Nitrogen Metabolism and Flower Symmetry of Petunia Corollas Treated with Glyphosate

Atsumi Shimada[a,*] and Yasuo Kimura[b]

[a] Department of Food and Nutrition, Faculty of Food and Nutrition Science, Beppu University, 82 Kita-ishigaki, Beppu, Oita 874-8501, Japan.
E-mail: shimada@nm.beppu-u.ac.jp
[b] Department of Biological and Environmental Chemistry, Faculty of Agriculture, Tottori University, Koyama, Tottori, Tottori 680-8553, Japan

* Author for correspondence and reprint requests

Z. Naturforsch. **62 c**, 849–856 (2007); received May 22/June 29, 2007

A change of flower shape was observed in petunia corollas treated with 0.5 mM glyphosate. Glyphosate changed the flower symmetry from the actinomorphic type to the zygomorphic type. Corollas treated with glyphosate showed an increased free amino acid content. Free amino acid profiles in petunia corollas revealed that glyphosate had no significant effect on aromatic amino acid levels but increased the level of proline. Soluble protein content in glyphosate-treated corollas did not cause any significant changes. The contents of soluble phenolics, lignin, and IAA in the corollas were not significantly affected by the glyphosate treatment. In contrast, glyphosate reduced the nitrate content and the RNA content of petunia corollas by 45% and 63% of the control, respectively. However, the DNA content in glyphosate-treated corollas was similar to that of the control.

Low concentrations of glyphosate did not show any phytotoxic effects on the whole plants and any remarkable changes on aromatic amino acid metabolism and protein synthesis. However, glyphosate reduced the RNA content of petunia corollas and changed the flower symmetry from the actinomorphic type to the zygomorphic type. The results of nonprotein nitrogen metabolism in glyphosate-treated petunia corollas suggested that glyphosate application at low concentration may influence the regulation of flower symmetry through the change of RNA biosynthesis.

Key words: Glyphosate, RNA, *Petunia hybrida*

Introduction

The flower is the reproductive structure for angiosperms and also shows morphological diversity in size, shape and colour. Much of the extant flower diversity results from evolutionary changes in the shape and number of floral organs. Appropriate molecular tools for the biodesign of the shape of floral organs are necessary for elucidation of the transitions occurred during angiosperm evolution and improvement of garden variety. For the biodesign of the flower shape, we need to understand the genetic mechanism that controls the flower morphogenesis (Hileman *et al*., 2003; Kim *et al*., 1999; Meyerowitz, 1998; Parcy *et al*., 1998; Bergbusch, 1999; van der Krol *et al*., 1993; Conner, 2002). In recent studies, clonal analysis showed that petal asymmetry of *Antirrhinum* (snapdragon) depends on the direction of growth rather than regional difference in the growth rate (Rolland-Lagan *et al*., 2003). However, the metabolism related to flower morphogenesis is still poorly understood, because the development of flowers is a complex phenomenon.

For the elucidation of the metabolism related to the flower shape, we need a flower model enabling to manipulate flower growth and shape. This study uses a technique of applying glyphosate (*N*-phosphonomethylglycine) (Jaworski, 1972) to petunia flower buds for characterizing the metabolism related to the flower shape. Application of glyphosate reveals the change of petunia flower shape from the actinomorphic type to the zygomorphic type (Shimada and Kimura, 2006).

Glyphosate is widely used as a nonselective, broad-spectrum, postemergence herbicide with particular potential for perennial weed control due to excellent translocation (Hollander and Amrhein, 1980; Haderlie *et al*., 1977). The primary mode of action of glyphosate is to competitively inhibit the binding of the substrate phosphoenolpyruvate to the active site of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase; 3-

0939–5075/2007/1100–0849 $ 06.00

phosphoshikimate 1-carboxyvinyltransferase; EC 2. 5.1.19). Glyphosate is thus thought to inhibit the plant growth by blocking the synthesis of aromatic amino acids which subsequently cause an inhibition of protein synthesis (Della-Cioppa *et al*., 1986). There have been reports on effects of glyphosate on the amino acid metabolism using axes of soybeans [*Glycine max* (L.) Merr.] (Duke *et al*., 1979, 1980) and maize (*Zea mays* L.) (Hoagland *et al*., 1978), suspension-cultured cells of carrot (*Daucus carota* L.) and tobacco (*Nicotiana tabacum* L.) (Haderlie *et al*., 1977), rhizome buds of quackgrass [*Elytrigia repens* (L.) Nevski], roots of wheat (*Triticum aestivum* L.) (Cole *et al*., 1980), inflated duckweed (*Lemna gibba* L.) (Cooley and Foy, 1992). However, there is not any information on effects of glyphosate on the amino acid metabolism using floral organs.

