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**Gene Note**

# Cloning and characterization of ACC oxidase genes from tulip

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**Abstract** Five cDNA clones encoding 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO) were isolated from tulip (*Tulipa gesneriana* L.) by differential screening of the petal cDNA library and were designated as *TgACO1*, *TgACO2*, *TgACO3*, *TgACO4* and *TgACO5*. The deduced amino acid sequences exhibited similarity to ACO proteins from other plant species. Among these proteins, TgACO1-4 have high similarity (90–94% identity) each other, whereas TgACO5 showed low similarity compared with the other four proteins (50% identity). Phylogenic analysis indicated that *TgACO1-4* and *TgACO5* are distant. Genomic analysis of *TgACO1-4* showed that they are organized into four exons and three introns, whereas *TgACO5* consists of three exons interrupted by two introns. Real-time RT-PCR analysis of gene expression revealed that *TgACO1*, *TgACO3* and *TgACO5* were expressed in wilting petals, leaves, and stems, respectively, whereas *TgACO2* and *TgACO4* were expressed only at basal levels in these tissues. Therefore, tulip ACO genes seem to be regulated differentially among the vegetative tissues and during flower senescence.

**Key words:** ACC oxidase, differential gene expression, ethylene, flower senescence, tulip.

Ethylene mediates numerous physiological aspects of plant growth and development and also serves as a signalling molecule to induce specific changes in genetic expression at certain stages of a plant's life cycle (Yang and Hoffman 1984; Abeles et al. 1992). The production of ethylene in most plant tissues is normally low; however, it can be induced by a wide range of developmental and environmental cues, including seed germination, fruit ripening, leaf and flower senescence and a multitude of biotic and abiotic stresses (Yang and Hoffman 1984; Abeles et al. 1992).

The ethylene biosynthetic pathway in plants has been well characterised. Ethylene is synthesized through the conversion of S-adenosylmethionine to ACC, which is then oxidized to ethylene (Kende 1993). In this pathway, two key enzymes, ACC synthase (ACS) and ACC oxidase (ACO), are involved. ACO, which catalyses the terminal step in ethylene biosynthesis, known to be encoded by a small multigene family in many species. A maximum of four genes were found in tomato (Nakatsuka et al. 1998), maize (Gallie and Young 2004) and petunia (Tang et al. 1993), and three genes are reported in melon (Lasserre et al. 1996). More recently, the presence of a fifth member of the ACO gene of tomato has been reported by Sell and Hehl (2005).

Furthermore, ACO gene families have been shown to be differentially expressed in various tissues and during the developmental stages. In tomato, *LeACO1* and *LeACO3* transcripts accumulate during the senescence of leaves, fruit, and flowers, whereas *LeACO2* is mainly expressed in the anther cone and *LeACO4* expression occurs during fruit ripening (Barry et al. 1996; Nakatsuka et al. 1998).

In tulips, petal abscission terminates the functional life of the flower; however, ethylene production rates remain low through the flower senescence, and the ethylene synthesis inhibitor failed to inhibit the process. Therefore, it has been concluded that tulip petal senescence may not be primarily regulated by ethylene (Sexton et al. 2000). To clarify the molecular mechanism of ethylene synthesis through the flower senescence of tulips, we tried to isolate the ACO gene; genomic organization and expression analysis of the ACO gene were studied.

Tulips, *Tulipa gesneriana* cv. Murasakizuisho, were grown under greenhouse conditions (20°C/15°C, 12-h cycle). To isolate the ACO gene from tulip, we performed screening of a petal cDNA library comprising $1\times10^6$ pfu using the petunia ACO as a probe. Total RNA (500 $\mu$g) was prepared from 1 g of petals at anthesis by the acid guanidium thiocyanate-phenol-chloroform (AGPC)

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; AGPC, acid guanidium thiocyanate-phenol-chloroform; CTAB, cetyltrimethylammonium bromide; MGB, minor groove binder

The nucleotide sequences reported in this paper have been submitted to DDBJ/GenBank/EMBL nucleotide sequence database under accession numbers AB232765 (*TgACO1*), AB232766 (*TgACO2*), AB232767 (*TgACO3*), AB232768 (*TgACO4*) and AB161946 (*TgACO5*).

