

Expression and Regulation of Senescence-related Genes in Carnation Flowers with Low Ethylene Production during Senescence

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We investigated ethylene production, ethylene biosynthesis genes, and senescence-related genes in flowers of a carnation (*Dianthus caryophyllus* L.) cultivar ‘Miracle Symphony’ (MS) and lines 006-13 and 62-2, which have a longer vase life than flowers of ‘White Sim’ (WS). WS flowers showed typical symptoms of senescence, but flowers of MS, 006-13, and 62-2 did not show symptoms of senescence, although they showed differences in vase life and ethylene production by day 15. The flowers of 006-13 and 62-2 produced small amounts of ethylene as a result of the low expression of two ethylene biosynthesis genes, *DcACS1* and *DcACO1*; those of MS produced extremely low levels of ethylene. By day 15, the flowers of 006-13 and 62-2 showed increased expression of some senescence-related genes (*DcCPI*, *DcbGal*, *DcGST1*, and *DcLip*) that were upregulated by exogenous ethylene, indicating that a low level of ethylene production could induce the senescence of petals. In contrast to the upregulation of these senescence-related genes, the expression of *DcCPI*, which was downregulated by exogenous ethylene decreased in petals of MS, 006-13, and 62-2 during flower senescence and was the same in all three lines at day 15. The results suggest that extended vase life depends on reduced levels of ethylene production, ethylene biosynthesis gene expression, and senescence-related gene expression.

Key Words: carnation, ethylene biosynthesis, flower life, senescence.

Introduction

The vase life of cut flowers is one of the most important factors that determine consumer satisfaction and repeat purchasing. Carnation is a typical ethylene-sensitive flower (Woltering and van Doorn, 1988), and its vase life is about 5 to 7 days in normal cultivars without the use of preservatives, such as silver thiosulfate (STS). During senescence, the flowers show climacteric ethylene production and subsequent petal inrolling (wilting). Autocatalytic ethylene production is accompanied by the expression of genes for 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO) (Jones and Woodson, 1999; ten Have and Woltering, 1997; Woodson et al., 1992). Three genes encoding ACS (*DcACS1*, *DcACS2*, and *DcACS3*) and 1 gene encoding ACO (*DcACO1*) have been identified (Henskens et al., 1994; Jones and Woodson, 1999; Park et al., 1992; Wang and Woodson, 1991). *DcACS1* (previously called *CARACC3*) is expressed in gynoecia and petals and is the most abundantly expressed of all

DcACS genes in petals (Jones and Woodson, 1999; Satoh and Waki, 2006). Its expression is enhanced by exogenous ethylene treatment, which induces ethylene production.

The genetic improvement of vase life would remove the need for chemical treatment. To this end, Japan's National Institute of Floricultural Science began a research breeding program in 1992 to lengthen the vase life of carnation flowers by conventional cross-breeding techniques (Onozaki et al., 2001). The resultant cultivars ‘Miracle Rouge’ and ‘Miracle Symphony’ (MS) were released in 2005. The expression of *DcACS1*, *DcACS2*, and (in particular) *DcACO1* is suppressed in the flowers of both cultivars, resulting in extremely low production of ethylene (Tanase et al., 2008).

The breeding process has produced these and many other lines with vase life extended to various degrees (Onozaki et al., 2001). These lines do not show an ethylene climacteric peak and subsequent inrolling, and some produce little ethylene at the end of flower life. All are progeny of the long-life cultivar ‘Sandrosa’, and the low-ethylene trait seems to be heritable. Although the lines differ in their extent of ethylene production, the effect of this remnant low level of ethylene

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production on flower longevity is not known.

Many genes regulate petal wilting in senescing carnation flowers (Hoeberichts et al., 2007; Otsu et al., 2007). Wilting may be caused by the degradation of cell components, which prompts cell death mediated by hydrolytic enzymes such as cysteine proteinase (CPase) (Jones et al., 1995) and lipase (Lip) (Hong et al., 2000; Kim et al., 1999a). Cysteine proteinase inhibitor (CPIIn) seems to suppress CPase and to regulate petal wilting (Kim et al., 1999b; Sugawara et al., 2002). Many senescence-related (SR) genes, including those encoding these enzymes, have been cloned from carnation petals. The expression levels of *DcCPI* (previously known as pDCCP1, isolated by Jones et al., 1995), β -galactosidase (*DcbGal*; previously called *SR12*) (Lawton et al., 1989), glutathione-S-transferase (*DcGST1*), and lipase (*DcLip*) increased during senescence and were upregulated by ethylene treatment (Verlinden et al., 2002). In contrast, levels of *DcCPIIn* expression decreased during senescence and were downregulated by ethylene (Sugawara et al., 2002). It is not clear whether these genes are involved in the wilting of petals of long-life carnations. To elucidate how the petals of long-life flowers senesce, we analyzed ethylene production and the expression profiles of SR genes during the senescence of untreated and ethylene-treated flowers of normal-life and long-life carnations. We also examined the effects of the remnant low level of ethylene production on flower longevity in long-life carnations.

