Effect of Localized Promotion of Cytokinin Biosynthesis on Flower Morphology in Flower Buds of *Torenia fournieri* Lind.

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To analyze the relationship between flower morphology and organ-specific promotion of cytokinin biosynthesis within flower buds, we introduced *Arabidopsis isopentenyltransferase 4* (*AtIPT4*) into *torenia* (*Torenia fournieri* L.) under the control of *APETALA1* (*API*) or *APETALA3* (*AP3*) promoter. *API::AtIPT4* plants had an increased number of petals, whereas *AP3::AtIPT4* plants had an expanded corolla, a paracorolla, and serrated petal margins along with an increased number of petals. In *AP3::AtIPT4* plants, marked receptacle enlargement was observed when the flower buds were in the early corolla development stage in which the paracorolla primordia differentiate. As expected, *AtIPT4* was expressed in the sepals and petals of *API::AtIPT4* plants, and in the petals and stamens of *AP3::AtIPT4* plants. Furthermore, the type-A response regulator (*TfRR1*) and cytokinin oxidase (*TfCKX5*) genes, which were used as indices of cytokinin signal, showed the same expression patterns as the transgene. These findings indicate that expansion of the corolla and development of the paracorolla and serrated petal margins after receptacle enlargement in *AP3::AtIPT4* plants are induced by localized elevated cytokinin signal in the petals and stamens. In contrast, localized elevated cytokinin signal in the sepals and petals only induced an increase in the number of petals. Therefore, an elevated cytokinin signal in the stamen may be important for inducing corolla expansion and for developing a paracorolla and serrated petal margins.

Key Words: flower morphology, genetic modification, isopentenyltransferase, paracorolla, spatial expression.

Introduction

Flower morphology is one of the most important commercial characteristics of floricultural plants. Flower morphologies such as double flowers, flowers with paracorollas and large flowers are attractive to consumers and are of great commercial value. Breeding programs to produce novel flower morphologies have mainly been based on mutant breeding, but this can be time consuming because the occurrence of desirable stable mutants is rare (Nishijima, 2007). Therefore, elucidation of the molecular mechanism underlying flower morphology may help produce a novel, more efficient breeding method by employing DNA markers and

genetic transformation.

Cytokinin reportedly plays an important role in the regulation of flower morphology. In *Arabidopsis*, the number of floral organs, such as petals, stamens, and pistils, is increased after *N*⁶-benzylaminopurine treatment of flower buds (Lindsay et al., 2006; Venglat and Sawhney, 1996). Such an increase in the number of floral organs is promoted by cytokinin-mediated induction of gene expression involved in meristematic activity and organ differentiation (Lindsay et al., 2006; Rupp et al., 1999). In *Petunia hybrida*, treatment with forchlorfenuron (CPPU), which inhibits cytokinin degradation through cytokinin oxidase/dehydrogenase (CKX) inhibition (Bilyeu et al., 2001), induces corolla expansion following an increase in the cell number (Nishijima, 2012; Nishijima et al., 2006). Furthermore, some genes involved in cytokinin biosynthesis and early signaling pathways are regulated by the *Grandiflora* gene, which is responsible for the large-flowered phenotype in *petunia* (Nishijima, 2012; Nishijima et al., 2011a, b).

In *torenia*, CPPU treatment induces several different flower morphologies, including an increase in the

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number of petals and development of the paracorolla and serrated petal margins (Nishijima and Shima, 2006). Of these flower morphologies, paracorollas are only observed in a few plant species, including *Narcissus*, *Asclepias*, *Antirrhinum majus*, and *Passiflora*, and contribute to the unique appearance of flowers. In addition, serrated petal margins, which are preferred as a ‘fringed flower’ type, also contribute to the increased diversity of flower morphologies. Varieties of new flowers could be produced if these flower morphologies could be universally induced in other floricultural plants.

The CPPU-induced changes in flower morphologies of torenia are dependent on the floral stage at which CPPU is applied; the petal-like wide paracorolla, which consists of wide extra petaloid organs, is induced when CPPU is applied at the sepal development stage (Stage 3), whereas the narrow paracorolla, which consists of narrow extra petaloid organs, is induced when CPPU is applied at the early corolla development stage (Stage 5). Serrated petal margins are induced when CPPU is applied at the middle corolla development stage (Stage 6; Nishijima and Shima, 2006). These morphological changes are induced by localized elevation of the cytokinin signal in flower buds (Niki et al., 2013).

Distribution of the cytokinin signal can be analyzed by monitoring the expression of cytokinin-responsive genes, i.e., type-A *response regulator* (*RR*) and *CKX* genes. The expressions of type-A *RR* and *CKX* genes are induced by cytokinin application and by an enhanced cytokinin signal (Brandstatter and Kieber, 1998; Brugière et al., 2003; D’Agostino et al., 2000; Kiba et al., 2005; Nishijima et al., 2011a, b; Taniguchi et al., 1998). Thus, expression of these genes reflects the extent of the cytokinin signal. In torenia, when CPPU application at Stage 3 induces wide paracorollas, the cytokinin signal is elevated on the abaxial side of the stamen primordia, which is the site of wide paracorolla initiation. When CPPU application at Stage 5 induces narrow paracorollas, the cytokinin signal is elevated in the middle part of young petals, which is the site of narrow paracorolla initiation. In contrast, the enhanced cytokinin signal by CPPU application at Stage 6 is localized from the middle to the apical parts of the petals when serrated petals are induced (Niki et al., 2013). Therefore, spatial distribution of the elevated cytokinin signal differs depending on the floral stage at CPPU application, which causes the induction of specific flower morphology. Further, morphological changes occur at the site of elevation of the cytokinin signal.

