The enzyme exhibited Michaelis-Menten kinetics with a Km of 500 μM, optimal pH 8.9 and an activation energy 1.9 kcal. The induced enzyme was sensitive to inhibition by phenylpropanoid intermediates involved in flavonol and anthocyanin biosynthetic pathway. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Oryza sativa; Gramineae; Rice; UV-B radiation; Phenylalanine ammonia-lyase

1. Introduction

The increasing amounts of UV-B radiation reaching the earth's surface due to depleted levels of stratospheric ozone layer poses a serious concern regarding the deleterious effects of these radiations to the biosphere (Anderson, Toohey, & Brune, 1991; Caldwell, Robberecht, & Billings, 1980). Several reports indicate various adverse effects, especially to plants. Prolonged exposure to UV-B light results in decreased dry weight, photosynthetic efficiency and growth rate of plants (Dai, Coronel, Vergava, Barnes, & Quintos; Teramura, Ziska, & Sztein, 1991; He, Huang, & Whitecross, 1994). However, plants possess a variety of mechanisms to ameliorate the damage caused by UV light; such as, on exposure to UV radiation, photo-repair of DNA is enhanced (Pang & Hays, 1991), synthesis of UV-B absorbing compounds is stimulated (Beggs, Stoltzer-Jehl, & Wellman, 1985; Li, Ou-Lee, Raba, Amundson, & Last, 1993), general phenylpropanoid and flavonoid metabolism increases (Kubasek, Shirley, Mckillop, Goodman, & Briggs, 1992) and morphological changes such as decreased plant height, leaf area and enhanced leaf thickness occur (Sisson & Caldwell, 1976, 1990). It is not clear whether the above responses occur as a consequence of UV-damage or these are integral part of the protective mechanism of plants.

Among these responses, the accumulation of UV-absorbing compounds such as flavones, flavonols, isoflavonoids and anthocyanins in the vacuoles of the epidermal cell layers seem to provide a selective way to attenuate the UV light. A role for these compounds in UV protection is apparent from the studies in *Arabidopsis*, where mutations that blocked the synthesis of specific group of flavonoids significantly reduced the UV-tolerance of these mutants (Li et al., 1993). However, information on the molecular regulation of flavonoids and their putative role in protection against the deleterious effects of UV light in crop plants is limited. Among the crop plants, rice is one of the largest staple food crops consumed all over the world. It has been observed that exposure to UV-B reduces the photosynthesis and growth of several rice cultivars resulting in severe reduction in yield (Teramura et al., 1991; Ziska & Teramura, 1992; Hidema, Kang, & Kumagai, 1996; Olszyk et al., 1996). Evidently, increase in ambient UV-B radiation due to ozone depletion would severely effect the yield of crop plants.

One of the strategies that can be employed to protect plants against increasing UV-B radiation is by providing effective sunscreens in the form of UV-absorbing compounds in epidermal cell layers (Tevini, Braun, & Fieser, 1991). The flavonoids, which can effectively screen the harmful UV radiation, are synthesized by a complex multi-step phenylpropanoid biosynthetic pathway. Phenylalanine ammonia-lyase (PAL), which is the first enzyme in this biosynthetic pathway catalyses the deamination of phenylalanine to *trans*-cinnamic acid, which in

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turn leads to an array of secondary metabolites. The studies concerning biosynthesis of flavonoids have shown that in several systems, biosynthesis of flavonoids is stimulated by light, and the photo-induced accumulation of flavonoids is preceded by photoinduction of the enzyme, phenylalanine ammonia lyase (Goud, Sharma, Kendrick, & Furuya, 1991; Reddy, Goud, Sharma, & Reddy, 1994).

The studies on transgenic tobacco harboring a bean PAL gene revealed that the regulation of PAL activity was one of the key steps of the phenylpropanoid pathway for the flux control and the channeling of metabolites into various branches (Howles et al., 1996). There are relatively few biochemical studies that have examined the role of PAL in flux control of phenylpropanoid pathway. In species such as *Oenothera*, two isoforms of PAL are present of which only PAL-2 was involved in flavonoid biosynthesis (Neumann & Schwemmle, 1993). It is likely that in species possessing multiple PAL isoforms, the flux into various branches of phenylpropanoid pathway may be regulated by these isoforms either individually or coordinately. However, other species such as sunflower, bamboo, etc, where PAL exists as a single isoform (Jorrin, Rafael, & Tena, 1988; Chen, Chang, & Liu, 1988), may require a more complex mechanism to regulate flux into various branches of phenylpropanoid pathway.

