Influence of Glyphosate on Flower Morphogenesis and Pigmentation in *Petunia hybrida*

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Glyphosate showed a remarkable effect inducing the change of flower symmetry from the actinomorphic to the zygomorphic type in *Petunia hybrida*. Glyphosate [*N*-(phosphonomethyl)glycine] reduced the anthocyanin content and showed a weak inhibitory effect against phenylalanine ammonia-lyase (PAL) activity. L-2-Aminooxy-3-phenylpropionic acid (APA), an inhibitor of PAL activity, reduced the anthocyanin content but had no effect on flower shape. Additional phenylalanine or *trans*-cinnamic acid, the intermediates of glyphosate inhibition against PAL activity, could not recover the change of flower shape induced by glyphosate. These results suggested that the reduction of PAL activity alone could not account for the two characteristic changes of flower symmetry and pigmentation induced by glyphosate. On the other hand, the results of application of glyphosate-related compounds suggested that the structure of glyphosate contributed to induce the morphological change of *Petunia* flower. Glyphosate may thus be a very useful agent in the elucidation of unresolved questions of flower morphogenesis and the related metabolism.

Key words: Glyphosate, Flower Symmetry, *Petunia hybrida*

Introduction

For the biodesign of flower shape, we need to understand the genetic mechanism that control flower morphogenesis (Hileman *et al.*, 2003; Bergbusch, 1999; van der Krol *et al.*, 1993; Conner, 2002). In recent studies, clonal analysis shows that petal asymmetry of *Antirrhinum* (snapdragon) depends on the direction of growth rather than regional difference in growth rate (Rolland-Lagan *et al.*, 2003). However, the molecular mechanism related to flower morphogenesis is still poorly understood, because the development of flowers is a complex phenomenon.

This study uses a technique of applying chemicals, whose mode of action is known, to *Petunia* flowers for developing a model plant enable to manipulate flower morphogenesis. We describe here the effect of glyphosate on flower symmetry and pigmentation in *Petunia hybrida*.

Materials and Methods

Plant material

Seeds from an F1 hybrid of *Petunia hybrida* purchased from Takii Nursery were sown in multipot plastic trays filled with peat and germinated under continuous light [100 μE/(m^2 s)] at 25 °C for 14 d. Uniform seedlings at the 4-leaf stage were transplanted into 20-cm pots filled with a commercial soil for flower, purchased from Hirata Nursery, and grown in a greenhouse (25/15 °C day/night) under a natural photoperiod. Plants were irrigated once a week with fertilization (4-6-7 NPK).

Chemicals

L-2-Aminooxy-3-phenylpropionic acid (APA), 5-azacytidine, phosphonomycin sodium, gibberellin (GA$_3$), glyphosate, indole-3-acetic acid (IAA), kinetin, paclobutrazol, L-phenylalanine, 3-phosphonopropionic acid (PPA), 4-phosphonobutyric acid (PBA), tetracycline hydrochloride, and L-tryptophan were purchased from Wako Pure

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Chemical Industries, Ltd, Osaka. Naringenin and *N*-(phosphonomethyl) iminodiacetic acid (PIA) were purchased from Sigma-Aldrich Co, USA. *trans*-Cinnamic acid was purchased from Tokyo Kasei Kogyo Co., Tokyo.

Application of chemicals

An aqueous solution to be tested contained 0.5 or 5 mM test compound, 0.1% Tween-80 as a wetting agent and 2% EtOH to aid its solubility. Each solution was sprayed on all leaves with an atomizer at the rate of 1 ml per pot. The chemicals were applied once in 2 d for a total of 3 treatments from 45 d after sowing. Triplicate experiments were conducted.

Determination of anthocyanin content

Anthocyanin analysis was carried out according to the method of Weiss *et al.* (Weiss *et al.*, 1988; Weiss and Halevy, 1989). Anthocyanin was extracted from fresh corollas (0.1 g) just after anthesis using 10 ml 1% HCl in MeOH. Concentrations were determined by absorbance measurements at 530 nm and 657 nm, using the formula ($A_{530} - 0.25 \times A_{657}$) to correct for chlorophyll and its degradation products present in the extract.

