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Expression Pattern and Gene Structure of Phenylalanine Ammonia-Lyase in *Pharbitis nil*

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Anthocyanin accumulation, phenylalanine ammonia-lyase (PAL) activity and *PAL* gene expression were examined in flower buds and irradiated hypocotyls in *Pharbitis nil*. PAL activity and transcript levels were correlated with the accumulation of anthocyanin. Both in flower buds and hypocotyls, transcript levels, PAL activity, and then the amount of anthocyanin, increased. The *PAL* transcript was abundant in flower buds for a few days before flower opening. But the increase in *PAL* transcript induced by irradiation was temporal in hypocotyls. Phytochrome was shown to be involved in inducing the accumulation of anthocyanin in hypocotyls. To examine the mechanism regulating the expression of the *PAL* gene, the gene was cloned and sequenced, and the promoter region was compared with that of other *PAL*s. The gene had two exons separated by an intron of 989 bp with consensus sequences at the intron/exon border. The predicted primary structure of the PAL protein consists of 711 amino acids. The promoter region was AT-rich and there were sequences similar to box 1, box 2, an AT-1 binding site and a G box. The role of PAL in the accumulation of anthocyanin is discussed.

Key words: Anthocyanin — *Pharbitis nil* — Phenylalanine ammonia-lyase — Phytochrome — Red light

Anthocyanins are the dominant pigments in plants that show red, orange, blue and purple colors. The accumulation of anthocyanin is affected by various environmental factors. The effect of light on the accumulation is well studied. Light induces the expression of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) in many plants (Beggs and Wellmann 1994). Phytochrome, Blue/UV-A receptor and UV-B receptor are involved in inducing the accumulation of anthocyanin (Mohr 1994). The pathway of anthocyanin biosynthesis is well studied and most of the enzymes and the genes involved have been characterized. The first step in the biosynthesis is a reaction by which phenylalanine is converted to *trans*-cinnamic acid. This step is catalyzed by PAL (EC 4.3.1.5). Because of its important role in the biosynthesis of flavonoids, lignins and phytoalexins, PAL and its gene are widely studied (Hahlbrock and Scheel 1989). The expression of PAL is regulated by various environmental factors such as light, low temperature, infection and wounding (Kuhn *et al.* 1984, Leyva *et al.* 1995, Lawton and Lamb 1987).

PAL is encoded by a small multigene family in many plants (Cramer *et al.* 1989, Kawamata *et al.* 1992, Wanner *et al.* 1995). Each member of the family shows a distinctive expression pattern. In bean, *PAL 1* is expressed in roots, shoots and leaves, *PAL 2* in roots, shoots and petals, and *PAL3* only in roots. *PAL 1* and *PAL 3* were induced by fungal infection and *PAL 1* and *PAL 2* were induced by light. All three genes are induced by mechanical wounding (Liang *et al.* 1989). In parsley, PAL is encoded by a small family of at least four genes and one of the *PAL* genes is expressed in response to UV irradiation and elicitor treatment. The kinetics of mRNA expression induced by light is completely different from that induced by elicitor treatment (Lois *et al.* 1989). Besides the expression pattern, the promoter region of the *PAL* gene is also characterized in several plants. Sequences such as box 1 (box L) and box 2 (box P) are reported to be conserved among promoters of the gene for the phenylpropanoid pathway (Lois *et al.* 1989, Ohl *et al.* 1990, Logemann *et al.* 1995).

Pharbitis nil (Japanese morning glory), which is a common garden flower in Japan, has been used for experiments on flowering (Takimoto and Hamner 1964), flower opening (Kaihara and Takimoto 1979) and flower pigmentation (Lu *et al.* 1991). Several genes involved in anthocyanin biosynthesis have been cloned by using transposon tagging (Inagaki *et al.* 1994, Fukada-Tanaka *et al.* 2000). Here, we examined the role of PAL in the course of anthocyanin accumulation in flower development and photo-induction in *Pharbitis nil*.

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Abbreviations: CHS, chalcone synthase; FR, far-red light; PAL, phenylalanine ammonia-lyase; R, red light; WL, white light.

Furthermore, we cloned a *PAL* gene and compared the promoter region with that of other *PAL*s.

