Gibberellin is not Associated with the Enhancing Effect of 2,4-Pyridinedicarboxylic Acid on Flower Opening of ‘Light Pink Barbara’ Carnation

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2,4-Pyridinedicarboxylic acid (2,4-PDCA) extends the vase life of cut flowers of spray-type carnations by accelerating flower opening as well as retarding senescence. Since 2,4-PDCA can inhibit 2-oxoglutarate-dependent dioxygenases, which include enzymes for gibberellin (GA) biosynthesis and catabolism, we hypothesized that GA might be involved in the enhancing effect of 2,4-PDCA on the flower opening of carnation. In this study, we tested this possibility by examining the changes in gene expression of DELLA protein (GAI), a negative regulator of GA signaling, and GA levels in carnation (*Dianthus caryophyllus* L. ‘Light Pink Barbara (LPB)’) flowers treated with 2,4-PDCA. We also analyzed the expression of cell expansion-related genes, xyloglucan endotransglucosylase/hydrolase (XTH), and expansin genes as markers of flower opening in the treated flowers. The transcript level of GAI gene was increased, whereas that of expansin was decreased, in petals of the 2,4-PDCA-treated flowers compared to those of the control, which was contrary to the enhancement of flower opening. Our results suggest that the changes in the expression of these genes are not associated with the enhancing effects of 2,4-PDCA. In addition, GA₃ content tended to be decreased by 2,4-PDCA treatment in the petals of opening flowers. Flower opening was not accelerated, but rather delayed, by treatment of flower buds with exogenous GA₃ and not affected by paclobutrazol, an inhibitor of GA biosynthesis, in ‘LPB’ carnation. These results suggest that endogenous GA is not associated with the enhancement of flower opening by 2,4-PDCA in carnation.

Key Words: DELLA protein, *Dianthus caryophyllus*, GAI gene, paclobutrazol, petal cell expansion.

Introduction

Carnation is a popular and economically important cut flower in the floriculture industry in many countries. During the senescence of carnation flowers, a climacteric increase in ethylene production occurs, resulting in in-rolling of petals and wilting of whole flowers (Abeles et al., 1992; Satoh, 2011). The effect of ethylene during senescence can be suppressed by treatment with inhibitors of ethylene biosynthesis or action

(as discussed in Satoh et al., 2014). These inhibitors, such as silverthiosulfate (STS), retard the senescence and prolong the vase life of cut carnation flowers.

2,4-Pyridinedicarboxylic acid (2,4-PDCA) is a structural analog of 2-oxoglutarate (OxoGA) and acts as a competitive inhibitor of OxoGA-dependent dioxygenases (Kivirikko and Myllyharju, 1998; Kivirikko and Pihlajaniemi, 1998; Vlad et al., 2010). 2,4-PDCA also inhibits 1-aminocyclopropane-1-carboxylate (ACC) oxidase from tomato and carnation (Fragkostefanakis et al., 2014; Satoh et al., 2014). The inhibition of ACC oxidase is supposed to be caused by a competition of 2,4-PDCA with ascorbate, a co-substrate of the enzyme. Treatment of carnation flowers with 2,4-PDCA results in the suppression of ethylene production and senescence as revealed by Vlad et al. (2010) in ‘White Sim’

Received; April 20, 2016. Accepted; December 22, 2016.

First Published Online in J-STAGE on February 22, 2017.

This study was supported financially by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS KAKENHI Grant no. 24580050 to S. Satoh).

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carnation. Also, we showed recently that 2,4-PDCA treatment delays senescence and prolongs the vase life of cut flowers of 'Light Pink Barbara (LPB)' and 'Mule', both of which are spray-type carnations (Satoh et al., 2014). In addition, 2,4-PDCA has enhancing effects on flower opening in 'LPB' as well as many cultivars of spray-type carnation (Sugiyama and Satoh, 2015; Satoh et al., 2016). The treatment with 2,4-PDCA leads to acceleration of flower opening and an increase in the total number of open flowers (Sugiyama and Satoh, 2015; Sugiyama et al., 2015). To our knowledge, this is the first example that a single chemical has both the activities to promote flower opening and to retard senescence in carnation flowers. 2,4-PDCA is a promising candidate compound for application as a flower care agent.

As mentioned above, 2,4-PDCA inhibits ethylene biosynthesis during senescence in carnation flowers (Vlad et al., 2010). However, it is unlikely that 2,4-PDCA affects ethylene production in opening flowers since ethylene production is under the detectable level during the flower opening process, which is only detected at the onset of senescence after the fully-open stage (Satoh et al., 2014). Also, we observed STS treatment caused no significant effects on flower opening in several carnation cultivars (Nomura and Satoh, unpublished data). These facts suggest that ethylene does not play a role in the flower opening process in carnation. Considering the action of 2,4-PDCA, inhibition of OxoGA-dependent dioxygenases might affect gibberellin (GA) levels in cells. OxoGA-dependent dioxygenases include the enzymes involved in GA biosynthesis and metabolism, such as GA 3 β -dioxygenase (GA 3 β -hydroxylase), GA-44 dioxygenase, and GA 2 β -dioxygenase (GA 2 β -hydroxylase) (Hedden and Kamiya, 1997; Lange et al., 1994a, b; Smith and MacMillan, 1983, 1986), which suggests a possibility that 2,4-PDCA treatment results in the changes in active GA levels. GA is suggested to be involved in the flower opening process of carnation since treatment of carnation buds with paclobutrazol (PBZ), an inhibitor of GA biosynthesis, prevented full opening of the buds (Saks et al., 1992). Therefore, we hypothesized the association of GA with the enhancing effects of 2,4-PDCA on flower opening in carnation flowers.

