

# Expression of Ethylene Biosynthetic Genes in the Gynoecium and Receptacle Associated With Sepal Abscission During Senescence in *Delphinium Grandiflorum*

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## Research Article

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# Abstract

*Delphinium* flowers are highly sensitive to ethylene and its sepals abscise during senescence, which is associated with increases in 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) activities and ethylene production in gynoecium and receptacle. Three *ACS* genes (*DgACS1*, *DgACS2*, and *DgACS3*) and three *ACO* genes (*DgACO1*, *DgACO2*, and *DgACO3*) were cloned from *Delphinium grandiflorum* cv. Super Grand Blue. To investigate the contribution of these genes to ethylene production, their expression was analyzed in these genes in the gynoecium and receptacle during natural senescence and following ethylene exposure and pollination. Ethylene production in the gynoecium and receptacle increased during natural flower senescence. The transcript levels of the *ACS* and *ACO* genes in these organs, excluding *DgACS2* in the receptacle, increased during senescence. Exposure to ethylene accelerated sepal abscission and more strongly increased ethylene production in the receptacle than in the gynoecium. *DgACS1* transcript levels in the gynoecium and *DgACS2* and *DgACO3* transcript levels in the receptacle were increased by ethylene exposure. Pollination accelerated sepal abscission and increased ethylene production in the gynoecium and receptacle. Pollination slightly affected *ACS* and *ACO* transcript levels in the gynoecium, whereas *DgACO3* transcript level in the receptacle were markedly increased. These results reveal that *ACS* and *ACO* gene expression is differently regulated in the gynoecium and receptacle, and some of these genes are more strongly upregulated by ethylene exposure and pollination in the receptacle than in the gynoecium, suggesting the significance of the receptacle to sepal abscission.

# Introduction

The phytohormone ethylene plays an important role in various stages of plant growth and development, including senescence. Exposure to ethylene accelerates the senescence of flowers in many species, and the primary symptom of senescence induced by ethylene is petal wilting or petal (sepal) abscission (Woltering and van Doorn 1988). In many ethylene-sensitive flowers, such as carnation (Nichols 1968) and *Eustoma grandiflorum* (Ichimura et al. 1998), the petals wilt in response to ethylene exposure. In *Digitalis* (Stead and Moore 1983), geranium (Hilioti et al. 2000), and torenia flowers (Goto et al. 1999), petals abscise upon exposure to ethylene. In these flowers, ethylene production in the gynoecium or pistil increases with the progression of flower senescence. However, this finding is not observed in the petals (Stead and Moore 1983; Goto et al. 1999; Hilioti et al. 2000). Therefore, ethylene produced by the gynoecium has been considered responsible for petal abscission.

Pollination accelerates petal wilting in many ethylene-sensitive flowers, including carnation (Jones and Woodson 1997), petunia (Gillisen and Hoekstra 1984; Whitehead et al. 1984), and gentian (Shimizu-Yumoto and Ichimura 2012). Similarly, pollination induces petal abscission in *Digitalis* (Stead and Moore 1979, 1983), geranium (Clark et al. 1997), and torenia (Goto et al. 1999). Pollination induces climacteric-like increases in ethylene production, and ethylene inhibitors suppress pollination-induced senescence in many plants, including orchids (O'Neill et al. 1993; Porat et al. 1995), *E. grandiflorum* (Ichimura and Goto

2000), and torenia (Goto et al. 1999), indicating that pollination-induced petal senescence is regulated by ethylene.

The biosynthetic pathway for ethylene in higher plants has been established. 1-Aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene, is produced by the conversion of S-adenosyl-L-methionine (SAM) by ACC synthase (ACS). ACC is converted to ethylene, carbon dioxide, and HCN by ACC oxidase (ACO). ACS is generally considered to be a rate-limiting enzyme for ethylene biosynthesis in plants because ACO activity is constitutive in many species (Yang and Hoffman 1984; Kende 1993). However, ACO is important for ethylene biosynthesis in some examples, including fruit maturation (Nakatsuka et al. 1998) and flower senescence (Tang et al. 1994). In cut carnation flowers, the biosynthesis of ethylene is regulated by the activities of ACS and ACO (Woodson et al. 1992; Woltering et al. 1993; Lee et al. 1997). ACS and ACO proteins are encoded by multi-gene families (Argueso et al. 2007; Lin et al. 2009), and these genes are differently regulated in response to developmental and environmental factors (Lin et al. 2009). Expression analyses of *ACS* and *ACO* genes have been performed in floral organs, using carnation (Jones and Woodson 1997, 1999; Jones 2003), petunia (Tang et al. 1994), tomato (Llop-Tous et al. 2000), and rose (Xue et al. 2008). In carnation flowers, three *ACS* genes, namely, *DcACS1*, *DcACS2*, and *DcACS3*, have been cloned, and their expression is differentially regulated (Jones and Woodson 1999). In tomato flowers, *SlACS2* is upregulated by pollination (Llop-Tous et al. 2000). However, few studies have analyzed *ACS* and *ACO* gene expression in flowers displaying petal abscission. In geranium, *ACS* and *ACO* gene expression has only been semi-quantified using RNA gel blot analysis (Clark et al. 1997).

*Delphinium* has long spikes with flowers of various colors, such as white, blue, and purple. The genus *Delphinium* consists of more than 300 species, and *D. elatum*, *D. grandiflorum* and *D. × belladonna*, which is a hybrid of *D. elatum* and *D. grandiflorum*, are widely produced as ornamental plants. Instead of *D. × belladonna*, which was mainly produced approximately a decade ago, *D. grandiflorum* is widely produced for cut flowers. *Delphinium* flowers are sensitive to ethylene, and their sepals rapidly abscise upon exposure (Woltering and van Doorn 1988; Ichimura et al. 2000), which is accompanied by signs of programmed cell death (Yamada et al. 2007). In *Delphinium*, sepals and petals are directly connected to the receptacle, but not to the gynoecium. Thus, ethylene produced by the receptacle should directly induce the abscission of the sepals (Ichimura et al. 2009). Ethylene production in the gynoecium and receptacle increases during flower senescence, but that in the other floral organs including petals and sepals, does not increase (Ichimura et al. 2009). ACS and ACO activity in the gynoecium and receptacle increases during flower senescence (Ichimura et al. 2009). Although genes for ethylene receptors and signal transduction components have been identified in *D. elatum* (Kuroda et al. 2003, 2004) and *D. × belladonna* (Tanase and Ichimura 2006), *ACS* and *ACO* genes have not yet been cloned in *Delphinium*. The quantification of *ACS* and *ACO* gene expression will help to clarify the effects of ethylene on sepal abscission.

In the present study, we cloned *ACS* and *ACO* genes and determined their nucleotide sequences in *D. grandiflorum*. Furthermore, we investigated their expression of *ACS* and *ACO* genes in the gynoecium and

receptacle during natural senescence, following exposure to ethylene, and after pollination in cut *D. grandiflorum* flowers.