It is difficult to intentionally produce a particular flower morphology using conventional mutant breeding strategies. Utilization of plant growth regulators is also difficult because the chemical has to be precisely applied at a specific floral stage to obtain the desired uniform flower morphology. Therefore, the production of genetically modified plants expressing a cytokinin biosynthesis gene under the control of a floral organ-

specific promoter would be an effective tool for more efficient breeding programs.

The biosynthetic pathways of cytokinins and their related genes in higher plants have been well documented (Frébert et al., 2011). The first step of cytokinin biosynthesis in higher plants is isopentenylolation of ATP and ADP catalyzed by isopentenyltransferase (IPT) using dimethylallyl diphosphate as a substrate (Kakimoto, 2001; Takei et al., 2001). This is thought to be a rate-limiting step of cytokinin biosynthesis. In *Arabidopsis*, nine genes encoding IPT have been cloned, and transgenic plants overexpressing these genes show elevated concentrations of endogenous cytokinins (Sakakibara et al., 2005). Furthermore, the application of a promoter of floral homeotic genes would be useful for inducing transgenes in a floral organ-specific manner. The floral homeotic genes *APETALA1* (*AP1*) and *APETALA3* (*AP3*) are specifically expressed in whorls 1 and 2 and whorls 2 and 3, respectively (Jack et al., 1992; Mandel et al., 1992). Therefore, transgenic plants expressing *IPT* under the control of the promoters of these genes would specifically elevate cytokinin concentration and cytokinin signal in the sepals and petals and in the petals and stamens, respectively.

In this study, we introduced *Arabidopsis IPT* (*AtIPT4*) into torenia under the control of *AP1* or *AP3* promoters to clarify the effects of floral organ-specific promotion of cytokinin biosynthesis on flower morphology. On the basis of our results, a strategy to produce decorative flower morphologies is discussed.

Materials and Methods

Plasmid construction and transformation of torenia

For cloning *AtIPT4* in *Arabidopsis thaliana* (AB061402), total RNA was isolated from flower buds and young flowers (Miyawaki et al., 2004) using an RNeasy Plant Mini Kit (Qiagen Sciences, Germantown, MD, USA) and RNase-Free DNase Set (Qiagen), and cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany), as described previously (Niki et al., 2012). For cloning *Arabidopsis AP1* (At1g69120) and *AP3* (At3g54340) promoters, genomic DNA was isolated from young *Arabidopsis* leaves using ISOPLANT II (Wako Pure Chemical Industries Ltd., Osaka, Japan). The open reading frame of *AtIPT4* and the 5'-upstream region of *Arabidopsis AP1* and *AP3* were amplified using PCR with KOD Plus DNA polymerase (Toyobo, Osaka, Japan) with the following primers including an *Eco* RI site (underlined): forward 5'-CTCAGAATTCGACATGAAGTGT-3' and reverse 5'-CTAGTTAAGACTTAAAAATCT-3' for *AtIPT4* (970 bp); forward 5'-TGTATCGTTTCAAACTCAGG-3' and reverse 5'-TACTGAATTCGAACCAACAAAAC-3' for *AP1* (1729 bp); and forward 5'-GACCAGATCAAGAGTGCCTG-3' and reverse 5'-GTTTGAATTCCTTTGTTGAAG-3' for *AP3* (1197 bp). All PCR fragments were digested using *Eco*

RI; subsequently, the *AtIPT4* fragment was ligated with the *AP1* or *AP3* fragment. The fragments produced were amplified by PCR using the following forward and reverse primers for *AtIPT4* described above: forward 5'-CACCTGTATCGTTTCAAACCTC-3' for *AP1::AtIPT4* (2689 bp), and forward 5'-CACCGACCAGATCAAGA GTGC-3' for *AP3::AtIPT4* (2157 bp). These fragments were cloned into a pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) and then transferred into a destination vector pGWB1 (Nakagawa et al., 2007), which was derived from the plant transformation vector pBI101, using the Gateway LR clonase reaction (Invitrogen).

Torenia fournieri 'Crown Violet' was used for transformation (Aida et al., 2000). Plant materials were vegetatively reproduced by herbaceous cuttings and grown at 25°C with illumination from fluorescent lamps (85 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD; 16 h light/8 h dark cycle). The vectors were introduced into *torenia* by *Agrobacterium*-mediated transformation as described by Aida and Shibata (1995, 2001). After selection with kanamycin and hygromycin, transformants were planted in horticultural soil (Kureha-Engei-Baido; Kureha Chemical Industry Co., Ltd., Tokyo, Japan) in plastic pots and grown in a greenhouse under natural light at 25°C/20°C (day/night). Plants showing any changes in flower morphology were selected, and their T_2 generations were obtained by selfing; the plants were used for subsequent investigations. Seeds of transgenic plants were germinated in another horticultural soil (Metro-Mix 350; Sun Gro Horticulture Canada Ltd, British Columbia, Canada), and the seedlings were transplanted into the horticultural soil in plastic pots and grown in the greenhouse as described above.