In order to examine the above-mentioned possibility, we investigated whether 2,4-PDCA treatment led to the changes in GA levels in carnation flowers in this study. Also, we analyzed the influences of 2,4-PDCA on the GA signaling pathway since alteration in GA levels should affect the GA signaling pathway to exert its action. For this purpose, we selected a DELLA protein, a negative regulator of the GA signaling pathway, for investigation. The DELLA proteins encoded by *GAI* (*GA-INSENSITIVE*) gene and its homologs are members of GRAS transcription factor (Pysh et al., 1999) and act as growth suppressors (Harberd et al., 2009). In opening

flowers of rose, transcriptional regulation of a *DELLA* gene, *RhGAIL*, is involved in the regulation of petal cell expansion (Luo et al., 2013). Therefore, we characterized the *GAI* (*DcGAI*) gene in carnation and analyzed its expression in 2,4-PDCA-treated flowers. In addition, we analyzed the expression of cell expansion-related genes as markers of the flower opening process. These genes encode proteins involved in cell wall expansion, such as xyloglucan endotransglucosylase/hydrolase (XTH) and expansin, both of which are expressed abundantly in opening flowers of carnation (Harada et al., 2011). Through these analyses, we examined the possibility of the involvement of GA with PDCA action in carnation.

Materials and Methods

Plant materials and chemical treatment

Cut carnation flowers (*Dianthus caryophyllus* L. 'LPB') that belong to the spray category of carnation flower were harvested when the first flower out of 6 to 8 flower buds was nearly open on a stem at the nursery of commercial growers. The flowers were transported without supply of water to the Biotechnology Research Department, Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center in Kyoto Prefecture or Faculty of Agriculture, Ryukoku University in Shiga Prefecture on the day after harvest. After arrival, they were put in plastic containers with their cut end in tap water under continuous light from white fluorescent lamps (14 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 23°C.

The flower opening process was separated into 6 opening stages (Os) and senescence process into 4 senescence stages (Ss) according to Harada et al. (2010) and Morita et al. (2011), respectively. Ten outermost petals per flower were sampled at the respective stages, and stored at -80°C for extraction of RNA. Other floral tissues, including calyx, style, receptacle, and ovary, were sampled from plants with fully opened flowers and stored as above. In carnation flowers, the stigma is along the surface of the style (rather than a distinct structure like in other flowers). Therefore, the term 'style' was used to describe the style plus stigma in this study. Three independent samples of each tissue were collected at each stage.

Carnation flowers at Os1 to Os2 were used for 2,4-PDCA treatment. For analysis of gene expression, three samples (bunches) of 5 flower stalks (trimmed to 60-cm length), with 28–32 flower buds in total per sample were put in 50-mL plastic tubes with 30 mL test solution (one sample per plastic tube). For measurement of GA content, triplicated samples of 8–9 flower stalks (trimmed to 60-cm length), with 15–27 buds per sample were put in 0.9-L glass jars with 300 mL of test solution (one sample per glass jar). The test solution contained 2 mM 2,4-PDCA in distilled water. The flowers were left under continuous light from white fluorescent lamps (14 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 23°C and 50–70% relative

humidity. Flower opening was scored daily by determining the flower opening stage for each flower. Flowers at Os4 to Os6 were regarded as flowers in vase life, and the ratio of these flowers to the total number of flowers per sample was calculated. Petals were sampled from the plants and stored at -80°C until use.

GA_3 and PBZ treatment was performed by a similar procedure to 2,4-PDCA treatment with modifications. Three samples of 5 flower stalks (trimmed to 60-cm length), each with 5 flower buds (25 buds in total per sample) were put in 0.9-L glass jars with 300 mL of test solution. The test solutions consisted of GA_3 or PBZ at 0 (control), 10, 30, and 100 μM dissolved in distilled water, and 100 $\text{mg}\cdot\text{L}^{-1}$ 8-hydroxyquinoline sulfate (8-HQS) was added to the solutions as a germicide. The flowers were kept under the same conditions as 2,4-PDCA treatment for 24 days. During this period the test solutions were replenished as necessary. Fully-open and non-senescent (not wilted and turgid) flowers at Os6 to Ss2 were counted daily and the percentage of these flowers to the total number of initial flower buds per sample was calculated.

Cloning of cDNA for carnation *GAI*

The amino acid sequences of five *DELLA* genes from *Arabidopsis* (*AtGAI*, *AtRGA*, and *AtRGL1-AtRGL3*; Harberd et al., 2009) were retrieved from The Arabidopsis Information Resource (TAIR) database (TAIR10; Lamesch et al., 2012; <http://www.arabidopsis.org>). Carnation open reading frames (ORFs) homologous to the *Arabidopsis* DELLAs were searched for by BLAST (Altschul et al., 1997) in the carnation genome sequence database (Carnation DB; Yagi et al., 2014; <http://carnation.kazusa.or.jp/index.html>). A primer set (see Table 1) was designed in the 5'- and 3'-ends of an ORF (Dca34377.1) found in the Carnation DB and used for amplification of the coding region of the ORF.

