Interaction of Microsomal Cytochrome *P*-450s and N-Phenylcarbamates that Induce Flowering in *Asparagus* Seedlings

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n-Propyl N-(3,4-dichlorophenyl)carbamate, which induces flowering while it inhibits a step or steps in the phenylpropanoid metabolism in *Asparagus officinalis* L. seedlings, was found to retard the conversion of *t*-cinnamic acid to *p*-coumaric acid by high-pressure liquid chromatography of the metabolites in the shikimic acid pathway. The concentrations of the metabolites preceding *t*-cinnamic acid on the pathway in treated and untreated seedlings were the same, but those of *p*-coumaric acid and later metabolites were significantly lower in treated plants. The carbamate inhibited phenylpropanoid metabolism when used to treat stem segments that included a shoot apex primordium, where flowers are induced, and when added to a 100,000 × *g* microsomal fraction prepared from such segments. NADPH-cytochrome *P*-450 and NADH-cytochrome b_5 reductases in the 100,000 × *g* fraction were not inhibited by the carbamate. The results showed that this compound has its site of action on the endoplasmic reticulum and that it inhibits cytochrome *P*-450s, including *t*-cinnamic acid 4-hydroxylase. We examined the flower-inducing activity of known cytochrome *P*-450 inhibitors, and found that piperonyl butoxide also causes flowering.

Introduction

A series of N-phenylcarbamates originally developed as anticytokinins induces flowering in seedlings of *Asparagus officinalis* L. when applied during shoot apex differentiation, 4 to 10 days after sowing [1]. Flowering occurs only once at the top of the seedlings, *i.e.* at the terminal bud. In search of the biochemical changes linked with the action of these chemicals, we have found that the concentrations of chlorogenic acid and other phenylpropanoids decrease with increasing concentrations of the chemicals and thus decrease with an increasing rate of flowering [2].

The concentration of chlorogenic acid is correlated with photoperiodic flowering in *Nicotiana tabacum*, and *N. sylvestris* [3], and *Lemna gibba* [4], with flowering during poor nutrition in *Pharbitis nil* [5], and with nights interrupted by light that inhibits flowering in *Xanthium* [6]. In these studies, the step at which the change in the metabolic pathway leading to chlorogenic acid biosynthesis occurs has not been identified, nor has a cause-effect relationship with flowering been established.

In this study, we used HPLC to examine plant extracts to find the step of phenylpropanoid metabolism inhibited by one of these carbamates, *n*-propyl N-(3,4-dichlorophenyl)carbamate (5091). Inhibition of the conversion of *t*-cinnamic acid to *p*-coumaric acid, catalyzed by *t*-cinnamic acid 4-hydroxylase (C4H, EC 1.14.13.11), was found. The carbamate inhibited the enzyme *in vitro* when applied to stem segments that included a shoot apex primordium, where flowers are induced [2], and also inhibited the enzyme in a microsomal fraction prepared from stem segments. The results suggested that the flower-inducing carbamate has its site of action in the endoplasmic reticulum (ER) for inhibition of cytochrome *P*-450s, including C4H. Based on this finding, we examined the flower-inducing activity of some cytochrome *P*-450 inhibitors and found that piperonyl butoxide caused flowering of asparagus seedlings as well as carbamate 5091.

Materials and Methods

Chemicals and plant

n-Propyl-N-(3,4-dichlorophenyl)carbamate (5091) was prepared as reported elsewhere [1].

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Piperonyl butoxide was purchased from Wakenyaku Co., Ltd. (Kyoto, Japan), metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was from Aldrich Chemical Co., Inc., SKF 525 A (2-diethylaminoethyl 2,2-diphenylvalerate) was from Funakoshi Co., Ltd. (Tokyo, Japan), glucose-6-phosphate dehydrogenase (Type XV) (EC 1.1.1.49) and cytochrome *c* (horse heart) were from Sigma Chemical Co. A phenylalanine ammonialyase (EC 4.3.1.5) inhibitor S-84702 [1-amino-1-hydroxycarbonylmethyliden-(3-chloro-2-methyl)aniline] was a gift from Sumitomo Chemical Co., Ltd. Seeds of *Asparagus officinalis* L. *cv.* Mary Washington 500 W were purchased from the Kaneko seed Co. (Gumma, Japan).

