

Cloning and Characterization of 1-Aminocyclopropane-1-carboxylate Oxidase Gene from Orchid (*Dendrobium* Spp.)

¹Titiporn Nagtong, ²Sudarat Thanonkeo, ³Preekamol Klanrit and ³Pornthap Thanonkeo

¹Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand

²Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham 44150, Thailand

³Department of Biotechnology and Fermentation Research Center for Value Added Agricultural Products (FerVAAP), Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand

Abstract: Cloning of the *DenACO* from *Dendrobium* hybrid cultivar Anna was performed by RT-PCR and nucleotide sequence analysis revealed that the open reading frame of this gene is 942 bp long, encoded for a protein of 313 amino acid residues. The calculated molecular mass of the deduced polypeptide is 35.6 kD and the predicted isoelectric point is 4.19. The deduced amino acid sequence of the *DenACO*-encoded protein showed a high degree of identity to those of the ACO from rose, geranium, carnation and tulip. RT-PCR analysis of gene expression revealed that the *DenACO* was expressed during the stage of flower development and in all floral tissues including petal, sepal, pedicel, labellum and stigma. In addition, the higher expression of the *DenACO* was observed in leaf of the orchid plants as compared to the root. These results suggested that the orchid *DenACO* plays a crucial role in the flower development process.

Key words: Ethylene Biosynthesis, 1-aminocyclopropane-1-carboxylic Acid (ACC), ACC oxidase (ACO), Gene expression, Orchid (*Dendrobium* spp.)

INTRODUCTION

Ethylene, one of the most important phytohormones, involves in various aspects of plant growth and development such as seed germination, flower and leaf senescence and abscission and fruit ripening [1]. It also serves as an important signaling molecule in plant responses to a range of both biotic and abiotic stresses such as pathogen attack, mechanical damage [2, 3], flooding [4] and chilling [5].

In higher plants ethylene is formed via a biosynthetic pathway involving the conversion of S-adenosylmethionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC) and of ACC to ethylene. In this pathway, two key enzymes are involved, i.e., ACC synthase (ACS), catalyzes the conversion of AdoMet to ACC and ACC oxidase (ACO), converts ACC to ethylene [6]. Although ACS has been the topic of much recent research, relatively little is known about the role of ACO in regulating ethylene biosynthesis especially in orchid flower. It has been shown in several systems that ACO is

encoded by a small multigene family. For example, five genes are reported in tomato [7] and tulip [8], four genes in petunia [9] and maize [10] and three genes in melon [11].

The ACO gene families have been shown to be differentially regulated in a tissue-specific manner during plant growth and development and its expression is regulated at the transcriptional level. In tulip, *TgACO1*, *TgACO3* and *TgACO5* are expressed in wilting petals, leaves and stems, respectively, whereas *TgACO2* and *TgACO4* are expressed only at basal levels in these tissues [8]. In tomato, the expression of *LeACO1* and *LeACO3* are occurred during the senescence of leaves, fruit and flowers, whereas *LeACO2* and *LeACO4* are mainly expressed in the anther cone and during fruit ripening, respectively [12, 13].

The opening and senescence of many kinds of flowers including carnation, petunia, rose and orchid are correlated tightly to ethylene [14]. Generally, orchids are classified as ethylene-sensitive, however the response to ethylene is dependent on the cultivars [15].

Dendrobium spp., one of the most important for ornamental industrial of Thailand, are member of the largest species of orchids containing about 1,200 species. Among them, *Dendrobium* hybrid cultivar Anna is one of the few cultivars that have been shown to be less sensitive to ethylene. To gain a better understanding of the ethylene response in this cultivar, isolation and expression analysis of the orchid *ACO* gene in different stages of flower development as well as in different floral tissues and plant organs were investigated in the present 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 different floral tissues and plant organs such as sepal, petal, pedicel, labellum, stigma, leaf and root 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 manufacturer's instruction except that RNase-free Dnase I was added to a final concentration of 0.1 mg ml⁻¹ to remove any contamination genomic DNA.