Total RNA was prepared from 2 g of frozen petals

according to Harada et al. (2005). Single strand cDNA was synthesized from the total RNA with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) using an oligo dT primer. A cDNA covering the entire sequence of the carnation *GAI* ORF was amplified by polymerase chain reaction (PCR) using a high-fidelity DNA polymerase, KOD plus (Toyobo). The amplified fragments were cloned into the pGEM-T vector (Promega, Madison, WI, USA) after A-tailing with Taq polymerase (Toyobo). The sequence of the cDNA from 'LPB' (*DcGAI*-LPB) was confirmed by sequencing on both strands. Multiple alignment of *DcGAI*-LPB and *DELLA* proteins from *Arabidopsis* and rice was performed by ClustalW (Larkin et al., 2007) on the website of the DDBJ (<http://clustalw.ddbj.nig.ac.jp>) with a default setting (ver 2.1). The sequence of *DcGAI*-LPB was deposited in the DDBJ, EMBL, and NCBI sequence databases under accession no. LC148053.

In order to search for *GAI* homologs further in the carnation genome, genomic sequences (scaffold sequences) were also searched for by BLASTN using the nucleotide sequence of *DcGAI*-LPB as a query in the Carnation DB. The sequences of scaffolds found by the search and that of Dca34377.1 were compared with GENETYX software (GENETYX, Tokyo, Japan). Database searches for *GAI* homologs in poplar (*Populus trichocarpa*), grape (*Vitis vinifera*), tomato, and soybean were performed in the Ensembl Genomes database (Release 30; Kersey et al., 2016; <http://ensemblgenomes.org/>).

Expression analysis by qRT-PCR

Total RNA was extracted from frozen samples and cDNA synthesis was performed as described above. To analyze the transcript levels of *DcGAI*, *DcXTH2*, and *DcEXPA2* genes, primer sets (listed in Table 1) were designed for each gene. Partial cDNA fragments of respective genes were amplified from petal cDNAs by PCR using the primer sets. They were cloned into the

Table 1. Primers used for PCR cloning and RT-PCR.

Purpose and target	Primer name	Sequence (5' to 3')
Full length cDNA amplification		
<i>DcGAI</i>	DcGAI-F3	ATGAAGAGAGAACATCCGTTGG
	DcGAI-R3	TCAGCACCGAGTTTTTTTACTG
qRT-PCR for expression analysis		
<i>DcGAI</i>	DcGAI-F1	AGACGTGCGAGAACAACGAA
	DcGAI-R1	TGCTCTTCCACACCGTAACC
<i>DcXTH2</i>	DcXTH2-F1	GTTCCAGGTCGAGATCGAA
	DcXTH2-R1	GGTTGTACAGTACAGTACGTAG
<i>DcEXPA2</i>	DcEXPA2-F4	CGTCCATGCCGTGTCAATT
	DcEXPA2-R3	CTGCCAGTTACTAGGAGCAA
<i>DcUbq3-7</i>	Ubq3F ^z	GTTGTTGGTTTCAGGGCTGGTTTG
	Ubq3R ^z	CTACGGTAATTGAGAATTCACACCGAAATG

^z These primers were designed by Nomura et al. (2012).

pGEM-T vector (Promega), and the resulting plasmids were used as a standard for quantification. Quantitative reverse transcription-PCR (qRT-PCR) was performed with a Thermal Cycler Dice Real Time System *Lite* (TaKaRa Bio Inc, Otsu, Japan) real-time PCR cycler using the KOD SYBR qPCR Mix (Toyobo) following the manufacturer's instructions. The transcript level was calculated using a dilution series of standard plasmids. The transcript level of ubiquitin genes (*DcUbg3* to *DcUbg7*; Nomura et al., 2012) was also measured and used for normalization of the transcript levels of *DcGAI*, *DcXTH2*, and *DcEXPA2*.

Measurement of GA₃ content by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Extraction, purification, and assay of GA₃ were performed according to Chiwocha et al. (2003), with modifications. Frozen samples (0.1–0.15 g FW) were ground in liquid N₂, and GA was extracted with 4 mL of isopropanol/acetic acid (99:1). An internal standard of 100 pmol of [²H₂]-GA₁ (OlChemIm Ltd., Olomouc, Czech Republic) was added to the extract, and the homogenate was clarified with centrifugation at 20000 × g for 10 min at 4°C. Extraction was performed again by resuspending the pellet with 1.8 mL of isopropanol/acetic acid (99:1). The extract was passed through a Sep-Pak tC18 Plus cartridge (400 mg sorbent; Waters Co., Milford, MA, USA), which was equilibrated with 4 mL of methanol followed by 4 mL of isopropanol/acetic acid (99:1), and dried with SpeedVac (Thermo Fisher Scientific K.K., Yokohama, Japan). The samples were dissolved in 200 µL of methanol, and passed through a 0.2 µm-membrane filter. GA₃ analysis was performed with a liquid chromatograph-tandem mass spectrometer (LCMS-8050; Shimadzu, Kyoto, Japan) equipped with an Inertsil ODS-3 column (3 µm, 2.1 mm i.d. × 150 mm, GL Sciences Inc., Tokyo, Japan). Samples were separated by a linear gradient of acetonitrile concentration from 1 to 22% over 5 min then to 45% over 10 min in the presence of 0.04% acetic acid at a flow rate of 0.25 mL·min⁻¹. GA₃ and [²H₂]-GA₁ was detected and quantitated by multiple reaction monitoring (MRM) in negative ion mode. Precursor ions and their product ions for GA₃ were *m/z* 345.20 > 239.20, and for [²H₂]-GA₁ *m/z* 349.20 > 261.25.

