

Generation and ethylene production of transgenic carnations harboring ACC synthase cDNA in sense or antisense orientation

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Abstract

Carnation (*Dianthus caryophyllus* L. cv. Nora) plants, transformed with carnation ACC synthase (*DC-ACS1*) cDNA in sense or antisense orientation (the transgenes are *sACS* transgene and *aACS* transgene, respectively) by *Agrobacterium*-mediated gene transfer were produced. Cut flowers of all the transgenic lines obtained, 2 lines transformed with *sACS* transgene and 3 lines with *aACS* transgene, showed suppressed ethylene production during natural senescence as compared with flowers of the non-transformed control line. Among 5 transgenic lines, the *sACS*-1 line harboring *sACS* transgene had the severest reduction in ethylene production from flowers, and the flowers lost their vase-life with drying and discoloration in the rim of petals, the ethylene-independent deterioration of carnation flowers. DNA gel blot analysis revealed that the loci of *sACS* transgenes in the *sACS*-1 line were different from those in the 16-0-66 line, which is another transgenic line of different origin harboring *sACS* transgene, although both lines have two copies of the transgenes.

Key words: ACC synthase, *Dianthus caryophyllus*, flower senescence, ethylene production, transgenic carnation

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AdoMet, S-adenosyl-L-methionine; DPSS, 1,1-dimethyl-4-(phenylsulfonyl) semicarbazide; STS, silver thiosulfate anionic complex

Introduction

Ethylene is a primary plant hormone involved in the senescence of cut flowers of carnation. A large amount of ethylene is synthesized, mostly from the petals, several days after full opening of the flower during senescence (Borochoy and Woodson, 1989, Abeles *et al.*, 1992, Reid and Wu, 1992, Woodson *et al.* 1992). The increased ethylene production accelerates in-rolling of petals resulting in wilting of the flower. Inhibition of the synthesis or action of ethylene delays the onset of senescence symptoms and increases flower longevity. Blockage of ethylene-induced senescence in flowers is of economical importance. Although preservatives that inhibit the synthesis or action of ethylene such as STS (Veen, 1979) or DPSS (Midoh *et al.*, 1996) are currently being used to lengthen the vase life of cut carnation flowers, it has been recognized that transgenic carnations with suppressed production or action of ethylene will be excellent alternatives for the preservation of flower longevity. So far, genetic transformation with transgenes related to the biosynthesis and action of ethylene has been successfully used to down-regulate ethylene production or responsiveness to ethylene in carnations (Savin *et al.*, 1995, Bovy *et al.*, 1999, Kosugi *et al.*, 2000). Cut flowers of these transgenic lines have a prolonged vase life compared with those of the non-transgenic plants.

Previous researchers generated carnation lines with various traits; e.g., the lines transformed with a carnation ACC oxidase cDNA in antisense orientation (Savin *et al.*, 1995) or in sense orientation (Kosugi *et al.* 2000), and the line harboring an *Arabidopsis thaliana etr1-1* allele capable of rendering ethylene insensitivity

in carnation (Bovy *et al.*, 1999). In addition, Florigene Ltd. (Melbourne, Victoria, Australia) allegedly generated many lines of carnation transformed with cDNAs for ethylene biosynthetic enzymes in the middle of 1990's, but few of them have been documented do date. With carnation plants, transgenes that were used for transformation have been limited and characteristics of the suppressed ethylene production in transgenic flowers have not been investigated thoroughly. Therefore, further research is needed to clarify the efficacy of different transgenes and mechanism of function of those genes.