We describe here the relationship between the flower symmetry and the nitrogen metabolism of petunia corollas treated with glyphosate.

Material and Methods

Plant growth and application of glyphosate

Seeds from an F1 hybrid of *Petunia hybrida* purchased from Takii Nursery, Kyoto, Japan, were sown in multipot plastic trays filled with peat and germinated under growth chamber conditions at 25 °C with 100 μmol photons $m^{-2}\,s^{-1}$ (white fluorescent lamps) for 14 d. Uniform seedlings at the 4-leaf stage were transplanted into 20-cm pots filled with a commercial soil for flowers purchased from Hirata Nursery, Fukuoka, Japan, 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). An 1 ml per pot aqueous solution containing 0.5 mM glyphosate and Tween-80 (1 g l^{-1}) was applied to all leaves once in 2 d for a total of three treatments from 45 d after sowing. Glyphosate was purchased from Wako Pure Chemical Industries Ltd, Osaka, Japan.

Free amino acid content of corollas

The fresh corollas (0.15 g) were homogenized in 5 ml cold 25 mM Na-borate buffer (pH 8.8) containing 2 mM Na-bisulfite with a Polytron equipment (Kinematica) at the medium-speed setting for 60 s, and the homogenate was centrifuged (4 °C, 12,000 × *g*, 10 min). The supernatant was analyzed for free amino acid content according to Moore and Stein (1948, 1954) and Moore (1968). The reaction mixture containing 2 ml supernatant and 2 ml ninhydrin reagent was heated for 15 min in a boiling water bath. After rapid cooling, ethanol and water (50 : 50, v/v; 3 ml) were added and the absorbance was measured at 570 nm using glutamic acid as the standard.

Free amino acid composition analysis of corollas

Free amino acid composition analysis was carried out according to the method of Chang *et al*. (Lin and Chang, 1975; Chang *et al*., 1981, 1982). The supernatant prepared for the measurement of the free amino acid content was adjusted to pH 7.0 with 0.1 M NaOH and then centrifuged (4 °C, 2,000 × *g*, 10 min). The clear supernatant was lyophilized and the residue was dissolved in 0.5 ml 0.1 M sodium carbonate buffer (pH 9.2). The reaction mixture containing 0.1 ml buffer solution and 0.1 ml dabsyl chloride solution was heated for 10 min at 70 °C. The mixture was concentrated *in vacuo* and redissolved in ethanol/water (70 : 30, v/v; 2 ml). The resulting solution was filtered through a Millex-LH filter (0.45 μm × 13 mm, Millipore) and analyzed by reversed-phase HPLC (LC-6A instrument, Shimadzu). The free amino acid solution was separated on a TSKgel ODS-80TS column (4.6 × 150 mm, Tosoh) at 40 °C. The mobile phase consisted of solvent A (10 mM citrate buffer, pH 6.2) and solvent B (90% acetonitrile in water), the following gradient procedure was used: 20% of B for 0 min; a linear gradient from 20 to 45% of B for 25 min; a linear gradient from 45 to 70% of B for 10 min; a linear gradient from 70 to 100% of B for 2 min; 100% of B for 13 min. The flow rate was 1.2 ml min^{-1}.

Soluble protein content of corollas

Soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard. The supernatant prepared for free amino acid measurements was used for the quantitative analysis of protein.

Lignin content of corollas

Quantitative analysis of lignin was carried out according to Li *et al*. (2003), Johnson *et al.* (1961) and Effland (1977). The corollas (0.2 g dry weight, DW) were extracted sequentially at room temperature twice with distilled water for 10 min, twice with 1 M NaCl containing Triton X-100

(20 g l^{-1}) for 10 min, and finally with ethanol/toluene (1:1, v/v) for 5 h. After drying, the sample was hydrolyzed with 2 M NaOH for 2 h at 70 °C, washed with distilled water for 10 min, dehydrated with acetone and dried. The dried sample (5 mg) was incubated in acetylbromide/glacial acetic acid (25:75, v/v; 0.5 ml) for 30 min at 70 °C. After a rapid cooling, 0.5 ml 2 M NaOH and 0.1 ml 7.5 M hydroxylamine-HCl were added and the volume was made up to 10 ml with glacial acetic acid. After filtration, the absorbance was measured at 280 nm using ferulic acid as the standard.

Total soluble phenol content of corollas

Total soluble phenol analysis was carried out according to the method of Camacho-Cristobal *et al.* (2002), Ruiz *et al.* (1999) and Arnaldos *et al.* (2002). The fresh corollas (0.3 g) were extracted with 3 ml methanol. The extracts were maintained on ice for 1 h and then centrifuged (4 °C, 4,000 × *g*, 15 min). Aliquots (0.25 ml) of the extracts were mixed with 2.5 ml 2% Na_2CO_3 in water and 0.2 ml Folin-Ciocalteu reagent. After the mixture was incubated for 15 min in the dark at room temperature, the absorbance was measured at 765 nm using caffeic acid as the standard.