Results and Discussion

Cloning and characterization of GAI homolog in carnation

In the Carnation DB, an ORF (Dca34377.1) was annotated as a DELLA gene in 'Francesco' carnation. Since there are five DELLA genes in *Arabidopsis*, there was a possibility that multiple DELLA genes existed in the carnation genome. In order to search for ORFs of carnation *GAI* homolog (*DcGAI*), a BLASTP search was performed using amino acid sequences of

Arabidopsis DELLAs as queries. The results revealed that Dca34377.1 was the top hit against either of the query sequences and no other full-length coding sequences were found. Therefore, we assumed Dca34377.1 was the only ORF encoding *DcGAI* in the Carnation DB. Based on the sequence of Dca34377.1, we amplified and cloned the cDNA of *DcGAI* from 'LPB'. The cDNA (*DcGAI*-LPB) encoded 595 amino acids, which was shorter by 6 amino acids than Dca34377.1. The homology between amino acid sequences of Dca34377.1 and DcGAI-LPB was 97.0%, and there were substitutions of 12 amino acids (Fig. 1). There are 2 conserved motifs, DELLA and VHYNP, in the N-terminal region of DELLA proteins that are essential for interaction of DELLA proteins with GA receptor, GIDs (Griffiths et al., 2006; Ueguchi-Tanaka et al., 2007). These motifs were conserved in DcGAI-LPB as well as DELLAs from *Arabidopsis* and rice (Fig. 1), suggesting that DcGAI-LPB is a functional DELLA protein in carnation.

In order to search for whether there are any other *DcGAI* genes, we performed a BLASTN search of the scaffold sequences using the nucleotide sequence of *DcGAI*-LPB as a query in the Carnation DB. Three scaffolds (scaffold3543, scaffold4311, and scaffold15839) were found, among which scaffold3543 encoded Dca34377.1 since the coding sequence was completely identical (data not shown). The sequence of scaffold15839 was highly similar to that of scaffold3543 in 3'-downstream region (760 bp) as well as the coding region, although the sequence of 5'-upstream region of scaffold3543 is unknown. This result suggests that these two scaffold sequences are alleles of an identical locus. In contrast, the polypeptide encoded by scaffold4311 lacked the DELLA motif in its N-terminal region and it was not supposed to be a functional *DELLA* gene. Although some species have multiple *GAI* homologs (5 genes in *Arabidopsis*, 4 in poplar and soybean, and 2 in grape), the rice genome contains only one *GAI* gene. Our results suggest that there is a single *GAI* gene encoded in the carnation genome, and that *DcGAI* plays a central role in the GA signaling pathway in carnation.

Expression profiles of DcGAI in carnation flowers

We performed qRT-PCR analysis of this gene in various tissues of carnation flowers (calyx, style, receptacle, ovary, and petals). *DcGAI* was expressed in all the tissues examined (Fig. 2A). A high level expression was observed in calyx, and the expression levels in style, receptacle, ovary, and petals were low. We also analyzed the changes in expression levels of *DcGAI* in petals of carnation flowers during the flower opening process. The transcript level of *DcGAI* was high at the early stages of flower opening (Fig. 2B), and it peaked at Os2 and then decreased at later stages (Os4–Os6).

Fig. 1. Alignment of amino acid sequences of DELLA proteins from carnation (‘LPB’, DcGAI-LPB; ‘Francesco’, DcGAI-Dca34377), *Arabidopsis*, and rice. The accession nos. of the DELLAs used in this study are as follows: *Arabidopsis thaliana* GAI, AT1G14920; *Arabidopsis* RGA, AT2G01570; and rice (*Oryza sativa*) SLR1, Os03g0707600. Inverted residues are conserved among all the sequences, and those shaded are residues with high similarity. Asterisks indicate the DELLA and VHYNP motifs highly conserved in DELLA proteins. Residues with plus signs show the substituted amino acids between DcGAI-LPB and DcGAI-Dca34377.

Expression of *DcGAI* in PDCA-treated carnation flowers

In order to test the possibility that 2,4-PDCA treatment affects the expression of *DcGAI* in opening flowers of carnation, we examined the expression of the gene in 2,4-PDCA-treated flowers. Flowers at Os1 to

Fig. 2. Expression of *DcGAI* in tissues of flowers at Os6 (fully-open stage) (A), and in petals during flower opening (B) of ‘LPB’ carnation. Total RNA was prepared from each sample and qRT-PCR was performed to quantify the transcript level of *DcGAI* gene, which was normalized with that of *DcUbg3-7*. The different letters indicate significant differences by Tukey’s test at $P < 0.05$. Data shown are mean $\pm$ SE ($n = 3-5$).

Os2 were treated with 2 mM 2,4-PDCA for 10 days. An increase in the number of flowers in vase life (Os4 to Os6) by PDCA treatment was observed compared with the control flowers (Fig. 3A), indicating the enhancement of flower opening as observed in our previous study (Sugiyama and Satoh, 2015).

