**ORIGINAL PAPER**

# Expression of ethylene biosynthesis genes in the gynoecium and receptacle associated with sepal abscission during senescence in *Delphinium grandiflorum*

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## Abstract

*Delphinium* flowers are highly sensitive to ethylene and its sepals abscise during senescence in association with an increase 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*) of *D. grandiflorum* cv. Super Grand Blue were cloned. To investigate the contribution of these genes to ethylene production, their expression in the gynoecium and receptacle was analyzed during natural senescence and after ethylene exposure and pollination. Ethylene production in the receptacle significantly increased, whereas that in the gynoecium exhibited only a slight increase during natural senescence. The transcript levels of *ACS* and *ACO* in these organs, excluding those of *DgACS2* in the receptacle, increased during senescence. Exposure to ethylene accelerated sepal abscission and increased ethylene production more markedly in the receptacle than in the gynoecium. *DgACS1* transcript levels in the gynoecium and *DgACS2* and *DgACO3* transcript levels in the receptacle increased after ethylene exposure. Pollination accelerated sepal abscission and increased ethylene production in the gynoecium and receptacle. Additionally, it slightly affected *ACS* and *ACO* transcript levels in the gynoecium, whereas *DgACO3* transcript levels in the receptacle markedly increased. These results reveal that *ACS* and *ACO* expression is differently regulated in the gynoecium and receptacle. Furthermore, some of these genes are upregulated by pollination only in the receptacle, indicating the significance of the receptacle in ethylene biosynthesis associated with sepal abscission.

**Keywords** 1-Aminocyclopropane-1-carboxylic acid synthase · 1-Aminocyclopropane-1-carboxylic acid oxidase · Ethylene exposure · Pollination

Communicated by Vijay Pratap Singh

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## Abbreviations

ACC   1-Aminocyclopropane-1-carboxylic acid
ACO   1-Aminocyclopropane-1-carboxylic acid oxidase
ACS   1-Aminocyclopropane-1-carboxylic acid synthase

## Introduction

The phytohormone ethylene plays an important role in various stages of plant growth and development, including senescence. Exposure to ethylene accelerates senescence in the flowers of many species, and the primary symptom of ethylene-induced senescence is petal wilting or petal (sepal) abscission (Woltering and van Doorn 1988). The petals of many ethylene-sensitive flowers, such as carnation (Nichols 1966) and *Eustoma grandiflorum* (Ichimura et al. 1998), wilt in response to ethylene exposure. In *Digitalis* (Stead and Moore 1983), *Pelargonium* (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, increased ethylene production in the gynoecium has been considered to cause 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). It also induces petal abscission in *Digitalis* (Stead and Moore 1979, 1983), *Pelargonium* (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 ethylene biosynthesis pathway of 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 by ACC synthase (ACS). ACC is converted into ethylene, carbon dioxide, and HCN by ACC oxidase (ACO). In plants, ACS is generally considered to be a rate-limiting enzyme in ethylene biosynthesis 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 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* in the floral organs of carnation (Jones and Woodson 1997, 1999; Jones 2003; Naiang et al. 2021; Norikoshi et al. 2022), petunia (Tang et al. 1994), tomato (Llop-Tous et al. 2000), and rose (Xue et al. 2008) have been performed. 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* expression in flowers displaying petal abscission. In *Pelargonium*, *ACS* and *ACO* 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. *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 use as cut flowers. *Delphinium* flowers are sensitive to ethylene. Their sepals rapidly abscise upon ethylene 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 and not the gynoecium. Thus, ethylene produced by the receptacle should directly induce sepal abscission (Ichimura et al. 2009). Ethylene production in the gynoecium and receptacle increases during flower senescence but that in 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* of *Delphinium* have not yet been cloned. Previously, Ichimura et al. (2009) highlighted the importance of the receptacle in sepal abscission during natural senescence. As observed in ethylene-sensitive flowers of many plants (van Doorn and Stead 1997), exogenous ethylene and pollination may accelerate sepal abscission in *D. grandiflorum*. Therefore, the quantification of *ACS* and *ACO* expression in the gynoecium and receptacle during natural senescence as well as after exposure to ethylene and pollination will elucidate the molecular mechanisms underlying ethylene biosynthesis in these organs associated with sepal abscission.

In the present study, we cloned *ACS* and *ACO* and determined their nucleotide sequences in *D. grandiflorum*. Furthermore, we investigated their expression in the gynoecium and receptacle during natural senescence, after 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 to 25 °C. On the day of flower opening, the anthers were removed, and individual flowers were covered with paper bags to prevent pollination. Flowers with peduncles of
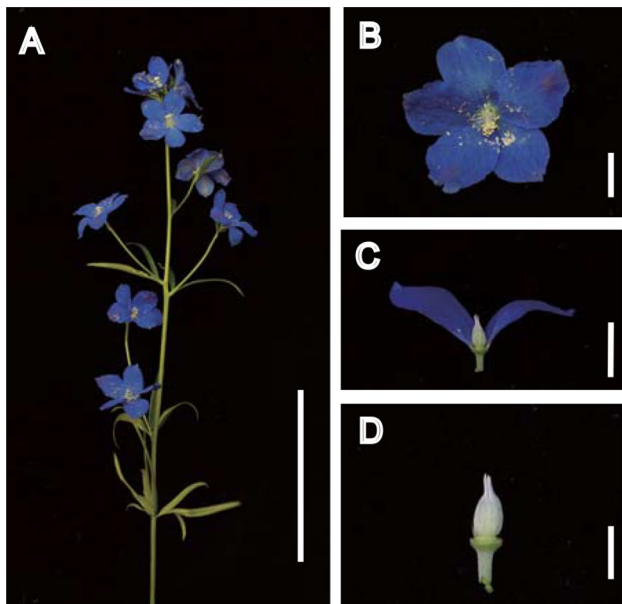
Fig. 1 Morphology of *Delphinium grandiflorum* flowers. **A** Flower spikes. The scale bar represents 10 cm. **B** Flower. The scale bar represents 1 cm. **C** Sepal, gynoecium, and receptacle. The scale bar represents 1 cm. **D** Gynoecium and receptacle. The scale bar represents 0.5 cm

3 cm in length were cut from the middle region of the flower spikes. *D. grandiflorum* flowers are protandrous, and the gynoecium matured approximately 4 days after emasculation. Next, the flowers were cut from the plant and transported to the laboratory. Cut peduncles were placed in a vessel with distilled water within 1 h of harvest and used for all subsequent experiments. Unless otherwise stated, the cut flowers were kept under a 12-h photoperiod at 23 °C, 70% relative humidity, and 10 µmol $m^{-2}$ $s^{-1}$ irradiance achieved using cool-white fluorescence lamps.

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

## Ethylene treatment

The cut flowers placed in individual vessels containing distilled water were transferred to a 70-L transparent acrylic box fitted with a septum, through which ethylene was introduced to achieve a concentration of 10 µL $L^{-1}$. The box was kept at 23 °C under dark conditions. The flowers were removed from the box 4, 8, 12, 16, 20, and 24 h after ethylene exposure and used for ethylene production assessment and RNA extraction.

## Pollination

The flowers were pollinated with fresh pollen collected from the same cultivar and kept under the aforementioned environmental conditions.

## Observation of pollen tube growth

The gynoecia were removed from the 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)]. Next, gynoecia were macerated in 8 M NaOH for 24 h, rinsed with tap water for 1 h, and stained with 0.1% aniline blue in 0.1 M $K_3PO_4$. The pollen tubes were observed microscopically under incident ultraviolet illumination.

## Measurement of ethylene production

Some flowers experienced sepal abscission during natural senescence on day 5. Sepals were abscised in most of the flowers 24 h after ethylene treatment or pollination. Abscised sepals were not used for ethylene measurements. The flowers were individually placed 20-mL Erlenmeyer flasks (23.5 mL), whereas the gynoecia and receptacles were individually placed in test tubes (14.8 mL). The Erlenmeyer flasks and test tubes were sealed and maintained at 23 °C. One hour later, a 1-mL gas sample was withdrawn using a syringe and used to determine 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 in terms of fresh weight.

