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Increase in the Activity of Phenylalanine Ammonia-Lyase during the Non-Photoperiodic Induction of Flowering in Seedlings of Morning Glory (*Pharbitis nil*)

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The activity of phenylalanine ammonia-lyase in the seedlings of *Pharbitis nil* strains 'Violet' and 'Kidachi' during the non-photoperiodic induction of flowering increased in close association with the accumulation of phenylpropanoids and the induction of flower buds. This observation suggested that the increased activity of phenyalanine ammonia-lyase caused the accumulation of the phenylpropanoids and that the activation of the biosynthesis of phenylpropanoids might be involved in the non-photoperiodic induction of flowering in the seedlings of 'Violet' and 'Kidachi'.

Key words: (Aminooxy)acetic acid — Flowering — Induction of flower buds — *Pharbitis nil* — Phenylalanine ammonia-lyase — Phenylpropanoid.

Seedlings of *Pharbitis nil* strain 'Violet', a short-day plant, are induced to flower under poor nutritional conditions (Shinozaki 1985), high-intensity light (Shinozaki 1972), and at low temperature (Shinozaki 1974, Shinozaki and Takimoto 1982) even in continuous light. A prior treatment with high-intensity light promotes the flower-inducing effect of low temperature (Shinozaki et al. 1982). We reported previously that the levels of chlorogenic acid, *p*-coumaroylquinic acid, and pinoresinol-*O*-*β*-D-glucoside in the cotyledons of 'Violet' seedlings were increased by culture under poor nutritional conditions (Shinozaki et al. 1988a, Hirai et al. 1993) or high-intensity light (Shinozaki et al. 1994). We also reported that the levels of 3-*O*-feruloylquinic acid and dehydrodiconiferyl alcohol-13-*O*-*β*-D-glucoside were increased by exposure to low temperature, with the levels being increased still further by prior exposure to high-intensity light (Hirai et al. 1994). The key enzyme in the biosynthesis of these phenylpropanoids is phenylalanine ammonia-lyase (PAL), which catalyzes the deamination of phenylalanine to give *t*-cinnamic acid (Hanson and Havir 1981). (Aminooxy)acetic acid (AOA), an inhibitor of PAL (Amrhein et al. 1976), inhibits the accumulation of phenylpropanoids and the induction of flowering in 'Violet' seedlings that are cultured either under poor nutritional conditions (Shinozaki et al. 1988a) or high-intensity light (Shinozaki et al. 1994). These findings strongly suggest that the activity of PAL is increased by non-photoperiodic flower-inducing conditions. This report describes the increase in the activity of PAL in cotyledons of seedlings of strain 'Violet' grown under non-photoperiodic flower-inducing conditions, such as poor nutrition, high-intensity light and low temperature, and the inhibition in vitro of the PAL activity by AOA. Seedlings of strain 'Kidachi' are scarcely induced to flower by poor nutritional conditions (Shinozaki et al. 1988b) but flowers are induced by low temperature (Shinozaki and Takimoto 1982). The activity of PAL in this strain was also examined under the various conditions.

Materials and Methods

Plant materials—Seedlings of *Pharbitis. nil* strain 'Violet' and 'Kidachi' were cultured in plastic containers (width, 15 cm; depth, 11 cm; height, 7 cm), with 12 seedlings in each container, as described elsewhere (Shinozaki et al. 1988a). For culture under conditions of poor nutrition, the seedlings were cultured in tap water at 20°C for 0–24 days under continuous light at 8 W m^{-2}. The control seedlings were cultured in Nakayama's solution (rich nutrition; Shinozaki et al. 1988a). For treatment with high-inten-

Abbreviations: AOA, (aminooxy)acetic acid; PAL, phenylalanine ammonia-lyase; mU, milliunits.

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sity light, seedlings cultured in Nakayama's solution were exposed to continuous light at 50 W m^{-2} at 25°C for 0–16 days. Control seedlings were exposed to continuous light at 8 W m^{-2}. For low-temperature treatment, seedlings were cultured in Nakayama's solution under continuous light at 8 W m^{-2} at 23°C for 3 days and then at 13°C for 0–12 days. For treatment with both high-intensity light and low temperature, seedlings were cultured in Nakayama's solution and were exposed to continuous light at 50 W m^{-2} at 23°C for 3 days, and then to continuous light at 8 W m^{-2} at 13°C for 0–12 days. After treatments, the seedlings were cultured in Nakayama's solution at 20°C under continuous light at 8 W m^{-2}, and the number of flower buds was counted five weeks after germination. For the short-day treatment, seedlings cultured in Nakayama's solution in continuous light at 10 W m^{-2} for 1 day were placed in darkness for 4, 8, 12 and 16 h at 25°C and then returned to continuous light as described above. The number of flower buds was counted two weeks after the short-day treatment.