In an earlier study, we observed that the sunlight induces anthocyanin synthesis in seedlings of purple puttu cultivar of rice, which is accompanied with a biphasic induction of PAL activity (Reddy et al., 1994). Of the two sunlight induced PAL peaks, the peaks at 4 and 12 h were induced by phytochrome and UV-B photoreceptor respectively. It was assumed that both photoreceptors induce the same PAL isoform on different temporal scale or two peaks of PAL represent induction of two PAL isoforms. In rice, PAL is encoded by a small multi-gene family and at least one gene of the PAL family possess a light responsive promotor (Minami, Ozeki, Matsuoka, Koizuka, & Tanaka, 1989; Zhu et al., 1995), therefore it is likely that the biphasic PAL induction may represent the induction of two PAL genes. Since PAL has not been purified and characterized so far from rice, there is no information on its physicochemical properties and isoform analysis. In the present study, we purified and characterized PAL from UV-B exposed rice seedlings. We report that UV-B light induces a single PAL isoform and its activity is regulated by specific phenylpropanoid metabolites that accumulated in response to UV exposure. We suggest that UV-B induced PAL plays an important role in the regulation of the biosynthesis of these metabolites.

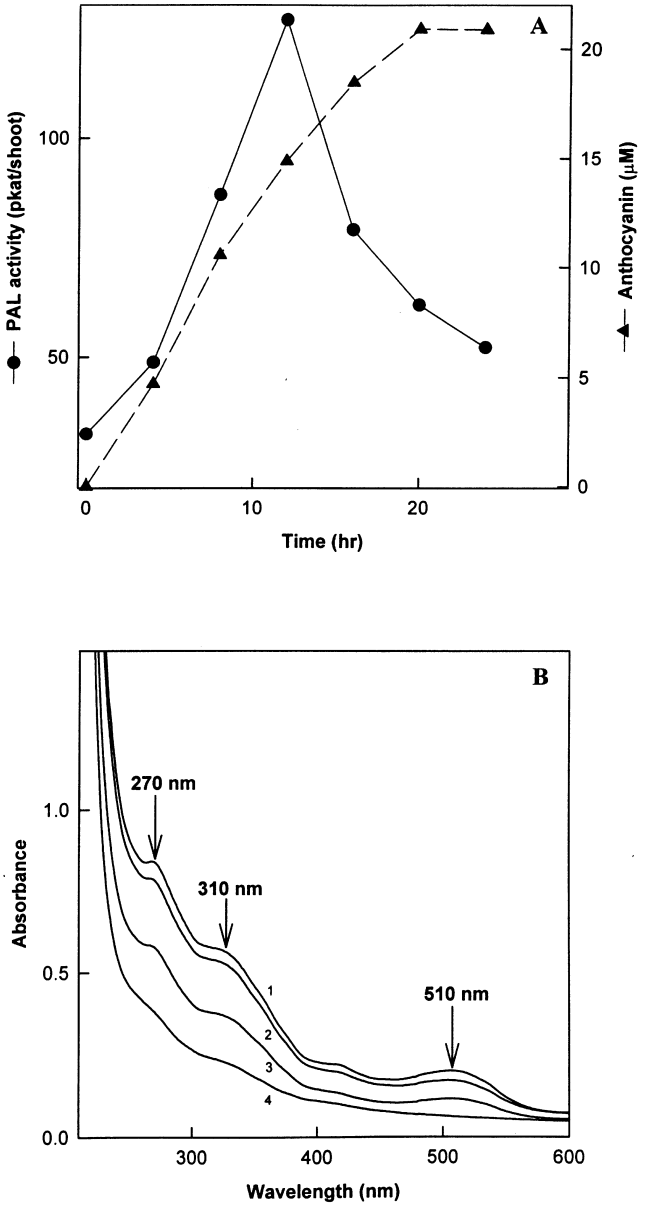

Fig. 1. (A) Time course of PAL activity and anthocyanin accumulation in rice seedlings in response to UV-B exposure. Six-day-old dark-grown seedlings were transferred to continuous UV-B radiation. At the time intervals indicated shoots were harvested and the PAL activity was determined. For anthocyanin determination at the time intervals indicated the seedlings were transferred back to darkness for further 24 h and then the anthocyanin level was determined. (B) For analysis of absorption spectra of total methanolic extracts, at the time intervals indicated the seedlings were transferred back to darkness for further 24 h before extracting the UV-B absorbing compounds and then the spectra's were recorded. (1) 24 h; (2) 16 h; (3) 8 h and (4) 0 h after UV-B exposure.