## RNA extraction

Frozen gynoecia and receptacles (0.1 g fresh weight) were powdered using liquid nitrogen and Shake Master Auto (Bio medical science, Tokyo, Japan). Next, 450 µL of RLT buffer containing 1% 2-mercaptoethanol was added to the obtained powder. The resulting mixture was incubated at 56 °C for 1 min. Total RNA was extracted using RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) according to the manufacture's instruction, with some modifications.

## First strand cDNA and PCR amplification

Synthesis of cDNA was carried out using random hexamer primers and 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′- CAIAYICKRAACCAICCIGSYTC-3′ (reverse); for *ACO*, 5′-GCITGYSAIAAYTGGGGGGTT-3′ (forward) and 5′-CCICCIGCRTCIGTRTGIGC-3′ (reverse); for Actin, 5′-TGGGAYGAYATGGARAARATHTGG-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-TeasyVecter (Promega) according to the manufacture's instructions.

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-T Easy Vecter. The full length cDNA was cloned and the DNA sequence was confirmed using specific primers.

## Determination of nucleotide sequence

DNA Sequencing was performed using the Applied Biosystems ABI Prism DNA sequencer (Model 377; Thermo Fisher Scientific) and 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 analysis was performed 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 using Phylogeny.fr (http://www.phylogeny.fr/).

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

The 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 using the RNase-free DNase

set. The quality and quantity of RNA were assessed using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). cDNA synthesis was performed with random hexamer primers using the Invitrogen Super-Script III 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′-ACCTCCTGGTGTTTATTTCACAGT C-3′ (reverse); for *DgACS3*, 5′-TTTTGCCAACATGAG CCAAC-3′ (forward) and 5′-TTGTGGCATCTTCTTTTC TCCA-3′ (reverse); for *DgACO1*, 5′-CCCAAATTTGTT TTCGAGGA-3′ (forward) and 5′-TAATAAGCCGTGGCG ATAGG-3′ (reverse); for *DgACO2*, 5′-GGTGAAGTTTCA AGCCAAGG-3′ (forward) and 5′-TTCTTCACTTGTTTG GGCACT-3′ (reverse); for *DgACO3*, 5′-GGGTGAAGT TTCAAGCCAAG-3′ (forward) and 5′-CAATTTGCTGAC CCATCTGA-3′ (reverse); and for *DgActin*, 5′-AGGGGA TACATGTTCACAACCAC-3′ (forward) and 5′-TTTCAA GCTCCTGCTCATAGTCC-3′ (reverse).

PCR reactions were performed using a 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 used: initial polymerase activation at 95 °C for 30 s and; then 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The specificity of PCR was checked using a heat dissociation protocol (from 95 to 60 °C) after the final cycle.

Absolute transcript levels were determined via the second derivative maximum method (Luu-The et al. 2005) using a dilution series of plasmid DNA containing the target sequence as external standards. To standardize the data, the ratio between the absolute transcript levels of the target and control genes was calculated for each sample.

## Statistical analysis

Student's *t*-test and Tukey–Kramer's multiple range test were performed using SigmaStat software (v.12.5; Systat Software, San Jose, CA, USA).

## Results

### Cloning and nucleotide sequences of ACS

Three *ACS* genes were cloned and designated *DgACS1*, *DgACS2* and *DgACS3* and deposited in the DDBJ with accession numbers LC603027, LC603028, and LC603029, respectively. The cDNAs of these genes were 1901, 1739, and 1721 bp in length, respectively. The predicted proteins

DgACS1, DgACS2, and DgACS3 consisted of 486, 485, and 444 amino acids, respectively. DgACS1 and DgACS2 had an amino acid homology of 80.9%, DgACS1 and DgACS3 had an amino acid homology of 55.8%, and DgACS2 and DgACS3 had an amino acid homology of 53.2%. Seven conserved domains were observed in the three genes (Fig. S1).

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

## Cloning and nucleotide sequences of ACO

Three *ACO* genes were cloned and designated *DgACO1*, *DgACO2* and *DgACO3*, and deposited in the DDBJ with accession numbers LC603030, LC603031, and LC603032, respectively. The cDNAs of these genes were 1182, 1159, and 1072 bp in length, respectively. The predicted protein DgACO1 consisted of 317 amino acids, DgACO2 consisted of 317 amino acids, and DgACO3 consisted of 319 amino acids (Fig. S3). DgACO1 and DgACO2 as well as DgACO1 and DgACO3 had amino acid homologies of 78.6%, whereas DgACO2 and DgACO3 had an amino acid homology of 94%. Twelve amino acids, which are known to be conserved in ACO proteins (Wang and Woodson 1991; Do et al. 2005; Momonoi et al. 2007), were found to be 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 highly similar, and they clustered into type 1 (Fig. S4).

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

The time until sepal abscission in all the tested flowers was 5 days. Ethylene production in the whole flowers was almost constant during the first 3 days and significantly increased on day 4 (Fig. 2). Ethylene production in the gynoecium was relatively high on day 0, decreased over the first 3 days and slightly increased on day 4. Ethylene production in the receptacle decreased during the first 3 days and significantly increased on day 4.

In the gynoecium, *DgACS1* transcript level, which was higher than that of the other two *ACS* transcript levels at harvest, decreased during the first 3 days and increased thereafter (Fig. 3). *DgACS2* transcript level in
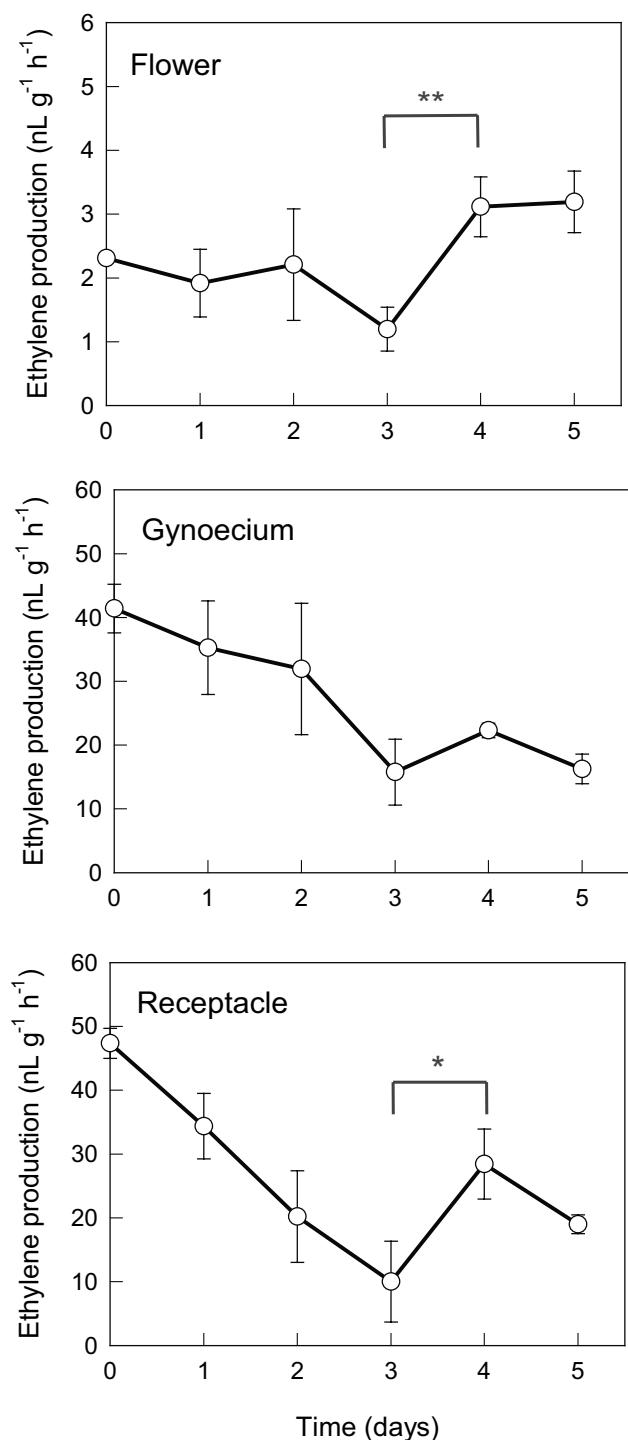