Using petals sampled from these flowers, expression of *DcGAI* was examined by qRT-PCR. The expression of the gene was decreased in accordance with the progression of the flower opening process in the control flowers (Fig. 3B). In contrast, the expression of *DcGAI* was maintained at a high level in 2,4-PDCA-treated flowers, and was significantly higher than that in the control flowers at day 10 (Fig. 3B). Thus, an increase in the transcript level of *DcGAI* was observed in 2,4-PDCA-treated flowers. GAI is a negative regulator of GA action and suppresses growth and elongation (Harberd et al., 2009). Therefore, it is supposed that the increase in *DcGAI* expression leads to suppression of petal cell growth, which does not coincide with the enhancement of flower opening. Therefore, it is likely that the alteration of *GAI* expression is not related to the enhancing effect of 2,4-PDCA.

Fig. 3. Gene expression analysis in 2,4-PDCA-treated flowers of 'LPB' carnation. (A) Time courses of flower opening of the samples used for qRT-PCR. Flowers at Os1 and Os2 were treated with (PDCA) or without (control) 2 mM 2,4-PDCA solution for 10 days. Number of flowers at Os4 to Os6 were counted and the percentage of these flowers is shown (mean $\pm$ SE of 3 replicates). ○, control; ■, 2,4-PDCA. Asterisks indicate a significant difference between the control and treated samples by *t*-test at $P < 0.05$. Expression of *DcGAI* (B), *DcXTH2* (C), and *DcEXPA2* (D) in petals of flowers treated with or without 2 mM 2,4-PDCA. Total RNA was prepared from petals at each time point and qRT-PCR was performed as Figure 2. White and grey bars indicate the control and treated samples, respectively. Data shown are mean $\pm$ SE ($n = 2-4$). Asterisks indicate a significant difference between control and treated samples by *t*-test at $P < 0.05$.

Changes in GA level by 2,4-PDCA in opening flowers of carnation

In order to examine whether 2,4-PDCA treatment affects GA levels, we measured GA content in petals of 2,4-PDCA-treated flowers. Although endogenous GAs acting in carnation have not been identified so far, we set out to measure the GA₁ content since several ornamental plants (*Chrysanthemum morifolium*, *Eustoma grandiflorum*, *Gentiana triflora*, *Phalaenopsis hybrid*, and petunia) contain GA₁ as a bioactive GA (Koshioka, 2004). However, GA₁ was not detected in carnation petals in our LC-MS/MS analysis, which was contrary to our expectation (data not shown). We found GA₃ accumulated instead, and determined the GA₃ content in petals of opening flowers.

In this experiment, all the flowers starting from Os2 reached Os4–Os6 in 4 days under control condition (without 2,4-PDCA treatment) (Fig. 4A). 2,4-PDCA treatment accelerated flower opening, and all the treated flowers reached Os4–Os6 in 2 days. We measured GA₃ content in the non-treated flowers at day 0 to day 4, and found that the GA₃ level tended to be decreased in the course of flower opening (Fig. 4B). We also measured GA₃ content in 2,4-PDCA-treated flowers at day 1, when the treated-flowers showed a significant increase in the number of open flowers. As a result, we observed a tendency that GA₃ content in the 2,4-PDCA treated-flowers was lower than that in the control (Fig. 4C).

Our results mentioned above indicate that 2,4-PDCA increases the gene expression of the growth suppressor, GAI (Fig. 3B), and decreases the GA level (Fig. 4C), suggesting that GA signaling and action are altered by 2,4-PDCA treatment. However, such changes are contradictory to the enhancement of flower opening, which suggests that GA is not associated with the enhancing effect of 2,4-PDCA in carnation flowers.

Effect of GA and PBZ on flower opening in carnation

Although our initial hypothesis that GA is associated with the action of 2,4-PDCA was denied, the involvement of GA in the flower opening of carnation was suggested by a previous study (Saks et al., 1992). We then tested whether application of GA or PBZ affected flower opening in our experimental system. We treated flowers at Os1 and Os2 with 10 to 100 μ M GA₃, and observed that flower opening was not accelerated but delayed by the treatment (Fig. 5A). Also the number of fully-open flowers showed no significant change by 10 μ M GA₃ and rather was decreased by 30 and 100 μ M GA₃ treatment (Fig. 5A). Although senescence was delayed as reported by a previous study (Saks et al., 1992), no enhancement of flower opening was achieved by exogenous application of GA₃. We also treated flowers at Os1 and Os2 with PBZ at 10 to 100 μ M, and monitored the opening and senescence of the flowers (Fig. 5B). We observed no significant effects of PBZ in the treated flowers at all the concentrations tested.

Fig. 4. GA₃ content in petals of opening flowers of 'LPB' carnation. (A) Time courses of flower opening of the samples used for GA₃ content measurement. Flowers at Os2 were treated with (PDCA) or without (control) 2 mM 2,4-PDCA solution for 4 days. Ratio of flowers at Os4 to Os6 is shown as Figure 3 (mean ± SE of 3 replicates). ○, control; ■, 2,4-PDCA. (B) Changes in the GA₃ content during flower opening in the control flowers. GA₃ content in the flowers treated with 2 mM 2,4-PDCA (C) or 100 μM PBZ (D) for 1 day. It should be noted that the control and the test solution for PBZ treatment shown in (D) contained 100 mg·L⁻¹ 8-HQS. Data shown are mean ± SE (n = 3–5) in (B)–(D). Asterisks indicate significant difference between the control and treated samples by *t*-test at *P* < 0.05 in (A) and (D). No significant differences were observed among the samples in (B) and (C) by Tukey's and *t*-test, respectively.

