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## Ethylene responsiveness in carnation styles is associated with stigma receptivity

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**Abstract** Sensitivity to ethylene increases as flowers mature, and this is associated with autocatalytic ethylene production that initiates petal senescence. The coordination of the senescence process within the flower requires interorgan communication. To determine if differential ethylene responsiveness among floral organs is involved in regulating senescence signaling, ethylene biosynthesis and the expression of ethylene biosynthetic genes were investigated in styles, ovaries, and petals from 6 stages of flowers following ethylene treatment. As flowers matured, all floral organs investigated became more responsive to ethylene. Ovaries were the first flower organ that had detectable increases in ethylene production following ethylene treatment. In stage 4 and 5 flowers, the highest levels of ethylene production were detected in petals, while styles had the highest ethylene production at stage 6. 1-Aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) genes were induced by ethylene treatment, and transcript abundance of DCACS1, DCACS2 and DCACO1 increased in all floral organs as flowers matured. DCACS3, a pollination-responsive ACS gene, did not show a significant increase in transcript levels following ethylene treatment except in styles. Patterns of gene expression in ethylene-treated styles and petals corresponded with increases in ethylene biosynthesis, with increases in ACS mRNA abundance detected at stage 1 or 2 in petals and at stages 4, 5, or 6 in styles. The largest increases in ethylene production and gene expression occurred in styles at stages 5 and 6 and corresponded to the stages at which the styles were first receptive to pollination. Reasons for differential ethylene sensitivity among the flower organs during development are discussed.

**Keywords** Autocatalytic ethylene · Carnation · Styles · Senescence

### Introduction

Senescence is a highly ordered process that is accompanied by an increase in ethylene production in carnation (*Dianthus caryophyllus* L. 'White Sim') flowers (Borochoy and Woodson 1989). In higher plants, two systems of ethylene production are believed to operate. System-1 represents the basal levels of ethylene production detected in all plant tissues including flowers, while system-2 functions during petal senescence and fruit ripening and represents a shift to an autostimulatory or autocatalytic production of ethylene (Yang and Hoffman 1984; Kende 1993; Woodson 1994). This autocatalytic ethylene production requires the induction of genes involved in ethylene biosynthesis. The regulatory control of this pathway occurs at the conversion of *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) catalyzed by the enzyme ACC synthase (ACS), and at the oxidation of ACC to ethylene by ACC oxidase (ACO) (Kende 1993; Fluhr and Mattoo 1996).

The conversion from system-1 to system-2 requires the tissue to develop an increased sensitivity to ethylene (Whitehead 1994; Lelievre et al. 1998). Both flowers and fruits have been shown to increase their responsiveness or sensitivity to ethylene as they mature (Nichols 1968; Barden and Hanan 1972; Mayak et al. 1977; Halevy and Mayak 1981; Liu et al. 1985; Woodson and Lawton 1988). While climacteric (system-2) ethylene production and ripening are accelerated by treating mature green tomatoes with ethylene, treatment of immature green fruit does not induce ACS activity or enhanced ethylene production (Liu et al. 1985). Similarly, the treatment of immature carnation petals with exogenous ethylene does not induce autocatalytic ethylene production or enhanced expression of senescence-related genes (Barden and Hanan 1972; Woodson and Lawton 1988). Recently, Barry et al. (2000) demonstrated that system-1 ethylene

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in green fruit and system-2 ethylene production during ripening were the result of differential expression of members of the tomato ACS gene family.

The mechanism by which ethylene sensitivity is regulated is unknown. In flowers, attempts to relate changes in sensitivity to ethylene binding have given variable results (Brown et al. 1986; Wu et al. 1991; Woltering et al. 1993). Consequently, changes in ethylene sensitivity during flower development or following pollination have been measured as the tissues capability to respond to exogenous ethylene application with petal senescence and increased ethylene production (Whitehead and Halevy 1989; Wu et al. 1991; Porat et al. 1994; Ketsa and Rugkong 2000).

