Allelic Nature of Two Carnation Variants 1-Aminocyclopropane-1-carboxylate (ACC) Synthase Genes, DcACS1a and DcACS1b

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We found a variant in the carnation genome, the 1-aminocyclopropane-1-carboxylate synthase gene (DcACS1) that had only DcACS1b, suggesting it is a homozygote of the gene in the carnation cultivars ‘Skyline’ and ‘Scarlett Plus’. The ‘Mini-tiara’ carnation is obtained by an interspecific cross between carnation and wild pink, one of which, Dianthus knappii. D. knappii, had three ACS1 genes orthologous to DcACS1b; i.e., DkACS1b.1, DkACS1b.2, and DkACS1b.3. The composition of ACS1 homologs was analyzed in sub-cultivars of the ‘Mini-tiara’ carnation by PCR amplification of intron 1, intron 2, and 3’-UTR. Each sub-cultivar of the ‘Mini-tiara’ carnation had two ACS1 genes with various combinations. ‘Mini-tiara 0707’ had only DcACS1a, suggesting it is a DcACS1a/DcACS1a homozygote. ‘Mini-tiara Topaz’ had DcACS1a and DcACS1b, ‘Mini-tiara Ruby’ had DcACS1a and DcACS1b, and ‘Mini-tiara Lilac’ had DcACS1b and DkACS1b.2. These findings suggest that the ‘Mini-tiara’ carnation has two genes from a virtual group of at least five ACS1 gene homologs of carnation and D. knappii. The discovery of a DcACS1b homozygotic carnation and inheritance of ACS1 homologous genes in the ‘Mini-tiara’ carnation from carnation and D. knappii suggests the allelic nature of carnation DcACS1a and DcACS1b genes.

Key Words: Dianthus caryophyllus, Dianthus knappii, inheritance, intron structure, PCR products.

Introduction

Ethylene plays a pivotal role in the regulation of carnation flower senescence. During senescence of carnation flowers, a climacteric rise in ethylene production occurs, and the evolved ethylene induces in-rolling of petals, resulting in wilting of whole flowers.

Ethylene production is accompanied by the expression of genes for 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (Jones and Woodson, 1999; ten Have and Woltering, 1997; Woodson et al., 1992). In carnation, three genes encoding ACS (DcACS1, DcACS2, and DcACS3) and one gene encoding ACC oxidase (DcACO1) have been identified (Henskens et al., 1994; Jones and Woodson, 1999; Park et al., 1992; Wang and Woodson, 1991). DcACS1 was found to be mainly responsible for autocatalytic ethylene production in carnation petals (Jones, 2003; Jones and Woodson, 1999; Satoh, 2011; Satoh and Waki, 2006; Shibuya and Ichimura, 2010; ten Have and Woltering, 1997). Therefore, DcACS1 plays the most important role in ethylene production in carnation petals undergoing senescence.

Harada et al. (2011) examined the genomic DNA structure of DcACS1 of the ‘Light Pink Barbara’ (‘LPB’) carnation by genomic PCR and nucleotide sequencing. Interestingly, in the genome of the ‘LPB’ carnation, two distinct nucleotide sequences, so-called variants, were found and were designated DcACS1a and DcACS1b. The former corresponded to a cDNA coding for the carnation DcACS1, which was first cloned as CARACC3 from the ‘White Sim’ carnation (Park et al., 1992), and later confirmed by Henskens et al. (1994), whereas the latter was a novel gene.

Both DcACS1a and DcACS1b have five exons and four introns, and have almost identical nucleotide sequences in exons, but not in some introns and 3’-
untranslated regions (3'-UTR). Both genes were expressed in senescing petals of the ‘LPB’ carnation. Genomic PCR analysis of 32 carnation cultivars showed that 25 cultivars had only $DcACS1a$ and seven cultivars had both $DcACS1a$ and $DcACS1b$; that is, cultivars such as ‘White Sim’, ‘Scania’, ‘Nora’, and so on had only $DcACS1a$, whereas others such as ‘LPB’, ‘Tanga’, ‘Kibo’ had both $DcACS1a$ and $DcACS1b$.

