The Large-flowered Petunia (*Petunia hybrida* Vilm.) Genotype Promotes Expressions of Type-A Response Regulator and Cytokinin Receptor Genes like Cytokinin Response

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Expressions of the genes involved in the cytokinin (CK) phosphorelay signaling circuitry in petunia (*Petunia hybrida* Vilm.) were investigated with regard to the large-flowered trait induced by the semidominant *Grandiflora* (*G*) gene. We isolated three cDNAs encoding type-A response regulators (RRs, *PhRR1-3*) and one cDNA encoding CK receptor histidine protein kinase (*PhHK*). Expression levels of *PhRR1-3* and *PhHK* in the developing corolla were markedly higher in large-flowered cultivars with the *Gg* genotype than in small- and medium-flowered cultivars with the *gg* genotype. Higher expression levels of *PhRR1-3* and *PhHK* in the *Gg* than in the *gg* genotypes was also consistent in BC₄ plants in which the genetic background except for *G* allele was mostly uniform. These results suggest that *Gg* genotype induces a high expression level of *PhRR1-3* and *PhHK*. These changes in expression caused by the *G* allele were the same as those caused by the CK response. Since type-A RRs repress CK signaling, the *G*-allele-induced high expression levels of *PhRR1-3* presumably have partial inhibitory effects on *G*-allele-induced corolla enlargement.

Key Words: feedback regulation, flower size, phosphorelay signaling circuitry, signal transduction.

Introduction

A large-flowered trait is an important breeding objective to improve the appearance of floricultural plants. This is clearly illustrated by the fact that many floricultural crops have much larger flowers than their corresponding wild species; however, large-flowered traits require a long time to develop, because of the rare occurrence of mutations responsible for large flowers (Nishijima, 2007).

Grandiflora (*G*) is the only major gene inducing a large-flowered phenotype in petunia (*Petunia hybrida* Vilm.) (Ewart, 1984). Although the *G* gene has not been molecularly identified, it is characterized as a semi-dominant gene, which mainly increases cell number rather than cell size to enlarge the corolla (Ewart, 1984; Nishijima et al., 2006). Commercial large-flowered cultivars are heterozygous, *Gg*, because the homozygous

GG genotype has the pleiotropic effect of extremely low vigor (Ewart, 1984). Although *GG* lines are necessary to produce commercial F₁ *Gg* seeds, the fertility of *GG* plants is usually low.

Large flowers of petunia can also be induced by cytokinin (CK) application to the young flower buds (Nishijima et al., 2006). Expression of the gene encoding a CK biosynthesis enzyme, adenosine phosphate-isopentenyltransferase (IPT), in the petunia corolla induces corolla enlargement through CK accumulation (Verdonk et al., 2008). In addition, the *G* allele and CK induce other common morphological changes. Corolla enlargement induced by CK is attributed mainly to cell number increase as observed in corolla enlargement by the *G* allele (Nishijima et al., 2006; Verdonk et al., 2008). The *G* allele induces paler leaf and sepal colors, which is also a characteristic of CK application to leaves and sepals (Ewart, 1984; Nishijima et al., unpublished data); however, the physiological implications of this similarity have not been investigated.

We previously reported that large-flowered cultivars have lower concentrations of endogenous CKs, including CK nucleobases, CK nucleosides, and CK glucosides, than small- and medium-flowered cultivars (Nishijima

Received; January 27, 2011. Accepted; March 8, 2011.

This work was supported by the National Agriculture and Food Research Organization (Development of innovative crop through molecular analysis of useful genes) and JSPS KAKENHI (22580045).

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et al., 2011). Thus, the large-flowered phenotype induced by the *G* allele is not attributable to high endogenous CK concentrations, although CK application effectively enlarges the corolla. The low CK concentration in large-flowered cultivars was caused by increased expression of the genes encoding CK oxidase/dehydrogenase (CKX) that degrade CKs through side-chain cleavage. This result enabled us to predict the involvement of CK signal transduction in *G*-allele-induced corolla enlargement because *CKX* expression is promoted by the CK response (Kiba et al., 2005; Nishijima et al., 2011).

