Possible Origin of Two Variants of a Carnation 1-Aminocyclopropane-1-carboxylate Synthase Gene, *DcACS1a* and *DcACS1b*, as Suggested by Intron Structure in Homologous Genes in *Dianthus* Species

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The intron structures of two variants of 1-aminocyclopropane-1-carboxylate synthase (ACS) genes (*DcACS1a* and *DcACS1b*) in carnation (*Dianthus caryophyllus*) and genes homologous to them (*ACS1* homologous genes) in other 10 *Dianthus* species (16 strains in total) were studied by comparing the sizes of the PCR amplicates and nucleotide sequence of the introns. All 16 sequenced homologous *ACS1* genes, including *DcACS1* genes themselves, had five exons and four introns. The exons had similar nucleotide sequences and consequently similar deduced amino-acid sequences. The sizes of three introns (intron-1, -2, -3) were variable among the homologous genes, whereas that of the fourth intron (intron-4) was almost identical. The variation in introns was probably caused by the insertion (and deletion) of nucleotide fragments of given lengths. Interestingly, the 3'-UTR of *DcACS1a* was different from that of *DcACS1b*, and the latter was similar to other 14 *ACS1* homologous genes. Moreover, the length of Thr repeat in the C-terminal region was long in *DcACS1a* protein but short in *DcACS1b* protein, and the latter resembled *ACS1* homologous proteins in other *Dianthus* species. The present findings suggest that (1) the variation in intron structure between two variants of carnation *DcACS1* is reminiscent of the variation that occurred universally in *Dianthus* species, (2) *DcACS1a* is probably a gene intrinsic to carnation, and (3) *DcACS1b* was acquired from another, as yet unknown, *Dianthus* species, in the course of breeding modern carnation cultivars.

Key Words: *ACS1* homologous genes, 1-aminocyclopropane-1-carboxylate synthase, carnation, *Dianthus* species, intron variation.

Introduction

Senescence in carnation (*Dianthus caryophyllus* L.) flowers is characterized by autocatalytic ethylene production, mostly in petals, of the flowers, and wilting of the petals induced by the evolved ethylene. Genus *Dianthus* belongs to the Caryophyllaceae and contains more than 300 species, including carnation and pinks (Galbally and Galbally, 1997). Woltering and van Doorn (1988) showed that flowers of all Caryophyllaceae plants investigated were highly sensitive to ethylene and their petals wilted after treatment with exogenous ethylene.

Satoh et al. (2008) confirmed this phenomenon in flowers of *D. superbus* var. *longicalycinus* 'Metia Pink'.

Ethylene is synthesized in plant cells via the following pathway: L-methionine → S-adenosyl-L-methionine (AdoMet) → 1-aminocyclopropane-1-carboxylate (ACC) → ethylene. The conversion of AdoMet to ACC is a rate-limiting step in ethylene biosynthesis and is catalyzed by ACC synthase (ACS) (Yang and Hoffman, 1984). ACS genes have been cloned from many plant species, including tomato (Rottmann et al., 1991) and *Arabidopsis* (Yamagami et al., 2003), and form a multigene family.

Three cDNAs encoding ACS have been cloned from carnation (Park et al., 1992; Henskens et al., 1994), indicating that ACS genes form a family in carnations. These three genes are named *DcACS1*, *DcACS2*, and *DcACS3*. *DcACS1* was shown to be mainly responsible

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for autocatalytic ethylene production in the petals (Jones, 2003; Jones and Woodson, 1999; Satoh, 2011; Shibuya and Ichimura, 2010; ten Have and Woltering, 1997); however, the genomic DNA structure of carnation ACS genes had not been reported until fairly recently when Harada et al. (2011) investigated the structure of *DcACS1* gene.

Harada et al. (2011) examined the genomic DNA structure of *DcACS1* isolated from 'Light Pink Barbara' carnation, and showed that *DcACS1* has two variants, distinguishing *DcACS1a* from *DcACS1b*. *DcACS1a* and *DcACS1b* genes consisted of five exons and four introns. Two genes had almost identical exons, but there were differences in the introns and 3'-UTR.

Moreover, they found heterogeneity of *DcACS1* gene variants among carnation cultivars; that is, some cultivars such as 'White Sim', 'Scania', 'Nora' and so on had only *DcACS1a*, whereas others such as 'Light Pink Barbara', 'Tanga', 'Kibo' and so on had both *DcACS1a* and *DcACS1b*. The cDNA coding for the carnation *DcACS1* was first cloned as *CARACC3* from 'White Sim' carnation (Park et al., 1992), and this was later confirmed by Henskens et al. (1994). Recently we confirmed that the structure and nucleotide sequence of genomic DNAs of *DcACS1* genes in 'Scania' and 'Nora' carnations, which belong to the 'Sim' group of carnation, are identical to those of *DcACS1a* gene in 'Light Pink Barbara' carnation (unpublished results).