Analysis of phenylpropanoids in seedlings

Methods of the experiments were basically the same as reported previously [2]. In brief, 80 seeds were incubated at 25 °C with 20 ml of test solution in a Petri dish (90 mm i.d. × 20 mm high) containing three layers of filter paper under four layers of tissue paper. The incubation was made at 25 °C with a 12 h period of light from fluorescent lamps. The chemicals, dissolved in dimethyl sulfoxide, were diluted to an appropriate concentration so that the final concentration of the organic solvent did not exceed 0.5% (v/v). The seedlings, from which seeds were detached, were extracted with boiling 2% acetic acid for 10 min. The extracts were analyzed on a CCPM HPLC system (Tosoh, Tokyo, Japan) equipped with a UV detector. The column (M&S C_{18}, 4.6 mm i.d. × 15 cm) was eluted with a gradient starting with a 1:1:2:96 (v/v) mixture of MeOH, *n*-BuOH, AcOH, and H_2O and ending with a 97:1:2 mixture of MeOH, *n*-BuOH, and AcOH at 35 °C and at a flow rate of 1 ml min^{-1}, with monitoring at 280 nm. The phenylpropanoids were identified as reported previously [5].

Assay of t-cinnamic acid 4-hydroxylase activity

Experiments with stem segments: Segments were prepared from seedlings of an appropriate age by removal of the seed, the root, and as much as possible of the lower part of the stem, leaving the bud (shoot apex) primordium. For feeding experiments, 15 segments were placed in a Petri dish (40 mm i.d. × 10 mm high) that contained 10 ml of the test solution and incubated at 25 °C in the dark. A *t*-cinnamic acid solution (2.2 mM) was prepared before each experiment by the dissolution of an appropriate amount in distilled water. The preparation of 5091 solution and the extraction and analysis of the *p*-coumaric acid produced were done as described above.

Experiments with cell-free preparations: A crude extract of seedlings was prepared and assayed for C4H activity by the method of Lamb and Rubery [7] with some modification. The seedlings, from which seeds had been detached, were homogenized with sea sand (1.5 g/g fr.wt.) in 50 mM sodium phosphate buffer (pH 7.6, 8 ml/g fr.wt.) containing 1 mM EDTA-2Na, Polycalar AT (0.15 g/g fr.wt.) with a chilled mortar and pestle. The extract was centrifuged at 500 × *g* for 10 min to remove debris and sea sand. To 0.8 ml of 50 mM sodium phosphate buffer (pH 7.6) containing 1 mM NADP (sodium salt), 2.5 mM glucose-6-phosphate (disodium salt), 1.5 mM 2-mercaptoethanol, glucose-6-phosphate dehydrogenase (2 units/ml) and 0.33 mM *t*-cinnamic acid solution (final concentration, 0.22 mM), 0.4 ml of the crude enzyme extract was added, and the mixture was incubated at 30 °C for 1 h with vigorous shaking. The reaction was stopped by the addition of 24 µl of acetic acid and boiling of the mixture for 1 min. The mixture was then cooled and centrifuged at 1,000 × *g* for 10 min. The concentration of *p*-coumaric acid in the supernatant was assayed as described above. The concentration of *p*-coumaric acid after the reaction was subtracted from the concentration before the reaction, and the C4H activity was calculated from the remainder in each experiment.

Experiments with microsomal fraction: The experiments were done as described elsewhere [8–11]. Seeds were incubated for 10 days under the conditions described above. The seedlings, from which seeds were detached (14 g fr.wt. from about 1300 seedlings), were homogenized with a Polytoron homogenizer (Kinematica PT 10 20 350 D) in two volumes (2 ml/g fr.wt.) of extraction buffer (50 mM Tris-HCl buffer, pH 8.0) containing 0.3 M D-sorbitol, 1 mM EDTA-2Na, 1% (wt./v) sodium isoascorbate, and Polyclar AT (0.05 g/g fr.wt.) at 2–4 °C. The homogenate was forced through four layers of cotton gauze, and the filtrate was centrifuged at 500 × *g* for 10 min to remove debris and the Polyclar AT. The supernatant was centrifuged at 20,000 × *g* for 15 min, and the precipitate suspended in 3 ml of the extraction buffer. The re-

sultant supernatant was centrifuged again at 100,000 × *g* for 60 min. The precipitate was suspended in 7 ml of the extraction buffer.