## Materials And Methods

### Plant materials

*Delphinium grandiflorum* cv. Super Grand Blue (Miyoshi, Tokyo, Japan) was grown in a greenhouse under natural day length conditions with temperatures ranging from 15°C to 25°C. On the day of anthesis, flowers with a peduncles of 3 cm in length were cut from the middle region of the flower spikes. Cut peduncles were placed in a vessel with distilled water within 1 h after harvest and used for all subsequent experiments. Cut flowers were held at 23°C, 70 % relative humidity, and 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance using cool-white fluorescence lamps under a 12-h photoperiod.

The morphology of *D. grandiflorum* flowers is shown in Figure 1. The spike features approximately 10 flowers (Fig. 1A), whereas petals lack in the flower, unlike other *Delphinium* species (Fig. 1B). Its gynoecium is covered with stamens (Fig. 1C). The receptacle lies below the gynoecium, and sepals are attached to the receptacle (Fig. 1D).

### Ethylene treatment

The cut flowers were placed in individual vessels containing distilled water and then transferred to a 70-L transparent acrylic box fitted with a septum, through which ethylene was introduced to achieve a concentration of 10  $\mu\text{l L}^{-1}$ . The box was kept at 23°C under a 12 h-photoperiod with 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance from cool-white fluorescent lamps for 24 h. After exposure to ethylene, flowers were removed from the chamber and kept under the same temperature and lighting conditions as above.

### Pollination

In the greenhouse, stamens were removed on the day of anthesis, and the individual flowers were enveloped with paper bags to prevent pollination. The stigma matured approximately 4 days after emasculation. Then, the flowers were cut from the plant, transported to the laboratory, and placed in water. The flowers were pollinated with fresh pollen collected from the same cultivar. The flowers were held under the aforementioned environmental conditions as above.

### Observation of the pollen tube growth

Gynoecia were removed from pollinated flowers 6, 12, 24, or 48 h after pollination and immersed in FAA solution (formalin, 80 % ethanol, acetic acid, 1:8:1 (v/v)). Gynoecia were macerated in 8 M NaOH for 24 h, rinsed in tap water for 1 h and stained with 0.1 % aniline blue in 0.1 M  $\text{K}_3\text{PO}_4$ . Pollen tubes were observed microscopically under incident ultraviolet illumination.

### Measurement of ethylene production

Gynoecia and receptacles were individually placed in test tubes (14.8 mL). They were sealed and maintained at 23°C. One hour later, a 1-mL gas sample was withdrawn using a syringe and used to determine the ethylene concentration using a gas-chromatograph (GC-14B, Shimadzu, Kyoto, Japan) equipped with an alumina column and a flame ionization detector. The results of ethylene production were expressed on a fresh weight basis.

## RNA extraction

Frozen gynoecium and receptacle (0.1 g FW) were powdered under liquid nitrogen by a Shake Master Auto (Bio medical science, Tokyo, Japan) and added to 450 µL of RLT buffer including 1 % 2-mercaptoethanol. The mixture was incubated at 56°C for 1 min. Total RNA was extracted using RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's manual with some modification.

## First strand cDNA and PCR amplification

Synthesis of cDNA was carried out with random hexamer primers using the Invitrogen SuperScript III first-strand synthesis system for RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA). To clone the partial-length of the target cDNA, degenerate primer pairs were designed based on highly conserved regions of the *ACS*, *ACO* or *Actin* genes: for *ACS*, 5'- CARATGGGIYTIGCIGARAAYCA -3' (forward) and 5'- GYICCIARIGGRTTIGAIGGRTT -3' or 5'- CAIAYICKRAACCAICIGSYTC -3' (reverse); for *ACO*, 5'- GCITGYSAIAAYTGGGGGTT -3' (forward) and 5'- CCICCGCRTCIGTRTGIGC -3' (reverse); for *Actin*, 5'- TGGGAYGAYATGGARAARATHHTGG -3' (forward) and 5'- CCDATIGTDATIACYTGICC -3' (reverse). PCR reactions were performed with Ex Taq (Takara bio, Kusatsu, Japan) and cycled 30 times at 94°C for 30 s, 50°C for 30 s and 72°C for 1 min. PCR products were separated electrophoretically onto 1 % (w/v) agarose gel, stained with GelRadTM (Biotium, Fremont, CA, USA) and candidate bands were excised. The excised bands were purified with a Wizard Plus SV Minipreps DNA Purification System (Promega, San Luis Obispo, CA, USA) and subcloned into the pGEM-TeasyVector (Promega) according to the manufacturer's manual.

To clone the 3' and 5' ends of the target cDNA, RACE-PCR was performed using 3'-Full RACE Core Set (Takara bio) and CapFishing Full-Length cDNA Premix Kit (Seegene, Seoul, Korea). Specific primers were designed based on the sequence data obtained from the partial-length of the target cDNA (Table S1). PCR products were purified and sub-cloned into the pGEM-TeasyVector. The full length cDNA was cloned and the DNA sequence was confirmed using specific primers.

## Determination of nucleotide sequence

Sequencing of DNA was performed using an Applied Biosystems ABI Prism DNA sequencer (Model 377; Thermo Fisher Scientific) and the Applied Biosystems Big Dye Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), employed with both T7HT and M3RP universal primers. Sequence analysis was performed using ClustalW2 (European Bioinformatics Institute, <http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Homology was obtained by comparing the sequenced clones with the existing translated DNA in the database of the National Center for Biotechnology

Information BLAST algorithm (<http://www.ncbi.nlm.nih.gov/nucleotide/>). Phylogenetic analysis was performed with Phylogeny.fr (<http://www.phylogeny.fr/>).

## Preparation of total RNA and real-time RT-PCR

Gynoecia and receptacles were collected from three individual flowers. Total RNA was isolated using RNeasy plant mini kit (QIAGEN). Genomic DNA included in the RNA sample was digested with the RNase-free DNase set. Quality and quantity of RNA were checked using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Synthesis of cDNA was performed with random hexamer primers using the Invitrogen SuperScriptIII first-strand system for RT-PCR. The following primers were used for real-time RT-PCR: for *DgACS1*, 5'-TGGACAACGAGACGATGGAG-3' (forward) and 5'-GAAGCTGAGACGGAGGTTGTT-3' (reverse); for *DgACS2*, 5'-GACTCCTCATACTCCTATCCCACAG-3' (forward) and 5'-ACCTCCTGGTGTATTTCACAGTC-3' (reverse); for *DgACS3*, 5'-TTTTGCCAACATGAGCCAAC-3' (forward) and 5'-TTGTGGCATCTTCTTTTCTCCA-3' (reverse); for *DgACO1*, 5'-CCCAAATTTGTTTTTCGAGGA-3' (forward) and 5'-TAATAAGCCGTGGCGATAGG-3' (reverse); for *DgACO2*, 5'-GGTGAAGTTTCAAGCCAAGG-3' (forward) and 5'-TTCTTCACTTGTTTGGGCACT-3' (reverse); for *DgACO3*, 5'-GGGTGAAGTTTCAAGCCAAG-3' (forward) and 5'-CAATTTGCTGACCCATCTGA-3' (reverse), for *DgActin* 5'-AGGGGATACATGTTTCAACAACCAC-3' (forward) and 5'-TTTCAAGCTCCTGCTCATAGTCC-3' (reverse).

