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Cultivar and anatomical analysis of corolla enlargement of petunia (*Petunia hybrida* Vilm.) by cytokinin application

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Abstract

We found that the corolla of petunia (*Petunia hybrida* Vilm.) could be conspicuously enlarged by the separate application of three cytokinins: forchlorfenuron (CPPU), N^6-benzylaminopurine (BA), and zeatin. To obtain the same enlargement as that achieved by CPPU, approximately 30 and 900 times the concentration of BA and zeatin, respectively, were required. CPPU at 3.2 μmol/L increased the limb area of the corollas of 15 cultivars to between 1.3 and 2.4 times (1.8 times on average) the size of the control area. The increase was negatively correlated ($R = 0.58$) with the "genetic" limb area (i.e., that of the untreated plant). The enlargement of the corolla caused by cytokinin application was mainly attributed to an increase in cell number in most cultivars. This increase resulted from a high rate of cell proliferation and from prolongation of the cell proliferation phase during corolla development. This anatomical change caused by cytokinin application was similar to the anatomical difference among cultivars because genetic differences in limb area resulted mainly from differences in cell number.
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1. Introduction

In floricultural plants, flower size is an important characteristic that can be controlled by breeding or cultivation methods. For example, major floricultural crops generally have larger flowers than the wild species as a result of breeding for high performance in appearance. However, small flower size caused by high summer temperatures is a problem in floricultural production (Durkin, 1992; Whealy, 1992). Thus, an important subject of floricultural research involves elucidating the physiological mechanisms responsible for flower size.

The effects of plant hormones on the size of leaves, which are homologous to petals, have been investigated in several plants, mostly by means of hormone-application experiments (reviewed in Kamisaka and Miyamoto, 1994; Sakurai, 1994). Cytokinins generally increase leaf size through cell enlargement (Kuraishi

and Okumura, 1956; Letham, 1969; Tsui et al., 1980). In contrast, gibberellins enlarge very young leaves by increasing the cell number, whereas enlargement of older leaves results from cell enlargement (Briant, 1974; Goodwin, 1978). In addition, gibberellins promote the growth of floral organs, including the corolla, in several species (reviewed in Pharis and King, 1985). Although the corolla of petunia (*Petunia hybrida* Vilm.) remains small when stamens are removed, the corolla size is restored when gibberellin is applied (Weiss et al., 1990). In *Arabidopsis thaliana* L., application of gibberellin increases the corolla size of gibberellin-deficient mutants, partially through promoting the expression of the floral homeotic genes *APETALA 3*, *PISTILLATA*, and *AGAMOUS* (Yu et al., 2004). In spite of these intensive studies of the positive effects of plant hormones on leaf size and of gibberellins on flower size, no plant hormones other than gibberellins have been shown to effectively increase flower size.

We have found that the application of cytokinins conspicuously increased flower size in petunia (*P. hybrida* Vilm.). This phenomenon may help us to understand the physiological

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mechanism underlying the differentiation of large-flowered cultivars and to develop a practical method of controlling flower size. Here, we report on a system for enlarging the corolla, the anatomical changes that occur during corolla development as a result of cytokinin application, and differences across cultivars in the plants' responses to cytokinin application. Finally, we discuss possible horticultural uses of this phenomenon.

2. Materials and methods

2.1. Plant material

We purchased seeds of several petunia cultivars from the Sakata Seed Co. (Yokohama, Japan): 'Fantasy' (small-flowered), 'Baccara' and 'Pearl' (medium-flowered), and 'Fulcon' (large-flowered) series. We purchased seeds of the 'Cutie' (small-flowered) and 'Carnival' (large-flowered) cultivars from the Takii Seed Co. (Kyoto, Japan). The small- and medium-flowered cultivars belong to the multiflora group, and the large-flowered cultivars belong to the grandiflora group (Ewart, 1984). Grandiflora cultivars have a single dominant *Grandiflora* (*G*) gene that is responsible for the large-flower phenotype. Cultivar 'Pearl White' was used in the detailed analysis of cytokinin effect, because it responded well to the cytokinin application. The other cultivars were used in the analysis of the cultivar difference.