CK early signal transduction consists of a two-component circuitry (reviewed in Mizuno (2005) and Oka (2005)). CK receptors are histidine protein kinases (HKs) that phosphorylate histidine phosphotransfer proteins (HPs) on receipt of biologically active CK molecules. Phosphorylated HPs move into the nucleus and phosphorylate type-B response regulators (RR). Phosphorylated type-B RRs function as transcription factors regulating the expression of target genes. Phosphorylated type-B RRs also induce the expression of type-A RRs. Accumulated type-A RRs act as repressors of CK signaling mediating a negative feedback loop in the signaling circuitry.

Expression of Type-A RRs is rapidly and greatly induced by the CK response (Brandstatter and Kieber, 1998; Taniguchi et al., 1998). Expression of type-A RRs is also regulated in response to light through phytochrome B (Sweere et al., 2001), by the WUSCHEL transcription factor involved in meristematic competence (Leibfield et al., 2005) and by the AUXIN RESPONSE FACTOR5/MONOPTEROS (MP) transcription factor

mediating auxin signalling (Zhao et al., 2010); however, the induction of type-A RR expression caused by the CK response is very conspicuous. Expression of *AHK4*, one of the genes encoding CK receptor HKs, is also conspicuously induced by the CK response (Kiba et al., 2004).

We investigated the effect of the petunia large-flowered *Gg* genotype on the regulation of CK phosphorelay signaling circuitry. Based on the results, the role of the CK phosphorelay signaling circuitry in *G* allele-induced corolla enlargement is discussed. Further, the potential modification of the CK phosphorelay signaling circuitry to produce the larger-flowered trait is discussed.

Materials and Methods

Plant materials

The cultivars listed in Table 1 were used in the experiment. Small- and medium-flowered cultivars belong to the multiflora group with the *gg* genotype of the *Grandiflora* (*G*) gene, while large-flowered cultivars belong to the grandiflora group with the *Gg* genotype (Ewart, 1984). Cultivars indicated by asterisks were used for detailed analysis. We also constructed backcross lines which mostly have an uniform genetic background except for *G* allele. A large-flowered cultivar 'Fulcon Midblue' with *Gg* genotype was crossed with a doubled haploid line 'Mitchell' with *gg* genotype. The *Gg* segregants were clearly distinguished from *gg* segregants by pale-colored shoots, and thick and large sepals and petals. *Gg* plants were selected and backcrossed to 'Mitchell'. Appearance of *gg* segregants at BC₂ and further generations became undistinguishable from

Table 1. *Petunia hybrida* cultivars used in the experiment.

Cultivar	Type	Corolla size ^y	Source
Fantasy Ivory ^z	multiflora	small	Sakata Seed Co.
Fantasy Blue*	multiflora	small	Sakata Seed Co.
Baccara White*	multiflora	medium	Sakata Seed Co.
Baccara Blue Picotee*	multiflora	medium	Sakata Seed Co.
Pearl White	multiflora	medium	Takii Seed Co.
Pearl Sky Blue	multiflora	medium	Sakata Seed Co.
Baccara Rose Morn	multiflora	medium	Sakata Seed Co.
Carpet Pink	multiflora	medium	M&B Flora Co., Ltd.
Rondo Pink	multiflora	medium	Takii Seed Co.
Rondo Violet Imp	multiflora	medium	Takii Seed Co.
Fulcon White*	grandiflora	large	Sakata Seed Co.
Fulcon Midblue*	grandiflora	large	Sakata Seed Co.
Fulcon Pink Morn	grandiflora	large	Sakata Seed Co.
Carnival Red Star	grandiflora	large	Takii Seed Co.
Carnival Brush Pink	grandiflora	large	Takii Seed Co.
Supercascade Blue	grandiflora	large	M&B Flora Co., Ltd.
Carnival Salmon	grandiflora	large	Takii Seed Co.
Dream White	grandiflora	large	M&B Flora Co., Ltd.

^z Cultivars indicated by asterisk were used for detailed analysis.