This article can be found at http://www.jspcmb.jp/

method (Chomczynski et al. 1987), and 5 μg of poly $(A)^+$ RNA was used for construction of the cDNA library in lambda ZAP (Stratagene). The petunia ACO was generated by RT-PCR, using the primers PETF, 5′-GGAGAACTTCCCAATTATCAGCTTGGACAAAG-3′ and PETR, 5′-GACAGTGGCAATTGGATCCATCTTG-ACATCAG-3′ that were constructed from the sequence data by Wang and Woodson (1992). The labelling and hybridization of probes and the detection of clones were performed using the Gene Image Random-Prime Labelling Module and CDP-Star Detection Module (GE Healthcare Bioscience).

Among the 10 clones obtained, DNA sequencing analyses by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using BigDye-terminator chemistry revealed that they represented 5 distinct ACO genes, *TgACO1-5* (GenBank accession nos. AB232765, AB232766, AB232767, AB232768 and AB161946). *TgACO1-3* contained 951 bp of the coding sequence, with *TgACO4* containing 936 bp and *TgACO5* containing 909 bp. Analysis of the predicted amino acid sequences revealed that *TgACO1-3* encode proteins of 316 amino acid residues, and *TgACO4* and *TgACO5* encode a protein of 311 and 302 amino acid residues, respectively (Figure 1). The amino acid sequence of clones *TgACO1-4* showed approximately 80% similarity with the other plant ACO clones such as petunia, while the amino acid sequence of *TgACO5* showed less than 70% identity. The best score found for TgACO5 is 70% identity with banana MhACO1 (Do et al. 2005). Among the tulip

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TgACO1      MASFPVINLE QLEGGERSTV MEALHDACAN WGFFELLNHG ISHELLDKVE
TgACO2      MASFPVINLE QLEGGERSKV MEVLHDACAN WGFFELLNHG ISNELLDKVE
TgACO3      MASFPVINLE KLEGGERSRV MEVLHDACAN WGFFELLHHG ISHELLDKVE
TgACO4      MASFPVINLE KLEGGERSKV MEVLHDACAN WGFFELLNHG ISHELLDKVE
TgACO5      -MAIPVIDFS MLNGSERTQT LAQIANGCEE WGFFELVNHG IPVELLDRVK
              ::***::.  *:*.**: . :  : :.* : ******::** *. ****:*:

TgACO1      RLTKDHYKKC MEERFREFAS KTLRDGSMVD VDNLDWESTF YLRHLPTSNM
TgACO2      RLTKDHYKKC MEERFREFAS KTLQDGSKVD VDNLDWESTF FLRHLPTSNM
TgACO3      RLTKDHYKKC MEERFREFAS KTLQDGSKVD VDNLDWESTF YLRHLPTSNM
TgACO4      KLTKGHYKKC MEERFREFAS KTLQDVSKVD VDNLDWESTF YLRHIPTSNM
TgACO5      KVCSECYKME REEGFKAASE KLLKKMENSG KEDVDWEDVF LLQ-----DD
            :: .  **    ** *:  :. * *:. .  .  :::***..*  *:     :

TgACO1      SEIPDLSDEY RETMKEFALR LEELAEQLLD LLCENLGLEK GYLKKAFSG-
TgACO2      SEIPDLSDEY RETMKDFVLR LEKLAEQLLD LLCENLGLEK GYLKKAFSG-
TgACO3      SEIPDLSDEY RETMKEFALR LEELAEQLLD LLCENLGLEK GYLKKAFSG-
TgACO4      SEIPDLSDEY RETMKEFVLS LEELAEQLLD LLCENLGLEK GYLKKAFSG-
TgACO5      NEWPSNPRDF KETMKAYRAE IKNLAERVME VMDENLGLDK GYINRAFCGG
            .* *. . :: :**** :    :::***:::: :: *****:* **:::**.*

TgACO1      --SKGPTFGT KVSNYPPCPK PKLIKGLRAH TDAGGLILLF QDDKVSGLQL
TgACO2      --SKGPTFGT KVSNYPPCPK PELIKGLRAH TDAGGLILLF QDDKVSGLQL
TgACO3      --SKGPTFGT KVSNYPPCPK PELIKGLRAH TDAGGLILLF QDDKVSGLQL
TgACO4      --SKGPTFGT KVSNYPPCPK PELIKGLRAH TDAGGLILLF QDDMVSGLQL
TgACO5      DTVQQPFFGT KVSHYPPCPR PDLVNGLRAH TDAGGVILLF QDDEVAGLQI
               : * *** ***:*****: *.*::***** ***** :**** *** *:***:

TgACO1      LKDGEWVDVP PMHHSIVINL GDQIEVITNG KYKSVMHRVL AQPDGTRMSI
TgACO2      LKDGEWVDVP PLHHSIVINL GDQIEVITNG KYKSVMHGVV AQPNGTRMSI
TgACO3      LKDGEWVDVP PMHHSIVINL GDQIEVITNG EYKSVLHRVL AQPDGTRMSI
TgACO4      LKDGEWVDVP PIHHSIVINL GDQIEVITNG KYKSVLHRVV AQPEGTRMSI
TgACO5      LKDGRWIDVQ PLPNSIVINT GDQIEVLSNG QYKSVRHRVL PTPDGNRRSI
            ****.*:**  *: :*****  ******::** :**** * *: . *:*.* **

TgACO1      ASFYNPGSDA VIYPAATLLE EAEKQSEVYP KFVFEDYMKL YAVQKFQAKE
TgACO2      ASFYNPGSDA VIYPAAALLK ETEKQSDMYP KFVFEDYMKL YAVQKFQPKE
TgACO3      ASFYNPGSDA VIYPAAALLE EVENQSEVYP KFMFEDYMKL YAVQKFQAKE
TgACO4      ASFYNPGSDA VIYPATTLLE EAKKKSEVYP KFVFEDYMKL YAIQKFQAKE
TgACO5      ASFYNPAMKA TIGPATKLVA QAAAVA-SYP DFVFGDYMDV YAKQKFLAKE
            ******. .* .* **: *:  :.   :  ** .*:* ***.: ** *** .**

TgACO1      PRFETMKTMK IADAQSIAT (316 a.a.)
TgACO2      PRFETMKTTE TEGVQPIAT (316 a.a.)
TgACO3      PRFETMKAMK IANVQPIAT (316 a.a.)
TgACO4      PRFETMKNME SAVI----- (311 a.a.)
TgACO5      PRFQAVRAM- --------- (302 a.a.)
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Figure 1. Alignment of amino acid sequences deduced from *TgACO* genes. The dashes indicate gaps that were introduced to maximize similarity. Perfectly conserved positions are indicated by asterisks and homologous positions, by dots. Amino acids conserved in all members of the Fe(II) ascorbate family of dioxygenases are boxed.