PCR reactions were performed with Thermal Cycler Dice Real Time System (Takara bio), using SYBR Premix Ex Taq II (Perfect Real Time, Takara bio). Each reaction was performed using 0.8 µL aliquot of 20 µL cDNA solution derived from 1 µg of total RNA. The following program was applied: initial polymerase activation at 95°C for 30 s; then 40 cycles at 95°C for 5 s, 60°C for 30 s. The specificity of the PCR was checked using a heat dissociation protocol (from 95°C to 60°C) after the final cycle.

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 sequence as external standards. To standardize the data, the ratio between the absolute transcript level of the target gene and the control gene was calculated for each sample.

## Results

### Cloning and nucleotide sequences of ACS genes

Three ACS genes were cloned and designated *DgACS1*, *DgACS2* and *DgACS3* and deposited in the DDBJ with accession nos LC603027, LC603028, and LC603029, respectively. The cDNAs of these genes were 1901, 1739, and 1721 bp in length, respectively. The predicted proteins consisted of 486 amino acids for *DgACS1*, 485 amino acids for *DgACS2*, and 444 amino acids for *DgACS3*. The amino acid homologies were 80.9 % between *DgACS1* and *DgACS2*, 55.8 % between *DgACS1* and *DgACS3*, and 53.2 % between *DgACS2* and *DgACS3*. Seven conserved domains were observed in the three genes (Supplementary Fig. S1).

ACS proteins have been categorized into three types based on the sequences of C-terminal region (Lin et al. 2009). Type 1 ACS proteins have been revealed to be phosphorylated by the mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK), whereas type 2 ACS proteins only have CDPK site. Type 3 proteins lack these two sites for phosphorylation. Based on these criteria, the predicted proteins of DgACS1 and DgACS2 belong to type 1, whereas that of DgACS3 belongs to type 3 (Supplementary Fig. S2).

## Cloning and nucleotide sequences of ACO genes

Three *ACO* genes were cloned and designated *DgACO1*, *DgACO2* and *DgACO3*, and deposited in the DDBJ with accession nos LC603030, LC603031, and LC603032, respectively. The cDNAs of these genes were 1182, 1159, and 1072 bp in length, respectively. The predicted proteins consisted of 317 amino acids for *DgACO1*, 317 amino acids for *DgACO2* and 319 amino acids for *DgACO3* (Supplementary Fig. S3). The amino acid homologies were 78.6 % between *DgACO1* and *DgACO2*, 78.6 % between *DgACO1* and *DgACO3*, and 94 % between *DgACO2* and *DgACO3*. Twelve amino acids, which are known to be conserved in ACO proteins (Do et al. 2005; Momonoi et al. 2007; Wang and Woodson 1991), were conserved in *DgACO1*, *DgACO2*, and *DgACO3*.

The phylogenetic analysis revealed that ACO proteins were clustered into three groups (Houben and Van de Poel, 2019). The amino acid sequences of the three genes were extremely similar, and they clustered into type 1 (Supplementary Fig. S4).

### *Ethylene production and ACS and ACO transcript levels in the gynoecium and receptacle during natural senescence*

Ethylene production in the gynoecium was relatively high on day 0, and they decreased over the first 3 days before increasing on day 4 (Fig. 2). Ethylene production in receptacle changed similarly to that in gynoecium.

In the gynoecium, *DgACS1* had the highest transcript levels among the three *ACS* genes at harvest, whereas its levels decreased during the first 3 days and increased thereafter (Fig. 3). *DgACS2* transcript level in gynoecium gradually increased during senescence, and *DgACS3* transcript level markedly increased from day 4. In receptacle, *DgACS2* transcript level was highest on day 0 among the three *ACS* transcripts, but its level decreased during senescence. *DgACS1* transcript level was relatively high on day 0, and it decreased over the first 3 days before increasing on day 4. *DgACS3* transcript level increased during senescence.

In the gynoecium, *DgACO1* transcript level was much higher than those of the other two *ACO* genes on day 0, and it increased during senescence (Fig. 4). *DgACO2* transcript level in the gynoecium gradually increased during senescence, whereas *DgACO3* transcript level decreased during the first 5 days before increasing thereafter. In receptacle, *DgACO1* had much higher transcript level than the other two *ACO* genes on day 0, and its level increased over the first 5 days and decreased thereafter. *DgACO2* level was

almost constant during the first 4 days before increasing thereafter. *DgACO3* level increased during senescence.

#### *Ethylene production and ACS and ACO transcript levels of gynoecium and receptacle after exposure to ethylene*

Exposure to ethylene accelerated sepal abscission, and the sepals abscised within 24 h. Ethylene production was more strongly increased in receptacle than in gynoecium after 8 h of ethylene exposure (Fig. 5).

In gynoecium, exposure to ethylene increased *DgACS1* and *DgACS2* transcript levels at most time points (Fig. 6). However, ethylene exposure only slightly affected *DgACS3* transcript level. In receptacle, *DgACS2* transcript level was highest among the three genes and higher after ethylene treatment than in the control at all time points. *DgACS1* transcript level was not noticeably increased by ethylene treatment versus control. *DgACS3* transcript level was decreased by ethylene treatment at all time points, and its level was lowest among the three transcripts.

In gynoecium, *DgACO1* transcript levels were highest among the three transcripts, but ethylene exposure only slightly increased its level (Fig. 7). Ethylene exposure did not increase *DgACO2* and *DgACO3* transcript levels. In receptacle, *DgACO1* transcript level was increased by ethylene treatment at all time points. *DgACO2* transcript level was not increased by ethylene treatment, whereas *DgACO3* transcript level was markedly increased by ethylene exposure.

## **Effect of pollination on sepal abscission and pollen tube growth**

*D. grandiflorum* flowers are protandrous, and the gynoecium matured 3 or 4 days after anthesis under the present experimental conditions. Pollination accelerated sepal abscission, which was observed within 24 h after pollination. Pollen tube growth in gynoecia was observed via fluorescent microscopy. Many pollen tubes reached the ovary 6 h after pollination (Supplementary Fig. S5).

#### *Ethylene production and ACS and ACO transcript levels in gynoecium and receptacle in unpollinated and pollinated flowers*

Ethylene production was extremely low in unpollinated whole flowers throughout the experimental period. Similarly, ethylene production in gynoecium and receptacle was also extremely low in unpollinated flowers. Pollination increased ethylene production in the whole flower. Similarly, pollination markedly increased ethylene production in gynoecium, and the two ethylene peaks were observed after pollination (Fig. 8). Pollination increased ethylene production in receptacles, with production peaking at 8 h after pollination.