Seeds were sown on horticultural soil (Metromix 350, Scotts-Sierra Horticultural Product Co., Marysville, OH, USA). We planted one seedling at the four- to five-leaf stage per pot. Pots were 15 cm in diameter by 18 cm in height, and were filled with a horticultural soil (Kureha-Engei-Baido, Kureha Chemical Industry Co. Ltd., Tokyo, Japan). We applied a slow-release coated fertilizer (Ecolong 70, $N:P_2O_5:K = 14:12:14$ (%, w/w), Chisso Co., Tokyo, Japan) as a top dressing at 5 g/pot. The plants were grown in a thermostatic glasshouse maintained at 25 °C from 6:00 a.m. to 6:00 p.m. and at 20 °C from 6:00 p.m. to 6:00 a.m. under natural sunlight. Plants were grown for 80–110 days after sowing before being used in the experiments.

2.2. Application of cytokinins

We dissolved forchlorfenuron (CPPU, Sigma–Aldrich Japan Co. Ltd., Tokyo, Japan), N^6-benzylaminopurine (BA, Wako Pure Chemical Industries Ltd., Osaka, Japan), and zeatin (Wako Pure Chemical Industries Ltd.) in 50% (v/v) aqueous acetone to produce the three cytokinin solutions. This solvent was chosen because it efficiently assisted the effect of applied cytokinin to appear without any inhibition of corolla growth (Nishijima, unpublished data). We applied 4 μL of solution to the apex of a corolla at 2-day intervals. The application began when corolla length reached 2 mm and ended when flowers began to open. Control flowers were treated with 50% (v/v) aqueous acetone.

2.3. Measurement of corolla

We measured the corolla dimensions 4 days after anthesis, when corolla size had nearly reached its maximum. Corolla area was measured with an automatic area meter (AAM-9, Hayashi Denko Co. Ltd., Tokyo, Japan). Very young corollas with an area of less than 50 mm^2 were measured using cross-section graph paper.

2.4. Anatomical observations

To prepare tissue samples for microscopic examination, we fixed corollas with a mixture of ethanol, formalin, and propionic acid (8:2:1 v/v/v) (FPA_{50}, 50%, v/v) and stored them at 4 °C. Before examination, we immersed the fixed corollas in 30% (v/v) ethanol for 30 min, then rendered them transparent by incubation for 2 h in a chloral hydrate solution (chloral hydrate, 8 g; glycerol, 1 mL; distilled water, 2 mL). We then observed the adaxial epidermis of the corolla using a differential interference microscope.

We measured the cell number and the projection area of the cells in the paradermal plane (i.e., cell area). To do so, we divided the corolla into the limb and the tube. The limb was further divided into three concentric rings. Width of the inner, middle, and outer rings was approximately in the ratio 5:3:2 to roughly equalize the area of the rings. The tube was also divided cross-sectionally into basal and apical truncated cones. Length of the basal and apical truncated cones was approximately in the ratio 7:3 to roughly equalize the area. The actual area of those sections was measured as described in Section 2.3. We used the central area of each section for microscopic observation. The cell area in a section equaled the area covered by the microscope's field of view divided by the cell number within this field. Cell number equaled the area of each section divided by the cell area. The average cell area of the limb or the tube was the mean weighted by the area of the sections. The cell number of the limb or the tube was the sum of cell number of the sections.

Before observation of the cross-section of the corolla, we dehydrated the fixed corolla using an ethanol series (50, 70, 90, 95, and 99.5%), and embedded the resulting tissue in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) following the manufacturer's instructions. We then sliced the embedded sample into sections with a thickness of 2 μm using a microtome. Sections were mounted on glass slides and stained with 1% (w/v) aqueous toluidine blue (Wako Pure Chemical Industries Ltd., Osaka, Japan). The stained sections were then observed with a bright-field microscope.