Furthermore, Harada et al. (2011) identified orthologous *ACS1* genes, *DsuACS1a* and *DsuACS1b* [hereafter, *DsACS1a(MP)* and *DsACS1b(MP)*], which are involved in autocatalytic ethylene production in petals of *D. superbus* var. *longicalycinus* 'Metia Pink'. The exons, except for exon-5, in *DsACS1a(MP)* and *DsACS1b(MP)* were almost identical to those in *DcACS1a* and *DcACS1b*, the four genes giving ACS proteins with almost identical deduced amino-acid sequences. On the other hand, the introns varied in size and nucleotide sequence, and could be classified into one or two groups depending on the respective introns. More interestingly, the nucleotide sequences in 3'-UTR differed between *DcACS1a* and *DcACS1b*, and that of the latter resembled closely those in *DsACS1a(MP)* and *DsACS1b(MP)*. These findings suggested a close relation between carnation *ACS1* genes and *ACS1* homologous genes in 'Metia Pink'.

Recently, Besse et al. (2009) investigated the intron length in caffeic acid *O*-methyltransferase (COMT) in *Vanilla* (Orchidaceae) and other plant species and discussed the evolution of the COMT genes. Elucidation of the variation and relationship of *ACS1* homologous genes in *Dianthus* species, in addition to carnation and *D. superbus* var. *longicalycinus*, would help elucidate the possible origin of *DcACS1a* and *DcACS1b* in carnation. In the present study, therefore, to gain further insight into the presence of *DcACS1* variants in carnation, we carried out a comparative investigation of

the exon and intron structures of homologous *ACS1* genes in *Dianthus* species by comparing the sizes of PCR amplicates and nucleotide sequences of the genes.

Combining the present findings with those obtained previously for carnation and *D. superbus* var. *longicalycinus* 'Metia Pink' (Harada et al., 2011), we considered the possible origin of two *DcACS1* variants in carnation.

Materials and Methods

Plant materials

The genus *Dianthus* consists of 300 species (Galbally and Galbally, 1997), and we examined 17 accessions of the genus *Dianthus* in the present study (Table 1). Carnation (*Dianthus caryophyllus* L.) 'Light Pink Barbara (LPB)' and *D. superbus* L. var. *longicalycinus* 'Metia Pink' (Sakata Seeds Corp., Yokohama, Japan) were the same as used previously (Harada et al., 2011). The other two accessions of *D. superbus* var. *longicalycinus*, TG-1 and TG-2, were collected in the field in Kyoto prefecture, Japan, and one accession of *D. chinensis* var. *laciniatus* KP, was purchased at a wildflower store in Kyoto prefecture. The other 12 accessions were obtained from the collection of *Dianthus* plants maintained at Kyoto Botanical Garden (KBG), Kyoto, Japan.

DNA extraction

Genomic DNA was extracted from petals or young leaves of *Dianthus* species according to the method described by Liu et al. (1995).

PCR amplification of introns and 3'-UTR regions in *ACS1* homologous genes in *Dianthus* species

PCR was performed using rTaq DNA polymerase (Takara, Otsu, Japan) with 25 ng genomic DNA as a template in a 10 µL reaction mixture. The previous paper (Harada et al., 2011) showed the forward and reverse primer pairs used for amplification of intron-1 and intron-2, and 3'-UTR region-1 (found only in *DcACS1a*) and 3'-UTR region-2 [found in *DcACS1b*, *DsACS1a(MP)* and *DsACS1b(MP)*]. The forward and reverse primer pairs used for amplification of intron-3 and intron-4 were newly designed in the present study. Figure 1 shows the positions on the *DcACS1a* gene as an example, where the forward and reverse primer pairs were set and their nucleotide sequences. 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 on 2% agarose gel by electrophoresis and visualized by ethidium bromide staining; however, the conditions for amplification of intron-3 were 94°C for 4 min followed by 35 cycles of 30 sec at 94°C, 30 sec at 53°C, and 3 min at 68°C. PCR products were separated on 1% agarose gel.

Cloning of genomic DNAs of *ACS1* homologous genes in *Dianthus* species

Previously, genomic DNAs for *DcACS1a* and *DcACS1b* in 'Light Pink Barbara' carnation and

Table 1. Summary of *Dianthus* accessions with species names and, if available, cultivar names and strain numbers, region of collection, and *ACS1* homologous genes sequenced.

