The Role of Aspterric Acid in Auxin-Regulated Reproductive Growth of *Arabidopsis thaliana*

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Application of 100 μM aspterric acid (AA), a pollen growth inhibitor, with different concentrations of indole-3-acetic acid (IAA) results in the recovery of normal pollen development of *Arabidopsis thaliana*. Treatment with 100 μM AA plus 5 mM IAA significantly induced the normal seed production. Treatment with 100 μM *N*-1-naphthylphthalamic acid (NPA), a polar auxin transport inhibitor, did not reduce the pollen growth but inhibited seed production. 100 μM NPA plus 5 mM IAA did not induce any seed production. The endogenous level of IAA in stems and leaves of *A. thaliana* treated with 100 μM AA was similar to that of the untreated control. In contrast to AA treatment, the IAA level by the treatment with 100 μM NPA was about twice as much as that of the untreated control. These results suggest that AA affects the *Arabidopsis* reproductive growth without inhibiting IAA biosynthesis and transport.

Key words: Aspterric Acid, Indole-3-acetic Acid, *Arabidopsis thaliana*

Introduction

Auxin, indole-3-acetic acid (IAA), causes important physiological effects on plant growth. IAA promotes root formation, and stem and leaf elongation by its role in inducing cell elongation (Li and Liu, 2003). However, the role of auxin in the reproductive organogenesis is still poorly understood, because the development of floral organs is a complex phenomenon (Martinez-Zapater *et al*., 1994; Clark and Meyerowitz, 1994). Previous studies indicated that auxin likely plays an important role in the developing gynoecium and androecium of *Arabidopsis thaliana* (Sessions *et al*., 1997; Nemhauser *et al*., 2000; Shimada *et al*., 2005).

Aspterric acid (AA), a pollen growth inhibitor, has been isolated from the fungus *Aspergillus terreus* (Tsuda *et al*., 1978; Shimada *et al*., 2002) (Fig. 1). Application of AA with IAA resulted in the recovery of the normal pollen development of *A. thaliana* in our study (Shimada *et al*., 2005). However, the relationship between AA and IAA through *Arabidopsis* reproductive growth including seed production has not been fully understood. The mode of action of AA on the reproductive growth of *A. thaliana* was investigated using HPLC quantification of IAA (Koshiba *et al*., 1995) and the technique of applying AA with IAA. We describe here the role of AA in the auxin-regulated reproductive growth of *A. thaliana*

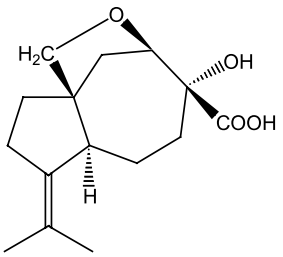

Fig. 1. Chemical structure of aspterric acid.

Materials and Methods

Plant material

Seeds of *A. thaliana* were sown in plastic pots (8 cm inner diameter) filled with a mixture of perlite and vermiculite (1:1, v/v) and germinated in a growth chamber maintained at 25 °C under continuous light (100 μE/m^2 s). Liquid fertilizer was

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applied once a week. The fertilizer contained 5 mM KNO_3, 2.5 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 μM Fe-EDTA, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4$, 10 μM NaCl, and 0.01 μM $CoCl_2$.

Treatment with AA and plant hormones

Chemicals to be tested were each formulated as an aqueous solution containing 0.1% Tween-80 as a wetting agent and 2% EtOH to aid their solubility. Each solution was sprayed on all leaves with an atomizer at the rate of 1 ml per three pots. The chemicals were applied once in 2 d for a total of 3 treatments from the period rosette leaf formed (17 d after sowing). In combined treatments with IAA, 5 mM IAA was applied alone twice in 4 d after 3 times of combined treatments with AA or *N*-1-naphthylphthalamic acid (NPA). Triplicate experiments were conducted.

Data collection

Stem length and the number of flower buds were measured everyday from the start of treatment. The stamens were stained with I_2-KI solution and observed under a light microscope after anthesis.