Fig. 5. Effect of GA₃ (A) and PBZ (B) treatment on flower opening of 'LPB' carnation. Flowers at Os1 and Os2 were treated with GA₃ or PBZ at 0 (○), 10 (●), 30 (▲), and 100 (■) μM. All the test solutions contained 100 mg·L⁻¹ 8-HQS. The percentage of fully-open and non-senescent flowers is shown by the mean of 3 replicated samples.

However, PBZ treatment unexpectedly caused a significant increase in the endogenous GA₃ level in petals, which was revealed by the measurement of GA₃ content (Fig. 4B). These results indicate that the increase in GA level has no effect on flower opening, and suggest that endogenous GA is not involved in flower opening in 'LPB' carnation.

These results are contrary to our hypothesis at the beginning of this study, and the result of PBZ treatment is inconsistent with the previous study by Saks et al. (1992). Although we have no experimental evidence, there are two possibilities for explaining the inconsistency between the current and previous studies. First, the discrepancy might be due to a difference in cultivars used. The action of plant hormones and growth regulators can vary among cultivars. Saks et al. (1992) used 'White Sim' as a material, which belongs to the standard type carnation. In contrast, we used the spray-type cultivar 'LPB'. PBZ treatment elevated the GA₃ level in petals of 'LPB' in our experiment, which was a completely opposite result to our expectation. The unexpected increase in the GA₃ level might be due to the feedback regulation of GA biosynthesis (Xu et al., 1999) that might surpass the inhibitory effect of PBZ. The second possible cause of the inconsistency between

the current and previous studies might be differences in experimental conditions. The effect of PBZ seems to be different according to the stage during the flower opening process. This notion is based on the result that differential effects of PBZ treatment were observed depending on the stage when the treatment started (Saks et al., 1992). The PBZ treatment of flower buds at stage II caused inhibition of flower opening, whereas it caused no effects to buds at stage III (Saks et al., 1992). Since Os2 in our study corresponds to stage III in the previous one, we might have failed to detect the expected effect of PBZ in our experiment. In any case, the current results demonstrated that the elevation of GA level caused no significant changes in flower opening, which implies that GA does not participate in flower opening in 'LPB'.

Expression of DcXTH and DcEXP in PDCA-treated carnation flowers

In order to gain further insights into the possible mechanism of 2,4-PDCA action, we examined the expression of cell expansion-related genes in 2,4-PDCA-treated flowers. We selected *DcXTH2* and *DcEXPA2* for this analysis since they were associated with petal growth during flower opening (Harada et al., 2011). *DcXTH2* is expressed abundantly in petals and its expression is higher at Os3 than at any other stages of the flower opening process. The expression level of *DcEXPA2* is highest among the three *DcEXPA* genes and peaks at Os2 and Os3 during flower opening. Although it is unclear that these genes are responsive to GA, it does not matter since the aim of this analysis is to clarify whether 2,4-PDCA exerts its action through modulation of genes associated with cell expansion.

We performed qRT-PCR of *DcXTH2* and *DcEXPA2* using the same cDNA samples as expression analysis of *DcGAI* in 2,4-PDCA-treated flowers (Fig. 3B). The transcript level of *DcXTH2* did not show significant changes by 2,4-PDCA treatment (Fig. 3C). In contrast, the expression of *DcEXPA2* was lower in 2,4-PDCA-treated flowers than the control at day 2 and thereafter, whereas the control flowers showed up-regulation of the gene (Fig. 3D). Thus, 2,4-PDCA caused a decrease in the transcript level of *DcEXPA2* in carnation flowers. However, the decrease in *DcEXPA2* expression can have a negative effect on the expansion of the cell wall that results in suppression of petal cell growth, which does not coincide with the enhancement of flower opening. Therefore, it is likely that the change in expression of *DcEXPA2* is not related to the enhancing effect of 2,4-PDCA.

Considering a possible mechanism of 2,4-PDCA action, OxoGA-dependent dioxygenases inhibited by 2,4-PDCA include prolyl 4-hydroxylases (P4Hs) (Kivirikko and Myllyharju, 1998; Kivirikko and Pihlajaniemi, 1998; Vlad et al., 2010). Proline hydroxylation catalyzed by P4Hs is a major posttranslational modification

of cell wall hydroxyproline-rich glycoproteins that are implicated in cell wall structure and function (Cassab, 1998). The inhibition of P4Hs by 2,4-PDCA was shown in carnation flowers (Vlad et al., 2010). In addition, silencing of tomato P4Hs resulted in enhanced growth of roots and shoots (Fragkostefanakis et al., 2014). Therefore, it is possible that 2,4-PDCA treatment might affect the growth of petal cells by inhibiting P4Hs in opening carnation flowers.

