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Research note

Effect of ethanol on ethylene biosynthesis and sensitivity in cut carnation flowers

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ABSTRACT

The vase life of cut carnation (*Dianthus caryophyllus* cv. Barbara) flowers was significantly extended by continuous treatment with 1% (v/v) ethanol. To investigate effects of ethanol on sensitivity to ethylene, flowers were pre-treated with aminoethoxyvinylglycine (AVG) prior to placement in ethanol solution and exposed to ethylene at 0.2 and 2 $\mu L L^{-1}$. AVG treatment delayed petal senescence for the 0.2 but not the 2 $\mu L L^{-1}$ ethylene treated flowers. Petal senescence was similarly accelerated by ethylene exposure, irrespective of ethanol treatment, suggesting that the effect of ethanol on sensitivity to ethylene is negligible. 1-Aminocyclopropane-1-carboxylic acid (ACC) levels, along with ACC synthase (ACS) and ACC oxidase (ACO) activities and the transcript levels of the corresponding genes in petals, *DcACS1* and *DcACO*, increased with flower senescence, and this increase was suppressed by ethanol treatment. These findings suggest that extension of vase life by ethanol treatment is attributable to the suppression of ethylene biosynthesis through the suppression of ACS and ACO at the transcriptional level.

1. Introduction

Carnation flowers are highly sensitive to ethylene, which increases during flower senescence (Wu et al., 1992). 1-Aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO), which catalyzes the final step of ethylene biosynthesis, contributes to the climacteric increase in ethylene production in carnation petals (Woodson et al., 1992). Three genes for ACS, *DcACS1*, *DcACS2* and *DcACS3* have been cloned from carnation (Jones and Woodson, 1999). Among these, *DcACS1* is expressed mainly in the petals whereas *DcACS2* and *DcACS3* are expressed in the gynoecium (Jones and Woodson, 1999). For ACO, one gene has been cloned and designated *DcACO1* (Wang and Woodson, 1991).

Continuous treatment with ethanol at 1 to 8% extends the vase life of cut carnations (Heins, 1980; Wu et al., 1992; Pun et al., 1999). Delay of senescence in cut carnation flowers by ethanol is attributable to the suppression of ethylene production (Wu et al., 1992). Ethanol treatment suppresses increases in ACC production and *in vivo* ACO activity in the petals of cut carnations (Wu et al., 1992). Similarly, ethanol suppresses increased *in vitro* ACO activity in broccoli (Suzuki et al., 2004). However, the effect of ethanol on ACS activity, on *in vitro* ACO activity and on the expression of genes responsible for ethylene biosynthesis has not previously been reported in carnations.

In terms of the effect of ethanol on sensitivity to ethylene, Wu et al. (1992) reported that time to petal wilting was shorter in ethanol-treated flowers than in untreated flowers when ethylene was supplied at concentrations lower than 0.6 $\mu L L^{-1}$, and sensitivity to ethylene was found to be slightly reduced by ethanol. As shown for the ethanol effect, inhibitors of ethylene biosynthesis, including AVG and aminoaxyacetic acid (AOA) appear to reduce flower sensitivity to ethylene (Broun and Mayak, 1981; Cook et al., 1985). However, these effects can be attributed to suppression of ethylene biosynthesis because autocatalytic ethylene production is known to affect ethylene sensitivity (Woodson and Lawton, 1988). Thus, ethylene inhibitors should be included in test solutions in order to adequately evaluate the effect of these compounds on sensitivity to ethylene. For instance, Porat et al. (1994) used AOA to evaluate ethylene sensitivity, but no ethylene inhibitors were included in the test solution in the study of Wu et al. (1992).

In the present study, we investigated the effect of ethanol on ethylene sensitivity, ethylene production, ACC concentration and

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on ACS and ACO activities and gene expression in order to clarify the mode of ethanol action on ethylene biosynthesis and sensitivity in cut carnation flowers.

2. Materials and methods

Carnations (*Dianthus caryophyllus*) cv. Barbara were grown under natural day length conditions in a greenhouse. When the outer petals became perpendicular to the stem axis, flowers were cut and immediately transported to the laboratory. The peduncles were trimmed to 5 cm, and treatment was started within 1 h. Ethanol (99.5%, Wako, Osaka, Japan) at various concentrations in distilled water (0, 1, 2, 3 and 5% v/v) was prepared in test tubes and covered with Parafilm (American Can, Menasha, WI, USA) to reduce evaporation. The cut end of the flower stem was inserted into the test solution through a hole in the Parafilm. Flowers were held at 23 °C, 70% RH throughout the experimental period with a photoperiod of 12 h at 10 μmol m^{-2} s^{-1} light intensity from cool-white fluorescent lamps. Each day, the ethanol treatment solutions were replenished. Vase life of the flower was determined as the time from the start of ethanol treatment to the time when petals showed inward rolling or wilting.

To evaluate sensitivity to ethylene, cut flowers were treated with 0 or 5 mM AVG (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 23 °C, 70% RH under 10 μmol m^{-2} s^{-1} PPFD, and the flowers were then transferred to 0 or 1% ethanol solution and placed in one of two 70 L transparent acrylic chambers held at 23 °C under continuous light at 10 μmol m^{-2} s^{-1} PPFD. Pure ethylene (GL Sciences, Tokyo, Japan) was introduced through the septum to produce a concentration of 0.2 or 2 μL L^{-1}. The flowers were photographed every 1 h by digital camera. Sensitivity to ethylene was evaluated based on the time from the start of ethylene treatment to the time when petals showed inward rolling or wilting.

