

# Reduced ethylene production in transgenic carnations transformed with ACC oxidase cDNA in sense orientation

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## Abstract

‘Lillipot’ carnation, which is usually cultivated as a potted ornamental, was transformed with a cDNA for carnation 1-aminocyclopropane-1-carboxylate (ACC) oxidase. Two lines, which harbor an *sACO* transgene, had a vase life of cut flowers more than twice longer than that of the non-transformed (NT) control. Flowers of the long vase life lines senesced with discoloring and browning in petal margins, which is typical to ethylene-independent senescence in carnation flowers. They produced negligible amount of ethylene for the first 8 day, whereas flowers of the NT control showed a climacteric ethylene production with a maximum on day 3. Transcripts for *DC-ACSI* and *DC-ACOI* were absent in petals of the long vase life flowers undergoing senescence. The present study revealed that transformation with *sACO* transgene may be useful to generate potted carnation plants with a long display time.

**Key words:** ACC oxidase gene (*DC-ACOI*), *Dianthus caryophyllus*, ethylene biosynthesis, flower senescence, potted carnation

## Introduction

Carnations are used as ornamentals as potted plants and as cut flowers. Ethylene is a primary plant hormone involved in the senescence of cut carnation flowers (Reid and Wu, 1992). It is synthesized in a large amount, mostly from the petals, at a later stage of flower senescence (Borochoy and Woodson, 1989; Abeles *et al.*, 1992; Reid and Wu, 1992; Woodson *et al.*, 1992). Increased ethylene production accelerates wilting of the petals. Inhibition of the synthesis or action of ethylene delays the onset of senescence and extends the vase life of flowers.

In senescing carnation flowers, ethylene is first produced in the gynoecium, and the ethylene evolved acts on petals and induces autocatalytic ethylene production in the petals. This results in petal wilting (Jones and Woodson, 1997; Shibuya *et al.*, 2000; ten Have and Woltering, 1997). Ethylene is synthesized through the pathway: L-methionine → S-adenosyl-L-methionine → ACC → ethylene. ACC synthase and ACC oxidase catalyze the last two reactions (Kende, 1993; Yang and Hoffman, 1984). So far, three genes encoding ACC synthase (*DC-ACSI*, *DC-ACS2* and *DC-ACS3*) and one gene encoding ACC oxidase (*DC-ACOI*) have been identified in carnations (Henskens *et al.*, 1994; Jones and Woodson, 1999; Park *et al.*, 1992; Wang and Woodson, 1991). Out of these genes, *DC-ACSI* and *DC-ACOI* have been shown to play a pivotal role in ethylene production in both the gynoecium and petals of senescing carnation flowers (Nukui *et al.*, 2004; Satoh and Waki, 2006).

Currently, prevention of senescence of carnation flower is being attained by treatment of the flower with chemical preservatives which inhibit the synthesis or action of ethylene (Veen, 1979; Midoh *et al.*, 1996). Another option for preventing senescence is the generation of transgenic flowers with suppressed production or action of ethylene. So far, the lines transformed with cDNAs for carnation 1-aminocyclopropane-1-carboxylate (ACC) oxidase (*DC-ACOI*) and ACC synthase (*DC-ACSI*) in sense or antisense

orientation (Savin *et al.*, 1995; Kosugi *et al.*, 2000, 2002; Iwazaki *et al.*, 2004) and a line harboring an *Arabidopsis thaliana etr1-1* allele capable of rendering ethylene insensitivity (Bovy *et al.*, 1999) have been generated. Cut flowers of the transgenic lines have a prolonged vase life compared with those of non-transgenic plants.