Moreover, it was revealed that three other wild-type Dianthus (wild pink) species had ACS genes orthologous to $DcACS1$ genes; they had a similar genomic structure with five exons and four introns, but with different intron nucleotide sequences (Harada et al., 2011; Satoh et al., 2011). Each Dianthus species had single to multiple variants of ACS1-like genes, and among wild pink species tested, $D. petraeus$ (KBG-2 strain) had four variants of ACS1-like genes. These findings raised several questions regarding why some Dianthus species have so many variants of ACS1 genes, whether all members of multiple gene variants are expressed and, if present, whether the ethylene production rate of senescing flowers is related to the number of ACS1 gene variants. The elucidation of these questions is important in carnation since it is one of the major ornamental flowers.

Harada and Satoh (unpublished) suspected that $DcACS1a$ and $DcACS1b$ genes were alleles in carnation, and attempted to examine them by the fluorescence in situ hybridization (FISH) technique. However, the size of the ‘LPB’ carnation chromosomes was too small to be analyzed by FISH. Meanwhile, if the two genes (variants) are actually alleles, homozygotes of $DcACS1b$ should be present in carnation cultivars. The discovery of homozygotes of $DcACS1b$ in carnation cultivars would support the allelic nature of $DcACS1a$ and $DcACS1b$ genes in carnation.

‘Mini-tiara’ carnations are interspecific hybrids of carnation and wild pink (wild-type Dianthus species) that were bred at Kagawa Prefectural Agricultural Experiment Station (KPAES), Japan (KPAES, 2016). Their parental species have not yet been officially disclosed. However, in addition to carnation, two wild pink species are thought to have been used; one was Dianthus knappii and the other remains undisclosed (Dr. Takako Narumi, Kagawa University, personal communication). ‘Mini-tiara’ carnations have several sub-cultivars with different petal colors. They also produce ethylene from petals during senescence as does carnation (Satoh et al., 2018).

We suspected that the ‘Mini-tiara’ carnation inherited two ACS1 gene homologs as alleles from a virtual group of ACS1 homologous genes consisting of carnation’s two $DcACS1$ genes, $DcACS1a$ and $DcACS1b$, and the corresponding ACS1 gene homologs of wild pinks. In the present study, we first searched for carnation cultivars having only the $DcACS1b$ gene, implying a homozygote of $DcACS1b$, and then analyzed the composition of ACS1 gene homologs in the ‘Mini-tiara’ carnation to clarify that $DcACS1a$ and $DcACS1b$ are actually alleles.

### Materials and Methods

#### Carnation and wild pink plants

Flowers of carnation ($Dianthus caryophyllus$ L.) ‘Carnet’, ‘Cherry Tessino’, ‘Light Pink Barbara’ (‘LPB’), and ‘Scarlett Oستera’ were obtained from the nursery of a commercial grower in Miyagi Prefecture, Japan. Flowers of cultivars ‘Barullo’, ‘Kirk’, ‘Toy’, ‘Scarlett Plus’, ‘Skyline’ were obtained from Glories Co., Ltd. (Ritto, Japan) and the latter two cultivars were imported from Colombia (Sagaro Flowers, Bogota, Colombia). The procedures for harvest at the nurseries and transportation to our laboratory at Ryukoku University were as described previously (Satoh and Nomura, 2019).

Flowers of the ‘Mini-tiara’ carnation were obtained from a nursery of a local grower in Miki-cho, Kagawa, Japan. ‘Mini-tiara’ carnations are separated into sub-cultivars according to their petal colors: ‘Mini-tiara Ruby’, ‘Mini-tiara Topaz’, ‘Mini-tiara Pink’, ‘Mini-tiara Lilac’, ‘Mini-tiara Baby pink’, ‘Mini-tiara Coral pink’, ‘Mini-tiara Milk white’, ‘Mini-tiara Star yellow’, ‘Mini-tiara Sunny’, and a strain ‘Mini-tiara 0707’. ‘Mini-tiara 0707’ is presently a strain and has not yet been released in the flower industry in Japan. Potted plants of $D. knappii$ were purchased from a commercial market in the middle of May 2018 and cultivated until flowering in August, 2018 in a greenhouse at the Faculty of Agriculture, Ryukoku University.