In gynoecium, *DgACS1* transcript level was slightly increased by pollination, whereas those of the other *ACS* genes were not increased (Fig. 9). In the receptacle, *DgACS2* transcript level was increased by pollination, but *DgACS1* and *DgACS3* transcript levels were not altered.

In gynoecium, *DgACO1* transcript level, which was highest among the three transcripts, was increased by pollination (Fig. 10). However, the levels of the other two *ACO* transcript levels were not increased by pollination. In receptacle, *DgACO1* transcript level was higher in pollinated flowers than in unpollinated flowers at all time points. Although *DgACO2* transcript level was slightly changed by pollination, *DgACO3* transcript level was markedly increased by pollination.

## Discussion

*Delphinium* flowers are highly sensitive to ethylene and the sepals abscise during senescence (Ichimura et al. 2009). In this study, we isolated three *ACS* and three *ACO* genes from *D. grandiflorum*, and the expression analysis in gynoecium and receptacle was performed during natural senescence and following ethylene exposure and pollination. *ACS* and *ACO* gene expression was differently regulated in the gynoecium and receptacle.

## Ethylene production of gynoecium and receptacle

Although ethylene production in gynoecium and receptacle at harvest was relatively high, its production increased in these organs during senescence (Fig. 2). This result somewhat differed from that in *D. belladonna*, in which ethylene production is relatively low at harvest in gynoecium and receptacle (Ichimura et al. 2009). The difference of ethylene production at harvest may be attributable to the different physiological properties of these species.

The trends of ethylene production in whole flowers following ethylene exposure and pollination differed from those in gynoecium and receptacle (Figs. 5 and 8). The sum of the gynoecium and receptacle fresh weight was one-tenth of that of the whole flower. Although ethylene production in floral organs other than gynoecium and receptacle was extremely low during natural senescence (Ichimura et al. 2009), ethylene production may decrease in these organs following ethylene exposure and pollination, resulting in differences in ethylene production between the whole flower and the two organs.

## ACS and ACO gene expression during natural flower senescence

In the carnation gynoecium, *DcACS2* and *DcACO1* transcript levels increase during senescence (ten Have and Woltering 1997; Jones and Woodson 1999). Similarly, *SIACS1A* transcript levels increased during senescence in the tomato (Llop-Tous et al. 2000). In petals displaying abscission, changes in *ACS* and *ACO* transcript levels in gynoecium during natural senescence have only been reported for geranium, but no substantial increases in their transcript levels were observed (Clark et al. 1997). In the present study, the peak *DgACS3* transcript level was much higher than those of the other *ACS* transcripts (Fig. 3).

*DgACO1* transcript levels were higher than those of the other *ACO* transcripts (Fig. 4). These results suggest that *DgACS3* and *DgACO1* expression appears to largely contribute to increased ethylene production in gynoecium.

To date, the expression of *ACS* and *ACO* genes in receptacle has only been investigated in carnation (Jones and Woodson 1999) and rose (Xue et al. 2008), but no obvious changes in these transcript levels were observed during senescence. In receptacle, *DgACS2* transcript level was much higher than those of the other *ACS* transcripts on day 0, but its level decreased during senescence (Fig. 3). Conversely, *DgACS1* and *DgACS3* transcript levels increased during senescence. Similarly, the levels of the three *ACO* transcripts increased during senescence, although some differences were observed (Fig. 4). Thus, the expression of these *ACS* and *ACO* genes appears to contribute to ethylene production in receptacle.

## **ACS and ACO gene expression following ethylene exposure**

Exposure to ethylene accelerated sepal abscission, which is consistent with previous findings (Ichimura et al. 2000, 2009). Ethylene exposure more strongly increased ethylene production in receptacle than in gynoecium (Fig. 5), suggesting that the responsiveness of ethylene production to ethylene exposure is higher in receptacle than in gynoecium. The receptacle, but not the gynoecium, is connected to sepals in *D. grandiflorum* flowers (Fig. 1). Thus, sepal abscission following ethylene exposure may be induced by increased ethylene production of the receptacle.

*ACS* and *ACO* gene expression in receptacle following ethylene exposure has only been reported in carnation (Jones and Woodson 1999) and rose (Xue et al. 2008). Among these genes, only *RhACS2* expression was slightly increased by ethylene exposure (Xue et al. 2008). *DgACS2* and *DgACO3* transcript levels in the receptacle were relatively high, and they were markedly increased by ethylene exposure (Figs. 6 and 7). This finding suggests that *DgACS2* and *DgACO3* may contribute to increased ethylene production in receptacle associated with sepal abscission.

*DgACS1* and *DgACS2* transcript levels in gynoecium were increased by ethylene exposure, whereas the expression of *DgACS3* and the three *ACO* genes did not increase (Figs. 6 and 7). These results suggest that *DgACS1* and *DgACS2* contribute to increased ethylene production in gynoecium in response to ethylene exposure. To date, the expression of *ACS* and *ACO* genes in pistil or gynoecium following ethylene exposure has been reported in carnation (Jones and Woodson 1997, 1999), petunia (Tang et al. 1994), rose (Xue et al. 2008), and tomato (Llop-Tous et al. 2000). In these plants, the transcript levels of some *ACS* genes, including *DcACS2* (Jones and Woodson 1997) and *SIACS2* (Llop-Tous et al. 2000) and *ACO* genes, including *PhACO1* (Tang et al. 1994), are increased by exposure to ethylene.

## **ACS and ACO gene expression in pollinated flowers**

As reported in many plants, including *Digitalis* (Stead and Moore 1979), geranium (Clark et al. 1997) and torenia (Goto et al. 1999), pollination accelerated sepal abscission in *D. grandiflorum*. Ethylene production in gynoecium markedly increased 4 h after pollination (Fig. 8). However, only *DgACO1* transcript level in the gynoecium was increased by pollination (Fig. 10). In *Delphinium* gynoecium, *ACO* activity at anthesis

is relatively high (Ichimura et al. 2009). The pollen of some plants, including petunia, contains large amounts of ACC (Whitehead et al. 1983). In petunia flowers, an early increase in ethylene production is associated with the ACC content in pollen (Singh et al. 1992). We confirmed that pollen tubes reached ovary 6 h after pollination (Supplementary Fig. S5). Thus, marked increase in ethylene production in gynoecium may be attributed to pollen-born ACC, which is converted to ethylene by ACO activity. Alternatively, other *ACS* and *ACO* genes may be specifically induced by pollination, although these types of *ACS* and *ACO* genes have not yet been identified in other plant species.

The coordination of senescence processes in pollinated flowers is involved in interorgan signaling (Jones and Woodson 1997). The transmissible factor for this signaling has been proposed to be ethylene (Woltering et al. 1995) or ACC (Reid et al. 1984). Ethylene production in receptacle increased 4 h after pollination (Fig. 8), suggesting that pollination generates some transmissible factor that induces ethylene production of the receptacles because receptacle lies below the gynoecium. Increased ethylene production was associated with increases in *DgACO1* and *DgACO3* transcript levels in receptacle (Fig. 10). Thus, ethylene produced by the gynoecium in response to pollination may increase the expression of these genes, resulting in increased ethylene production in receptacle.