In summary, we revealed that 2,4-PDCA affects expression of *GAI* and expansin genes in opening flowers of carnation, but it seems that this effect is not related to the enhancement of flower opening by 2,4-PDCA. In addition, our results suggest that GA is not associated with the enhancing effect of 2,4-PDCA since the GA level was not elevated by 2,4-PDCA treatment in opening flowers of carnation. The GA level in carnation tended to be decreased by 2,4-PDCA treatment, which might be caused by the inhibition of enzymes involved in GA biosynthesis. It is possible that 2,4-PDCA could have a similar influence on the GA level in other species as well. Also, there is a possibility that 2,4-PDCA has pleiotropic effects by inhibiting OxoGA-dependent dioxygenases such as P4Hs and/or ACC oxidase. The examination of the effects of 2,4-PDCA in other ornamental and crop plants is a subject of future study.

Literature Cited

- Abeles, F. B., P. W. Morgan and M. E. Saltveit, Jr. 1992. Ethylene in plant biology. 2nd ed. Academic Press, San Diego, CA.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* 25: 3389–3402.
- Cassab, G. I. 1998. Plant cell wall proteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49: 281–309.
- Chiwocha, S. D. S., S. R. Abrams, S. J. Ambrose, A. J. Cutler, M. Loewen, A. R. S. Ross and A. R. Kermode. 2003. A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. *Plant J.* 35: 405–417.
- Fragkostefanakis, S., K. E. M. Sedeek, M. Raad, M. S. Zaki and P. Kalaitzis. 2014. Virus induced gene silencing of three putative prolyl 4-hydroxylases enhances plant growth in tomato (*Solanum lycopersicum*). *Plant Mol. Biol.* 85: 459–471.
- Griffiths, J., K. Murase, I. Rieu, R. Zentella, Z.-L. Zhang, S. J. Powers, F. Gong, A. L. Phillips, P. Hedden, T. Sun and S. G. Thomas. 2006. Genetic characterization and functional analysis of the *GID1* gibberellin receptors in *Arabidopsis*. *Plant Cell* 18: 3399–3414.
- Harada, T., S. Satoh, T. Yoshioka and K. Ishizawa. 2005. Expression of sucrose synthase genes involved in enhanced elongation of pondweed (*Potamogeton distinctus*) turions under anoxia. *Ann. Bot.* 96: 683–692.
- Harada, T., Y. Torii, S. Morita, T. Masumura and S. Satoh. 2010. Differential expression of genes identified by suppression subtractive hybridization in petals of opening carnation flowers. *J. Exp. Bot.* 61: 2345–2354.
- Harada, T., Y. Torii, S. Morita, R. Onodera, Y. Hara, R.