IAA content in stems and leaves of A. thaliana

Extraction of IAA from stems and leaves was carried out according to a modified method of Koshiba *et al.* (1995). Fresh stems and leaves (*ca*. 0.1 g) just before anthesis were collected, frozen in liquid N_2 and ground in 1 ml 80% acetone containing 0.1 mg/ml butylated hydroxytoluene (BHT) using a mortar and pestle. The suspension was shaken for 1 h on ice in the darkness and then centrifuged (5 °C, 1200 × *g*, 10 min). The pellet was re-extracted for 1.5 h with 1.5 ml 80% acetone by shaking on ice. Acetone was evaporated from the combined supernatants, and the residual aqueous solution (1 ml) was adjusted to pH 3 with 1 M HCl. The aqueous phase was then partitioned 3 times with 1 ml cold diethyl ether containing 0.01 mg/ml BHT. The ether was evaporated under a stream of N_2 gas from the combined diethyl ether phase, and the residual diethyl ether solution (0.8 ml) was then partitioned 3 times with 2% $NaHCO_3$. The aqueous phase was adjusted to pH 3 with 1 M HCl. The aqueous phase was then partitioned 3 times with 1 ml cold diethyl ether containing 0.01 mg/ml BHT. The combined ether phase was evaporated under a stream of N_2 gas and then 0.05 ml MeOH was added. The MeOH solution was used for IAA quantification.

IAA in each sample was quantified chromatographically by HPLC (Model LC-6A; Shimadzu, Tokyo) with a Nucleosil $5N(CH_3)_2$ column (6 mm i.d. × 100 mm; Senshu, Tokyo) at a flow rate of 1 ml/min with 0.3% AcOH in MeOH as the mobile phase. The excitation wavelength was 280 nm, and the emission wavelength was 355 nm; triplicate experiments were conducted.

Results and Discussion

Comparative effects of AA, NPA and combined treatments with IAA

The effects of AA, NPA and combined treatments with IAA on the reproductive growth and seed production of *A. thaliana* are shown in Table I. The anthers treated with 100 μM AA plus 5 mM IAA contained pollen grains, and the siliques treated with 100 μM AA plus 5 mM IAA contained seeds. In contrast to combined treatment, the anthers treated with 100 μM AA alone contained no pollen. Application of 100 μM NPA alone did not show any inhibitory activity against

Table I. Effects of AA, NPA and combined treatments with IAA on the growth of *A. thaliana.*

Treatment	Pollen formation	Seed production	Stem length* [mm]	Flower buds* (No)
Control	+	+	98[b]	22[b]
100 μM AA	–	–	104[b]	17[b]
100 μM AA + 1 mM IAA	+	–	90[b]	16[b]
100 μM AA + 5 mM IAA	+	+	116[b]	18[b]
100 μM NPA	+	–	43[a]	10[a]
100 μM NPA + 1 mM IAA	+	–	48[a]	8[a]
100 μM NPA + 5 mM IAA	+	–	57[a]	9[a]

Data of stem length and flower buds were collected 14 days after the first application.
* Within the same row, values with different superscripts (a and b) are significantly different, $p < 0.05$.

Table II. Effects of AA and NPA on the endogenous level of *A. thaliana.*

Treatment	IAA level* [ng/g fresh weight]
Control	10.1[a]
100 μM AA	14.6[a]
100 μM NPA	29.3[b]

Each sample was collected just before anthesis.
* Within the same row, values with different superscripts (a and b) are significantly different, $p < 0.05$.

pollen development, but the treatment inhibited the reproductive growth and seed production of *A. thaliana*. Similarly, 100 μM NPA plus 5 mM IAA inhibited the growth and seed production of *A. thaliana* without pollen growth inhibition.

Effect of AA on the endogenous level of IAA

The endogenous level of IAA in stems and leaves of *A. thaliana* treated with 100 μM AA was similar to that of the untreated control. In contrast to AA treatment, the treatment with 100 μM NPA increased the IAA level by 186% of the untreated control (Table II).

AA inhibited *Arabidopsis* pollen development at meiosis (Shimada *et al.*, 2002). In contrast to the treatment with AA alone, combined treatment with AA and IAA resulted in the recovery of the normal pollen development of *A. thaliana* (Shimada *et al.*, 2005). Furthermore, combined treatment with AA plus 5 mM IAA resulted in the recovery of the seed production. NPA, a polar auxin transport inhibitor, did not inhibit *Arabidopsis* pollen development but reduced the normal reproductive growth including seed production. Similarly, application of NPA with IAA resulted in the inhibition of the normal reproductive growth. Loss of polar auxin transport in developing *Arabidopsis* leaves, following NPA treatment, reduced the flow of auxin from leaf margins to reproductive organs (Nemhauser *et al.*, 2000). In contrast to NPA treatment, the inhibitory effects against the reproductive growth by AA treatment released by IAA application simultaneously. AA did not reduce the endogenous IAA level. These results suggest that AA is likely to bind the promoters of auxin-regulated genes (Sessions *et al.*, 1997) or an auxin receptor such as the F-box protein TIR1 (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005) and affects the *Arabidopsis* reproductive growth without inhibiting IAA biosynthesis and transport. The mode of action of AA is thus different from that of NPA toward auxin-regulated reproductive growth of *A. thaliana*.

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