This microsomal suspension (0.4 ml) was mixed with 3 µl of dimethyl sulfoxide containing a test chemical and 0.4 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 3 mM NADP (sodium salt), 10 mM glucose-6-phosphate (disodium salt), 3 mM 2-mercaptoethanol, and glucose-6-phosphate dehydrogenase (4 units/ml), and incubated first at 30 °C for 15 min. To the mixture was added 0.4 ml of 2 mM *t*-cinnamic acid solution in the buffer, bringing the final concentration to 0.66 mM, and the mixture was incubated at 30 °C for 1 h with vigorous shaking. The reaction was stopped as described above, and *p*-coumaric acid was assayed by HPLC.

Assay of NADPH-cytochrome c (P-450) and NADH-cytochrome c (b_5) reductases

To 50 mM potassium phosphate buffer (pH 7.7) containing 50 µM horse heart cytochrome *c*, 1 mM KCN, and an appropriate amount of the chemical, the microsomal fraction prepared as above but without sodium isoascorbate was added so that there was a total of 100 µg of protein. For the assay of NADPH-cyt. *P*-450 reductase (EC 1.6.2.4), the reaction was started by the addition of 25 mM NADPH (or NADH) solution in 10 mM Tris (pH 10.2) to a final concentration of 100 µM, and the increment of optical density at 550 nm was measured at 25 °C with a Shimadzu UV-3000 spectrophotometer. For the assay of NADH-cyt. b_5 reductase (EC 1.6.2.2), the microsomal preparation was added to the reaction mixture so that there was a total of 20 µg of protein. Both cytochrome *c* and potassium ferricyanide were used as electron acceptors; when potassium ferricyanide was used, reduction was monitored at 420 nm. The milimolar extinction differences (cm^{-1} mM^{-1}) between the reduced and oxidized electron acceptors used are 21.1 at 550 nm for cytochrome *c* [12] and 1.02 at 420 nm for potassium ferricyanide [13]. The reductase activity was expressed as µmol min^{-1} mg^{-1} protein.

The protein in the microsomal preparation was measured as described elsewhere [14]. A 0.1 ml portion of the fraction was mixed with Coomassie Brilliant Blue reagent (0.01% dye, 4.7% ethanol, and 8.5% phosphoric acid, wt./v), and the absorbance at 595 nm was measured. Bovine serum albumin was taken as the standard.

Flowering experiments

Fourty seeds were incubated as above, and, ten days after being sown, the germinated seeds were washed in running water, planted in Vermiculite, and grown for 13 days at 25 °C with 12 h of light every 24 h. The rate of flowering (%) was calculated as the (number of plants with flowers/number of plants that had emerged from the Vermiculite) × 100.

In figures, the means of at least four runs of experiments are shown, and vertical bars express the standard errors.

Results

Site of retardation of the secondary metabolism

Eight-day-old seedlings, from which seeds were detached, were extracted. The concentrations of phenylalanine and *t*-cinnamic acid were little affected by the use of 5091 in the concentration range that induce flowering (Fig. 1 A). The same was observed for phenylpyruvic acid and shikimic acid, in the early parts of the pathway (data not shown). However, the concentrations of *p*-coumaric acid, caffeic acid, and chlorogenic acid in the lower parts decreased as the concentration of 5091 increased (Fig. 1 B), indicating that the compound inhibits the step of the conversion of *t*-cinnamic acid to *p*-coumaric acid.

Effects on t-cinnamic acid 4-hydroxylase activity in stem segments and cell-free preparations

When stem segments prepared from 8-day-old seedlings were incubated with 200 µM *t*-cinnamic acid, the concentration of *p*-coumaric acid in the segments increased with incubation time up to 2 h and then decreased to reach a stationary phase (Fig. 2 A, open circles). When the segments were first incubated with 400 µM 5091 for 2 h and then 200 µM *t*-cinnamic acid was added, inhibition of *p*-coumaric acid production occurred (Fig. 2 A, closed circles). Similary when segments prepared from seedlings grown for 8 days in the presence of 400 µM 5091 were incubated with *t*-cinnamic acid but without 5091, inhibition was observed (Fig. 2 B, closed circles).