^y Corolla diameters of small-, medium-, and large-flowered cultivars were 33.8–43.3 mm, 57.1–65.2 mm, and 80.9–86.7 mm (average of 10 flowers per cultivar) under our growth conditions.

‘Mitchell’. BC₄ plants with *gg* and *Gg* genotypes were used for the experiment.

We planted single seedlings in plastic pots (15 cm in diameter, 18 cm in height) filled with a horticultural medium (Kureha-Engei-Baido, Kureha Chemical Industry Co. Ltd., Tokyo, Japan). Plants were grown in an incubator set at 22.5°C with a 16-h photoperiod provided by fluorescent lamps (150 µmol·m⁻²·s⁻¹ PPFD).

Corollas at the following growth stages were used for the analysis of gene expression: stage 1, early cell proliferation stage; stage 2, late cell proliferation stage; stage 3, cell expansion stage; and stage 4, flower opening. The growth of the corolla is attributed mainly to cell proliferation in stages 1 and 2, while it is solely attributed to cell expansion in stage 3. Corolla lengths at stages 1, 2, and 3 were 4.5–5.5, 7.5–8.5, and 24–28 mm, respectively. Excised corollas were immediately frozen in liquid nitrogen and stored at –80°C until analysis. Stems, and young and mature leaves of BC₄ plants were also used for analysis. Length of young leaves was 5–7 mm, while that of mature leaves was more than 30 mm.

Molecular cloning of genes involved in CK phosphorelay signaling circuitry

Total RNA was obtained from young corollas of petunia (*P. hybrida* cv. ‘Baccara White’ and ‘Fulcon Midblue’) using an RNeasy Plant Mini Kit (QIAGEN K. K., Tokyo, Japan). cDNA was synthesized using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche Applied Science, Indianapolis, USA). Degenerate primers corresponding to the amino acid sequences HVLAVDD (forward 5'-CAYGTIYTIGCIGTIGAYG AY-3') and (E/Q)AGEELC (reverse 5'-YTSIGCICCYTC YTCIARRCA-3') were used to isolate partial sequences of type-A RR (*PhRR*) cDNAs. To isolate partial sequences of CK receptor *HK* (*PhHK*) cDNAs, two sets of degenerate primers corresponding to the amino acid sequences T(A/S/T)FERPL (forward 5'-ACIDVITYTG ARMGICCIYT-3')/MPTRIEHS (reverse 5'-CATIGGIG TICKDATYTCTRG-3') for the first PCR and YAPVIF(A/S)Q (forward 5'-GARTAYGCICCI GTIATH TWYDS-3')/TALFQSKA (reverse 5'-GTIGCIARRAA YTGISWYTTIGC-3') for the second PCR were used as nested PCR. 5'- and 3'-RACE-PCR (Frohman et al., 1988) were performed using the CapFishing™ Full-length cDNA Premix Kit (Seegene, Seoul, South Korea) based on the partial cDNA sequences. Full-length cDNA was obtained by PCR using the following primers: forward 5'-ACATACACATTACACACACTCCTCTCTT-3' and reverse 5'-TGTGATTAAACAATATCATCGATG TGTT-3' for *PhRR1*, forward 5'-ATAAGCACAACCCC ATGTTCTAAG-3' and reverse 5'-TCTAGGCCCTCTT AGATTAGCAA-3' for *PhRR2*, and forward 5'-CTTTAC ACTTTTCATTCTTGATTCTTCTT-3', reverse 5'-CAGG CATTGCTTGATCTACACAATA-3' for *PhRR3*, and forward 5'-AGAATAGTGAGAAGTGTTAGTAGTGAT GGG-3' and reverse 5'-TGGACCTCTCCTCCCCCTTCT

RTAT-3' for *PhHK*. Phylogenetic analysis of the deduced protein was conducted by the neighbor-joining method (Saitou and Nei, 1987).

