

Full Length Research Paper

# Cloning and differential expression of 1-aminocyclopropane-1-carboxylate synthase gene in different floral tissues of *Dendrobium* 'anna' flowers

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Cloning of the *DenACS* from *Dendrobium* hybrid cultivar Anna was performed by RT-PCR and nucleotide sequence analysis revealed that the open reading frame of this gene was 1,308 bp in length, encoded for a protein of 435 amino acid residues. The calculated molecular mass of the deduced polypeptide is 48.5 kDa and the predicted isoelectric point is 5.84. The deduced amino acid sequence of the *DenACS*-encoded protein showed a high degree of identity to those of the ACS from petunia, geranium, carnation and rose. The expression of *DenACS* was examined by RT-PCR and the results revealed that this gene was highly expressed in flower stage 2 (partially opened flower) and stage 3 (full opened flower) during flower development. It was also expressed in all floral tissues and organs including petal, sepal, pedicel, labellum, stigma, young leaves and roots, but the maximum expression was observed in petal, sepal and pedicel. These results indicated that the orchid *DenACS* is involved in the flower opening, flower senescence as well as in vegetative cell growth and development.

**Key words:** Ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid (ACC), ACC synthase (ACS), gene expression, orchid (*Dendrobium* spp.).

## INTRODUCTION

The phytohormone ethylene plays a key role in various aspects of plant growth and development, e.g., seed germination, stem elongation, leaf and flower senescence and fruit ripening and abscission (Abeles et al., 1992). It also acts as an important signaling molecule in plants responses to a range of both biotic and abiotic stimuli such as pathogen attack, mechanical damage (Johnson and Ecker, 1998; Bleecker and Kende, 2000), flooding (Olson et al., 1995) and chilling (Lelievre et al., 1997).

Ethylene is synthesized in plant tissues via the conversion of methionine (Met) to S-adenosylmethionine (AdoMet), catalyzed by the enzyme S-adenosylmethionine synthase. AdoMet serves as an intermediate in a number of biosynthetic pathways, including the production of polyamines. AdoMet is then transformed to 1-aminocyclopropane-1-carboxylate (ACC) by the ACC synthase (ACS, S-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), which is most likely a cytoplasmic enzyme. In the last step, ACC is converted to ethylene, CO<sub>2</sub> and HCN by the ACC oxidase (ACO, EC 1.4.3), formerly known as the ethylene-forming enzyme (EFE) (Kende, 1993). Ethylene biosynthesis is mainly regulated through the expression of ACS genes (Yang and Hoffman, 1984), but in some cases the regulation can also be through the expression of ACO genes, e.g., in melon (Yamamoto et al., 1995; Lasserre et al., 1996), tomato (Barry et al., 1996), marsh dock (Vriezen et al., 1999), peruvian lily (Wagstaff et al., 2005) and plum (Fernandez-Otero et al., 2006).

ACC synthases have been cloned from various plant

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**Abbreviations:** PCR, Polymerase chain reaction; RT-PCR, reverse transcriptase -PCR; ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; Met, methionine; AdoMet, S-adenosylmethionine; EFE, ethylene-forming enzyme; ORF, open reading frame; CDP, cytidine diphosphate; dNTP, deoxyribonucleoside 5'-triphosphate; PLP, pyridoxal 5'-phosphate.

species, including winter squash (Nakajima et al., 1990), tomato (Olson et al., 1991; Rottmann et al., 1991), zucchini (Huang et al., 1991), apple (Dong et al., 1991), *Arabidopsis* (Liang et al., 1992; Van Der Straeten et al., 1992), mung bean (Botella et al., 1992), tobacco (Bailey et al., 1992), carnation (Park et al., 1992), rice (Zarembinski and Theologis, 1993), orchid (Bui and O'Neill, 1998) and rose (Wang et al., 2004; Ma et al., 2006). It has been shown in several cases that ACC synthase genes are encoded by a multigene family and they are differentially expressed in response to various signals such as wounding, fruit ripening, flower senescence and auxin (Nakajima et al., 1990; Van Der Straeten et al., 1990; Dong et al., 1991; Olson et al., 1991; Rottmann et al., 1991; Bui and O'Neill, 1998; Ma et al., 2006). The expression of ACC synthase genes in some systems is also regulated in a tissue-specific manner during plant growth and development, e.g., in carnation, the *CARACC3* mRNA is mostly abundant in the petals while the *CARAS1* mRNA is mostly abundant in the styles (Park et al., 1992; Henskens et al., 1994). In rose, three ACC synthase genes have been isolated and the *Rh-ACS1* is transcribed specifically in response to wounding whereas the *Rh-ACS2* is detectable only in senescent petals. With respect to the *Rh-ACS3*, formerly known as *RKacc7*, it is specifically expressed in petals, ovary and sepals and its expression is increased dramatically as the flower matured to senescence (Wang et al., 2004; Ma et al., 2006). According to the orchid flower, little is known about tissue and cell specificity of expression of ACC synthase gene during flower development.

Previously, we have cloned and analyzed the expression of *ACO* gene, *DenACO*, from *Dendrobium* hybrid cultivar Anna, one of the most important cut flower for ornamental industrial of Thailand and found that *DenACO* is highly expressed in petal, labellum and stigma during flower opening, suggesting that it plays a crucial role in the flower development process (Nagtong et al., 2009). To gain a better understanding of the ethylene response in different stages of flower development as well as in different floral tissues, cloning and expression of ACC synthase gene was investigated in this study.

## MATERIALS AND METHODS

### Plant materials

Orchid flowers (*Dendrobium* hybrid cultivar Anna) were harvested from a local commercial greenhouse (Nakhon Pathom, Thailand). The flowers were immediately put in tap water after harvested and then transported to the laboratory within 2 h. After being cut to 20 cm under water, the flowers were placed in deionized water (DW). Flowers at different stages and floral tissues including sepal, petal, pedicel, labellum and stigma were collected, immediately frozen in liquid nitrogen and stored at -20°C for further processing.

### Total RNA extraction

For cDNA cloning, total RNA was extracted from petals using

RNeasy Plant Mini Kit (QIAGEN, Germany). The extraction of RNA was carried out essentially as recommended by the manufacturer's instruction except that RNase-free DNase I was added to a final concentration of 0.1 mg/ml to remove any contamination genomic DNA.