## Significance of the receptacle and possible model for sepal abscission

The significance of gynoecium to petal senescence has been emphasized for petal wilting in petunia (Whitehead et al. 1984) and carnation (Shibuya et al. 2000) and for petal abscission in *Digitalis* (Stead and Moore 1983) and geranium (Clark et al. 1997). Although changes in some *ACS* and *ACO* transcript levels in receptacle were reported in carnation (Jones and Woodson 1999) and rose (Xue et al. 2008), the significance of this organ to petal wilting was only mentioned by Hsieh and Sacalis (1986), who reported ACC levels in receptacle during senescence in cut carnation. The significance of receptacle to sepal abscission has been described only for *Delphinium* flowers (Ichimura et al. 2009). In our study, ethylene exposure and pollination increased ethylene production more strongly in receptacle than in gynoecium, and this finding was associated with marked increases in some *ACS* and *ACO* transcript levels in receptacle. These results suggest the significance of receptacle to sepal abscission. Thus, the receptacle is likely to be a primarily organ that regulates sepal abscission in *Delphinium*.

We thus propose a model for sepal abscission based on the present results (Fig. 11). During natural flower senescence, increases in *DgACS1*, *DgACS3*, *DgACO1* and *DgACO3* transcript levels are primarily responsible for ethylene production in receptacle, leading to sepal abscission. However, whether increase in ethylene production in gynoecium affects ethylene biosynthesis is unclear. Exposure to ethylene increases *DgACS2* and *DgACO3* transcript levels in receptacle, leading to sepal abscission. Receptacle should directly respond to exogenous ethylene. Pollination increases ethylene production in gynoecium without inducing *ACS* and *ACO* gene expression. The increase in ethylene production in gynoecium may lead to increased *DgACS2* and *DgACO3* transcript levels in receptacle, resulting in sepal abscission.

# Conclusion

Ethylene production in gynoecium and receptacle in *D. grandiflorum* flowers somewhat increased during natural senescence. Three *ACS* genes (*DgACS1*, *DgACS2*, and *DgACS3*) and three *ACO* genes (*DgACO1*, *DgACO2*, and *DgACO3*) were cloned. The transcript levels of the *ACS* and *ACO* genes in these organs increased during senescence, excluding *DgACS2* in receptacle. Exposure to ethylene more strongly increased ethylene production in receptacle than in gynoecium and induced sepal abscission. *DgACS1* transcript level in gynoecium and *DgACS2* and *DgACO3* transcript levels in receptacle were increased by ethylene exposure. Pollination increased ethylene production of gynoecium and receptacle and markedly accelerated sepal abscission. However, pollination only slightly affected *ACS* and *ACO* transcript levels in gynoecium, whereas *DgACO3* transcript level in receptacle was markedly increased. These results suggest that increases in the transcript levels of some *ACS* and *ACO* genes in gynoecium and receptacle may contribute to the increases in ethylene production associated with sepal abscission. Ethylene exposure and pollination more strongly increased the expression of these genes in receptacle than in gynoecium, suggesting the significance of receptacle to sepal abscission.

# Declarations

## Author contribution statement

MO, KS and KI conceived and designed the research. MO and AS performed physiological experiments. MO and TN performed molecular experiments. KI wrote the manuscript. All authors read and approved the manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

**Data Availability Statement** The data that supports the findings of this study are available in the supplementary material of this article.

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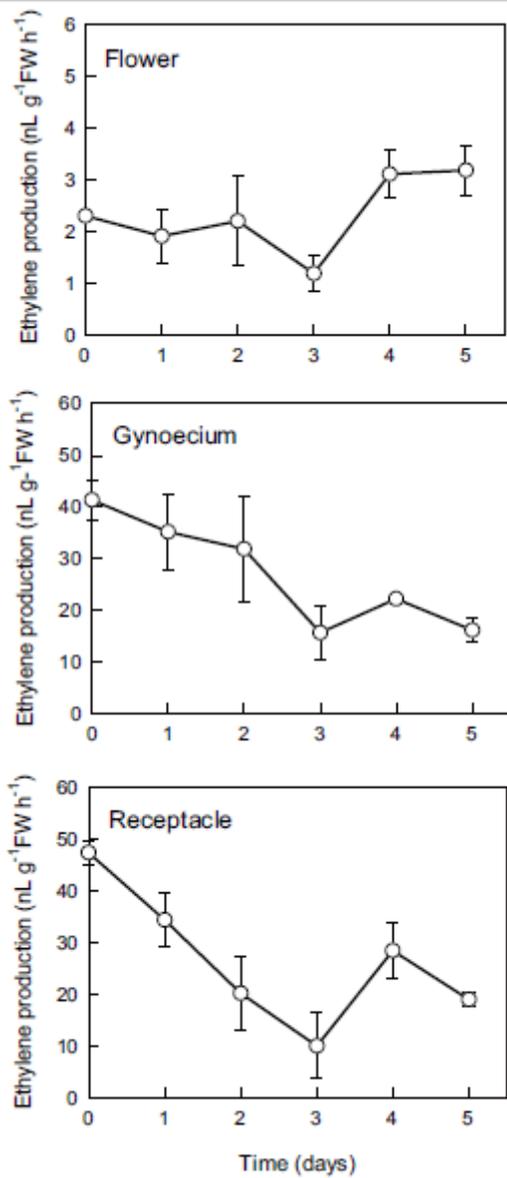
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## Figures

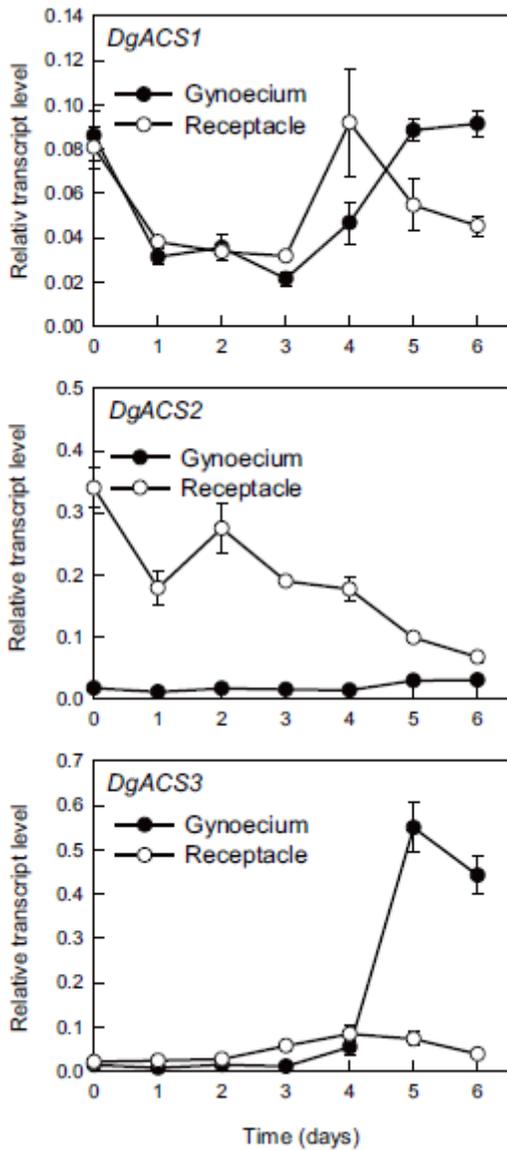
### Figure 1

Morphology of *Delphinium grandiflorum* flowers. A. Flower spikes. The scale bar represents 10 cm. B. Flower. The scale bar represents 1 cm. C. Sepal, gynoecium and receptacle. The scale bar represents 1 cm. D. Gynoecium and receptacle. The scale bar represents 0.5 cm.