**Fig. 2** Ethylene production in the flowers, gynoecium, and receptacle during natural flower senescence. Values are means of five biological replicates $\pm$ SE. * and ** indicate significance at $P < 0.05$ between stages 4 and 5, respectively, based on a *t*-test

the gynoecium gradually increased during senescence, and *DgACS3* transcript level markedly increased from day 3, peaking on day 5. In the receptacle, *DgACS2* transcript level was the highest on day 0 among the three transcript
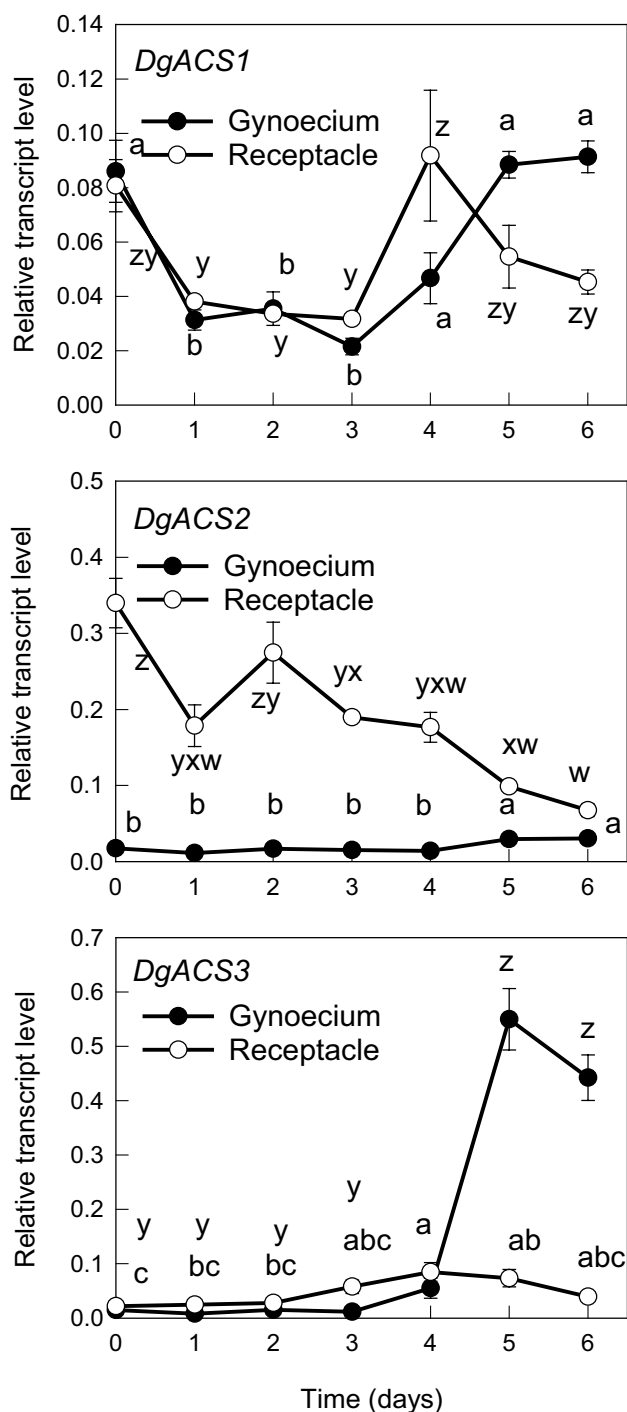
Fig. 3 *DgACS1*, *DgACS2* and *DgACS3* transcript levels in gynoecium and receptacle during natural flower senescence. Values are means of three biological replicates ± SE. Different letters in each organ indicate significant differences ($P < 0.05$) using Tukey–Kramer's multiple range test

levels; however, its level decreased during senescence. *DgACS1* transcript level decreased during the first 3 days and increased on day 4. *DgACS3* transcript level peaked on day 4.

In the gynoecium, *DgACO1* transcript level, which was higher than the other two *ACO* transcript levels on day 0, increased during senescence (Fig. 4). *DgACO2* and *DgACO3* transcript levels in the gynoecium were almost constant during the first 4 days and increased thereafter. In the receptacle, *DgACO1* transcript level increased during the first 4 days and decreased thereafter. *DgACO2* transcript level was almost constant during the first 3 days, increased thereafter, and peaked on day 5. *DgACO3* transcript level increased starting from day 1 and peaked on day 4.

### Ethylene production and ACS and ACO transcript levels in the gynoecium and receptacle after exposure to ethylene

Exposure to ethylene accelerated sepal abscission; the sepals began to abscise 20 h after ethylene exposure and were abscised in most of the flowers 24 h after exposure. In the control and ethylene-treated whole flowers, there were no significant differences in terms of ethylene production at most time points. There was a greater increase in ethylene production in the receptacle than in the gynoecium after 8 h of ethylene exposure (Fig. 5).

In the gynoecium, exposure to ethylene significantly increased *DgACS1* and *DgACS2* transcript levels at several time points (Fig. 6). However, ethylene exposure had only a slight effect on *DgACS3* transcript level. In the receptacle, *DgACS2* transcript level was the highest among the three *ACS* transcript levels and was higher in ethylene-treated flowers than in control flowers at all time points. *DgACS1* transcript level did not noticeably increase in ethylene-treated flowers compared with control flowers. *DgACS3* transcript level decreased after ethylene treatment at all time points and was the lowest among the three transcript levels.

In the gynoecium, *DgACO1* transcript level was higher than that of the other two transcript levels; however, ethylene exposure only slightly increased it (Fig. 7). Ethylene exposure did not increase *DgACO2* and *DgACO3* transcript levels. In the receptacle, *DgACO1* transcript level significantly increased after 12 h of ethylene exposure. After ethylene treatment, *DgACO2* transcript level did not increase whereas *DgACO3* transcript level markedly increased.

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

Pollination accelerated sepal abscission. The sepals began to abscise at 12 h and were abscised in all the flowers 24 h after pollination. Pollen tube growth in the gynoecia was observed via fluorescent microscopy. Many pollen tubes reached the ovary 6 h after pollination (Fig. S5).

