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Full Paper

Cloning and sequence of cDNA encoding 1-aminocyclo-propane-1-carboxylate oxidase in *Vanda* flowers

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Abstract: The 1-aminocyclopropane-1-carboxylate oxidase (ACO) gene in the final step of ethylene biosynthesis was isolated from ethylene-sensitive *Vanda* Miss Joaquim flowers. This consists of 1,242 base pairs (bp) encoding for 326 amino acid residues. To investigate the specific divergence in orchid ACO sequences, the deduced *Vanda* ACO was aligned with five other orchid ACOs. The results reveal that the ACO sequences within *Doritaenopsis*, *Phalaenopsis* and *Vanda* show highly conserved and almost 95% identical homology, while the ACOs isolated from *Cymbidium*, *Dendrobium* and *Cattleya* are 87–88% identical to *Vanda* ACO. In addition, the 2-oxoglutarate-Fe(II)_oxygenase (Oxy) domain of orchid ACOs consists of a higher degree of amino acid conservation than that of the non-haem dioxygenase (DIOX_N) domain. The overall homology regions of *Vanda* ACO are commonly folded into 12 α-helices and 12 β-sheets similar to the three dimensional template-structure of *Petunia* ACO. This *Vanda ACO* cloned gene is highly expressed in flower tissue compared with root and leaf tissues. In particular, there is an abundance of *ACO* transcript accumulation in the column followed by the lip and the perianth of *Vanda* Miss Joaquim flowers at the fully-open stage.

Keywords: 1-aminocyclopropane-1-carboxylate oxidase, Miss Joaquim vanda, vanda orchid, ethylene, flower senescence

INTRODUCTION

The flower of *Vanda* Miss Joaquim (*Papilionanthe* Miss Joaquim, *Papilionanthe hookeriana* × *Papilionanthe teres*), the national flower of Singapore, is particularly striking in shape and style. However, it is difficult to use them as cut flowers because the removal of the pollinia (emasculation) induces severe senescence-related phenomena such as the destruction of

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anthocyanin (colour fading) within 24 hours due to the release of endogenous ethylene. The flower of *Vanda* Miss Joaquim produces ethylene at a high rate and is very sensitive to ethylene [1]. Fading of the petal, also found in *Vanda* Rose Marie, first becomes evident after 8–12 hours in self-pollinated flowers, and after approximately 24–30 hours in emasculated flowers [2, 3]. In contrast, control flowers with no self-pollination or emasculation stay fresh for about 1 week and produce no ethylene. The production of ethylene in *Vanda* Miss Joaquim flower can reach the peak level of 3.442 nL/gf/hr at 32 hours after emasculation [4]. At the same ethylene concentration, *Dendrobium* Pompadour flowers, which are less sensitive to ethylene, can produce ethylene at a peak of 4.5 nL/gf/hr on the 25th day after anthesis [5] and do not display obvious senescent symptoms in the early stage of flower senescence [3, 4]. The endogenous ethylene production in ethylene-sensitive ornamental species such as the *Phalaenopsis* orchid, rose and petunia is often induced by pollination [6], and the treatment of these flowers with exogenous ethylene accelerates flower senescence or programmed cell death [7].

The ethylene biosynthetic pathway has been well documented by Yang and Hoffman [8]. Ethylene is basically synthesised by two major enzymes, namely 1-aminocyclopropane-1-carboxylic acid synthase (ACC synthase, ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO). ACO is an ethylene-forming enzyme that oxidises the intermediate ACC to ethylene in the final step. During fruit ripening or flower senescence and wounding or elicitor treatment, the endogenous ethylene biosynthesis is highly regulated by an increases in ACS and ACO activity [9-11]. Although ethylene biosynthesis is mainly regulated by ACS, in some cases it has been suggested that the ethylene biosynthetic pathway is highly regulated by increased ACO activity during flower senescence [11]. Unlike ACS gene expression patterns, the level of ACO gene expression is suggested in terms of a developmentally regulated and tissue-specific manner [12]. Consequently, ACO has been one of the molecular markers for both ethylene formation and ethylene responsiveness [13].

ACO is classified as a member of the large 2-oxoglutarate (OG) Fe(II)–dependent dioxygenase superfamily which require ferrous ions and ascorbate for enzyme activity [14]. To date, homologous *ACO* genes have been isolated from flowers of a range of orchid species such as the self-pollinated flower of *Phalaenopsis* [15], the senescing perianth of *Doritaenopsis* [16], the necrotic flower of *Cymbidium* [17] and the fully-open flowers of *Dendrobium* [18, 19] and *Cattleya* [20]. The increasing abundance of ACO transcripts in the gynoecium and labellum of *Phalaenopsis* flower is coordinately regulated by emasculation, auxin and ethylene [12]. In addition, the dramatically increased level of ACO transcripts in half-open and fully-open *Dendrobium* flowers suggests that ACO may play a crucial role in flower opening and the senescence process [19, 21].