Ethylene is synthesized in plant tissues through the following pathway: L-methionine→AdoMet→ACC→ethylene. ACC synthase and ACC oxidase catalyze the last two reactions (Yang and Hoffman, 1984, Kende, 1993). Three genes encoding ACC synthase (*DC-ACS1*, *DC-ACS2* and *DC-ACS3*) and one gene encoding ACC oxidase (*DC-ACO1*) have been identified from carnation (Wang and Woodson, 1991, Park *et al.*, 1992, Henskens *et al.*, 1994, Jones and Woodson, 1999). These genes are regulated in a tissue-specific manner during flower senescence; *DC-ACO1* is expressed in both the gynoecium and petals of carnation flowers that are undergoing senescence, and *DC-ACS1* is expressed also in both the gynoecium and petals, but mainly in the latter, whereas *DC-ACS2* and *DC-ACS3* occur in the gynoecium (Henskens *et al.*, 1994, ten Have and Woltering, 1997, Jones and Woodson, 1999). *DC-ACS1* and *DC-ACO1* genes play a crucial role in massive ethylene production from petals of carnation flowers undergoing senescence. Thus it is possible to use ACC synthase cDNA in addition to ACC oxidase cDNA to generate transgenic carnations with repressed ethylene production. In the present

study, we aimed to generate carnation plants transformed with ACC synthase (*DC-ACS1*) cDNA in sense or antisense orientation and to examine ethylene production from their flowers during senescence. We also detailed one of transformants (sACS-1 line), harboring ACC synthase cDNA in sense orientation, by comparing with a similar transgenic carnation (16-0-66 line) of different origin.

Materials and methods

Plant materials: Transgenic carnation (*Dianthus caryophyllus* L.) lines transformed with carnation ACC synthase (*DC-ACS1*) cDNA in sense or antisense orientation were generated with a parent cv. Nora as described below. The 16-0-66 line transformed with ACC synthase (*DC-ACS1*) cDNA in sense orientation and its parent cv. Ashley were supplied from Florigene Ltd. (Melbourne, Victoria, Australia).

The transgenic lines and the respective parent cultivars (controls) were cultured in a containment greenhouse. Lateral shoots of about 8 cm in length were detached from parent carnation plants and planted in a commercial potting medium (Horticultural Soil, Kureha Chemical Industry Co., Ltd., Tokyo) in a plastic container (25 cm in width, 60 cm in length and 15 cm in depth; 18 liter soil). The plants were grown under 14-h days of 23°C with average irradiance of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 10-h nights of 18°C. Plants were fertilized monthly with 7 g per container commercial fertilizer granules (12N-12P-12K-1Mg). Flowering started 4-5 months after the planting depending on cultivars and transgenic lines, and flowers were harvested during the following 5-6 months. Only the first and second flowers opening on each stem were used.

***DC-ACS1* cDNA constructs and transformation of carnation:** Cloning of carnation ACC synthase cDNA (*DC-ACS1*) was described previously (Kosugi *et al.*, 2000). The cDNA was 1566 bp in size and contained an open reading frame (1554-bp) that shares 99.6% similarity with *DC-ACS1* cDNA (formerly CARACC3, GenBank Accession No. M66619, Park *et al.*, 1992). For the transformation of carnation plant, binary vectors in

combination with *DC-ACS1* cDNA in sense or antisense orientation (*sACS* transgene and *aACS* transgene, respectively) and a vector pIG121-Hm (Ohta *et al.*, 1990) were prepared as shown in Fig. 1. *DC-ACS1* cDNA fragment was recovered from the pBluescript plasmid harboring *DC-ACS1* cDNA (Kosugi *et al.*, 2000) after digestion with *Spe* I, then inserted in sense or antisense orientation into pIG121-Hm at the *Xba* I/*Sac* I site created by removing the GUS region. Correct orientation of the insert was confirmed by PCR. The constructed plasmids were introduced into *Agrobacterium tumefaciens* EHA101 by the freeze-thaw method (An *et al.*, 1988). Transformation by *Agrobacterium*-mediated gene transfer and regeneration of carnation plants were performed similarly to the method used for the generation of sACO-1 transgenic carnation (Kosugi *et al.*, 2000).