2. Results and discussion

The profile of PAL induction was examined in rice seedlings exposed to UV-B light. Figure 1 shows that continuous UV-B exposure stimulates the PAL activity with a maxima at 12 h and after that the activity declines to basal level in next 12 h. This observation confirms our earlier result, where we observed that sunlight mediated induction of PAL is mainly due to activation of PAL synthesis by UV-B light. The observed profile of PAL

Table 1
Purification of PAL from UV-B exposed rice seedlings

Purification step	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Purification (fold)	Yield (%)
Protamine sulphate	61	500	0.122	1.0	100
Phenyl Sepharose	19	16	1.187	9.73	31.1
DEAE Sepharose	8.74	5	1.748	14.3	14.3
Chromatofocussing	3.7	0.8	4.625	40.9	6.0

induction is similar to sunlight induced PAL profile albeit without the first peak induced by phytochrome (Reddy et al., 1994). While this result highlights that sunlight mediated PAL induction results from independent stimulation of PAL activity by phytochrome and UV-B photoreceptor on different temporal scale, the question whether the phytochrome-induced and UV-B-induced PAL activity is also spatially separated at tissue/cell level needs to be examined.

A substantial increase in the levels of anthocyanins in the shoots of the seedlings was observed with longer UV-B exposure. The examination of absorption spectra of methanolic extracts of UV irradiated seedlings at different time intervals showed a significant elevation of UV-absorbing pigments. The comparison of spectra of dark-grown seedling extracts with extracts of seedlings exposed to 24 h UV-B shows that in addition to an appearance of anthocyanin peak (510 nm), the spectra also have two distinct shoulders at 270 and 310 nm. On comparing with the known UV-B absorbing compounds extracted in acidified MeOH (Markham, 1982), it was apparent that these shoulders likely arise from increase in the levels of two flavonoid groups; flavonones and dihydroflavonols (Figure 1B). The induction of these compounds were found to be UV-B specific, since seedlings exposed to UV-B light through a 4 mm thick window glass that specifically cuts-off UV-B light (Reddy et al., 1994) showed no increase in PAL activity, anthocyanins and the other UV-B absorbing compounds (data not shown). Thus, the above results indicate a correlation between PAL induction and the accumulation of UV-B absorbing compounds.

The likelihood of the role of PAL in regulating flux into different branches in phenylpropanoid pathway can be examined by monitoring the effect of intermediates or end products of pathway on in vitro activity of purified PAL. Since a 12-h UV-B exposure induces maximal PAL level, these seedlings were used for PAL purification. Table 1 outlines the protocol used for PAL purification. The crude extract was clarified of phenolics by protamine sulphate treatment and subsequently PAL was purified by three sequential chromatographic steps on, phenyl-Sepharose, DEAE-Sepharose and chromatofocussing on PBE-94 Table 1. On phenyl-Sepharose column, the bound PAL activity eluted as a single peak at the beginning of ethylene glycol (EG) gradient. The further increase in the EG concentration did not reveal any additional PAL peaks as observed in several other systems (Jorrin & Dixon, 1990; Sarma, Sreelakshmi, & Sharma, 1998). The phenyl-Sepharose chromatography purified PAL by 10-fold, with a recovery of 31%. Subsequent chromatography of pooled active PAL fractions on DEAE-Sepharose and chromatofocussing by PBE-94 enriched the enzyme by 40-fold. The purified PAL had a specific activity of 4.6 nkat/mg, which is similar to the specific activity values reported for PAL from other sources (Bolwell, Bell, Cramer, Schuch, & Dixon, 1985; Jorrin & Dixon, 1990). The electrophoretic analyses of purified PAL protein obtained from chromatofocussing on both native as well as SDS-PAGE respectively, showed a single band on the gels indicating that the above protocol purified PAL to homogeneity (Figure 2). The fact that the single protein band (inset, Fig. 2B) observed on non-denaturing PAGE belonged to PAL was established by activity staining of PAL in the gel and it corresponded with the position of PAL protein band (data not shown).

The molecular weight of the purified native PAL protein was determined by gel filtration on Sepharose CL 6B gel filtration column (Figure 4A). The PAL protein eluted as a single peak corresponding with an estimated molecular mass of 320 kDa. Electrophoresis on SDS-PAGE showed a single protein band with estimated M_r of 83 ± 2 kDa (inset, Fig. 2A). The comparison of the molecular weights obtained by these methods reveals that PAL holoenzyme is likely to be a 320 kDa tetramer with 4 ca. 80 Kda subunits. The purified rice PAL was found to be an inherently unstable protein, as a consequence of which, lower M_r partial degradation products (50.28 kDa) appear during SDS-PAGE (data not shown). The fragmentation intensifies with excess incubation at high temp, on storage and repeated freeze-thaw. Similarly, in several other systems, native PAL enzyme (Jorrin & Dixon, 1990; Bolwell et al., 1986) is reported to be inherently unstable protein and is prone to fragmentation to lower M_r products during purification and storage.