Preparation of solutions of crude enzyme—Cotyledons of ten plants that had been cultured under a particular set of conditions were collected and stored at −80°C until use. The cotyledons were homogenized with 0.1 M borate-HCl buffer [8 ml (g fr wt)$^{-1}$; pH 8.8] that contained 0.5 mM 2-mercaptoethanol, quartz sand [1.5 g (g fr wt)$^{-1}$] and Polyclar-AT [0.2 g (g fr wt)$^{-1}$; International Specialty Products, Inc., Wayne, NJ, U.S.A.] in an ice-chilled mortar with a pestle. A clear supernatant was obtained by successive centrifugations of the homogenate at 5,000 × *g* for 10 min and 16,000 × *g* for 30 min at 4°C, and it was used as the solution of crude enzyme.

Assay of the activity of PAL in solutions of crude enzyme—The activity of PAL in each solution of crude enzyme was assayed by the method of Zucker (1965) with minor modifications. The reaction mixture contained 0.2 ml of 0.1 M phenylalanine, 0.8 ml of water, 0.1–1.0 ml of a solution of crude enzyme, and 0.9–0 ml of the borate-HCl buffer. A reaction mixture containing 0.2 ml of water instead of 0.1 M phenylalanine was used as a control. The mixture was incubated at 24°C for 15 h, and then extracted with 5 ml of diethyl ether for 1 min with mixing on a Vortex-Genie (Scientific Industries, Inc., Bohemia, NY, U.S.A.) after addition of 0.1 ml of 5 M HCl. Three ml of the diethyl ether layer were evaporated to dryness under a stream of nitrogen gas and the residue was dissolved in 1.2 ml of 50 mM NaOH. Absorbance at 268 nm of the alkaline solution, namely, at the wavelength that corresponds to maximum absorption by *t*-cinnamic acid, was measured using the alkaline solution of the control reaction mixture as a blank. The total molar amount of *t*-cinnamic acid formed in the reaction mixture was calculated from the absorbance, based an extinction coefficient for *t*-cinnamic acid of 19,800 that was measured at 268 nm in 50 mM NaOH. The amount was divided by the reaction time to yield μmoles per min (units), and the activity is presented as mU (g fr wt)$^{-1}$. The activity of PAL was measured at least three times for each solution of crude enzyme. The effect of a solution of crude enzyme with low PAL activity on the activity of PAL in a solution of crude enzyme with high PAL activity was examined by the same method. Table 1 shows the volumes of the solutions used.

Inhibition of PAL activity by AOA—An aqueous solution (0.8 ml) of AOA hemihydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to the reaction mixture that contained 0.2 ml of 1 or 10 mM phenylalanine, 0.2 ml of the solution of crude enzyme obtained from 'Violet' seedlings that had been cultured under poor nutritional conditions for 14 days, and 0.8 ml of the borate-HCl buffer. The final concentration of AOA was adjusted to 0.1, 0.3, 1.0, 3.0 and 10 mM. The activity of PAL was measured as described above.

Results

Determination of PAL activity—The activity of PAL was examined with a solution of crude enzyme obtained from the seedlings of 'Violet' that had been cultured under poor nutritional condition for 11 days since the amount of phenylpropanoids in cotyledons is known to increase from day 8 to day 16 under such conditions (Hirai et al. 1993). When the volume of the solution of crude enzyme was 0.1, 0.3 and 1.0 ml, the activities of PAL calculated after a 15-h incubation were 2.77, 2.62 and 2.54 mU (g fr wt)$^{-1}$, respectively. The activity scarcely changed with changes in the volume of the enzyme solution, showing that the amount of *t*-cinnamic acid formed was almost directly related to the volume of the solution of crude enzyme. Thus, the enzymatic reaction was a zero-order reaction during the incubation, and the molar amount of *t*-cinnamic acid formed per min could be regarded as the rate of the reaction. For subsequent assays, 0.1–0.3 ml of solutions of crude enzyme were used. There was no decrease in the activity of PAL in a solution of crude enzyme prepared after storage of cotyledons at −80°C for 4 weeks, while 15% of the activity was lost after storage of the solution of crude enzyme at −80°C for 2 weeks.