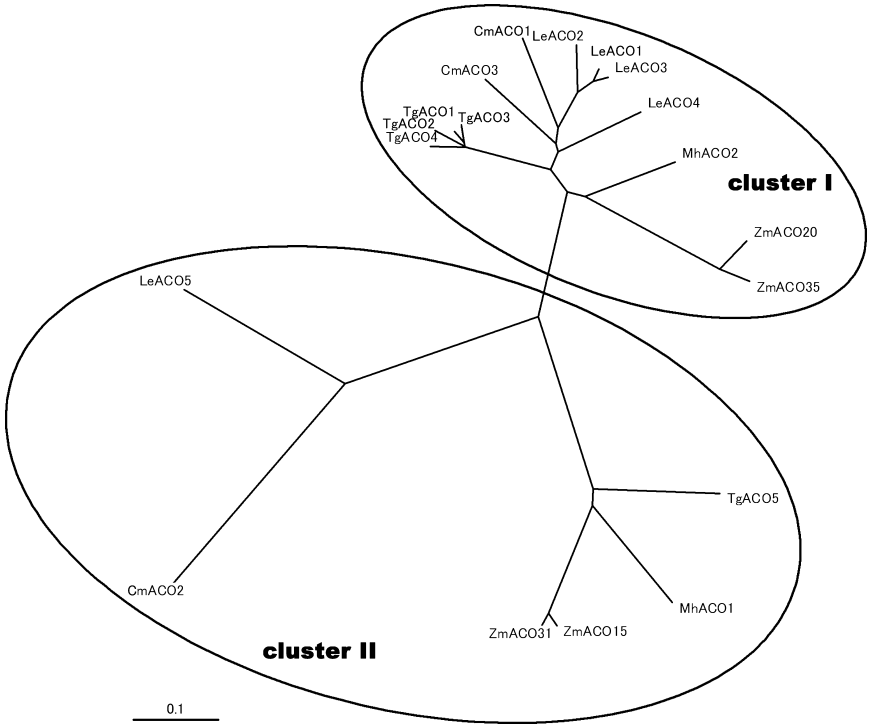


Figure 2. Phylogenetic analysis of ACO deduced amino acid sequences. Phylogenetic tree constructed from the optimal alignment of proteins using the CLUSTALW program of the DNA Data Bank of Japan (DDBJ). The bar indicates the distance corresponding to 10 changes/100 amino acid positions. Accession numbers: melon, *Cucumis melo* (CmACO1, X95551; CmACO2, X95552; CmACO3, X95553); tomato, *Lycopersicon esculentum* (LeACO1, X58273; LeACO2, Y00478; LeACO3, Z54199; LeACO4, AB013101; LeACO5, AJ715790); banana, *Musa acuminata* (MhACO1, AF030411; MhACO2, U86045); tulip, *Tulipa gesneriana* (TgACO1, AB232765; TgACO2, AB232766; TgACO3, AB232767; TgACO4, AB232768; TgACO5, AB161946); maize, *Zea maize* (ZmACO15, AY359572; ZmACO20, AY359575; ZmACO31, AY359573; ZmACO35, AY359576)

ACO proteins, TgACO1-4 share high similarity (90–94% identity); TgACO1 and TgACO3 especially are highly similar (94% amino acid identity). These similarities were also detected within the multigene family of petunia PhACO1, PhACO3 and PhACO4 (Tang et al. 1993), and tomato LeACO1 and LeACO3 (Barry et al. 1996). On the other hand, TgACO5 shares 51% and 50% identity with TgACO1 and TgACO3, respectively. This shows a strong resemblance to the case of melon CmACO2, which is only 43% and 44% identical to CmACO1 and CmACO3 (Lasserre et al. 1996), respectively, and the case of tomato LeACO5, which shares 50% identity with LeACO4 (Sell and Hehl 2005).

To examine the evolutionary relationships between TgACO1-4 and TgACO5, a phylogenetic analysis based on the deduced amino acid sequences within the ACO gene families was characterized (Figure 2). Since TgACO1-4 and TgACO5 showed phylogenetically distance, they divided into two different clusters designated as cluster I (to which TgACO1-4 belong) and cluster II (to which TgACO5 belongs). ACO requires Fe(II) and ascorbate as cofactors for enzymatic activity (McGarvey and Christoffersen 1992), and a certain 12 amino acid residues of ACO participate in the interaction with these cofactors (Zarembinski and Theologis 1994). According to the alignment of the amino acids sequences of TgACO1-5, 12 amino acid residues were well conserved in all members except for the substitution of alanine-27 to glycine in TgACO5 (Figure 1). Recently, Do et al. (2005) have suggested that this alanine is not required for ACO activity. Therefore, each tulip ACO protein might possess the enzyme activities required for ethylene synthesis.

To reveal the genomic organization of the ACO genes, we designed specific primers corresponding to the coding region of the *TgACO* clones and performed genomic PCR. Genomic DNA was extracted from the mature green leaves by the modified cetyltrimethyl-ammonium bromide (CTAB) methods (Murray and Thompson 1980). ACO genomic clones were amplified using the ACO gene specific primer pairs, *TgACO* forward (*TgACO1-4* commonness), 5′-ATGGCATC-TTTTCCGGTGATCAA-3′, and *TgACO1* reverse, 5′-TCAGGTAGCAATCGATTGGGCA-3′; *TgACO2* reverse, 5′-TCAGGTGGCAATCGGCTGAA-3′; *TgACO3* reverse, 5′-TCAGGTAGCAATCGGCTGGACATT-3′; *TgACO4* reverse, 5′-GCAGTCTGCTAGATTACTGCA-3′; *TgACO5* forward, 5′-ATGGCGATTCCTGTCATCG-3′ and *TgACO5* reverse, 5′-CTACATTGCCCTAACTGCC-3′. LA Taq (Takara) was used to amplify the fragments.