2.5. Experimental design

We examined 12 flowers from 3 plants (i.e., 4 flowers per plant) per treatment in the all experiments. The plants and the treatments were arranged based on the completely randomized design.

3. Results

3.1. Effect of cytokinin application on corolla size

CPPU application enlarged the corolla of the medium-flowered cultivar 'Pearl White' (Figs. 1 and 2). The extent of the

Fig. 1. Corolla enlargement in response to the application of CPPU to the 'Pearl White' cultivar. Measurement was done 4 days after anthesis. Numbers indicate CPPU concentration (μmol/L). CPPU at 10 μmol/L caused the development of noticeable creases and chlorophyll pigmentation. In this treatment, 4 μL of CPPU dissolved in 50% (v/v) aqueous acetone was applied to each corolla apex at 2-day intervals. Applications began when corolla length reached 2 mm and ended when flower opening began.

enlargement increased with increasing CPPU concentration. The highest concentration that we tested (i.e., 10 μmol/L) caused the development of creases and chlorophyll pigmentation in the corolla. The highest concentration that did not generate these side effects was 3.2 μmol/L. This concentration of CPPU increased the limb area to 2.4 times that of the control.
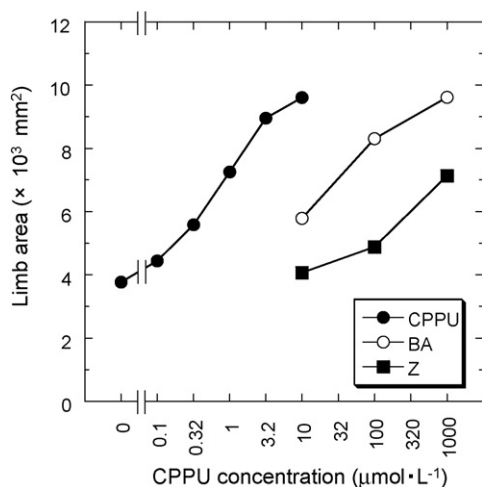

Fig. 2. Corolla enlargement in the 'Pearl White' cultivar in response to cytokinin application. Measurement was done 4 days after anthesis. The method of cytokinin application is described in the caption of Fig. 1. Data represent the mean ± S.E. ($n = 12$). Error bars were smaller than the symbols used to plot the data.

BA and zeatin also enlarged the corolla (Table 1 and Fig. 2). To obtain effects similar to those produced by CPPU, approximately 30 and 900 times the concentrations of BA and zeatin, respectively, were necessary. BA at a concentration of 1000 μmol/L caused the same side effects that were visible in corollas treated with 10 μmol/L of CPPU. The highest BA concentration that could be applied without causing these side effects was 100 μmol/L. This concentration of BA increased the limb area to 2.2 times that of the control. Zeatin did not cause any side effects within the range of concentrations tested (i.e., up to 1000 μmol/L). Zeatin at 1000 μmol/L increased the limb area to 1.9 times that of the control.

Table 1 shows the detailed effects of CPPU, BA, and zeatin applied at the highest concentrations without any side effects. Separate application of the three cytokinins enlarged both the limb and the tube of the flowers, although the extent of the enlargement was much greater in the limb than in the tube. Cytokinins did not affect the timing of anthesis (data not shown).

3.2. Anatomical changes in the corolla resulting from cytokinin application

Application of CPPU, BA, and zeatin increased both cell number and cell area in the 'Pearl White' cultivar. However, the increase in cell number was much greater than the increase in cell area (Table 1).