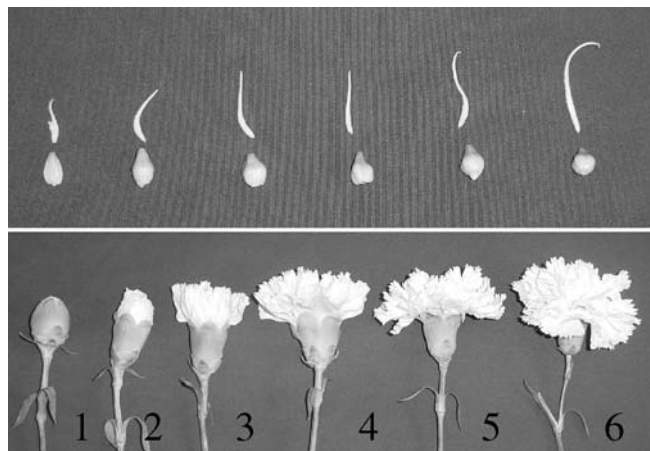
The flower is a complex organ composed of many tissues senescing at different rates. Ethylene treatment of carnation flowers results in differential regulation of ACS gene expression within the different flower organs (Woodson et al. 1992; ten Have and Woltering 1997; Jones and Woodson 1997, 1999a). Previous studies have demonstrated that the style has a central role in coordinating the senescence process within the flower following pollination (Gilissen 1977; Jones and Woodson 1997). Recent studies by Shibuya et al. (2000), have demonstrated that the careful removal of the gynoecium prolongs the vase life of carnations, suggesting that the style and/or the ovary is involved in the initiation of senescence during natural aging.

To determine if carnation flower organs develop responsiveness to ethylene at similar stages of development, the capacity to produce autocatalytic ethylene and induce ACS and ACO genes following ethylene exposure was investigated in styles, ovaries, and petals from 6 flower stages. In carnation flowers the post pollination response, including accelerated corolla senescence and autocatalytic ethylene production from the petals, is dependent on autocatalytic ethylene production by the style, and is prevented when ethylene perception is inhibited in the style prior to pollination (Jones and Woodson 1997). To determine if styles were more responsive to ethylene when receptive to pollination, stigma receptivity was assessed in styles from the 6 flower stages.

## Materials and methods

### Plant material and ethylene treatment

Greenhouse-grown carnations (*D. caryophyllus* L. 'White Sim') were used in all experiments. Carnations were grown at the Colorado State University Holley Plant Environmental Research Center in a fiberglass-reinforced plastic greenhouse under natural photoperiod at 25/16°C (day/night). Flowers were harvested at 6 developmental stages (Fig. 1). Stems were recut to 13 cm and placed in deionized H<sub>2</sub>O in the laboratory. Flowers were sealed in a 24 l chamber, and ethylene was injected to give a final concentration of 10 µl l<sup>-1</sup>. Control flowers were held in a chamber with ethylene-free air. Flowers were treated for 24 h. After treatment, flowers were removed from the chambers and placed in air for 1 h to let exogenously applied ethylene diffuse away before ethylene production from stigma/styles, ovaries, and petals was determined.



**Fig. 1** Stages of carnation flower development. Stages: 1 Petals are showing color but have not emerged beyond the calyx; 2 petals emerged 12 mm from calyx; 3 petals separated and forming a 30° angle with the axis of the calyx; 4 petals fully open forming a 90° angle with the axis of the calyx, style just starting to elongate; 5 petals open slightly beyond 90°, style elongated and base of style expanded; 6 petals open beyond 90°, style elongated, tip starting to curl, base of style fully expanded. The visual differences between the corollas of stage 4 and stage 5 flowers is very slight and their stages are most accurately determined by visualizing the style