Correlation between the activity of PAL and induction of flowering in 'Violet' seedlings—Under poor nutritional conditions, the activity of PAL increased from day 4, reaching a maximum on day 16 (Fig. 1A), while flowering was induced after day 16. When the seedlings were transferred to Nakayama's solution, a nutrient-rich medium, on day 14, the PAL activity decreased markedly, falling to the level in control seedlings, and no flowering was induced. In control seedlings, the activity of PAL remained low throughout the culture period and flowering was not induced. Irradiation with high-intensity light increased the activity of PAL from day 1, but the activity decreased gradu-

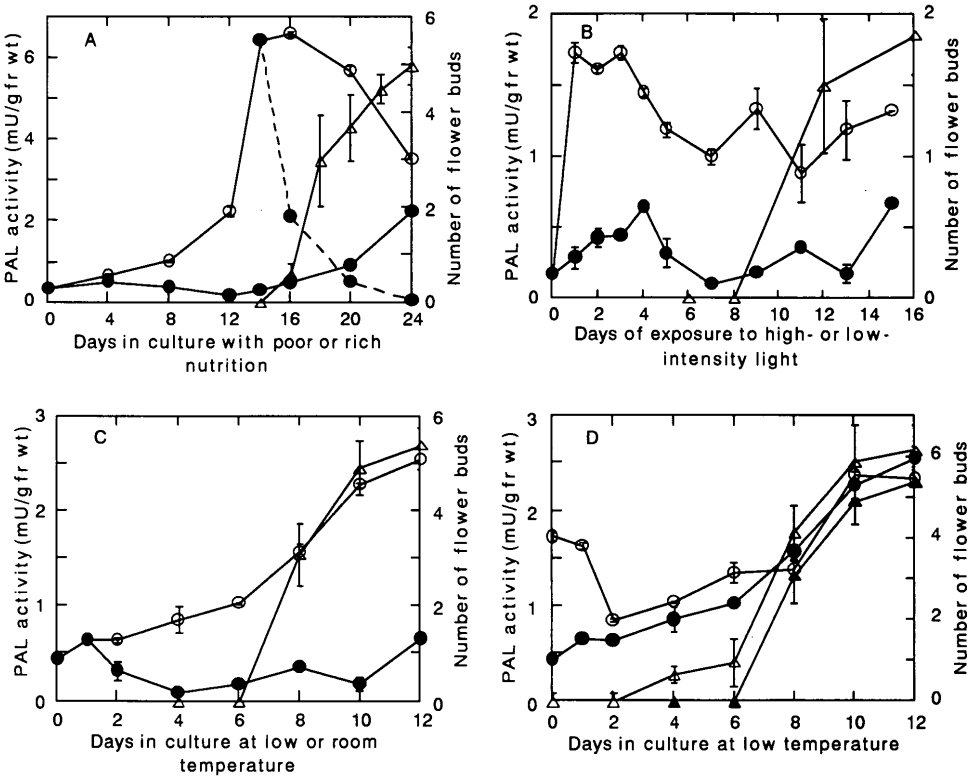

Fig. 1 Changes in the activity of PAL (○ and ●) in solutions of crude enzyme obtained from cotyledons of 'Violet' seedlings during culture under poor nutritional conditions, under high-intensity light, at a low temperature and under high-intensity light followed by low temperature, as well as the flowering response (number of flower buds per plant) of the seedlings (△ and ▲) determined two weeks after each treatment. A: poor nutrition (○ and △; water); rich nutrition (●; Nakayama's solution); transfer from poor nutrition to rich nutrition on day 14 (● with dashed line). B: high-intensity light (○ and △); low-intensity light (●). C: low temperature (○ and △; 13°C) and room temperature (●; 23°C) preceded by culture without high-intensity light for three days. D: low-temperature treatment with (○ and △) and without (● and ▲) prior exposure to high-intesity light for three days. Flowering was not induced under conditions of rich nutrition and transfer from poor nutrition to rich nutrition on day 14 (A), by low-intensity light (B), and at room temperature (C). Each point for number of flower buds and PAL activity represents the mean of results from 10 seedlings and the mean of results of three measurements with a solution of crude enzyme from 10 seedlings, respectively, with standard errors indicated by vertical lines. All experiments were repeated at least twice and typical data are shown.