Gene Amplification, Cloning and Sequencing: Amplification of the *Dendrobium ACO* gene was performed by Reverse Transcriptase-PCR (RT-PCR) using degenerated oligonucleotide primers designed based on the *ACO* sequences of the *Dendrobium* hybrid cultivar Pompadour (GenBank accession number: EF487342), Karen (GenBank accession number: EF487343) and Sonia (GenBank accession number: EF061081). The forward ACOFor1 (5'-ATGGAGCTTCT(C/T)(G/C)AGGGTTC-3') and reverse ACORor1 (5'-CTTCTCCTCCGC (C/T) TCT (C/T) TCT-3') were used to amplify the 5' of the *ACO* gene and the forward ACOFor2 (5'-CCGCCGTGTCCGAAGCC(A/G)GA-3') and reverse ACORor2 (5'-TCAAGCAGTAGGAATC(A/G)GCT-3') were used to amplify the 3' of the *ACO* gene. The

RT-PCR reaction was carried out using OneStep RT-PCR Kit (QIAGEN, Germany). 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. 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 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). The complete full length open reading frame (ORF) of the *ACO* gene was RT-PCR amplify using the ACOFor1 and ACORor2 primers. The amplified product was separated on the gel, purified, cloned and sequenced as previously described. The sequence of the *ACO* gene and deduced amino acid sequence were analyzed using GENETYX (Software Development, Tokyo, Japan). Homology searching was performed using FASTA and BLAST program in the GeneBank and DDBJ databases.

RT-PCR Analysis: The expression of the orchid *ACO* gene was detected from the transcript by RT-PCR. Total RNA was isolated from petal and sepal of orchid flowers at different developmental stages (stage 1-3) and from different floral tissues including sepal, petal, pedicel, labellum and stigma (Fig. 1). In order to analyze the expression of the orchid *ACO* gene in the plant organs, total RNA from leaf and root of the plants was extracted. RT-PCR was carried out using OneStep RT-PCR Kit (QIAGEN, Germany) with the forward ACOFor1 and reverse ACORor2 synthesized based on the 5' and 3'-region of the orchid *ACO* 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)

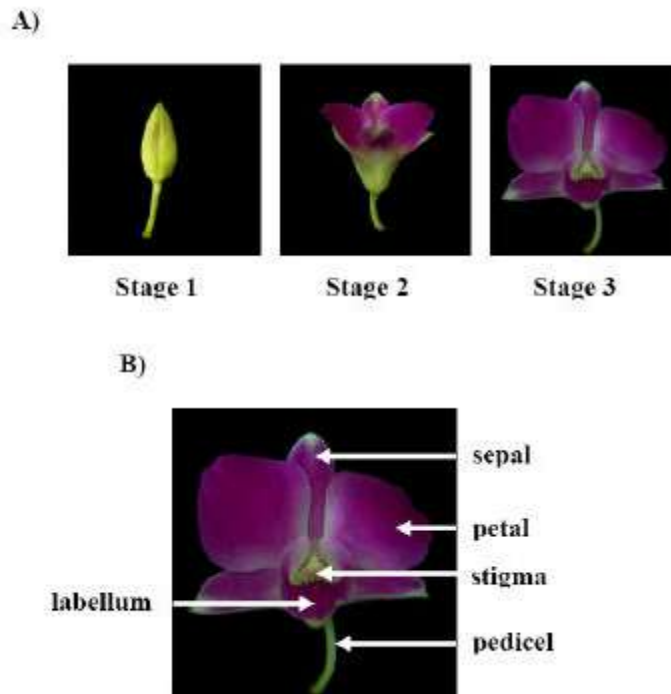


Fig. 1: Development stages of the orchid flowering (A) and different floral tissues of orchid flower (B). Stage 1, unopened bud; 2, partially opened flower and 3, full opened flower

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 30 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C. The amplified product at the 15th, 18th, 21st, 24th, 27th and 30th cycle were electrophoresed on a 0.9% agarose gel and 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 our 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 Sequence Analysis of the ACO gene from Dendrobium Hybrid Cultivar Anna: The ACO gene of *Dendrobium* hybrid cultivar Anna was cloned and sequenced as described in materials and methods. Sequence analysis revealed the presence of an open reading frame encoding the entire amino acid sequence of the ACO gene product. The nucleotide sequence of this open reading frame, designated as *DenACO*, its

flanking region and the deduced amino acid residues are shown in Fig. 2. The *DenACO* contained 942 bp of the coding sequence encoded a protein of 313 amino acid residues. The calculated molecular mass of the deduced polypeptide is 35.6 kD and the predicted isoelectric point is 4.19. The nucleotide sequence of the *DenACO* gene has been deposited in the GenBank nucleotide sequence database under the accession number GQ332400.