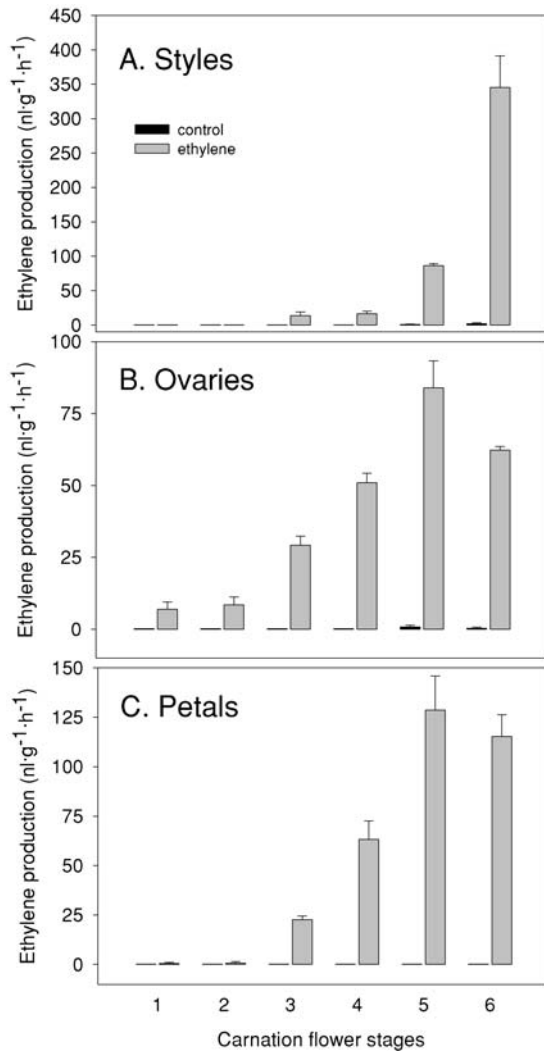
Hereafter, the term style will be used to refer to the floral organ that includes the style and the stigmatic surface, tissues that are not morphologically distinct in carnation flowers. For each flower, the ovary, all of the styles (2–4) and three petals were enclosed in individual 6-ml vials with a rubber septum. Following a 15 min incubation period, 1-ml gas samples were withdrawn from the vials and analyzed for ethylene using a gas chromatograph equipped with a Haysep R packed column and flame ionization detector (Varian, Walnut Creek, Calif.). All experiments used a replication of 12 flowers and the graphed values represent the mean ethylene production ±SE. All experiments were conducted a minimum of three times with similar results. Following ethylene measurements, floral organs were weighed, quick frozen in liquid N<sub>2</sub>, and stored at -80°C for RNA extraction.

### RNA extraction and gel blot analysis

RNA extraction and gel blots were conducted as described previously (Jones et al. 1995; Jones and Woodson 1999a). Probes used for the detection of ACC synthase were gene-specific probes containing the 3'-untranslated regions of three carnation ACC synthase cDNAs (DCACS1, DCACS2, DCACS3) as described by Jones and Woodson (1999a). ACC oxidase expression was detected with the carnation cDNA DCACO1 (Wang and Woodson 1991). To demonstrate equal loading of RNA samples, membranes were re-probed with ribosomal RNA (Goldsbrough and Cullis 1981).

### Stigma receptivity

Stigma receptivity was determined utilizing the alpha-naphthyl acetate test for esterase activity (Dafni 1992). The presence of esterase activity has been found to correlate with stigma receptivity (Dafni 1992). Esterase activity is indicated by the production of a deep blue color in the papillae. Styles were visualized using a Leica MZ8 microscope and images were captured with an Optronics Magnafire digital camera.

