Cloning and Characterization of a cDNA Encoding Sucrose Synthase Associated with Flower Opening through Early Senescence in Carnation (*Dianthus caryophyllus* L.)

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Flower opening in carnations (*Dianthus caryophyllus* L.) is the result of the enlargement of petal cells, which requires sugar metabolism. A cDNA encoding sucrose synthase (*DcSUS1*) was isolated from carnation petals as a candidate gene acting in the initial step of sugar metabolism in petal cells. *DcSUS1* transcripts were detected abundantly in floral tissues of flowering carnation plants; the transcripts accumulated most in the petals and style followed by the ovary, whereas only small accumulation were found in stems, leaves, and calyces. Moreover, nearly constant accumulation of *DcSUS1* transcripts was found in the petals during flower opening, fully open, and early senescence periods, whereas decreasing accumulation was detected in petals when senescence progressed. These findings suggested the involvement of *DcSUS1* expression in petal cell growth during the opening of carnation flowers.

Key Words: carnation, *Dianthus caryophyllus*, flower opening, petal cell growth, sucrose synthase.

Introduction

The vase life of cut ornamental flowers is the period from flower opening to senescence. To prolong this period, many investigators have studied the mechanism of flower opening (Harada et al., 2010; van Doorn and van Meeteren, 2003; Yamada et al., 2009a, b), and senescence (Hoeberichts et al., 2007; Hunter et al., 2004; Satoh, 2011; Shimizu-Yumoto and Ichimura, 2010; van Doorn and Woltering, 2008). We have been studying the molecular mechanism of flower opening using cut carnation flowers as a model ornamental.

Flower opening involves elongation, expansion, and outward bending of petals, which result from the enlargement of petal cells (Evans and Reid, 1988; Kenis et al., 1985; Koning, 1984). Sugar accumulation in petal

cells reduces the petal water potential, and promotes water influx into the petal cells, resulting in cell enlargement (Evans and Reid, 1988; Ho and Nichols, 1977; Ichimura et al., 2003).

Sugar accumulation in petal cells is caused by a combination of sugar uptake by the cells and degradation of various polysaccharides stored in young petals in buds (van Doorn and van Meeteren, 2003). In general, sucrose is one of the prevailing sugars involved in these processes, and is metabolized by invertase and sucrose synthase (Harada et al., 2005). Invertase cleaves sucrose into glucose and fructose, and sucrose synthase converts sucrose into UDP-glucose and fructose by its reverse reaction (Giegenberger and Stitt, 1993), therefore, these enzymes probably accompany flower opening. Yamada et al. (2007) analyzed sucrose metabolism during petal growth of cut roses and suggested the involvement of invertase activities in that process. On the other hand, sucrose synthase was reported to be involved in sucrose metabolism in other plant cell growth, such as that responsible for enhanced elongation of pondweed turions (Harada et al., 2005).

To gain more insight into the sugar metabolism responsible for flower opening in carnations, we tried to clone cDNAs encoding candidate enzymes from

Received; August 24, 2010. Accepted; December 1, 2011.

This study was supported financially by a Grant-in-Aid (19380024 to S.S.) for Scientific Research from the Japan Society for the Promotion of Science.

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carnation flowers. In the present study, we cloned and characterized the cDNA of sucrose synthase (*DcSUS1*) from carnation (*Dianthus caryophyllus* L.), and examined gene expression during the growth and development of the petals. We also searched for possible inhibitors of sucrose synthase, which are expected to be safe for humans, with easy practical use.

Materials and Methods

Plant materials and sample preparation

Cut flowers of carnation (*Dianthus caryophyllus* L. 'Light Pink Barbara') belonging to the spray category of carnation flowers were harvested when the first flower out of five to six flower buds was nearly open at the nursery of a commercial grower in Miyagi prefecture. The flowers were transported dry to the laboratory at Kyoto Prefectural Institute of Agricultural Biotechnology in Kyoto prefecture on the day after harvest. They were placed in plastic containers with their cut end in water under continuous light from white fluorescent lamps ($14 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) at 23°C . The flower-opening process was separated into 6 stages according to Harada et al. (2010): stage 1, petals just emerged from buds; stage 2, petals elongated vertically; stage 3, petal

clusters expanded; stage 4, outer petals start to reflex (bend outwards); stage 5, outer petals have reflexed; stage 6, fully open flower with outer petals at right angles to the stem. In addition, the flower senescence process was separated into 4 stages: senescence stage 1, the last phase of fully opened flowers; senescence stage 2, petal in-rolling and wilting started; senescence stage 3, petal wilting culminated; senescence stage 4, wilted petals markedly shrunk. The profiles of flower opening and senescence are shown in Figure 2.