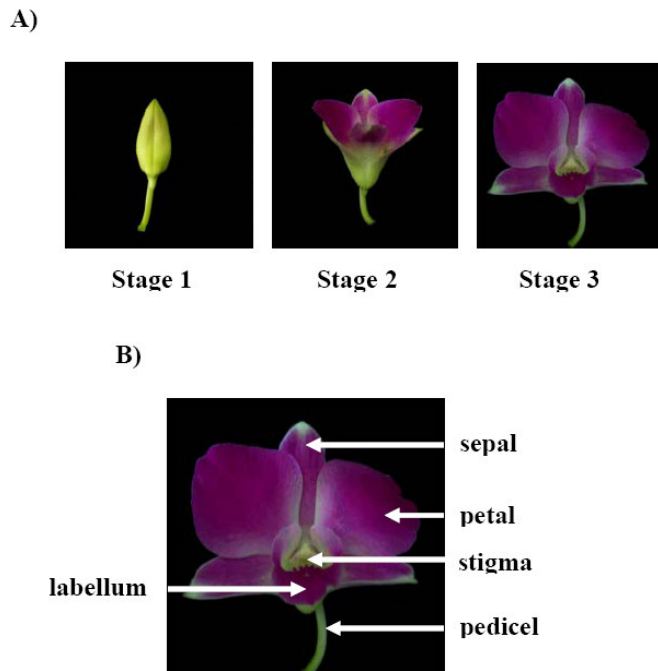
### Gene amplification, cloning and sequencing

Amplification of the *Dendrobium* ACS gene was performed by Reverse Transcription-PCR (RT-PCR) using degenerated oligonucleotide primers designed based on the ACS sequences of the *Dendrobium* hybrid cultivar Pompadour (EF488013), Karen (EF488014), *Dendrobium crumenatum* (U64031) and *Cattleya* spp. (AY504664). An internal fragment of the ACS gene was amplified using the forward ACSFor1 (5'-ATCTTAGCCGATCCGGCGA-3') and reverse ACSRor1 (5'-ACAC(A/G)CAATCCCTGCTTCCT-3'). The RT-PCR reaction was carried out using OneStep RT-PCR Kit (QIAGEN, Germany). The reaction mixture (50 µl) consisted of 10 µl of 5xQIAGEN OneStep RT-PCR buffer, 400 µM dNTP, 0.6 µM of each primer (forward and reverse primers), 2 µl of QIAGEN OneStep RT-PCR enzyme mix and 1 µg of RNA template. The RT reaction was carried out at 45°C for 45 min. Following an initiation denaturation of template cDNA at 95°C for 15 min, 40 cycles of the following temperatures were used: denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. The amplified product was separated on 0.7% agarose gel, then target DNA was excised from the gel and purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Germany). The purified PCR product was cloned into pGEM T-easy vector (Promega, Madison, WI, USA) and was transformed into the competent *E. coli* JM109 using the Rapid DNA Ligation and Transformation Kit (Fermentas, USA). After screening, target DNA was sequenced by the dideoxynucleotide chain termination method using the MegaBACE 1000 automated DNA sequencer (Pharmacia Biotech, Sweden). In order to obtain the sequence of 5'- and 3'-end of the ACS gene, the SMART™ RACE cDNA Amplification Kit (ClonTech, USA) was used. The reaction was carried out essentially as recommended by the manufacturer's instruction. The complete full length open reading frame (ORF) of the ACS gene was confirmed by RT-PCR using the forward ACSFor2 (5'-ATGTCCAAAGAGTTTGGGATGG-3') and reverse ACSRor2 (5'-CTAAATATTCTTCGTCTTCTT-3'). After amplification reaction, the amplified product was separated on the gel, purified, cloned and sequenced as previously described. The sequence of the ACS gene and deduced amino acid sequence was analyzed using GENETYX (Software Development, Tokyo, Japan), while homology searching was performed using FASTA and BLAST program in the GenBank and DDBJ databases.

### Southern hybridization analysis

The ACS gene(s) in the orchid genome was investigated by Southern blotting. Genomic DNA was extracted from petals of orchid flowers using DNeasy Plant Mini Kit (QIAGEN, Germany). The extraction of genomic DNA was carried out essentially as recommended by the manufacturer's instruction. DNA samples (20 µg) were digested with *Bam*HI, *Eco*RI, *Hind*III or *Pst*I, electrophoresed on 0.7% agarose gel and transferred to Hybond N<sup>+</sup> positively charged nylon membrane (Schleicher and Schuell) by overnight capillary transfer. The DNA was cross-linked to the membrane by UV irradiation and baked at 80°C for 2 h. All procedures were performed according to the standard methods as described by Sambrook and Russell (2001).

PCR fragment (approximately 650-bp in length) containing part of the coding region of ACS gene of orchid was used as a DNA probe. Labeling of the DNA probe with alkaline phosphatase for use in conjunction with chemiluminescent detection with CDP-*Star* was



**Figure 1.** Developmental stages of flowering (A) and different floral tissues of orchid flowers (B). Unopened buds (stage 1), partially opened flower (stage 2) and fully opened flower (stage 3).

performed using Gene Images AlkPhos Direct Labeling and Detection System (AlkPhos Direct™, Amersham). All procedures for DNA labeling were carried out as recommended by the manufacturer. The Hybond N<sup>+</sup> positively charged nylon membrane with cross-linked DNA was prehybridized at 55°C for 2h in hybridization oven (Hybrid limited equipment class I) with gently rotation. After incubation, the labeled-DNA probe was added and subsequently incubated at 55°C an overnight. The membrane was washed according to the standard procedure for Southern blot analysis. After washing, the CDP-*Star* detection reagent (AlkPhos Direct™, Amersham) was added onto the membrane and the membrane was left at room temperature for 5 min. The membrane was then exposed to X-ray film (hyper film, Amersham) for 1 h, thereafter the film was developed and fixed with developer and fixer solution (Kodak), respectively.