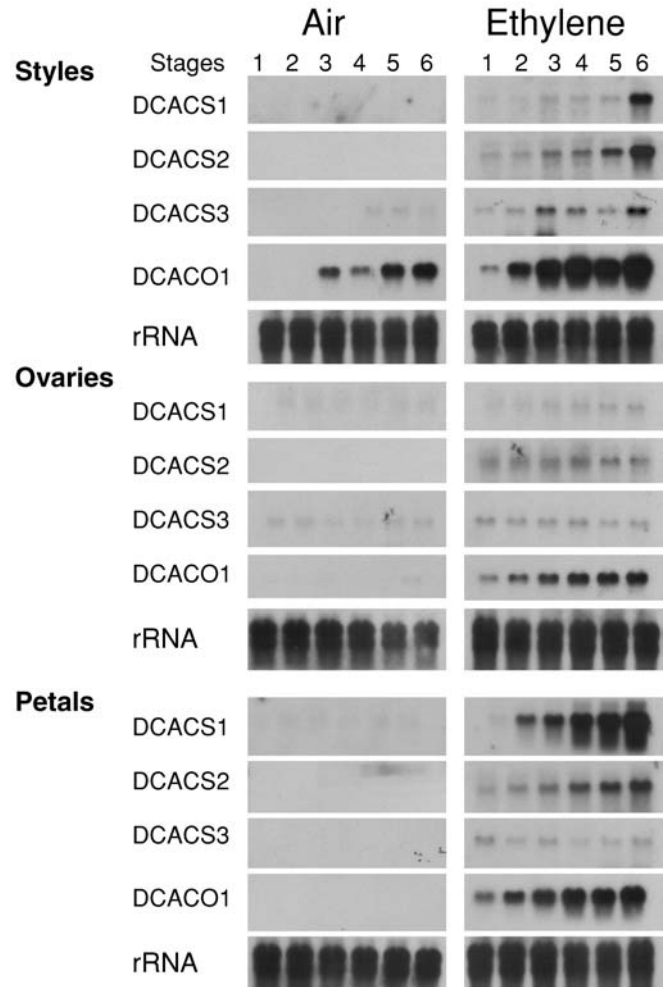


**Fig. 2** Ethylene production by **a** styles, **b** ovaries, or **c** petals from flowers treated with air (control) or  $10 \mu\text{l l}^{-1}$  ethylene for 24 h. Values represent means  $\pm$ SE of 12 flowers

## Results and discussion

Flower senescence is controlled by both an increase in ethylene production and an increase in sensitivity to ethylene (Borochoy and Woodson 1989). Responsiveness of carnation flower organs to ethylene changed during aging, with styles, ovaries, and petals from immature flowers producing less ethylene following a 24 h treatment with  $10 \mu\text{l l}^{-1}$  ethylene than organs from mature flowers (Fig. 2). In ethylene-treated flowers from stages 1 and 2, only ovaries produced more ethylene than controls held in air. By stage 3, ethylene production by all flower organs was higher in ethylene-treated than control organs.

Styles from ethylene-treated flowers had little increase in ethylene biosynthesis until stages 5 and 6. At stage 6, styles were producing  $345 \text{ nl g}^{-1} \text{ h}^{-1}$  compared to rates of  $86 \text{ nl g}^{-1} \text{ h}^{-1}$  at stage 5. Ethylene treatment resulted in up-regulation of both ACS and ACO transcripts



**Fig. 3** Expression of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) (DCACS1, DCACS2, and DCACS3) and ACC oxidase (ACO) (DCACO1) genes in styles, ovaries, and petals from flowers treated with air (control) or  $10 \mu\text{l l}^{-1}$  ethylene for 24 h. Blots were probed with rRNA to demonstrate equal loading. Numbers at the top of the blots indicate the flower stage

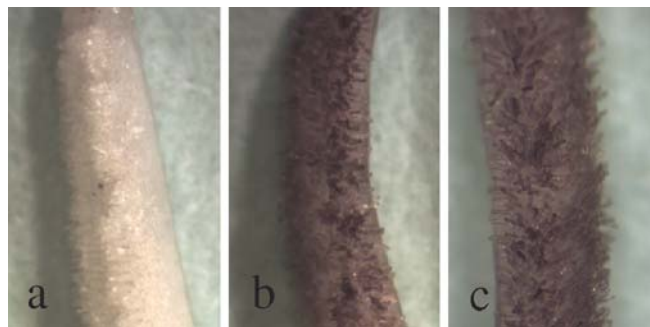
in styles (Fig. 3). DCACS3 was induced by ethylene to a similar level in styles from stage 3 and 4 flowers with a decrease detected in stage 5 and a subsequent increase in mRNA abundance in stage 6 flowers. DCACS3 is a pollination-responsive gene that is also up-regulated in styles by LiCl and 2,4-dichlorophenoxyacetic acid treatment (Jones and Woodson 1999a). DCACS2 expression steadily increased in styles from stage 1 to 6, with the largest increases detected from stage 5 to 6. Increases in DCACS1 were not detected until stage 6. DCACS1 and DCACS2 are both ethylene-responsive genes (Woodson et al. 1992; Jones and Woodson 1997, 1999a) and their increased expression at stage 6 correlated with the large increases in ethylene production detected from stage 6 styles. DCACO1 transcript accumulation also increased following ethylene treatment in styles, but mRNAs were equivalent in mature stages 4–6. This suggests that ACS limits autocatalytic ethylene production in styles. Ethyl-