Table 1
Detailed effect of the three cytokinins applied at the most effective concentrations to the corolla enlargement of the 'Pearl White' cultivar

Application[a]	Limb diameter (mm)	Limb area (mm^2)	Cell number (million cells)	Cell area (μm^2)	Tube length (mm)	Tube area (mm^2)
Control	70.9 ± 0.6	377 ± 7	7.1 ± 0.2	532 ± 11	31.9 ± 0.3	40 ± 1
CPPU (3.2 μmol/L)	105.3 ± 1.4[**]	895 ± 30[**]	14.7 ± 0.7[**]	614 ± 14[**]	32.0 ± 0.6	52 ± 2[**]
BA (100 μmol/L)	100.7 ± 0.9[**]	831 ± 12[**]	13.8 ± 0.3[**]	606 ± 12[**]	30.7 ± 0.6	51 ± 2[**]
Z (1000 μmol/L)	92.0 ± 1.0[**]	714 ± 14[**]	11.3 ± 0.3[**]	637 ± 9[**]	30.6 ± 0.3	51 ± 2[**]

Values represent the mean ± S.E. ($n = 12$).

[a] Values in the parentheses are cytokinin concentrations that caused the maximum corolla enlargement without side effects (i.e., creases and chlorophyll pigmentation) from a range of 0.1–1000 μmol/L.

[**] Values differ significantly from that of the control based on Dunnett's test at $P \leq 0.01$.

In the control corolla, cells of the adaxial epidermis were conical in shape, and the parenchyma cells lay flat and parallel to the paradermal direction (Fig. 3). CPPU applied at 3.2 μmol/L did not change the corolla anatomy, except that the cells of the adaxial epidermis expanded slightly in the paradermal direction. CPPU applied at 10 μmol/L, which caused the side effects described above, changed the corolla anatomy remarkably. Cells of the adaxial epidermis were smaller and more globular than in the untreated corolla. Furthermore, the parenchyma cells were swollen perpendicular to the paradermal direction.

3.3. Effect of cytokinin application on corolla development

CPPU application increased corolla length above that in the control starting at 10 days after the initial application (Fig. 4). However, the area of the CPPU-treated corolla started to become larger than that of the control within 4 days after the start of the application (Fig. 4, inset section of the lower graph).

The difference in area between the CPPU-treated and untreated corollas reached its maximum around 16 days after the start of the application. The number of days to anthesis was not significantly affected by CPPU (see the caption of Fig. 4).

Corolla development consisted of two phases (Fig. 5). The cell number increases dramatically at first (i.e., the cell proliferation phase), followed by a rapid increase in cell area and volume (the cell expansion phase). In the control, the cell proliferation phase ended at around day 8 after the start of the application, and the cell expansion phase began at around the same time. In contrast, the cell proliferation phase continued until 12 days after the start of the CPPU application, which is 4 days longer than that of the control. In addition, the increase in cell number during the cell proliferation phase was greater than in the control. However, the cell expansion phase of the CPPU-treated corolla started to increase at around the same time (i.e., 8 days after the start of the application) as in the untreated corolla. The final cell area was slightly increased by CPPU application.
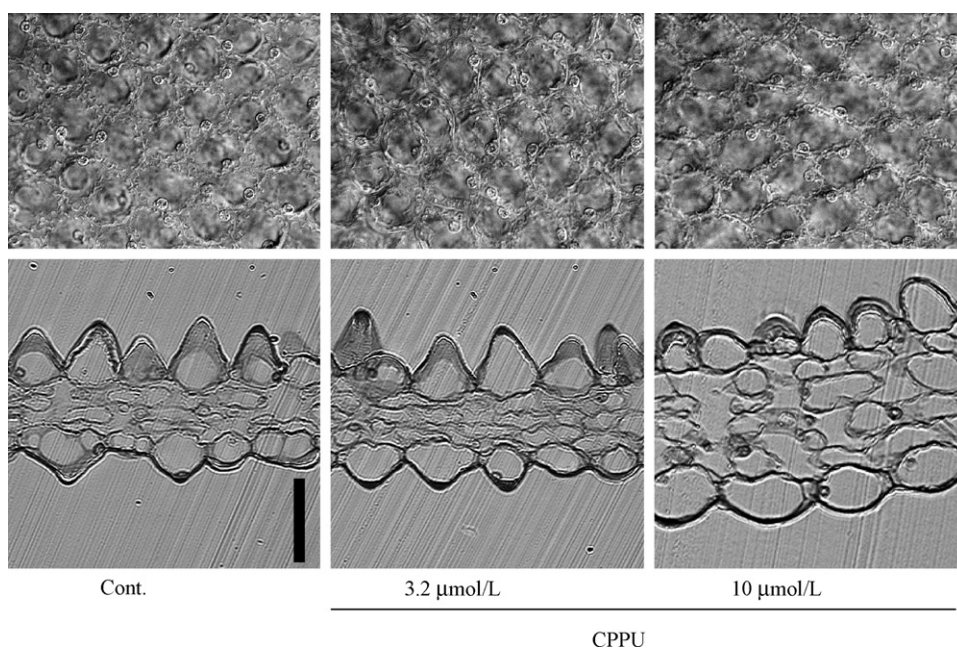