IAA content of corollas

Quantitative analysis of indole-3-acetic acid (IAA) in petunia corollas was carried out according to the modified method of Akiyama *et al.* (1983), Shimada *et al.* (2000) and Crozier *et al.* (1980). The fresh corollas (0.3 g) were homogenized in 25 ml 80% acetone in water with a Polytron equipment (Kinematica) at the medium-speed setting for 60 s, and the homogenate was centrifuged (4 °C, 2,000 × *g*, 5 min). The clear supernatant was concentrated *in vacuo* to give an aqueous solution. The volume of the aqueous solution was made up to 10 ml with distilled water and was adjusted to pH 3.5 with 0.1 M tartaric acid. The solution was washed with an equal volume of petroleum ether and partitioned twice with an equal volume of diethyl ether. The diethyl ether fraction was partitioned twice with an equal volume of 0.1 M potassium phosphate buffer (pH 8.0). The aqueous fraction, adjusted to pH 3.5 with 0.1 M HCl, was partitioned twice with an equal volume of diethyl ether. The organic fraction was concentrated *in vacuo*, and the residue was dissolved in acetonitrile. IAA in each sample was quantified chromatographically on a HPLC instrument (Model LC-6A, Shimadzu) with a TSKgel ODS-80Ts column (4.6 × 250 mm, Tosoh). Each sample was eluted with 20% acetonitrile solution plus 20 mM sodium acetate buffer (pH 3.5) at a flow rate of 0.8 ml min^{-1}. The elute was monitored with a spectrofluorometer (Model RF-550, Shimadzu). The excitation wavelength was 280 nm and the emission wavelength was 355 nm. Triplicate experiments were conducted.

Nitrate content in petunia corollas

Quantitative analysis of nitrate in petunia corollas was carried out according to the method of Cataldo *et al.* (1974, 1975). The solution prepared for the measurement of the ammonia content was used for measuring the nitrate content. To 50 μl sample solution in a 20-ml test tube, 200 μl of 6% salicylic acid and conc. H_2SO_4 were added. The solution was mixed and incubated for 20 min at room temperature. After 5 ml 2 M NaOH were added, the solution was mixed thoroughly and incubated for 20 min at room temperature. The absorbance was measured at 410 nm using $NaNO_3$ as the standard. Triplicate experiments were conducted.

DNA content of corollas

Extraction of DNA from petunia corollas was carried out according to the modified method of Murray and Thompson (1980). The fresh corollas (1 g) were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. After the extract had been transferred to a 20-ml test tube, 3 ml extraction buffer [1.4 M NaCl, 20 g l^{-1} cetyltrimethylammonium bromide (CTAB), 100 mM Tris (2-amino-2-hydroxymethyl-1,3-propanediol)-HCl buffer (pH 8.0), 20 mM EDTA] were added. The mixture had been emulsified by gentle inversion, and then was incubated for 30 min at 65 °C. The solution was emulsified for 5 min by gentle mixing with an equal volume of chloroform/isoamyl alcohol (24:1, v/v), and then centrifuged (20 °C, 12,000 × *g*, 15 min). The supernatant was emulsified once more with an equal volume of chloroform/isoamyl alcohol and centrifuged (20 °C, 12,000 × *g*, 15 min). After 1.5 volume of CTAB solution [10 g l^{-1} CTAB, 50 mM Tris-HCl buffer (pH 8.0), 10 mM EDTA] was added, the solution was incubated for 1 h at room temperature. After centrifugation (20 °C, 8,000 × *g*, 10 min), the

supernatant had been removed. The precipitate was dissolved in 2 ml 1 M CsCl, and then 4 ml of ethanol were added. The solution was mixed by gentle inversion and incubated for 20 min at –20 °C. After centrifugation (20 °C, 12,000 × *g*, 5 min), an supernatant had been removed, and 2 ml 70% ethanol in water were added. After centrifugation (20 °C, 12,000 × *g*, 15 min), the precipitate was dissolved in 2 ml 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA.

Quantitative analysis of DNA in petunia corollas was carried out according to the method of Burton (1956). After 2 ml sample solution were mixed with an equal volume of diphenylamine reagent, the solution was incubated for 18 h at 30 °C. The absorbance was measured at 600 nm using DNA from salmon sperm as the standard.