ene production from air treated (control) styles was below  $2 \text{ nl g}^{-1} \text{ h}^{-1}$  at all developmental stages. The constitutive DCACO1 mRNA expression, which has previously been reported in styles (Woodson et al. 1992; Jones and Woodson 1997, 1999a), was not observed until stage 3. DCASCS3 mRNAs were barely detectable in stage 4, 5, and 6 styles.

Ethylene production by ovaries from ethylene-treated flowers was higher than controls in both stage 1 and 2 flowers. Ethylene production then increased from stages 3 to 5 and decreased at stage 6 (Fig. 2). In ovaries, ethylene production did not correlate as well with the expression of ethylene biosynthetic genes (Fig. 3). While DCACO1 expression was induced by ethylene treatment and mRNA abundance increased as carnation ovaries became more mature, substantial increases in ACS transcripts were not observed. Previous experiments have reported a lack of ACS transcript and activity increases following pollination, despite large increases in ethylene evolution from the ovary (Jones and Woodson 1999a, 1999b). These results led to the hypothesis that ACC substrate may be translocated to the ovary to sustain autocatalytic ethylene production from this organ. It is also possible that other, as yet unidentified ACS genes are responsible for catalyzing this ethylene production. In control organs, ethylene production was always below  $2 \text{ nl g}^{-1} \text{ h}^{-1}$  and low constitutive levels of DCACS3 transcripts were detected in ovaries from all developmental stages.

In petals, the largest increases in ethylene production were observed between stage 3 and 4 (2.8 $\times$ ) and 4 and 5 (2 $\times$ ) (Fig. 2). Ethylene treatment resulted in induction of DCACS1, DCACS2 and DCACO1 transcripts in petals (Fig. 3). Transcript abundance increased as flowers became more mature. While DCACS1 and DCACS2 transcript abundance was similar in stage 6 styles, DCACS1 was the predominant ACS in stage 6 petals. It has previously been reported that DCACS1 is preferentially expressed in the petals and DCACS2 and DCACS3 in the gynoecium (Woodson et al. 1992; Jones and Woodson 1997, 1999a). Increases in expression of the ethylene-responsive genes DCACS1 and DCACS2 also increased much earlier than in styles, with significant up-regulation detected in stage 2 petals. While petals just emerging from the bud (stage 1) had little detectable expression of DCAC1 or DCACS2, DCACO1 was significantly increased by ethylene treatment in these immature petals. Similar to styles, ACS rather than ACO appears to be limiting ethylene biosynthesis in petals. Petals from air-treated flowers had ethylene production rates below  $1 \text{ nl g}^{-1} \text{ h}^{-1}$  and no ACS or ACO transcripts were detectable at any of the developmental stages.