Materials and Methods

Plant materials

Many lines have been selected for vase life in our institute's research breeding programs to lengthen the vase life of carnation flowers by conventional cross-breeding techniques (Onozaki et al., 2001). For the experiments here, we chose the cultivar 'Miracle Symphony' (MS) and lines 006-13 and 62-2 as long-life carnations (hereafter, both cultivars and breeding lines are referred to as 'lines'). These materials were obtained through a process of repeated crossing and selection across three generations: MS and 62-2 were from selected second-generation lines, and 006-13 was a selected third-generation line. The breeding of these lines is shown in Figure 1. Line 006-13 was derived from a cross between MS and line 62-2. These three lines showed low ethylene production at the senescence stage and were as sensitive to exogenous ethylene as normal cultivars (Onozaki et al., 2006). We grew the long-life carnations and the control (normal-life) cultivar 'White Sim' (WS) under natural-daylight conditions in a greenhouse. Flowers were harvested when the outer petals were horizontal (full-open stage: day 0).

Determination of flower longevity and sample preparation

Flower life was determined as the number of days from day 0 until the flowers lost their ornamental value,

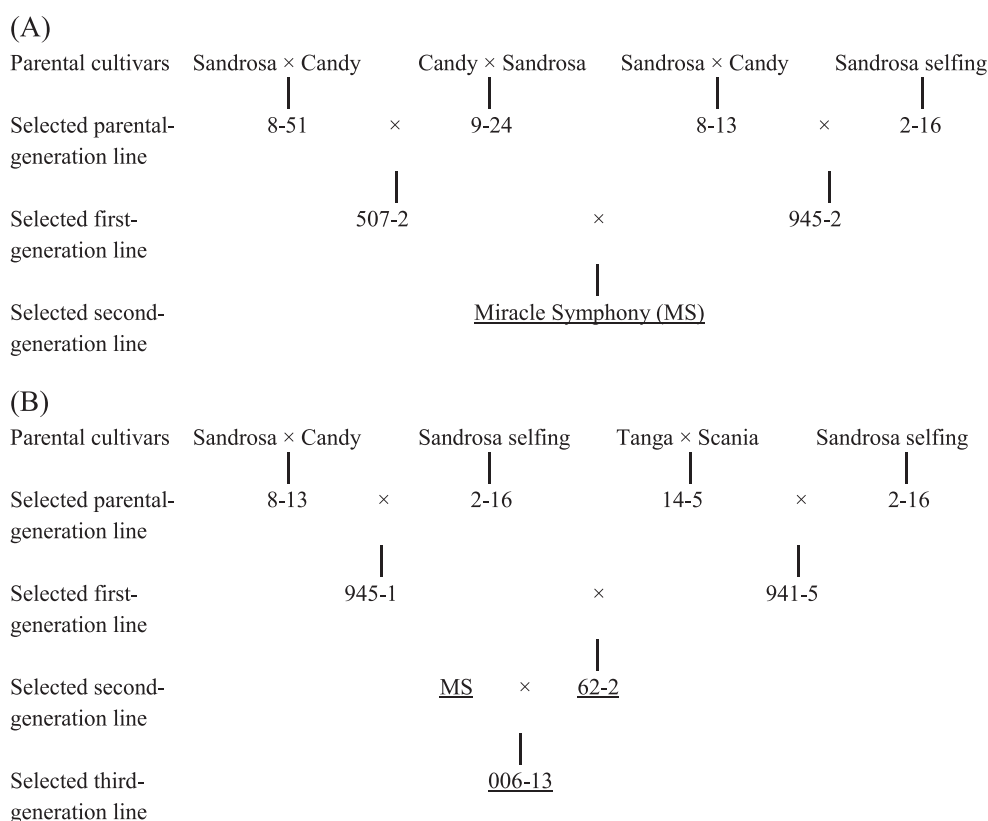


Fig. 1. Pedigrees of 'Miracle Symphony' (MS) (A) and lines 006-13 and 62-2 (B).

defined as when a flower showed wilting with inrolling, browning of the petal margins without inrolling, or otherwise desiccated petals without inrolling. Unless otherwise noted, flowers were placed in distilled water at the time of harvest. Flowers were held at a constant air temperature of 23°C and 70% relative humidity, under a 12 h photoperiod under cool white fluorescent lamps ($10 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), and were evaluated daily. For real-time RT-PCR analysis, gynoecia and petals were detached from 3 flowers at 0, 5, 6, and 15 days after harvest, and stored at -80°C until RNA extraction.

For STS treatment, stems were harvested and placed in a solution containing 2 mM STS for 20 h and then transferred to a glass beaker containing distilled water for the remainder of the experiment. These were grown and evaluated for flower longevity as described above.

Ethylene treatment and sample preparation

On day 0, flower stems were cut to 30 cm and placed in distilled water, and the flowers were held at a constant air temperature of 23°C under continuous light (as above) in a 70-L chamber with $2 \mu\text{L}\cdot\text{L}^{-1}$ ethylene. The flowers were photographed every 1 h by digital camera (Caplio GX; Ricoh, Tokyo, Japan) to determine the beginning of petal inrolling. For real-time RT-PCR analysis, the flowers were held in a 70-L chamber with $2 \mu\text{L}\cdot\text{L}^{-1}$ ethylene for 12 h, then gynoecia and petals were detached from 3 flowers and stored at -80°C for RNA extraction.