The preservatives described above are considered to be not applicable to potted plants, since they are usually administered to cut flowers through vascular transport by immersing the cut stem end in solutions containing the preservative. Therefore, the generation of transgenic plants is a promising way to retard senescence of flowers of potted carnations, *i.e.*, to lengthen their display time. Kinouchi *et al.* (2006) recently generated potted carnation plants transformed with cDNAs for carnation ACC synthase (*DC-ACSI*, *s/aACS* transgenes) or ACC oxidase (*DC-ACOI*, *s/aACO* transgenes) in sense or antisense orientation or mutated carnation ethylene receptor cDNA (*DC-ERS2'*) by *Agrobacterium*-mediated gene transfer. They partly characterized the transformants by investigating the conversion of exogenously-applied ACC to ethylene in leaflet segments. A performance test of the transformants as potted plants remains to be carried out. However, we should first know the synthesis and action of ethylene in flowers in each transformant to select the best line for the large scale performance test. Therefore, in this study, we cultivated several lines of the transformants on soil until flowering, and characterized their senescence, ethylene production and gene expression in cut flowers.

## Materials and methods

**Plant materials:** Plantlets of the transgenic lines of carnation (*Dianthus caryophyllus* L. cv. Lillipot), generated previously (Kinouchi *et al.*, 2006), and the NT control were grown *in vitro* to about 5 cm in height, were transplanted into a commercial horticulture soil in a plastic container, under conditions described previously (Iwazaki *et al.*, 2004) in a containment green house

at Tohoku University. Out of 39 transgenic carnation lines generated previously (Kinouchi *et al.*, 2006), 6 transgenic and the non-transformed (NT) control lines were used since these lines flowered one year after transplanting to soil. Three transgenic lines studied were pMLH-sACO-2, -3 and -12, which were transformed with carnation *DC-ACO1* cDNA in sense orientation (*sACO* transgene) in a pMLH2113 vector. Two other transgenic lines, pIG-sACS-1 and pIG-sACO-1, were transformed with carnation *DC-ACSI* and *DC-ACO1*, respectively, in sense orientation (*sACS* and *sACO* transgenes) using the pIG121 vector. Finally, pIG-DC-ERS2'-2 was transformed with a mutated carnation ethylene receptor cDNA (*DC-ERS2'*) in the pIG121 vector. Flowering started around one year after transplanting. Flowers were harvested during the following 5-6 months. Only the first and second flowers opening on each stem were used.

**Analysis of vase life of cut flowers:** Three to ten flowers, depending on the line, of each of the transgenic and NT control lines were harvested at the full opening stage (day 0; their outermost petals were at right angles to the stem of flower). Stems were trimmed to 0.5 cm in length, and placed with their cut end in distilled water in 5-ml plastic vials. The flowers were left at 23°C under a 16-h photoperiod using white fluorescent light (20-30  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ ). The water was replaced daily. Senescing flowers were observed and photographed daily to record in-rolling and subsequent wilting of petals, the desiccation, and discoloration of the petal margins. Vase life in days is expressed as the mean  $\pm$  SE of given numbers of flowers.

**Assay of ethylene production:** Ethylene production from carnation flowers was monitored daily by enclosing individual flowers in plastic vials in 140-mL glass containers (1 flower per container) for 1 h at 23°C. A 1-mL gas sample was taken with a hypodermic syringe from inside the container through a rubber septum of a sampling port on the container and injected into a gas chromatograph (Shimadzu GC-14A, Kyoto, Japan), equipped with an alumina column and a flame ionization detector to determine ethylene content.

**Treatment with exogenous ethylene of flowers of the transgenic lines:** Two to five cut flowers each of the respective transgenic lines and the NT control were enclosed in a 60 L glass chamber and exposed to ethylene at 10  $\mu\text{l L}^{-1}$  for 16 h at 23°C under white fluorescent light (50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). After the treatment, the flowers were held in open air for 1 h to let exogenous ethylene diffuse. They were subsequently encapsulated and their ethylene production was determined by gas-chromatography. The petals and gynoecia were immediately excised and prepared for total RNA extraction.