Plant No.	<i>Dianthus</i> species	Cultivar or strain	Region of collection		<i>ACS1</i> homologous genes sequenced ^z
			Place where the accession was obtained ^y	Origin of the accession	
1	<i>D. caryophyllus</i>	‘Ligh Pink Barbara’	Miyagi Prefecture		<i>DcACS1a</i> , <i>DcACS1b</i>
2	<i>D. superbus</i> var. <i>longicalycinus</i>	‘Metia Pink’	KAFTC		<i>DsACS1a(MP)</i> , <i>DsACS1b(MP)</i>
3		KBG-1	KBG	Japan	<i>DsACS1(KBG-1)</i>
4		KBG-2	KBG	Japan	<i>DsACS1(KBG-2)</i>
5		TG-1	Kyoto Prefecture	Japan	<i>DsACS1a(TG-1)</i> , <i>DsACS1b(TG-1)</i>
6		TG-2	Kyoto Prefecture	Japan	— ^w
7	<i>D. chinensis</i> var. <i>laciniatus</i>	KP	Kyoto Prefecture	—	—
8		KBG	KBG	China	<i>DchACS1(KBG)</i>
9	<i>D. caesisus</i>	Blaureif SB-475	KBG	—	—
10	<i>D. petraeus</i>	KBG-1	KBG	Hungary	<i>DpACS1a(KBG-1)</i> , <i>DpACS1b(KBG-1)</i> , <i>DpACS1c(KBG-1)</i>
13		KBG-2	KBG	Southwest Europe	<i>DpACS1a(KBG-2)</i> , <i>DpACS1b(KBG-2)</i> , <i>DpACS1c(KBG-2)</i> , <i>DpACS1d(KBG-2)</i>
11	<i>D. giganteus</i>	— ^x	KBG	Balkan peninsula	—
12	<i>D. plumaris</i>	—	KBG	Southeast Europe	—
14	<i>D. alwoodii alpines</i>	—	KBG	—	—
15	<i>D. integer</i>	—	KBG	—	—
16	<i>D. haematocalyx</i>	—	KBG	Balkan peninsula	—
17	<i>D. gallicus</i>	—	KBG	Southwest Europe	—

^z*DcACS1* homologous genes for which nucleotide sequences were determined.^yKAFTC, Kyoto Agriculture and Forestry Technology Center; KBG, Kyoto Botanical Garden.^{x,w} —, not available or not determined.

DsACS1a(MP) and *DsACS1b(MP)* in *D. superbus* var. *longicalycinus* ‘Metia Pink’ were obtained by PCR cloning (Harada et al., 2011). Additionally, 12 other genomic DNAs for *ACS1* homologous genes in *Dianthus* species were cloned in the present study. In brief, to obtain full-length composite genomic DNAs for *ACS1* homologous genes, we used a nearly 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, full-length genomic DNAs were amplified with primers derived from both ends of the composite genomic DNAs and total DNAs obtained from corresponding *Dianthus* species. The forward primer was 5'-CTT ATA AGG GTG TTT ACG ACC GTG A-3', and the reverse primer was 5'-GGC ATC AAC AAT TAA TCA CTC T-3'; however, another reverse primer, 5'-CAA TGA TCT TAC ACA TGA CTA C-3', was used for cloning *DcACS1a* genomic DNA (Harada et al., 2011) (Fig. 1). PCR products covered from exon-1, actually seven nucleotides downstream from the A of starting codon ATG, until 3'-UTR. The amino-acid sequences of *ACS1* homologs

were deduced from the nucleotide sequence of the PCR products combined with the nucleotide sequence obtained by 5'-RACE (Fig. 2).

Sequence analysis and accession numbers

Nucleotide sequencing was carried out using the BigDye terminator v3.1 Cycle Sequencing Kit (PE Biosystems, Foster City, USA) and ABI PRISM 310 Genetic Analyzer (PE Biosystems). Nucleotide sequences were edited and analyzed with GENETYX-WIN and homology search was conducted with the BLASTX program on 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 with accession numbers from AB597941 to AB597948.

Accession numbers and names of respective genes whose nucleotide sequences were determined in this study, are as follows: AB597941 for *DpACS1a(KBG-2)*, AB597942 for *DpACS1b(KBG-2)*, AB597943 for *DpACS1c(KBG-2)*, AB597944 for *DpACS1d(KBG-2)*, AB597945 for *DpACS1a(KBG-1)*, AB597946 for *DpACS1b(KBG-1)*, AB597947 for *DpACS1c(KBG-1)*, AB597948 for *DchACS1(KBG)*. In addition, the

Fig. 1. Structure of *DcACS1a* and positions where the forward and reverse primer pairs were set for amplifying respective introns, 3'-UTR, nearly full-length DNAs, and 5'-UTR of genomic DNAs for *ACS1* homologous genes in *Dianthus* species and their nucleotide sequences. Primer pairs were aligned on carnation *DcACS1a* gene.