ally after day 3 and started to increase again from day 12 (Fig. 1B). Under such conditions, flowering was induced after day 12. The activity of PAL remained low under the control conditions and the seedlings did not flower. At low temperature, PAL activity increased gradually from the beginning of the treatment, and flowering was induced after 8 days of low-temperature treatment (Fig. 1C). During exposure of seedlings to high-intensity light for 3 days, the activity of PAL increased but it decreased rapidly within 2 days after transfer to light at 8 W m^{-2} and a low temperature (Fig. 1D). The activity started to increase again on day 4 of the low-temperature incubation and remained higher than that in seedlings that had not been exposed to high-intensity light during the period from day 2 to day 6. Flowering of seedlings was induced from day 4, which was 4 days earlier than that of the seedlings without prior exposure to high-intensity light (see Fig. 1C). The seedlings that had been placed in darkness for 16 h developed six floral buds per plant, but there was no change in PAL activity during the dark treatment (data not shown).

Correlation between the activity of PAL and induction of flowering in 'Kidachi' seedlings—Under poor nutritional conditions, PAL activity in 'Kidachi' seedlings was as low as that in control seedlings, and flowering was not induced (Fig. 2A). During culture at a low temperature, PAL activity increased slightly from day 2 and remained higher than that in control seedlings throughout the experimental period (Fig. 2B). Clear evidence of induction of flowering was apparent from day 6 under these conditions.

Effects of a solution of crude enzyme with low PAL activity on the activity of PAL in a solution of crude enzyme with high PAL activity—We determined whether or not the PAL activity in solutions of crude enzyme might be influenced by inhibitors contained in such solutions. A solu-

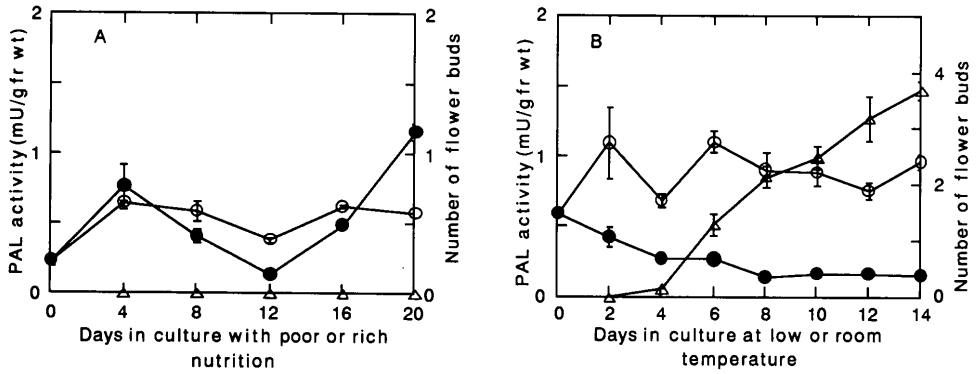

Fig. 2 Changes in the activity of PAL (○ and ●) in solutions of crude enzyme obtained from cotyledons of 'Kidachi' seedlings during culture under poor nutritional conditions or at a low temperature (13°C) and the flowering response (number of flower buds per plant) of the seedlings (△) determined two weeks after the treatment. A: poor nutrition (○); rich nutrition (●). B: low temperature (○; 13°C), room temperature (●; 23°C). The number of flower buds is shown only for poor nutrition (A) and low temperature (B). Flowering was not induced under the other conditions. For other details, see legend to Fig. 1.

tion of crude enzyme from 'Violet' seedlings that had been cultured under poor nutritional conditions for 14 days was used as a solution with high PAL activity (Fig. 1A, day 14). Three solutions with low PAL activity were prepared, and each was mixed separately with the solution with high activity. Table 1 shows the activity of PAL in each mixture. Addition of the solution of crude enzyme (0.1, 0.2 and 0.4 ml) from 'Violet' seedlings that had been cultured under poor nutritional conditions for 4 days (Fig. 1A, day 4) and from 'Violet' seedlings that had been cultured under poor nutritional conditions for 14 days and then in a nutrient-rich solution for 6 days (Fig. 1A, day 20 of dashed line) did not affect the activity of PAL. A solution of crude enzyme from 'Kidachi' seedlings cultured under poor nutritional conditions for 16 days (Fig. 2A, day 16) also did not decrease the activity.

Inhibition of PAL activity by AOA in vitro—As shown in Fig. 3, AOA inhibited the activity of PAL in the solution of crude enzyme from 'Violet' seedlings that had been cultured under poor nutritional conditions for 14 days (Fig. 1A, day 14). The concentrations giving 50% inhibition were 1.8 and 8.4 mM for reaction mixtures that contained 1 and 10 mM phenylalanine, respectively.