**Northern blot analysis:** Total RNA was isolated by the SDS-phenol method (Palmiter, 1974) from the petals and with RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) from gynoecium of cut flowers of the respective transgenic lines and the NT control line at given time after the full opening of flowers (day 0). Pistils and petals were detached from one to three flowers, depending on lines, at the given time and combined to make one sample each. Also, total RNA was isolated from the gynoecium and petals of the flowers treated with ethylene as described above. Ten  $\mu\text{g}$  of total RNA was denatured, separated on a 1.0% agarose gel, transferred onto nylon membranes (Hybond N<sup>+</sup>, Amersham Pharmacia Biotech, Tokyo, Japan) and hybridized with the DNA

probes for *DC-ACSI* and *DC-ACO1* transcripts. The DNA probe for *DC-ACSI* transcript was 560 bp which corresponded to the position 1 bp to 560 bp of the coding region of *DC-ACSI* cDNA (GenBank Accession No. M66619), and that for *DC-ACO1* transcript 560 bp corresponded to the position 261 bp to 820 bp of the coding region of *DC-ACO1* cDNA (GenBank Accession No. M62380). The DNA probes were labeled with HRP and hybridized with the blot by using ECL Direct<sup>TM</sup> (Amersham Pharmacia Biotech) according to the manufacturer's instruction. Hybridization signals were detected by exposure to X-ray film (RX-U, Fuji Photo Film, Tokyo, Japan).

## Results

**Vase-life and senescence profile of the transgenic flowers:** Cut flowers of each of the transgenic lines had vase-life of various length varying from  $3.0 \pm 0.4$  to  $7.6 \pm 0.4$  days, whereas that of the NT control line was  $2.8 \pm 0.2$  days (Table 1). Vase lives of the pMLH-sACO-2 and -12 lines were  $7.6 \pm 0.4$  and  $6.3 \pm 0.5$  days, respectively (significantly different from the NT control, at  $P=0.001$  by *t* test). Other transgenic lines had vase lives that were not significantly different from that of the NT control, except for the pIG-DC-ERS2'-2 line. We did not investigate further the pIG-DC-ERS2'-2 line.

Flowers of the NT control remained turgid until day 3, showed in-rolling of petals on day 4, and completely wilted thereafter. On contrast, flowers of the pMLH-sACO-2 and -12 lines remained turgid without petal in-rolling until day 6 or more, but eventually began to show desiccation and discoloration in the rim of petals. Petal in-rolling at the onset of wilting is a well-known characteristic of ethylene-dependent senescence of carnation flowers. Desiccation, discoloration, and browning of the rim of petals are characteristics of ethylene-independent senescence of carnation flowers. These findings suggested little or no function of ethylene during the senescence of petals of the pMLH-sACO-2 and -12 flowers. In the following experiments, we characterized ethylene production, expression of genes for ethylene biosynthesis and response to exogenous ethylene of flowers of the pMLH-sACO-2 and -12 lines by comparison with those of the NT control.

**Ethylene production of the transgenic flowers:** Flowers of the NT control showed a climacteric rise in ethylene production, attaining a maximal rate on day 3 (Fig. 1). Flowers of the pMLH-sACO-2 and -12 lines produced very small amounts of ethylene during senescence period of 8 days. Their maximum ethylene production rates were around 10% that of the NT control. The

Table 1. Senescence of flowers of the NT control and transgenic carnations

Lines	Number of flowers tested	Vase life <sup>a</sup> (days)	Senescence pattern <sup>b</sup>
NT control	6	2.8 $\pm$ 0.2	W
pMLH-sACO-2	7	7.6 $\pm$ 0.4	D
pMLH-sACO-3	6	3.2 $\pm$ 0.4	W
pMLH-sACO-12	4	6.3 $\pm$ 0.5	D
pIG-aACS-1	10	3.0 $\pm$ 0.4	W
pIG-aACO-1	3	3.3 $\pm$ 0.9	W
pIG-DC-ERS2'-2	4	4.5 $\pm$ 1.0	W

<sup>a</sup> Each value is the mean  $\pm$  SE.

<sup>b</sup> W, in-rolling and wilting of the petals; D, desiccation, discoloration and necrosis of the petals.

lack of petal in-rolling and prolonged vase-life in flowers of the pMLH-sACO-2 and -12 lines coincided with a marked reduction in ethylene production.

**Transcript levels for *DC-ACS1* and *DC-ACO1* in the gynoecium and petals of the transgenic flowers:** As described in introduction, *DC-ACS1* and *DC-ACO1* play a pivotal role in ethylene production in both the gynoecium and petals of senescing carnation flowers (Nukui *et al.*, 2004; Satoh and Waki, 2006). We examined the transcript levels for *DC-ACS1* and *DC-ACO1* in the gynoecium and petals of the transgenic flowers undergoing senescence.