Morphological analysis of flower bud development

For observation of the morphological changes that occurred in the developing flower buds, flower buds at the sepal development stage (Stage 3), the stamen and pistil initiation stage (Stage 4), the early corolla development stage (Stage 5), and the middle corolla development stage (Stage 6), which are defined by Nishijima and Shima (2006), were fixed in FAA [50% (v/v) ethanol, 10% (v/v) formaldehyde, 5% (v/v) acetic acid] at 4°C overnight, then dehydrated through a graded ethanol series [50%, 70%, 90%, and 99.5% (v/v)]. The ethanol was replaced with 2-methyl-2-propanol. The samples were freeze-dried in 2-methyl-2-propanol and analyzed by scanning electron microscopy (SEM; VE-7800; Keyence, Osaka, Japan).

Quantitative real-time PCR analysis

Total RNA was separately isolated from the sepals, petals, stamens, and pistils of the flower buds at Stage 6 from transgenic and non-transgenic plants using the same procedure used for cDNA cloning. Gene-specific primers for *AtIPT4*, *torenia TjRR1* (AB740033), *TjCKX5*

(AB740039), and an actin gene (*TjACT3*; AB330989), which was used as an internal standard, were designed for the 3'-terminal regions of the open reading frame and the 3'-untranslated regions of each gene. Primer sequences and the lengths of the PCR products used for quantitative real-time PCR reactions of *AtIPT4* were forward 5'-ACAGCATCGTTTCGAGAGG-3' and reverse 5'-GTGGCTCCTGACAATCTTCAC-3' and 120 bp, respectively. The primers for *TjRR1*, *TjCKX5*, and *TjACT3* were the same as those described previously (Niki et al., 2013). Gene expression was quantified using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) and quantitative real-time PCR (LightCycler; Roche). The PCR reactions were performed with an initial denaturation step of 10 s at 95°C followed by 50 cycles of 5 s at 95°C, 10 s at 60°C, and 5–7 s at 72°C. To avoid calculating non-specific PCR products, fluorescence was measured at the end of the extension phase at 76°C for *TjRR1*, at 77°C for *TjACT3*, at 78°C for *TjCKX5*, and at 80°C for *AtIPT4*. Raw data were analyzed with LightCycler software version 3.5 (Roche). Plasmids harboring full-length cDNA of the *AtIPT4*, *TjRR1*, or *TjCKX5* or a partial cDNA fragment of *TjACT3* were used to obtain standard curves. The ratio of the expression of each gene to that of *TjACT3* was calculated. Expression analyses were conducted independently and in triplicate.

Analysis of endogenous cytokinins

Endogenous cytokinins were extracted separately from the sepals, petals, stamens, and pistils at the late corolla development stage (Stage 7) from transgenic and non-transgenic plants. The methods used for cytokinin analysis were the same as those described by Dobrev and Kaminek (2002) and Nishijima et al. (2011a). Each floral organ (0.2–0.6 g FW) was homogenized in liquid nitrogen and extracted in ice-cold MeOH/water/formic acid (15/4/1, v/v/v), which includes 1 ng of each internal standard, [$^2\text{H}_6$] *N*⁶-(Δ^2 -isopentenyl)adenine (iP), [$^3\text{H}_5$] *trans*-zeatin (tZ), [$^2\text{H}_6$] iP riboside (iPR), and [$^3\text{H}_5$] tZ riboside (tZR) (OChemim, Olomouc, Czech Republic). After overnight extraction at –20°C, solids were separated using centrifugation. The extract was passed through a Sep-Pak tC₁₈ cartridge (Waters, Milford, MA, USA) and evaporated to near dryness. The residue was dissolved in 1 M formic acid and applied to an Oasis MCX column (Waters). The column was sequentially eluted with MeOH, 0.35 M NH₄OH, and 0.35 M NH₄OH in 60% (v/v) aqueous MeOH. The eluent with 0.35 M NH₄OH in 60% (v/v) aqueous MeOH comprising cytokinin nucleobases, cytokinin nucleosides, and cytokinin glucosides was evaporated to dryness. The dried sample was then dissolved in 10% aqueous MeOH containing 0.05% acetic acid and analyzed using a liquid chromatography–tandem mass spectrometry system (LC/MS/MS, model 2695/TSQ7000; Waters/Thermo Fisher Scientific, Waltham, MA, USA) coupled with

positive ion electrospray ionization. Cytokinins were separated using an ODS column (MD, 5 μ m, 2.5 mm $\times$ 250 mm; Shiseido Fine Chemicals, Tokyo, Japan) at a flow rate of 0.2 mL·min⁻¹ with gradients of solvents A (MeOH containing 0.05% acetic acid) and B (water containing 0.05% acetic acid) as follows: 0 min, 10% A + 90% B; 45 min, 80% A + 20% B; and 55 min, 100% A. The column temperature was 35°C. Quantification was performed in selected ion recording mode. The ionization voltage was 4.7 kV, the capillary temperature was 200°C, and the collision energy was -22 to -34 V depending on the cytokinin species. Data were analyzed using Xcaliber software (Thermo Fisher Scientific). Endogenous cytokinins were quantified by the internal standard method using the corresponding deuterated cytokinins. Quantification of endogenous cytokinins was conducted independently and in triplicate.