Ten outermost petals were detached from 5 flowers at the respective stages, mixed to make one sample, and stored at -80°C for extraction of RNA. Other tissues, leaves, stem, calyx, ovary, and style were detached 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 as in other flowers); therefore, the term 'style' was used to describe the style plus stigma in the following. A series of samples collected at each stage during flower opening and senescence was prepared and used for real-time PCR analysis.

Measurement of ethylene production

To determine ethylene evolution from flowers undergoing senescence, flowers were cut leaving 10 mm of stem and enclosed in a 240-mL glass vessel for 1 h. A 1-mL gas sample was then taken from the vessel and used for measurement of ethylene concentration with a gas chromatograph (GC-8A, Shimadzu, Kyoto, Japan), equipped with an alumina column (operating temperature, 80°C) and a flame ionization detector.

RNA extraction

Total RNA was isolated by the cetyltrimethylammonium bromide (CTAB) method from petal tissues (Chang et al., 1993; Murry and Thompson, 1980). and precipitated with LiCl (Nakamura et al., 1991), or by the phenol-chloroform method, as described in Harada et al. (2005). RNA was also extracted by the phenol-chloroform method from other tissues. Briefly, in the phenol-chloroform method, a sample of about 1 g frozen petals, or other tissues, was pulverized in liquid N_2 with a mortar and pestle, and extracted with 5 mL extraction buffer [1% SDS, 50 mM Tris-HCl (pH 7.5), 50 mM EDTA] plus an equal volume of water-saturated phenol. Total RNA was recovered in the supernatant after centrifugation, purified by treatment with phenol-chloroform solution (1 : 1 v/v) followed by chloroform, and finally precipitated with isopropanol at -20°C .

PCR cloning of cDNA for sucrose synthase

To obtain full-length composite cDNA for sucrose synthase, we obtained three partial-length cDNAs by ordinary RT-PCR, 3'-rapid amplification of cDNA end (3'-RACE) (Frohman et al., 1988) and 5'-RACE techniques, and then the cDNAs were combined to make

Fig. 1. Southern blot analysis of *DcSUS1* gene. Genomic DNA samples were obtained from petals of 'Light Pink Barbara' carnation. Each lane contained 10 μg genomic DNA digested with *EcoR* V (E), *Hind* III (H), and *Xba* I (X). The bolts were hybridized with the DIG-labeled DNA probe and detected with a CSPD-Star kit (Roche Diagnostics).

Fig. 2. Profiles of flower opening and senescence in 'Light Pink Barbara' carnation (A), separation of the petal into four parts (B), and ethylene production during senescence (C). The petal was separated into four parts as shown in Figure 1 of Harada et al. (2010) and used for *DcSUS1* expression analysis in Figure 3C. More than 14 flowers at each senescence stage were used for measurement of ethylene production, and date are shown as the means $\pm$ SE.

a composite cDNA. Briefly, a partial length cDNA (687 bp) was amplified by RT-PCR with total RNA obtained from opening carnation petals (stage 5) and the forward (SUS1F, 5'-CCY GAY ACY GGW GGB CAG GTT GTY TAC-3') and reverse (SUS1R, 5'-CCA GGR GAS ACR ATG TTG AAC TTN GG-3') degenerate primers derived from sequences of sucrose synthase cDNAs reported in public databases. RT-PCR was performed according to the standard procedure with necessary optimization. The PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, USA) for sequencing, and a nucleotide sequence was obtained. Using the nucleotide sequences, sequence-specific primers were designed. Then, a 3'-downstream cDNA of 1137 bp was obtained by 3'-RACE with the forward SUSRACE1 primer (5'-CTT CTT GGG CTC TAC CGA GTG GTT C-3') and the reverse RT1 primer (Invitrogen, Carlsbad, USA; 5'-AAC TGG AAG AAT TCG CGG CCG GAG GAA-3'). Finally, a 5'-upstream cDNA of 1106 bp was obtained by 5'-RACE with the forward GeneRacer 5'primer (Invitrogen; 5'-CGA CUG GAG

CAC GAG GAC ACU GA-3') and the reverse DcSUS1-RACE primer (5'-CTG CGT CAG GGA GCA GAC GAG TTA CAA-3'). The three partial cDNAs were reconstituted to make the composite cDNA (*DcSUS1*) of 2790 bp, including polyA sequence. Then, to confirm complete nucleotide sequences, a full-length cDNA (2690 bp) was amplified from total RNA obtained from opening carnation petals as templates with primers derived from both ends of the composite cDNA (forward DcSUS1-F1 primer, 5'-ACA CTC CTC TAC TCT TCC CT-3'; reverse DcSUS1-R1 primer, 5'-ATC AAA CAC AAA TGC CTC AAA GAG C-3'). The nucleotide sequence of the cDNA was obtained, and the complete sequence was confirmed to be identical with the composite sequence.