On day 0 (at the time of full opening of flowers), *DC-ACS1* and *DC-ACO1* transcripts were absent in both the gynoecium and petals of carnation flowers (Fig. 2). With the NT control flowers, *DC-ACS1* and *DC-ACO1* transcripts accumulated abundantly in the gynoecium on day 2 (the day before petal in-rolling), and significantly in the petals on day 3 when a maximum ethylene production from flowers occurred (Fig. 1). In the pMLH-sACO-2 and -12 lines, tissue sampling was conducted on days 0, 3 and 6. In gynoecia of the pMLH-sACO-2 line, *DC-ACS1* and *DC-ACO1* transcripts accumulated, although to small amounts in the latter, on day 3. These transcripts diminished on day 6. Both transcripts were absent in the petals on days 3 and 6. Similarly, with pMLH-sACO-12 line, *DC-ACS1* and *DC-ACO1* transcripts accumulated abundantly in the gynoecium on day 3 and diminished on day 6, but they were absent in the petals on both days. Absence of *DC-ACS1* and *DC-ACO1* transcripts in the petals of pMLH-sACO-2 and -12 lines coincided with the negligible amount of ethylene production from flowers of these lines (Fig. 1).

**Responses to exogenous ethylene of flowers of the transgenic lines:** In carnation flowers, the expression of *DC-ACS1* and *DC-ACO1* genes in petals can be induced by exogenously applied ethylene and by ethylene produced endogenously from the gynoecia (Shibuya *et al.*, 2000). The response of the transgenic lines and the NT control to exogenously applied ethylene was investigated after treatment with ethylene at  $10 \mu\text{l L}^{-1}$  for 16 h. Ethylene evolution was determined from flowers at the beginning

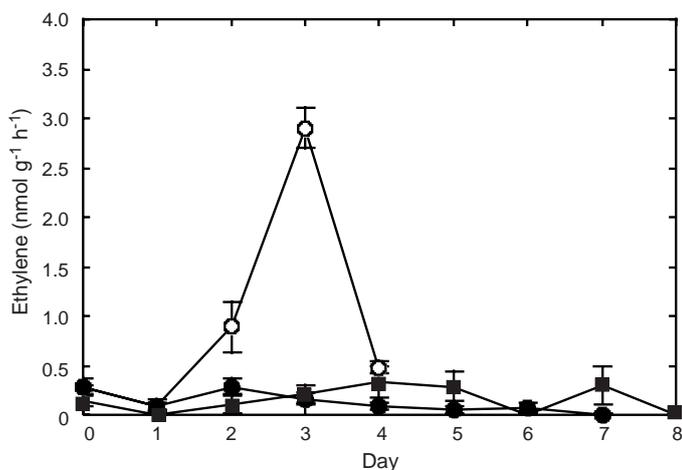


Fig. 1. Ethylene production from cut carnation flowers during the senescence period in the NT control and the transgenic lines. Flowers (numbers shown in Table 1) of three lines were harvested at full opening stage (day 0) and their ethylene production was monitored daily. Data are shown by the mean  $\pm$  SE.

○, NT control; ●, pMLH-sACO-2; ■, pMLH-sACO-12.