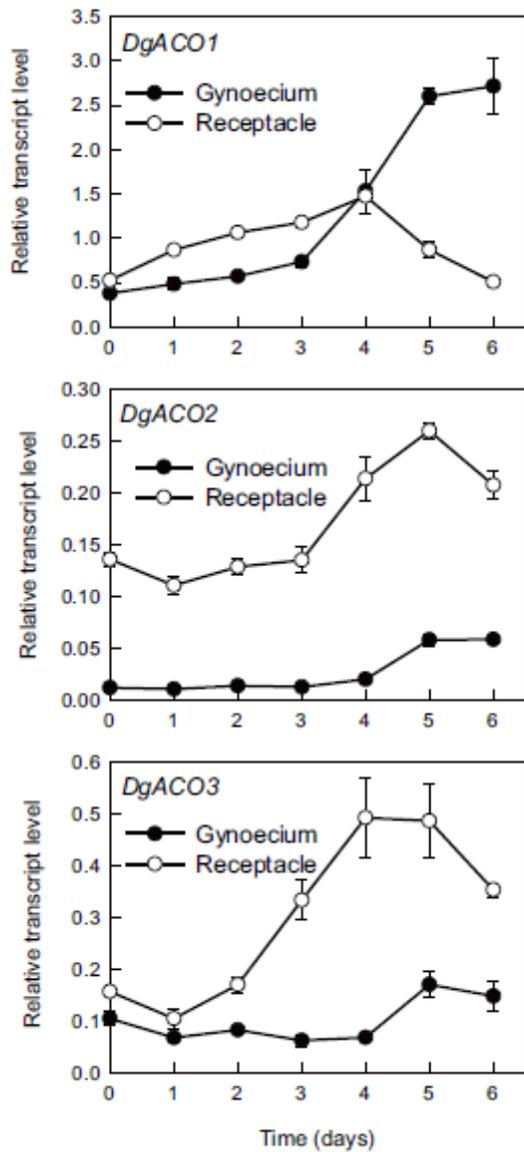
**Figure 2**

Ethylene production in flowers, gynoecium and receptacle during natural flower senescence. Values are means of five biological replicates  $\pm$  SE.



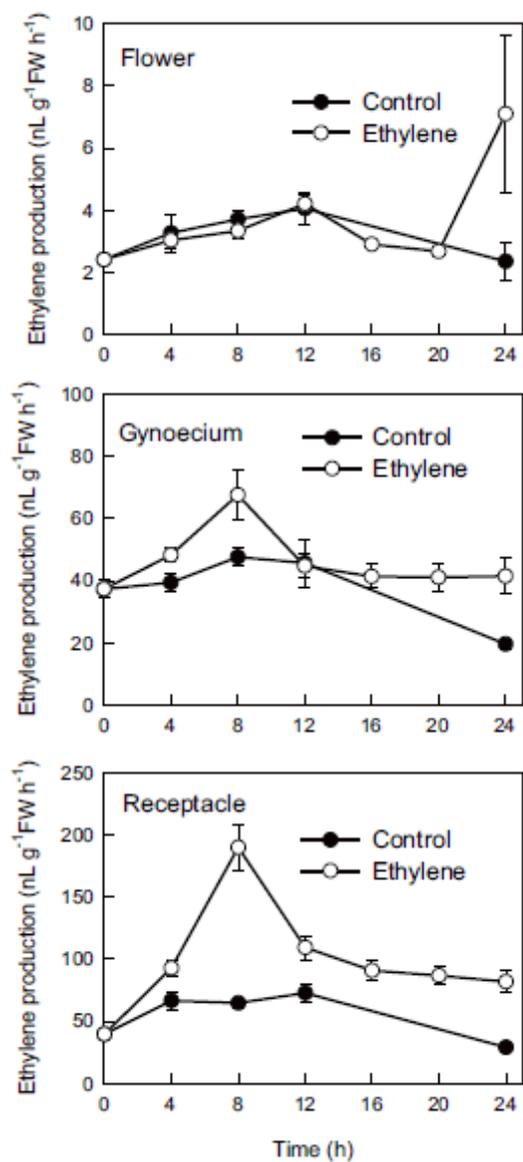
**Figure 3**

*DgACS1*, *DgACS2* and *DgACS3* transcript levels in gynoecium and receptacle during natural flowers senescence. Values are means of three biological replicates  $\pm$  SE.