Assay of ethylene production: Flowers of the non-transformed control and transgenic lines were harvested when fully opened (day 0). Stems were trimmed to 2-cm long and flowers were placed in 30-ml glass vials with their basal end in distilled water and kept at 23°C under 14 h d⁻¹ white fluorescent light (15 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The water was replaced daily. Ethylene production from carnation flowers was monitored daily by enclosing individual flowers in 350-ml glass containers (one flower per container) for 1 hour at 23°C. A 1-ml gas sample was taken into a hypodermic syringe from the inside of the container through a rubber septum of a sampling port on the container, and analyzed for ethylene with a gas-chromatograph (263-30, Hitachi) equipped with an alumina column and a flame ionization detector. Fifteen flowers per each line were subjected to ethylene production assay, but 7 and 11 flowers were used for ‘Ashley’ and the 16-0-66 flowers, respectively. Data are given by the mean \pm SE. During ethylene production assay, senescence behavior of flowers was monitored and photographed daily to examine in-rolling and subsequent wilting of petals or desiccation and discoloration of the rim of petals.

PCR analysis of *s/aACS* transgenes: Genomic DNA was isolated from carnation leaves with the Plant Geno-DNA-Template kit (Geno Technology, St. Louis, USA). PCR was conducted in a reaction mixture containing 2 μl of the genomic DNA extract (1 ng DNA), 7 pmol each of the upstream and

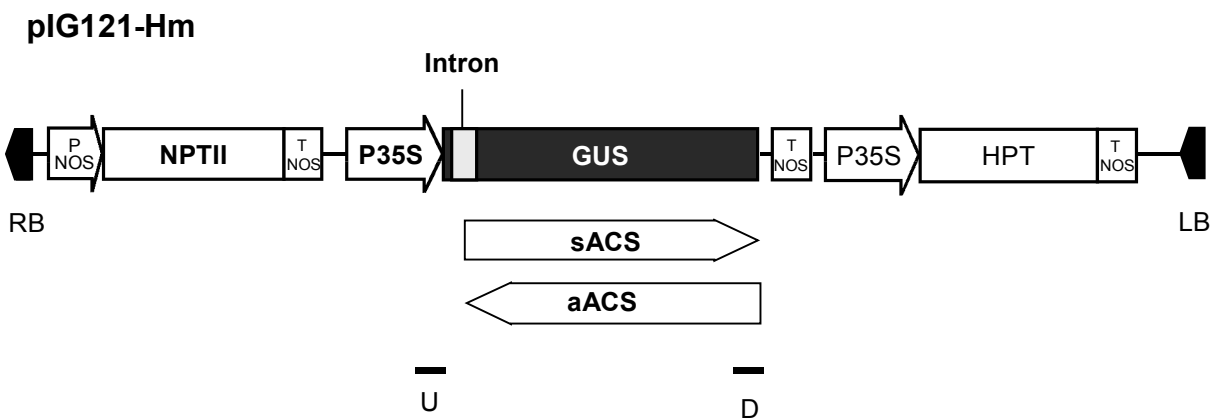


Fig. 1. Schematic representation of transformation vectors. GUS gene in pIG121-Hm was replaced with *DC-ACS1* cDNA in sense or antisense orientation. Positions corresponding to upstream and downstream primers are shown by closed rectangular boxes. RB and LB, right and left border of T-DNA, respectively; NPTII, neomycin phosphotransferase II gene; P35S, cauliflower mosaic virus 35S promoter; Intron, a modified intron of the castor bean catalase gene; PNOS, the promoter of the nopalyn synthase gene; TNOS, the terminator of the nopalyn synthase gene; HPT, hygromycin phosphotransferase gene; sACS and aACS, *DC-ACS1* cDNA in sense or antisense orientation, respectively; U, the upstream primer, 35S1F; D, the downstream primers, DCACS1-R for sACS transgene and DCACS1-F for aACS transgene.