Extraction and assay of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5)

Phenylalanine ammonia-lyase (PAL) assay was carried out according to the method of Weiss and Halevy (1989) and Zucker (1969). PAL was extracted at 4 °C from fresh corolla tissue (150 mg) by homogenizing with 5 ml cold 25 mM Na-borate buffer (pH 8.8) containing 2 mM Na-bisulfite. A clear supernatant was obtained by centrifugation of the homogenate at 12,000 × *g* for 10 min at 4 °C. Samples were taken for protein determination according to the method of Bradford (1976) with bovine serum albumin as a standard. The reaction mixture contained 30 mM borate at pH 8.8, 2 mM L-phenylalanine and 0.2 ml enzyme extract. The final volume was 3.7 ml. The rate of the enzymatic reaction was measured at 35 °C by following the increase in absorbance at 290 nm over a period of 2 h. The activity was linear for 2 h with time and with protein concentration up to 60 μg/ml.

Results

Development and pigmentation of Petunia flower

Development of *Petunia* flower was divided into five stages according to the modified method of Weiss and Halevy (1989) and Zucker (1969) (Fig. 1). Stage 1 was a small flower bud with the corolla. Corollas at stages 1 and 2 were green, and their anthocyanin content was negligible. The average bud lengths at stage 1 and stage 2 were 4 mm and 20 mm, respectively. Corolla at stage 3 was characterized by the advent of anthocyanin accumulation. The bud continued growing throughout stages 3 and 4 with an increase in pigmentation. Anthesis occurred at the end of stage 4 and flower length was 45 mm at this stage. At stage 5 there was still additional growth and anthocyanin content increased, actinomorphic flower with a pinkish corolla was observed, and the flower length was 60 mm.

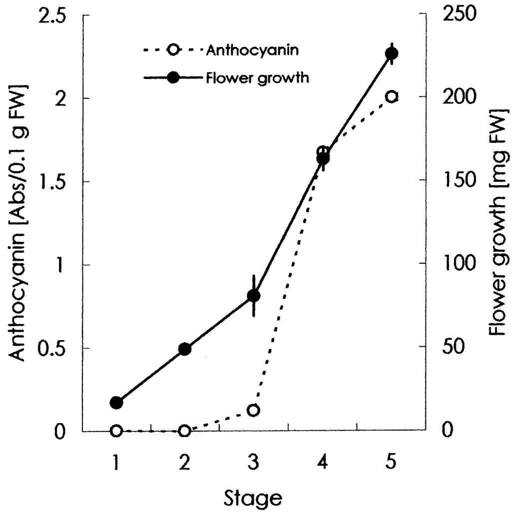

Fig. 1. Changes in fresh weight and anthocyanin content of *Petunia* flowers with developmental stage. Bars indicate standard error of the mean.

Effects of chemical treatments on flower development and pigmentation

The nine chemicals to be tested were applied on all buds and leaves at a concentration of 5 mM except for 0.5 mM glyphosate, since glyphosate inhibited flower bud formation at a concentration above 1 mM (Table I). Flowers treated with glyphosate, an inhibitor of aromatic amino acid biosynthesis (Jaworski, 1972; Amrhein *et al.*, 1980; Rubin *et al.*, 1984), showed a similar growth pattern for the control flowers, but glyphosate reduced the anthocyanin content of *Petunia* corollas to 17% of control at a concentration of 0.5 mM. In addition, glyphosate showed a change of flower

Table I. Effect of chemical treatments on growth and pigmentation of *Petunia* flowers. Data were collected 10 days after the first application. Means in the column followed by the same letters are not significantly different at the 5% level of significance by Duncan's multiple-range test.

Treatment	[mM]	Number of flower buds at each developmental stage					Anthocyanin
		1	2	3	4	5	[Abs/0.1 g FW]
Control		6	1	0	2	7^{a}	1.97^{a}
Glyphosate	0.5	9	0	1	2	6^{a}	0.33^{d}
Phosphonomycin	0.5	9	3	0	0	4^{b}	1.28^{c}
PPA	0.5	10	1	1	0	6^{a}	1.58^{bc}
PBA	0.5	7	2	1	1	3^{bc}	1.72^{ab}
PIA	0.5	9	1	1	2	7^{a}	1.67^{ab}
5-Azacytidine	5	10	0	1	0	3^{bc}	2.06^{a}
Tetracycline	5	9	0	0	0	2^{cd}	1.66^{ab}
APA	5	10	2	2	2	4^{b}	0.43^{d}
Naringenin	5	7	2	0	1	3^{bc}	2.10^{a}
Paclobutrazol	5	9	2	1	1	4^{b}	1.55^{bc}
GA_3	5	7	3	2	1	6^{a}	1.60^{bc}
IAA	5	10	0	0	0	0^{e}	–
Kinetin	5	12	1	0	1	1^{de}	1.23^{c}

Fig. 2. Effect of glyphosate on flower symmetry; (a) untreated flower; (b) flower treated with 0.5 mM glyphosate.