On chromatofocussing, PAL activity resolved as a single peak bearing an isoelectric point 5.01, there was no evidence for existence of any other minor peaks of PAL in the eluate of this column (Figure 3C). The measure-

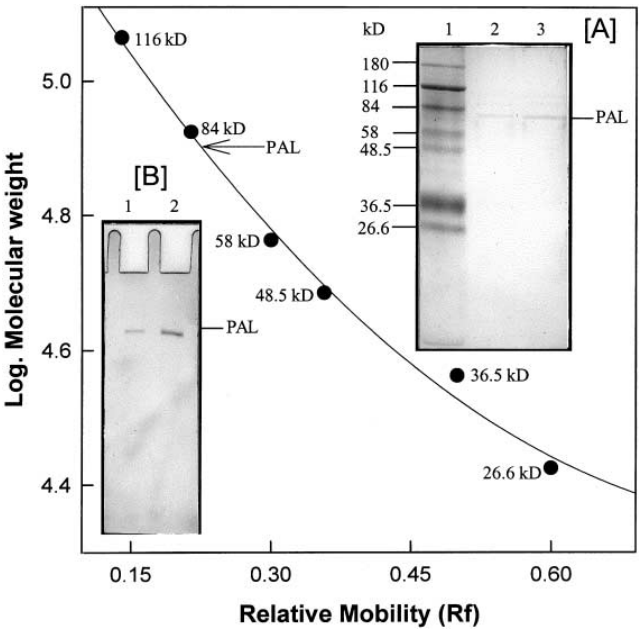

Fig. 2. Nondenaturing and denaturing polyacrylamide gel electrophoresis of purified PAL protein obtained after chromatofocussing step of purification process. The figure shows a calibration curve obtained for relative mobility of known molecular weight markers run on 12% denaturing SDS acrylamide gel. (Inset A) Nondenaturing PAGE (7.5%) of PAL. (Inset B) SDS-PAGE (12%) of purified PAL. For both the analyses, after the electrophoresis gels were stained for protein with coomassie blue R-250.

ment of purified PAL activity over a pH range of 6–11 showed an optimum value at pH 8.9 (Figure 4C). The optimum temperature for PAL activity was determined as 50°C, any further increase in temp declined the activity. The Arrhenius plot yielded a straight line between 10–50°C with activation energy constant as approx. 18 kcal mol^{-1} (Figure 4D). All these above properties are closely similar to the PAL isoforms from various other species (Jones, 1984).

Substrate saturation experiments performed with both purified and partially purified PAL showed no deviation from Michealis–Menten kinetics and exhibited a straight line on a Lineweaver–Burk plot (Figure 4B). A K_m value of 500 μM for phenylalanine was obtained from this plot. This is in contrast to the behaviour reported for PAL from most of the higher plants, which usually exhibit negative cooperativity with high affinity for phenylalanine at low concentration (Jones, 1984). Several reports attributed this phenomenon as a consequence of the presence of PAL isoforms that differed with respect to their K_m values (Bolwell et al., 1985; Jorrin & Dixon, 1990; Sarma et al., 1998). However, the nature of purification procedure used also seems to play a role in exhibiting this deviation. Overall, the results indicate that the single K_m for PAL is consistent with the single isoform induced and suggest that cooperative interactions probably may not play an important role in regulating the rice PAL activity in vivo.