Materials and Methods

Growth conditions

Seeds of *Pharbitis nil* Chois. (cv. violet) were imbibed with distilled water for 24 hr and sown on a filter paper in petri dishes. The petri dishes were kept in darkness for 48 hr then seedlings were used in the experiments. Flower buds were collected from plants grown in the experimental field of Shinshu University. White light (WL) and red light (R) were obtained from fluorescent tubes (FL20SD; Toshiba, Tokyo, Japan). Acryrite No. 102 (Mitsubishi Rayon Co. Ltd., Tokyo, Japan) was used for the generation of R. Far-red light (FR) was obtained from fluorescent tubes (FL20SFR74; Toshiba) with a resin-film filter (IR-1; NEC, Tokyo, Japan). Light energy was measured with a photodiode (S1406-03; Hamamatsu TV Co., Hamamatsu, Japan) which was calibrated with a thermopile (MIR-100Q; Mitsubishi Yuka Co., Tokyo, Japan).

Quantification of anthocyanin

Anthocyanin was extracted from 10 pieces of hypocotyl or a bud with 5 ml of 0.5% HCl-methanol. The extract was centrifuged and the absorbance of the supernatant was measured with a spectrophotometer (Ubest-30, Japan Spectroscopic Co., Tokyo). Absorption by chlorophyll was compensated for using the equation $A_{530}-0.33\times A_{657}$ (Mancinelli *et al.* 1975).

Measurement of PAL activity

Proteins were extracted from hypocotyls and flower buds by grinding fresh tissues in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% sodium isoascorbate and 10% Polyclar AT (Gokyo-Sangyo Co. Ltd., Osaka). The crude extract was centrifuged and the supernatant was loaded onto a Sephadex G-25 (fine) column. The effluent was used for measuring PAL activity.

PAL activity was measured in a reaction mixture containing 10 mM phenylalanine and 50 mM Tris-HCl (pH 8.5). The reaction was stopped by adding perchloric acid and the absorbance at 280 nm was measured with a spectrophotometer.

RNA gel blot analysis and slot blot analysis

Total RNA was extracted from 10 seedlings by a standard phenol extraction method. Total RNA (20 μg per lane) was separated on 0.66 M formaldehyde gel and transferred to a nitrocellulose membrane. The membrane was hybridized with Dig-labeled probe (Boehringer) overnight at 42 C in the presence of 50% formamide. cDNA was used as a template for preparing the probe. After hybridization, the membrane was washed twice with 2xSSC, 0.1% SDS and once with 0.1xSSC, 0.1% SDS for 20 min at 68 C. Signals were detected by using a Dig nucleic acid detection kit (Boehringer). For slot blot analysis, total RNA (7 μg for hypocotyl, 2.5 μg for flower bud) was fixed on a nitrocellulose membrane. Hybridization and washing conditions were the same as for RNA gel blot analysis.

Cloning and sequencing of cDNA and genomic clones

A cDNA library was constructed using a cDNA synthesis kit (Pharmacia) and λgt11 phage vector (Stratagene) according to the manufacturer's instructions. A genomic library was constructed with *Sau*3AI-partially digested genomic DNA in λDASH II (Stratagene) according to the manufacturer's instructions. The cDNA library and the genomic library (6×10^5 clones) were screened by using pPAL02 of sweet potato (Tanaka *et al.* 1989) as a probe. Positive cDNA clones were subcloned into pBluescript SK$^+$. A kit (Stratagene) was used for the mapping of the genomic clone. A 5.1 kbp *Eco*RI fragment was subcloned in pBluescript SK$^+$. A series of deletion fragments to obtain various sized deletion clones was made using an ExoIII exonuclease deletion kit (Takara). A DNA sequencer model 373S (Applied Biosystems) was used for sequencing. All cloning procedures were done by standard methods (Sambrook *et al.* 1989).