Ethylene measurement

Flowers from each treatment (i.e. non-treated flowers, and ethylene-treated flowers) were exposed to fresh air for 1 h before incubation. Whole flowers were placed in a 143- or 433-mL glass bottle, which was then closed with a silicone cap and kept at 23°C for 1 or 2 h. Gynoecia and petals were separately placed in a 15-mL glass vial or bottle, which was then closed with a silicone cap and kept at 23°C for 1 h. Gas samples (1 mL) were taken from the headspace and injected into a gas chromatograph (GC-13A; Shimadzu, Kyoto, Japan) equipped with an alumina column and a flame ionization detector, for ethylene measurement.

Real-time RT-PCR analysis

We investigated changes in the expression levels of ethylene-signaling-pathway genes to understand how these genes are involved in the decrease in autocatalytic ethylene production. The levels of *DcACS1*, *DcACO1*, *DcCPI1*, *DcCPI2*, *DcCPIIn*, *DcbGal*, *DcGST1*, and *DcLip* mRNAs were determined in flowers by real-time RT-PCR analysis. Total RNA was extracted from the gynoecia and petals of flowers sampled on days 0, 5, 6, and 15 using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and 1 μg was used for the synthesis of first-strand cDNA using an oligo (dT) primer and performing reverse transcription using an Advantage RT-for-PCR kit (BD Bioscience Clontech, Palo Alto, CA, USA). The

amount of total RNA in each sample was normalized to that of the actin gene, *DcACT1-2* (Tanase et al., 2008).

Real-time PCR was performed in a LightCycler model 3.1 system (Roche Diagnostics, Mannheim, Germany) as described (Tanase et al., 2008). The sequences of primers for *DcACS1*, *DcACO1*, and *DcACT1-2* (actin), and duration condition are described in a previous paper (Tanase et al., 2008), and primers for the other genes are listed in Table 1. Amplification of PCR products was monitored via the intercalation of SYBR Green dye. Primer specificity was verified by PCR and melting curve analyses. Each assay with the gene-specific primers amplified a single product of the correct size at >90% efficiency. Linear dynamic-range assays were performed using serial dilutions of purified DNA as the template in accordance with the method in a previous report (Bustin et al., 2009). The levels of *DcACS1*, *DcACO1*, *DcCPI1*, *DcCPI2*, *DcCPIIn*, *DcbGal*, *DcGST1*, and *DcLip* transcripts were expressed relative to that of the *DcACT1-2* transcript in each sample.

Results

Flower life, fresh weight changes, and ethylene sensitivity of flowers

Vase life differed greatly among the lines. The vase life of untreated flowers of MS, 006-13, and 62-2 was significantly longer than that of WS (Table 2). Flowers of WS showed inrolling at senescence, but those of MS, 006-13, and 62-2 did not. Instead, they slowly faded and started browning from the petal edges, most slowly in

Table 1. Primers used for real-time RT-PCR.

Gene name	Forward primer sequence	Reverse primer sequence
<i>DcCPI1</i>	gatttcccagcaagctcgt	aaaacaaagccgagataaacg
<i>DcCPI2</i>	tacaaatcccgcattgaag	gatgttgctgcccctttcac
<i>DcCPIIn</i>	cgactgatggcggagttaag	gcatcagcatcatggtcttc
<i>DcbGal</i>	agcttttagatttgggcagca	agtagattccacgcgggttg
<i>DcGST1</i>	agatcatagcatcaatcacgg	cgacaagaataacttcacgtcg
<i>DcLip</i>	aagggggagtttaaggtgtgaaa	caaaaccactcaccatcct

Table 2. Flower life and senescence symptoms in carnation flowers during senescence.

Cultivar	Treatment	Flower life (days) ^x
White Sim	—	6.9 ± 0.1 ^a
White Sim	STS ^z	13.5 ± 0.2 ^b
Miracle Symphony	—	22.5 ± 0.9 ^{cd}
Miracle Symphony	STS	23.8 ± 0.7 ^c
006-13	—	14.8 ± 0.6 ^b
006-13	STS	18.2 ± 1.9 ^d
62-2	—	13.6 ± 0.6 ^b
62-2	STS	20.7 ± 0.9 ^d

Values are the means ± SE of 9 flowers.

^z Flower stems were held in a solution of 2 mM silver thiosulfate for 20 h and were then transferred to distilled water.

^x Different letters indicate a significant difference with $P \leq 0.05$ by Tukey's test.

MS. STS nearly doubled the vase life of WS flowers (Table 2). It also significantly extended the vase life of 006-13 and 62-2 flowers (by 3.4 and 7.1 days, respectively), but not that of MS flowers. There was no difference between the vase life of STS-treated 006-13 and 62-2 flowers and that of untreated MS flowers (Table 2).