Results and Discussion

Changes to the flower morphology of transgenic torenia

Several morphological changes were induced in the

flowers of both *AP1::AtIPT4* and *AP3::AtIPT4* plants, and these traits were inherited. The number of petals in *AP1::AtIPT4* plants was increased to 6–7 (Fig. 1b) from the characteristic 5 petals of the normal type (NT) plants (Fig. 1a). The number of sepals was increased to 5–7 in *AP1::AtIPT4* plants, whereas the NT plants had 5 sepals. In contrast, the number of stamens and pistils remained unchanged. In *AP3::AtIPT4* plants, the number of petals increased to 5–6 (Fig. 1c, d), although the increase was moderate compared with *AP1::AtIPT4* plants (Fig. 1b). In addition, marked petal expansion and serrated petal margins were observed in *AP3::AtIPT4* plants (Fig. 1c, d), but not in NT plants (Fig. 1a). Furthermore, extra floral organs resembling petals were observed on the corolla (Fig. 1d). *AP3::AtIPT4* plants had 4–5 stamens compared with the 4 stamens of NT plants, but the number of sepals and pistils was unchanged.

In Stage 3 of floral development, both NT and *AP3::AtIPT4* plants had 5 sepals (Fig. 2a, o); however, *AP1::AtIPT4* plants had more than 6 sepals (Fig. 2h). In the initiation stage of the stamen and pistil (Stage 4),

Fig. 1. Morphological changes in the flowers of torenia. a, Normal type (NT); b, *AP1::AtIPT4*; c and d, *AP3::AtIPT4*. Pc, Paracorolla; Sp, Serrated petal.

Fig. 2. Floral development of transgenic torenia. a–g, Normal type (NT); h–n, *AP1::AtIPT4*; o–u, *AP3::AtIPT4*. Floral stages were defined as described by Nishijima and Shima (2006). Closed and open triangles represent lobes from a petal primordium and the site of paracorolla initiation, respectively. Pe, Petal; Sn, Staminoid; Sp, Serrated petal; St, Stamen. Scale bars = 100 μ m.

the receptacle was larger in both *AP1::AtIPT4* and *AP3::AtIPT4* plants (Fig. 2i, p) than in NT plants (Fig. 2b). Furthermore, the receptacle of *AP3::AtIPT4* plants became markedly larger than those of NT and *AP1::AtIPT4* plants at Stage 5 (Fig. 2c, j, q). In Stage 5 and early Stage 6, an increase in the number of petal primordia, was observed in *AP1::AtIPT4* plants (Fig. 2c, d, g, j, k, n). The staminoid of *AP3::AtIPT4* plants was also larger than that of the NT and *AP1::AtIPT4* plants (Fig. 2c, d, j, k, q, r). In late Stage 6, primordia of the extra floral organs were initiated and corolla expansion and serrated petal margins were observed in *AP3::AtIPT4* plants (Fig. 2s). These extra floral organs were formed on the abaxial and lateral sides of the basal part of the stamen, which corresponded to the middle part of the petal (Fig. 2t, u). The morphology of the extra floral organs resembled the narrow paracorollas induced by CPPU treatment (Niki et al., 2012; Nishijima and Shima, 2006; Figs. 1d and 2u).

The paracorolla originates from the stipule of the stamen in *Narcissus*, *Asclepias*, and *A. majus*, and from the receptacle in *Passiflora* (Troll, 1957; Yamaguchi et al., 2010). The site of paracorolla differentiation depends on its origin. Paracorolla primordia differentiate on the abaxial and lateral sides of the stamen primordia when the paracorolla originates from the stipule of the stamen in *A. majus* (Yamaguchi et al., 2010) and the CPPU-treated *torenia* flowers (Nishijima and Shima, 2006). In *AP3::AtIPT4* plants of *torenia*, the site and the floral stage in which the extra floral organ initiation occurred coincided with those of the narrow paracorollas induced by CPPU treatment and those of the *A. majus* paracorolla (Fig. 2s, t). Therefore, together with the morphological similarity to the narrow paracorollas as described above, the extra floral organs formed in *AP3::AtIPT4* flowers may be paracorollas originating from the stipule of the stamen.

Cytokinin biosynthesis and signal transduction in transgenic *torenia*

As expected, the *AtIPT4* transgene was mainly expressed in the sepals and petals of *AP1::AtIPT4* plants and in the petals and stamens of *AP3::AtIPT4* plants (Fig. 3).