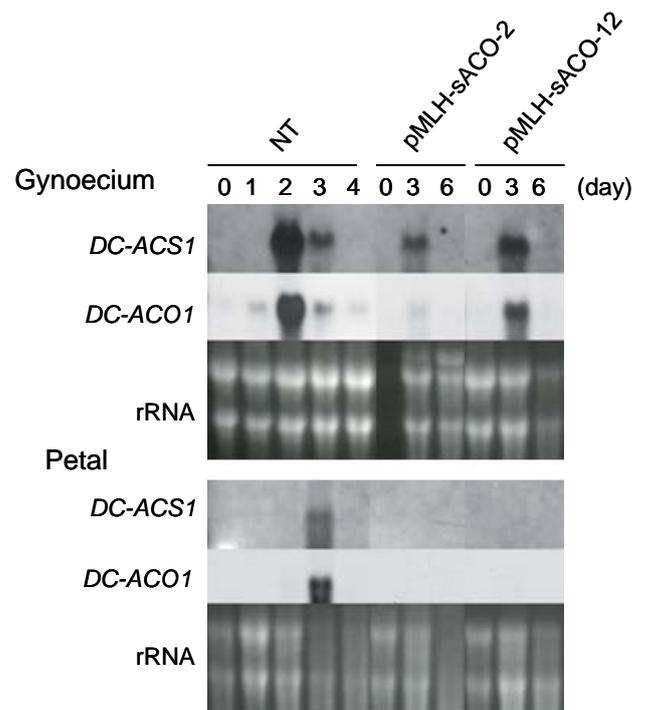


Fig. 2. RNA gel blot analysis of *DC-ACO1* and *DC-ACS1* transcripts in the gynoecium and petals of the NT control and in two transgenic lines during natural senescence. Gynoecium and petals were isolated from cut flowers at given days after full opening of flowers; days 0, 1, 2, 3, and 4 for the NT control, but days 0, 3 and 6 for pMLH-sACO-2 and -12 transgenic lines which did not show wilting. Ten  $\mu\text{g}$  of total RNAs isolated from respective flower tissues were separated on an agarose gel and hybridized to DIG-labeled *DC-ACS1* and *DC-ACO1* probes. Equal loading of total RNAs was checked by ribosomal RNAs visualized by ethidium bromide staining of the agarose gel. No data for the gynoecium of pMLH-sACO-2 on day 0.

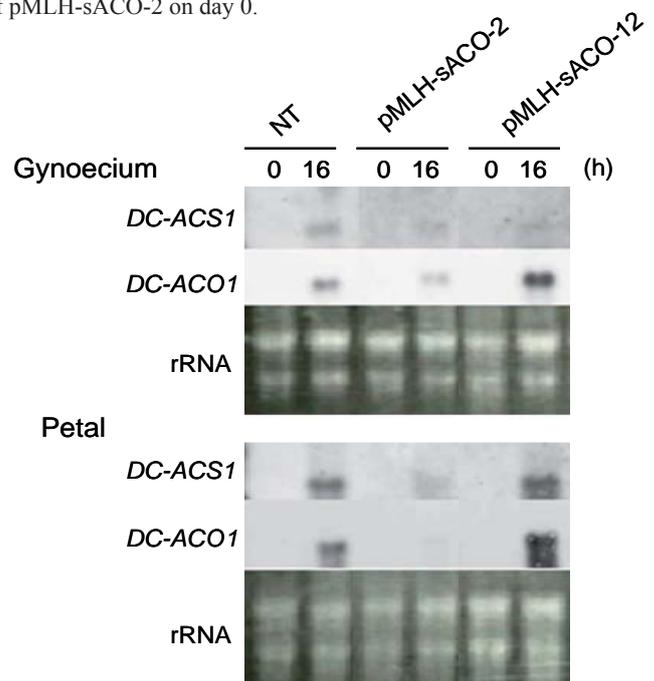


Fig. 3. RNA gel blot analysis of *DC-ACS1* and *DC-ACO1* transcripts in gynoecium and petals of the NT control and two transgenic flowers before and after ethylene treatment. Cut carnation flowers of respective lines were treated with  $10 \mu\text{l L}^{-1}$  ethylene for 16 h. Ethylene production from flowers was measured before and after the ethylene treatment by enclosing them for 1 h and measuring ethylene produced. Numbers of flowers used were 2, 5 and 3 for the NT, pMLH-sACO-2 and -12 in this order. After ethylene assay, the flowers were subjected to analysis of amounts of *DC-ACS1* and *DC-ACO1* transcripts as described in the legend to Fig. 2.

and end of ethylene treatment. We also determined transcript levels for *DC-ACSI* and *DC-ACO1* in the gynoecia and petals. Treatment with exogenous ethylene for 16 h caused petals of the transgenic lines and the NT control to wilt, indicating that flowers of the transgenic lines were responsive to ethylene.