Southern blot analysis

Genomic DNA was isolated from 'Light Pink Barbara' carnation petals by the CTAB method (Hasebe and Iwatsuki, 1990). Ten micrograms of genomic DNA were digested with *EcoR* V, *Hind* III, *Xba* I and separated on

a 0.8% agarose gel and transferred to a nylon membrane (Hybond N⁺, Amersham Pharmacia Biotech, Piscataway, USA). A *DcSUS1* cDNA fragment, 725 bp, was amplified by PCR with the forward SUS2F primer (5'-GTG GAT CTC TGC GCA GAT GAA CCG-3') and the reverse RT1 primer, and used as the probe for gel blot analysis. The DNA probe was labeled with DIG using the DIG DNA labeling kit (Roche Diagnostics, Mannheim, Germany). After hybridization with the blot, the hybridization signals were detected by chemiluminescence using CSPD-Star (Roche Diagnostics) as a substrate, and recorded on an X-ray film (RX-U, Fuji Photo Film, Tokyo, Japan).

Real-time PCR

Real-time RT-PCR for the quantification of transcripts for *DcSUS1* was conducted using the LightCycler FastStart DNA Master SYBR Green I Kit and the LightCycler Instrument (Roche Diagnostics). cDNA was synthesized using ReverTra Ace (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The primer sets for real-time PCR were the forward SUS3F and the reverse SUS3R for carnation *DcSUS1*, and the forward DcACT1P51F (5'-CGT CAC CAA CTG GGA TGA CA-3') and the reverse DcACT1P229R (5'-GAG AGA ACG GCC TCC ATG GC-3') primers for carnation actin [*DcACT1*, accession number (acc. no.) AY0073415] mRNA (Waki et al., 2001). The sizes of amplicates were 196 bp and 198 bp for *DcSUS1* and *DcACT1*, respectively. PCR conditions were initial heating for 10 min at 95°C followed by 40 cycles of 5 s at 95°C, 5 s at 53°C, 8 s at 72°C, in which the extension time was adopted according to the length of amplicates (1 s per 25 mer). The absolute transcript level was calculated using a dilution series of a target sequence on LightCycler Software Ver. 3.5 (Roche Diagnostics).

DcACT1 had an almost constant transcript level throughout the flower-opening stages and tissues, and was used to normalize the transcript level (Fig. 3). On the other hand, the amount of *DcACT1* transcripts was found to decrease markedly in the later stages of petal senescence, suggesting that the use of *DcACT1* transcript as a normalizing standard would result in an over-estimation of *DcSUS1* transcript (Jones, 2004); therefore, in the case of senescing carnation petals, we showed the transcript levels of *DcSUS1* on the basis of total RNA (Fig. 4). Three RNA preparations per stage were independently used for analyses, and data are shown as the mean $\pm$ SE of the three preparations for each stage.

Effects of sucrose analogs on flowering

Flowers of 'Light Pink Barbara' carnation at opening stage 2 with 10 cm stems were put in 50-mL plastic tubes (3 flowers per tube), dipping their stem ends in 20-mL test solution containing sucrose and its analogs at 0.1 M. The sucrose analogs used were trehalose, palatinose, and sucralose. The control was distilled water. The test solution contained isothiazolinone derivatives (0.01% Legend MK[®], Cresta Japan, Chiba, Japan) as a bactericide. Experiments were repeated twice. Four replicated plastic vessels were used per treatment for experiment 1, but three replicates for experiment 2. The flowers were left at 21°C under continuous light from white fluorescent lamps (14 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD). Flower opening was observed daily, and days from flower-opening stage 2 until stage 4 were determined for each vessel by averaging the values obtained with 3 flowers. Data are shown as the mean $\pm$ SE of four or three replicated vessels. Data were analyzed by Dunnett's multiple range test.