#### DNA extraction

Genomic DNA was extracted from petals or leaves of carnation cultivars and $D. knappii$ according to the method described by Liu et al. (1995). Genomic DNA was extracted using commercially available kits for DNA extraction: DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), Simple DNA extraction kit v. 2 (Kaneka Corp., Tokyo, Japan), and DNAzol Reagent (Thermo Fisher Scientific K.K., Tokyo, Japan) as instructed by each manufacturer.

#### PCR amplification of regions including intron 1, intron 2 and 3'-UTR in ACS1 homologous genes in Dianthus species

PCR was performed using rTaq DNA polymerase (Takara Bio Inc., Shiga, Japan) with 25 ng genomic DNA as a template in a 10 μL reaction mixture. The forward and reverse primer pairs used for amplification of regions 1–4 including intron 1, intron 2, 3'-UTR found in $DcACS1a$, and 3'-UTR in $DcACS1b$, and the PCR products were the same size as described previously (Harada et al., 2011; Satoh et al., 2011). Conditions were 94°C for 4 min followed by 30 cycles of...
30 sec at 94°C, 30 sec at 53°C, and 1 min at 72°C. PCR products were separated in 2% agarose gel by electrophoresis and visualized by ethidium bromide or SYBR Green (SAFELOOK Post-Green Nucleic Acid Stain; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) methods.

Cloning of genomic DNAs for ACS1 homologous genes of D. knappii and ‘Mini-tiara’ carnations

Previously, genomic DNAs for DcACS1a and DcACS1b in the ‘LPB’ carnation were obtained by PCR cloning (Harada et al., 2011). Genomic DNAs for ACS1 homologous genes in D. knappii and the ‘Mini-tiara’ carnation were cloned in the present study. In brief, to obtain full-length composite genomic DNAs for ACS1 homologous genes, we used an almost identical strategy in which several partial-length genomic DNAs were obtained by ordinary PCR and PCR similar to the rapid amplification of the cDNA end (5'-RACE) (Frohman et al., 1988) and 3'-RACE techniques. The genomic DNAs were combined to make composite genomic DNAs. Then, to confirm complete nucleotide sequences, we amplified full length genomic DNAs with primers derived from both ends of the composite genomic DNAs and total DNAs obtained from corresponding Dianthus species. The forward and reverse primer pair for full length cloning of DcACS1 corresponding genes was as described in Satoh et al. (2011). PCR products covered from 5'-UTR until 3'-UTR. The amino-acid sequences of ACS1 homologs were deduced from the nucleotide sequence of the determined genomic DNAs.

Sequence analysis and accession numbers

Nucleotide sequencing was outsourced to Fasmac Co., Ltd. (Atsugi, Japan). Nucleotide sequences were edited and analyzed with GENETYXWIN and a homology search was conducted with the BLASTX program on the DDBJ (the DNA Data Bank of Japan) website. Similarly, a homology search was conducted for respective introns with the BLAST program. Nucleotide sequences obtained in this study were deposited in DDBJ.

