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Physiology

Ethylene production associated with petal senescence in carnation flowers is induced irrespective of the gynoecium

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SUMMARY

To clarify whether climacteric-like increases in ethylene production of senescing petals are also induced in the absence of the gynoecium in cut carnation (*Dianthus caryophyllus* cv. Barbara) flowers, we compared ethylene production and expression of ethylene-biosynthesis genes in detached petals and in petals, which remained on flowers (attached petals). No significant difference in longevity was observed between the attached and detached petals when held in distilled water, and both showed the inward rolling typical of senescing flowers. Treatment with silver thiosulfate complex (STS), an ethylene inhibitor, similarly delayed senescence of attached and detached petals. Climacteric-like increases in ethylene production of petals and gynoecium started on the same day, with similar bursts in attached and detached petals. Transcript levels of *DcACS1* and *DcACO1* were very low at harvest and increased similarly during senescence in both petal groups. Removal of the gynoecium did not significantly delay wilting of attached petals. In flowers with the gynoecium removed, the petals produced most of the ethylene while production by the other floral organs was very low, suggesting that wound-induced ethylene is not the reason for the ineffectiveness of gynoecium-removal in inhibiting flower senescence. These results indicate that ethylene biosynthesis is induced in carnation petals irrespective of the gynoecium.

Introduction

Carnation flowers are highly sensitive to ethylene (Wu et al., 1991; Onozaki et al., 2004) and ethylene production increases in flowers during senescence (Nichols, 1966). The application of ethylene inhibitors including silver thiosulfate complex (STS) (Veen, 1979), aminoethoxyvinylglycine (Baker et al., 1977) and aminooxyacetic acid (Broun and Mayak, 1981) delays senescence of carnation flowers, indicating that the senescence is regulated by endogenous ethylene.

Following pollination, ethylene production in carnation increases sequentially in the order style, ovary and petals (Jones and Woodson, 1997, 1999a). This suggests that some signals generated by pollination induce ethylene production. For unpollinated carnation flowers, it has been postulated that ethylene produced by the gynoecium regulates petal senescence (ten Have and Woltering, 1997). This assumption is based on data showing that the climacteric increase in ethylene production of the gynoecium is preceded by an ethylene burst in petals (Woodson and Brandt, 1991) and that a climacteric increase is absent in detached petals (ten Have and Woltering, 1997). Furthermore, removal of the gynoecium, which is accompanied by suppression of ethylene production, markedly delayed petal senescence (Shibuya et al., 2000). These findings suggest that ethylene produced by the gynoecium may trigger ethylene production in petals, thereby inducing petal wilting in unpollinated flowers. However, whether petal senescence of carnation flowers occurs independently or is influenced by other organs remains controversial. Mor et al. (1980) reported no difference in longevity of attached and detached carnation petals. In addition, removal of the gynoecium did not delay senescence of attached petals (Mor et al., 1980; Sacalis and Lee, 1987).

The biosynthetic pathway of ethylene has been elucidated in higher plants as follows: methionine → S-adenosyl-L-methionine (SAM) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene. ACC is produced from SAM by ACC synthase (ACS). The precursor of ethylene, ACC, is converted to ethylene, carbon dioxide and HCN by ACC oxidase (ACO). In general, the conversion by ACS was identified as the rate-limiting step because ACO activity is constitutive in many plant tissues (Kende, 1993). Similarly, ACS and ACO were found to contribute to the climacteric

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; DW, distilled water; STS, silver thiosulfate complex.

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increase in ethylene production of carnation petals (Woodson et al., 1992; Woltering et al., 1993; Lee et al., 1997).

ACS has been shown to be encoded by a multi-gene family (Kende, 1993). In carnation, three genes encoding ACS have been isolated and are designated *DcACS1*, *DcACS2* and *DcACS3* (Park et al., 1992; Jones and Woodson, 1999b). The expression of *DcACS1* in petals increased during senescence of carnation flowers (Woodson et al., 1992). ACO is known to be encoded by a multi-gene family, but only one ACO gene, *DcACO1*, has been cloned in carnation (Wang and Woodson, 1991). In cut carnation flowers, the expression of *DcACO1* is very low in petals and ovary at harvest, but increases during senescence (Jones and Woodson, 1997).

In the present study, we investigated the longevity, ethylene production and transcript levels of *DcACS1* and *DcACO1* of attached and detached petals to clarify whether a climacteric-like increase in senescence-associated ethylene production is induced in the absence of the gynoecium in cut carnations.

Materials and methods

Plant materials

Carnation (*Dianthus caryophyllus* L.) cv. Barbara was grown in a greenhouse under natural sunlight. When outer petals were perpendicular to the stem axis in the morning, flowers were harvested and transported immediately to a controlled-environment room. The peduncles were trimmed to 50 mm, and treatment was started within 1 h.