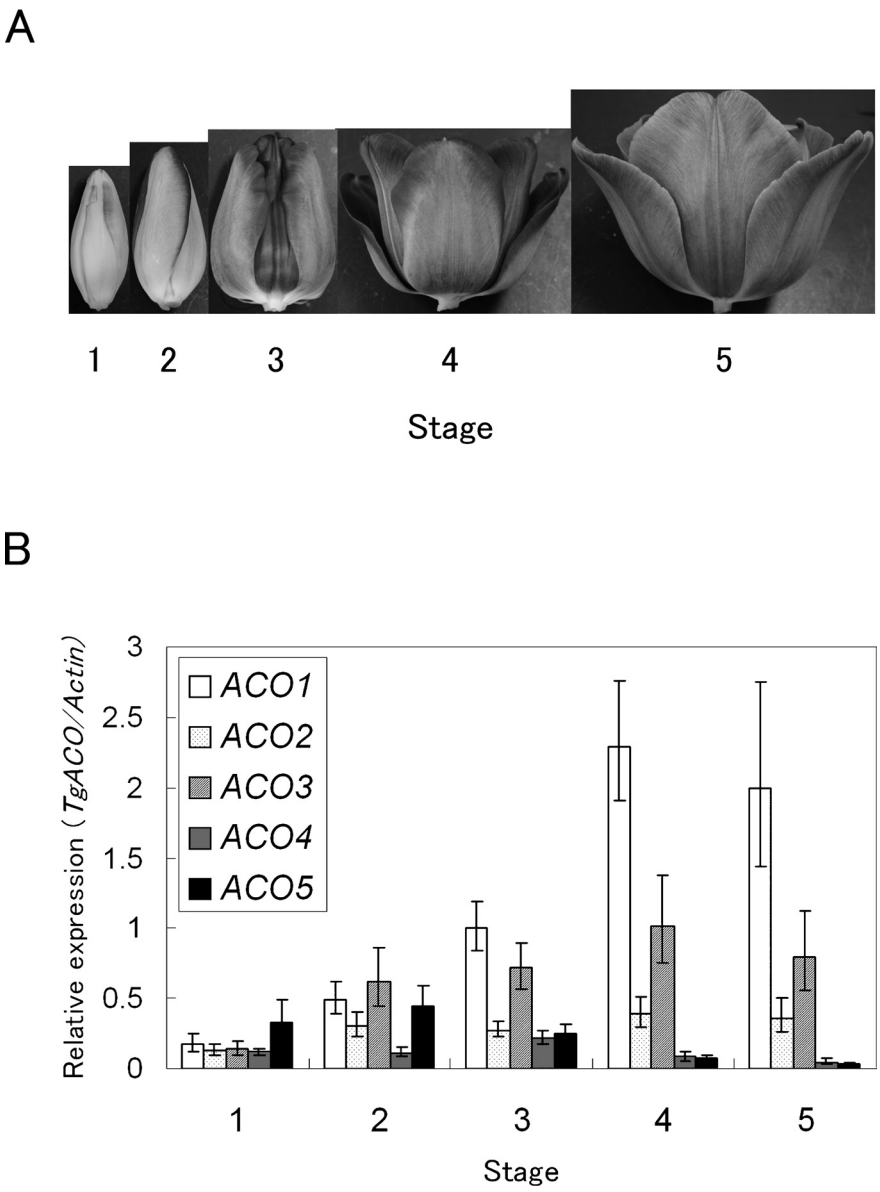


Figure 3. Differential expressions of *TgACO* genes in petals. (A) Flower developmental stages are divided into five stages as follows: Stage 1, small buds with green petals, 2, buds whose petals are beginning to colour, 3, the time of anthesis, 4, the onset of senescence, 5, the late stage of senescence. (B) Real-time RT-PCR analyses of *TgACO* genes in petals. Real-time RT-PCR amplification of actin was used to normalize the expression of the genes under identical conditions. The vertical bars represent SE of the means of three replications.

From genomic analysis, *TgACO1-4* consists of 4 exons interrupted by 3 introns. This organization, including the location of the exon/intron junction, is also identical to the other plants, whereas *TgACO5* consisted of three exons interrupted by two introns (data not shown). The same organization was found in *CmACO2* of melon and *MhACO1* of banana (Lasserre et al. 1996; Do et al. 2005). This result supports the idea that ACO subfamilies belong to two different clusters, and that tulip ACO genes also belong to these clusters.