downstream primers, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 μM dNTP mixture and 1 unit of *Taq* DNA polymerase (Sigma-Aldrich) in a total volume of 20 μl. PCR was carried out for 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, and supplemental incubation for 15 min at 72°C with an automatic thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer/Cetus). The PCR amplicates were separated on a 1.0% agarose gel and visualized by ethidium bromide staining. The upstream primer for PCR was 5'-CGC AAG ACC CTT CCT CTA TAT AAG GAA GTT-3' (35S1F), which corresponded to the downstream sequence of CaMV 35S promoter (Fig. 1). The downstream primer was 5'-TTA TGT TCT TGC TTT AAC AAG GGG TGA TTG-3' (DCACS1-R) for the amplification of *DC-ACS1* cDNA transgene in sense orientation, but the primer 5'-ATG GGT TCT TAT AAG GGT GTT TAC GAC CGT-3' (DCACS1-F) was used for amplification of *DC-ACS1* cDNA transgene in antisense orientation (Fig. 1). The sizes of expected amplified cDNA fragments were 1.5 kbp in both cases.

DNA gel blot analysis: Genomic DNA was isolated from carnation leaves by CTAB method (Murray and Thompson, 1980). 40 μg of the DNA was digested with *Hind* III, separated on a 0.8% agarose gel and transferred to a nylon membrane filter (Hybond N⁺, Amersham Pharmacia Biotech). The *DC-ACS1* cDNA fragment, 560 bp in size and corresponded to the position 1 to 560 bp of the coding region of *DC-ACS1* cDNA (Park *et al.*, 1992), was amplified by RT-PCR and used as the probe for gel blot analysis. The DNA probe was labeled with HRP and hybridized with the blot using ECL Direct™ (Amersham Pharmacia Biotech) according to the manufacturer's instruction. Hybridization signals were detected using X-ray film (RX-U, Fuji Photo Film, Tokyo).

Results and discussion

Generation of transgenic carnations transformed with *DC-ACS1* cDNA in sense or antisense orientation: We used pIG121-Hm to construct binary vectors (Fig. 1). This vector has an NPTII gene and an HPT gene, which enable selection of

transformants by kanamycin or hygromycin. We used hygromycin at 10 mg l⁻¹ initially and at 20 mg l⁻¹ thereafter, since we preliminary observed that kanamycin allowed escapes in many cases of carnation plants as reported by Firoozabady *et al.* (1995). After selection on a medium containing hygromycin, we obtained 3 regenerated lines of shoot clusters out of 400 leaf explants infected with *A. tumefaciens* EHA 101 harboring the pIG121 vector with *sACS* transgene, and 5 regenerated lines out of 920 leaf explants with *aACS* transgene. All of the regenerated lines grew and flowered normally like the non-transformed control plants.

PCR analysis with genomic DNAs extracted from the regenerated lines and two combinations of primers, corresponding to the downstream sequence of CaMV35S promoter (35S1F primer) and 5'- or 3'-marginal sequences of *DC-ACS1* cDNA (DCACS1-R and DCACS1-F primers, respectively) gave amplicates of 1.5 kbp in size with 2 lines transformed with *sACS* transgene (*sACS*-1 and *sACS*-2) and 3 lines transformed with *aACS* transgene (*aACS*-1, *aACS*-2 and *aACS*-3). These findings indicated the presence of *sACS* transgene in the former two lines, and that of *aACS* transgene in the latter three lines.

The yield of transformants was about 0.4% in total. Previously, Bovy *et al.* (1999) reported that carnation plants, transformed to be insensitive to ethylene with *Arabidopsis etr1-1* gene, had a decreased transformation-regeneration capacity and a decreased viability that caused a loss of transformants in tissue culture and subsequent growth in a greenhouse. In the present study also, the low yield of transformants after transformation with *a/sACS* transgenes might result from a negative effect of transgenes which caused modified ethylene production in the transformed leaf explants and resultant shoot clusters.