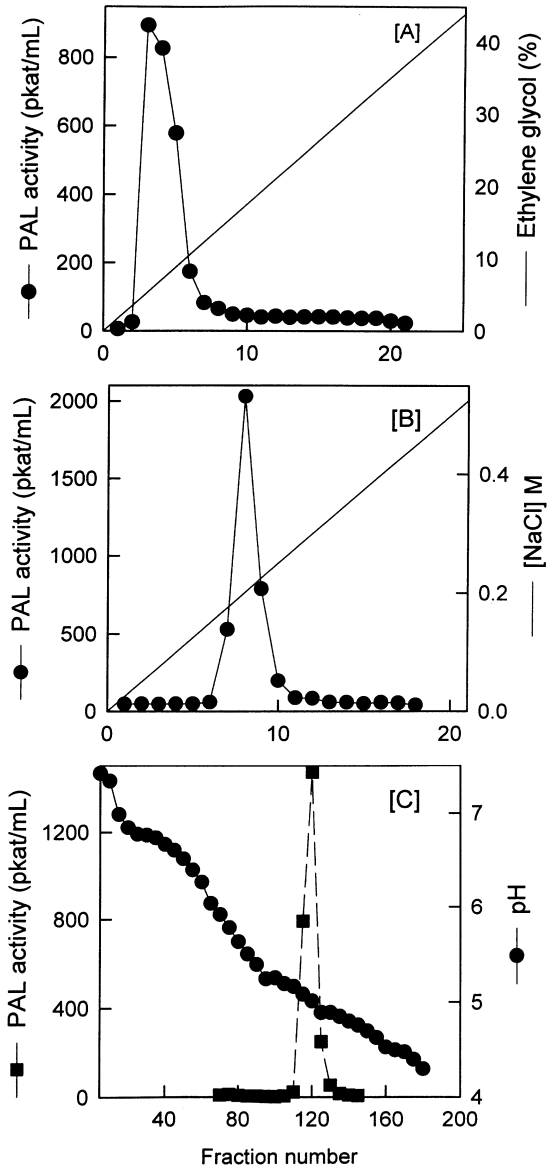

Fig. 3. The elution profiles of phenylalanine ammonia lyase (PAL) activity on different chromatography columns. (A) Elution profile of PAL from phenyl-Sepharose; protein sample containing PAL enzyme was applied to a 1.2×5 cm column and was eluted with a linear gradient of buffer A and buffer A containing 50% (v/v) ethylene glycol. (B) Elution profile of PAL from DEAE-Sepharose; protein sample containing PAL enzyme was loaded onto a DEAE-Sepharose column and the enzyme was eluted with a linear gradient of buffer C containing NaCl (0.0-0.5 M). (C) Elution profile of PAL activity from chromatofocussing on PBE-94 column over a pH range of 7–4.

In several species, PAL consists of multiple isoforms, few of which are constitutively expressed and others are induced specifically by a stimulus or show tissue localization (Liang, Dron, Cramer, Dixon, & Lamb, 1989; Shufflebottom, Edwards, Schuch, & Bevan, 1993). It is generally considered that these isoforms represents the gene products of different members of the PAL gene family. In addition to contribution by multiple genes, isoforms can also arise due to several posttranslational