RNA content of corollas

Extraction of RNA from petunia corollas was carried out according to the modified method of Lopez-Gomez and Gomez-Lim (1992) and Shirzadegan *et. al*. (1991). The fresh corollas (1.5 g) were homogenized with a Polytron at medium speed for 30 s by the addition of 15 ml extraction buffer and 15 ml phenol solution. The extraction buffer contained 200 mM NaCl, 50 mM Tris-HCl buffer (pH 8.5), and 10 mM EDTA. The phenol solution contained water-saturated phenol/chloroform/isoamyl alcohol (50:50:1, v/v/v). The homogenate was emulsified by quickly inversion for 10 min and centrifuged (10 °C, 7,000 × *g*, 20 min). The supernatant had been emulsified with an equal volume of phenol solution and centrifuged (10 °C, 7,000 × *g*, 20 min). The supernatant was emulsified and centrifuged once more. The clear supernatant was mixed with an equal volume of isopropanol and incubated for 20 min at –80 °C. After centrifugation (4 °C, 7,000 × *g*, 20 min), a supernatant had been removed. The precipitate was dissolved in 2 ml 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. After 80 µl 5 M NaCl and 5 ml cold EtOH had been added, the solution was mixed by gentle inversion. The solution had been incubated for 20 min at 20 °C and was centrifuged (4 °C, 7,000 × *g*, 20 min). An aqueous phase had been removed, and the precipitate was redissolved in 2 ml 50 mM Tris buffer. After 0.5 ml 8 M urea and 0.5 ml 10 M LiCl had been added, the solution was mixed gently. The solution had been incubated overnight at 4 °C and was centrifuged (4 °C, 7,000 × *g*, 20 min). A supernatant had been removed, and the precipitate was redissolved in 2 ml 50 mM Tris buffer.

Quantitative analysis of RNA in petunia corollas was carried out according to the method of Mejbaum (1939). After 2 ml sample solution were mixed with an equal volume of orcinol reagent, the solution was incubated for 20 min in a boiling water bath. After rapid cooling, the absorbance was measured at 670 nm using RNA from yeast as the standard.

Results

Effects of glyphosate on free amino acids and soluble protein of petunia corollas

The corollas treated with glyphosate increased the free amino acid content to 136% of the control. Free amino acid profiles in petunia corollas revealed that glyphosate had no significant effect on the aromatic amino acid levels but increased the level of proline (Table I). Soluble protein content in glyphosate-treated corollas did not cause any significant changes. Glyphosate caused a reduction of anthocyanin formation and PAL activity (Shimada and Kimura, 2006) but did not affect the aromatic amino acid and protein biosynthesis. Phenylalanine and tyrosine are the important precursors in the production of phenolic compounds and lignin (Duke *et al*., 1979). Tryptophan is an

Table I. Free amino acid profile of petunia corollas treated with 0.5 mM glyphosate.

Amino acid	Control (mol%)	Glyphosate (mol%)
Ala	6.1	8.8
Leu	2.2	1.3
Val	5.1	3.2
Phe	1.5	1.2
Tyr	0.4	0.8
Trp	1.3	1.0
His	1.7	1.8
Gly	3.3	3.1
Ser	7.5	7.4
Cys1	24.3	30.4
Cys2	0.9	0.3
Glu	15.6	12.4
Arg	5.2	4.1
Pro	3.1	6.4*
Asp	12.5	11.9
Thr	2.1	2.2
Met	3.0	0.7*
Ile	2.4	1.2
Lys	2.0	1.6

* $P < 0.05$ compared with the control.