Fresh weight changes corresponded closely to vase life (Fig. 2). The fresh weight of whole flowers of all cultivars and lines increased during the first 4 days and decreased thereafter. The fresh weight of WS decreased quickly after day 4, but those of MS, 006-13, and 62-2 decreased slowly.

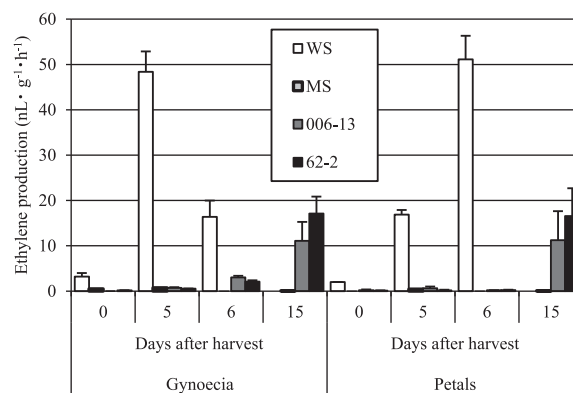
Ethylene sensitivity was measured as the time to the beginning of inrolling in the presence of exogenous ethylene. The mean response time of all lines was almost

the same, although that of MS was slightly longer (data not shown).

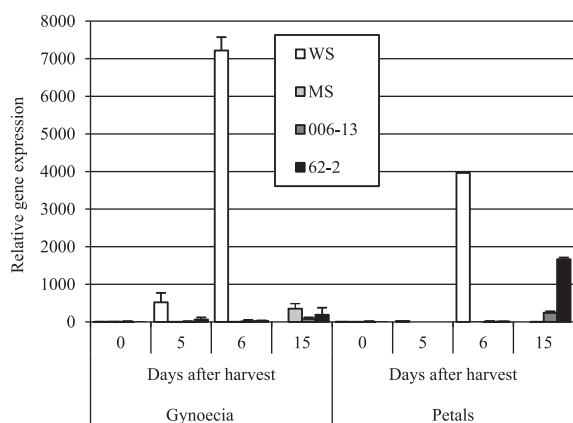
Ethylene production from flowers during senescence

Ethylene production by whole flowers of WS peaked

(A: ethylene production)



(B: *DcACSI*)



(C: *DcACO1*)

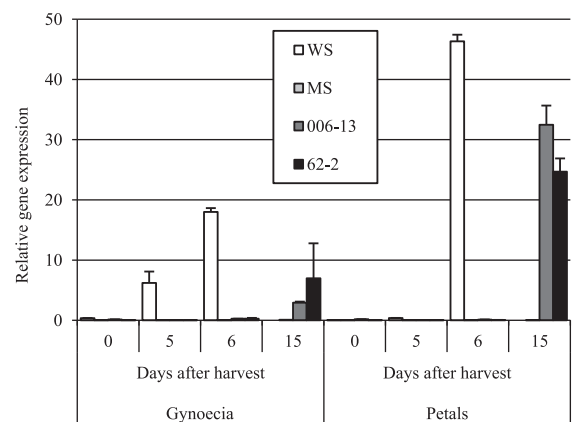


Fig. 4. (A) Ethylene production and levels of (B) *DcACSI* and (C) *DcACO1* transcripts in gynoecium and petal during flower senescence. Values shown in B and C are expressed relative to the expression of the actin gene *DcACT1-2*. Values are the means $\pm$ SE of 3 flowers. WS, 'White Sim'; MS, 'Miracle Symphony'.

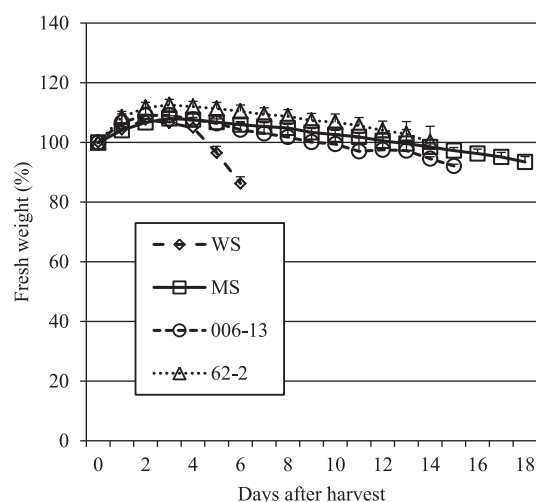


Fig. 2. Fresh weights of cut carnation flowers held in distilled water relative to the fresh weight on day 0. Values are the means $\pm$ SE of 6 replications. WS, 'White Sim'; MS, 'Miracle Symphony'.