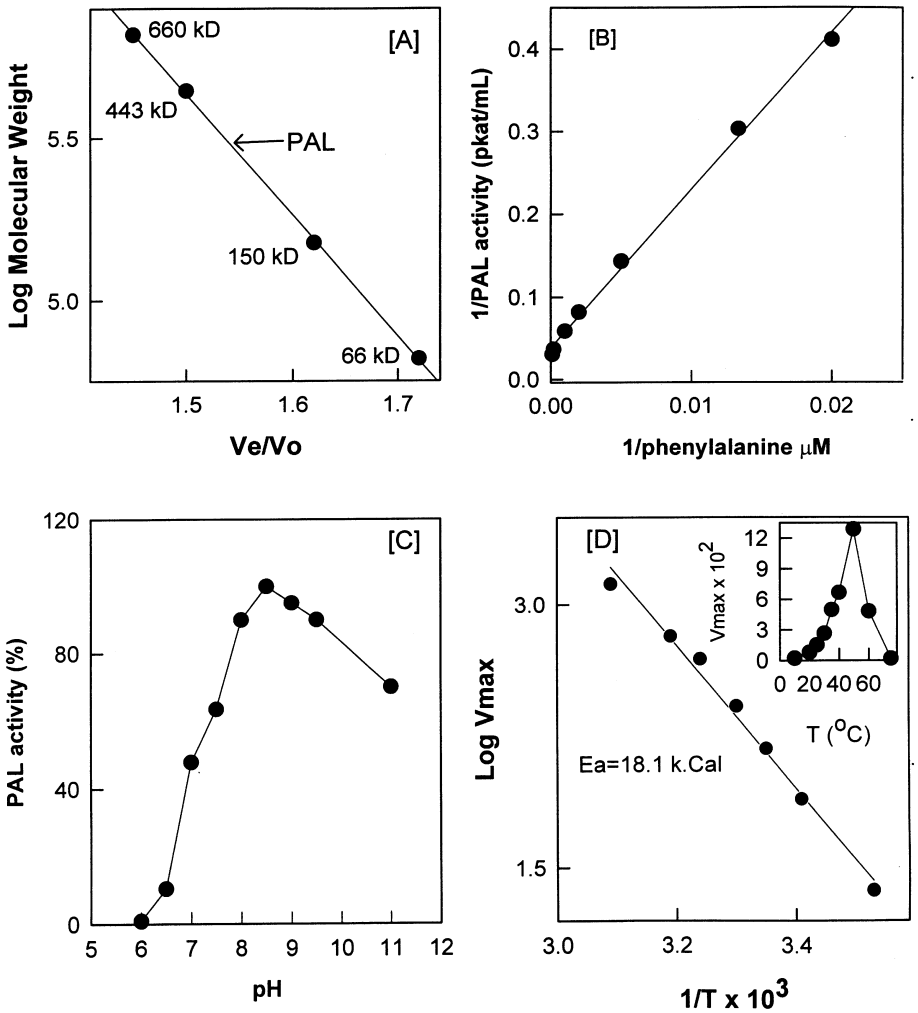

Fig. 4. Physicochemical characterization of UV-B induced rice PAL. (A) The native molecular weight of PAL by gel-filtration chromatography on a Sepharose CL-6B column. (B) Lineweaver–Burk plot of initial rate data for PAL. The initial rate is the value obtained at A_{290}/15 min at varying concentrations of phenylalanine against a blank without phenylalanine. (C) Activity–pH profile of PAL, assayed for the optimal pH for activity using different buffers ranging from pH 6–11. PAL activity is represented as percentage of maximum activity obtained at optimal pH. (D) Arrhenius plot for PAL obtained at 10–50°C . The Inset shows the activity profile assayed for the optimal temperature at varying temp.

modifications, aggregation and/or conformational changes of the enzyme subunits. In rice, PAL gene family consists of at least five to six genes (Minami et al., 1989; Zhu et al., 1995). Two PAL genes from rice; *GP-1* and *GP-28* have been isolated and characterized. The only expression information available about these forms is that, during greening, the transcription of *GP-1* is enhanced while the transcript levels of *GP-28* remain low. In addition, a third form *ZB-8* is also recently been isolated and a thorough examination of expression of this gene as fusion product of reporter gene GUS revealed that it is both stress and developmentally regulated.

Though rice PAL is encoded by a multi-gene family, whether the gene products of these contributes to different PAL isoforms has not been experimentally ascertained. We tested this possibility by analyzing the rice PAL activity on at least three different chromatographic columns, which includes hydrophobic, ion-exchange and by chromatofocussing (Figure 3A–C). However, exposure of rice seedlings to UV-B radiation exhibited only one isoform of PAL. Furthermore, UV-B exposure did not result in the production of new, chromatographically distinct isoforms, but rather resulted in an increase in PAL activity that was chromatographically indistinguishable from that seen in unexposed seedlings and seedlings grown in continuous white light (data not shown). These data suggest that rice PAL is constitutively present as single form during development and that the levels of this form are elevated on UV-B exposure.

PAL being a tetrameric enzyme and also encoded by multiple genes, the individual genes coding for distinct isoforms can account for differential regulation of PAL activity. However a study conducted by Appert, Logemann, Hahlbrock, Schmid, and Amrhein (1994) wherein they expressed the individual cDNA clones of parsley PAL in *E. coli*, the expressed PAL polypeptides

assembled into homo-tetramers. Interestingly irrespective of the fact that cDNA belonging to different PAL genes were used, the assembled PAL homo-tetramers exhibited identical kinetic properties (K_m, temperature and pH optima) in vitro. These results indicated that though different genes may encode PAL isoforms, the final assembled enzymes may bear identical kinetic properties. Alternatively, differential expression of PAL genes may account for differential PAL regulation e.g. in transgenic tobacco, potato and *Arabidopsis* the differential expression patterns of bean *gPAL2* and *gPAL3* genes resulted from the sequence divergence of their promotors (Shufflebottom et al., 1993). It has been shown that individual members of the PAL gene family are expressed differently during plant development and in response to different stress stimulus. In rice, analyses of sequences of available three PAL genes indicate that these three genes show 80% homology in their coding region. It is therefore likely that differences in the expression patterns of rice PAL genes may be regulated by having stimulus specific promotors. Thus, it is reasonable to speculate that probably in rice, individual PAL genes codes for the PAL proteins which bear similar kinetic properties but the expression of these PAL proteins is tissue or stimulus specific.

The studies on regulation of PAL activity have revealed the operation of multiple regulatory mechanisms to modulate the flux into metabolic pathway such as, feedback activation or inhibition by products from branches of phenylpropanoid pathway (Alibert, Ranjeva, & Boudet, 1972; Sarma et al., 1998), enzyme inactivation (Creasy, 1987) or modifications like glycosylation/phosphorylation (Shaw, Bolwell, & Smith, 1990; Bolwell, 1992). It is likely that UV-B induced PAL in vivo functions to channelize the products into a particular branch of phenylpropanoid metabolism, which specifically enhances the synthesis of UV-B absorbing compounds. Light induction studies of flavonoid biosynthetic genes (PAL), chalcone synthase (CHS), chalcone isomerase (CHI) and dihydroflavonol reductase (DFR) in *Arabidopsis* (Kubasek et al., 1992) indicated a sequential accumulation of mRNA's for these genes. This order of induction was the same as the order of the biosynthetic steps in flavonoid biosynthesis. One way to achieve this regulation is by feedback activation/inhibition mechanisms utilizing phenylpropanoid intermediates themselves, as it not only ensures sufficient levels of precursor molecules for efficient enzyme functioning but also independent regulation of the enzyme would provide a wide variety of stress related compounds. This possibility was examined by incubating and assaying the purified UV-B induced rice PAL in the presence and absence of several phenolic compounds. Table 2 shows that the enzyme was very sensitive to metabolites of flavonol and anthocyanin