However, there is no information available on the *ACO* gene from *Vanda* Miss Joaquim, whose flowers are highly sensitive to endogenous ethylene, leading to the fading of flower colour within 24 hours after removal of the pollinia [1]. The *Vanda* flower has been considered to be the highest ethylene-producing tissue [22]. Thus, in the paper the *ACO* gene from *Vanda* Miss Joaquim flowers is cloned and identified as representing the mRNA transcript that is produced in ethylene-sensitive flowers. The differences in sequence homology of the *Vanda* ACO and five orchid ACOs reported in GenBank database are also analysed to determine the relationships among orchid ACOs.

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MATERIALS AND METHODS

Total RNA Extraction

Samples of the flower of *Vanda* Miss Joaquim were purchased from an orchid nursery, Pathum Thani, Thailand. Four hours after removal of pollinia, the fully-open flowers were used for total RNA extraction using lithium chloride precipitation method [23]. A sample of 200 mg of flower tissue was ground to a fine powder in liquid nitrogen and then extracted with 50 µL of an extraction buffer (0.2 M Tris-HCl (pH 7.5), 0.1 M LiCl, 5 mM EDTA and 1% (w/v) sodium dodecyl sulphate). After vortexing for 30 sec., 50 µL of phenol:chloroform:isoamyl (25:24:1) was added and the mixture was incubated at room temperature. Cellular debris and denatured proteins were collected by centrifugation at 3,000×g for 20 min. at 4°C and then the supernatant was adjusted to a final concentration of 3M LiCl by adding an equal volume of 6M LiCl, whereupon the RNA precipitated overnight at 4°C. The RNA was collected by centrifugation as described above. The pellet obtained was suspended in 3M LiCl after it was collected by centrifugation; then, the pellet was resuspended in diethylpyrocarbonate-treated water. The total RNA was precipitated with ethanol and resuspended in a small volume of diethylpyrocarbonate-treated water.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The total RNA (1 µg) was reverse transcribed to cDNA by Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, USA) with two primers, namely RACE-T (5'-GAC TCG AGT CGA CAT CG $(T)_{17}$-3') and 5'ACO-R (5'-AAG GAG CCG AGG TTT GAG GCC-3'). The reaction mixture (25 µL) consisted of 2.5 µL of 10× PCR buffer, 7.5 µM of each primer (forward and reverse primers), 0.5 µL of i-Taq^{TM} DNA polymerase (iNtRON Biotechnology, Korea) and 50 ng of RNA template. By amplifying the 927 bp-5' region and 579 bp-3' region of the *Vanda* ACO gene, two pairs of primers, viz. 5'ACO-F (5'-AGC CAT GGA GAG CGG AAG C-3') and 5'ACO-R; and 3'ACO-F (5'-GGG GAT CAG CTC GAG GTT ATA ACA-3') and RACE-T, were respectively generated using the following conditions: denaturising for 5 min. at 95°C followed by 10 cycles of amplification with 30 sec. of denaturising at 95°C, 1 min. of annealing at 50°C, 2 min. of extension at 72°C followed by 20 cycles of amplification with 30 sec. of denaturising at 95°C, 1 min. of annealing at 55°C, 2 min. of extension at 72°C and a final extra extension step of 10 min. at 72°C for cycle completion. The PCR products were cloned into pGEM®-T Easy (Promega Co., USA) using the manufacturer's instructions and then were transformed into *Escherichia coli* XL1-blue strain and confirmed to be *ACO* by DNA sequencing. The full-length cDNA sequence data for genes in this article have been deposited at GenBank [24]

Primary Structure Analysis

An ACO monomer is known to have activity, although this ACO protein generally functions as a dimer. Hence, six orchid ACO sequences of *Cattleya bicolor* (GenBank ID: AAT02192), *Cymbidium* hybrid cultivar (GenBank ID: BAF36562), *Dendrobium* hybrid cultivar (GenBank ID: ABK32881), *Doritaenopsis* sp. (GenBank ID: AAA21611), *Phalaenopsis* hybrid cultivar (GenBank ID: AAR00506) and *Vanda* Miss Joaquim ACO (GenBank ID: ACS34759), and an out-group *Petunia* ACO (GenBank ID: Q08506) were retrieved from the NCBI protein database [24]. The protein sequences were aligned using Muscle alignment [25], and the phylogenetic tree of plant ACO amino acid sequences was performed with the MEGA package version 5.1 using the neighbour-joining method. The bootstrap consensus tree inferred from 1,000 replicates and

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branches corresponding to partitions was reproduced in less than 50% bootstrap collapsed replicates. The evolutionary distances were computed using the Dayhoff matrix–based method and were in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option) [26].