In NT plants, iP and iPR were not detected in the sepals and petals, while the stamens contained high concentrations of iP (Fig. 4a, b). The concentrations of iP in the pistils and iPR in the stamens and pistils were slight in NT plants (Fig. 4a, b). Both iP and iPR accumulated in the sepals and petals in *AP1::AtIPT4* plants, whereas changes in iP and iPR concentrations in the stamens and pistils compared with those of NT plants were obscure (Fig. 4a, b). On the other hand, both iP and iPR accumulated in the petals of *AP3::AtIPT4* plants, but were undetected in the sepals, whereas changes in iP and iPR concentrations in the stamens and pistils compared with those of NT plants were obscure (Fig. 4a,

Fig. 3. *AtIPT4* expression in the floral organs of transgenic *torenia*. The expression levels are shown as a value relative to that of *TfACT3*, which was used as an internal standard. Vertical bars represent $\pm$ SE (n = 3).

b). The other cytokinin species quantified, tZ and tZR, were accumulated mainly in the stamen in NT plants, although they were also detected in the other floral organs (Fig. 4c, d). The accumulations of tZ and tZR among the floral organs in *AP1::AtIPT4* and *AP3::AtIPT4* plants showed mostly the same pattern as those of NT plants, except that tZR was higher in the sepals of *AP1::AtIPT4* plants than in those of NT plants (Fig. 4c, d).

Because the changes of cytokinin concentrations in the stamens of *AP3::AtIPT4* plants from those of NT plants were obscure, we analyzed the expressions of *TjRR1* and *TjCKX5*, which reflect the extent of the cytokinin signal as described above. The expressions of *TjRR1* and *TjCKX5* were low or undetectable in all floral organs of NT plants (Fig. 5). In *AP1::AtIPT4* plants, the expressions of *TjRR1* and *TjCKX5* in the sepals and petals were more than 10-fold that in NT plants (Fig. 5). On the other hand, the expressions of *TjRR1* and *TjCKX5* were more than 10-fold higher in the petals and stamens of *AP3::AtIPT4* than in NT plants (Fig. 5). These expression patterns among floral organs coincided with those of the introduced *AtIPT4*.

Why was endogenous cytokinin concentration in the stamens of *AP3::AtIPT4* plants not markedly different from that in NT plants (Fig. 4a, b)? The stamen is the organ synthesizing cytokinins because cytokinin is necessary for pollen development (Huang et al., 2003; Sawhney and Shukla, 1994). At the same time, the development of the anther and pollen is inhibited by elevated cytokinin concentrations in transgenic tobacco plants overexpressing *IPT* in an anther-specific manner (Geng et al., 2002). This suggests that normal development of the stamen would be inhibited by excess cytokinin signal in the anther. Therefore, in the stamens of *AP3::AtIPT4* *torenia* plants, intensive expression of *AtIPT4* possibly had an inhibitory effect on stamen

Fig. 4. Concentration of endogenous cytokinins in the floral organs of normal type (NT) and transgenic torenia. iP, isopentenyladenine; iPR, isopentenyladenine riboside; tZ, *trans*-zeatin; tZR, *trans*-zeatin riboside. Vertical bars represent $\pm$ SE (n=3).

Fig. 5. Expression of *TfRR1* and *TfCKX5* in the floral organs of normal type (NT) and transgenic torenia. The expression levels are shown as values relative to that of *TfACT3*, which was used as an internal standard. Vertical bars represent $\pm$ SE (n=3).

development, which may have been a barrier to cytokinin biosynthesis.

Taken together, these results indicate that enhanced cytokinin signal in the sepals and petals of *AP1::AtIPT4* plants and in the petals and stamens of *AP3::AtIPT4* plants induced flower morphologies specific to the transgenes.

In *Arabidopsis*, the number of sepals, petals, and stamens increases when *AtIPT4* is expressed under the control of *API* promoter (Li et al., 2010). Some morphological changes, such as the development of a small petal on top of the filament, a lobed petal, and the formation of the filament and a small petal between the sepal and petal, are observed in transgenic *Arabidopsis* overexpressing *IPT* in an anther-specific manner (Geng et al., 2002). Furthermore, *IPT* expression under the control of *AP3* promoter leads to an increase in the size of the corolla in petunia (Verdonk et al., 2008). However, there has been no research analyzing the relationship between spatial distributions of elevated cytokinin and flower morphologies using the same plant material under the same experimental condition. In our study, the increase in petal number, corolla expansion, and the development of paracorollas and serrated petal margins were induced by localized expression of *AtIPT4* in the petals and stamens of torenia plants, whereas only the petal number increased when *AtIPT4* was expressed in the sepals and petals (Figs. 1 and 2). These results indicate that an elevated cytokinin signal in the stamen may be critical for corolla expansion, development of paracorollas and serrated petal margins (Fig. 6).