Table 2
Effect of various phenylpropanoid compounds on activity of rice PAL

Inhibitor type	[Inhibitor] (μM)	[Substrate] (mM)	Residual PAL activity (%)
Cinnamic acid	250	1.0	25.5
	100	1.0	86.2
	50	1.0	87.2
p-Coumaric acid	250	1.0	0.64
	100	1.0	45.2
	50	1.0	60.0
Ferulic acid	250	1.0	000.0
	100	1.0	102.4
	50	1.0	95.0
Caffeic acid	250	1.0	000.0
	100	1.0	92.9
	50	1.0	94.6
3,4-Dihydroxybenzoic acid	250	1.0	87.0
	100	1.0	91.2
	50	1.0	95.0
Quercetin	250	1.0	32.8
	100	1.0	73.7
	50	1.0	88.0
Naringenin	250	1.0	19.1
	100	1.0	63.0
	50	1.0	74.4

UV-B induced PAL form was assayed in presence of 1.0 mM phenylalanine and varying concentrations of individual metabolites. The PAL activity is expressed as percent residual PAL activity with respect to control enzyme sample without phenolic compounds.

biosynthetic pathways, namely *p*-coumaric acid, naringenin and quercetin, which strongly inhibited the enzyme with increasing concentrations. The preliminary analysis of the levels of UV-B induced metabolites revealed that the UV-B stimulates the levels of metabolites belonging to this group of flavonoids (Figure 1B). Since PAL is the first enzyme for the general phenylpropanoid pathway, the strict feedback control of UV-B induced PAL provides a selective way for its regulation and thus may be a key locus in the regulation of overall flux into this pathway. It is therefore likely that the accumulation of these compounds results in a feedback inhibition of the enzyme to regulate the flux of the pathway.

In summary, exposure of rice seedlings to UV-B light induces a PAL isoform similar to that constitutively present in the seedlings. The above results indicate that though the rice genome has 5–6 PAL genes, these genes may be under strict stimulus specific regulation. The induction of PAL was associated with accumulation of phenylpropanoid secondary products (flavonoids) which could provide specific means to protect the plant from UV damage. The activity of induced PAL isoform was found to be under negative feedback control to some of these products, indicating that the induction of this PAL isoform may be an important step in determining the channeling of metabolites associated with flavonoid synthesis.

3. Experimental

3.1. Plant growth conditions

Rice (*Oryza sativa* cv. purple puttu) seeds were dehusked and surface sterilized for 5 min with 0.01% (w/v) sodium hypochlorite soln, followed by extensive washing with distilled water. The seeds were further soaked in distilled water for 24 h and then sown in soilrite (vermiculite and peat mixture) and grown at 25°C in the dark. The six-day-old etiolated seedlings were exposed to UV-B radiations (0.6 W m^{-2} s^{-1}) obtained using two Philips tubelights (TL40/12, Holland). For analysis of the induction profile of PAL, the shoots of equal length from five seedlings were harvested at different time intervals. For anthocyanin determination, after the different time intervals of UV-B exposure, the seedlings were retransferred to darkness and the amount of anthocyanin was estimated after 24 h of dark incubation. For PAL purification, the 6-day old seedlings were exposed to UV-B light for 12 h and shoots were immediately harvested and were frozen in liquid N_2 before processing as described below.

3.2. Buffers

The following buffers were used. Buffer A: 0.1 M K–Pi, pH 7.0 containing 1.0 mM PMSF and 10 mM β-mercaptoethanol. Buffer B: buffer A containing $(NH_4)_2SO_4$ (25% w/v). Buffer C: 20 mM Tris–HCl, pH 8.0 containing 1.0 mM PMSF and 10 mM β-mercaptoethanol. Buffer D: 50 mM Tris–HCl, pH 8.0 containing 1.0 mM PMSF and 10 mM β-mercaptoethanol. Buffer E 25 mM imidazole-HCl, pH 6.3. Buffer F: polybuffer-74 which was diluted 1:8 with H_2O and adjusted to pH 4.0.

3.3. Data analysis

All the experiments were repeated minimum 3–5 × and the representative figures are presented.