**Fig. 4** *DgACO1*, *DgACO2* and *DgACO3* transcript levels in the gynoecium and receptacle of during natural flower senescence. Values are means of three biological replicates ± SE. Different letters in each organ indicate significant differences (*P* < 0.05) using Tukey–Kramer's multiple range test
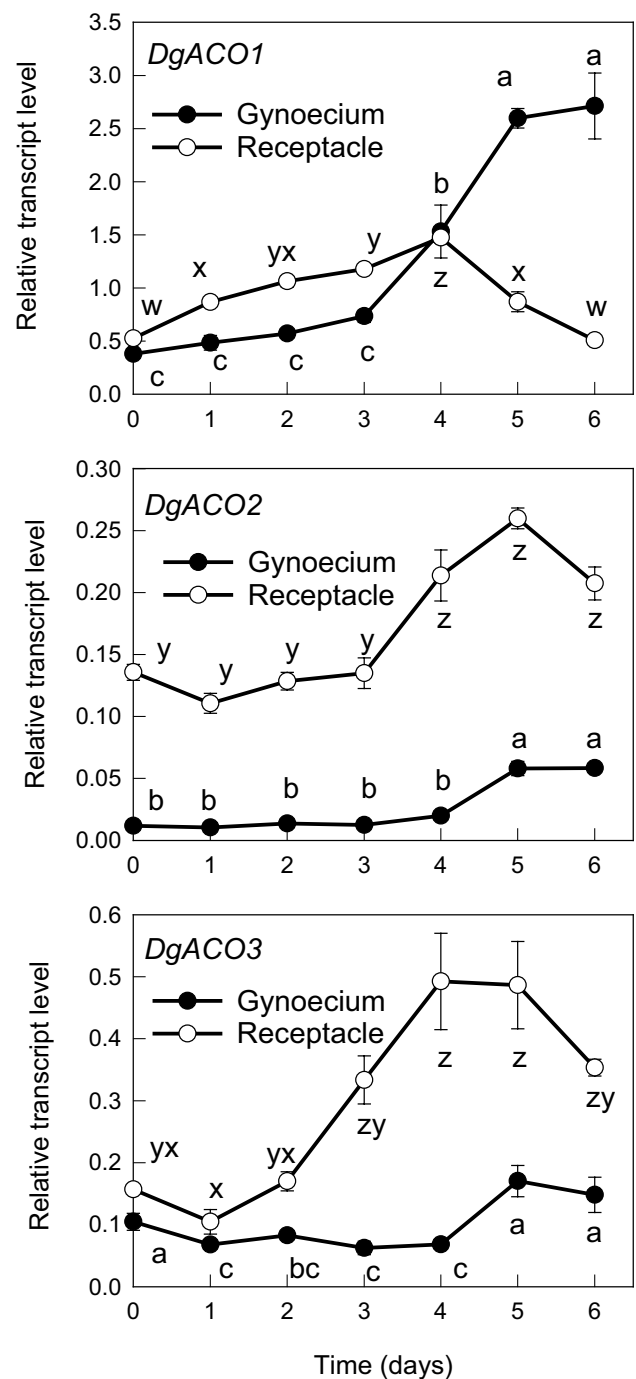


## Ethylene production and ACS and ACO transcript levels in the gynoecium and receptacle in unpollinated and pollinated flowers

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

In the gynoecium, *ACS* transcript levels did not significantly increase after pollination (Fig. 9). In the receptacle, *DgACS2* transcript level increased after pollination, but *DgACS1* and *DgACS3* transcript levels did not.

In the gynoecium, the three *ACO* transcript levels did not significantly increased after pollination (Fig. 10). In the receptacle, *DgACO1* transcript level was significantly
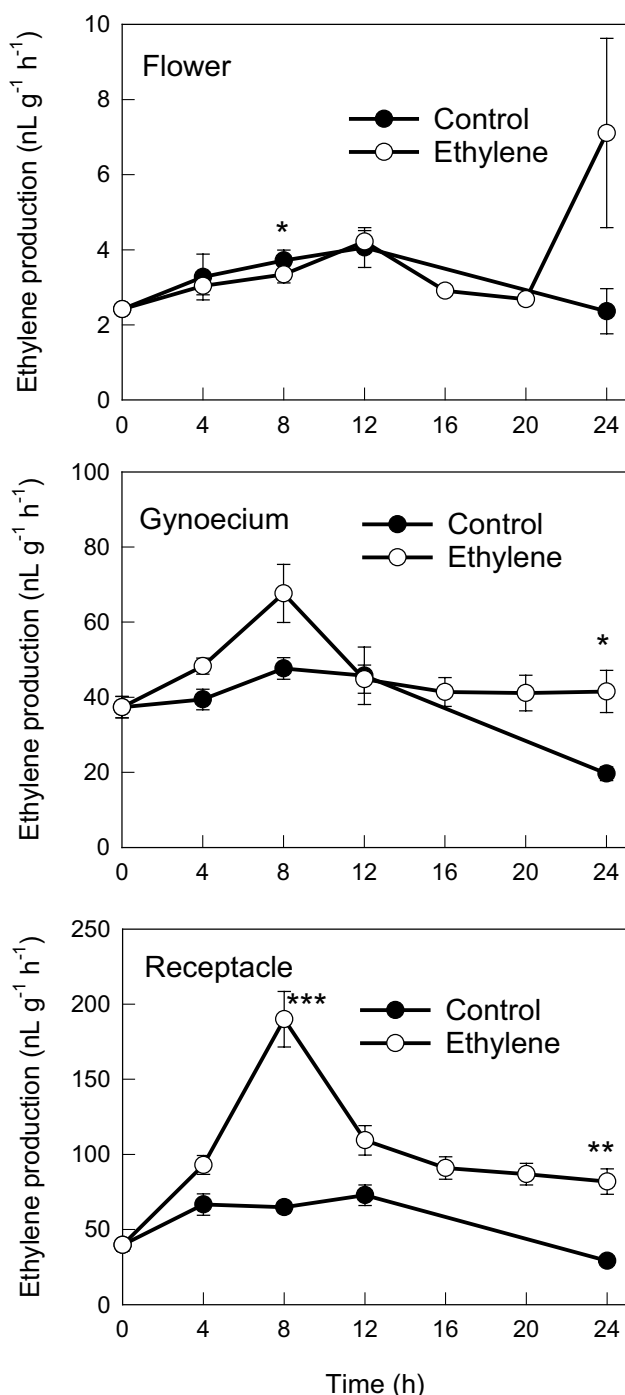
**Fig. 5** Ethylene production in the flowers, gynoecium and receptacle during ethylene exposure. Values are means of five biological replicates ± SE. * and ** at each time point indicate significant differences at $P < 0.05$, 0.01, and 0.001, respectively, using a $t$-test

higher in the pollinated flowers than in the unpollinated flowers at 4, 8, and 12 h after pollination. Although *DgACO2* transcript level slightly changed after pollination, *DgACO3* transcript level markedly increased after pollination.

## Discussion

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

### Ethylene production and ACS and ACO expression during natural senescence

Ethylene production in the gynoecium and receptacle was relatively high on day 0 (Fig. 2). This result somewhat differed from that obtained for *D. × belladonna* in which ethylene production in the gynoecium and receptacle was relatively low at harvest (Ichimura et al. 2009). In our study, the flowers were emasculated on the day of flower opening and harvested approximately 4 days later. Emasculation increased ethylene production in the gynoecium and receptacle, and it peaked 3 days later (Ichimura et al. 2009). It is known that wounding increases ethylene production (Yang and Hoffman 1984). Therefore, high levels of ethylene production at day 0 may be attributed to wounding possibly caused by emasculation.

In the gynoecium of carnation, *DcACS2* and *DcACO1* transcript levels increase during senescence (ten Have and Woltering 1997; Jones and Woodson 1999). Similarly, *SlACS1A* transcript levels increased during senescence in tomato flowers (Llop-Tous et al. 2000). In petals displaying abscission, changes in *ACS* and *ACO* transcript levels in the gynoecium during natural senescence have only been reported for *Pelargonium*; however, no substantial increase in their transcript levels was observed (Clark et al. 1997). In the present study, based on the observed expression levels and trends, *DgACS3* and *DgACO1* were found to produce the highest levels of transcripts (Figs. 3 and 4), and the expression of these genes appears to contribute to increased ethylene production in the gynoecium.

To date, the expression of *ACS* and *ACO* in the receptacle has only been investigated in carnation (Jones and Woodson 1999) and rose (Xue et al. 2008); however, no obvious change in these transcript levels was observed during senescence. In our study, increases were observed in the expression of *DgACS1*, *DgACS3*, and the three *ACO* genes during senescence (Fig. 4). Thus, the expression of these *ACS* and *ACO* genes appears to contribute to ethylene production in the receptacle.