Fig. 3. The effects of CPPU on the histology of the corolla in the 'Pearl White' cultivar. Upper row: the adaxial surface of the limb. The transparent samples were photographed under a differential-interference microscope. Lower row: cross-sections. The sections (2 μm thick) were stained with toluidine blue and photographed under a bright-field microscope. Corolla samples were collected 4 days after anthesis. The method of CPPU application is described in the caption of Fig. 1. The vertical bar represents 50 μm for all photographs.
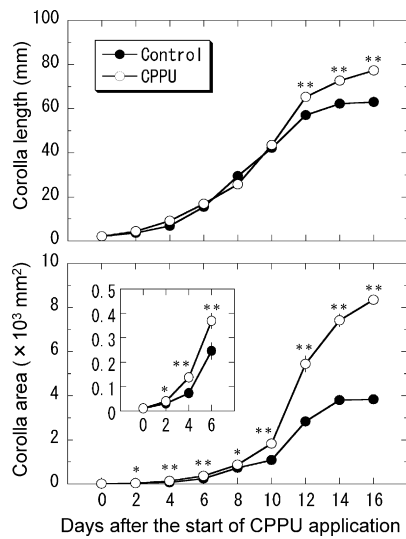
Fig. 4. The effects of CPPU on corolla length (upper) and corolla area (lower) in the 'Pearl White' cultivar. In the lower figure, the inset graph provides a magnified view of the results of the early stage. CPPU at 3.2 μmol/L was applied as described in the caption of Fig. 1. Data represents the mean ± S.E. ($n = 12$). Most error bars were smaller than the symbols used to plot the data. Values significantly different from those of the control flowers based on Dunnett's test are indicated by $^*P \leq 0.05$ and $^{**}P \leq 0.01$. The mean ± S.E. ($n = 12$) of the number of days to anthesis was 13.0 ± 0.2 in the control flowers and 13.2 ± 0.2 in the CPPU-treated flowers; these values did not differ significantly (t-test, $P = 0.05$).
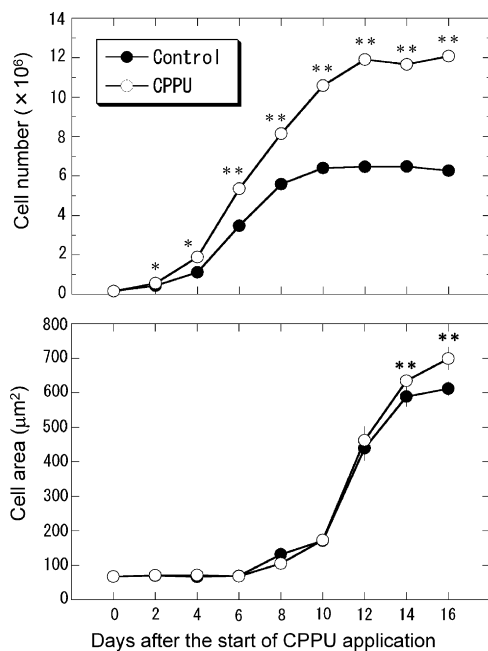

Fig. 5. The effects of CPPU on corolla development in the 'Pearl White' cultivar in terms of cell number (upper) and cell area (lower). CPPU at 3.2 μmol/L was applied as described in the caption of Fig. 1. Data represents the mean ± S.E. ($n = 12$). Most error bars were smaller than the symbols used to plot the data. Values significantly different from those of the control flowers based on Dunnett's test are indicated by $^*P \leq 0.05$ and $^{**}P \leq 0.01$. The number of days to anthesis did not differ significantly among treatments (see caption of Fig. 4).