#### RT-PCR analysis

The expression level of the orchid *ACS* gene was determined by RT-PCR. Total RNA was isolated from petals of orchid flowers at different developmental stages (stages 1 - 3) (Figure 1A) and from different floral tissues including sepal, petal, pedicel, labellum, stigma (Figure 1B), young leaves and roots. RT-PCR was carried out using OneStep RT-PCR Kit (QIAGEN, Germany) with the forward ACSFor2 and reverse ACSRor2 primers synthesized based on the 5'- and 3'-region of the orchid *ACS* gene. The reaction mixture (50 µl) consisting of 10 µl of 5xQIAGEN OneStep RT-PCR buffer, 400 µM dNTP, 0.6 µM of each primer (forward and reverse primers), 2 µl of QIAGEN OneStep RT-PCR enzyme mix and 1 µg of RNA template. As a control, 10 µg samples of total RNA were subjected to agarose gel electrophoresis (0.9% agarose) and stained with ethidium bromide. Actin gene was used as an internal control. A thermocycler was used to perform 1 cycle of 45 min at

50°C for reverse transcription followed by 40 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. The amplified products at the 28<sup>th</sup>, 31<sup>st</sup>, 34<sup>th</sup>, 37<sup>th</sup> and 40<sup>th</sup> cycle were electrophoresed on a 0.9% agarose gel. After staining with ethidium bromide, the relative amounts of the products were compared using the Gel Image Master (Pharmacia Biotech). The experiment was repeated at least twice. Under these conditions, the OneStep RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

## RESULTS AND DISCUSSION

### Cloning and sequencing analysis of the *ACS* gene from *Dendrobium* 'Anna' flower

The *ACS* gene of the *Dendrobium* hybrid cultivar Anna was cloned as described in materials and methods. After sequencing, a complete full length open reading frame encoding the entire amino acid sequence of the *ACS* gene product was obtained. The nucleotide sequence of this open reading frame, designated as *DenACS*, its flanking region and the deduced amino acid residues were shown in Figure 2. The *DenACS* sequence has been deposited in the GenBank nucleotide sequence database under the accession number GU138671. The *DenACS* contained 1,308 bp of the coding sequence, encoded a protein of 435 amino acid residues. The calculated molecular mass of the deduced polypeptide is 48.5 kDa and the predicted isoelectric point is 5.84. The highly conserved regions among all *ACS* sequences

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ATGTCCAAAGAGTTTGGGATGGAAGCTCCATTATCAAAAATTGCAGTCTCCAAAGCTCAT      60
M S K E F G M E A P L S K I A V S K A H
GGAGAGGACTCCCCATACTTTGCTGGCTGGAAAGCTTATGAAGAAAATCCTTATGATGCT      120
G E D S P Y F A G W K A Y E E N P Y D A
GTAGATAATCCTAATGGAGTTATACAGATGGGCTTGGCTGAGAATCAGCTTCTTTTGAT      180
V D N P N G V I Q M G L A E N Q L S F D
CTACTGGAAGAGTACCTGGAGCAGCACCCAGAAGCTTCCAGCTGGGGCCCTGCCATGCT      240
L L E E Y L E Q H P E A S S W G P A M S
ACTTTCAGAGAAAATGCTTTGTTTTCAGGACTATCATGGCCTCCGATCTTTTAGAAAGGCA      300
T F R E N A L F Q D Y H G L R S F R K A
ATGGCCAGCTTTATGGAGAAAATAAGAGGGCGCCGTTCAAATTCGATCCCAACCGGGTC      360
M A S F M E K I R G G R S K F D P N R V
GTCCTCACCGCCGGAGCCACTGCCGCCAACGAGCTCATTACCTTCATCTTAGCCGATCCT      420
V L T A G A T A A N E L I T F I L A D P
GGCGACGCTTGCTTGTCCCGACTCCTTATTACCCAGGGTTCTTGAGAGACCTGCAATGG      480
G D A L L V P T P Y Y P G F L R D L Q W
AAAACGGGAGTTACCATCTTCCCCGTCCTTTCGACAGGTCCAAAGGCTTGCAACTCACA      540
K T G V T I F P V L C D R S K G L Q L T
CTCTCCTCCCTCGGATCAGCCTACGCCGACGCGAAAGCTTCCAACCTTAAATGTAAAGGGT      600
L S S L G S A Y A D A K A S N F N V K G
CTTCTAATAACCAATCCCTGTAATCCTCTCGGCACCGCCGCTCTCTCTCTCTCTCTCCAA      660
L L I T N P C N P L G T A A S L S L L Q
GATATAATTTTCTTCAATTCAGACAAAAACATCCATTTGATCTCCGACGAGATCTACTTT      720
D I I F F I S D K N I H L I S D E I Y F
GGCTGTGTCTTCTTCCACAACTTATTCAGACTTTCAGATCTCATCACCACGCGTC      780
G C V F S S T N L F S I S D L I T N A V
TCGGAACAGATTATATTGTTTATAGCTTATCGAAAGACTTAGGCCTTCTGGTTTCAGA      840
S E Q I H I V Y S L S K D L G L P G F R
GTTGGAGCTCTATATTCTTATAATGACAGAGTTGTTAAACAGCAAGAAGAATGTCAAGT      900
V G A L Y S Y N D R V V K T A R R M S S
TTCAGTCTTGTCTTCTCAGACTCAGAAGTTGCTAGCTTTTATGTTATCAGCTGAGGAA      960
F S L V S S Q T Q K L L A F M L S A E E
TTTACAGTGAAGTATATAAAGAAGAATAGAGAGAGATTGAGAGAGCGATATGAATTAGTT      1020
F T V K Y I K K N R E R L R E R Y E L V
GTCGACGGATTAAAGGAAGCAGGGATTGCGTGTGTTGAAAGGGGAAGCAGGATTGTTCTGC      1080
V D G L K E A G I A C L K G E A G L F C
TGGGTGGATGGAGACGTTGATGGACGAGAAGACGCGGGAAGGAGAGCTGAGGCTTTGG      1140
W V D M E T L M D E K T P E G E L R L W
AAGGTGATAGTTGATGAATTGAAGCTTAATATATCTCCTGGTTCTTCTGTTGCTGTTCT      1200
K V I V D E L K L N I S P G S S C C C S
GAACCAGGTTGGTTTCAGAGTTTGCTTTGCTAATATGAGCAGAGAAACATTGGAGGTGGCG      1260
E P G W F R V C F A N M S R E T L E V A
TTGAAGAGATTGAAGGATTTGCTCagaagaagacgaagaatatttag      1310
L K R L K D F A Q K K T K N I *

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**Figure 2.** Nucleotide and deduced amino acid sequences of the orchid *DenACS*. Nucleotides are numbered from the first nucleotide from 5' end of the sequence. Amino acids are indicated below the nucleotide sequence in single-letter codes. Translation stop codon is indicated by an asterisk. Seven conserved regions observed in ACS are shaded, and the residues in the boxes are the conserved residues in all ACS and amino acid transferases. The active site lysine which binds to pyridoxal 5'-phosphate (PLP) and AdoMet is indicated as a bold letter in the box.

were identified in the *DenACS* (Henskens et al., 1994). One of the conserved regions contained the active site lysine residue (YLSLK**DLGLP**GRV**G**) which binds pyridoxal 5'-phosphate (PLP) and AdoMet (Yip et al., 1990). The amino acids conserved among amino-transferases and ACC synthases (Huang et al., 1991; Rottmann et al., 1991) were also found in the *DenACS*

gene product (Figure 2).