Fig. 3. Comparison of transcript levels of the *DcSUS1* gene among different tissues (A), and their changes in whole petals at each stage (B) and different parts of petals (C) during flower opening. Transcript levels for *DcSUS1* and *DcACT1* were determined by real-time RT-PCR, and *DcSUS1* transcript level relative to that of *DcACT1* are shown. Data are the means $\pm$ SE of three separate samples. Abbreviations in the abscissa of the panel A: S, stem; L, leaf; C, calyx; O, ovary; SI, style (actually style plus stigma); P, petal. Photographs of flowers at each stage during flower opening for (B) and petal portions for (C) are shown in Figure 2A and 2B, respectively.

Results and Discussion

Cloning and characterization of *DcSUS1*

We isolated a 2769-bp long cDNA fragment encoding a putative sucrose synthase from 'Light Pink Barbara' carnation using a combination of RT-PCR, 3'-RACE and 5'-RACE techniques. The cDNA had an entire open reading frame of 2406 bp, and 91-bp long 5'- and 272-bp long 3'-marginal sequences. The deduced protein consisted of 801 amino acid residues and had a molecular mass of 91 kDa. The protein had an amino acid sequence of DTGGQVVY at amino acid 299–306, which is conserved among sucrose synthases from different plants. These findings combined with those by homology analysis shown below indicated that the cDNA encoded a putative sucrose synthase. The cDNA was designated *DcSUS1* (*Dianthus caryophyllus* sucrose synthase 1) and deposited in DDBJ (acc. no. AB543810).

DcSUS1 had an amino acid sequence similar to other sucrose synthases; similarity revealed by BLAST analysis was 90.1% for chenopodium CSS1 (*Chenopodium rubrum*, acc. no. X82504) (Godt et al., 1995), 89.9% for beet SBSS2 (*Beta vulgaris*, acc. no. AY457173) (Haagensohn et al., 2006), 81.9% for mandarin orange CitSUS1 (*Citrus unshiu*, acc. no. AB022092) (Komatsu et al., 2002), 82.5% for watermelon WSUS (*Citrullus lanatus*, acc. no. AB018561), and 82.5% for eucalyptus SuSy3 (*Eucalyptus grandis*, acc. no. DQ22794). The homology was markedly high among carnation *DcSUS1*, chenopodium CSS1, and beet SBSS2. This finding suggests a close relationship among carnation, chenopodium, and beet in terms of sucrose synthase proteins. This agrees with the fact that carnation (*Dianthus caryophyllus*), chenopodium (*Chenopodium rubrum*), and sugar beet (*Beta vulgaris*) belong to the order Caryophyllales.

Southern blot analysis of genomic DNA obtained from 'Light Pink Barbara' carnation showed two or more bands after digestion with three restriction enzymes (Fig. 1). These findings suggested that there are at least two *DcSUS1* copies in 'Light Pink Barbara' carnation.

Flower opening, senescence, and ethylene production in 'Light Pink Barbara' carnation

Figure 2A shows the profile of flower opening and senescence. The flower-opening process was separated into six stages according to Harada et al. (2010), whereas the flower senescence process was separated into four stages. Duration from flower-opening stage 6 to senescence stage 1 could be regarded as the fully open stage.

Ethylene production from whole flowers was not detected at senescence stage 1, but became detectable at senescence stage 2 and then increased at senescence stages 3 and 4 (Fig. 2C). The maximum rate of ethylene production ($0.91 \pm 0.29 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) at senescence stage 3 was comparable to that observed previously, such as $1.42 \pm 0.33 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ (Otsu et al., 2007).

Expression analysis of *DcSUS1*

Figure 3 shows the relative transcript levels of *DcSUS1* in different tissues of carnation plants with opening flowers (A), in whole petals at each opening stage (B), and in different parts of petals at opening stages 1 and 5 (C). Large amounts of *DcSUS1* transcript accumulated in the petals and style (actually style plus stigma), followed by the ovary. In contrast, there was little accumulation of *DcSUS1* transcript in the stems, leaves, and calyces. These findings suggested that *DcSUS1* is expressed largely in floral tissues but scarcely, if any, in vegetative green tissues. As shown in Figure 3B, in whole petals, *DcSUS1* transcript was already accumulated at flower-opening stage 1 (buds), and its level was maintained nearly constant until flower-opening stage 6 (fully open flower). Figure 3C shows that *DcSUS1* transcripts were present in four longitudinally successive parts of petals (Fig. 2B) at flower-opening stages 1 and 5. There was little difference in the *DcSUS1* transcript level among petal parts at both flower-opening stages.