^z used for amplifying 3'-UTR of *DcACS1a*

^y used for amplifying 3'-UTR of *DcACS1b* and *ACS1* homologous genes of *Dianthus* species other than carnation

^x used for amplifying nearly full-length genomic DNA of *ACS1* homologous genes of *Dianthus* species

^w used for amplifying 5'-UTR, including the start codon, of *ACS1* homologous genes of *Dianthus* species

Fig. 2. PCR amplification of intron-1, -2, -3, -4 and 3'-UTR of *ACS1* homologous genes in *Dianthus* species. Sample numbers, as indicated in Table 1, are shown above each lane. Results for 'Light Pink Barbara' carnation (No. 1) and *D. superbis* var. *longicalycinus* 'Metia Pink' were the same as shown previously (Harada et al., 2011). Each set of forward and reverse primers, as described in Figure 1, was used for PCR amplification of the respective introns, but for 3'-UTR amplification we used two sets of forward and reverse primers, which were originally designed for separate amplification of 3'-UTRs of *DcACS1a* and *DcACS1b* (Harada et al., 2011).

accession numbers and names of the genes, which were reported in the previous paper (Harada et al., 2011), are as follows: AB543556 for *DcACS1a*, AB543557 for *DcACS1b*, AB543544 for *DsACS1(MP)*, previously *DsuACS1a*, AB543546 for *DsACS1b(MP)*, previously *DsuACS1b*, AB543548 for *DsACS1a(TG-1)*, previously

DsuACS1c, AB543550 for *DsACS1b(TG-1)*, previously *DsuACS1d*, AB543552 for *DsACS1(KBG-1)*, previously *DsuACS1e*, AB543554 for *DsACS1(KBG-2)*, previously *DsuACS1f*. The gene names of *D. superbis* var. *longicalycinus* were changed to show the plants from which respective genes were obtained.

Results

Intron amplification and size variations in different Dianthus species

Genes homologous to carnation *DcACS1* (*ACS1* homologous genes) might exist as a multigene family (in terms of introns) in all *Dianthus* species, as indicated by the previous observation with carnation (*D. caryophyllus*) and *D. superbus* var. *longicalycinus* ‘Metia Pink’ (Harada et al., 2011). We amplified DNA fragments containing the respective introns and 3'-UTR region with 11 *Dianthus* species (17 accessions in total), including those used in the previous study (Harada et al., 2011). Each set of forward and reverse primers was designed from the nucleotide sequences in the flanking exons and 3'-UTR of *DcACS1a* and *DcACS1b* genes (Fig. 1). PCR amplification was repeated twice and the patterns were highly reproducible. PCR amplification revealed one to three size variants for intron-1, one to four size variants for intron-2, one to three size variants for intron-3, one size variant for intron-4, and one to two nucleotide sequence variants for 3'-UTR (Fig. 2).

Size variants of intron-1, -2, and -3 were found among and within species. Roughly speaking, for intron-1, each *Dianthus* plant had two size variants (ca. 200 and 300 bp PCR products) or had only one of the two size variants. As reported previously (Harada et al., 2011), ‘Light Pink Barbara’ carnation and *D. superbus* var. *longicalycinus* ‘Metia Pink’ had two size variants (ca. 200 and 300 bp PCR products) for intron-1. Interestingly, *D. superbus* var. *longicalycinus* KBG-1 and *D. superbus* var. *longicalycinus* KBG-2 had only one size variant (ca. 200 bp PCR product) and *D. superbus* var. *longicalycinus* TG-1 and *D. superbus* var. *longicalycinus* TG-2 another size variant (ca. 300 bp PCR product) for intron-1.

For intron-2, ‘Light Pink Barbara’ carnation and *D. superbus* var. *longicalycinus* ‘Metia Pink’ had two size variants (ca. 500 and 750 bp PCR products) as reported previously (Harada et al., 2011). *D. superbus* var. *longicalycinus* KBG-1, *D. superbus* var. *longicalycinus* KBG-2, and *D. superbus* var. *longicalycinus* TG-1 had one size variant (ca. 500 bp PCR product), whereas *D. superbus* var. *longicalycinus* TG-2 had both size variants. Other *Dianthus* species showed the co-existence of two size variants or existence of one of the two size variants.