3.4. Determination of PAL activity

PAL activity was assayed using a method described (Saunders & McClure, 1975), in a reaction mixture containing 0.1 M borate buffer, pH 8.8, 10 mM phenylalanine, and an aliquot of enzyme in total volume of 1.5 ml. The reaction was carried out for 15 min at 40°C and the rate of formation of *trans*-cinnamic acid was taken as a measure of enzyme activity using increase in 0.01 A at A_{290} nm as 3.09 nmol of *trans*-cinnamic acid formed. The PAL activity was expressed in pkat (pmol *trans*-cinnamic acid formed per second). Protein levels were determined by the Bradford dye-binding assay using BSA as a standard (Bradford, 1976).

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) EtOH to make stock solns (10 mM). The final concentration of metabolites in the PAL assay mixture was 50–250 μM and that of phenylalanine 1.0 mM. In the control assay mixture an equivalent amount of EtOH was added. The PAL assay was carried out as described above.

3.5. Purification of PAL

All purification steps were carried out at 4°C unless otherwise specified. 11 g of six-day old rice seedlings (shoots) exposed to UV-B radiation for 12 h was ground in liquid N_2. The powder was homogenized in 50 ml of buffer A in the presence of 5.0 g of acid washed sea sand and 4.0 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 (20,000*g*, 30 min). To the resulting supernatant, 10% (w/v) protamine sulphate was added with constant stirring to reach a final conc. of 1.0% (w/v) and the suspension was centrifuged at 20,000*g* for 30 min. Solid $(NH_4)_2SO_4$ was then added to the supernatant to reach 25% (w/v) saturation and the soln was applied at a flow rate of 2.0 ml min^{-1} to a phenyl Sepharose column (1.2 × 5 cm) preequilibrated with buffer B at 25°C. The

column was washed with 50 ml of buffer B, and subsequently eluted with a linear gradient of buffer A and buffer A containing 50% (v/v) ethylene glycol. Fractions (2.0 ml) exhibiting PAL activity were pooled and concentrated by ultrafiltration to about 1.0 ml. The concentrate was then applied to a DEAE-Sepharose column (1×12 cm) preequilibrated with buffer C. Protein was eluted with a linear gradient of NaCl (0.0–0.25 M). The amount of protein in the fractions was monitored at A_{280} nm and the PAL activity by the method described above. Fractions (2.0 ml) exhibiting PAL activity were pooled and concentrated by ultrafiltration to about 1.0 ml. The concentrate was mixed with buffer F and then applied to a PBE-94 column (1.3×18 cm) preequilibrated with buffer E. Elution was carried out at a flow rate of 30 ml h^{-1} with buffer F. Fractions of 1.5 ml each were collected, protein, PAL activity and pH were monitored.

3.6. Determination of molecular weight

The M_r of PAL was estimated by gel-filtration chromatography on a Sepharose CL-6B column (1.0×100 cm). The column was preequilibrated with buffer D and was calibrated with the following protein standards: thyroglobulin (660 kDa), apoferrtin (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 molecular weights, and by comparing the elution peak of PAL activity, its M_r was determined. The M_r of PAL subunits was determined by SDS-PAGE using a 12% (w/v) separation gel. A standard plot was made using the following prestained proteins: α-macroglobulin, human plasma (180 kDa), β-galactosidase, *E. coli* (116 kDa), fructose-6-phosphate kinase, rabbit muscle (84 kDa), pyruvate kinase, chicken muscle (58 kDa), fumarase, porcine heart (48.5 kDa), lactic dehydrogenase, rabbit muscle (36.5 kDa) and triose phosphate isomerase, rabbit muscle (26.6 kDa).

3.7. Electrophoresis

Nondenaturing electrophoresis was performed on 7.5% (w/v) polyacrylamide gels and SDS-PAGE was performed with 12% (w/v) acrylamide in the resolving gel according to the method described in (Davies, 1964; Laemmli, 1970). Following electrophoresis, proteins were stained with coomassie brilliant blue R-250.

3.8. Determination of pH optima

The pH optima for PAL activity was obtained by using the following buffers at 100 mM final concentration in the assay mixture: sodium citrate or acetate buffer (pH 4.0–5.0), MES (pH 6.0), MOPS (pH 6.5), Na–Pi buffer (pH 7.0–7.5), Tris–HCl (pH 8.0–8.5), potassium borate (pH 9.0–9.5), sodium carbonate–bicarbonate (pH 10–11).

3.9. Determinations of anthocyanins and other UV absorbing compounds

Three seedlings of uniform height were harvested and anthocyanins were extracted in 2.0 ml of acidified (1.0% v/v HCl) MeOH for 24 h at 4°C with occasional shaking. The anthocyanins were quantitatively estimated by measuring the A_{535} ($\varepsilon_{535} = 31{,}625$). Similarly, UV-B absorbing compounds were extracted and quantitated as described (Dangl, Hauffe, Lipphardt, Hahlbrock, & Scheel, 1987).

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