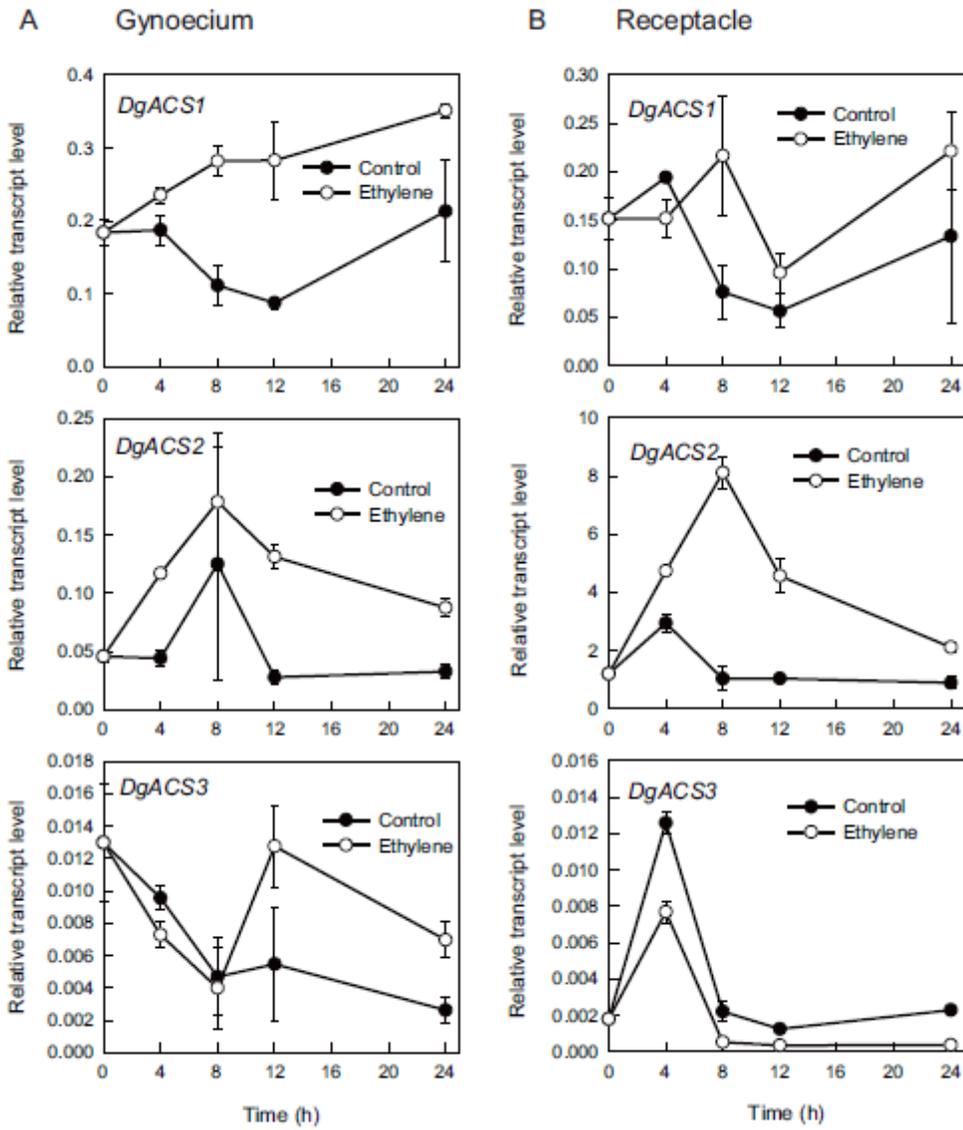
**Figure 4**

*DgACO1*, *DgACO2* and *DgACO3* transcript levels in gynoecium and receptacle during natural flowers senescence. Values are means of three biological replicates  $\pm$  SE.



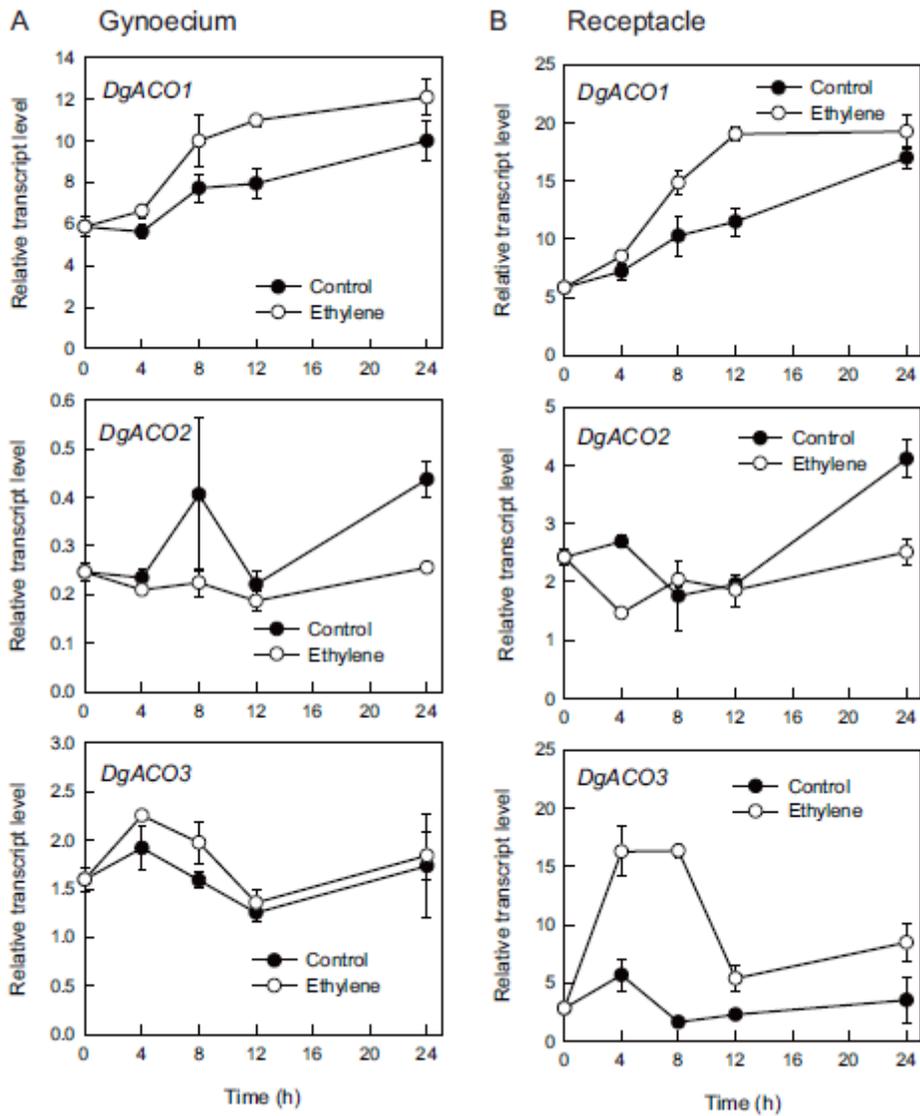
**Figure 5**

Ethylene production in flowers, gynoecium and receptacle during ethylene exposure. Values are means of five biological replicates  $\pm$  SE.



**Figure 6**

*DgACS1*, *DgACS2* and *DgACS3* transcript levels in gynoecium (A) and receptacle (B) during ethylene exposure. Values are means of three biological replicates  $\pm$  SE.