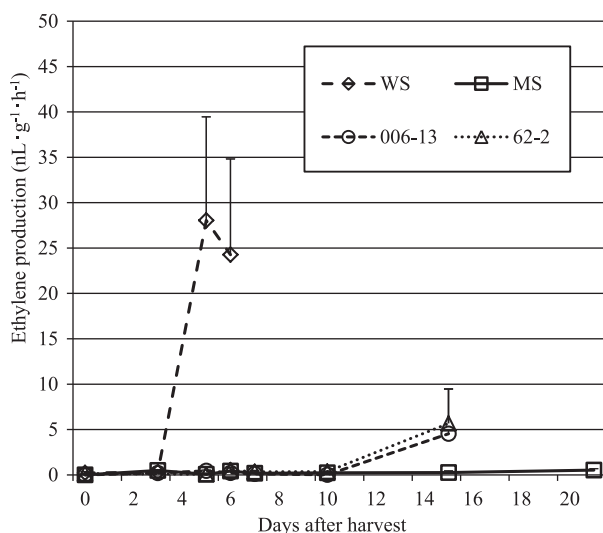


Fig. 3. Ethylene production in whole carnation flowers during senescence. Values are the means $\pm$ SE of 3 flowers. WS, 'White Sim'; MS, 'Miracle Symphony'.

at day 5 (Fig. 3). That by 006-13 and 62-2 stayed low until rising slightly by day 15 (to 4.5 and 5.7 $\text{nL}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$, respectively). That by MS stayed very low throughout the experiment. Ethylene production by WS gynoecia increased markedly at day 5 and decreased again at day 6, and that by petals increased until day 6 (Fig. 4A). That by gynoecia and petals of 006-13 and 62-2 was low from day 0 to day 6, but increased by day 15. That

by MS was very low in both tissues throughout the experiment.

Gene expression during flower senescence

Levels of *DcACS1* transcripts in gynoecia and petals of WS were much higher than in MS, 006-13, and 62-2 at day 6 (Fig. 4B). Those in gynoecia of MS, 006-13, and 62-2 increased by day 15, and those in petals of