For intron-3, ‘Light Pink Barbara’ carnation showed two size variants (ca. 2 k and 3 kbp PCR products); however, *D. superbus* var. *longicalycinus* exhibited complex patterns for intron-3; *D. superbus* var. *longicalycinus* ‘Metia Pink’ had a size variant (ca. 3 kbp PCR product), whereas *D. superbus* var. *longicalycinus* KBG-2 and *D. superbus* var. *longicalycinus* TG-2 had another size variant (ca. 2 kbp PCR product). Moreover, *D. superbus* var. *longicalycinus* KBG-1 showed two size variants of similar sizes (both around 2 kbp PCR products), and *D. superbus* var. *longicalycinus* TG-1 had three size

variants (one 3 kbp and two ca. 2 kbp PCR products). Furthermore, in most other *Dianthus* species, size variation for intron-3 ranged between 2 kbp and 3 kbp, as found in ‘Light Pink Barbara’ carnation and *D. superbus* var. *longicalycinus*.

For intron-4, all *Dianthus* species and strains showed the same amplification pattern as carnation, giving ca. 120 bp PCR product (Fig. 2). For 3'-UTR, two sets of PCR primers gave PCR products of 250 bp and 200 bp only with ‘Light Pink Barbara’ carnation; however, only one set of PCR primers could amplify a 3'-UTR fragment giving 200 bp PCR products in other *Dianthus* species.

Comparison of nucleotide sequences of exons and introns in ACS1 homologous genes in different Dianthus species

Nucleotide sequencing of the introns will certainly allow the molecular basis of the observed intron length variation; therefore, we cloned genomic DNAs of *ACS1* homologous genes from *Dianthus* species and determined their nucleotide sequences.

Previously Harada et al. (2011) determined the nucleotide sequences of eight *ACS1* homologous genes: two genes (*DcACS1a* and *DcACS1b*) in ‘Light Pink Barbara’ carnation; two genes [*DsACS1a*(MP) and *DsACS1b*(MP)] in *D. superbus* var. *longicalycinus* ‘Metia Pink’; two genes [*DsACS1a*(TG-1) and *DsACS1b*(TG-1)] in *D. superbus* var. *longicalycinus* TG-1; one gene [*DsACS1*(KBG-1)] in *D. superbus* var. *longicalycinus* KBG-1, and one gene [*DsACS1*(KBG-2)] in *D. superbus* var. *longicalycinus* KBG-2 (Table 1).

In the present study, we further determined the nucleotide sequences of another eight *ACS1* homologous genes in *D. chinensis* var. *laciniatus* KBG and *D. petraeus* KBG-1 and KBG-2, since their *ACS1* homologous genes were expected to exhibit heterogeneity among and within species. The genes determined were *DchACS1*(KBG) from *D. chinensis* var. *laciniatus* KBG; *DpACS1a*(KBG-1), *DpACS1b*(KBG-1), and *DpACS1c*(KBG-1) from *D. petraeus* KBG-1; *DpACS1a*(KBG-2), *DpACS1b*(KBG-2), *DpACS1c*(KBG-2), and *DpACS1d*(KBG-2) from *D. petraeus* KBG-2 (Table 1). Nucleotide numbers of predicted exons, introns, and 3'-UTRs for respective genomic DNAs are summarized in Table 2.

All the *ACS1* homologous genes sequenced so far had five exons and four introns, as reported previously (Harada et al., 2011). The predicted cDNAs were deduced from these genomic sequences by splicing out the introns and, in turn, the deduced amino-acid sequences were aligned to the 518 amino acids of *DcACS1a* (Fig. 3). *ACS1* homologous proteins consisted of 498 to 518 amino acids and their amino-acid sequences were highly conserved; similarities to *DcACS1a* ranged from 90.0% [*DpACS1d*(KBG-2)] to 97.7% (*DcACS1b*). The amino-acid residues around the active-site lysine (Lys-278 in *DcACS1a*) were conserved among 16 *ACS1*

Table 2. Comparison of genomic DNA structures of *ACS1* homologous genes in *Dianthus* species.