The deduced amino acid sequence of the *Dendrobium* hybrid cultivar Anna, *DenACO*, showed 98% identity to the ACO from the *Dendrobium* hybrid cultivar Karen (EF487343), Pompadour (EF487342), Sonia (EF061081) and Missteen (EU151724) (Fig. 3). Although all these cultivars are belonged to the same genus, *Dendrobium* spp., but a little difference in amino acid sequences may be due to a different in plant genotype. A comparison of the ACO from other plant species in the GenBank database with *DenACO* shows that *DenACO* has 71% amino acid sequence identity to the ACO from rose (DQ077712) and geranium (PHU19856), 67% to that from carnation (L35152) and 51% to that from tulip (AB161946) (Fig. 4). ACO requires Fe(II) and ascorbate as cofactors for enzymatic activity [16] and a certain 12 amino acid

ATGGAGCTTCTTGAGGGTTCTCAGCGCTCAGACGCCATGGCTGTTCTTCGAGACGCCCTGT	60
M E L L E G S Q R S D A M A V L R D A C	
GAGAACTGGGGATTCTTCGAGCTACTGAACCACGGAATCTCCACGACCTAATGAACAGA	120
E N W G F F E L L N H G I S H D L M N R	
GTCGAAACTGTCAACAAAGAACATTACCGCCGATTCCGCGAGCAGCGATTCAAAGAATTC	180
V E T V N K E H Y R R F R E Q R F K E F	
GCTGCGAAAACCTTAGATTCCGGCGAAAATGTCGTCGCCGATAATCTCGATTGGGAAAGC	240
A A K T L D S G E N V V A D N L D W E S	
ACCTTCTTCTGCGCCATCTCCCAACCTCCAACATCTCACAAATCCCCGATCTGGACAAG	300
T F F L R H L P T S N I S Q I P D L D K	
GATAGCCCGCAGCAGGATTAAAGAATTTGCTCTAGAGCTCGAGAAATTGGCGGAGAGATTG	360
D S R S T I K E F A L E L E K L A E R L	
CTGGATCTGTTGTGCGAGGATTTGGGGCTTGAGAAGGGGTATTTAAAAAGAGTTTCTGC	420
L D L L C E D L G L E K G Y L K R V F C	
GGCGGATCGGACGGATTGCCGACTTTTGGAAACGAAGTTAGTAATTATCCGCCGTGTCCG	480
G G S D G L P T F G T K V S N Y P P C P	
AAGCCGAGCTGATAAAGGGACTGAGAGCTCATACGGATGCGGGAGGGATTATTCTGCTG	540
K P E L I K G L R A H T D A G G I I L L	
TTTCAGGATGATAAGGTCAAGTGGGCTTCAGTTGCTTAAGGATGGTGAATGGGTTGATGTT	600
F Q D D K V S G L Q L L K D G E W V D V	
CCTCCGATGCGCCATTCCATTGTTGTTAATATCGCGATCAGCTGGAGGTGATAACAAAT	660
P P M R H S I V V N I G D Q L E V I T N	
GGAAGATACAAGAGTGTGATGCACAGGGTGGTGGCGCAAACCAACGCAACCGCATGTG	720
G R Y K S V M H R V V A Q T N G N R M S	
ATTGCATCGTTCTACAACCCCGGCAGCGACGCTGTCATCTTCCCGCGCCCGGAGCTGGTG	780
I A S F Y N P G S D A V I F P A P E L V	
GAGAAAGAAGCGGAGGAGAAGAAGGAAGTTTATCCAAAGTTTGTGTTTGAGGACTACATG	840
E K E A E E K K E V Y P K F V F E D Y M	
AAGCTTTATGTTCCGCAGAAGTTTGAGGCTAAGGAGCCAGGTTTGAGGCTATGAAGACT	900
K L Y V R Q K F E A K E P R F E A M K T	
ATGGATACTGTTATCAGCTCTCAGCCGATCTCTACTGCTTGA	942
M D T V I S S Q P I P T A *	

Fig. 2: Nucleotide and deduced amino acid sequences of the orchid *DenACO*. 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

residues of ACO participate in the interaction with these cofactors [17]. In tulip, twelve conserved amino acid residues are proline-5, alanine-27, glycine-32, histidine-39 and -177, aspartate-179, leucine-195, glutamine-196, glycine-218, histidine-234, arginine-244 and serine-246 [8]. However, no such the twelve conserved amino acid residues were found in the orchid *DenACO*, suggested that the *DenACO* may be subject to fewer structural constraints than most other ACC oxidases [11].