Expression analysis of genes involved in CK phosphorelay signaling circuitry

To investigate *PhRRs* and *PhHK* expression levels in cultivars with different corolla sizes, cDNAs were obtained from corollas at stages 1–4, as defined above. To investigate the effect of CK application on *PhRRs* and *PhHK* expressions, 30 µmol·L⁻¹ *N*-benzylaminopurine (BA) was dissolved in 20% aqueous acetone containing 0.05% Triton X-100 (Wako Pure Chemical, Osaka, Japan). Corollas at stage 2 were treated with 4 µL/corolla of BA solution. cDNAs were obtained from the corollas 0–48 h after BA application. For total RNA extraction for cDNA synthesis, we treated the samples with DNase to avoid potential contamination with genomic DNA.

Primers were designed to amplify 107–259 bp fragments of the 3' regions of the cDNAs. Primer sequences used for the quantitative real-time PCR reaction were as follows: forward 5'-CCCTCGCCATCA CTTGACCT-3' and reverse 5'-GATTGAGGGGGTTTG AGAACC-3' for *PhRR1*, forward 5'-AAGAAGAAGTA GCTGAGAAAATAGTCACAG-3' and reverse 5'-TCAT TAAGTCAAATAGCAGTAAGGCC-3' for *PhRR2*, forward 5'-GAGCACTCCACAAATGGCAA-3' and reverse 5'-CGGCATTGATTAGTTGACATCGT-3' for *PhRR3*, and forward 5'-AAATGGCGTAAATGGCATAT GC-3' and reverse 5'-TGAGAGCAGCTGAATTGGTAG AATC-3' for *PhHK*. The primers used for expression analysis of the internal standard *PhUBQ* were as described previously (Nishijima et al., 2011). Gene expression was quantified using a quantitative real-time PCR machine (LightCycler, Roche Diagnostics, Mannheim, Germany) and SYBR Premix Ex Taq (TakaraBio, Ohtsu, Japan). The reactions were cycled with a preincubation step of 10 min at 95°C followed by 50 cycles of 5 s at 95°C, 10 s at 55°C, and 20 s at 72°C, each. The raw data were analyzed with LightCycler software version 3.5 (Roche Diagnostics). The plasmids (pGEM-T Easy vector, Promega, Madison, USA) harboring cDNAs of *PhRR1*, *PhRR2*, *PhRR3*, *PhHK*, and *PhUBQ* were used to obtain standard curves. The ratio of the expression of each gene to that of *PhUBQ* was calculated. Expression analysis was conducted independently in triplicate.

Results and Discussion

Isolation of genes encoding type-A RR and CK receptor HK and their response to CK

Three full-length cDNAs of type-A RR-like gene (*PhRR1*, AB610787; *PhRR2*, AB610788; *PhRR3*, AB610789) and a CK receptor *HK*-like gene (*PhHK*, AB610790) were isolated from young corollas of petunia. The deduced PhRR proteins had a structure typical of type-A RR that consisted of a receiver domain

and short C-terminal sequence (Brandstatter and Kieber, 1998; Taniguchi et al., 1998). The receiver domain had two critically conserved aspartates and a lysine (i.e., D-32, D-86, and K-137 in PhRR1; D-17, D-80, and K-132 in PhRR2; D-40, D-94, and K-146 in PhRR3) (Imamura et al., 1999). The phylogenetic relationship between PhRRs and *Arabidopsis* type-A RRs (ARRs) is shown in Figure 1A. PhRR1 belonged to the same clade as ARR3 and ARR4. PhRR2 belonged to the same clade as ARR8 and ARR9. The deduced amino acid sequence of PhRR3 was identical to that of ARR3, although the nucleotide sequence of *PhRR3* was substantially different from that of *ARR3*.