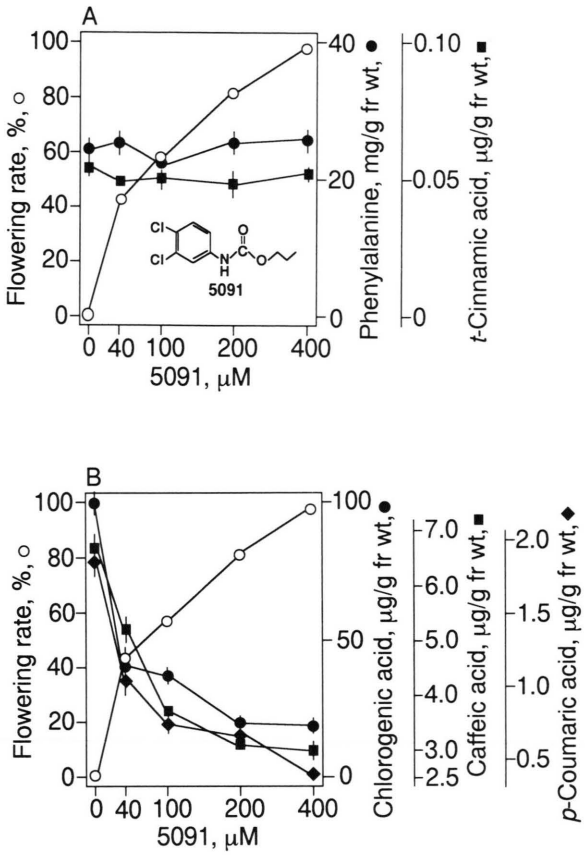

Fig. 1. Flowering response and changes in concentrations of secondary metabolites. Flowering occurred in plants treated with 5091 for 12 days after sowing and grown in Vermiculite for the next 13 days (open circles). The phenylpropanoids were assayed in 8-day-old seedlings with seeds detached. A: Relationship between flowering and concentrations of phenylalanine and *t*-cinnamic acid. B: Relationship between flowering and contents of chlorogenic acid, caffeic acid, and *p*-coumaric acid.

The cell-free preparations were prepared from seedlings grown for 8–14 days from sowing with and without 5091, and their C4H activities were compared. As the results, the enzyme activity per plant tended to decrease gradually with the growth of the plants, but a fall due to growth in the presence of 5091 was not observed (Fig. 3). The set of results showed that the retardation of the phenylpropanoid metabolism was by virtue of inhibition of C4H activity, not due to inhibition of C4H biosynthesis.

Effects on t-cinnamic acid 4-hydroxylase activity in microsomes

The 100,000 × *g* microsomal fraction was prepared from stem segments of 10-day-old plants. The fraction converted *t*-cinnamic acid to *p*-coumaric acid at a linear rate for at least 2 h of incubation under the present experimental condition (inset, Fig. 4). Thus, the reaction for 1 h was studied in the experiments in which the effects of 5091 were examined. The production of *p*-coumaric acid was inhibited with increasing concentrations of the chemical (Fig. 4).

Effects on NADPH-cytochrome c (P-450) and NADH-cytochrome c (b_5) reductases

NADPH-cytochrome *c* (*P*-450) and NADH-cytochrome *c* (b_5) reductases in microsomal fraction were not inhibited by 5091 (Fig. 5), indicating that 5091 did not retard the action of C4H by inhibiting these reductases. In the NADH-cyt. b_5 reductase assay, when potassium ferricyanide was used as an electron acceptor, the results were the same as with cytochrome *c* (data not shown).

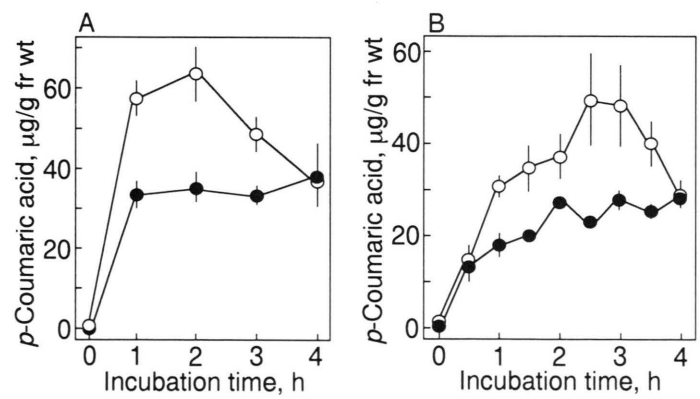

Fig. 2. Effects of 5091 on the metabolism of *t*-cinnamic acid in stem segments. A: Stem segments prepared from 8-day-old seedlings grown without 5091 were incubated with 400 μM 5091 for 2 h at 25 °C in the dark, and then 200 μM *t*-cinnamic acid was added to the medium. B: Stem segments prepared from 8-day-old seedlings grown with (closed circles) and without (open circles) 400 μM 5091 were incubated with 200 μM *t*-cinnamic acid at 25 °C in the dark.