Expression Analysis of the Orchid ACO Gene:

It has been reported in several plant species that the expression of *ACO* gene occurs during different developmental stages of flowers [18, 19]. Therefore, the *ACO* gene expression in orchid

flowers was compared during the three developmental stages from unopened bud to full opened flower (senescence stage) (Fig. 1). As shown in Figure 5, the accumulation level of the *DenACO* transcripts in stage 1 (unopened buds) and stage 3 (full opened flower) of the flower development was approximately 3 times lower than that observed in stage 2 (partially opened flower). These results are consistent with that of Ma *et al.* (2005) who reported that the accumulation of the *Rh-ACO1* mRNA in rose was detected in the partially opened flowers and peaked markedly in the stage of full opened flowers (beginning of flower senescence). The major accumulation of the *ACO* transcripts has also been observed at the start of flower senescence in tulip [8], tomato [12] and petunia [20].

Sonia	MEERSEFFVINMELLEGSQSDAMAVLRDACEWGFELLNHGISHDLAMRVETVMKEHYR	60
Missteven	-----MELLEGSQSDAMAVLRDACEWGFELLNHGISHDLAMRVETVMKEHYR	50
Pompadour	-----MELLEGSQSDAMAVLRDACEWGFELLNHGISHDLAMRVETVMKEHYR	50
Karen	-----MELLEGSQSDAMAVLRDACEWGFELLNHGISHDLAMRVETVMKEHYR	50
Anna	-----MELLEGSQSDAMAVLRDACEWGFELLNHGISHDLAMRVETVMKEHYR	50

Sonia	RFREQRFRFAAKTLDGSENVVADNLDEWSTFFLRHLPTSNISQIFDLEDORSTNKFA	120
Missteven	RFREQRFRFAAKTLDGSENVVADNLDEWSTFFLRHLPTSNISQIFDLEDORSTNKFA	110
Pompadour	RFREQRFRFAAKTLDGSENVVADNLDEWSTFFLRHLPTSNISQIFDLEDORSTNKFA	110
Karen	RFREQRFRFAAKTLDGSENVVADNLDEWSTFFLRHLPTSNISQIFDLEDORSTNKFA	110
Anna	RFREQRFRFAAKTLDGSENVVADNLDEWSTFFLRHLPTSNISQIFDLEDORSTNKFA	110

Sonia	LELEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPELKLGLA	160
Missteven	LELEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPELKLGLA	170
Pompadour	LELEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPELKLGLA	170
Karen	LELEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPELKLGLA	170
Anna	LELEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPELKLGLA	170

Sonia	HTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYKSVHRV	240
Missteven	HTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYKSVHRV	230
Pompadour	HTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYKSVHRV	230
Karen	HTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYKSVHRV	230
Anna	HTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYKSVHRV	230

Sonia	VAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYVRQGF	300
Missteven	VAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYVRQGF	290
Pompadour	VAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYVRQGF	290
Karen	VAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYVRQGF	290
Anna	VAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYVRQGF	280

Sonia	AKEDRFRANKTMDTVISSQDIFTA	314
Missteven	AKEDRFRANKTMDTVISSQDIFTA	314
Pompadour	AKEDRFRANKTMDTVISSQDIFTA	314
Karen	AKEDRFRANKTMDTVISSQDIFTA	314
Anna	AKEDRFRANKTMDTVISSQDIFTA	313

Fig. 3: Comparison of the deduced amino acid sequence of DenACO from *Dendrobium* hybrid cultivar Anna with ACO from *Dendrobium* hybrid cultivar Sonia, Missteven, Pompadour and Karen. Amino acids residues identical and similar between each other are shown by asterisks and dots, respectively. Gaps introduced for alignment are indicated by a horizontal dash