3.4. Cultivar differences in the response to cytokinin application

The limb area of the untreated corolla ranged from 94 to 158 mm^2 in the small-flowered cultivars, from 242 to 311 mm^2 in the medium-flowered cultivars, and from 242 to 311 mm^2 in the large-flowered cultivars (Fig. 6). The ratio of the limb area after the application of 3.2 μmol CPPU to that of the control ranged from 1.5 to 2.5 in the small-flowered cultivars, 1.4 to 2.3 in the medium-flowered cultivars, and 1.3 to 1.8 in the large-flowered cultivars. Thus, the increase varied greatly even within cultivars that had approximately similar limb areas. However, a significant negative correlation was observed between the "genetic" limb area (i.e., the limb area of the untreated corolla) and the increase: the magnitude of the increase decreased with increasing size of the genetic limb area.

The genetic cell number of the limb (i.e., that of the untreated control) increased with increasing genetic limb area (Fig. 7). However, no correlation was observed between the genetic limb area and the cell area. The increase in cell number caused by the application of 3.2 μmol CPPU ranged from 1.3 to 1.8 in the small-flowered cultivars, 1.3 to 1.9 in the medium-flowered cultivars, and 1.3 to 1.9 in the large-flowered cultivars. There was no correlation between the genetic limb area and the increase in cell number, nor between the genetic limb area and the increase in cell area.

In the cultivar 'Fantasy Salmon', the increase in limb area caused by CPPU application was mainly attributed to increased cell area (Fig. 7). The number and area of the cells increased to
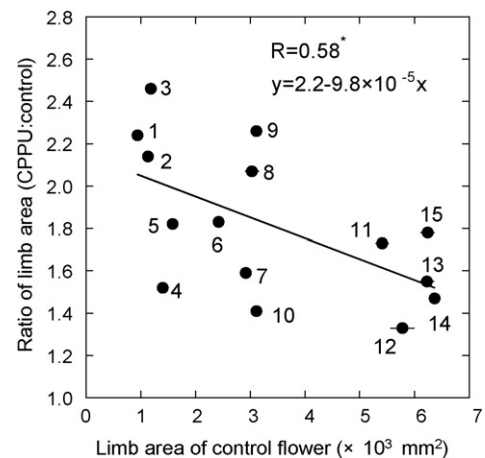