The deduced PhHK proteins had a structure typical of CK receptor HK that was composed of the following

Fig. 1. Phylogenetic relationships of type-A response regulator (A) and histidine protein kinase (B) in petunia and *Arabidopsis*. Medium-flowered 'Baccara White' and large-flowered 'Fulcon Midblue' (Table 1) were used for isolation of petunia full-length cDNA. PhRRs, petunia type-A response regulators; ARRs, *Arabidopsis* type-A response regulators (ARR3, At1g59940; ARR4, At1g104700; ARR5, At3g48100; ARR6, At5g62920; ARR7, At1g19050; ARR8, At2g41310; ARR9, At3g57040; ARR15, At1g74890; ARR16, At2g40670; ARR17, At3g56380); PhHK, petunia cytokinin receptor histidine protein kinase; AHK2-4 (At5g35750, At1g27320, At2g01830, respectively), *Arabidopsis* cytokinin receptor histidine protein kinases. AtHK1 (At2g17820), CKI1 (At2g47430), AHK5 (At5g10720), EIN4 (At3g04580), ERS1/2 (At2g40940, At1g04310, respectively), and ETR1/2 (At1g66340, At3g23150, respectively) are *Arabidopsis* histidine protein kinases with functions other than cytokinin receptors. Gray arrows indicate petunia proteins. Values in parentheses indicate P-distance.

domains sequentially from the N-terminal: hydrophobic membrane-spanning domain, CHASE domain, hydrophobic membrane-spanning domain, histidine kinase domain, receiver-like domain, and receiver domain (Ueguchi et al., 2001). The phylogenetic relationship among PhHK, *Arabidopsis* CK receptor HKs, and other HKs is shown in Figure 1B. PhHK belonged to the same clade as *Arabidopsis* CK receptor HKs (i.e., AHK2-4), and was different from HKs with other functions. PhHK had the closest relationship with AHK4 among the three *Arabidopsis* CK receptor HKs. Deduced amino acid sequences unique to large-flowered cultivars were not detected in PhRR1-3 and PhHK.

Expression of *PhRR1-3* was upregulated within 1 h of BA application and then gradually decreased (Fig. 2). Expression of *PhHK* was also upregulated within 1 h of BA application, and the upregulation continued for at least 24 h after BA application. These rapid upregulations of type-A RRs and CK receptor HK are common in *Arabidopsis* type-A RRs (Brandstatter and Kieber, 1998; Taniguchi et al., 1998) and a CK receptor HK, *AHK4* (Kiba et al., 2004; Rashotte et al., 2003). The structural similarity to the corresponding proteins in *Arabidopsis* and the rapid response to CK suggest that *PhRR1-3* and *PhHK* function as type-A RRs and CK receptor HK, respectively.

Expression analysis of type-A RR and CK receptor HK genes among cultivars and developmental stages

Expressions of *PhRR1-3* and *PhHK* were not markedly different between small- and medium-flowered gg cultivars throughout corolla development (Fig. 3); however, expression levels of *PhRR1* and *PhRR2* were conspicuously higher in large-flowered Gg cultivars than in small- and medium-flowered gg cultivars. These high

Fig. 2. Effect of application of *N*⁶-benzylaminopurine (BA) on the expression of genes encoding type-A response regulator and cytokinin receptor histidine protein kinase. *PhRRs*, petunia type-A response regulator genes; *PhHK*, petunia cytokinin receptor histidine protein kinase gene. Symbols indicate control (●) and BA-treated (○) corollas. Vertical bars represent $\pm$ SE ($n=3$).

Fig. 3. Expression of genes encoding type-A response regulator and cytokinin receptor histidine protein kinase during corolla development in small-, medium-, and large-flowered cultivars. *PhRRs*, petunia type-A response regulator genes; *PhHK*, petunia cytokinin receptor histidine protein kinase gene. Developmental stage of corolla: stage 1, early cell proliferation stage; stage 2, late cell proliferation stage; stage 3, cell expansion stage; stage 4, flower opening. Dark gray column, small-flowered cultivars (left, 'Fantasy Ivory'; right, 'Fantasy Blue'); light gray column, medium-flowered cultivars (left, 'Baccara White'; right, 'Baccara Blue Picotee'); open column, large-flowered cultivars (left, 'Fulcon White'; right, 'Fulcon Midblue'). Vertical bars represent $\pm$ SE ($n=3$).