Ethylene production from flowers of transgenic carnations:

Flowers of the 'Nora' used as control showed a climacteric rise in ethylene production, attaining the maximal rate of 4.6 nmol g⁻¹ h⁻¹ on day 4. Whereas, flowers of all the transformants produced a less amount of ethylene during the senescence period. The *sACS*-1 line did not show a distinct profile of climacteric ethylene production and its maximum ethylene production rate observed on day 5 was only 11% that of the control on day 4. Similarly the *aACS*-3 line produced ethylene in a small amount with a vague climacteric manner; the maximum rate of ethylene production on days 4 and 5 being 11% that of the control on day 4. Other 3 transgenic lines, *sACS*-2, *aACS*-1 and *aACS*-2, produced ethylene in a climacteric manner; the maximal ethylene production was seen on day 3 through 6 and their maximum ethylene production rates were 40-50% that of the control on day 4.

Flowers of the control and transgenic lines, except for *sACS*-1 line, showed in-rolling of the petals simultaneously with the increase of ethylene production, and wilted completely thereafter. These findings suggested that the amount of ethylene produced from the transgenic flowers was sufficient to induce in-rolling and wilting of petals. By contrast, the majority of the *sACS*-1 flowers remained turgid for almost 10 days, then lost their vase-life with desiccation and discoloration in the rim of petals, although the rest of the flowers showed in-rolling and wilting as seen in the control flowers. Petal in-rolling at the onset of wilting is a well-known characteristic of ethylene-dependent senescence of carnation flowers. By contrast, desiccation, discoloration and browning of the rim of petals are characteristics of ethylene-

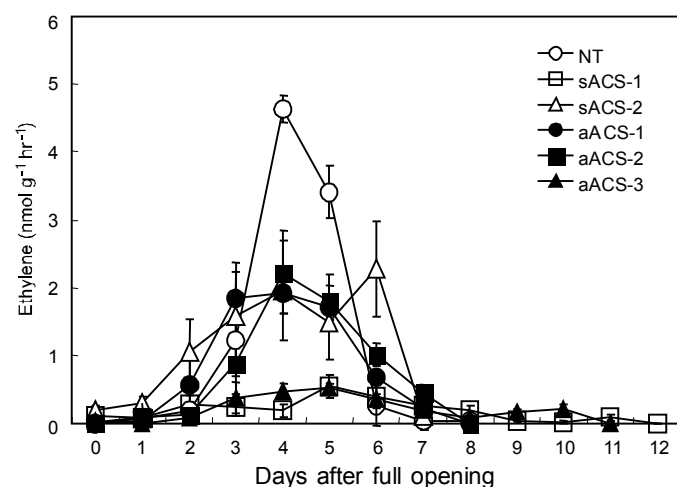


Fig. 2. Ethylene production from cut carnation flowers during the senescence period in the non-transformed 'Nora' control (NT) and transgenic lines. Fifteen flowers of respective lines were harvested at full opening stage (day 0) and their ethylene production monitored daily. Data are the mean \pm SE of 15 flowers.

independent senescence of the flowers. The present findings suggest no or little function of ethylene during the senescence of the majority of sACS-1 flowers. The findings suggested that the amount of ethylene produced from the sACS-1 flowers was below the threshold level required to induce in-rolling and wilting of petals. Previously, Jones and Woodson (1997) presented the idea that there is a threshold level of ethylene production from the style required to induce senescence of petals in the pollination-induced senescence of carnation flowers.