Fig. 6. Correlation between the genetic limb size (i.e., the size in the absence of cytokinin treatment) and enlargement of the limb by CPPU treatment in 15 cultivars. CPPU at 3.2 μmol/L was applied as described in the caption of Fig. 1. The ratio of the increase in limb area was calculated based on the average limb area of 12 untreated and 12 CPPU-treated flowers. Measurements were done 4 days after anthesis. Cultivars (ordered from small- to large-flowered ones): 1, 'Fantasy Ivory'; 2, 'Cutie Crystal Red'; 3, 'Fantasy Pink Morn'; 4, 'Fantasy Blue'; 5, 'Fantasy Salmon'; 6, 'Pearl Sky Blue'; 7, 'Baccara Rose Morn'; 8, 'Baccara White'; 9, 'Pearl White'; 10, 'Baccara Blue Picotee'; 11, 'Fulcon Pink Morn'; 12, 'Carnival Red Star'; 13, 'Fulcon White'; 14, 'Fulcon Midblue'; 15, 'Carnival Pink Flash'. Cultivars 1–5, 6–10, and 11–15 are small-, medium-, and large-flowered ones, respectively. Data for the limb area of control flower represent the mean ± S.E. ($n = 12$). Most error bars are smaller than the symbols used to plot the data.
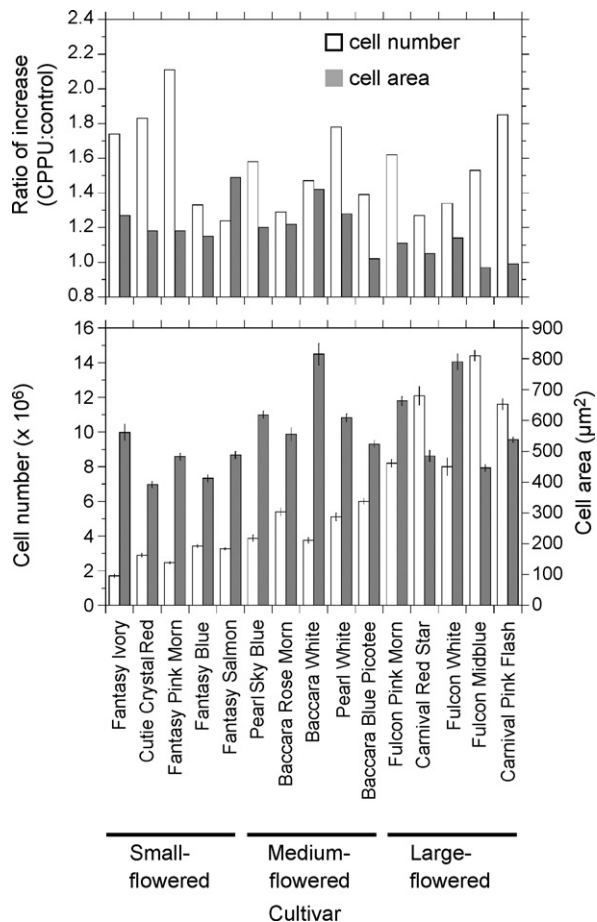
Fig. 7. Correlations between the genetic cell number and limb area (lower) and their increase in response to CPPU treatment (upper). CPPU at 3.2 μmol/L was applied as described in the caption of Fig. 1. The data for cell number and area represent the mean ± S.E. (*n* = 12). The increase in cell number and area represent the means for 12 untreated and 12 CPPU-treated flowers. Measurements were done 4 days after anthesis.

similar extents in the 'Baccara Rose Morn' and 'Baccara White' cultivars. In all other cultivars, however, the increase in limb area was mainly attributed to the cell number increase.

4. Discussion

Based on their chemical structures, the three cytokinins used in our experiment belong to different classes of cytokinins. Zeatin, BA, and CPPU are isoprenoid, aromatic, and phenylurea cytokinins, respectively (Mok and Mok, 2001). Although all of these chemicals were effective in enlarging the petunia corolla, the mode of action may be different. Molecules of zeatin and BA have cytokinin action in themselves (Mok and Mok, 2001). However, CPPU inhibits metabolism of endogenous cytokinins through inhibition of the oxidation of the isoprenoid side chain (Bilyeu et al., 2001). This inhibition promotes accumulation of endogenous cytokinins in plant tissue.

We also applied 0.1–1000 μmol/L of several other plant hormones (indole-3-acetic acid [IAA], 1-naphthylacetic acid [NAA], gibberellins in the form of GA_3, and brassinolide) to petunia corollas in the same manner as for our other applications. With the exception of GA_3, these hormones had no effect on corolla enlargement in the 'Pearl White' cultivar. The effect of GA_3 was weaker than that of the cytokinins in the present study; the maximum increase in limb area was 1.4 times that of the control at 1000 μmol/L versus values of 1.9–2.4 for the cytokinins.