At the beginning of experiment, ethylene production from flowers was not detected or negligible ( $< 0.2 \text{ nmol g}^{-1} \text{ h}^{-1}$ ). In flowers of the NT control treated with ethylene for 16 h, ethylene production was  $9.38 \pm 0.27 \text{ nmol g}^{-1} \text{ h}^{-1}$ . Ethylene evolution from the transgenic lines was less than that of the NT control;  $1.96 \pm 0.54$  and  $4.46 \pm 1.34 \text{ nmol g}^{-1} \text{ h}^{-1}$  for the pMLH-sACO-2 and -12 lines, respectively. Exogenous ethylene treatment caused an accumulation of *DC-ACSI* and *DC-ACO1* transcripts in the gynoecium and petals of the NT control and the transgenic lines, although the level of *DC-ACSI* and *DC-ACO1* transcripts in pMLH-sACO-2 line was lower than that in the NT control (Fig. 3).

## Discussion

In this study we used six transgenic lines, which flowered a year after transplanting and cultivation, in soil, out of 39 transgenic lines generated previously (Kinouchi *et al.*, 2006). In the six transgenic lines, two lines transformed with pMLH2113-Hm/*sACO* construct, pMLH-sACO-2 and -12 lines, had a vase life more than twice longer than that of the NT control. The *sACO* transgene in the pMLH2113 vector efficiently suppressed ethylene production, which resulted in longer-lasting flowers.

Flowers of the pMLH-sACO-2 and -12 lines did not show petal in-rolling and wilting which are typical for ethylene-dependent senescence in carnation petals. Instead, the flowers showed browning and drying in the rim of petals, which spread out to all the portion of the petals, and eventually the flowers faded out at the late stage of vase life, which was about twice that of the NT control flowers. These are typical of ethylene-independent senescence of carnation flowers.

*DC-ACSI* and *DC-ACO1* transcripts were absent in the petals of both pMLH-sACO-2 and -12 flowers undergoing natural senescence (Fig. 2). This explained the reduced ethylene production in flowers of the two lines (Fig. 1). Previously, Kosugi *et al.* (2002) suggested that the *sACO* transgene integrated into carnation inhibited ethylene production in the flowers by cosuppression of expression of endogenous *DC-ACO1* gene in flower tissues. This seems also true in the present *sACO* transgenes with a long vase life. Kosugi *et al.* (2002) showed that the integrated *sACO* transgene might act first in the gynoecium, inhibiting the expression of *DC-ACO1* and suppressing ethylene production in the gynoecium and, subsequently, the expression of *DC-ACO1* and *DC-ACSI* in the petals. This was also found as an explanation for the absence of *DC-ACSI* transcript in the petals of both pMLH-sACO-2 and -12 flowers undergoing natural senescence.

The accumulation of *DC-ACSI* and *DC-ACO1* transcripts in flower tissues of the transgenic carnations (pMLH-sACO-2 and -12 lines) after treatment with exogenous ethylene ( $10 \mu\text{L L}^{-1}$  for 16 h), indicated that the integrated *sACO* transgene did not impair their responsiveness to ethylene. The accumulation of *DC-ACO1* transcripts in the petals of the pMLH-sACO-2 and -12 flowers

after exogenous ethylene treatment indicated that exogenous ethylene treatment overcame the effect of the integrated *sACO* transgene in these two lines. Kinouchi *et al.* (2006) tested *in vivo* ACC oxidase activity using leaflet segments of the transgenic plants harboring *sACO* transgene. The *in vivo* ACC oxidase activities of the pMLH-sACO-2 and -12 lines were similar to and lower than the NT control, respectively.

These results suggested that the integrated *sACO* transgene exerted its effect in the leaflet segments of pMLH-sACO-12 line, but not pMLH-sACO-2 line. In this study, however, *DC-ACO1* transcript accumulated in low amounts in the gynoecium and was absent in the petals of pMLH-sACO-2 flowers. It was present abundantly in the gynoecium but also absent in the petals of pMLH-sACO-12 flowers on day 3. These differences suggest different action (expression) of the integrated *sACO* transgene between the leaflet and flower tissues, and also a difference between the two transgenic lines.

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