- Yokoyama, K., Nishitani, S. and S. Satoh. 2011. Cloning, characterization, and expression of xyloglucan endotransglucosylase/hydrolase and expansin genes associated with petal growth and development during carnation flower opening. *J. Exp. Bot.* 62: 815–823.
- Harberd, N. P., E. Belfield and Y. Yasumura. 2009. The angiosperm gibberellin-GID1-DELLA growth regulatory mechanism: how an “inhibitor of an inhibitor” enables flexible response to fluctuating environments. *Plant Cell* 21: 1328–1339.
- Hedden, P. and Y. Kamiya. 1997. Gibberellin biosynthesis: enzymes, genes and their regulation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48: 431–460.
- Kersey, P. J., J. E. Allen, I. Armean, S. Boddu, B. J. Bolt, D. Carvalho-Silva, M. Christensen, P. Davis, L. J. Falin, C. Grabmueller, J. Humphrey, A. Kerhornou, J. Khobova, N. K. Aranganathan, N. Langridge, E. Lowy, M. D. McDowall, U. Maheswari, M. Nuhn, C. K. Ong, B. Overduin, M. Paulini, H. Pedro, E. Perry, G. Spudich, E. Tapanari, B. Walts, G. Williams, M. Tello-Ruiz, J. Stein, S. Wei, D. Ware, D. M. Bolser, K. L. Howe, E. Kulesha, D. Lawson, G. Maslen and D. M. Staines. 2016. Ensembl genomes 2016: more genomes, more complexity. *Nucl. Acids Res.* 44: D574–D580.
- Kivirikko, K. I. and J. Myllyharju. 1998. Prolyl 4-hydroxylases and their protein disulfide isomerase subunit. *Matrix Biol.* 16: 357–368.
- Kivirikko, K. I. and T. Pihlajaniemi. 1998. Collagen hydroxylases and the protein disulfide isomerase subunit of prolyl 4-hydroxylases. *Adv. Enzymol. Relat. Areas Mol. Biol.* 72: 325–398.
- Koshioka, M. 2004. Gibberellin metabolism and its regulation in horticultural plants. *Regulation of Plant Growth & Development* 39: 1–9 (In Japanese).
- Lamesch, P., T. Z. Berardini, D. Li, D. Swarbreck, C. Wilks, R. Sasidharan, R. Muller, K. Dreher, D. L. Alexander, M. Garcia-Hernandez, A. S. Karthikeyan, C. H. Lee, W. D. Nelson, L. Ploetz, S. Singh, A. Wensel and E. Huala. 2012. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. *Nucl. Acids Res.* 40: D1202–D1210.
- Lange, T., P. Hedden and J. E. Graebe. 1994a. Expression cloning of a gibberellin 20-oxidase, a multifunctional enzyme involved in gibberellin biosynthesis. *Proc. Natl. Acad. Sci. USA* 91: 8552–8556.
- Lange, T., A. Schweimer, D. Ward, P. Hedden and J. Graebe. 1994b. Separation and characterisation of three 2-oxoglutarate-dependent dioxygenases from *Cucurbita maxima* L. endosperm involved in gibberellin biosynthesis. *Planta* 195: 98–107.
- Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson and D. G. Higgins. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947–2948.
- Luo, J., N. Ma, H. Pei, J. Chen, J. Li and J. Gao. 2013. A *DELLA* gene, *RhGAIL*, is a direct target of EIN3 and mediates ethylene-regulated rose petal cell expansion via repressing the expression of *RhCesA2*. *J. Exp. Bot.* 64: 5075–5084.
- Morita, S., Y. Torii, T. Harada, M. Kawarada, R. Onodera and S. Satoh. 2011. Cloning and characterization of a cDNA encoding sucrose synthase associated with flower opening through early senescence in carnation (*Dianthus caryophyllus* L.). *J. Japan. Soc. Hort. Sci.* 80: 358–364.
- Nomura, Y., S. Morita, T. Harada and S. Satoh. 2012. Cloning, characterization and expression of carnation (*Dianthus caryophyllus* L.) ubiquitin genes and their use as a normalization standard for gene expression analysis in senescing petals. *J. Japan. Soc. Hort. Sci.* 81: 357–365.
- Pysh, L. D., J. W. Wysocka-Diller, C. Camilleri, D. Bouchez and P. N. Benfey. 1999. The GRAS gene family in Arabidopsis: sequence characterization and basic expression analysis of the *SCARECROW-LIKE* genes. *Plant J.* 18: 111–119.
- Saks, Y., J. Van Staden and M. T. Smith. 1992. Effect of gibberellic acid on carnation flower senescence: evidence that the delay of carnation flower senescence by gibberellic acid depends on the stage of flower development. *Plant Growth Regul.* 11: 45–51.
- Satoh, S. 2011. Ethylene production and petal wilting during senescence of cut carnation (*Dianthus caryophyllus*) flowers and prolonging their vase life by genetic transformation. *J. Japan. Soc. Hort. Sci.* 80: 127–135.
- Satoh, S., Y. Kosugi, S. Sugiyama and I. Ohira. 2014. 2,4-Pyridinedicarboxylic acid prolongs the vase life of cut flowers of spray carnations. *J. Japan. Soc. Hort. Sci.* 83: 72–80.
- Satoh, S., Y. Nomura, S. Morita and S. Sugiyama. 2016. Further characterization of the action of pyridinedicarboxylic acids: multifunctional flower care agents for cut flowers of spray-type carnation. *J. Appl. Hort.* 18: 3–6.
- Smith, V. A. and J. MacMillan. 1983. Purification and partial characterization of a gibberellin 2 β -hydroxylase from *Phaseolus vulgaris*. *J. Plant Growth Regul.* 2: 251–264.
- Smith, V. A. and J. MacMillan. 1986. The partial purification and characterisation of gibberellin 2 β -hydroxylases from seeds of *Pisum sativum*. *Planta* 167: 9–18.
- Sugiyama, S. and S. Satoh. 2015. Pyridinedicarboxylic acids prolong the vase life of cut flowers of spray-type ‘Light Pink Barbara’ carnation by accelerating flower opening in addition to an already-known action of retarding senescence. *Hort. J.* 84: 172–177.
- Sugiyama, S., S. Morita and S. Satoh. 2015. Three criteria for characterizing flower opening profiles and display values in cut spray-type carnation flowers. *J. Appl. Hort.* 17: 92–95.
- Ueguchi-Tanaka, M., M. Nakajima, E. Katoh, H. Ohmiya, K. Asano, S. Saji, X. Hongyu, M. Ashikari, H. Kitano, I. Yamaguchi and M. Matsuoka. 2007. Molecular interactions of a soluble gibberellin receptor, GID1, with a rice DELLA protein, SLR1, and gibberellin. *Plant Cell* 19: 2140–2155.
- Vlad, F., P. Tiainen, C. Owen, T. Spano, F. B. Daher, F. Oualid, N. O. Senol, D. Vlad, J. Myllyharju and P. Kalaitzis. 2010. Characterization of two carnation petal prolyl 4 hydroxylases. *Physiol. Plant.* 140: 199–207.
- Xu, Y.-L., L. Li, D. A. Gage and J. A. D. Zeevaert. 1999. Feedback regulation of *GA5* expression and metabolic engineering of gibberellin levels in Arabidopsis. *Plant Cell* 11: 927–935.
- Yagi, M., S. Kosugi, H. Hirakawa, A. Ohmiya, K. Tanase, T. Harada, K. Kishimoto, M. Nakayama, K. Ichimura, T. Onozaki, H. Yamaguchi, N. Sasaki, T. Miyahara, Y. Nishizaki, Y. Ozeki, N. Nakamura, T. Suzuki, Y. Tanaka, S. Sato, K. Shirasawa, S. Isobe, Y. Miyamura, A. Watanabe, S. Nakayama, Y. Kishida, M. Kohara and S. Tabata. 2014. Sequence analysis of the genome of carnation (*Dianthus caryophyllus* L.). *DNA Res.* 21: 231–241.