Cell wall hydrolases, including cellulases and pectinases, are believed to be involved in petal abscission (Brown 1997; van Doorn and Stead 1997). Petal abscission in rose is associated with enhanced expression of genes encoding
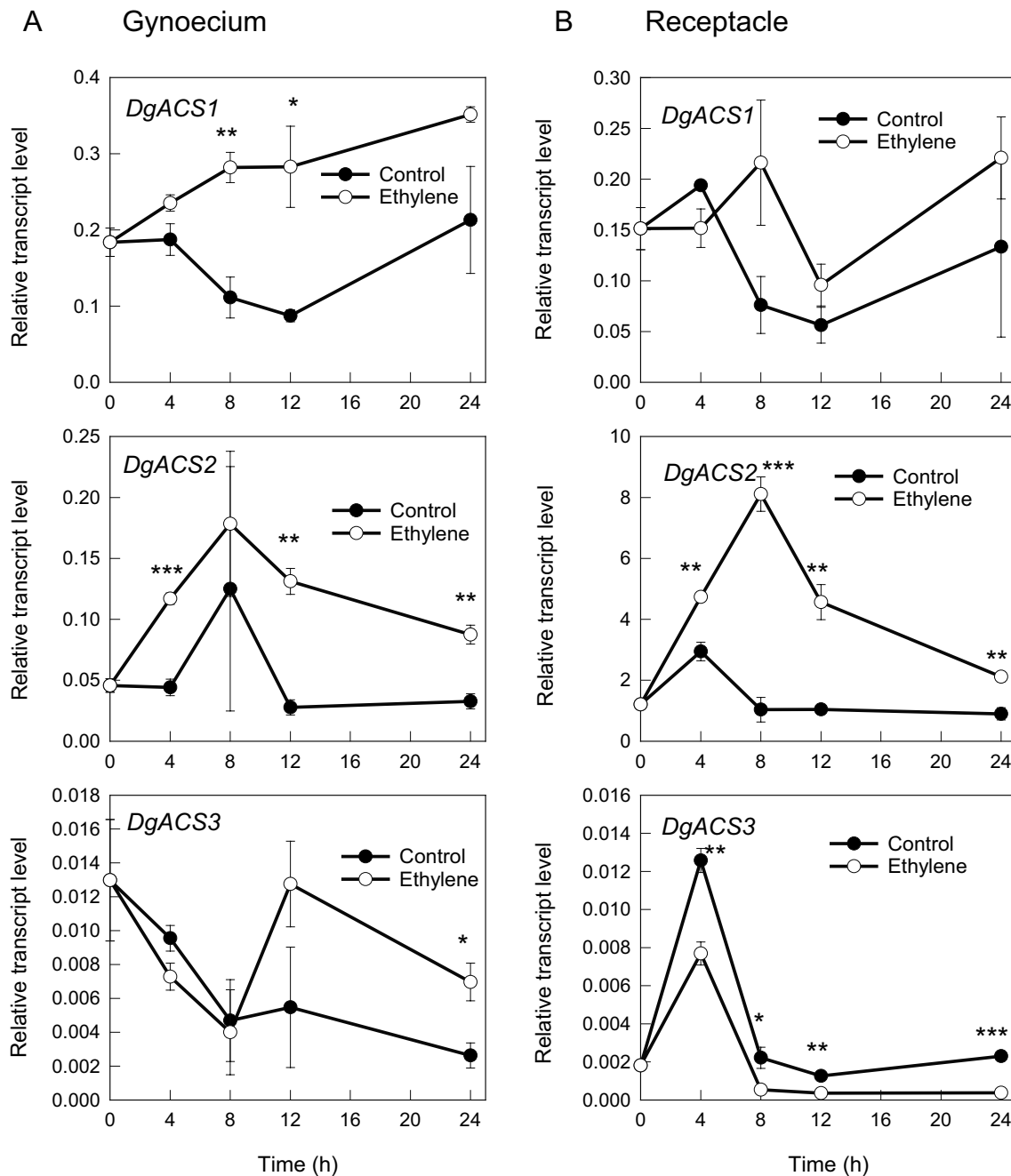
**Fig. 6** *DgACS1*, *DgACS2* and *DgACS3* transcript levels in the gynoecium (**A**) and receptacle (**B**) during ethylene exposure. Values are means of three biological replicates ± SE. *, **, and *** at each time point indicate significant differences at $P < 0.05$, 0.01, and 0.001, respectively, using a *t*-test

expansin, pectate lyase, and xyloglucan endotransglucosylase/hydrolase (Sane et al. 2007; Singh et al. 2011ab). Sepals are directly connected to the receptacle. Therefore, an increase in ethylene production mediated by the expression of *ACS* and *ACO* in the receptacle may increase the expression of these genes, leading to sepal abscission.

In the receptacle, the expression of *DgACS2*, which produced the highest transcript levels on day 0, decreased during senescence (Fig. 3). In the *D.* × *belladonna* receptacle, wounding caused by emasculation induced a climacteric-like increase in ethylene production, which reached a peak 3 days later
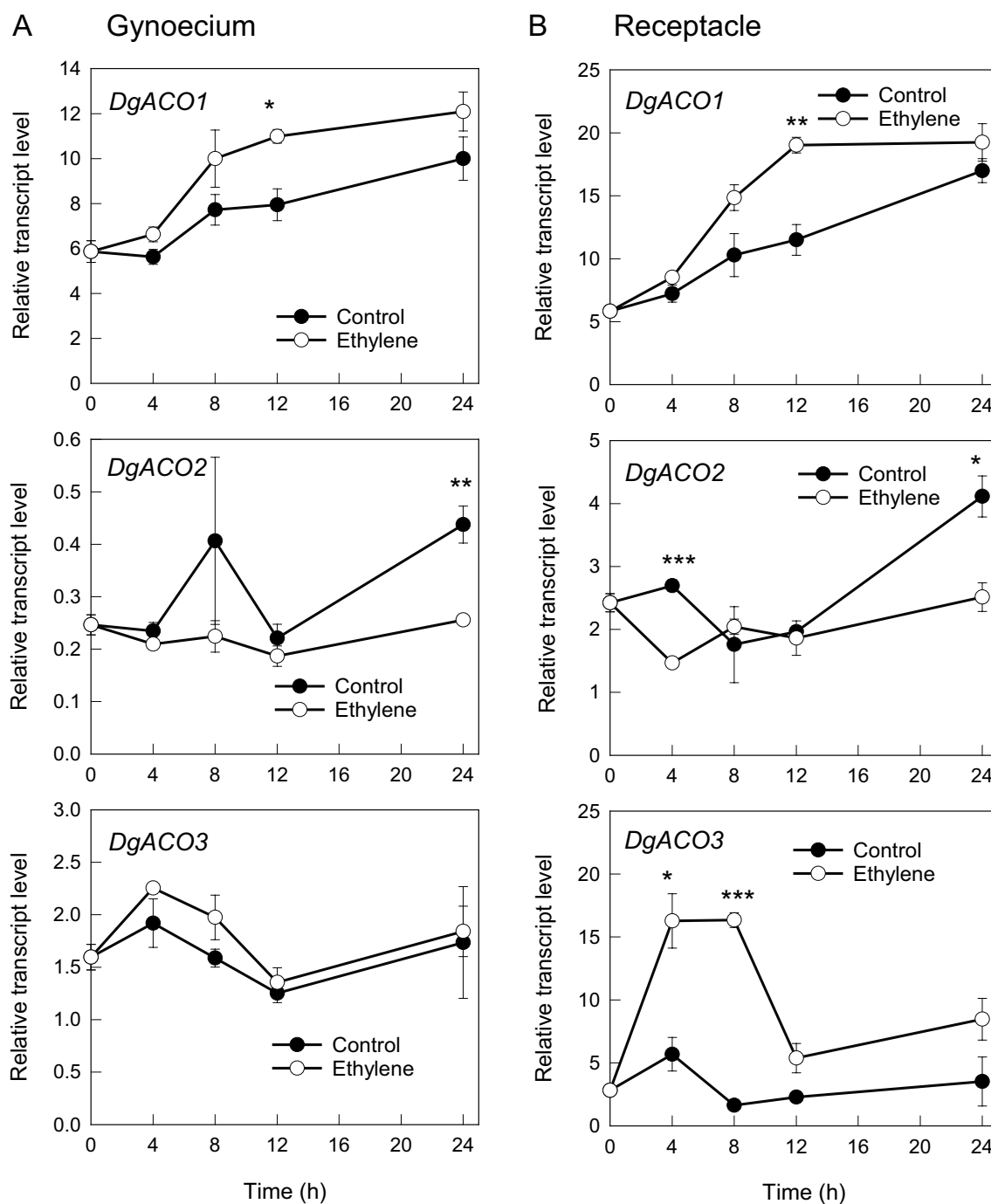
**Fig. 7** *DgACO1*, *DgACO2* and *DgACO3* transcript levels in the gynoecium (**A**) and receptacle (**B**) during ethylene exposure. Values are means of three biological replicates ± SE. *, **, and *** at each time point indicate significant differences at $P < 0.05$, 0.01, and 0.001, respectively, using a *t*-test