The deduced amino acid sequence of the *Dendrobium* hybrid cultivar Anna, *DenACS*, showed 95, 94, 92, 88 and 85% amino acid identity to the ACS from *Dendrobium* hybrid cultivar Karen (EF488014), Pompadour (EF488013), *Dendrobium crumenatum* (U64031), *Cattleya* spp. (AY 504664) and *Phalaenopsis* spp. (AF004663), respectively

(Figure 3). Although *Dendrobium* hybrid cultivar Anna is belonged to the same genus with *Dendrobium* hybrid cultivar Karen, Pompadour and *D. crumenatum*, 5 - 8% difference in amino acid sequences was detected. This might be due to a difference in the plant genotype, since all these cultivars are hybrid lines. A comparison of the ACS from other plant species in the GenBank database with DenACS showed that DenACS had 55% amino acid sequence homology to ACS from petunia (AF049711), 54% to ACS from geranium (U88971) and carnation (X66605) and 39% to ACS from rose (AY378152) (Figure 4). The amino acid sequences of the highly conserved active site region of ACS from various species were found. This region contained the lysine residue responsible for pyridoxal 5'-phosphate binding which is probably essential for functioning of the ACS protein (Yip et al., 1990). However, four amino acid changes (G/S, L/I, L/M and L/F) were observed between the species, suggesting that the ACS(s) from these species are different (Figure 4) (Kim et al., 1992).

A multigene family for ACS sequences has been reported in zucchini (Huang et al., 1991), tomato (Olson et al., 1991), *Arabidopsis* (Liang et al., 1992), muangbean (Botella et al., 1992), rice (Zarembinski and Theologis, 1993), carnation (Henskens et al., 1994; Ten Have and Woltering, 1997), orchid '*Phalaenopsis*' (Bui and O'Neill, 1998), melon (Ishiki et al., 2000) and rose (Ma et al., 2006). With respect to the ACS gene in *Dendrobium* hybrid cultivar Anna, one to two hybridization bands were observed after Southern hybridization analysis (Figure 5). These results raise the possibility that a multiple gene family may also encode for ACS in *Dendrobium* hybrid cultivar Anna.

### Expression analysis of the *DenACS* gene

It has been reported in several plant species that the expression of ACS gene occurs during different developmental stages of flowers (Henskens et al., 1994; Bui and O'Neill, 1998; Wang et al., 2004; Ma et al., 2006). Therefore, the *DenACS* gene expression in orchid 'Anna' flowers was compared during three developmental stages from unopened bud to fully opened flower (Figure 1). As shown in Figure 6, the accumulation level of the *DenACS* transcripts in the flower stage 2 (partially opened flower) and stage 3 (full opened flower) was approximately 3 - 4 times higher than that observed in the flower stage 1 (unopened bud). In the previous study, we also found that the expression level of ACC oxidase gene, *DenACO*, is dramatically increased in the flower stages 2 and 3 (Nagdong et al., 2009). These findings suggested that *DenACS* and *DenACO* play a crucial role in flower opening and senescence, as previously described by Yamamoto et al. (1994), Shibuya et al. (2000) and Nukui et al. (2004). Our results are consistent with that of Wang et al. (2004) who reported that the accumulation of

the rose ACS gene, *RKacc7*, is increased at the onset of flower senescence. The major accumulation of the ACS transcripts has also been observed at the start of flower senescence in carnation (Park et al., 1992) and *Arabidopsis* (Van Der Straeten et al., 1992).

Various expression levels of the ACS gene in the floral tissues have been reported in several plant species. In carnation, for example, the accumulation of the *CARACC3* transcripts is mostly abundant in the petals whereas that of the *CARAS1* transcripts is mostly abundant in the styles (Park et al., 1992; Henskens et al., 1994). In rose, the *Rh-ACS1* is transcribed specifically in response to wounding whereas the *Rh-ACS2* is detectable only in senescent petals. In addition, the *Rh-ACS3* is specifically expressed in petals, ovary and sepals and its expression is increased dramatically as the flower matured to senescence (Wang et al., 2004; Ma et al., 2006). With respect to the present study, the expression of the *DenACS* was detected in all floral tissues including petal, sepal, pedicel, labellum and stigma, but the maximum expression was illustrated in petal, sepal and pedicel. The accumulation level of the *DenACS* transcripts in these tissues was approximately 3 times higher than those observed in labellum and stigma (Figure 7). The induction of the *DenACS* in these floral tissues may be triggered by the aging and senescence of the orchid flowers, like that observed in carnation (Henskens et al., 1994).

The accumulation of the *DenACS* transcripts was detected in both young leaves and roots of the orchid plants, but their accumulation levels were not significantly different (Figure 8). Our results are similar to that of Rodrigues-Pousada et al. (1993) who reported that the expression of ACS is high in young tissues. Therefore, we speculated from these results that the *DenACS* is involved in cell growth and development process.