Ethylene is produced by the flowers of several species during senescence (Woltering et al. 1994). Therefore, ACO gene expression in tulip flowers was compared during the five developmental stages from immaturity to late senescence (Figure 3A). To clarify the *TgACO1-5* expression patterns, we performed quantitative real-time RT-PCR using gene-specific primers. Total RNA (25 $\mu$g) was treated with RNase-free DNase I (Takara) according to the manufacturer's specifications. Total RNA (500 ng) was conducted for cDNA synthesis using the Exscript RT reagent kit (Takara) with random 6mers as the primer. Quantitative real-time RT-PCR was performed using TaqMan minor groove binder (MGB) probes, and FAM dye-labelling (Applied Biosystems) was performed using Premix Ex Taq (Takara). The reactions contained 1×buffer, 2 $\mu$l of the reverse transcription reaction (equivalent to 10 ng of total RNA) and 1×assay mix, containing forward and reverse primers at 0.9 $\mu$M and

Table 1. Oligonucleotide primers and probe used for the amplification of cDNAs by real-time RT-PCR.

| Name | Oligonucleotide | DNA sequence |
|---|---|---|
| *TgACO1* | forward primer | GTTCAAAAGTTCCAGGCAAAGGA |
| | reverse primer | CCAGTCTTTCAGGTAGCAATCGA |
| | probe | TTTGAGACCATGAAAACTAT |
| *TgACO2* | forward primer | CCAGCGGCAGCGTTATTG |
| | reverse primer | ACACAAACTTCGGGTACATGTCA |
| | probe | AAGGAAACAGAGAAACAAAG |
| *TgACO3* | forward primer | CCAGGTTTGAGACCATGAAAGC |
| | reverse primer | CAACAGTCAACGACCAAGTCTTT |
| | probe | AAATGTCCAGCCGATTGC |
| *TgACO4* | forward primer | CACAGCCAGAAGGGACTAGAATG |
| | reverse primer | GCTGGGTAAATGACTGCATCACT |
| | probe | TCCATTGCATCGTTTTACA |
| *TgACO5* | forward primer | GCAATGGCCAGTACAAGAGTGT |
| | reverse primer | GCGATTACCGTCCGGAGTAG |
| | probe | CCGGCACCGCGTGCT |
| *Actin* | forward primer | GTGCCGGCCATGTATGTTG |
| | reverse primer | TGTTCGTCCACTGGCATACAG |
| | probe | CCATTCAGGCTGTTCTC |

probe at 0.25 $\mu$M, 1×ROX reference dye, in a total reaction volume of 20 $\mu$l (Table 1). The reactions were performed using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems) under the following thermal cycling conditions: 95°C for 10 s followed by 95°C for 5 s , 60°C for 31 s for 40 cycles. Relative expression values and corresponding standard deviations for the transcripts were calculated from at least three experimental replicates. The efficiency of the primers was tested in preliminary experiments with dilutions of the input cDNA amounts. The expression of genes was normalized to actin. All samples were measured in triplicate, every run included the actin control for each sample, and the experiments were repeated. The difference between the cycle threshold (Ct) of the target gene and Ct of actin, $\Delta Ct = Ct_{Target} - Ct_{Actin}$, was used to obtain the normalized expression of target genes, which corresponds to $2^{-\Delta Ct}$.

As shown in Figure 3B, there was a major accumulation of the *TgACO1* transcript at the start of flower senescence (Stage 4), and then the abundance of the transcript was slightly reduced at the later stage of flower senescence (Stage 5). Although *TgACO3* transcript is less than that of *TgACO1*, the expression pattern was similar. These major accumulations of ACO transcripts at the start of flower senescence have also been observed in *LeACO3* of tomato (Barry et al. 1996) and petunia (Tang et al. 1994). In previous study, tulip was thought as an ethylene-insensitive species because the flowers produce only a small amount of ethylene during flower senescence and exogenous ethylene have no effect on abscission (Sexton et al. 2000). However, the observation of gene expressions of *TgACO1* and *TgACO3* suggested that the ethylene may participate to the flower development and senescence.

From real-time RT-PCR analyses of expression in stems, leaves and roots, *TgACO3* transcript was found in leaves, and a major accumulation of *TgACO5* was observed in stems as well as in roots, but the expression was lower than that of the other tissues (data not shown). *TgACO3* and *TgACO5* seem to be specifically associated with growth in leaves and stems, respectively. The number of ACO genes in a family and their expression are various in plant species. So, numerous ACO isoforms might be useful in plants to ensure the first response of ethylene production under various conditions.

This is the first report that a multigene family of tulip ACC oxidase consists of five genes, and each of them is differentially regulated during flower development and senescence and in the vegetative tissues.

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