Results

Search for carnation cultivars having only the DcACS1b gene

Previously, a search for carnation cultivars that are possible homozygotes of the DcACS1b gene was not successful (Harada et al., 2011). However, in the present study, genomic PCR analysis revealed two carnation cultivars, ‘Skyline’ and ‘Scarlett Plus’, which had only the DcACS1b gene (Fig. 1). PCR amplification revealed one or two size variants for region 1 (ca. 510 nt and 590 nt), region 2 (ca. 950 nt and 630 nt) and regions 3 and 4 (ca. 730 nt and 670 nt), respectively (Fig. 1). Respective cultivars were classified into three groups according to the band patterns of PCR products. The first group showed the same band pattern as that of ‘LPB’, which includes ‘Cherry Tessino’, ‘Scarlett Ostera’, and ‘Carnet’. ‘LPB’ was shown to have DcACS1a and DcACS1b (Harada et al., 2011; Satoh et al., 2011). In this group, PCR products of 510 nt, 950 nt and 730 nt for region 1, region 2, and region 3, respectively, came from DcACS1a, whereas those of 590 nt, 630 nt, and 670 nt, for region 1, region 2, and region 4, respectively, came from DcACS1b (Harada et al., 2011; Satoh et al., 2011). The second group, including the ‘Toy’ cultivar, was one in which the bands corresponding to the DcACS1b in ‘LPB’ were not detected, suggesting the ‘Toy’ cultivar had only DcACS1a. This band pattern, derived from genomic DcACS1a, was previously shown with 25 carnation cultivars including ‘Scania’, ‘Nora’, and ‘Francesco’ (Harada et al., 2011). The third group consisting of ‘Skyline’ and ‘Scarlett Plus’ cultivars showed a unique band pattern that lacked bands corresponding to those derived from DcACS1a. The detected PCR products were clearly derived from genomic DcACS1b, implying that these two cultivars had only the DcACS1b gene. In another experiment, the ‘Barullo’ and ‘Kirk’ cultivars produced a band pattern similar to that of ‘LPB’ (data not shown).

Cloning and structure of genomic DNAs for ACS1 homologous genes of D. knappii

Full length nucleotide sequencing of genomic ACS1-like genes cloned from D. knappii confirmed the pres-
ence of three ACS1 homologous genes that had a 3'-UTR nucleotide sequence similar to that of DcACS1b. The three genes were named DkACS1b.1, DkACS1b.2 and DkACS1b.3 and deposited at DDBJ with Ac. Nos, LC511683, LC511684, and LC511685, respectively. The full length genomic DNA was 4,298 nt for DkACS1b.1, 4,532 nt for DkACS1b.2 and 4,540 nt for DkACS1b.3 (Suppl. Fig. 1). Table 1 summarizes the genomic DNA structure of the three DkACS1b genes from D. knappii in addition to two carnation genes DcACS1a and DcACS1b. The sizes in terms of nucleotide number of 5'-UTR, 3'-UTR, exons 1–5 were almost the same among three genomic DNAs, but the sizes of introns 2 and 4 were similar and those of introns 1 and 3 were markedly different among them. Among three genomic DkACS1b genes, the nucleotide sequences of 5'-UTR were completely identical, and the homologies in the nucleotide sequence of introns 2 and 4 and 3'-UTR were almost identical (94.2–95.7%, 96.7–99.2%, and 97.7–100.0%, in this order), but those of introns 1 and 3 were fairly conserved (63.6–93.4% and 73.8–88.3%, respectively) (Table 2).

The predicted cDNAs were deduced from these genomic sequences by splicing out the introns. Nucleotide sequences of exons 1–3 in DkACS1b.1, DkACS1b.2 and DkACS1b.3 were almost identical to those in DcACS1b, but deletion and substitution of several nucleotides were found in exons 4 and 5 (Suppl. Fig. 1). The deduced amino-acid sequences were aligned to the 518 amino acids of DcACS1a (Fig. 2). ACS1 homologous proteins of D. knappii consisted of 489, 488, and 486 amino acids, for DkACS1b.1, DkACS1b.2, and DkACS1b.3 proteins, respectively, and these numbers of amino acids were shorter by 29–32 amino acids than those of DcACS1a and by 23–26 amino acids than those of DcACS1b proteins. The amino-acid sequences were highly conserved between carnation DcACS1 proteins and D. knappii DkACS1b proteins, and within the three DkACS1b proteins themselves, except for a 12 amino-acid substitution over whole regions of the protein in three DkACS1b proteins (Fig. 2). The amino-acid residues around the active-site lysine (Lys-278 in DcACS1a protein) were conserved among three DkACS1b proteins. There were fewer Thr residues of the threonine stretch unique to Dianthus ACS1 protein, and fewer DkACS1b proteins than DcACS1a and DcACS1b proteins (Fig. 2).