Species	Gene	Exon-1	Intron-1	Exon-2	Intron-2	Exon-3	Intron-3	Exon-4	Intron-4	Exon-5	3'-UTR
<i>D. caryophyllus</i> 'LPB'	<i>DcACS1a</i>	174	217	132	769	161	2962	558	119	529	248
	<i>DcACS1b</i>	174	296	132	445	161	2891	558	119	511	225
	<i>DsACS1a(MP)</i>	174	225	132	495	161	3096	558	119	487	209
	<i>DsACS1b(MP)</i>	174	305	132	763	161	3090	558	119	487	209
<i>D. superbis</i> var. <i>logicarycinus</i>	<i>DsACS1(KBG-1)</i>	174	225	132	502	161	2121	558	119	487	209
	<i>DsACS1(KBG-2)</i>	174	225	132	504	161	1959	558	119	484	209
	<i>DsACS1a(TG-1)</i>	174	305	132	755	161	3094	558	119	487	209
	<i>DsACS1b(TG-1)</i>	174	305	132	1188	161	1905	558	119	484	209
<i>D. chinensis</i> var. <i>lacinatus</i>	<i>DchACS1(KBG)</i>	174	228	132	502	158	ca. 3 k	555	119	487	226
	<i>DpACS1a(KBG-1)</i>	174	203	129	741	158	ca. 2 k	549	119	496	207
	<i>DpACS1b(KBG-1)</i>	174	209	132	502	156	ca. 2 k	546	119	496	202
	<i>DpACS1c(KBG-1)</i>	174	223	132	419	161	ca. 2 k	537	119	490	213
<i>D. petraeus</i>	<i>DpACS1a(KBG-2)</i>	174	203	132	544	161	1980	558	119	493	206
	<i>DpACS1b(KBG-2)</i>	174	209	132	525	161	1908	558	113	493	217
	<i>DpACS1c(KBG-2)</i>	174	203	132	767	161	ca. 2 k	558	119	496	205
	<i>DpACS1d(KBG-2)</i>	174	191	132	514	158	ca. 2 k	547	119	496	200

Fig. 3. Alignment of amino-acid sequences deduced from the predicted cDNAs of *ACS1* homologous genes in *Dianthus* species. Amino-acid sequences of *ACS1* homologous proteins are aligned to that of *DcACS1a*. Common amino acids are shown by dots and gaps by hyphens. The straight line shows the conserved amino acid residues for the enzymes' active site, and the dotted line shows Thr repeat sequences unique to *ACS1* homologous proteins.

homologous proteins. Interestingly, the major difference between *DcACS1a* in carnation and other ACS1 homologous proteins was in the length of the Thr repeat in the C-terminal region, which was coded in exon-5 of the respective genes. The Thr repeat of *DcACS1a* was the longest (18 Thr residues), followed by *DcACS1b* (13 residues), *DpACS1a*(KBG-1) (12 residues), *DpACS1b*(KBG-1) (12 residues), and *DpACS1c*(KBG-2) (12 residues) and so on, making carnation *DcACS1a* distinct; however, the role, if any, of these Thr repeats in ACS1 homologous proteins remains unknown.

Nucleotide sequences of intron-1 were aligned to that of *DcACS1a*, which was temporarily used as a control (Fig. 4). The introns were grouped into shorter (191–225 bp) and longer (296–305 bp) introns. All of the shorter introns appeared similar when indels less than 12 bp were ignored. Three of the four longer introns, those of *DsACS1b*(MP), *DsACS1a*(TG-1), and

DsACS1b(TG-1), had an identical insertion of 96 bp, but the longer intron of *DcACS1b* was different.

The nucleotide sequence profiles of intron-2 were separated into two groups of 445–544 and 749–769 bp, except for a very long intron-2 (1188 bp) in *DsACS1b*(TG-1) and another intron-2 with distinct insertion in *DpACS1a*(KBG-1) (Fig. 5). Interestingly, about two-thirds of the genes had insertion of 61–69 bp of almost identical sequences. The corresponding shorter introns appeared similar, except for these insertions. On the other hand, the longer introns had insertions of around 250 bp, although their nucleotide sequences were different.

Regarding intron-3, whole nucleotide sequences were determined for 10 ACS1 homologous genes, and partial nucleotide sequences for the remaining 6 genes (Fig. 6). Among 10 genes sequenced, intron-3 was presented as two forms; the intron of 1.9–2.1 kbp and that of 2.9–3.1 kbp. Intron-3s of *DcACS1a* and *DcACS1b*, *DsACS1a*(MP) and *DsACS1b*(MP), and *DcACS1a*(TG-1) fell into the larger group. The intron-3s in two carnation genes, *DcACS1a* and *DcACS1b*, were ca. 2.9 kbp, but had a different nucleotide sequence (variation within a species), and were different from those of *D. superbis* var. *longicalycinus* [*DsACS1a*(MP), *DsACS1b*(MP), and *DsACS1a*(TG-1)] (variation between species).

Intron-4s of all of the sequenced ACS1 homologous genes were 119 bp long, except for those of

Fig. 4. Intron-1 structure of different ACS1 homologous genes in *Dianthus* species. The sequenced genes were named after the species and strains of *Dianthus*, as shown in Table 1. Alignments of intron sequences were analyzed using ClustalW. Regions with nearly identical nucleotide sequences (>90% similarity) among introns were marked with the same color, whereas the regions (around or less than 50% similarity) unique to one intron are shown by different colors. Numbers and arrowheads on the diagram show where the unique sequence, insertion, or deletion starts. Nucleotide numbers for insertions in introns are shown in white, as judged by comparing to the intron of *DcACS1a* (a tentative control for alignment), and deletions in introns are shown by red with minus symbols.