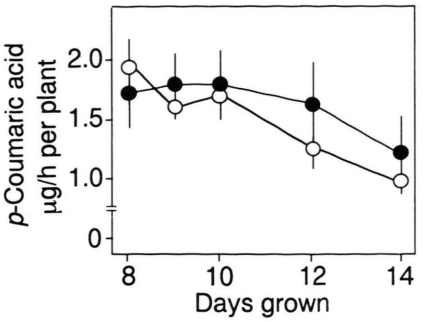

Fig. 3. *t*-Cinnamic acid 4-hydroxylase activity of cell-free preparations from seedlings with seeds detached. Seedlings grown with (closed circles) or without (open circles) 200 μM 5091 for the time shown after sowing were used, and the cell-free preparations were incubated with 0.22 mM *t*-cinnamic acid for 1 h.

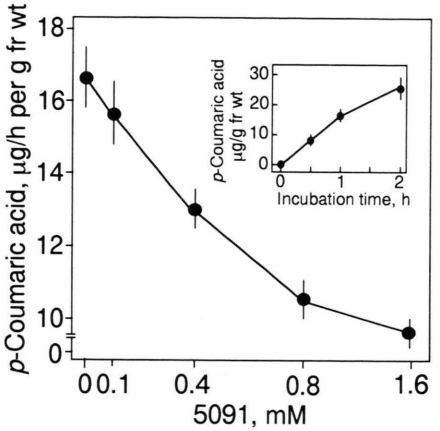

Fig. 4. Effects of 5091 on *t*-cinnamic acid 4-hydroxylase activity in the microsome preparation. Medium containing 0.66 mM *t*-cinnamic acid was incubated for 1 h at 30 °C. The incubation period was set at 1 h, when *t*-cinnamic acid was converted to *p*-coumaric acid at a linear rate, on the basis of the results shown in the inset.

Effects of cytochrome P-450 inhibitors on flowering and C4H activity

Among the *P*-450 inhibitors tested, piperony butoxide, SKF 525 A, and metyrapone, piperonyl-butoxide caused flowering, at a rate as high as or slightly less than that with 5091: flowering was 0%, 28%, 41%, 72%, and 85% at 0, 40, 100, 200, and 400 μM, respectively. Piperonyl butoxide inhibited C4H activity in the microsomal fraction slightly less than 5091 did (data not shown).

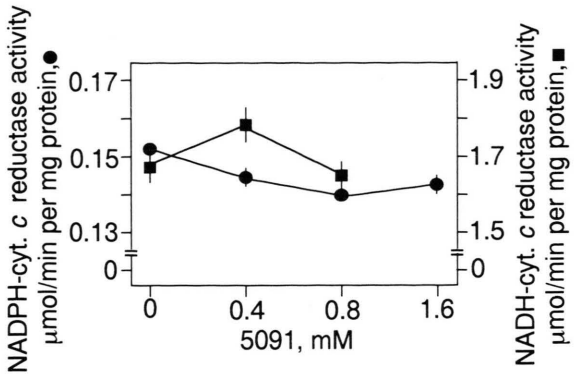

Fig. 5. Effects of 5091 on NADPH-cytochrome *c* (*P*-450) and NADH-cytochrome *c* (b_5) reductase activities. The microsomal fraction containing cytochrome *c* as an electron acceptor was incubated with NADPH or NADH, and the increment of absorption at 550 nm was measured at 25 °C.

Effects of a phenylalanine ammonia-lyase inhibitor on flowering and phenylpropanoid metabolism

We incubated seeds with S-84702, a phenylalanine ammonia-lyase inhibitor recently developed by Sumitomo Chemical Industries, Co., Ltd. S-84702 decreased the levels of the metabolites assayed as much as 5091 did (Fig. 6, closed circles), but flowering did not occur (closed squares), sug-