Results

Anthocyanin accumulation, PAL activity and PAL *gene expression in flower buds*

Petals of *Pharbitis nil* accumulate large amounts of anth-

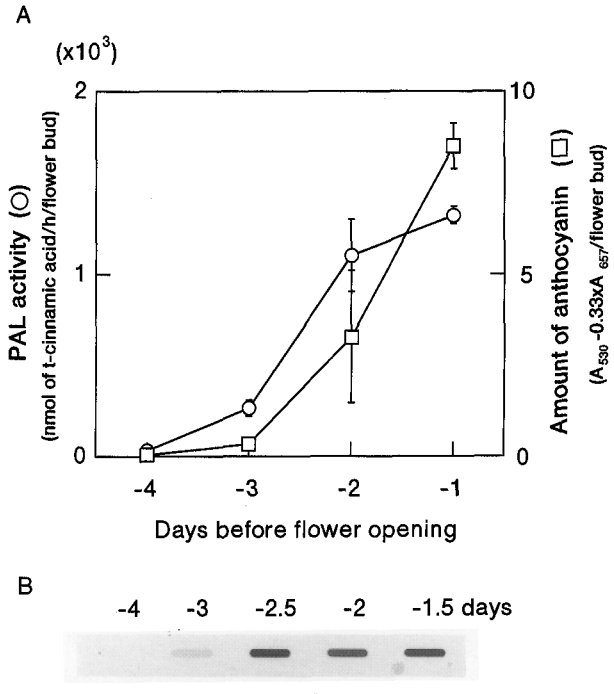

Fig. 1. Amount of anthocyanin, PAL activity and *PAL* transcript in flower buds. A: Flower buds were harvested 1-4 days before opening and anthocyanin accumulation and PAL activity were examined. B: Flower buds were harvested 1. 5-4 days before opening and the level of *PAL* transcript was examined by slot blot analysis. Plots show the average value for two experiments. Bars indicate the maximum and minimum values.

ocyanin during the development of flowers. First, we examined the relation between PAL expression and anthocyanin accumulation during flower development. Figure 1 shows the time course of anthocyanin accumulation, PAL activity and *PAL* gene expression in flower buds. The accumulation of anthocyanin in flower buds started 2-3 days before flower opening. PAL activity started to increase before anthocyanin was accumulated. Transcript levels of *PAL* started to increase 3 days before flower opening and remained high for more than 24 hr.

Relation between PAL expression and anthocyanin accumulation in hypocotyls

Stem and hypocotyl also accumulate much anthocyanin in *Pharbitis*. Next, we examined the relation between PAL expression and anthocyanin accumulation in hypocotyls (Fig. 2). Hypocotyls of dark-grown seedlings accumulated small amounts of anthocyanin but showed relatively strong PAL activity. WL (16 W/m²) stimulated both anthocyanin accumulation and PAL expression in hypocotyls. The accumulation of anthocyanin started 3-6 hr after the start of irradiation. The PAL activity increased 0-3 hr and reached a maximum 9 hr after the start of irradiation. The transcript level of the *PAL* gene reached a maximum at 2 hr and gradually decreased thereafter. After 12 hr, the level of *PAL* transcript was reduced although the activity of PAL was still strong.

To determine whether phytochrome is involved in inducing the accumulation of anthocyanin, we examined R-FR reversibility in the photo-induction of anthocyanin (Fig. 3). Seedlings grown for 2 days in darkness were irradiated with R for 5 min or R for 5 min followed by FR for 5 min. R induced the accumulation of anthocyanin and the effect of R was reversed by FR, showing the involvement of phytochrome.

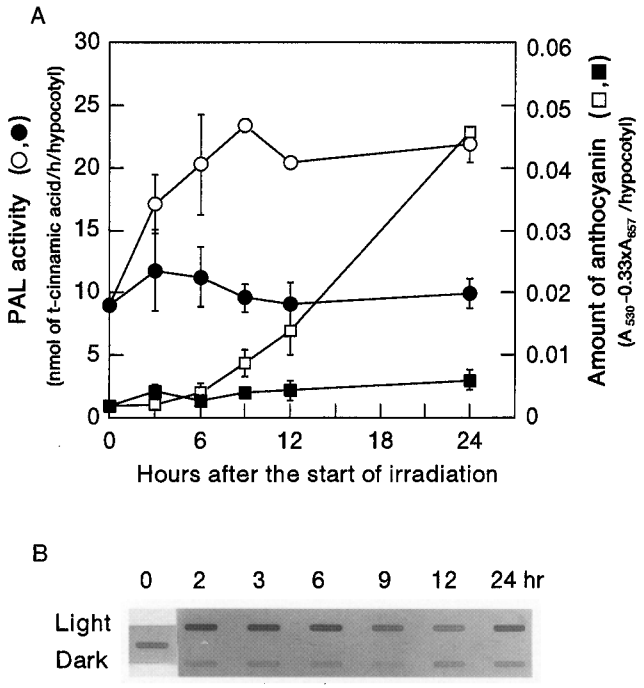

Fig. 2. Amount of anthocyanin, PAL activity and *PAL* transcript in hypocotyls. Seedlings grown for 2 days in darkness were irradiated continuously with WL (16 W/m²). A: Anthocyanin accumulation and PAL activity were evaluated at 0-24 hr after the start of irradiation. B: The level of *PAL* transcript was determined by slot blot analysis. Plots show the average value for two experiments. Bars indicate the maximum and minimum values.