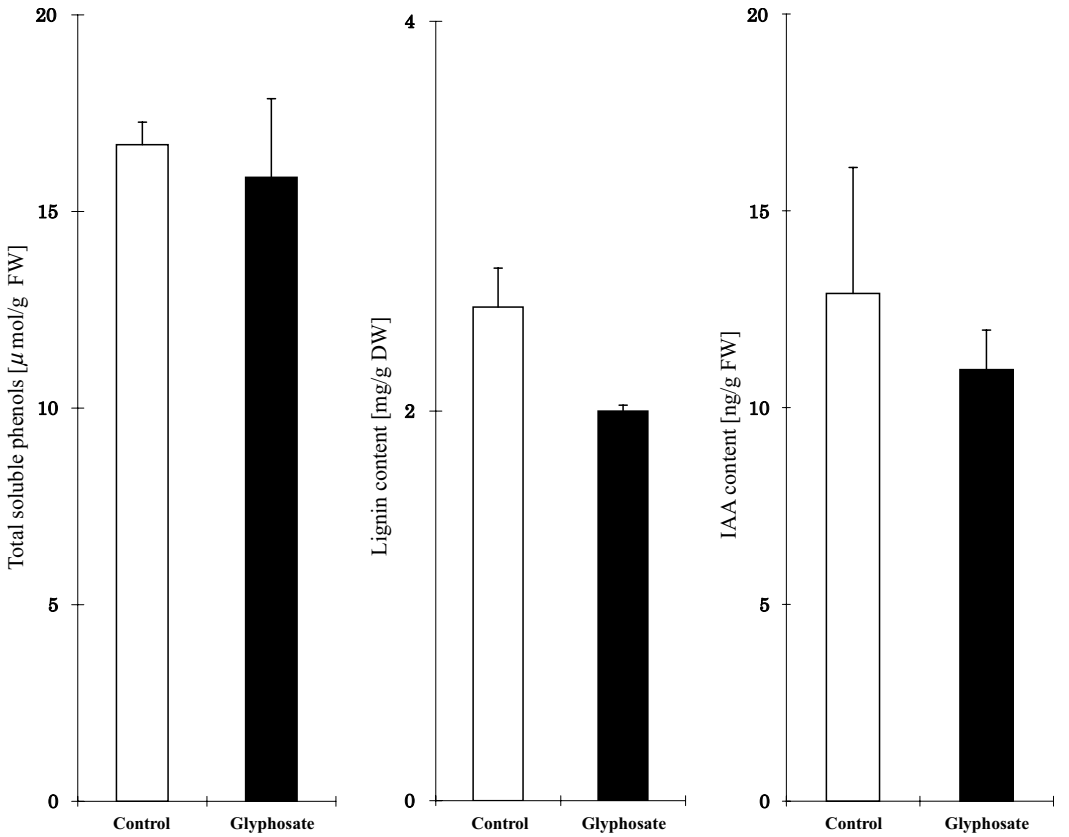

Fig. 1. Effects of glyphosate on total soluble phenols, lignin and IAA contents of petunia corollas. Values are means ± SE; $n = 3$; * $P < 0.05$ compared with the control.

important precursor in the production of IAA (Hoagland *et al.*, 1978; Duke *et al.*, 1979). Thus, the effect of glyphosate on aromatic amino acid metabolism was the next examination.

Effect of glyphosate on total soluble phenolics, lignin and IAA contents of petunia corollas

Methanol extracts of the corollas treated with glyphosate were analyzed for total soluble phenolic compounds. There was no significant difference in the amount of soluble phenolic compounds between the control tissue and the tissue treated with glyphosate (Fig. 1). The effect of glyphosate on the lignin content in the corollas is shown in Fig. 1; it was not significantly affected by the glyphosate treatment. The effect of glyphosate on the IAA content is also shown in Fig. 1. The amount of IAA, a metabolite of tryptophan, in the corollas was not significantly affected by the glyphosate treatment.

Glyphosate also did not affect the aromatic amino acid metabolism. Since no information seems to be available on the role of glyphosate in the regulation of flower symmetry in petunia corollas, the effect of glyphosate on nonprotein nitrogen was examined next.

Effects of glyphosate on the content of nitrate of petunia corollas

Glyphosate reduced the nitrate content of petunia corollas by 45% of the control (Fig. 2), but did not affect the contents of amino acid and protein. Thus, the effect of glyphosate on the contents of DNA and RNA was examined next.

Effects of glyphosate on the contents of DNA and RNA of petunia corollas

DNA content of petunia corollas increased to 113% of the control by the treatment of glypho-

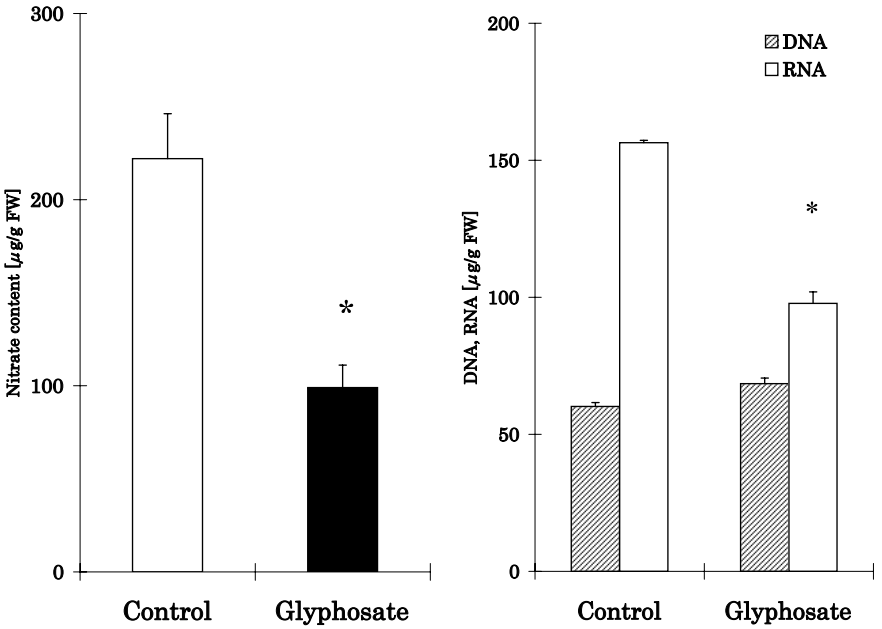

Fig. 2. Effects of glyphosate on the nitrate, DNA and RNA contents of petunia corollas. Values are means ± SE; $n = 3$; * $P < 0.05$ compared with the control.

sate. In contrast, RNA content decreased by 63% of the control by the treatment of glyphosate (Fig. 2).