**Analysis of composition of ACS1 gene homologs in ‘Mini-tiara’ carnation**

As described in Introduction, we anticipated that the ‘Mini-tiara’ carnation inherited two ACS1 genes from a virtual group of several ACS1 genes consisting of carnation genes (DcACS1a and DcACS1b) and D. knappii (DkACS1b.1, DkACS1b.2, and DkACS1b.3) and an undisclosed wild pink.

In the previous study, Satoh et al. (2011) revealed that primer sets used for PCR amplification of regions 1 to 4 from DcACS1 gave amplificates with comparable sizes to genomic DNAs from three other wild-type Dianthus species, including seven accessions in total, when they were used as templates. Therefore, we could...
expect that PCR amplification with the same primer sets would give amplificates of sizes almost similar to those from *DcACS1a* and *DcACS1b*. Figure 3 shows the PCR product profiles of region 1, region 2, region 3, and region 4, which contained intron 1, intron 2, and 3'-UTR of *DcACS1a* or *DcACS1b*, obtained from genomic PCR of various ‘Mini-tiara’ sub-cultivars. Band patterns of PCR products for regions 1 to 4 differed from the ‘Mini-tiara’ sub-cultivar. The band patterns could be explained by assuming that PCR products were derived from the combination of two *ACS1* genes as shown below. The results of analyses for the combination of two *ACS1* genes are summarized in Table 3, by comparing the predicted sizes of the respective PCR products with the actual sizes of carnation (*DcACS1a* and *DcACS1b*) and *D. knappii* (*DkACS1b.1*, *DkACS1b.2*, and *DkACS1b.3*) *ACS1* genes.

‘Mini-tiara 0707’ gave PCR products of 510 nt, 950 nt, and 730 nt for regions 1, 2, and 3, respectively. This of PCR product profile appearance indicated that ‘Mini-tiara 0707’ had only one gene, *DcACS1a*. ‘Mini-tiara Ruby’ gave a PCR product of 510 nt for region 1, two PCR products of 680 and 950 nt for region 2, a PCR product of 730 nt for region 3, and a PCR product of 650 nt for region 4. This PCR product pattern appearance could be explained when ‘Mini-tiara Ruby’ had *DcACS1a*, as well as *DkACS1b.1* and/or *DkACS1b.3*. ‘Mini-tiara Topaz’ gave two PCR products of 510 nt and 590 nt for region 1, two PCR products of 950 nt and 630 nt for region 2, and a PCR product of 730 nt for region 3, and a PCR product of 650 nt for re-
region 4. From these PCR product patterns appearance, ‘Mini-tiara Topaz’ was thought to contain DcACS1a and one unknown gene. Later, by DNA sequencing, this unknown gene was verified to be DcACS1b. Therefore, ‘Mini-tiara Topaz’ had DcACS1a and DcACS1b. ‘Mini-tiara Lilac’ gave a PCR product of 590 nt for region 1, two PCR products of 630 nt and 680 nt for region 2, and two PCR products of 650 nt and 670 nt for region 4. This PCR product profile suggested that ‘Mini-tiara Lilac’ had DcACS1b and DkACS1b.2. The remaining sub-cultivars ‘Mini-tiara Pink’, ‘Mini-tiara Baby pink’, ‘Mini-tiara Coral pink’, ‘Mini-tiara Milk white’, and ‘Mini-tiara Star yellow’, except for ‘Mini-tiara Sunny’, gave two PCR products of 510 nt and 590 nt for region 1, two PCR products of 630 nt and 590 nt for region 2, and two PCR products of 650 nt and 670 nt for region 4. This PCR product pattern suggested that these ‘Mini-tiara’ sub-cultivars had DcACS1b, as well as DkACS1b.1 and/or DkACS1b.3. Only for ‘Mini-tiara Sunny’, regions 1, 2, and 4 were PCR-cloned using genomic DNA obtained from this sub-cultivar as a template, and their nucleotide sequences and numbers were determined. ‘Mini-tiara Sunny’ gave two PCR products of 589 nt and 520 nt for region 1, two PCR products of 629 nt and 692 nt for region 2, and two PCR products of 670 nt and 682 nt for region 4. The combination of PCR products of 589 nt, 629 nt, and 670 nt for regions 1, 2, and 4, respectively, suggested the presence of DcACS1b in this sub-cultivar. On the other hand, the combination of PCR products of 520 nt, 693 nt, 682 nt for regions 1, 2, and 4, respectively, did not fit any of the ACS1 genes of carnation and D. knappii. The corresponding genomic gene may come from the second undisclosed wild pink used for the interspecific cross.