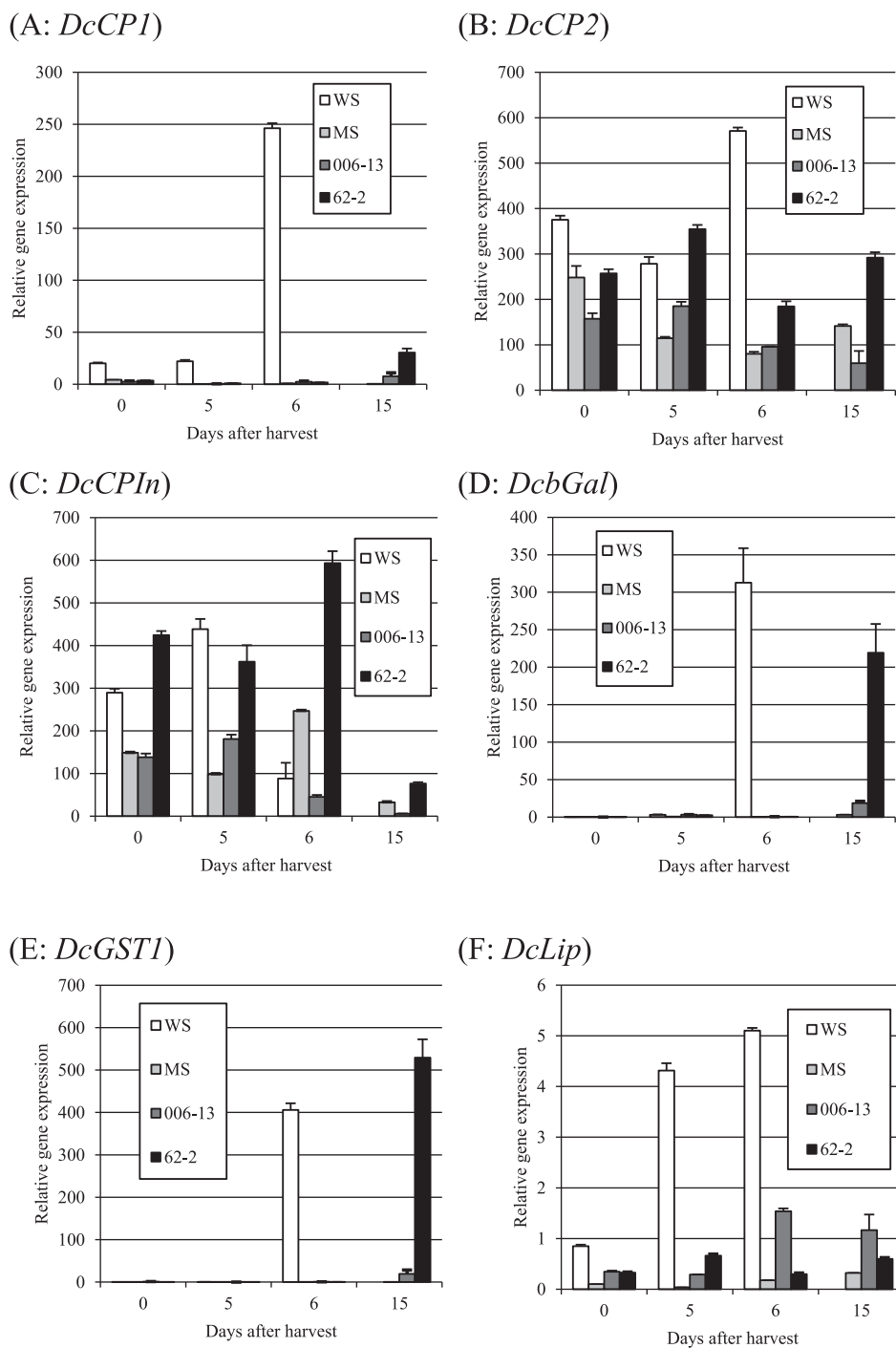


Fig. 5. Levels of (A) *DcCPI1*, (B) *DcCPI2*, (C) *DcCPIIn*, (D) *DcbGal*, (E) *DcGST1*, and (F) *DcLip* transcripts in petals during flower senescence. Values shown are expressed relative to the expression of *DcACT1-2*. Values are the means $\pm$ SE of 3 replications. WS, 'White Sim'; MS, 'Miracle Symphony'.

006-13 and 62-2 (but not MS) increased by day 15. Levels of *DcACO1* transcripts in WS flowers were high in gynoecia at days 5 and 6 and in petals at day 6 (Fig. 4C). Those in gynoecia and petals of 006-13 and 62-2 were low at days 5 and 6 and increased by day 15; however, no *DcACO1* expression was found in the gynoecia or petals of MS throughout the experiment.

Levels of *DcCPI*, *DcbGal*, and *DcGST1* transcripts

increased by day 6 in petals of WS and by day 15 in 006-13 and 62-2, but they were very low in MS (Fig. 5A, D–E). That of *DcLip* was high at days 5 and 6 in WS, and gradually increased in 006-13 and 62-2 during the experiment, but it remained low in MS (Fig. 5F). That of *DcCP2* was high in all cultivars and lines on day 0, and followed a different pattern in each (Fig. 5B). That of *DcCPI* in WS increased at day 5 and decreased markedly at day 6 (Fig. 5C). Those in MS and 62-2 decreased by day 15, but that in 006-13 decreased at day 6.

Effects of exogenous ethylene treatment on gene transcripts in WS, MS, and 006-13

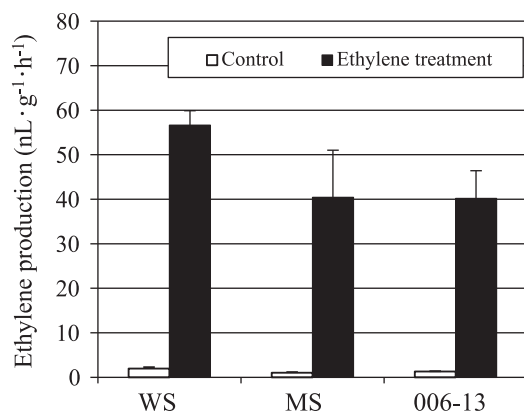
Unfortunately, line 62-2 wilted before flowers could be harvested for ethylene treatment. In the other line, treatment of petals with exogenous ethylene induced ethylene production, slightly more so in WS than in MS and 006-13 (Fig. 6A). Levels of *DcACS1* and *DcACO1* transcripts in ethylene-treated gynoecia and petals were higher than those in controls in all lines (Fig. 6B, C). Levels of *DcCPI*, *DcbGal*, *DcGST1*, and *DcLip* were higher in the ethylene-treated petals than in the control petals in all lines, but levels of *DcCPI* and *DcLip* expression were the same or lower in ethylene-treated petals of MS and 006-13 than in control petals of WS (Fig. 7A, D–F). Ethylene treatment increased the levels of *DcCP2* expression in WS and 006-13, but had no effect in MS (Fig. 7B). In contrast, ethylene treatment suppressed *DcCPI* expression in all lines to near zero (Fig. 7C). In particular, the level of *DcCPI* expression in control petals of WS was twice that in control petals of MS and 006-13.

Discussion

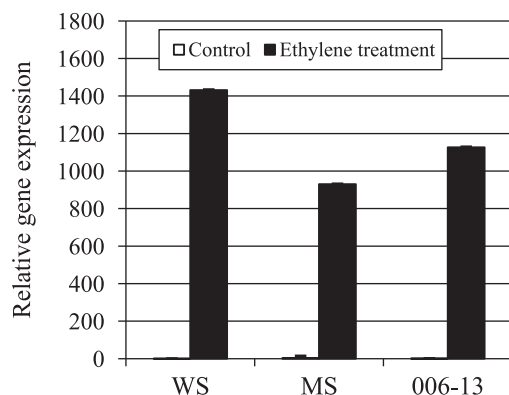
Genotypic variation in the vase life of cut carnation flowers results from variation in the flowers' ability to synthesize ethylene during senescence and in the petals' sensitivity to ethylene (Onozaki et al., 2001). Flowers of 006-13 and 62-2 produced low levels of ethylene during the experiment, and flowers of MS produced extremely low levels (Fig. 3). The genes responsible for low ethylene production were introduced from 'Sandroza' (Onozaki et al., 2001). Many long-life carnation lines with low ethylene production have been developed through cross-breeding, because the trait of low ethylene production is heritable.