Rose	-----HWGFELVNHGLPIELLOTVERMTK	20
Geranium	---MENVFVIDHEMLN---GEERSAANDMKDACHNNGFFELNHGISHDLAMRVETVMKEHYR	54
Carnation	MANIVNFDIDMEFLNNYNGVRSIVLDQIFDACHNNGFFELVNHGISHDLAMRVETVMKEHYR	60
Orchid	-----MELLE---GSQSDAMAVLRDACEWGFELLNHGISHDLAMRVETVMKEHYR	46
Tulip	---MRFVIDFEMLN---GSRTQTLAGIANGCHENGFFELVNHGIFVELLRVKKVCS	53

Rose	CHYKNCLEGRFELVASKOLEAVNTEVED--MDWESTFHLKHLPTSNISEVTDLEDEYKRV	84
Geranium	EHYKFNDEGRFEMVASKOLEDATQSEIDD--LDWESTFHLKHLPTSNISEVTDLEDEYKRV	113
Carnation	EHYKFNDEGRFEMVASKOLEDATQSEIDD--LDWESTFHLKHLPTSNISEVTDLEDEYKRV	119
Orchid	EHYKFNDEGRFEMVASKOLEDATQSEIDD--LDWESTFHLKHLPTSNISEVTDLEDEYKRV	105
Tulip	ECYKMEREGCFGAASEKLEKHNESKED--VMDWDFLODD-----NEWTONPDEKET	107

Rose	MREFALFLEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPEL	142
Geranium	MREFALFLEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPEL	170
Carnation	MREFALFLEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPEL	176
Orchid	MREFALFLEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPEL	164
Tulip	MREFALFLEKLAERLLDLDLCELDGLEKGYLKRVTFCGSSDGLPTFGTKVSNYPPCPKPEL	167

Rose	IKGLRAHTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYK	202
Geranium	IKGLRAHTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYK	230
Carnation	IKGLRAHTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYK	236
Orchid	IKGLRAHTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYK	234
Tulip	YNGLRHTDAGGIIILLFQDDKVSGLQLLEDGSENVVDFPMRHSIVVNIQGLEVITNGRYK	227

Rose	SVNHRVIAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYV	210
Geranium	SVNHRVIAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYV	208
Carnation	SVNHRVIAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYV	205
Orchid	SVNHRVIAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYV	204
Tulip	SVNHRVIAQTNGNRMSIASFYNGSDAVIFPAPELVEKEAEKKREYVPRFVFDYMKLYV	205

Rose	GLKFOAKERFRANKTMDTVISSQDIFTA	323
Geranium	GLKFOAKERFRANKTMDTVISSQDIFTA	321
Carnation	GLKFOAKERFRANKTMDTVISSQDIFTA	313
Orchid	GLKFOAKERFRANKTMDTVISSQDIFTA	313
Tulip	GLKFOAKERFRANKTMDTVISSQDIFTA	302

Fig. 4: Comparison of the deduced amino acid sequence of the DenACO with ACO from rose (*Rosa roxburghii*), geranium (*Pelargonium hortorum*), carnation (*Dianthus caryophyllus*) and tulip (*Tulipa gesneriana*). Amino acids residues identical and similar between each other are shown by asterisks and dots, respectively. Gaps introduced for alignment are indicated by a horizontal dash

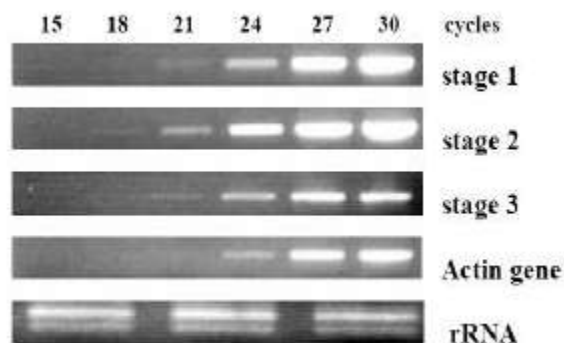


Fig. 5: RT-PCR analysis of the orchid *DenACO* expression in different stages of flowering. Total RNAs were prepared from orchid flowers at stage 1, 2 and 3 and subjected to RT-PCR analysis with primers specific for the *DenACO* as described in Materials and Methods. Actin gene was used as an internal control. The numbers above the lanes represent cycles of PCR. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNA (10 µg).