The effects of exogenously applied cytokinins on leaf enlargement have been reported in several plants (reviewed in Kamisaka and Miyamoto, 1994; Sakurai, 1994). In contrast, overproduction of cytokinins due to overexpression of the *IPT* gene encoding isopenthenyl transferase decreased or did not change leaf size in tobacco (Medford et al., 1989; Wang et al., 1997) and *Arabidopsis* (Medford et al., 1989; Rupp et al., 1999; Graaff et al., 2001). In those experiments, expression of the transgene was not leaf-specific. Thus, the high concentration of cytokinin is more localized at the leaf when cytokinins were applied directly to the leaf than in the transgenic experiments. Consequently, leaf enlargement may occur only when high cytokinin concentration is localized at the leaf.

The enlargement of petunia corolla was observed when a cytokinin was applied to the flower bud. Spray application to the entire shoot did not remarkably enlarge the corolla (data not shown). Thus, as in the case of leaf tissue, localized increase of cytokinin concentration in the corolla is probably necessary for effective enlargement of the corolla.

Cytokinins increase leaf size through cell enlargement (Kuraishi and Okumura, 1956; Letham, 1969; Tsui et al., 1980). In contrast with these previous studies, cytokinins increased corolla area of most petunia cultivars mainly through an increase in cell number in our study (Fig. 7). In *Arabidopsis*, cell size increased when normal organ size was not attained due to inhibition of cell proliferation (Wang et al., 2000). In our experiment, the cytokinin-induced increase in cell area in the corolla of the 'Pearl White' cultivar was greater in the results shown in Fig. 7 than in Table 1. Thus, a compensatory system between cell size and cell number might have influenced the results, thereby introducing some inaccuracy in our interpretation of the cytokinin effect on cell proliferation and expansion. Nonetheless, because in no case did cytokinin application fail to increase cell number (Fig. 7), our results confirmed that cytokinins promote cell proliferation in the petunia corolla.

Cytokinin application increased the cell proliferation rate and prolonged the cell proliferation phase during corolla development (Fig. 5). These results suggest that cytokinins promote both cell division and the maintenance of meristematic ability in the petunia corolla.

The main factor contributing to the genetic control of size in plant organs is cell number rather than cell size (Niklas, 1994). Cultivar differences in petunia corolla size also were attributed mainly to differences in cell number (Fig. 7), and enlargement of the corolla in response to cytokinin application resulted mainly from an increase in cell number (Fig. 7). Thus, the observed enlargement of the corolla in response to hormone treatment resulted from the same anatomical changes that seem to be responsible for the cultivar differences in genetic corolla size.

Furthermore, the extent of the increase in corolla area in response to cytokinin application was negatively correlated with the genetic corolla size (Fig. 6), although the magnitude of the increase varied greatly even among cultivars with similar genetic corolla size. This correlation was not observed when GA_3 was applied to the same 15 cultivars (Nishijima, unpublished data). Thus, it is unlikely that the correlation was caused by underestimation of the magnitude of the increase in large-flowered cultivars (i.e., due to the large genetic corolla area that served as the denominator in calculating the proportional increase). These results might indicate that a cytokinin-related physiological mechanism is involved in differentiation of the genetic corolla size in petunia. The cause of the negative correlation might be cultivar differences in activity of cytokinin biosynthesis and metabolism, because CPPU strongly inhibits metabolism of cytokinins as described above. This action promotes accumulation of endogenous cytokinins in plant tissue. Thus, the accumulation level of endogenous cytokinin may differ among cultivars if the rates of cytokinin biosynthesis and metabolism are different.

Because flower size is an important factor in the attractiveness of floricultural plants, it would be worthwhile to investigate the practical use of the cytokinin-induced enlargement of the corolla in horticulture. However, simple application of CPPU by spraying did not enlarge the flower. The best way to bring about flower enlargement would be to create transgenic plants in which biosynthesis of the endogenous cytokinin is promoted specifically in the corolla. The target cultivar should be cautiously selected, however, because large differences in the response to cytokinin exist among cultivars (Fig. 6). This transgenic strategy might be effective in extending the range of variation in corolla size of petunia, because the limb area of the large-flowered cultivar was increased up to 1.8 times the control value by CPPU application (Fig. 6).

Acknowledgments

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