Normal-life carnation flowers, such as WS, increase their ethylene production at the same time as they rapidly lose weight (Figs. 2 and 3) (Brandt and Woodson, 1992). MS, 006-13, and 62-2 all gradually lost fresh weight and produced little ethylene by day 15. In addition, STS treatment, which has been shown to slow the evolution of ethylene (Woodson and Lawton, 1988), extended the vase life of 006-13 and 62-2. Therefore, the low level of ethylene production during senescence in MS, 006-13, and 62-2 relative to WS was associated with longer

(A: ethylene production)



(B: *DcACS1*)



(C: *DcACO1*)

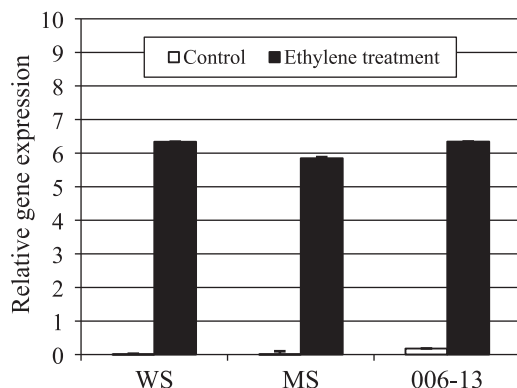


Fig. 6. (A) Ethylene production and levels of (B) *DcACS1* and (C) *DcACO1* transcripts in petal after exogenous ethylene treatment. Values shown in (B) and (C) are expressed relative to the expression of *DcACT1-2*. Values are the means $\pm$ SE of 3 flowers. WS, 'White Sim'; MS, 'Miracle Symphony'.

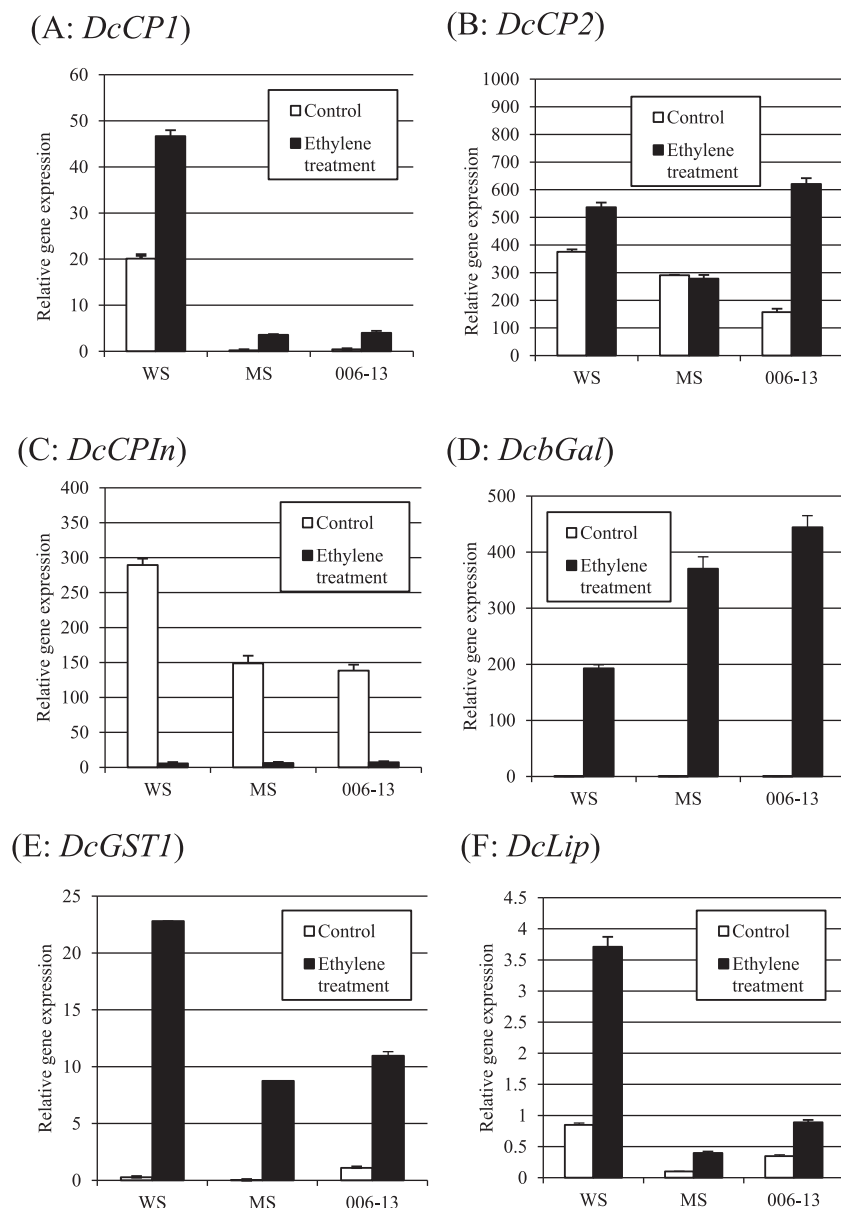


Fig. 7. Levels of (A) *DcCPI1*, (B) *DcCPI2*, (C) *DcCPIin*, (D) *DcbGal*, (E) *DcGST1*, and (F) *DcLip* transcripts in petals after exogenous ethylene treatment. Values shown are expressed relative to the expression of *DcACT1-2*. Values are the means $\pm$ SE of 3 replications. WS, 'White Sim'; MS, 'Miracle Symphony'.

vase life and lower loss of fresh weight, which delayed petal wilting.