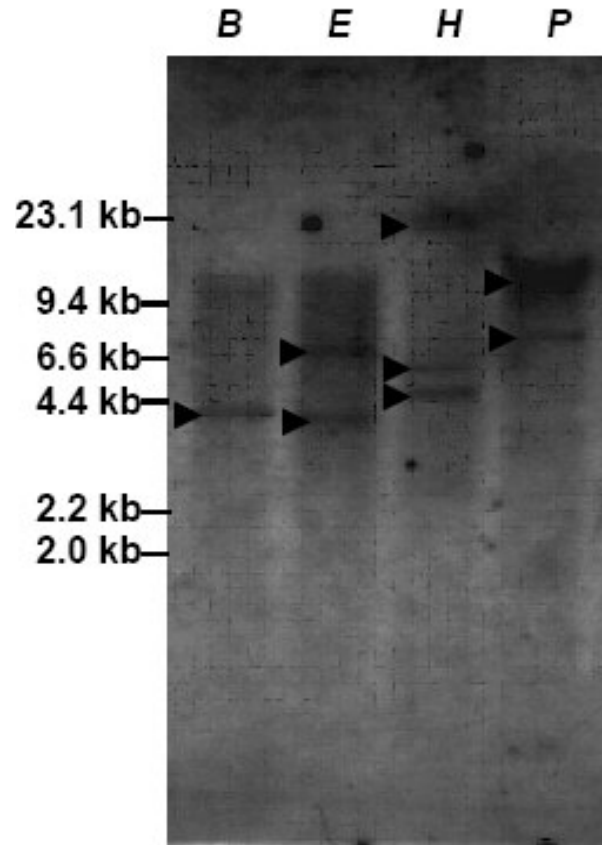
There is a diverse group of factors that have been described in numerous plant species that modulate differential expression of the ACS genes, e.g. flooding, drought, wounding, pathogen attack, flower pollination and senescence and auxin (Nakajima et al., 1990; Van Der Straeten et al., 1990; Dong et al., 1991; Olson et al., 1991; Rottmann et al., 1991; Abeles et al., 1992; Woeste et al., 1999; Bui and O'Neill, 1998; Ma et al., 2006). Whether the *DenACS* expression is regulated by these factors remains to be investigated.

The opening and senescence of the orchid flowers are complex processes, involving several internal developmental factors such as ethylene (Yamamoto et al., 1994; Van Doorn, 2002). Based on data studies carried out in our laboratory, we hypothesize that ethylene (or unknown internal developmental factors) induces the expression of *DenACS*, leading to increased ACC synthase activity. Newly formed ACC is then translocated throughout the floral organs, including petal, sepal, pedicel, labellum and stigma, resulting in the induction of *DenACO* in these organs. As a result, ACC is finally oxidized to ethylene by

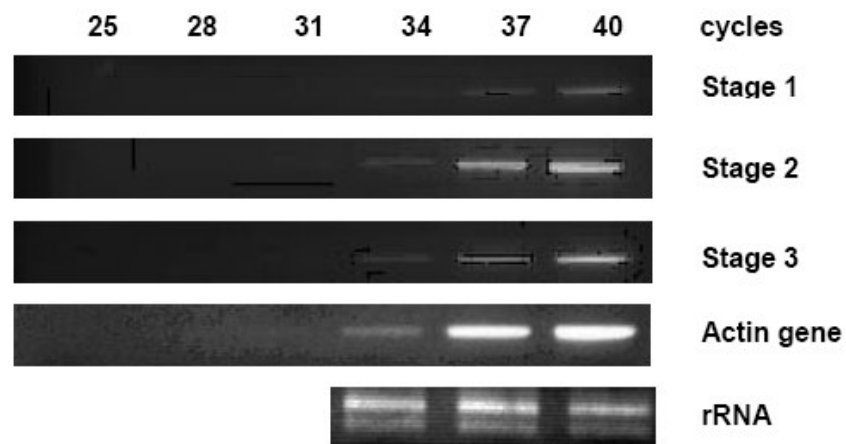
Anna	MSKEFGMEAPLSKIAVSKAHGEDSPYFAGWKAYEENPYDAVDNPNNGVIQMGLAENQLSFD	60
Karen	MSKEFGMEAPLSKIAVSKAHGEDSPYFAGWKAYEENPYDAVDNPNNGAIQMGLAENQLSFD	60
Pompadour	MSKEFGIEAPSSKIAVSKAHGEDSPYFAGWKAYEENPYDAEDNPDGVIQMGLAENQLSFD	60
D.crumenatum	MSKEFGIEAPLSKIAVSKAHGEDSPYFAGWKAYEENPYDAVDNPNNGVIQMGLAENQLSFD	60
Cattleya	MSKVFGIEAPLSKIALSKAHGEDSPYFAGWKAYEENPYDAMNPNNGVIQMGLAENQLSFD	60
Phalaenopsis	MSKMFGEVPLSKMAVSKAHGEGSPYFAGWKAYEENRYDAVGNPDGVIQMGLAENQLSFD	60
	*** ** *.* **:*.*:*****.***** ** *.*.*:*****	
Anna	LLEEYLEQHPEASSWGPAMSTFRENALFQDYHGLRSFRKAMASFMEKIRGGRSKFDPNRV	120
Karen	LLEEYLEQHPEASSWGPAMSTFRENALFQDYHGLRSFRKAMASFMEKIRGGRSKFDPNRI	120
Pompadour	LLEEYLEQHPEASSWGSMSSTFRENALFQDYHGLQTFRKAMASFMEKIRGGRSKFDPNRI	120
D.crumenatum	LLEEYLEQHPEASSWGSMSSTFRENALFQDYHGLQTFRKAMASFMEKIRGGRSKFDPDRI	120
Cattleya	LLEEYLEQHPDAFGWGSDLSGFRENALFQDYHGLQTFRKAMASFMEKIRGGRSKFDPNRI	120
Phalaenopsis	LLEEYLELHPEAFSWADSSSFRENALFQDYHGLQTLRQALASFMEKIRGGRSKFDANRI	120
	***** **:*.*. * *****:.*:*****.*****.***:	
Anna	VLTAGATAANELITFILADPGDALLVPTPYYPGFRLDLQWKTGVTIFPVLCDRSKGLQLT	180
Karen	VLTAGATAANELITFILADPGDALLVPTPYYPGFRLDLQWRTGVRIFPVHCDSSNGFQLT	180
Pompadour	VLTAGATAANELITFILADPGDALLVPTPYYPGFRLDLQWRTGVTIFPVHCDSSNGFQLT	180
D.crumenatum	VLTAGATAANELLTFILADPGDALLVPTPYYPGFRLDLQWRTGVTIFPVHCHSSNGFQLT	180
Cattleya	ILTAGATAANELLTFILANPGDAILVPTPYYPGFRLDLQWRTGVTIFPVHCDSSNGFQLT	180
Phalaenopsis	VLTAGATAANEILTIFILADRGDALLVPTPYYPGFRLDLQWRTGVTIFPVHCYSSNGFQLT	180
	.*:*****:.*:*****.***:*****.*** ** *.*.*:*****	
Anna	LSSLGSAYADAKASNFNVKGLLITNPCNPLGTAASLSLLQDIIFVISDKNIHLISDEIYF	240
Karen	LSSLESAYADAKASNFNVKGLLITNPCNPLGTTASPILLSQDIILFISDKNIHLISDEIYF	240
Pompadour	LSSLESAYADAKASNFNVKGLLITNPCNPLGTAASLSLLQDIIFVISDKNIHLISDEIYF	240
D.crumenatum	LASLESAYADAKASNFNVKGLLITNPCNPLGTVASLSLLQDIILFISDKNIHLISDEIYF	240
Cattleya	LSSLEKAYADAKASNFVKGLLITNPCNPLGTTASFSLLQDIILFISDKNIHLISDEIYS	240
Phalaenopsis	LSSLEKAYAEAKASNFVNRGLLMTNPCNPLGTSASLSLLQDIIFHVISDKNIHLISDEIYS	240
	*.*.*.***:*****:.*:***.***** ** ***** *****	
Anna	GCVFSSTNLFISIDLITNAVSEQIHIVYSLSKDLGLPGFRVGLYSYNDRVVKTARRMSS	300
Karen	GCVFSSTNLFISIDLITNAISDQIHIVYSLSKDLGLPGFRVGLYSYNDRVVKTARRMSS	300
Pompadour	GCVFSSTNLFISIDLITNAVSDQIHIVYSLSKDLGLPGFRVGLYSYNDRVVKTARRMSS	300
D.crumenatum	GCVFSSTNLFISIDLITNAVSEQIHIVYSLSKDLGLPGFRVGLYSYNDRVVKTARRMSS	300
Cattleya	GSVFSSTNLFISIDLITDAVSEQIHIVYSLSKDLGLPGFRVGLYSYNDRVVKTARRMSS	300
Phalaenopsis	GSVFSSTNLFISIDLITDAISEQVHIVYSLSKDLGLPGFRVGLYSYNDRVVKTARRMSS	300
	*.*:*****:.*:***.*****:*****:*****:*****:*****	
Anna	FSLVSSQTQKLLAFMLSDEEFTVKYIEKNRERLRERYELVVDGLKEAGIACLKEAGLFC	360
Karen	FSLVSSQTQKLLAFMLSDEEFTVKYIEKNRERLRERYELVVDGLKEAGIACLKAEAGLFC	360
Pompadour	FSLVSSQTQKLLAFMLSDEEFTVKYIEKNRERLRERYELVVEGLKEAGIECLKEAGLFC	360
D.crumenatum	FSLVSSQTQKLLAFMLSDEEFTVNIYIEKNRERLRERYELVVGGLKEAGIECLKEAGLFC	360
Cattleya	FSLVSSQTQKLLAFMLSDEEFTVRYIEKNRERLTERYELVVEGLKEARIECLKEAGLFC	360
Phalaenopsis	FSLVSSQTQRLLSFMLSDEEFTVRYIEKNRERLRERYELVVGGLKEAGIECLKEAGLFC	360
	*****:.*:*** ** *.*.***:***** ***** ***** * *.*.*****	
Anna	WVDMETLMDEKTPGELRLWKVIVDELKLNISPGSSCCCEPWFVRCFANMSRETLEVA	420
Karen	WVNMTLMDEKTPGELRLWKVIVDELKLNISPGSSCCCEPWFVRCFANMSRETLEVA	420
Pompadour	WVNMTLMDEKTPGELRLWKVIVDELKLNISPGSSCCCEPWFVRCFANMSRETKEVA	420
D.crumenatum	WVNMEELMEDKTEGELRLWKVIVDELKLNISPGSSCCCEPWFVRCFANTSRETLEVA	420
Cattleya	WVNMEELMEEKTMEELKLVKIVEELKLNITPGSSCCCEPWFVRCFANMSRETLEVA	420
Phalaenopsis	WVNMEKLMEEETKEGEAELWKVIDDLKLNISPGSSCCAEPWFRLCFANMSRETLEVA	420
	*.*:.*: **:*.* ** *.*.***:.*:*****:*****:*****:*****:*****	
Anna	LKRLKDFQAQKTKNI----- 435	
Karen	LKRLKDFQAQKTKNI----- 435	
Pompadour	LRRLKDFQAQKTKNI----- 435	
D.crumenatum	LRRLKDFARKTKNI----- 435	
Cattleya	LRRLKDFQAQKDCR----- 434	
Phalaenopsis	LKRLKDFQAQKVAAKKKMNNVYFC 445	
	*.*:*****:***	