In *Arabidopsis*, the increase in the number of floral organs in response to the application of cytokinin has

Fig. 6. Model of the effect of localized elevated cytokinin concentration in flower buds on flower morphology in transgenic torenia. Whorls with elevated cytokinin levels are represented in gray. Pc, Paracorolla; Pe, Petal; Pi, Pistil; Se, Sepal; Sn, Staminoid; Sp, Serrated petal; St, Stamen.

been shown to be caused by an enlarged floral meristem, and consequently by an enlarged receptacle (Bartrina et al., 2011; Lindsay et al., 2006). In addition, in *Arabidopsis*, an enlarged receptacle is observed in *AP1::AtIPT4* plants with an increase in the number of floral organs (Li et al., 2010). Because the organ primordia tend to differentiate at constant intervals, the number of organs increases when the receptacle is enlarged. In our study, receptacle enlargement was observed at Stage 4 in both *AP1::AtIPT4* and *AP3::AtIPT4* torenia plants (Fig. 2i, p). This suggests that elevated cytokinin signal in whorl 2 causes receptacle enlargement, resulting in an increase in the number of petals (Fig. 6). However, only *AP3::AtIPT4* plants showed enhanced receptacle progressively in Stage 5 (Fig. 2q). Enlargement preferably occurs in whorl 3. When enhanced receptacle enlargement continues to Stage 5, the distance between each stamen is increased markedly (Fig. 6). Because the stipule of the stamen, which develops into paracorolla, is located on the lateral sides of the stamen and included in whorl 3, sufficient space allowing paracorolla primordia to be initiated may have been supplied. The same phenomenon is observed when the paracorollas are induced by CPPU treatment (Nishijima and Shima, 2006). That is, the paracorollas are generated synchronously with receptacle enlargement at Stage 5. In *Arabidopsis*, receptacle enlargement by cytokinin has been shown to be caused through

coordinated regulation of *WUSCHEL* (*WUS*) and *CLAVATA* (*CLV*) (Bartrina et al., 2011; Clark et al., 1993; Lindsay et al., 2006). *WUS*, which maintains meristematic activity of the shoot apical meristem (SAM), expressed in the central zone in SAM. *WUS* expression is restricted in the central zone by *CLV1*, which promotes organ differentiation (Schoof et al., 2000). Cytokinin expands the *WUS* expression area and consequently meristem size through repression of *CLV1* expression (Bartrina et al., 2011; Lindsay et al., 2006). This regulation mechanism of SAM size is thought to function also in the floral meristem, which regulates receptacle size (Bartrina et al., 2011; Lindsay et al., 2006). In our transgenic torenia, if *CLV* expression was repressed at the site where the cytokinin signal was elevated, it could be presumed that elevation of the cytokinin signal in whorl 3 would be more effective for receptacle enlargement than that in whorl 2. This is because whorl 3 is located more closely to the central zone of the floral meristem than whorl 2. Meanwhile, it can't be ruled out that the elevated cytokinin signal in whorl 3 of *AP3::AtIPT4* torenia induced the development of the stipule of the stamen independent of any receptacle enlargement. This is because cytokinin promotes organ differentiation and development by directly promoting the meristematic activity of the organ (Dewitte et al., 1999; Mok and Mok, 2001; Pernisová et al., 2009).

Serrated petal margins have been shown to be generated by changes in the arrangement of the vascular bundle induced by an elevated cytokinin signal at the petal limb (Niki et al., 2013; Nishijima and Shima, 2006). In contrast, we demonstrated that an elevated cytokinin signal in the stamen was critical for inducing a serrated petal margin, as described above. A serrated petal margin is also induced in a transgenic torenia in which the function of *AGAMOUS* (*AG*), a floral homeotic gene expressed in whorls 3 and 4, is inhibited by a plant-specific transcriptional repression domain (*AG-SRDX*, Narumi et al., 2008). This shows that a functional change in a gene expressed in whorls 3 and 4 may affect the arrangement of the vascular bundle in whorl 2. The elevated cytokinin signal in whorl 3, which inevitably alters the gene expression profile, remotely affects the vascular bundle arrangement in the petal.

In our study, corolla expansion and a serrated petal margin were stable phenotypes in transgenic torenia (Figs. 1 and 6), which suggests that an elevated cytokinin signal in the petals and stamens can be practically applied for the development of novel flower morphologies. However, the induction of the narrow paracorolla observed in *AP3::AtIPT4* plants was unstable (Figs. 1d and 2u). On the other hand, CPPU treatment induced a petal-like wide paracorolla, which makes a stronger impression than the narrow paracorolla (Niki et al., 2012; Nishijima and Shima, 2006; Figs. 1d and 2u). The delicate regulation of spatial and temporal accumulation of the cytokinin signal is necessary to induce a wide

paracorolla (Niki et al., 2013). Because the transgene was expressed in the whole area of whorls 2 and 3 by the *AP3* promoter in this study, more precisely and intensively targeted cytokinin accumulation in floral organs may be necessary to induce a wide paracorolla. As described, the type of CPPU-induced flower morphology is highly dependent on the floral stage at which CPPU is applied (Nishijima and Shima, 2006); however, such floral stage-dependent regulation of cytokinin accumulation is impossible in the *AP3::AtIPT4* construct, thereby making paracorolla induction unstable. Therefore, a floral stage-specific promoter may be needed to ensure the stable induction of paracorollas. In the future, novel breeding methods for producing particular flower morphologies may be established if new transgenic technologies enabling spatial and temporal localization of cytokinin signal in the manner described above are developed.