(Ichimura et al. 2009). Therefore, the high levels of *DgACS2* expression observed in this study may be a result of emasculation.

It is unclear whether ethylene produced in the gynoecium affects ethylene production in the receptacle; additional research is needed to clarify this relationship.

### 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). In some ethylene-sensitive flowers, including
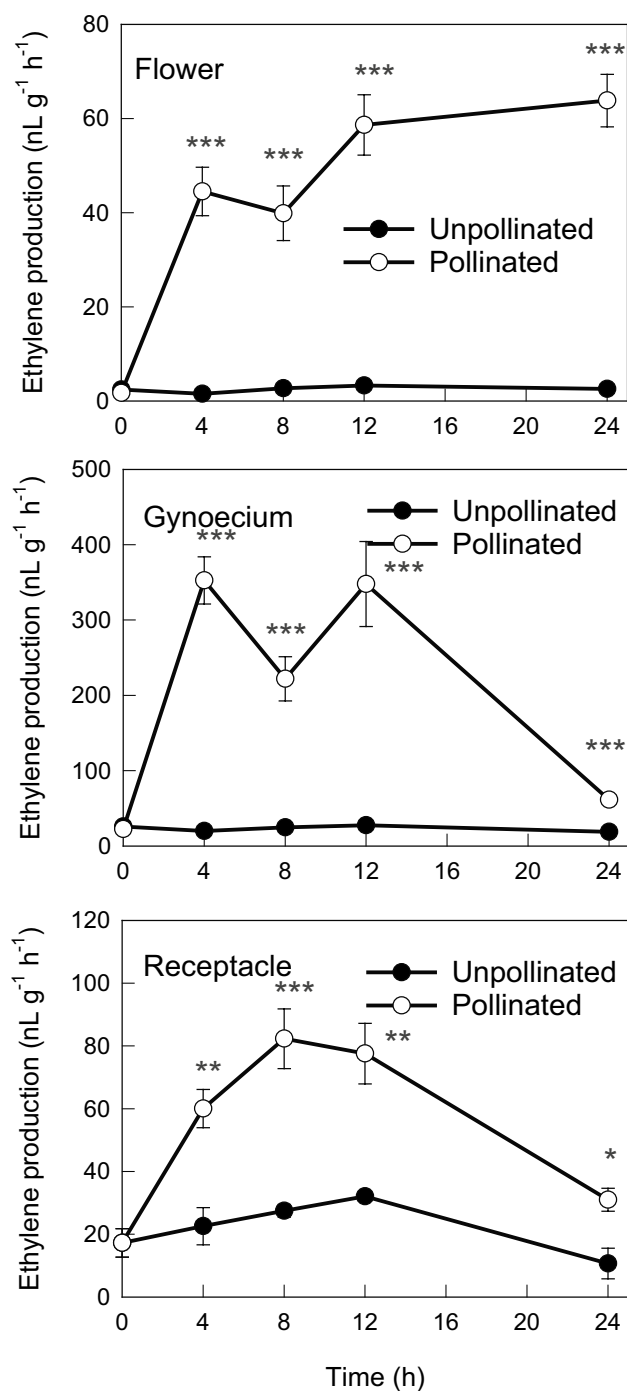
**Fig. 8** Ethylene production in flowers, gynoecium and receptacle following pollination. Values are means of five biological replicates ± SE. *, **, and *** at each time point indicate significant differences at $P < 0.05$, 0.01, and 0.001, respectively, using a $t$-test

carnation (Jones 2003; Norikoshi et al. 2022) and *Tweedia caerulea* (Pun et al. 2013), exogenous ethylene induces autocatalytic ethylene production. However, ethylene exposure did not increase ethylene production in the whole flower but significantly increased ethylene production in the receptacle

(Fig. 5). Ethylene production might decrease in the sepals and filaments following ethylene exposure, resulting in different trends of ethylene production in the whole flower and receptacle. In the whole flowers, high ethylene production was observed 24 h after ethylene exposure, when the sepals were abscised. The total fresh weight of the gynoecium and receptacle was one-tenth of that of the whole flower, which includes the sepals and filaments. The observed increase in ethylene production in whole flower after sepal abscission can be explained by the fact that ethylene production in the sepals is very low (Ichimura et al. 2009).

The expression of *DgACS1* and *DgACS2* in the gynoecium was increased after ethylene exposure (Figs. 6 and 7), suggesting that the expression of these genes may have contributed to the slight increase in ethylene production in this organ in response to ethylene exposure. The expression of *ACS* in carnation (Jones and Woodson 1997) and tomato (Llop-Tous et al. 2000), and that of *ACO* in petunia (Tang et al. 1994) increased in response to ethylene.

*ACS* and *ACO* expression in the receptacle following ethylene exposure has been reported in carnation (Jones and Woodson 1999) and rose (Xue et al. 2008). In rose, *RhACS2* expression slightly increased after ethylene exposure (Xue et al. 2008). In this study, the expression of *DgACS2* and *DgACO3* markedly increased after ethylene exposure (Figs. 6 and 7), suggesting that the expression of these genes may contribute to increased ethylene production in the receptacle.

In carnation, exogenous ethylene markedly accelerates petal senescence, irrespective of treatment with aminoethoxyvinylglycine, an inhibitor of ethylene biosynthesis (Pun et al. 2016). This finding suggests that exogenous ethylene directly induces petal senescence. However, pollination accelerated sepal abscission more than high-concentration ethylene exposure did. Therefore, endogenous ethylene production in the receptacle induced by exogenous ethylene exposure may be involved in sepal abscission.