**Figure 3.** Comparison of the deduced amino acid sequence of DenACS from *Dendrobium* hybrid cultivar Anna with ACS from *Dendrobium* hybrid cultivar Karen (EF488014), Pompadour (EF488013), *Dendrobium crumenatum* (U64031), *Cattleya* spp. (AY504664) and *Phalaenopsis* spp. (AF004663). Amino acids residues identical and similar to each other are illustrated by asterisks and dots, respectively. Gaps introduced for alignment are indicated by a horizontal dash. The conserved active site regions are underlined and the active site lysine that bind to pyridoxal 5'-phosphate (PLP) and AdoMet is indicated by bold letter.



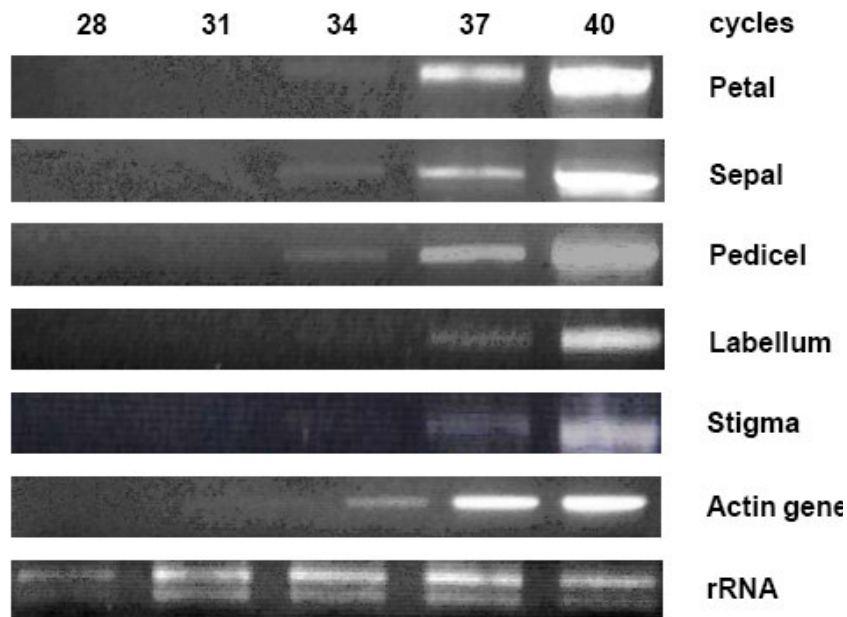


**Figure 5.** Southern hybridization analysis of *DenACS*. Genomic DNA (20  $\mu$ g) isolated from petal of the orchid flowers was digested with *Bam*HI (*B*), *Eco*RI (*E*), *Hind*III (*H*) and *Pst*I (*P*), electrophoresed on 0.7% agarose gel and transferred to nylon membrane. The membrane was hybridized with a 0.65-kb internal fragment of *ACS* gene.