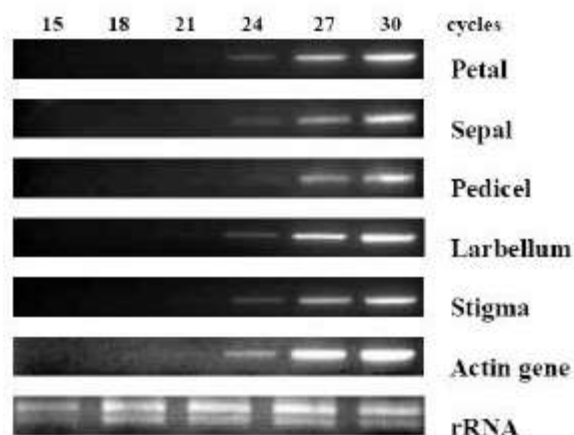


Fig. 6: RT-PCR analysis of the orchid *DenACO* expression in different floral tissues. Total RNAs were prepared from petal, sepal, pedicel, larbellum and stigma of orchid flowers and subjected to RT-PCR analysis with primers specific for the *DenACO* as described in Materials and Methods. Actin gene was used as an internal control. The numbers above the lanes represent cycles of PCR. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNA (10 µg).

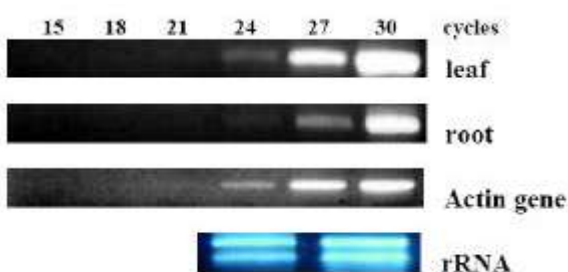


Fig. 7: RT-PCR analysis of the orchid *DenACO* expression in leaf and root tissues. Total RNAs were prepared from leaf and root of orchid plants and subjected to RT-PCR analysis with primers specific for the *DenACO* as described in Materials and Methods. Actin gene was used as an internal control. The numbers above the lanes represent cycles of PCR. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNA (10 µg).

Various expression levels of the *ACO* gene in the floral tissue have been reported in several plant species. In tomato, for example, the *Le-ACO1* is predominantly expressed in the petals, stigma and style, whereas the *Le-ACO3* is accumulated in all of the floral organs [12]. In *Phalaenopsis* orchid flowers, the *ACO* transcripts accumulate dramatically after pollination in the stigma, petal, sepal and larbellum tissues [21]. According to the present study, the expression of the orchid *DenACO* was detected in all floral tissues tested including petal, sepal, pedicel, larbellum and stigma, but the maximum expression was observed in larbellum, stigma and petal. The accumulation level of the *DenACO* transcripts in these floral tissues was approximately 3 times higher than those observed in sepal and pedicel (Fig. 6). The induction of the *DenACO* in all floral tissues may be due to a translocation of ACC throughout the flower and that can trigger the expression of the *DenACO* which is localized in all these floral tissues [22, 23].

Considering the expression of the *DenACO* in leaf and root of the orchid plants, the accumulation level of its transcripts in leaf was approximately 3-4 times higher than that observed in root (Fig. 7). These data together with the expression results of the *DenACO* gene in floral tissues suggested that this *DenACO* plays a crucial role in the flower development.

As demonstrated in this study, the *DenACO* isolated from *Dendrobium* hybrid cultivar Anna is 942 bp long encoded for a polypeptide of 313 amino acid residues with the calculated molecular mass of 35.6 kD. The deduced amino acid sequence of the orchid *DenACO* showed a high degree of identity with other ACOs. RT-PCR analysis suggested that the induction of the *DenACO* is regulated at the transcriptional level and this *DenACO* seem to be involved in flower development and senescence. These data would be expected to be useful for future work such as creation of the new cultivars with a strong resistance to senescence caused by ethylene through the modification of the *ACO* gene using the modern biotechnology techniques such as antisense or RNAi technology.

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