Discussion

Changes of flower shape and pigmentation were observed in petunia corollas treated with glyphosate (Amrhein *et al.*, 1980). Although the decrease in anthocyanin content was a result of the reduction of PAL activity caused by glyphosate (Hollander and Amrhein, 1980), the reduction of PAL activity alone could not account for the two independent processes of flower shape and pigmentation.

Many studies have given strong support to the hypothesis that inhibition of the shikimate pathway enzyme EPSP synthase is the primary site-of-action of glyphosate and other metabolic effects are secondary in nature. According to this hypothesis, inhibition of EPSP synthase may lead to decreased levels of aromatic amino acids that subsequently cause an inhibition of protein synthesis (Amrhein *et al.*, 1980; Rubin *et al.*, 1984). However, our findings showed that glyphosate increased the free amino acid content but did not affect the aromatic amino acid levels and soluble protein content. Furthermore, glyphosate had no significant effect on the aromatic amino acid metabolism such as phenolic compounds, lignin and IAA. In contrast, glyphosate reduced the nonprotein nitrogen of nitrate and RNA.

High concentrations of glyphosate showed phytotoxic effects on a number of plant species by the inhibition of EPSP synthase. This inhibition led to decreased levels of aromatic amino acids that subsequently cause an inhibition of protein synthesis (Amrhein *et al.*, 1980; Rubin *et al.*, 1984). In addition, glyphosate induced high levels of PAL activity (Duke *et al.*, 1979, 1980; Cooley and Foy, 1992; Amrhein and Gödeke, 1977). High levels of PAL activity produced toxic levels of ammonia and increased the levels of growth-inhibiting phenolic compounds (Duke *et al.*, 1980). Furthermore, glyphosate acted as a respiratory inhibitor through uncoupling mitochondrial phosphorylation and reduced photosynthesis in the whole plants (Haderlie *et al.*, 1977; Ali and Fletcher, 1977; Olorunsogo *et al.*, 1979). In contrast, low concentrations of glyphosate did not show any phytotoxic effects on the whole plants and any remarkable changes on the aromatic amino acid metabolism and protein synthesis. However, glyphosate reduced the RNA content of petunia corollas and changed the flower symmetry from the actinomorphic type to the zygomorphic type.

In petunia, expression of the gene encoding EPSP synthase is petal-specific, and the expression level increases dramatically during flower opening (Hirai *et al*., 1994). EPSP synthase is a key enzyme of the shikimate pathway which is necessary for the production of aromatic compounds to provide material such as phenylpropanoids and IAA for flower pigmentation and development (Hirai *et al*., 1994; Gasser *et al*., 1988). This enzyme is the target of the nonselective herbicide glyphosate. PETHy;ZPT2–1, one of the zinc-finger transcription factors of petunia, regulates the petal-specific expression of the gene for EPSP synthase and the floral organ formation (Takatsuji *et al*., 1994; Kobayashi *et al*., 1998). Overexpression of cDNA for another zinc-finger protein of petunia, designated lateral shoot-inducing factor (LIF), in transgenic petunia plants results in the morphology of flowers (Nakagawa *et al*., 2005). The flower shape of LIF-overexpressed petunia is similar to that treated with glyphosate. In addition, glyphosate inhibits RNA transcription in the sea urchin's early development (Marc *et al*., 2005). Thus, the results of nonprotein nitrogen metabolism in glyphosate-treated petunia corollas suggests that low concentration of glyphosate might affect the expression of the transcription factors related to the flower development through the reduction of RNA biosynthesis.

Acknowledgements

This work was financially supported by the grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to A. Shimada (No. 16580094).

Akiyama M., Sakurai N., and Kuraishi S. (1983), A simplified method for the quantitative determination of indoleacetic acid by high performance liquid chromatography with a fluorometric detector. Plant Cell Physiol. **24**, 1431–1439.

Ali A. and Fletcher R. A. (1977), Phytotoxic action of glyphosate and amitrole on corn seedlings. Can. J. Bot. **56**, 2196–2202.

Amrhein N. and Gödeke K. (1977), α-Aminooxy-β-phenylpropionic acid. A potent inhibitor of L-phenylalanine ammonia-lyase *in vitro* and *in vivo*. Plant Sci. Lett. **8**, 313–317.