Structure of the PAL gene

Examining the regulatory mechanism of PAL expression requires the cloning of the *PAL* gene. Therefore, we isolated the gene and determined its structure. A genomic library of *Pharbitis nil* was screened using a PAL02 clone of sweet potato as a probe and one clone which is presumed to contain the whole coding region was isolated (Fig. 4). The 5.2 kb *Eco*RI fragment was subcloned and sequenced

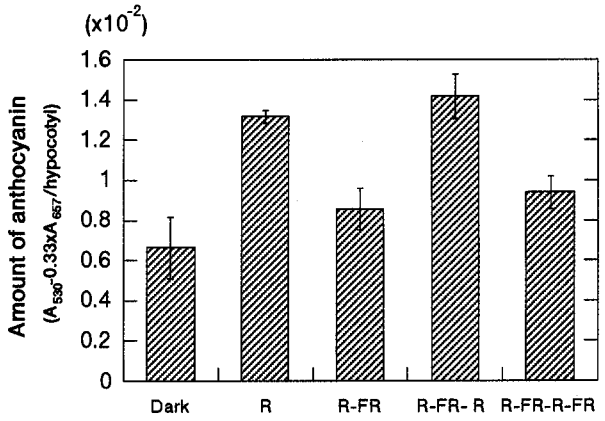

Fig. 3. R-F reversibility of anthocyanin accumulation. Seedlings grown for 2 days in darkness were irradiated with R (1.9 W/m²) and FR (2.6 W/m²) as indicated. Each irradiation lasted 5 min. The amount of anthocyanin was determined at 48 hr after the irradiation. Bars indicate SE (n=3).

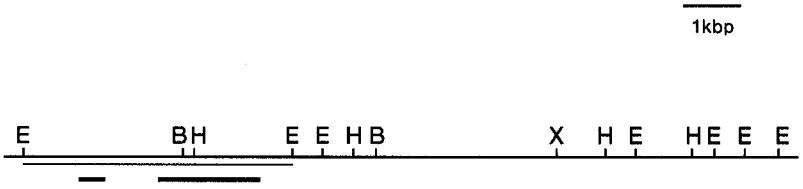

Fig. 4. Restriction enzyme map of the *PAL* gene. A fine line shows the sequenced region. Bold lines show exon regions. B: *Bam*HI, E: *Eco*RI, H: *Hin*dIII, X: *Xba*I

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Fig. 5. Nucleotide and amino acid sequences of *PAL*. Several consensus sequences in the promoter region, putative start and stop codons and a putative poly A signal are underlined. The intron sequence is omitted. Genbank accession number is AF325496.

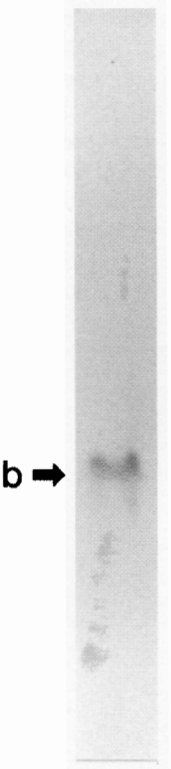

Fig. 6. RNA gel blot of *PAL* transcript. Seedlings grown for 2 days in darkness were irradiated with WL for 12 hr, then total RNA was extracted. *PAL* expression was examined by RNA gel blot analysis using a cDNA clone as a probe.

(Fig. 5). The clone contained a 1.0 kb promoter region and the entire coding region. The *PAL* gene consisted of two exons and a intron of 989 bp that was inserted between the 2nd and 3rd bases of the Arg^{128} codon. The intron was inserted at the same site as in other plants (Lois *et al*. 1989, Minami *et al*. 1989). There was consensus sequence in the intron/exon border. Comparing the sequence with sweet potato *PAL* and considering the size of the transcript (Fig. 6), there was only one candidate for the start codon. The predicted primary structure of the PAL protein had 711 amino acids and showed high homology with sweet potato PAL (94% with pPAL02 (M29232) and 87% with PAL 2-8(D78640)). The promoter region was AT-rich. There were several sequences similar to box 1 and box 2 which are conserved among promoters of genes for the phenylpropanoid pathway (Lois *et al*. 1989, Ohl *et al*. 1990). Sequences similar to an AT-1 binding site and a G box were also found in the promoter region of the *Pharbitis PAL* gene.