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Literature Cited

- Aida, R. and M. Shibata. 1995. *Agrobacterium*-mediated transformation of torenia (*Torenia fournieri*). *Breed. Sci.* 45: 71–74.
- Aida, R. and M. Shibata. 2001. Transgenic *Torenia fournieri* Lind (torenia). p. 294–305. In: Y. P. S. Bajaj (ed.). *Biotechnology in agriculture and forestry*, vol 48 transgenic crops III. Springer, Berlin.
- Aida, R., S. Kishimoto, Y. Tanaka and M. Shibata. 2000. Modification of flower color in torenia (*Torenia fournieri* Lind.) by genetic transformation. *Plant Sci.* 153: 33–42.
- Bartrina, I., E. Otto, M. Strnad, T. Werner and T. Schmülling. 2011. Cytokinin regulates the activity of reproductive meristems, flower organ size, ovule formation, and, thus, seed yield in *Arabidopsis thaliana*. *Plant Cell* 23: 69–80.
- Bilyeu, K. D., J. L. Cole, J. G. Laskey, W. R. Riekhof, T. J. Esparza, M. D. Kramer and R. O. Morris. 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. *Plant Physiol.* 125: 378–386.
- Brandstatter, I. and J. J. Kieber. 1998. Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in *Arabidopsis*. *Plant Cell* 10: 1009–1019.
- Brugière, N., S. Jiao, S. Hantke, C. Zinselmeier, J. A. Roessler, X. Niu, R. J. Jones and J. E. Habben. 2003. Cytokinin oxidase gene expression in maize is localized to the vasculature, and is induced by cytokinins, abscisic acid, and abiotic stress. *Plant Physiol.* 132: 1228–1240.
- Clark, S. E., M. P. Running and E. M. Meyerowitz. 1993. *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development* 119: 397–418.
- D'Agostino, I. B., J. Deruère and J. J. Kieber. 2000. Characterization of the response of the *Arabidopsis* response regulator gene family to cytokinin. *Plant Physiol.* 124: 1706–1717.
- Dewitte, W., A. Chiappetta, A. Azmi, E. Witters, M. Strnad, J. Rembur, M. Noin, D. Chriqui and H. Van Onckelen. 1999. Dynamics of cytokinins in apical shoot meristems of a day-neutral tobacco during floral transition and flower formation. *Plant Physiol.* 119: 111–121.
- Dobrev, P. I. and M. Kamínek. 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid phase extraction. *J. Chromatogr. A* 950: 21–29.
- Frébort, I., M. Kowalska, T. Hluska, J. Frébortová and P. Galuszka. 2011. Evolution of cytokinin biosynthesis and degradation. *J. Exp. Bot.* 62: 2431–2452.
- Geng, S., M. Ma, H-C. Ye and G-F. Li. 2002. Anther-specific expression of *ipt* gene in transgenic tobacco and its effect on plant development. *Transgenic Res.* 11: 269–278.
- Huang, S., R. E. Cerny, Y. Qi, D. Bhat, C. M. Aydt, D. D. Hanson, K. P. Malloy and L. A. Ness. 2003. Transgenic studies on the involvement of cytokinin and gibberellin in male development. *Plant Physiol.* 131: 1270–1282.
- Jack, T., L. L. Brockman and E. M. Meyerowitz. 1992. The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. *Cell* 68: 683–687.
- Kakimoto, T. 2001. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. *Plant Cell Physiol.* 42: 677–685.
- Kiba, T., T. Naitou, N. Koizumi, T. Yamashino, H. Sakakibara and T. Mizuno. 2005. Combinatorial microarray analysis revealing *Arabidopsis* genes implicated in cytokinin responses through the His→Asp phosphorelay circuitry. *Plant Cell Physiol.* 46: 339–355.
- Li, X. G., Y. H. Su, X. Y. Zhao, W. Li, X. Q. Gao and X. S. Zhang. 2010. Cytokinin overproduction-caused alteration of flower development is partially mediated by *CUC2* and *CUC3* in *Arabidopsis*. *Gene* 450: 109–120.
- Lindsay, D. L., V. K. Sawhney and P. C. Bonham-Smith. 2006. Cytokinin-induced changes in *CLAVATA1* and *WUSCHEL* expression temporarily coincide with altered floral development in *Arabidopsis*. *Plant Sci.* 170: 1111–1117.
- Mandel, M. A., C. Gustafson-Brown, B. Savidge and M. F. Yanofsky. 1992. Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. *Nature* 360: 273–277.
- Miyawaki, K., M. Matsumoto-Kitano and T. Kakimoto. 2004. Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. *Plant J.* 37: 128–138.
- Mok, D. W. and M. C. Mok. 2001. Cytokinin metabolism and action. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52: 89–118.
- Nakagawa, T., T. Kurose, T. Hino, K. Tanaka, M. Kawamukai, Y. Niwa, K. Toyooka, K. Matsuoka, T. Jinbo and T. Kimura. 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J. Biosci. Bioeng.* 104: 34–41.
- Narumi, T., R. Aida, T. Niki, T. Nishijima, N. Mitsuda, K. Hiratsu, M. Ohme-Takagi and N. Ohtsubo. 2008. Chimeric *AGAMOUS* repressor induces serrated petal phenotype in *Torenia fournieri* similar to that induced by cytokinin application. *Plant Biotechnol.* 25: 45–53.
- Niki, T., M. Hirai, T. Niki, A. Kanno and T. Nishijima. 2012. Role of floral homeotic genes in the morphology of forchlorfenuron-induced paracorollas in *Torenia fournieri* Lind. *J. Japan. Soc. Hort. Sci.* 81: 204–212.
- Niki, T., T. Mahesumu, T. Niki and T. Nishijima. 2013. Localized high expression of type-A response regulator and cytokinin oxidase/dehydrogenase genes in relation to forchlorfenuron-induced changes in flower morphology in *Torenia fournieri*