Discussion

The method used in the present study to determine the activity of PAL in a solution of crude enzyme from seedlings of *P. nil* was proved to be useful by quantitation of the *t*-cinnamic acid formed per min by the method of Zucker (1965). The activity in solutions of crude enzyme from the cotyledons was relatively low, so the reaction mixtures were incubated for 15 h.

There was a good correlation between PAL activity and the induction of flowering in 'Violet' seedlings grown under poor nutritional conditions, high-intensity light, and low temperature (Figs. 1A, 1B, and 1C, respectively), and the PAL activity was well correlated with the amounts of

Table 1 Effects of solutions of crude enzyme with low PAL activity on the activity of PAL in a solution of crude enzyme with high PAL activity

Source of solution of crude enzyme		Relative activity of PAL (%)			
Strain	Culture conditions	Without mixing	With mixing[a]		
			0.1 ml	0.2 ml	0.4 ml
'Violet'	Poor nutrition for 14 days	100	—	—	—
	Poor nutrition for 4 days	6	108	106	103
	Poor nutrition for 14 days followed by rich nutrition for 6 days	7	98	98	107
'Kidachi'	Poor nutrition for 16 days	9	100	102	103

[a] 0.2 ml of a solution of crude enzyme from 'Violet' seedlings that had been cultured in tap water for 14 days was mixed with 0.1, 0.2, and 0.4 ml of the solutions of crude enzyme indicated in the Table.

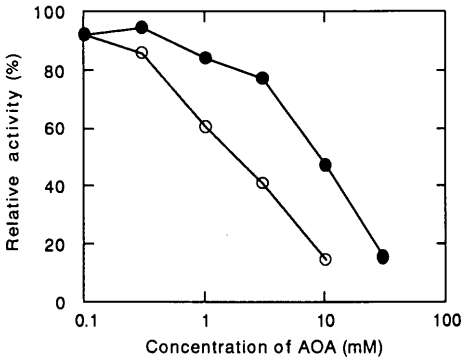

Fig. 3 Effect of AOA on the activity of PAL in a solution of crude enzyme obtained from 'Violet' seedlings that had been cultured under poor nutritional conditions for 14 days. The concentration of phenylalanine was 1 mM (○) and 10 mM (●). Each point represents the mean of results of three measurements and standard errors were less than 4% in every case. For details of the assay, see Materials and Methods.

phenylpropanoids in seedlings (see Fig. 3 in Hirai et al. 1993, Fig. 1 in Shinozaki et al. 1994). The promotive effect of a prior treatment with high-intensity light on the induction of flowering and on the accumulation of phenylpropanoids upon exposure to low temperature (see Fig. 2 in Hirai et al. 1994) was also observed when the activity of PAL was examined (Fig. 1D). These findings suggest that the non-photoperiodic induction of flowering in 'Violet' seedlings is correlated with an increase in the activity of PAL in the cotyledons, which causes the accumulation of phenylpropanoids.

The phenylpropanoids that accumulated under poor nutritional conditions and at low temperature were different: chlorgenic acid, coumaroylquinic acid and pinoresinol-*O*-β-D-glucoside were accumulated under poor nutritional conditions (Hirai et al. 1993); and feruloylquinic acid and dehydrodiconiferyl alcohol-13-*O*-β-D-glucoside were accumulated at a low temperature (Hirai et al. 1994). This difference suggests that the enzymes whose activities increased under the two conditions might be different forms of PAL. The isozymes of PAL in *P. nil* have not been reported, but the presence of PAL isozymes has been demonstrated in other plants: spinach leaves have three forms of PAL which differ in terms of both localization and sensitivity to thioredoxin (Nishizawa et al. 1979), and polymorphic forms have been observed in three divergent classes of PAL genes in the bean genome (Cramer et al. 1989).