On the other hand, the *DcSUS1* transcript was detected at flower senescence stages 1 and 2, and markedly decreased at senescence stages 3 and 4 (Fig. 4). The presence of the transcript in petals in senescence stages 1 and 2 suggested that *DcSUS1* was expressed in the petals of carnation flowers at fully open and early senescence stages.

The present findings are slightly different from those reported by Panavas et al. (1999), who showed that the mRNA level of the sucrose synthase gene increased during the growth and development of tepals of daylily flowers. We conclude that the *DcSUS1* gene expression

Fig. 4. Changes in transcript levels of *DcSUS1* gene in whole petals of carnation flowers undergoing senescence. Transcript levels are determined by real-time RT-PCR on the basis of given amounts of total RNA, since amounts of *DcACT1* mRNA decreased markedly at later senescing stages. Data for opening-stage 6 were the data used in Figure 3B, but before normalizing with the amount of *DcACT1* mRNA. Photographs of flowers at each senescing stage are shown in Figure 2A. Data are the means $\pm$ SE of three separate samples. Op st 6 stands for opening-stage 6.

at high level is associated with the progress of petal development in carnation flower opening and to maintain them fully open.

Trial search for possible inhibitors of sucrose synthase in carnation petals

Based on the present findings we considered that if the action of *DcSUS1* could be prevented, the progress of flower opening would be retarded, resulting in lengthening the flower display time. This may be achieved by inhibition of *DcSUS1* protein synthesis itself by application of cycloheximide (a protein synthesis inhibitor) and perhaps actinomycin D (an RNA synthesis inhibitor). In the past, many reports showed that cycloheximide prevented the senescence of carnation flowers or their isolated petals, although its mechanism of action was explained differently (Halaba and Rudnicki, 1985; Taverner et al., 2000; Wulster et al., 1981, 1982a, b). To our best knowledge, there have been no reports in which the effects of these inhibitors on carnation flower opening were tested. On the other hand, cycloheximide and actinomycin D are very toxic to humans and livestock, which allowed them to be used only for experimental tools and never as preservatives used practically in the flower industry. This situation will not change in the future.

Therefore, we took another approach to find possible inhibitors of sucrose synthase with the expectation that they, if active, would retard the progress of flower opening. We searched for possible inhibitors in sucrose analogs, keeping the following expectations in mind: (1) the sucrose analogs would compete with sucrose at the active site of the enzyme, resulting in inhibition of the enzyme's action, and (2) they would be safe for humans and livestock when used practically in the flower industry since they are already used as food additives or, if not, they are expected to be metabolized easily by humans and livestock.

We tested the effects of trehalose, palatinose, and sucralose, as well as sucrose itself (Table 1). The days from flower-opening stage 2 to stage 4 varied from 3.7 days to 4.9 days in experiment 1, and from 4.3 days to 6.3 days in experiment 2. There was no significance in the duration of flower opening (days from stage 2 to

stage 4) between the control flowers and flowers treated with sucrose and its analogs, except those treated with sucralose at 0.1 M. However, severe injury occurred in the calyces of flowers treated with 0.1 M sucralose for 2 days, indicating that the delay of flower opening might have been caused by side negative effects of the chemicals other than the inhibition of sucrose synthase. In addition to the above experiment, we preliminarily tested sucrose analogs at lower or higher concentrations, i.e., isomaltose at 29.2 mM, trehalose at 29.2, 87.6, and 292 mM, palatinose 29.2, 87.6, and 292 mM, and sucralose at 0.3, 1, and 10 mM; however, these chemicals at given concentrations did not delay the rate of flower opening (data not shown). Although the sucrose analogs used in the present experiment did not affect the flower opening of carnations, we believe that it is worth conducting this trial with different kinds of flowers and chemicals to pursue sucrose analogs which inhibit sucrose synthase activity.

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Table 1. Effects of sucrose and its analogs applied exogenously at 0.1 M on the progress of flowering in cut carnation flowers.

Treatment	Days from stage 2 to stage 4	
	Experiment 1	Experiment 2
Control (H_2O)	4.0 ± 0.3	4.8 ± 0.3
Sucrose	3.7 ± 0.3	4.3 ± 0.2
Trehalose	4.7 ± 0.2	5.9 ± 0.4
Palatinose	3.6 ± 0.5	5.2 ± 0.1
Sucralose ^z	4.7 ± 0.6	6.3* ± 0.9

^z Severe injury was caused to calyces.

* Significantly different by Dunnett's multiple range test ($P < 0.05$).

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