Attached and detached petals

Five outer petals were detached from an individual flower (detached petals) on the day of harvest and were compared to petals remaining intact on flowers (attached petals).

STS treatment

A 0.2 mM silver thiosulfate complex (STS) solution was prepared by mixing 0.1 M $AgNO_3$ and 0.1 M $Na_2S_2O_3$ at a volume ratio of 1:8 and dilution of the mix with distilled water (DW) at 1–55. Harvested flowers ($n = 8$) were treated with STS or DW (control) at 23 °C, 70% relative humidity at 10 μmol m^{-2} sec^{-1} light intensity for 4 h prior to petal detachment and the start of observation.

Removal of gynoecium

In a separate examination of intact flowers (attached petals), the gynoecium was left intact (gynoecium intact, $n = 20$) or removed on the day of harvest (gynoecium removed, $n = 20$) as described by Shibuya et al. (2000). Briefly, the calyx was stripped, and the gynoecium was snapped off from each flower by hand.

Incubation conditions and evaluation of petal longevity

Following manipulations and/or STS treatment, flowers and detached petals were transferred to DW and held at 23 °C, 70% relative humidity with a photoperiod of 12 h at 10 μmol m^{-2} sec^{-1} light from cool-white fluorescent lamps. Petal longevity was determined as the duration from the start of incubation to the petals showing inward rolling, wilting or necrosis. For the evaluation of detached petal longevity, the appearance of senescence symptoms in three of five petals from an individual flower was taken as the endpoint.

Ethylene production

Petals, gynoecium, stamen, receptacle and calyx were collected at 0, 2, 3, 4, 5, 6, 7 and 8 d after harvest. All of the petals removed from the attached petal group were enclosed in a glass bottle (148 mL), and detached petals and the other organs were separately enclosed in test tubes (15 mL). After incubation at 23 °C for 1 h, an aliquot of gas (1 mL) was withdrawn with a hypodermic syringe and injected into a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) equipped with an alumina column and flame ionization detector. The GC conditions used were: 100 °C injection temperature, 80 °C column temperature, and 40 mL min^{-1} carrier gas flow rate.

Preparation of total RNA and real-time RT-PCR

Two petals were collected from each of three flowers at 0, 4, 5, 6, 7 and 8 d after harvest. Petals from each time point were pooled and total RNA was isolated using ISOGEN (Nippongene, Toyama, Japan). Extractions were digested with an RNase-free DNase kit (Qiagen, Hilden, Germany) and purified using an RNeasy mini kit (Qiagen). The quality and quantity of RNA were checked using a DU640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) and agarose gel electrophoresis.

Total RNA samples (1 μg) were reverse-transcribed using the PrimeScript RT Master Mix (Takara Bio, Otsu, Japan) in 20 μL reactions. Synthesized cDNA (1 μL aliquot) was amplified using SYBR Premix Ex Taq II (Takara Bio) and the following primer pairs: *DcACS1* (forward, 5′-CAG TGT GAC GCC ATT GAA AC-3′ and reverse, 5′-TTA AGT TCA ATG TTT TGT TGA GAA GA-3′); *DcACO1* (forward, 5′-GCC AAC ATT GGT GGA AAA AG-3′ and reverse, 5′-CAA TCC ATA GGA CAT GGA ACA-3′); and *DcUbq3-7*, a control gene (forward, 5′-GTT GTT GGT TTC AGG GCT GGT TTG-3′ and reverse, 5′-CTA CGG TAA TTG AGA ATT CAC ACC GAA ATG-3′). Amplification was performed using the Thermal Cycler Dice Real Time System (TP800, Takara Bio) with an initial polymerase activation at 95 °C for 30 s followed by the following gene-specific programs: *DcUbq3-7* and *DcACS1*, 40 cycles at 95 °C for 5 s and 60 °C for 30 s; *DcACO1*, 40 cycles at 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. The specificity of the PCR was checked using a heat dissociation protocol (from 60 to 95 °C) after the final cycle. In addition, the PCR products were run on agarose gel electrophoresis to confirm that the amplification products were of the target length. The specificity of each primer pair was confirmed by direct DNA sequencing of the PCR products.

The absolute transcript level was determined by the second derivative maximum method (Luu-The et al., 2005) using a dilution series of plasmid DNA containing target sequences as external standards. The transcript levels of *DcUbq3-7* were almost constant during petal senescence as reported by Nomura et al. (2012). To standardize the data, the ratio between the absolute transcript level of the target gene and the control gene was calculated for each sample. The mean absolute transcript level and ratios were obtained for three independent experiments.

Statistical analysis

The significance of pairwise differences in means was analyzed by the *t*-test using Stat View software (v.5.0, SAS Institute Inc., Cary, NC, USA).

Results

Longevity of attached and detached petals

Longevity was 26.5 h longer for the detached petal group than the attached petal group, but the difference in longevity was not