Amrhein N., Deus B., Gehrke P., and Steinrücken H. C. (1980), The site of the inhibition of the shikimate pathway by glyphosate. II. Interference of glyphosate with chorismate formation *in vitro* and *in vivo*. Plant Physiol. **66**, 830–834.

Arnaldos T. L., Munoz R., Ferrer M. A., and Calderon A. A. (2001), Changes in phenol content during strawberry (*Fragaria* X *ananassa*, cv. Chandler) callus culture. Physiol. Plant. **113**, 315–322.

Bergbusch V. (1999), A note on the manipulation of flower symmetry in *Antirrhinum majus*. Ann. Bot. **83**, 483–488.

Bradford M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Burton K. (1956), A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**, 315–323.

Camacho-Cristobal J. J., Anzellotti D., and Gonzalez-Fontes A. (2002), Changes in phenolic metabolism of tobacco plants during short-term boron deficiency. Plant Physiol. Biochem. **40**, 997–1002.

Cataldo D. A., Schrader L. E., and Youngs V. L. (1974), Analysis by digestion and colorimetric assay of total nitrogen in plant tissues high in nitrate. Crop Sci. **14**, 854–856.

Cataldo D. A., Haroon M., Schrader L. E., and Youngs V. L. (1975), Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. **6**, 71–80.

Chang J., Knecht R., and Braun D. G. (1981), Amino acid analysis at the picomole level. Biochem. J. **199**, 547–555.

Chang J., Knecht R., and Braun D. G. (1982), A complete separation of dimethylaminoazobenzenesulphonyl-amino acids. Biochem. J. **203**, 803–806.

Cole D. J., Dodge A. D., and Caseley J. C. (1980), Some biochemical effects of glyphosate on plant meristems. J. Exp. Bot. **31**, 1665–1674.

Conner J. K. (2002), Genetic mechanisms of floral trait correlations in a natural population. Nature **420**, 407–410.

Cooley W. E. and Foy C. L. (1992), Effects of SC-0224 and glyphosate on free amino acids, soluble protein, and protein synthesis in inflated duckweed (*Lemna gibba*). Weed Sci. **40**, 345–350.

Crozier A., Loferski K., Zaerr J. B., and Morris R. O. (1980), Analysis of picogram quantities of indole-3-acetic acid by high performance liquid chromatography-fluorescence procedures. Planta **150**, 366–370.

Della-Cioppa G., Christopher Bauer S., Klein B. K., Shah D. M., Fraley R. T., and Kishore G. M. (1986), Translocation of the precursor of 5-enolpyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants *in vitro*. Proc. Natl. Acad. Sci. USA **83**, 6873–6877.

Duke S. O., Hoagland R. E., and Elmore C. D. (1979), Effects of glyphosate on metabolism of phenolic compounds. IV. Phenylalanine ammonia-lyase activity,

free amino acids, and soluble hydroxyphenolic compounds in axes of light-grown soybeans. Physiol. Plant. **46**, 307–317.

Duke S. O., Hoagland R. E., and Elmore C. D. (1980), Effects of glyphosate on metabolism of phenolic compounds. V. L-α-Aminooxy-β-phenylpropionic acid and glyphosate effects on phenylalanine ammonia-lyase in soybean seedlings. Plant Physiol. **65**, 17–21.

Effland M. J. (1977), Modified procedure to determine acid-insoluble lignin in wood and pulp. TAPPI **60**, 143–144.

Gasser C. S., Winter J. A., Hironaka C. M., and Shah D. M. (1988), Structure, expression, and evolution of the 5-enolpyruvylshikimate-3-phosphate synthase genes of petunia and tomato. J. Biol. Chem. **263**, 4280–4289.

Haderlie L. C., Widholm J. M., and Slife F. W. (1977), Effect of glyphosate on carrot and tobacco cells. Plant Physiol. **60**, 40–43.

Hileman L. C., Kramer E. M., and Baum D. A. (2003), Differential regulation of symmetry genes and the evolution of floral morphologies. Proc. Natl. Acad. Sci. USA **100**, 12814–12819.

Hirai N., Yamamuro M., Koshimizu K., Shinozaki M., and Takimoto A. (1994), Accumulation of phenylpropanoids in the cotyledons of morning glory (*Pharbitis nil*) seedlings during the induction of flowering by low temperature treatment, and the effect of precedent exposure to high-intensity light. Plant Cell Physiol. **35**, 691–695.

Hoagland R. E., Duke S. O., and Elmore C. D. (1978), Effects of glyphosate on metabolism of phenolic compounds. II. Influence on soluble hydrooxyphenolic compound, free amino acid and soluble protein levels in dark-grown maize roots. Plant Sci. Lett. **13**, 291–299.

Hollander H. and Amrhein N. (1980), The site of the inhibition of the shikimate pathway by glyphosate. I. Inhibition by glyphosate of phenylpropanoid synthesis in buckwheat (*Fagopyrum esculentum* Moench). Plant Physiol. **66**, 823–829.