The change in the activity of PAL in seedlings during exposure to high-intensity light (Fig. 1B) differed from that during culture under poor nutritional conditions or at a low temperature. The increase in the activity under high-intensity light was more rapid than that under the latter two conditions. However, no accumulation of phenylpropanoids was observed during the early period of high-intensity light treatment (Shinozaki et al. 1994), an indication that this increase in activity might not be involved in the synthesis of phenylpropanoids. The substrate for PAL, namely, phenylalanine, might not be available, or the activity of the enzyme necessary for the next step in the biosynthesis of phenylpropanoids, cinnamate 4-hydroxylase, might be low during this period. At present, it is not clear why the increase in PAL did not cause an increase in levels of phenylpropanoids during the early period of exposure to high-intensity light, but the PAL whose activity increased during this period seems to differ from the PAL whose activity increased later. The promotive effect of high-intensity light on the accumulation of phenylpropanoids and on the flower-inducing effect of low temperature suggests that the PAL whose activity increased early during exposure to high-intensity light was the same as the PAL whose activity increased at a low temperature. The PAL whose activity increased during the later period of the irradiation with high-intensity light might be the same form of PAL as the PAL whose activity increased under poor nutritional conditions, since the same phenylpropanoids accumulated under both sets of conditions. The localization and the activity of other enzymes in the biosynthesis of phenylpropanoids and lignans (Hahlbrock and Scheel 1989, Lewis and Yamamoto 1990) might be also involved in the control of the kind of phenylpropanoids that are accumulated.

The activity of PAL in 'Kidachi' seedlings was low throughout the period of poor nutrition, which did not induce flowering in these seedlings. This result coincides with the absence of any accumulation of phenylpropanoids under poor nutritional conditions in this strain (Shinozaki et al. 1988b, Hirai et al. 1993). The activity of PAL in the seedlings cultured at a low temperature, namely, flower-inducing conditions, was higher than that in control seedlings, although the activity was lower than that of 'Violet' seedlings cultured at a low temperature. Thus, there was a good correlation between the activity of PAL and induction of flowering in 'Kidachi' seedlings as well as in 'Violet' seedlings.

The activity of PAL is inhibited by *t*-cinnamic acid and *p*-coumaric acid (Camm and Towers 1973). However, the activity of PAL in the solution of crude enzyme from 'Violet' seedlings grown under poor nutritional conditions for 14 days was unaffected by the addition of solutions of crude enzyme with low PAL activity. This result indicates that the concentration of putative inhibitors in the solution of crude enzyme was too low to inhibit the activity of PAL (Hirai et al. 1993). The increase or decrease in the activity of PAL in the seedlings examined here would seem, therefore, to have been caused by a change in the concentration of PAL protein rather than a change in the concentration of an inhibitor of PAL. This observation suggests, moreover, that the PAL protein is synthesized under non-

photoperiodic conditions. The increase in PAL activity in elicitor-treated bean cells has been shown to be caused by stimulation of the synthesis of PAL mRNA (Edwards et al. 1985). The decrease in the activity of PAL in 'Violet' seedlings transplanted from poor nutritional conditions to Nakayama's solution might be caused by interruption of the transcription of PAL mRNA and degradation of the PAL protein.

The inhibition of the activity of PAL by AOA was confirmed with a solution of crude enzyme from 'Violet' seedlings; the extent of inhibition was dependent on the molar ratio of phenylalanine to AOA. Therefore, the inhibition was competitive. This finding suggests that the inhibition of the accumulation of phenylpropanoids in vivo and inhibition of the induction of flowering by AOA (Shinozaki et al. 1988a, 1994) is caused by the inhibition of PAL in the seedlings.

Dilution of suspension cultures, light, low temperature and pathological events induce an increase in the activity of PAL in many species of plants (Hanson and Havir 1981, Jones 1984). The former three treatments are similar to the non-photoperiodic conditions that induce flowering of *P. nil* seedlings, but PAL activities have not yet been examined precisely in relation to the induction of flowering. Although the increase in the activity of PAL might be a side effect linked to the process of induction of flowering in seedlings of *P. nil*, the close correlation between the increase in PAL activity and the induction of flowering in *P. nil* seedlings does not exclude the possibility that the accumulation of phenylpropanoids might be associated with some process that is essential for the induction of flowering. The inhibition of induction of flowering by AOA provides a circumstantial evidence in support of this possibility. It is unlikely that the ammonium ion released from phenylalanine by PAL, rather than the phenylpropanoids, is critical in the induction of flowering. Exogenous ammonium ions have been shown to inhibit the induction of flowering in *Lemna* (Hillman and Posner 1971, Tanaka and Takimoto 1975).

A short-day treatment did not cause changes in the activity of PAL or in the concentration of phenylpropanoids (Hirai et al. 1993) in seedlings of either strain of *P. nil*. AOA, supplied to the root (Shinozaki et al. 1988a) and to the cotyledon (Amagasa et al. 1992), also did not inhibit the photoperiodic induction of flowering in 'Violet' seedlings. The process of photoperiodic induction seems, therefore, to be rather different from that of the non-photoperiodic induction of flowering.

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