**Figure 7**

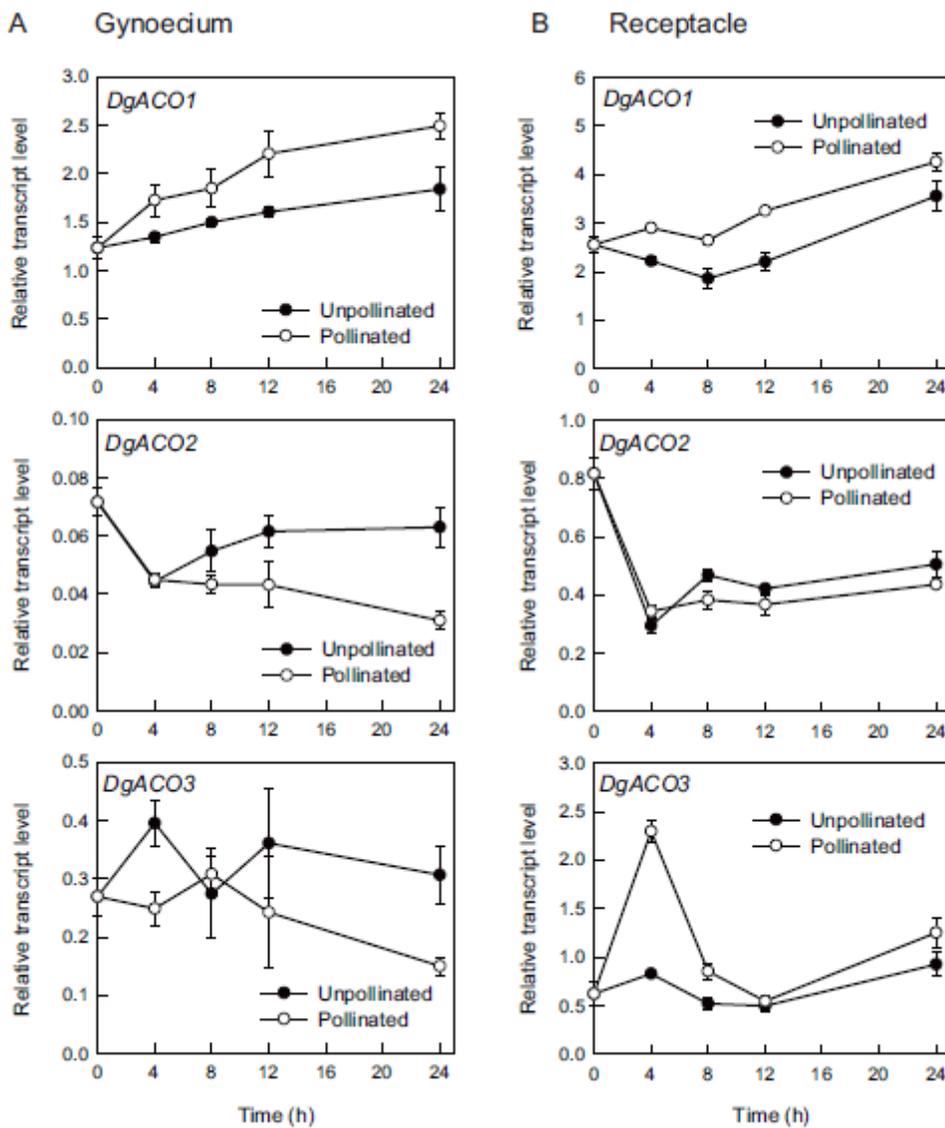
*DgACO1*, *DgACO2* and *DgACO3* transcript levels in gynoecium (A) and receptacle (B) during ethylene exposure. Values are means of three biological replicates  $\pm$  SE.

**Figure 8**

Ethylene production in flowers, gynoecium and receptacle following pollination. Values are means of five biological replicates  $\pm$  SE.

**Figure 9**

*DgACS1*, *DgACS2* and *DgACS3* transcript levels in gynoecium (A) and receptacle (B) following pollination. Values are means of three biological replicates  $\pm$  SE.



**Figure 10**

*DgACO1*, *DgACO2* and *DgACO3* transcript levels in gynoecium (A) and receptacle (B) following pollination. Values are means of three biological replicates  $\pm$  SE.

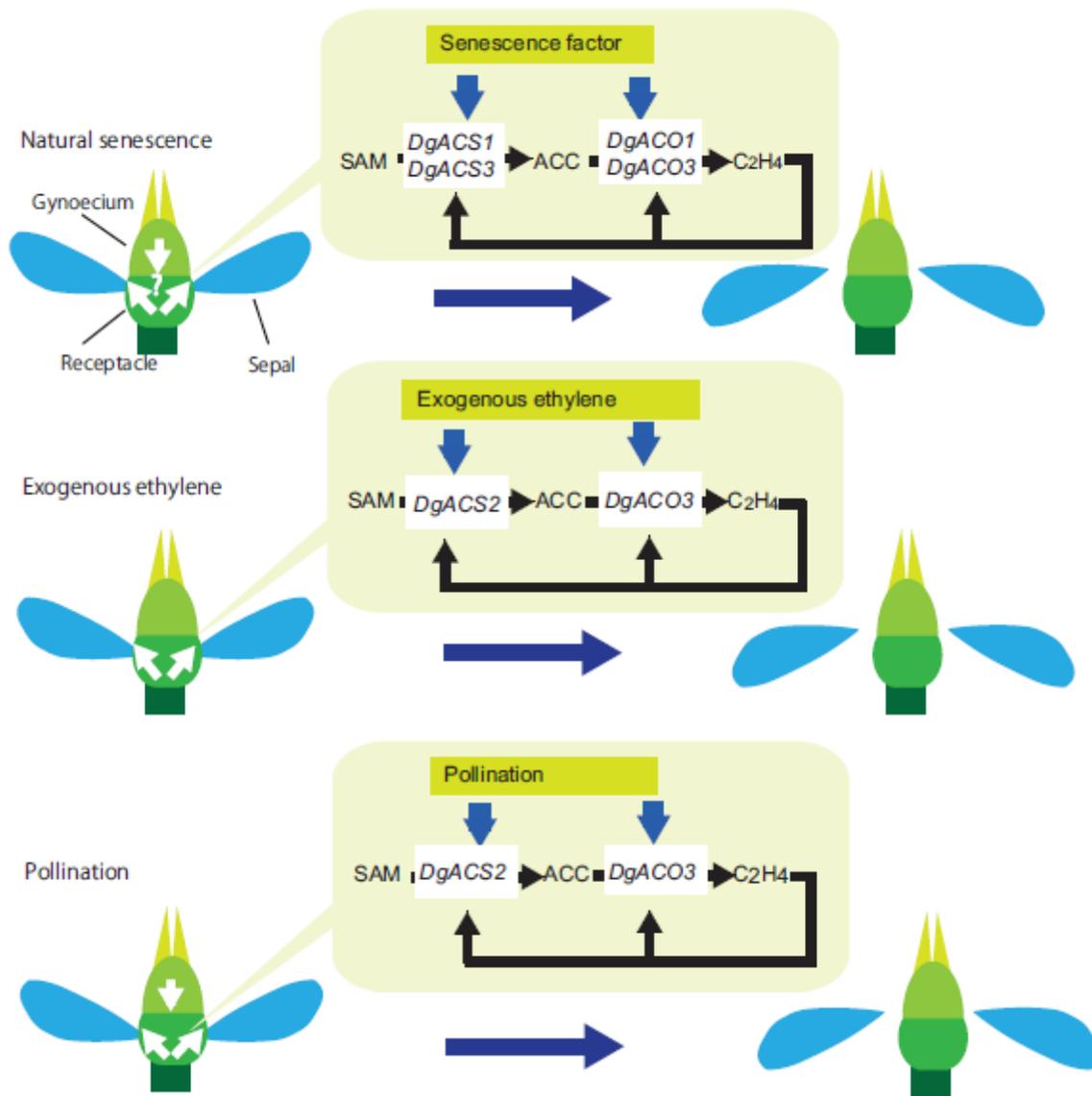


Figure 11

Proposed model for sepal abscission during natural senescence, following exposure to ethylene and pollination in *D. grandiflorum*. White arrows indicate ethylene production. Balloon diagrams indicate the regulation of ethylene biosynthesis in receptacle.

## Supplementary Files

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