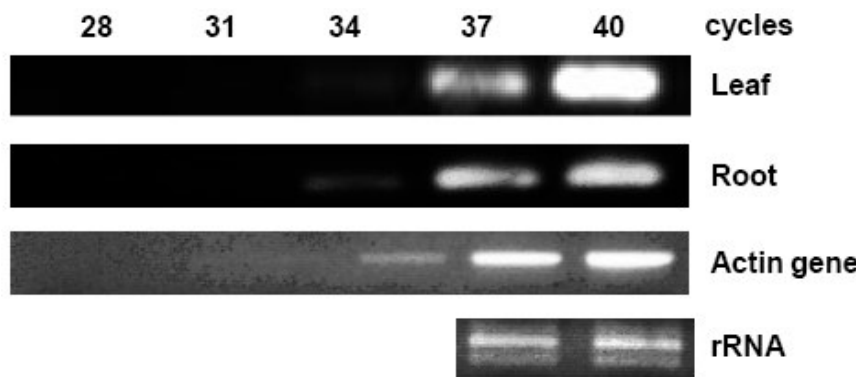


**Figure 6.** RT-PCR analysis of the orchid *DenACS* expression in different stages of flowering. Total RNAs were prepared from orchid flowers at stages 1, 2 and 3 and subjected to RT-PCR analysis with primers specific for the *DenACS* as described in materials and methods. Actin gene was used as an internal control. The numbers above the lanes represent the number of PCR cycles. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNA (10  $\mu$ g).





**Figure 7.** RT-PCR analysis of the orchid *DenACS* expression in different floral tissues. Total RNAs were prepared from petal, sepal, pedicel, labellum and stigma of orchid flowers and subjected to RT-PCR analysis with primers specific for the *DenACS* as described in materials and methods. Actin gene was used as an internal control. The numbers above the lanes represent the number of PCR cycles. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNA (10  $\mu$ g).



**Figure 8.** RT-PCR analysis of the orchid *DenACS* expression in young leaves and roots of the orchid plants. Total RNAs were prepared from young leaves and roots of orchid plants and subjected to RT-PCR analysis with primers specific for the *DenACS* as described in materials and methods. Actin gene was used as an internal control. The numbers above the lanes represent the number of PCR cycles. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNA (10  $\mu$ g).

the ACC oxidase activity. These data would be expected to be beneficial for future work such as the creation of the new cultivars that have strong resistance to senescence caused by ethylene through the modification of the *DenACS* or *DenACO* gene using the modern biotechnological techniques, such as antisense or RNAi technology.