The flower organ producing the most ethylene following ethylene treatment was dependent on the developmental stage of the flower. Following ethylene treatment, ethylene production was highest in ovaries from stage 1, 2, and 3 flowers, while in stage 4 and 5 flowers, petals produced the most ethylene. The largest differences were observed in flower organs from stage 6 flowers, where



**Fig. 4** Styles from **a** stage 4, **b** stage 5, and **c** stage 6 flowers were stained for esterase activity to determine receptivity of the stigmatic surface to pollination. When receptive to pollination, styles have endogenous esterase activity that is visualized by the formation of a blue color in the papillae

styles produced 5.5 $\times$  and 3 $\times$  more ethylene than ovaries and petals, respectively. It is very likely that the large differences in the ethylene production rates from stage 4 and stage 6 styles accounts for the different production rates reported in the literature for styles from ethylene-treated flowers (ten Have and Woltering 1997; Jones and Woodson 1999a). In general, ethylene production was highest in styles, then petals, and lastly ovaries. This correlated well with overall expression levels of both ACS and ACO genes.

In carnation flowers, the post-pollination response includes enhanced autocatalytic ethylene production from styles, ovaries, and petals, and results in accelerated corolla senescence. This ethylene production can be prevented by treating the flower with inhibitors of ethylene action prior to pollination (Jones and Woodson 1997). To observe all components of the post pollination response, (i.e., ethylene production and corolla senescence) and get successful fertilization and seed set, carnations must be pollinated when the stigma is receptive to pollination. The activity of enzymes like esterase has been correlated developmentally with stigma receptivity (Dafni 1992). Esterase activity, as indicated by the formation of a deep blue color in stained styles, was observed in styles from stage 5 and 6 flowers but not styles from stages 1 to 4 (Fig. 4). While both stage 5 and stage 6 styles stained for esterase activity, the percentage of successful pollinations as determined by accelerated corolla senescence, and the number of pollen tubes growing through the styles, were both greatest in styles from stage 6 flowers (data not shown).

Interestingly, treatment of only the style with an ethylene action inhibitor, prior to pollination, prevents pollination-induced corolla senescence but does not inhibit pollen tube growth or fertilization (Jones and Woodson 1997). These experiments suggest that autocatalytic ethylene production from the style functions in pollination-induced senescence signaling within the flower. While ethylene perception and responsiveness of the gynoecium may not be required for successful fertilization in carnations, the inhibition of pollination-induced petal

senescence has the potential to influence fruit set and yield by decreasing mobilization of reserves from senescing petals to the developing ovary. In petunias that are insensitive to ethylene and do not exhibit pollination-induced corolla senescence, fruit ripening is delayed and % fruit set is lower than in wild type ethylene-sensitive flowers (Gubrium et al. 2000). In flowers like *Phalaenopsis*, where the ethylene induced by pollination is required for the development of both male and female gametophytes, the development of tissue sensitivity to ethylene would appear to play an even more important role in successful reproduction (Zhang and O'Neill 1993).

## Conclusions

These experiments have indicated that ethylene responsiveness within the flower occurs at different developmental stages. While immature carnation petals are less responsive to ethylene, petals from stage 3 flowers produce significant amounts of ethylene following ethylene exposure. The function of petals is to attract pollinators. Once this has been achieved, or the flower is beyond the stage at which it is receptive to pollination, autocatalytic ethylene production causes the flower to senesce. Carnation petals develop sensitivity to ethylene before the style is receptive to pollination. This may represent a mechanism by which the open flower can respond to environmental stresses, insects, or disease, via flower senescence when conditions are no longer ideal for seed production. Ovaries from carnation flowers are responsive to ethylene at even earlier stages, with increases in ethylene detected in stage 1 ovaries. This may reflect the role of ethylene in ovary development, and further investigations are required to correlate this sensitivity to ethylene with the maturation of ovules within the ovary. The styles develop ethylene sensitivity later in development, when the flower is past anthesis and the stigma is receptive to pollination. The role of the stigma/style in sexual reproduction is pollen reception and pollen tube growth. The role of autocatalytic ethylene production by the style has been proposed to be that of stimulation of pollen tube growth, but treatment with ethylene action inhibitors and pollen tube growth experiments in transgenic plants insensitive to ethylene suggest that this is not the role of ethylene in the style (Jones and Woodson 1997; Clevenger 2000). In contrast, ethylene responsiveness in the style is required for pollination-induced senescence and the timing of the development of this sensitivity supports the role of stylar ethylene in the propagation of the senescence signal.

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