symmetry from the actinomorphic to the zygomorphic type (Fig. 2) (Bergbusch, 1999). This change is caused in this experiment only by glyphosate treatment. Furthermore, flowers treated with glyphosate set seed when fertilized with pollen from untreated plants, and pollen from flowers treated with glyphosate fertilized emasculated flowers from untreated plants. 5-Azacytidine as an inhibitor of DNA methylation (Horváth *et al.*, 2003) and tetracycline as an inhibitor of protein biosynthesis (Shimada *et al.*, 1992) had no effect on the anthocyanin content, but these chemicals showed a weak inhibitory effect on the flower development. APA, an inhibitor of PAL activity (Amrhein and Gödeke, 1977; Duke *et al.*, 1980), showed a weak inhibitory effect on the flower development and reduced the anthocyanin content of *Petunia* corollas to 22% of control at a concentration of 5 mM. Naringenin, an inhibitor of lignin biosynthesis (Rakwal *et al.*, 2000), showed a weak inhibitory effect on the flower development but had no effect on the anthocyanin content. GA_3 and paclobutrazol, an inhibitor of gibberellin biosynthesis (Lee *et al.*, 1985), did not show any effect on the flower development and pigmentation. In contrast, IAA and kinetin showed a marked inhibitory effect on the flower development.

Effects of combined treatments with glyphosate and plant hormones or phenolic compounds on flower development and pigmentation

Treatments by glyphosate plus IAA or kinetin completely inhibited the flower development at stage 3 (Table II). Treatment by glyphosate plus *trans*-cinnamic acid slightly promoted flower pigmentation more than glyphosate alone, but glyphosate plus GA_3 or L-phenylalanine showed a similar anthocyanin content to that of glyphosate alone. Treatments by glyphosate plus a phenylalanine or *trans*-cinnamic acid showed a weak inhibitory effect on the flower development. Combined treatments with glyphosate and those chemicals did not recover the flower shape to the actinomorphic type.

Effects of glyphosate and related compounds on flower development and pigmentation

Flowers treated with phosphonomycin (1,2-epoxypropylphosphonic acid), 3-phosphonopro-

Table II. Effect of combined treatments with glyphosate and plant hormone or phenolic compound on growth and pigmentation of *Petunia* flowers. Data were collected 10 days after the first application. Means in the column followed by the same letters are not significantly different at the 5% level of significance by Duncan's multiple-range test.

Treatment	[mM]	Number of flower buds at each developmental stage					Anthocyanin
		1	2	3	4	5	[Abs/0.1 g FW]
Glyphosate	0.5	9	0	1	2	6^{a}	0.33^{b}
plus GA3	5	9	1	2	1	2^{b}	0.24^{b}
plus IAA	5	9	1	1	0	0^{c}	–
plus kinetin	5	9	1	2	0	0^{c}	–
plus L-phenylalanine	5	8	2	0	2	2^{b}	0.26^{b}
plus cinnamic acid	5	6	2	2	2	3^{b}	0.45^{a}

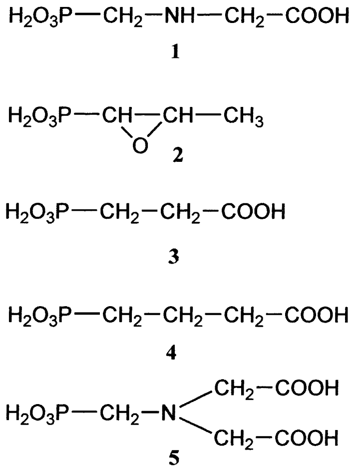

Fig. 3. Structures of glyphosate [*N*-(phosphonomethyl)-glycine] (**1**), phosphonomycin (1,2-epoxypropylphosphonic acid) (**2**), PPA (3-phosphonopropionic acid) (**3**), PBA (4-phosphonobutyric acid) (**4**), and PIA [*N*-(phosphonomethyl) iminodiacetic acid] (**5**).

pionic acid (PPA), 4-phosphonobutyric acid (PBA), and *N*-(phosphonomethyl) iminodiacetic acid (PIA) did not show any inhibitory effect on the anthocyanin content but phosphonomycin and PBA slightly reduced the flower development as compared to those treated with glyphosate alone (Table I, Fig. 3). All glyphosate-related compounds to be tested did not change the *Petunia* flower shape.

Effects of glyphosate on PAL activity of Petunia corollas

Since phenylalanine ammonia-lyase (PAL) was related to the anthocyanin synthesis, PAL activity was measured in corolla tissue treated with glyphosate. Glyphosate reduced the PAL activity to 65% of control at a concentration of 0.5 mM.

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