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## REFERENCES

- Abeles FB, Morgan PW, Saltveit ME (1992). Ethylene in plant biology. 2<sup>nd</sup> edn Academic Press, New York.
- Bailey BA, Avni A, Li N, Mattoo AK, Anderson JD (1992). Nucleotide sequence of the *Nicotiana tabacum* cv Xanthi gene encoding 1-aminocyclopropane-1-carboxylate synthase. *Plant Physiol.* 100: 1615-1616.
- Barry CS, Blume B, Bouzayen M, Cooper W, Hamilton AJ, Grierson D (1996). Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. *Plant J.* 9: 525-535.
- Bleecker AB, Kende H (2000). Ethylene: a gaseous signal molecular in plants. *Annu. Rev. Cell Dev. Biol.* 16: 1-18.
- Botella JR, Schiaghnauffer CD, Arteca RN, Phillips AT (1992). Identification and characterization of three putative genes for 1-aminocyclopropane-1-carboxylate synthase from etiolated mung bean hypocotyls segments. *Plant Mol. Biol.* 18: 793-797.
- Bui AQ, O'Neill SD (1998). Three 1-amino-cyclopropane-1-carboxylate synthase gene regulated by primary and secondary pollination signals in orchid flowers. *Plant Physiol.* 16: 419-428.
- Dong JG, Kim WT, Yip WK, Thompson GA, Li L, Bennett AB, Yang SF (1991). Cloning of a cDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression of its mRNA in ripening apple fruit. *Planta.* 185: 38-45.
- Fernandez-Otero C, Matilla AJ, Rasori A, Ramina A, Bonghi C (2006). Regulation of ethylene biosynthesis in reproductive organs of damson plum (*Prunus domestica* L. subsp. *Syriaca*). *Plant Sci.* 171: 74-83.
- Henskens JAM, Rouwendal GJA, Ten Have A, Woltering EJ (1994). Molecular cloning of two different ACC synthase PCR fragments in carnation flowers and organ-specific expression of the corresponding genes. *Plant Mol. Biol.* 26: 453-458.
- Huang PL, Parks JE, Rottmann WH, Theologis A (1991). Two genes encoding 1-aminocyclopropane-1-carboxylate synthase in zucchini (*Cucurbita pepo*) are clustered and similar but differentially regulated. *Proc. Natl. Acad. Sci. USA.* 88: 7021-7025.
- Ishiki Y, Oda A, Yaegashi Y, Orihara Y, Arai T, Hirabashi T, Nakagawa H, Sato T (2000). Cloning of an auxin-responsive 1-aminocyclopropane-1-carboxylate synthase gene from melon and expression of ACS genes in etiolated melon seedlings and melon fruits. *Plant Sci.* 159:173-81.
- Johnson P, Ecker JR (1998). The ethylene gas signaling pathway in plants: a molecular perspective. *Ann. Rev. Genet.* 32: 227-254.
- Kende H (1993). Ethylene biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44: 283-307.
- Kim WT, Silverstone A, Yip WK, Dong JG, Yang SF (1992). Induction of 1-aminocyclopropane-1-carboxylate synthase mRNA by auxin in mung bean hypocotyls and cultured apple shoots. *Plant Physiol.* 98: 465-471.
- Lasserre E, Bouquin T, Hernandez JA, Bull J, Pech JC, Balague C (1996). Structure and expression of three genes encoding ACC oxidase homologs from melon (*Cucumis melo* L.). *Mol. Gen. Genet.* 251: 81-90.
- Lelievre JM, Tichit L, Dao P, Fillion L, Nam YW, Pech JC, Latche A (1997). Effects of chilling on the expression of ethylene biosynthetic genes in passé-crassane pear (*Pyrus communis* L.) fruits. *Plant Mol. Biol.* 33: 847-855.
- Liang XW, Abel S, Keller JA, Shen NF, Theologis A (1992). The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA.* 89: 11046-11050.
- Ma N, Tan H, Liu X, Xue J, Li Y, Gao J (2006). Transcriptional regulation of ethylene receptor and *CTR* genes involved in ethylene-induced flower opening in cut rose (*Rosa hybrida*) cv. *Samantha*. *J. Exp. Bot.* 57: 2763-2773.
- Nagtong T, Thanonkeo S, Klanrit P, Thanonkeo P (2009). Cloning and characterization of 1-aminocyclopropane-1-carboxylate oxidase gene from orchid (*Dendrobium* spp.). *World Appl. Sci. J.* 7: 11-18.
- Nakajima N, Mori H, Yamazaki K, Imaseki H (1990). Molecular cloning and sequence of a complementary DNA encoding 1-aminocyclopropane-1-carboxylate synthase induced by tissue wounding. *Plant Cell Physiol.* 31: 1021-1029.
- Nukui H, Kudo S, Yamashita A, Satoh S (2004). Repressed ethylene production in the gynoecium of long-lasting flowers of the carnation 'White Candle': role of the gynoecium in carnation flower senescence. *J. Exp. Bot.* 55: 641-650.
- Olson DC, Oetiker JH, Yang SF (1995). Analysis of *LE-ACS3*, a 1-aminocyclopropane-1-carboxylic acid synthase gene expressed during flooding in the roots of tomato plants. *J. Biol. Chem.* 270: 14056-14061.
- Olson DC, White JA, Edelman L, Harkins RN, Kende H (1991). Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruits. *Proc. Natl. Acad. Sci. USA.* 88: 5340-5344.
- Park KY, Drory A, Woodson WR (1992). Molecular cloning of an 1-aminocyclopropane-1-carboxylate synthase from senescing carnation flower petals. *Plant Mol. Biol.* 18: 377-386.
- Rodrigues-Pousada RA, De Rycke R, Dedonder A, Van Caeneghem W, Engler G, Van Montagu M, Van Der Straeten D (1993). The *Arabidopsis* 1-aminocyclopropane-1-carboxylate synthase gene 1 is expressed during early development. *Plant Cell.* 5: 897-911.
- Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campbell AD, Theologis A (1991). 1-aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. *J. Mol. Biol.* 222: 937-961.
- Sambrook J, Russell DW (2001). *Molecular Cloning: A laboratory manual*. 3<sup>rd</sup> ed, Cold Spring Harbor Laboratory Press, New York.
- Shibuya K, Yoshioka T, Hashiba T, Satoh S (2000). Role of the gynoecium in natural senescence of carnation (*Dianthus caryophyllus* L.) flowers. *J. Exp. Bot.* 51: 2067-2073.
- Ten Have A, Woltering EJ (1997). Ethylene biosynthetic genes are differentially expressed during carnation (*Dianthus caryophyllus* L.) flower senescence. *Plant Mol. Biol.* 34: 89-97.
- Van Der Straeten D, Wiemeersch LV, Goodman HM, Montagu MV (1990). Cloning and sequence of two different cDNAs encoding 1-aminocyclopropane-1-carboxylate synthase in tomato. *Proc. Natl. Acad. Sci. USA.* 87: 4859-4863.
- Van Der Straeten D, Rodrigues-Pousada RA, Villarroel R, Hanley S, Goodman H, Van Montagu M (1992). Cloning, genetic mapping, and expression analysis of an *Arabidopsis thaliana* gene that encodes 1-aminocyclopropane-1-carboxylate. *Proc. Natl. Acad. Sci. USA.* 89: 9969-9973.
- Van Doorn W (2002). Effect of ethylene on flower abscission: a survey. *Annals Bot.* 89: 689-693.
- Vriezen WH, Hulzink R, Mariani C, Voeselek LACJ (1999). 1-aminocyclopropane-1-carboxylate oxidase activity limits ethylene biosynthesis in *Rumex palustris* during submergence. *Plant Physiol.* 121:189-195.
- Wagstaff C, Chanasut U, Harren FJM, Laarhoven LJ, Thomas B, Rogers HJ, Stead AD (2005). Ethylene and flower longevity in *Alstroemeria*: relationship between sepal senescence, abscission, and ethylene biosynthesis. *J. Exp. Bot.* 56: 1007-1016.
- Wang D, Fan J, Ranu RS (2004). Cloning and expression of 1-aminocyclopropane-1-carboxylate synthase cDNA from rose (*Rosa x hybrida*). *Plant Cell Rep.* 22: 422-429.
- Woeste KE, Vogel JP, Kieber JJ (1999). Factors regulating ethylene biosynthesis in etiolated *Arabidopsis thaliana* seedlings. *Physiol. Plant.* 105: 478-484.
- Yamamoto K, Komatsu Y, Yokoo Y, Furukawa T (1994). Delaying flower opening of cut roses by *cis*-propenylphosphonic acid. *J. Jpn. Soc. Hort. Sci.* 63: 159-166.
- Yamamoto M, Miki T, Ishiki Y, Fujinami K, Yanagisawa Y, Nakagawa H, Ogura N, Hirabayashi T, Sato T (1995). The synthesis of ethylene in melon fruit during the early stage of ripening. *Plant Cell Physiol.* 36: 591-596.
- Yang SF, Hoffman NE (1984). Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev. Plant Physiol.* 35: 155-189.
- Yip WK, Dong JG, Kenny JW, Thompson GA, Yang SF (1990). Characterization and sequencing of the active site of 1-aminocyclo-

propane-1-carboxylate synthase. Proc. Natl. Acad. Sci. USA, 87: 7930-7934.

Zarebinski TI, Theologis A (1993). Anaerobiosis and plant growth hormones induce two genes encoding 1-aminocyclopropane-1-carboxylate synthase in rice (*Oryza sativa* L.). Mol. Biol. Cell. 4: 363-373.