expression levels, unique to large-flowered *Gg* cultivars, were marked at stages 1 and 2, and persisted until stage 3, while they were not obvious at stage 4. Expression level of *PhRR3* was also higher in large-flowered *Gg* cultivars than in small- and medium-flowered *gg* cultivars in stages 1 and 2, while this high expression level was not obvious in stages 3 and 4. Large-flowered *Gg* cultivars also exhibited a higher expression level of *PhHK* than small- and medium-flowered *gg* cultivars in stages 1–3, while it was not obvious in stage 4. Therefore, *Gg*-cultivar-specific high expression of type-A *RRs* and CK receptor *HK* was conspicuous in the cell proliferation stages. This high expression continued until the cell expansion stage, although there was an exception (i.e., expression of *PhRR3* in this stage).

To examine whether the high expression levels of these genes is a common phenomenon among large-flowered *Gg* cultivars, we also analyzed the corollas of

Fig. 4. Expression of genes encoding type-A response regulator and cytokinin receptor histidine protein kinase in corollas of various medium- and large-flowered cultivars. Corollas at stage 2, late cell proliferation stage, were used. *PhRRs*, petunia type-A response regulator genes; *PhHK*, petunia cytokinin receptor histidine protein kinase gene. Gray column, medium-flowered cultivars (1, 'Pearl White'; 2, 'Pearl Sky Blue'; 3, 'Baccara Rose Morn'; 4, 'Carpet Pink'; 5, 'Rondo Pink'; 6, 'Rondo Violet Imp'); open column, large-flowered cultivars (7, 'Fulcon Pink Morn'; 8, 'Carnival Red Star'; 9, 'Carnival Brush Pink'; 10, 'Supercascade Blue'; 11, 'Carnival Salmon'; 12, 'Dream White'). Vertical bars represent $\pm$ SE ($n=3$).

12 other cultivars at stage 2 (Fig. 4). In several large-flowered *Gg* cultivars, some *PhRRs* did not show marked higher expression than in medium-flowered *gg* cultivars (i.e., *PhRR1* and *PhRR3* in 'Supercascade Blue', *PhRR2* in 'Carnival Salmon', and *PhRR3* in 'Carnival Red Star'); however, at least one of *PhRR1-3* had markedly higher expression levels in each of the large-flowered *Gg* cultivars than in the medium-flowered *gg* cultivars. In *Arabidopsis*, 10 type-A *RRs* function in partial redundancy as negative regulators of CK signaling (Imamura et al., 1999; To et al., 2004), suggesting that *PhRR1-3* also functions redundantly. Even if the expression of a certain type-A *RR* is inhibited by mutation, other type-A *RRs* will compensate for the function, as shown by To et al. (2004). Thus, high expression of type-A *RRs* is probably a common phenomenon among large-flowered *Gg* cultivars; nevertheless, some type-A *RRs* have lost the response to the large-flowered genotype, possibly through mutation. On the other hand, expression of *PhHK* was consistently higher in large-

Fig. 5. Expression of genes encoding type-A response regulator and cytokinin receptor histidine protein kinase in corolla of 'Mitchell' BC₄ plants with *gg* (gray column) and *Gg* (open column) genotypes. Corollas at stage 2, late cell proliferation stage, were used. *PhRRs*, petunia type-A response regulator genes; *PhHK*, petunia cytokinin receptor histidine protein kinase gene. Vertical bars represent $\pm$ SE ($n=3$).

flowered *Gg* cultivars than in medium-flowered *gg* cultivars, suggesting that high expression of *PhHK* is also a common phenomenon among large-flowered *Gg* cultivars.

To confirm that high expressions of *PhRRs* and *PhHK* are caused by the *Gg* genotype, we performed expression analysis employing BC₄ plants in which the genetic backgrounds except for the *G* allele were mostly as same as that of a doubled haploid line 'Mitchell'. All of the 9 BC₄ plants with *Gg* genotype exhibited higher expression levels of *PhRR1*, *PhRR2*, and *PhHK* than the 9 plants with *gg* genotype (Fig. 5). The expression level of *PhRR3*, which was much lower than *PhRR1* and *PhRR2*, was also conspicuously higher in most *Gg* plants than in *gg* plants, although some *Gg* plants had about the same expression levels as those of *gg* plants. These results strongly suggest that high expression levels of type-A *RRs* and CK receptor *HK* are induced by the *G* allele.