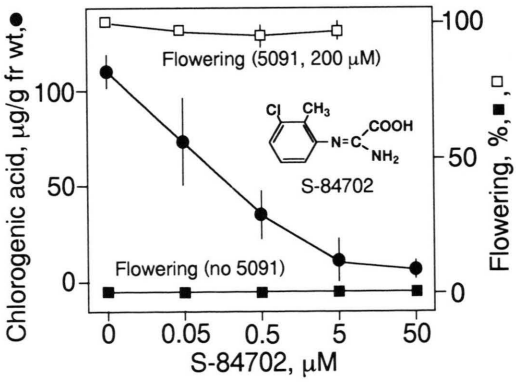

Fig. 6. Effects of S-84702 on flowering. Seeds were incubated with various concentrations of the phenylalanine ammonia-lyase inhibitor for 8 days at 25 °C with a 12 h light period, stem segments prepared from the germinated seeds were extracted, and chlorogenic acid was assayed (closed circles). For observation of flowering, seeds germinated on a medium containings various concentration of S-84702 and zero (closed squares) or 200 μM (open squares) 5091 were planted in Vermiculite and grown for 13 days.

gesting that the fall in the phenylpropanoid metabolism is not the cause of flower induction. When seeds were incubated with a medium containing both 200 μM 5091 and various concentrations of S-84702, the seedlings had flowers as well as those treated with 5091 only did (Fig. 6, open squares), denying the possibility that S-84702 might act at a later step to obstruct the formation or development of flower buds after induction.

Discussion

The flower-inducing 5091 was shown in intact plants as well as in steg segments to retard the phenylpropanoid metabolism at the step in which *t*-cinnamic acid is converted to *p*-coumaric acid by C4H. Since the C4H activity was little different between the cell-free preparations prepared from the seedlings grown with an without 5091, the retardation is thought to be not due to the inhibition of biosynthesis of C4H but by virtue of the inhibition of action of C4H. The site of action of the chemical was identified to be in the microsomal fraction, in which the C4H activity has been found to be localized [9, 15]. Flower induction is thought to be triggered in the endoplasmic reticulum (ER).

Young and Beevers [16] have shown that C4H is a NADPH-dependent cytochrome *P*-450 enzyme principally associated with the ER in castor bean endosperm tissue, and Benveniste *et al.* [17, 18] have made the same suggestion about Jerusalem artichoke tubers and pea seedlings. C4H is said to be associated with the ER in *Sorghum* seedlings [9] and potato tubers [19], as well. In our study, the activities of NADPH-cytochrome *P*-450 reductase (a marker enzyme of the ER [17]) and NADH-cytochrome b_5 reductase were found in the microsomal fraction. These reductases were not inhibited by 5091, so it seems to be a cytochrome *P*-450 inhibitor.

The possibility that known cytochrome *P*-450 inhibitors cause flowering as well was examined. Piperonyl butoxide caused flowering comparable to that caused by 5091, but SKF 525A and metyrapone had little activity. SKF 525A and metyrapone did not inhibit phenylpropanoid metabolism in intact seedlings, but piperonyl butoxide did. As expected, piperonyl butoxide inhibited C4H in the microsomal fraction. This flower-inducing activity of the compound known to be a cytochrome *P*-450 inhibitor further evidences our conclusion that 5091 acts on the ER to cause flowering.

The process in which the chemical acts is thought to be the process responsible for the triggering of flower induction. However, the fall in phenylpropanoid metabolism caused by the inhibition of C4H is ruled out as the cause of flower induction. This conclusion is drawn from the fact that flowering did not occur even if the phenylpropanoid pathway was prevented by an inhibitor of phenylalanine ammonia-lyase, the enzyme involved in the step preceding that catalyzed by C4H. The fall in the phenylpropanoid contents is due to the inhibition of C4H by 5091, so it cannot be the results of flowering. It is thought to be an event linked to flower induction.

Piperonyl butoxide is a broad-spectrum inhibitor of animal cytochrome *P*-450s [15, 20]. Its spectrum of effects on plant *P*-450s has not been reported, but in instances in which *P*-450 inhibitors (all of which were synthesized to inhibit mixed-function oxidases of animal origin) have been found to inhibit plant enzymes, they inhibit more than one. 1-Aminobenzotriazole inhibits C4H strongly and lauric acid 12-hydroxylase weakly [11]; 11-dodecanoate inhibits C4H but inhibits lauric acid inchain hydroxylase more strongly [21, 22]; and metyrapone inhibits the hydroxylation of cytokinins, N^6-isopentenyladenine and -adenosine in the microsomal fraction of cauliflower [23], and also inhibits flavon synthase and C4H of soybean callus cells [24]. In the same way, 5091 and piperonyl butoxide probably inhibit other cytochrome *P*-450s besides C4H, and one seems to be responsible for triggering the action of the flowering machinary.

Acknowledgements

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