Discussion

The biosynthesis of flavonoids and phenylpropanoids is regulated by various environmental factors (Kuhn *et al*. 1984, Lawton and Lamb 1987) and several regulatory steps are identified in the biosynthetic pathway. In parsley cells, enzymes of the general phenylpropanoid pathway are induced by light and fungal elicitors but enzymes of the

flavonoid pathway are not induced by elicitors (Chappell and Hahlbrock 1984), suggesting that the regulatory mechanisms of these two pathways are different. Even in the flavonoid biosynthetic pathway, the regulatory mechanism in the early steps is different from that in the late steps (Shirley *et al.* 1995). Because the mechanism regulating the biosynthesis of anthocyanin is complicated, PAL activity is not always correlated with anthocyanin accumulation (Hrazdina and Parsons 1982, Mohr 1972). In *Pharbitis*, PAL activity was correlated with the accumulation of anthocyanin, suggesting that PAL is a rate limiting enzyme of anthocyanin biosynthesis. One exception is that PAL activity was relatively intense in dark-grown seedlings which contain only a little anthocyanin. The PAL in dark-grown seedlings may have other functions such as the supply of lignins to vascular tissues.

Transcript levels of *PAL* in flower buds remained 3 to 1.5 days before flower opening. This pattern is different from that in *Antirrhinum* in which *PAL* expression during flower development is temporal (Jackson *et al.* 1992). The stability in the level of transcript may enable *Pharbitis* flowers to accumulate large amounts of anthocyanin. On the other hand, *PAL* expression induced by irradiation was temporal and decreased to the control level 12 hr after the start of irradiation in hypocotyls. Thus, the kinetics of transcription during flower development was different from that during light induction. The regulatory mechanism in flower development is probably different from that for the induction by light. Further study is required to determine whether the difference in mRNA kinetics is due to the stability of PAL mRNA or to transcriptional activity.

R induced the accumulation of anthocyanin and this effect was reversed by FR. These results showed that phytochrome is at least one of the photoreceptors needed for induction of anthocyanin synthesis. Preliminary data showed that continuous WL was more effective than continuous R of similar fluence rate (data not shown), indicating the involvement of other photosystem(s).

We isolated the *PAL* gene of *Pharbitis nil* and determined its structure. The predicted amino acid sequence had high similarity to that of other plants. The site of intron insertion was the same as in other plants (Lois *et al.* 1989, Minami *et al.* 1989). In the promoter region, there were sequences similar to box 1 and box 2 (Ohl *et al.* 1990, Logemann *et al.* 1995). The sequences CCTTACCTTGC and CTACACCTACC are similar to the box 1 sequence (T/C)C(T/C)(C/T)ACC(T/A)ACC. The sequence CCACCAACCCCC was similar to the box 2 sequence (T/C)(C/T)(A/C)(A/C)C(A/C)A(A/C)C(C/A)(C/A)C. These sequences are conserved within genes for the phenylpropanoid pathway (Lois *et al.* 1989, Ohl *et al.* 1990). The sequences TATAAAAATAAT and AATAAAATAA were similar to the sequence of the complimentary strand of the AT-1 binding site (AATATTTTTATT). The AT-1 binding site has a positive role in the expression of the gene *rbcS* (Datta and Cashmore 1989, Ueda *et al.* 1989). The sequence CCCACGTAAG is similar to the G-box in the promoter region of *Pharbitis PAL*. A G-box is found in several light-regulated genes such as *CHS* and *rbcS* as well as in genes which are not light-regulated (Williams *et al.* 1992). It remains to be determined whether these sequences in *Pharbitis PAL* have similar functions to those boxes.

PAL constitutes a gene family in most plants and the expression pattern of each member of the family is different (Liang *et al.* 1989). Here, we examined only one *PAL* gene. However, there are likely to be other members of the *PAL* gene family in *Pharbitis*. Further studies on the other *PAL* species are required to understand the regulatory mechanism of *PAL* expression in *Pharbitis*.

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