Fig. 5. Intron-2 structure of different ACS1 homologous genes in *Dianthus* species. Details are similar to those shown in the legend to Figure 4, but the tentative control for alignment was intron-2 of *DcACS1b*.

Fig. 6. Intron-3 structure of different *ACS1* homologous genes in *Dianthus* species. Details are similar to those shown in the legend to Figure 4, but the tentative control for alignment was intron-3 of *DcACS1a*. Only partial sequences were determined for genes such as *DchACS1(KBG)*, and the diagrams for the introns are shown with dashed boxes with the sizes predicted from the sizes of PCR amplicates (Fig. 2).

DpACS1b(KBG-2) (113 bp). Nucleotide sequences had high similarity of more than 89.2%. 3'-UTRs varied in size from 202 to 225 bp among *ACS1* homologous genes, except for *DcACS1a*, and the similarity to the 3'-UTR of *DcACS1b* ranged from 77.9 to 90.1%, whereas the 3'-UTR of *DcACS1a* was 248 bp and the similarity to *DcACS1b* was 55.8% (Fig. 7, Table 2).

Discussion

The present observation revealed that the structure of intron-1, -2 and -3 in *Dianthus* species varied in size and nucleotide sequence. Variation in each intron was thought to be caused by the insertion and deletion of short nucleotide sequences and the insertion of long nucleotide fragments. On the other hand, variation in each intron occurred independently among the four introns. Based on these findings, we suggest that variations in intron structures between the two carnation *DcACS1a* and *DcACS1b* genes are reminiscent of those in other *ACS1* homologous genes in *Dianthus* species.

It is interesting to speculate the possible origin of *DcACS1a* and *DcACS1b* in carnation plants. As described in **Introduction**, *DcACS1a* was present in all the carnation cultivars investigated so far, whereas *DcACS1b* was found in restricted cultivars (Harada et

Fig. 7. Comparison of 3'-UTR structure among different *ACS1* homologous genes in *Dianthus* species.

al., 2011), suggesting that carnation plants originally had *DcACS1a*, or more precisely its ancestor gene, and *DcACS1b* appeared as the second gene in some cultivars during the establishment of modern carnation cultivars. The nucleotide sequences in 3'-UTR were different considerably between *DcACS1a* and *DcACS1b*, and that of the latter resembled closely those in *ACS1* homologous genes in other *Dianthus* species. A similar relation was found in the length of the Thr repeat in the C-terminal region of *ACS1* homologous proteins (Fig. 3). *DcACS1a* has a Thr repeat consisting of 18 Thr residues, whereas *DcACS1b* has a Thr repeat consisting of 13 Thr residues. In *ACS1* homologous proteins in other *Dianthus* species, the number of Thr residues ranged from 7 to 12; *DpACS1a(KBG-1)*, *DpACS1b(KBG-1)*, and *DpACS1c(KBG-2)* having Thr repeats consisting of 12 Thr residues. These results suggest that *DcACS1b* did not derive from *DcACS1a* itself, but from other *ACS1* homologous genes in other *Dianthus* species. Based on these findings, we suggest that *DcACS1a* is a gene derived from an ancestor gene intrinsic to carnation, and *DcACS1b* is a gene acquired from another, as yet unknown, *Dianthus* species, in the course of breeding modern carnation cultivars.

Previously, Iwazaki et al. (2004) showed by Southern blotting analysis that 'Nora' carnation, which is a diploid plant (Yagi et al., 2007), has only one copy of the *DcACS1* gene, corresponding to the findings shown later by Harada et al. (2011) that this cultivar had the *DcACS1a*

gene only. Harada et al. (2011) investigated 32 carnation cultivars, including ‘Light Pink Barbara’ and ‘Nora’, and revealed that 25 cultivars have only *DcACSIa* and the remaining 7 cultivars have both *DcACSIa* and *DcACSIb*. Previously, Yagi et al. (2007) showed that 25 of the corresponding 32 cultivars were diploid; the ploidy of the remaining 7 cultivars, all of which had *DcACSIa* only, were not investigated because these cultivars are new and were not available at the time of investigation.