## ACS and ACO expression in pollinated flowers

As reported in many plants, including *Digitalis* (Stead and Moore 1979), *Pelargonium* (Clark et al. 1997) and *Torenia* (Goto et al. 1999), pollination accelerated sepal abscission in *D. grandiflorum*. Although a marked increase in ethylene production in the gynoecium was observed (Fig. 8), the expression of *ACS* and *ACO* in the gynoecium did not significantly increase after 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, an early increase in ethylene production is associated with the ACC content of the pollen (Singh et al.
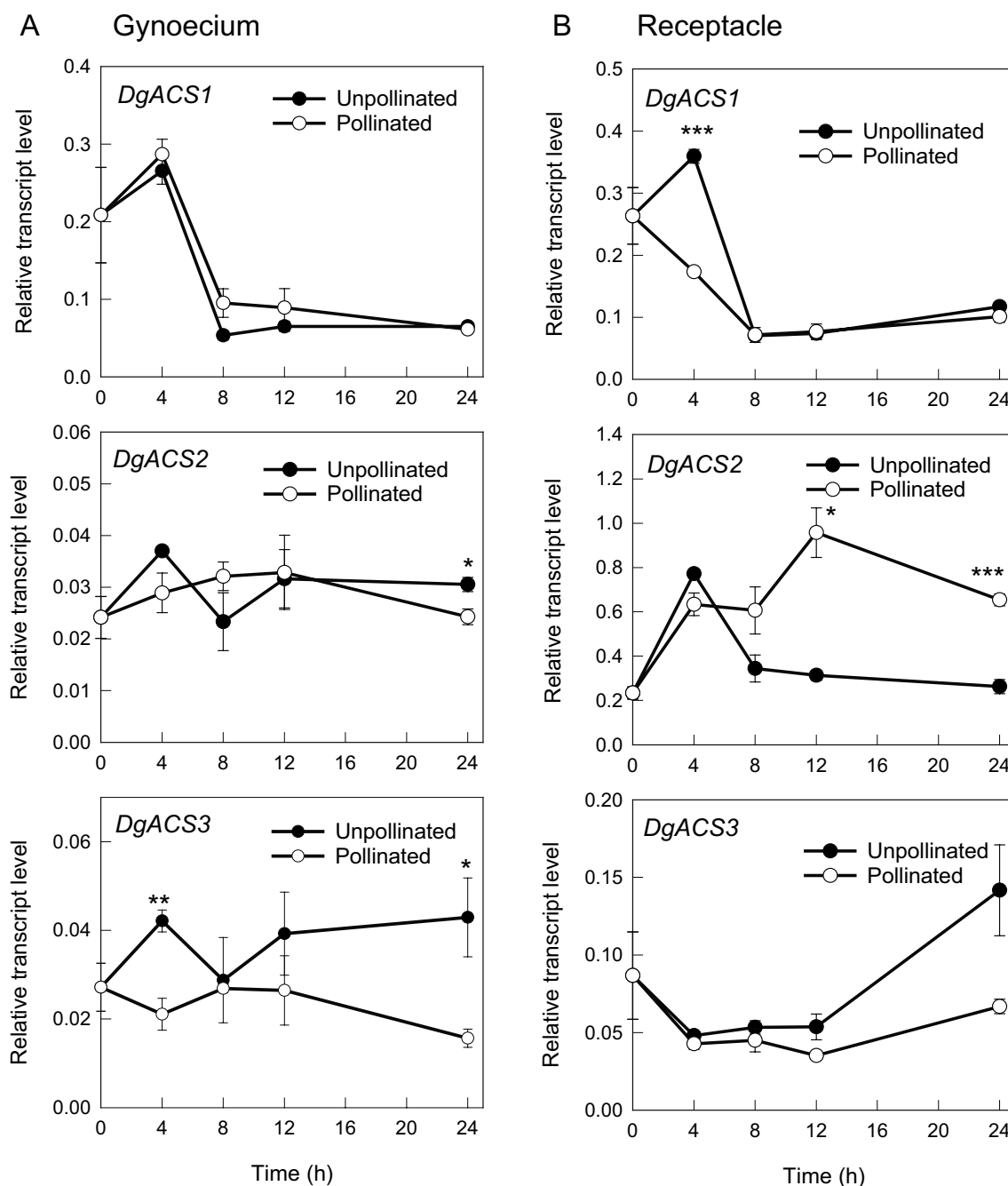
**Fig. 9** *DgACS1*, *DgACS2* and *DgACS3* transcript levels in gynoecium (**A**) and receptacle (**B**) following pollination. Values are means of three biological replicates ± SE. *, **, and *** at each time point indicate significant differences at $P < 0.05$, 0.01, and 0.001, respectively, using a *t*-test

1992). We confirmed that pollen tubes reached the ovary 6 h after pollination (Fig. S5). Thus, the marked increase in ethylene production in the gynoecium may be attributed to pollen-borne ACC, which is converted to ethylene via 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 involves interorgan signaling (Jones and Woodson 1997). Two transmission factors for this signaling have been proposed: ethylene (Woltering et al. 1995) and ACC (Reid et al. 1984). The increase in ethylene production in the receptacle after pollination was associated with an increase in the expression of *DgACO1* and *DgACO3* (Figs. 8 and 10). These results suggest that pollination
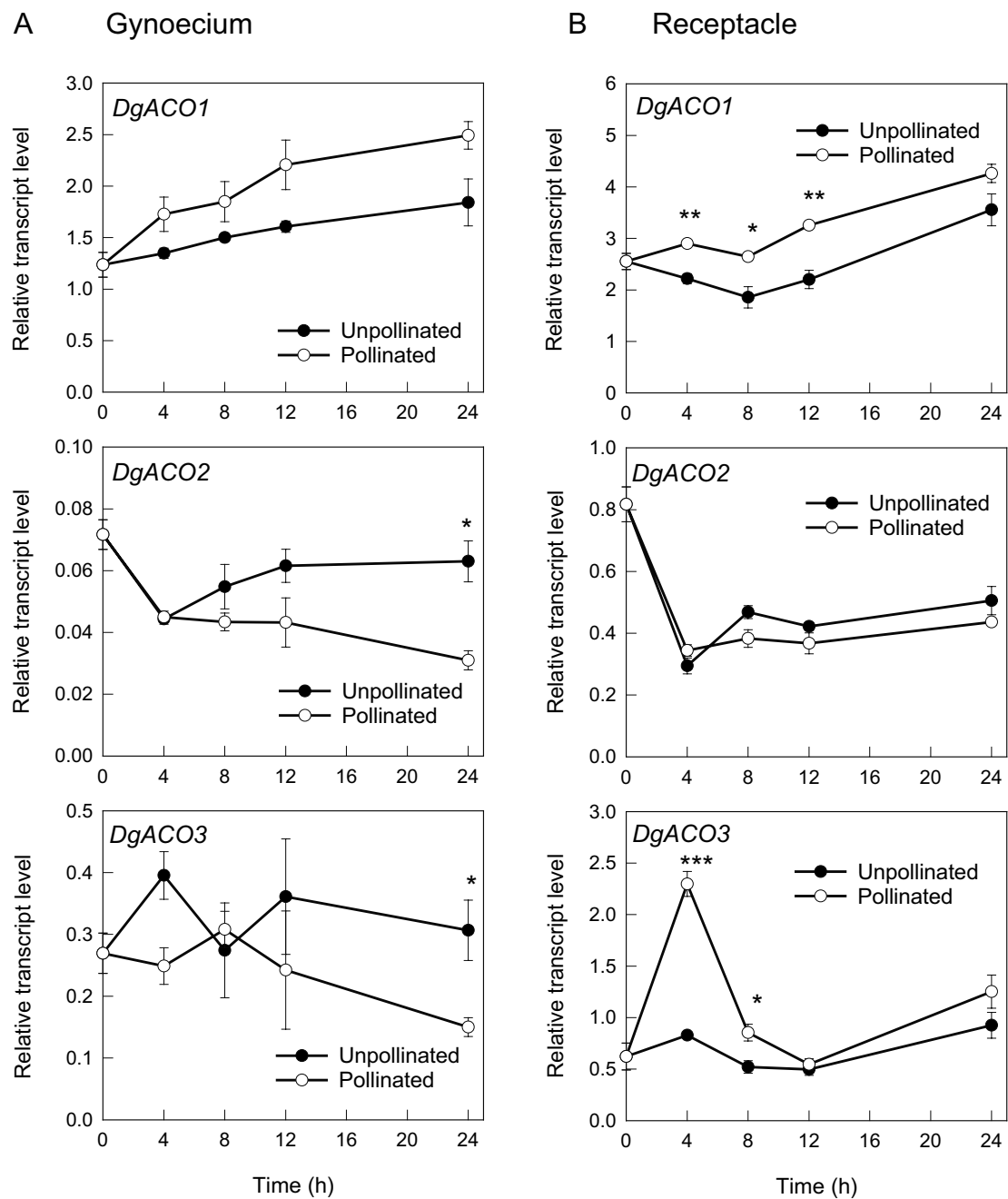
## A Gynoecium



## B Receptacle



**Fig. 10** *DgACO1*, *DgACO2* and *DgACO3* transcript levels in gynoecium (**A**) and receptacle (**B**) following pollination. Values are means of three biological replicates ± SE. One, two, and three asterisks at each time point indicate significantly different at $P < 0.05$, 0.01, and 0.001, respectively by *t*-test

generates some transmission factor that induces ethylene production in the receptacles because the receptacle lies below the gynoecium. From these findings, we propose that ethylene produced in the gynoecium in response to pollination increases the expression of *DgACO1* and *DgACO3*, resulting in increased ethylene production in the receptacle.