### Discussion

Previously, Harada et al. (2011) revealed that carnation has two variants (paralogous genes) of the DcACS1 gene, DcACS1a, and DcACS1b. Both genes have five exons and four introns, and have almost identical nucleotide sequences in exons, but not in some introns and 3’-UTR. Genomic PCR analysis of 32 carnation cultivars showed that 25 cultivars had only DcACS1a and DcACS1b. Previous analysis of transcript accumulation revealed that both DcACS1a and DcACS1b are expressed at a similar level in senescing petals of the ‘LPB’ carnation, suggesting that DcACS1a and DcACS1b behave as codominant genes (Harada et al., 2011).

The two variants have been suspected to be alleles, but direct evidence has not been obtained until recently. In the present study, we aimed to obtain collateral evidence to support their allelic nature by analyzing the inheritance of two variant genes. A previous study showed that there were two groups of carnation cultivars in which one group had only the DcACS1a gene and the other had both the DcACS1a and DcACS1b.

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**Table 3.** Estimation of ACS1 genes contained in sub-cultivars of ‘Mini-tiara’ carnation by sizes of PCR products.

| Species and subspecies | Region 1 | Region 2 | Regions 3 and 4 | Gene          |
|------------------------|---------|---------|-----------------|--------------|
| Carnation and wild pink |         |         |                 |              |
| D. caryophyllus ‘LPB’  | 510     | 950     | 730             | DcACS1a      |
|                        | 589     | 629     | 670             | DcACS1b      |
| D. knappii             | 492     | 694     | 655             | DkACS1b.1    |
|                        | 584     | 686     | 652             | DkACS1b.2    |
|                        | 504     | 688     | 646             | DkACS1b.3    |
| ‘Mini-tiara’ sub-cultivars |       |         |                 |              |
| 0707                   | 510     | 950     | 730             | DcACS1a      |
| Ruby                   | 510     | 950     | 730             | DcACS1a      |
|                        | 510     | 680     | 650             | DkACS1b.1 and/or DkACS1b.3 |
| Topaz                  | 510     | 950     | 730             | DcACS1a      |
|                        | 590     | 630     | 650             | Unknown*     |
| Lilac                  | 590     | 630     | 670             | DcACS1b      |
| Pink, Baby pink, Coral pink, Milk white, Star yellow | 590 | 630 | 670 | DcACS1b |
|                        | 510     | 680     | 650             | DkACS1b.2    |
| Sunny                  | 589     | 629     | 670             | DcACS1b      |
|                        | 520     | 692     | 682             | Unknown*     |

* revealed separately as DcACS1b by DNA sequencing.