On the other hand, the identification of three *ACSI* homologous genes in *D. petraeus* KBG-1 and four genes in *D. petraeus* KBG-2 (Figs. 4–6, Table 1) may be related to the fact that *D. petraeus* is hexaploid (Ushio et al., 2002). In wild *Dianthus* species, which are hexaploid (Ushio et al., 2002), the multiple presence of *ACSI* homologous genes may be caused by the presence of allelic genes.

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Literature Cited

- Besse, P., D. Da Silva, S. Bory, M. Noirot and M. Grisoni. 2009. Variation in intron length in caffeic acid *O*-methyltransferase (COMT) in *Vanilla* species (Orchidaceae). *Plant Sci.* 176: 452–460.
- Frohman, M. A., M. K. Dush and G. R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* 85: 8998–9002.
- Galbally, J. and E. Galbally. 1997. Carnations and pinks for garden and greenhouse. Timber Press, Portland.
- Harada, T., Y. Murakoshi, Y. Torii, K. Tanase, T. Onozaki, S. Morita, T. Masumura and S. Satoh. 2011. Analysis of genomic DNA of *DcACSI*, a 1-aminocyclopropane-1-carboxylate synthase gene, expressed in senescing petals of carnation (*Dianthus caryophyllus*) and its orthologous genes in *D. superbus* var. *longicalycinus*. *Plant Cell Rep.* 30: 519–527.
- Henskens, J. A. M., G. J. A. Rouwendal, A. ten Have and E. J. Woltering. 1994. Molecular cloning of two different ACC synthase PCR fragments in carnation flowers and organ-specific expression of the corresponding genes. *Plant Mol. Biol.* 26: 453–458.
- Iwazaki, Y., Y. Kosugi, K. Waki, T. Yoshioka and S. Satoh. 2004. Generation and ethylene production of transgenic carnations harboring ACC synthase cDNA in sense or antisense orientation. *J. Appl. Hort.* 6: 67–71.
- Jones, M. L. 2003. Ethylene biosynthetic genes are differentially regulated by ethylene and ACC in carnation styles. *Plant Growth Regul.* 40: 129–138.
- Jones, M. L. and W. R. Woodson. 1999. Differential expression of three members of the 1-aminocyclopropane-1-carboxylate synthase gene family in carnation. *Plant Physiol.* 19: 755–764.
- Liu, Y.-G., N. Mitsukawa, T. Oosumi and R. F. Whittier. 1995. Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* 8: 457–463.
- Park, K. Y., A. Drory and W. R. Woodson. 1992. Molecular cloning of an 1-aminocyclopropane-1-carboxylate synthase from senescing carnation flower petals. *Plant Mol. Biol.* 18: 377–386.
- Rottmann, W. H., G. F. Peter, P. W. Oeller, J. A. Keller, N. F. Shen, B. P. Nagy, L. P. Taylor, A. D. Campbell and A. Theologis. 1991. 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. *J. Mol. Biol.* 222: 937–961.
- 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., N. Suetome, S. Namura and H. Kuwabara. 2008. Prolonged vase life of *Dianthus superbus* by 2-aminoisobutyric acid and *cis*-propenylboric acid. *Flor. Orn. Biotech.* 2: 5–8.
- Shibuya, K. and K. Ichimura. 2010. Depression of autocatalytic ethylene production by high-temperature treatment in carnation flowers. *J. Japan. Soc. Hort. Sci.* 79: 97–102.
- ten Have, A. and E. J. Woltering. 1997. Ethylene biosynthetic genes are differentially expressed during carnation (*Dianthus caryophyllus* L.) flower senescence. *Plant Mol. Biol.* 34: 89–97.
- Ushio, A., T. Onozaki and M. Shibata. 2002. Estimation of polyploidy levels in *Dianthus* germplasms by flowcytometry. *Bull. Natl. Inst. Flor. Sci.* 2: 21–26.
- Woltering, E. J. and W. G. van Doorn. 1988. Role of ethylene in senescence of petals—morphological and taxonomical relationships. *J. Exp. Bot.* 39: 1605–1616.
- Yagi, M., Y. Fujita, T. Yoshimura and T. Onozaki. 2007. Comprehensive estimation of polyploidy level in carnation cultivars by flow cytometry. *Bull. Natl. Inst. Flor. Sci.* 7: 9–16.
- Yamagami, T., A. Tsuchisaka, K. Yamada, W. F. Haddon, L. A. Harden and A. Theologis. 2003. Biochemical diversity among the 1-aminocyclopropane-1-carboxylate synthase isozymes encoded by the *Arabidopsis* gene family. *J. Biol. Chem.* 278: 49102–49112.
- Yang, S. F. and N. E. Hoffman. 1984. Ethylene biosynthesis and its regulation in higher plants. *Ann. Rev. Plant Physiol.* 35: 155–189.