Expression levels of *PhRR1-3* and *PhHK* in each organ were compared between BC₄ plants with *Gg* and *gg* genotypes. Higher expression levels of *PhRR1-3* and *PhHK* in *Gg* than in *gg* plants were observed in the sepals and stamens (Fig. 6). These genes, except for

Fig. 6. Expression of genes encoding type-A response regulator and cytokinin receptor histidine protein kinase in various organs of 'Mitchell' BC₄ plants with *gg* (gray column) and *Gg* (open column) genotypes. *PhRRs*, petunia type-A response regulator genes; *PhHK*, petunia cytokinin receptor histidine protein kinase gene. YL, young leaf; ML, mature leaf; S, stem; Se, sepal; St, stamen; C, carpel. The floral organs were collected from the flower bud in stage 2. Vertical bars represent $\pm$ SE ($n=3$).

PhRR1, were also promoted by the *Gg* genotype in the carpels. In contrast, ambiguous results were obtained for their expression in leaves and stems. In leaves, the expression of *PhRR1* was higher in *gg* than in *Gg* plants, although the *Gg* genotype promoted *PhHK* expression. In stems, the expression levels of *PhRR1-3* and *PhHK* were not markedly different between *gg* and *Gg* plants. Thus, the *G*-allele-induced high expression of type-A *RR* and CK receptor *HK* was consistent in floral organs, but to some extent, it was more ambiguous in vegetative organs.

Accumulation of type-A *RR* serves as a negative regulator of CK signaling in the phosphorelay circuitry (Rashotte et al., 2003). In contrast, the effects of a high expression of CK receptor *HK* have not been well documented. *HK* is activated by phosphorylation of the receiver domain by the *HK* domain (Stock et al., 2000). The *HK* and receiver domains also have phosphatase activity that dephosphorylate the receiver domain and inactivate *HK* (Stock et al., 2000). Owing to these contradictory actions, the possibility that a high expression level of *PhHK* induces the enhanced CK response and enlarge corolla can not be ruled out; therefore, we constructed transgenic plants overexpress-

ing *PhHK* under the control of CaMV 35S promoter. However, overexpression of *PhHK* did not induce the enlargement of any organs, including the corolla (Nishijima et al., unpublished data), suggesting that a high expression level of *PhHK* is not the cause of the large-flowered phenotype induced by the *G* allele.

Role of the G-allele-induced promotion of type-A RR and CK receptor HK genes in corolla enlargement

The effects of CK application and the *G* allele on the CK phosphorelay signaling circuitry and corolla enlargement are summarized in Figure 7. The *G*-allele-induced high expression of type-A RRs in the petunia corolla probably represses the CK response in CK signaling as described above, although the function of the high expression of *PhHK* remains unclear. The *G* allele also decreased endogenous CK concentration in the corolla through enhanced expression of CKX genes (Nishijima et al., 2011). These changes in CK biosynthesis and the phosphorelay signaling circuitry resemble the changes induced by the CK response (Kiba et al., 2005). Thus, it can be assumed that the *G* gene has a mutation that promotes the CK response (Fig. 7, arrow 1). Although the mutation might be present in the CK phosphorelay signaling circuitry, *PhHK* may not be the causal gene because overexpression of *PhHK* had no effect on corolla enlargement, as mentioned above. It is also possible that the mutation is present in the factor promoting the CK response in CK signaling *in trans*, as shown in the overexpression of *Arabidopsis CKII* (Kakimoto, 1996).

On the other hand, the concept that the *G* allele enlarges the corolla independently of the CK phospho-

relay signaling circuitry cannot be excluded (Fig. 7, arrow 2). This is because the repression of CK signaling possibly occurs as negative feedback from the hypothesized systemic checkpoint of the organ size that senses excess organ enlargement (Weiss et al., 2005). In either case, it is clear that the *G* allele causes feedback regulation of the CK phosphorelay circuitry to partially repress CK signaling through promotion of type-A RRs.