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

The significance of the gynoecium in 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
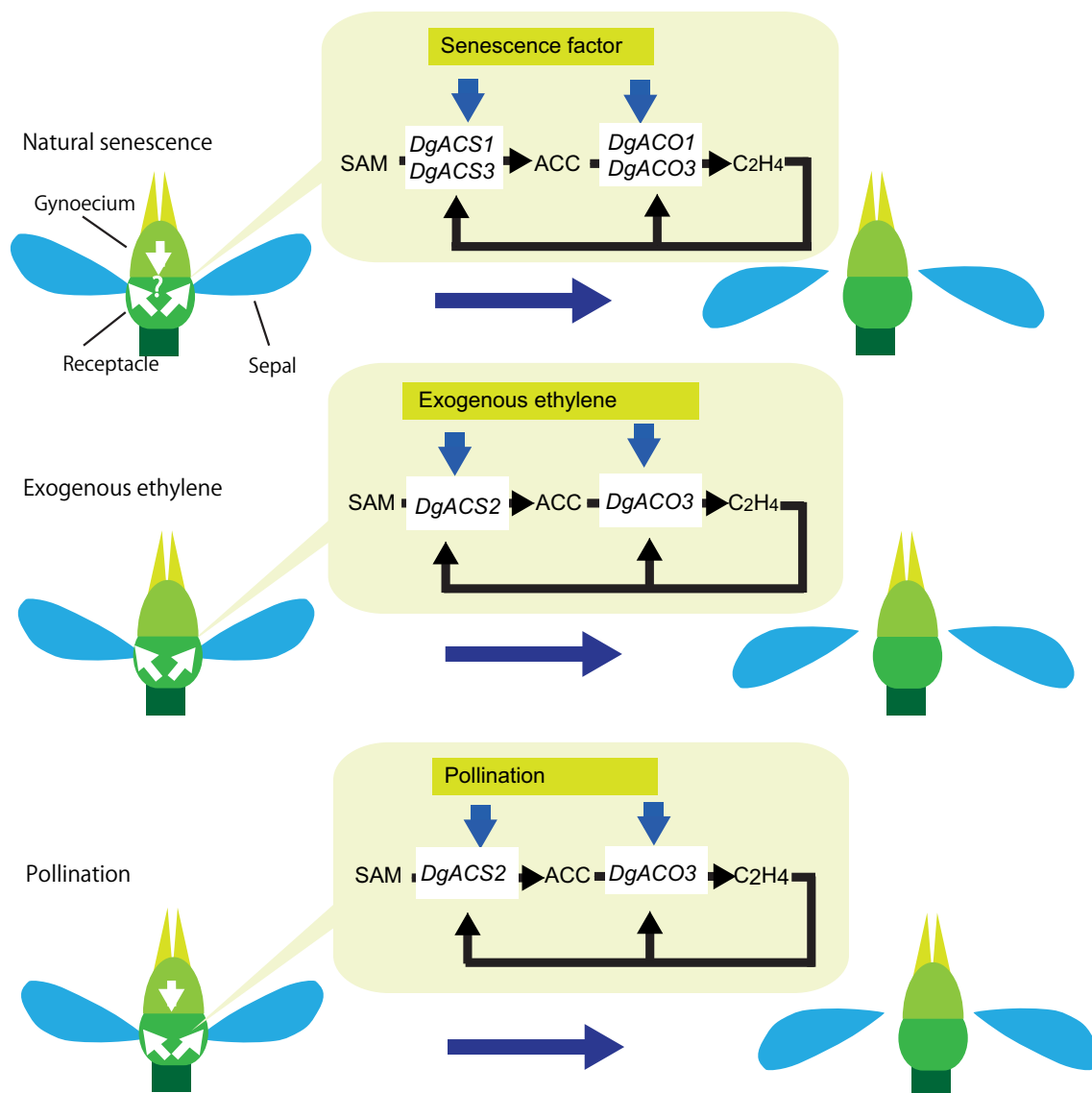
**Fig. 11** Proposed model for sepal abscission during natural senescence, following exposure to ethylene and pollination in *D. grandiflorum*. White arrows indicate ethylene production. Balloon diagrams indicate the regulation of ethylene biosynthesis in receptacle

*Pelargonium* (Clark et al. 1997). In *Digitalis* (Stead and Moore 1983) and *Pelargonium* (Clark et al. 1997), pollination markedly accelerates petal abscission. In our study, however, pollination did not significantly increase the expression of *ACS* and *ACO* in the gynoecium.

Changes in the expression of *ACS* and *ACO* in the receptacle have been reported in carnation (Jones and Woodson 1999) and rose (Xue et al. 2008). However, the significance of this organ in petal wilting has only been mentioned by Hsieh and Sacalis (1986) who reported ACC levels in the receptacle during senescence in cut carnation. Although the significance of the receptacle in sepal abscission has been described for *D. × belladonna* flowers, the activity

of ACS and ACO during natural senescence has only been investigated (Ichimura et al. 2009). In our study, ethylene exposure increased ethylene production more markedly in the receptacle than in the gynoecium, and this finding was associated with marked increases in the expression of some *ACS* and *ACO* genes in the receptacle. This suggests that the responsiveness of the receptacle to ethylene exposure is higher than in that of the gynoecium. Moreover, the expression of *ACS* and *ACO* after pollination increased only in the receptacle. Therefore, the receptacle is likely to be the primary organ that regulates ethylene biosynthesis through the expression of *ACS* and *ACO*, leading to sepal abscission in *D. grandiflorum*.

Thus, we propose a model for sepal abscission based on these results (Fig. 11). During natural flower senescence, increases in the expression of *DgACS1, DgACS3, DgACO1,* and *DgACO3* are responsible for ethylene production in the receptacle, leading to sepal abscission. However, whether an increase in ethylene production in the gynoecium affects ethylene biosynthesis remains unclear. Furthermore, exposure to ethylene increases the expression of *DgACS2* and *DgACO3* in the receptacle, leading to sepal abscission. Therefore, the receptacle may directly respond to exogenous ethylene. Pollination increases ethylene production in the gynoecium without inducing *ACS* and *ACO* expression. Therefore, increase in ethylene production in the gynoecium may lead to increases the expression of *DgACS2* and *DgACO3* in the receptacle, resulting in sepal abscission.

## Conclusions

Three *ACS* genes (*DgACS1*, *DgACS2*, and *DgACS3*) and three *ACO* genes (*DgACO1*, *DgACO2*, and *DgACO3*) were cloned. The expression of *ACS* and *ACO* in the gynoecium and receptacle increased during senescence, with the exception of *DgACS2* expression in the receptacle. Exposure to ethylene increased ethylene production more markedly in the receptacle than in the gynoecium and induced sepal abscission. Additionally, the expression of *DgACS1* in the gynoecium and that of *DgACS2* and *DgACO3* in the receptacle increased after ethylene exposure. Pollination increased ethylene production in the gynoecium and receptacle and markedly accelerated sepal abscission. However, pollination only slightly affected the expression of *ACS* and *ACO* in the gynoecium, whereas the expression of *DgACO3* in the receptacle was markedly increased. In addition to the fact that the sepals are directly connected to the receptacle, we conclude that the receptacle is the primary organ that regulates ethylene biosynthesis associated with sepal abscission.

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1007/s10725-022-00822-z.

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

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

## Declarations

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

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