Comparison of sACS-1 line with another line harboring sACS transgene: Since our objective in this study was to obtain transgenic carnations whose flowers had suppressed ethylene production and the vase-life longer than that of the non-transformed control, we concentrated subsequent investigation on the sACS-1 line. In the course of this study, by courtesy of Florigene Ltd., we could use a transgenic carnation plant (16-0-66 line, its parent cultivar is 'Ashley') that was transformed with *DC-ACS1* cDNA in sense orientation (*sACS* transgene). Thus, we compared ethylene production from cut flowers between the sACS-1 and the 16-0-66 lines. The 16-0-66 flowers produced ethylene in an undetectable amount during the 12-day senescence period, whereas the control 'Ashley' flowers showed a climacteric rise in ethylene production, attaining a maximal rate on day 6 (Fig. 3). Thus, it was obvious that *sACS* transgene caused suppressed ethylene production more severely in the flowers of the 16-0-66 line than those of the sACS-1 line. Cut flowers of 'Ashley' remained turgid until day 4, showed in-rolling of petals on day 5 and completely wilted on day 7. By contrast, cut flowers of the 16-0-66 line remained turgid without petal in-rolling until day 10, but showed desiccation and discoloration in the rim of petals on day 11 or later.

We investigated the number and loci of *sACS* transgenes present in tissues by DNA gel blot analysis of the restriction fragments containing *DC-ACS1* cDNA sequence in *Hind* III digests of genomic DNAs of the sACS-1 and 16-0-66 lines (Fig. 4). The DNA probe for the *DC-ACS1* gene (DC-ACS1-560 probe) detected only one DNA fragment of 8.5 kbp in *Hind* III digests

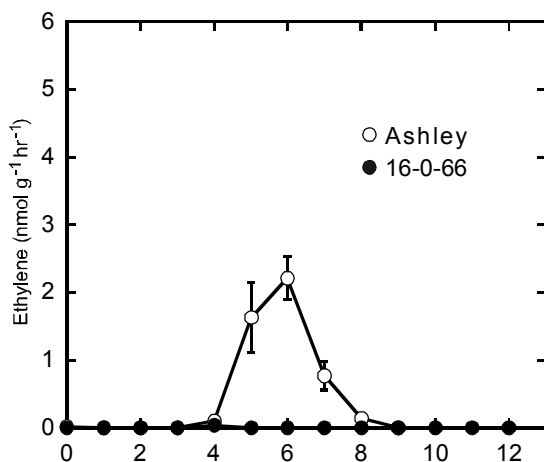


Fig. 3. Ethylene production from cut carnation flowers of the 'Ashley' control and the 16-0-66 line during the senescence period. Seven and 11 flowers for 'Ashley' and the lines, respectively, were harvested at full opening stage (day 0) and their ethylene production monitored daily. Data are shown by the mean \pm SE.

of both 'Nora' and 'Ashley' genomic DNAs. The 8.5-kbp fragment was probably derived from the endogenous *DC-ACS1* gene. However, these findings were contrary to the result of our previous experiments, in which the DC-ACS1-560 probe detected two DNA fragments of 8.5 and 3.8 kbp in *Hind* III digests of 'Nora' genomic DNA. The appearance of two DNA fragments in the *Hind* III digests coincided with the presence of a *Hind* III cleavage site in the *DC-ACS1* genomic DNA, which was deduced from the *Hind* III cleavage site at the position of 98-103 bp of the *DC-ACS1* cDNA. Our separate experiments suggested that 3.8 kbp and 8.5 kbp fragments were the upstream and downstream portions of the *DC-ACS1* genomic DNA, respectively. Combining together the present and preceding findings, we think that in the present experiment the DC-ACS1-560 probe could detect only the 8.5-kbp DNA fragment, which has a 400 bp-long sequence hybridizable to the probe, but failed to detect the 3.8-kbp DNA fragment, which has only a 100 bp-long sequence hybridizable to the probe. We did not investigate further the cause for absence of 3.8 kbp fragments in the *Hind* III digests. Anyway, detection of only one DNA fragment suggested that the DC-ACS1-560 probe was specific to *DC-ACS1* genomic DNA.