In general, autocatalytic ethylene production in normal carnation flowers begins in the gynoecia and is subsequently induced in the petals (Sato and Waki, 2006; Shibuya et al., 2000; ten Have and Woltering, 1997). It is still unclear whether the low level of ethylene production by flowers of 006-13 and 62-2 is induced by ethylene from the gynoecia. Because the production was very low, it was difficult to characterize by gas chromatography. The use of a laser-driven photoacoustic detector system allowed the detection of low levels of ethylene and the real-time measurement of ethylene evolution from a single flower (Woltering et al., 1988). This technique will allow further investigation.

There were small differences in ethylene production by MS, 006-13, and 62-2. The late production of ethylene by 006-13 and 62-2 was associated with the upregulation of *DcACS1* and *DcACO1* (Fig. 4). MS, which produced extremely low levels of ethylene, had extremely low levels of *DcACS1*, *DcACO1*, *DcCPI1*, *DcbGal*, and *DcGST1*. *DcCPI1*, *DcbGal*, *DcGST1*, and *DcLip* are upregulated by exogenous ethylene (Fig. 7A, D–F) (Hong et al., 2000; Jones et al., 1995; Verlinden et al., 2002). Genomic analysis for characteristic *cis*-element motifs of promoter sequences identified the ethylene-responsive element (ERE) of carnation or ERE-like sequences in the 5'-upstream regions of these genes (Itzhaki and Woodson, 1993; Itzhaki et al., 1994; Kosugi et al., 2007; Verlinden et al., 2002). The low level of

ethylene production may be the cause of the delayed upregulation of *DcCP1*, *DcbGal*, *DcGST1*, and *DcLip*, which promoted senescence, in petals of 006-13 and 62-2.

Wilting of petals can be caused by hydrolytic enzymes, which collapse cellular components, and result in the degradation of cell structure and cell death (Jones et al., 1995; Jones et al., 2005; Sugawara et al., 2002). *DcCP1*, one such hydrolytic enzyme, probably plays an important role in protein degradation in carnation petals. The expression of *DcCP1* in petals increased at the onset of flower senescence: yet it was already present at detectable levels at the full open stage (Fig. 5) (Jones et al., 1995; Otsu et al., 2007); therefore, an unknown mechanism may suppress the action of CPase at the full open stage. One candidate for the CPase suppressor is *DcCPIn*, which inhibited the activity of proteinase *in vitro* (Sugawara et al., 2002). The expression of *DcCP1* increased in the petals of 006-13 and 62-2 on day 15, although only to the same low level as in WS on day 0 (Fig. 5A). The expression of *DcCPIn*, in contrast, fell in 006-13 and 62-2 by day 15 (Fig. 5C). It also fell in petals of MS, which did not show any senescence symptoms by day 15. These results suggest that the senescence of petals is related to the induction of *DcCP1* expression, but the relationship with the reduction of *DcCPIn* expression is less clear.

In general, carnation flowers are highly sensitive to exogenous ethylene (Onozaki et al., 2001; Woltering and van Doorn, 1988). The vase life of the tested carnation lines in this study was obviously shortened by exogenous ethylene, due to accelerated senescence of petals, which resulted in in-rolling and wilting (data not shown). Expression of *DcCP1*, *DcbGal*, *DcGST1*, and *DcLip* was upregulated by exogenous ethylene; in contrast, *DcCPIn* expression was downregulated by exogenous ethylene (Fig. 7A, C–F). These results suggest that senescence of petals accelerated by exogenous ethylene is related to the regulation of SR gene expression in carnation flowers.

The expression of another CPase gene, *DcCP2*, which was isolated from petals of normal-life carnations, remained steady from full open to wilting and was not responsive to ethylene or dehydration (Kosugi et al., 2007). We detected *DcCP2* on day 0, when senescence was not evident (Fig. 5). In addition, each of the long-life carnations showed a different pattern of *DcCP2* expression. These results suggest that *DcCP2* is not involved in petal wilting. As its role remains unclear, further study of its role in developing flowers is required.

These analyses confirm that the suppression of ethylene production prolongs the vase life of carnation flowers. The level of ethylene production was related to the levels of expression of *DcCP1*, *DcbGal*, *DcGST1*, and *DcLip*. Although *DcCPIn* expression was downregulated by exogenous ethylene, its level decreased in all lines during senescence. Other suppressors may also

regulate its expression in petals. Recently, an ultra-long-life carnation line was reported with a vase life of about a month, 5 times that of WS (Onozaki et al., 2011). We plan further research on the regulation of SR genes involved in ethylene biosynthesis to establish the basis of the ultra-long-life trait.

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