- Lind. J. Japan. Soc. Hort. Sci. 82: 69–77.
- Nishijima, T. 2007. Hanagata. p. 37–43. In: Nosangyosonbunkakyokai (ed.). Nogyojijututaikei Kakihen 5 (Tsuiroku) 9 (In Japanese). Nosangyosonbunkakyokai, Tokyo.
- Nishijima, T. 2012. Large flower size: Molecular basis and role of cytokinin. J. Japan. Soc. Hort. Sci. 81: 129–139.
- Nishijima, T. and K. Shima. 2006. Change in flower morphology of *Torenia fournieri* Lind. induced by forchlorfenuron application. Sci. Hortic. 109: 254–261.
- Nishijima, T., H. Miyaki, K. Sasaki and T. Okazawa. 2006. Cultivar and anatomical analysis of corolla enlargement of petunia (*Petunia hybrida* Vilm.) by cytokinin application. Sci. Hortic. 111: 49–55.
- Nishijima, T., T. Niki and T. Niki. 2011a. Corolla of the large-flowered petunia (*Petunia hybrida* Vilm.) cultivars exhibit low endogenous cytokinin concentration through enhanced expression of the genes encoding cytokinin oxidases. J. Japan. Soc. Hort. Sci. 80: 334–342.
- Nishijima, T., T. Niki and T. Niki. 2011b. The large-flowered petunia (*Petunia hybrida* Vilm.) genotype promotes expressions of type-A response regulator and cytokinin receptor genes like cytokinin response. J. Japan. Soc. Hort. Sci. 80: 343–350.
- Pernisová, M., P. Klíma, J. Horák, M. Válková, J. Malbeck, P. Soucek, P. Reichman, K. Hoyerová, J. Dubová, J. Friml, E. Zařmalová and J. Heřátko. 2009. Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. Proc. Natl. Acad. Sci. USA 106: 3609–3614.
- Rupp, H. M., M. Frank, T. Werner, M. Strnad and T. Schmölling. 1999. Increased steady state mRNA levels of the *STM* and *KNATI* homeobox genes in cytokinin overproducing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem. Plant J. 18: 557–563.
- Sakakibara, H., H. Kasahara, N. Ueda, M. Kojima, K. Takei, S. Hishiyama, T. Asami, K. Okada, Y. Kamiya, T. Yamaya and S. Yamaguchi. 2005. *Agrobacterium tumefaciens* increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant. Proc. Natl. Acad. Sci. USA 102: 9972–9977.
- Sawhney, V. K. and A. Shukla. 1994. Male sterility in flowering plants: Are plant growth substances involved? Am. J. Bot. 81: 1640–1647.
- Schoof, H., M. Lenhard, A. Haecker, K. F. X. Mayer, G. Jürgens and T. Laux. 2000. The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. Cell 100: 635–644.
- Takei, K., H. Sakakibara and T. Sugiyama. 2001. Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. J. Biol. Chem. 276: 26405–26410.
- Taniguchi, M., T. Kiba, H. Sakakibara, C. Ueguchi, T. Mizuno and T. Sugiyama. 1998. Expression of *Arabidopsis* response regulator homologs is induced by cytokinins and nitrate. FEBS Lett. 429: 259–262.
- Troll, W. 1957. Praktische Einführung in die Pflanzenmorphologie 2. Fischer, Jena. (Troll, W. 2004. Zusetu-shokubutukeitaigaku-handobukku 2 (In Japanese). Translated by S. Nakamura and H. Tobe. Asakurashoten, Tokyo.)
- Venglat, S. P. and V. K. Sawhney. 1996. Benzylaminopurine induces phenocopies of floral meristem and organ identity mutants in wild-type *Arabidopsis* plants. Planta 198: 480–487.
- Verdonk, J. C., K. Shibuya, H. M. Loucas, T. A. Colquhoun, B. A. Underwood and D. G. Clark. 2008. Flower-specific expression of the *Agrobacterium tumefaciens* isopentenyltransferase gene results in radial expansion of floral organs in *Petunia hybrida*. Plant Biotechnol. J. 6: 694–701.
- Yamaguchi, H., T. Niki, T. Niki and T. Nishijima. 2010. Morphological property and role of homeotic genes in paracorolla development of *Antirrhinum majus*. J. Japan. Soc. Hort. Sci. 79: 192–199.