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

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Z. Naturforsch. 48c, 879–885 (1993); received May 21, 1993

N-Phenylcarbamates, Flower Induction, Asparagus officinalis, Microsomes, Cytochrome P-450s

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 r-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 r-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 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 r-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 anticytokininins 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 Lemma 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 r-cinnamic acid to p-coumaric acid, catalyzed by r-cinnamic acid 4-hydroxylase (C 4H, 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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Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0939–5075/93/1100–0879 $ 01.30/0

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Piperonyl butoxide was purchased from Waken-yaku Co., Ltd. (Kyoto, Japan), metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was from Aldrich Chemical Co., Inc., SKF 525A (2-diethylaminoethyle 2,2-diphenylvalerate) was from Funa-koshi 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-hydroxy-2-methyl-1,2-di-3-pyridyl-1-propanone] was from Funakoshi Co., Ltd. (Tokyo, Japan), glucose-6-phosphate dehydrogenase (2 units/ml) and 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 x 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 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, Polyclar AT (0.15 g/g fr.wt.) with a chilled mortar and pestle. The extract was centrifuged at 500 x 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 x 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 Polytron 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 isoaasorbate, and Polyclar AT (0.05 g/g fr.wt.) at 2–4 °C. The homogenate was forced through a polyethylene tubing, and then cooled and centrifuged at 1,000 x g for 10 min to remove debris and the Polyclar AT. The supernatant was centrifuged at 20,000 x 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 (b5) 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. b5 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, caffeeic 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. 2A, 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. 2A, closed circles). Similarly 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).
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 x 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 (b5) reductases**

NADPH-cytochrome c (P-450) and NADH-cytochrome c (b5) 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. b5 reductase assay, when potassium ferricyanide was used as an electron acceptor, the results were the same as with cytochrome c (data not shown).

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**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.

**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 \( \mu \text{M} \) 5091 for the time shown after sowing were used, and the cell-free preparations were incubated with 0.22 \( \text{mM} \) \( t \)-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 \( \text{mM} \) \( t \)-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 \( C_4 H \) activity

Among the \( P \)-450 inhibitors tested, piperonylbutoxide, SKF 525A, and metyrapone, piperonylbutoxide 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 \( \mu \text{M} \), respectively. Piperonylbutoxide inhibited \( C_4 H \) 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 \( c (b_6) \) 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 containing various concentrations of S-84702 and zero (closed squares) or 200 \( \mu \text{M} \) (open squares) 5091 were planted in Vermiculite and grown for 13 days.
suggesting 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 or 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 b5 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 SKF525A and metyrapone had little activity. SKF525A 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-dodecanoyl inhibits C4H but inhibits lauric acid inchain hydroxylase more strongly [21, 22]; and metyrapone inhibits the hydroxylation of cytokinins, N6-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 machinery.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Special Research on Priority Areas (Project No. 0166005; Cellular and Molecular Basis for Reproductive Processes in Plants) from the Ministry of Education, Science, and Culture, Japan.
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