Ethylene production from petals and gynoecium was determined as described by Ichimura et al. (2009). For the extraction of ACC, ACC synthase and ACC oxidase, petals (0.5 g) obtained from three flowers were extracted as described by Ichimura et al. (2009). ACC concentration was determined by the method of Lizada and Yang (1979). ACC synthase activity was measured by incubating a standard reaction mixture containing 0.4 mL of enzyme preparation, and 0.1 mL each of 4 mM HEPES-KOH (pH 8.5), 0.3 mM S-adenosylmethionine (Sigma-Aldrich) and 0.4 μM pyridoxal 5-phosphate (Sigma-Aldrich) at 30 °C for 30 min and stopping the reaction by the addition of 0.1 mL of 20 mM $HgCl_2$. ACC was assayed according to Lizada and Yang (1979), and ACC oxidase activity was measured as described by Ichimura et al. (2009).

To determine *DcACS1* and *DcACO1* transcript levels, petals were collected from three individual flowers. Total RNA was isolated from six petals using ISOGEN (Nippongene, Toyama, Japan), treated with an RNase-free DNase set (Qiagen, Hilden, Germany) to digest genomic DNA, and purified using an RNeasy mini kit (Qiagen). Synthesis of cDNA was carried out with random hexamer primers using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). Preparation of the primer pairs and PCR reactions were performed as described in Tanase et al. (2008). The following thermalcycler program was applied: initial polymerase activation at 95 °C for 10 min; then for *DcACT1-2*, a constitutively expressed control gene, 30 cycles of 94 °C for 10 s, 60 °C for 10 s, 72 °C for 15 s; for *DcACS1*, 50 cycles of 94 °C for 10 s, 55 °C for 15 s, 72 °C for 20 s; and for *DcACO1*, 40 cycles of 94 °C for 10 s, 60 °C for 10 s, 72 °C for 15 s. Transcript levels of the target genes were normalized to the transcript levels of *DcACT1-2* and reported as relative transcript level based on the method of Shibuya and Ichimura (2010).

Table 1
Effect of ethanol and AVG on ethylene-induced flower senescence of cut carnation flowers.

Ethanol (%)	AVG (mM)	Ethylene (μL L^{-1})	Vase life (h)
0	0	0	158.4 ± 10.2c
0	0	0.2	38.9 ± 1.3a
0	0	2	7.9 ± 0.2a
0	5	0	328.8 ± 8.8d
0	5	0.2	99.8 ± 15.4b
0	5	2	8.0 ± 0.4a
1	0	0	336.0 ± 8.8d
1	0	0.2	41.9 ± 0.7a
1	0	2	8.3 ± 0.3a
1	5	0	314.4 ± 12.6d
1	5	0.2	91.8 ± 14.9b
1	5	2	8.3 ± 0.3a

Values are means of 10 flowers ± S.E. and those with the same letter are not significantly different by the Tukey-Kramer's multiple range test ($P<0.05$).

3. Results and Discussion

The vase life of control flowers was 6.7 days and 1% ethanol significantly increased vase life to 12.8 days. There were no significant differences in vase life among ethanol treatments (1%, 2%, 3% and 5%) and 1% ethanol was used for subsequent experiments. As observed in the present study, the effective concentrations are lower for shorter flower stems although effective ethanol concentrations have been reported to vary from 1% to 8% (Heins, 1980; Wu et al., 1992; Podd and van Staden, 1999).

To exclude the known effect of autocatalytic ethylene production on ethylene responsiveness (Woodson and Lawton, 1988), ethylene inhibitors should be included in the test solution. Here, flowers were treated with AVG prior to being subjected to ethanol and ethylene treatments in order to clarify whether exposure to ethanol reduces sensitivity to ethylene. Irrespective of ethanol treatment, AVG treatment significantly delayed petal wilting of flowers exposed to 0.2 μL L^{-1} ethylene by 61 h (Table 1), which is consistent with findings reported by Cook et al. (1985). These findings together with our results suggest that the delay may be due to the inhibition of ethylene biosynthesis. However, AVG treatment did not delay petal wilting in the 2 μL L^{-1} ethylene treatment (Table 1). Without AVG treatment, the control and ethanol-treated flowers exhibited similar wilting patterns following exposure to 0.2 and 2 μL L^{-1} ethylene, suggesting that the effect of ethanol on sensitivity to ethylene is negligible in cut carnation 'Barbara'. In the present study, control flowers were not placed in the chambers to avoid molding. Separately, we confirmed that flower senescence was not affected by the chamber condition (data not shown).

As reported in previous studies (Wu et al., 1992), ethanol treatment reduced ethylene production from petals (data not shown). In control flowers, ACC concentration in the petals gradually increased during the first 6 days (Fig. 1), whereas ACC concentration in ethanol-treated flowers was low until day 12. Ethanol suppressed and delayed ACS activity and almost completely suppressed the increase in ACO activity in petals. Transcript levels and respective enzyme activities were similar for *DcACS1* and *DcACO1*. In control flowers, the peak in activity of ACS and ACO on day 7 was well matched to the peak in transcript level of *DcACS1* and *DcACO1* on day 7 (Fig. 2). Ethanol treatment suppressed the increase in the level of these transcripts. These results suggest that suppression of ACS and ACO activities is mainly regulated at the transcriptional level. In accordance with suppression of *in vitro* ACO activity, expression of *DcACO1* was suppressed by ethanol. *DcACT1-2* was used as an internal standard because this gene has been used in many papers (Shibuya and Ichimura, 2010; Harada et al., 2011; In et al., 2013). However, we observed that transcript level of *DcACT1-2* showed