The *G* allele induces large and thick vegetative organs (i.e., leaf and stem) as well as large and thick floral organs (i.e., sepal and corolla) (Ewart, 1984), however, the *G*-allele-induced high expression levels of *PhRRs* and *PhHK* were more consistent in floral organs than in vegetative organs (Fig. 5). This result may have different implications depending on the assumptions described above. Based on the former assumption that the *G* allele promotes a CK response, the floral organ-preferable high expression of *PhRRs* and *PhHK* might reflect the greater CK response in floral organs than in vegetative organs. This presumption corresponds with the observation that *G* allele enlarges floral organs like sepals and corolla to a greater extent than leaves.

In contrast, based on the latter assumption that *G* allele induces organ enlargement independently of CK phosphorelay signaling circuitry and high expression of *PhRRs* and *PhHK* is caused by feedback regulation from the systemic organ size checkpoint, the floral organ-preferable high expression of *PhRRs* and *PhHK* might reflect strict feedback regulation of floral organ size. In wild petunia species, including *P. axillaries* and *P. integrifolia*, which are ancestors of *P. hybrida*, the flower size and shape are critical evolutionary traits for adaptation to specific pollinators (Ando et al., 2001; Galliot and Hoballah, 2006); therefore, “incorrect” flower size causes severe disadvantage in propagation. This evolutionary background might have conferred the strict regulation of flower size.

Potential modification of CK phosphorelay signaling in petunia breeding

The *G* gene is the only major gene inducing large-flowered phenotype in petunia (Ewart, 1984). Although this gene effectively induces a large-flowered phenotype, it has several pleiotropic effects, as described above. To establish larger-flowered phenotypes of petunia, it is necessary to find another large-flower mutation or a modification mutant that enhances the *G*-allele-induced large-flowered phenotype. As a modification mutation, the repression of *G*-allele-induced feedback regulation of CK biosynthesis and phosphorelay signaling may be effective, because this feedback regulation represses the CK response, which induces the large-flowered phenotype (Nishijima et al., 2006). A possible way to repress the feedback regulation of CK biosynthesis was described previously (Nishijima et al., 2011). To repress the feedback regulation of the CK phosphorelay signaling circuitry, the presence of type-A RRs that have

Fig. 7. Cytokinin (CK) phosphorelay signaling circuitry regulated by *Grandiflora* and cytokinin application in petunia corolla. HK, histidine protein kinase; PhHK, petunia CK receptor HK; HP, histidine phosphotransfer protein; RR, response regulator; PhRR, petunia type-A RR. Black arrows indicate promotion (→) and inhibition (—|) of signaling mediated by direct molecular interaction. Open arrows indicate promotion of gene expression. Gray arrows indicated by 1 and 2 show hypothetical pathways regulating CK phosphorelay signaling circuitry by *Grandiflora*. Note that target of the pathway 1 is not either certain protein or gene but CK phosphorelay signaling circuitry. The illustration of the CK signaling phosphorelay circuitry is based on Oka (2005) and Mizuno (2005).

lost their response to the *G* allele (Fig. 4) will be key tools. In *Arabidopsis*, a reduction in type-A RR function enhanced the CK response (To et al., 2004). Since there are 10 type-A RRs in *Arabidopsis* with partial functional redundancy (Imamura et al., 1999), accumulation of multiple mutant genes was necessary to enhance the CK response (To et al., 2004). Type-A RRs, other than *PhRR1-3*, are likely to be present in petunia. Analysis of all of the type-A RRs expressed in the corolla may enable us to find and accumulate type-A RRs which are unresponsive to the *G* allele. Large-flowered cultivars actually have the potential for further corolla enlargement through increased CK response, because CK application further enlarges the corolla of large-flowered cultivars with *Gg* genotype (Nishijima et al., 2006).

Acknowledgements

We thank Mrs. T. Kurobe for the technical assistance.

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