The sizes for regions 1–4 were determined by DNA sequencing for D. caryophyllus ‘LPB’ (Harada et al., 2011), D. knappii and ‘Mini-tiara Sunny’. Whereas, those for other ‘Mini-tiara’ sub-cultivars, excepting ‘Mini-tiara Sunny’, were estimated from the sizes of PCR products by genomic PCR shown in Figure 3. With ‘Mini-tiara Sunny’, regions 1, 2, and 4 were PCR-cloned and their nucleotide sequences were determined.
genes (Harada et al., 2011), suggesting that the former group is a homozygote of the \textit{DeACS1a} gene and the latter is a heterozygote of \textit{DcACS1a} and \textit{DcACS1b}. In the present study, we first looked for the third group with only \textit{DcACS1b}. Genomic PCR analyses showed that the ‘Skyline’ and ‘Scarlett Plus’ cultivars had only the \textit{DcACS1b} gene (Fig. 1), and suggested that these two cultivars were homozygotes of the \textit{DcACS1b} gene. The probable presence of homozygotes, \textit{DcACS1a/ACACS1a} and \textit{DcACS1b/DcACS1b}, and a heterozygote of \textit{DcACS1a/DcACS1b} suggest that both genes are alleles, and inherited or segregated according to Mendelian heredity.

The ‘Mini-tiara’ carnation is a group of hybrid carnations and their descendants obtained by an interspecific cross between carnation and two species of wild pink. One of the wild pink species was thought to be \textit{Dianthus knappii}. We cloned three \textit{ACS1} genes from \textit{D. knappii} homologous to \textit{DcACS1b}; i.e., \textit{DkACS1b.1}, \textit{DkACS1b.2}, and \textit{DkACS1b.3}. All three genomic genes consisted of five exons and four introns, similar to those cloned previously from \textit{Dianthus} species (Harada et al., 2011). These three genes were almost identical and resembled \textit{DcACS1b} in genomic DNA structure (Tables 1 and 2) and predicted amino acid sequence (Fig. 2).

The fact that carnation \textit{DcACS1a} and \textit{DcACS1b} genes are alleles implies that the three \textit{DkACS1b} genes may also be alleles. On the assumption that \textit{D. knappii} is also a diploid plant and the three genes are alleles, two out of three genes board on one of two homologous chromosomes and the remaining gene on its counterpart chromosome. The impossible discrimination between \textit{DkACS1b.1} and \textit{DkACS1b.3} in ‘Mini-tiara’ sub-cultivars, such as ‘Ruby’, ‘Pink’, ‘Baby pink’, ‘Coral pink’, ‘Milk white’, and ‘Star yellow’ suggested that these two genes were on the same chromosome and were formed by duplication of an original gene on the same chromosome.

The composition of \textit{ACS1} homologs was analyzed in ‘Mini-tiara’ carnations by genomic PCR amplification of regions containing intron 1, intron 2, and 3’-UTR. Each sub-cultivar of ‘Mini-tiara’ carnation had two \textit{ACS1} homologous genes with various combinations; for instance, ‘Mini-tiara 0707’ had only \textit{DcACS1a} (suggesting a \textit{DcACS1a/DcACS1a} homozygote), ‘Mini-tiara Topaz’ had \textit{DcACS1a} and \textit{DcACS1b}, ‘Mini-tiara Ruby’ had \textit{DcACS1a} and \textit{DkACS1b.1} (or \textit{DkACS1b.3}), and ‘Mini-tiara Lilac’ had \textit{DcACS1b} and \textit{DkACS1b.2}. These findings suggest that the ‘Mini-tiara’ carnation had two genes from a virtual group of at least five \textit{ACS1} gene homologs of carnation and \textit{D. knappii}.

In conclusion, two carnation \textit{DcACS1} gene variants, \textit{DcACS1a} and \textit{DcACS1b}, were confirmed to be alleles by analysis of inheritance characteristics, that is, the discovery of probable \textit{DcACS1b} homozygotes of the carnation cultivars ‘Skyline’ and ‘Scarlett Plus’, and the inheritance of these two genes in the ‘Mini-tiara’ carnation, which is an interspecific carnation between carnation and wild pink.

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