The DNA probe detected two extra DNA fragments of 13.0 kbp and 3.7 kbp, in addition to the 8.5-kbp DNA fragment, in *Hind* III digest of the sACS-1 genomic DNA. The DNA probe also detected two extra DNA fragments of 6.2 kbp and 2.9 kbp, in addition to the 8.5-kbp DNA fragment, with the 16-0-66 genomic DNA. The extra DNA fragments were thought to result from *sACS* transgene present in the transgenic lines. Judging from the intensity of hybridization signals, we thought the extra DNA

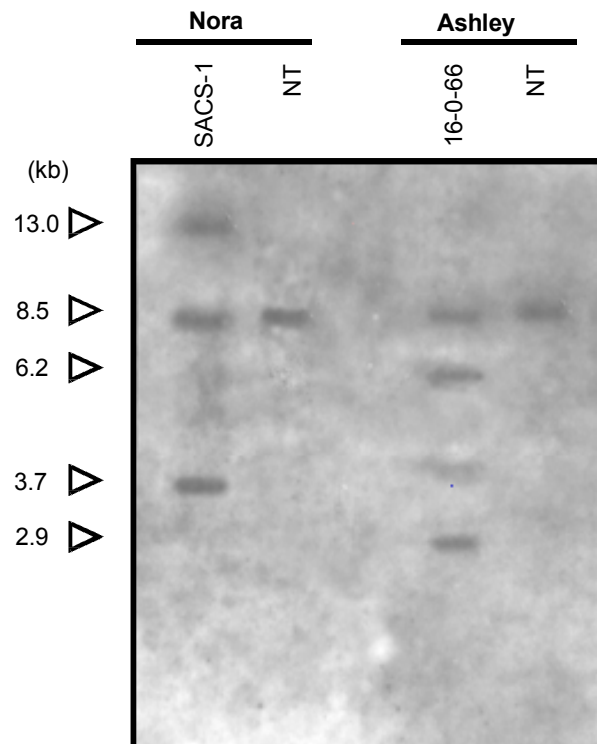


Fig. 4. DNA gel blot analysis of *sACS* transgenes in the sACS-1 and 16-0-66 lines. The *Hind* III digests of genomic DNAs from the sACS-1 line and its parent line 'Nora' as well as the 16-0-66 line and its parent line 'Ashley' were separated on agarose-gel and hybridized with the DC-ACS1-560 probe. NT, non-transformant.

fragments were those of the downstream portion of *sACS* transgene, which corresponds to the 8.5 kbp fragment of the endogenous *DC-ACSI* genomic DNA. The present results suggested the presence of two copies of *sACS* transgene in both the *sACS*-1 and the 16-0-66 lines. However, difference in the sizes of the DNA fragments in *Hind* III digests implied that *sACS* transgenes were present in different loci in genomic DNAs of the *sACS*-1 and 16-0-66 lines. Thus, the difference in the extent of suppression of ethylene production found between the *sACS*-1 and 16-0-66 lines might result from the difference in positions (loci) of integrated *sACS* transgene, rather than the difference in numbers of integrated transgenes. However, alternative explanation is that it might be caused by the difference in genetic background for ethylene production between two parent cultivars, which was seen by the difference in the maximum ethylene production rates during senescence (Figs 2 and 3).

Previously, it was demonstrated that ethylene production in carnation flowers was suppressed by transformation with a carnation ACC oxidase (*DC-ACO1*) cDNA in sense orientation (Kosugi *et al.* 2000). It was speculated that the ACC oxidase cDNA in sense orientation inhibited ethylene production in the flowers by cosuppression of expression of endogenous *DC-ACO1* gene in flower tissues (Kosugi *et al.*, 2002). On the analogy of the action of *sACO* transgene, we suspected that *sACS* transgene inhibited the expression of endogenous *DC-ACSI* gene by cosuppression.

In conclusion, the present investigation indicated that the transgenes derived from *DC-ACSI* cDNA, in addition to those derived from *DC-ACO1* cDNA, are useful to prolong flower longevity of carnation by transgenic approach.

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