Jaworski E. G. (1972), Mode of action of *N*-phosphonomethylglycine: inhibition of aromatic amino acid biosynthesis. J. Agric. Food Chem. **20**, 1195–1198.

Johnson D. B., Moore W. E., and Zank L. C. (1961), The spectrophotometric determination of lignin in small wood samples. TAPPI **44**, 793–798.

Kim G., Tsukaya H., Saito Y., and Uchimiya H. (1999), Changes in the shapes of leaves and flowers upon overexpression of cytochrome P450 in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **96**, 9433–9437.

Kobayashi A., Sakamoto A., Kubo K., Rybka Z., Kanno Y., and Takatsuji H. (1998), Seven zinc-finger transcription factors are expressed sequentially during the development of anther in petunia. Plant J. **13**, 571–576.

Li X., Li S., and Lin J. (2003), Effect of GA_3 spraying on lignin and auxin contents and the correlated enzyme activities in bayberry (*Myrica ruba* Bieb.) during flower-bud induction. Plant Sci. **164**, 549–556.

Lin J. and Chang J. (1975), Chromophoric labeling of amino acids with 4-dimethylaminoazobenzene-4′-sulfonyl chloride. Anal. Chem. **47**, 1634–1638.

Lopez-Gomez R. and Gomez-Lim M. A. (1992), A method for extracting intact RNA from fruits rich in polysaccharides using ripe mango mesocarp. Hort. Sci. **27**, 440–442.

Marc J., Le Breton M., Cormier P., Morales J., Bell R., and Mulner-Lorillo O. (2005), A glyphosate-based pesticide impinge on transcription. Toxicol. Appl. Pharmacol. **203**, 1–8.

Mejbaum W. (1939), Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. Z. Physiol. Chem. **258**, 117–120.

Meyerowitz E. M. (1998), Genetic and molecular mechanisms of pattern formation in *Arabidopsis* flower development. J. Plant Res. **111**, 233–242.

Moore S. (1968), Amino acid analysis: Aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J. Biol. Chem. **243**, 6281–6283.

Moore S. and Stein W. H. (1948), Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. **176**, 367–388.

Moore S. and Stein W. H. (1954), A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. **211**, 907–913.

Murray M. G. and Thompson W. F. (1980), Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res. **8**, 4321–4325.

Nakagawa H., Jiang C., Sakakibara H., Kojima M., Ajisaka H., Nishijima T., Koshioka M., Homma T., Mander L. N., and Takatsuji H. (2005), Overexpression of a petunia zinc-finger gene alters cytokinin metabolism and plant forms. Plant J. **41**, 512–523.

Olorunsogo O. O., Bababunmi E. A., and Bassir O. (1979), Uncoupling of corn shoot mitochondria by *N*-(phosphonomethyl)glycine. FEBS Lett. **97**, 279–282.

Parcy F., Nilsson O., Busch M. A., Lee I., and Weigel D. (1998), A genetic framework for floral patterning. Nature **395**, 561–566.

Rolland-Lagan A., Bangham J. A., and Coen E. (2003), Growth dynamics underlying petal shape and asymmetry. Nature **422**, 161–163.

Rubin J. L., Gaines C. G., and Jensen R. A. (1984), Glyphosate inhibition of 5-enolpyruvylshikimate 3-phosphate synthase from suspension-cultured cells of *Nicotiana silvestris*. Plant Physiol. **75**, 839–845.

Ruiz J. M., Garcia P. C., Rivero R. M., and Romero L. (1999), Response of phenolic metabolism to the application of carbendazim plus boron in tobacco. Physiol. Plant **106**, 151–157.

Shimada A. and Kimura Y. (2006), Influence of glyphosate on flower morphogenesis and pigmentation in *Petunia hybrida*. Z. Naturforsch. **61c**, 578–582.

Shimada A., Takeuchi S., Nakajima A., Tanaka S., Kawano T., and Kimura Y. (2000), Phytotoxicity of indole-3-acetic acid produced by the fungus, *Pythium aphanidermatum*. Biosci. Biotechnol. Biochem. **64**, 187–189.

Shirzadegan M., Christie P., and Seemann J. R. (1991), An efficient method for isolation of RNA from tissue cultured plant cells. Nucleic Acid Res. **19**, 6055.

Takatsuji H., Nakamura N., and Katsumoto Y. (1994), A new family of zinc finger proteins in petunia: structure, DNA sequence recognition, and floral organ-specific expression. Plant Cell **6**, 947–958.

van der Krol A. R., Brunelle A., Tsuchimoto S., and Chua N. (1993), Functional analysis of petunia floral homeotic MADS box gene pMADS1. Genes Dev. **7**, 1214–1228.