3D Structure Homology Modelling

To identify the homologous regions of the deduced protein structure, the *Vanda* ACO sequence was transformed into a 3D structure using the SWISS-MODEL repository of homology models of annotated 3D protein structures by employing the most homologous of the amino acid sequences resulting from the former 1W9Y_A sequence as standard model [27]. Figure construction and Deepview/Swiss-PdbViewer 2.SP5 were performed for superimposing [28]. The 3D structures of the *Vanda* and *Petunia* ACOs were constructed by the Accelrys DS Visualiser [29].

Gene Expression Analysis by Qualitative Real-Time PCR (qPCR)

To evaluate the *ACO* transcripts in the various tissues of *Vanda* Miss Joaquim, qPCR was performed and *5.8S rRNA* was used as the reference gene. One microgram of total RNA from each of the root, leaf, fully-open (senescing) flower, lip, perianth and column of *Vanda* Miss Joaquim was reverse transcribed to cDNA with two primers, specifically RT-ACO(R) (5′-ATG GCG GAG GAA GAA GGT GCT-3′) and 5.8S rRNA(R) (5′-GCT TGA AGC CCA GGC AGA CG-3′), using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, USA). Using the SuperScript III One-Step RT-PCR System with a Platinum® *Taq* DNA Polymerase kit (Invitrogen, USA), the 207 bp of *ACO* and 198 bp of *5.8S rRNA* were generated together using two pairs of primers: RT-ACO(F) (5′-GAC GCC TGT GAG AAC TGG GG-3′) and RT-ACO(R) for *ACO*; and 5.8S rRNA(F) (5′-ATG ACT CTC GAC AAT GGA TTT-3′) and 5.8S rRNA(R). Meanwhile, the standard curve of *ACO* gene copy numbers was constructed from six serial dilutions with final concentrations of 6.9×10^4, 4.4×10^4, 1.4×10^4, 9.2×10^3, 4.2×10^3 and 2.2×10^3 copies of the cloned *ACO* gene in pACOJ9 of pGEM-T Easy vector (Promega, USA). Each orchid sample reaction containing 0.6 µL of cDNA template along with 7.5 µM primers in a final reaction volume of 10 µL was set up in triplicate to ensure the reproducibility of the results. The real-time PCRs were generated using the following conditions: denaturising for 5 min. at 94°C followed by 35 cycles of amplification, 20 sec. of denaturising at 95°C, 15 sec. of annealing at 60°C and 30 sec. of extension at 72°C by Eppendorf Mastercycle® ep realplex real-time PCR (Eppendorf, USA). At the end of the PCR run, a melting curve was generated and analysed using the following conditions: denaturising for 15 sec. each at 95°C, 60°C and 95°C. The mean and standard deviation of the copy number of the genes were calculated. Statistical analysis at 95% significance level was performed using one-way analysis of variance, and multiple comparisons were done using Duncan's multiple range test [30].

RESULTS AND DISCUSSION

Vanda ACO cDNA and Its Organisation

cDNAs corresponding to the induced *ACO* gene were isolated from the fully-open flower of *Vanda* Miss Joaquim 4 hr after emasculation, whereupon the flowers developed colour fading within 24 hr (Figure 1). A full-length clone of *Vanda ACO* was constructed from two RT-PCR fragments of the 5′-end and the 3′-end cDNA and further subjected to sequence analysis. The obtained full-length *Vanda ACO* cDNA sequence is composed of 1,242 bp, encoding a polypeptide of 326 amino acids (Figure 2) with a predicted molecular weight of 37.2 kDa and a calculated

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isoelectric point of 5.16. The *Vanda ACO* accession number submitted to the GenBank database is GQ140315 for the ACO nucleotide (*Papilionanthe hookeriana* × *Papilionanthe teres*) and ACS34759 for its deduced amino acids. Apart from the highly conserved sequence among orchid ACOs (88–95% amino acid identity), *Vanda* ACO has sequence similarity to other monocotyledon species according to a Blastp search: it shares 77, 76 and 76% amino acid identity with the ACO of Moso bamboo (*Phyllostachys pubescens*) (GenBank ID: BAB32502), Hardy sugar cane (*Saccharum arundinaceum*) (GenBank ID: ABM74187) and rice (*Oryza sativa*) (GenBank ID: EEC84681) respectively.

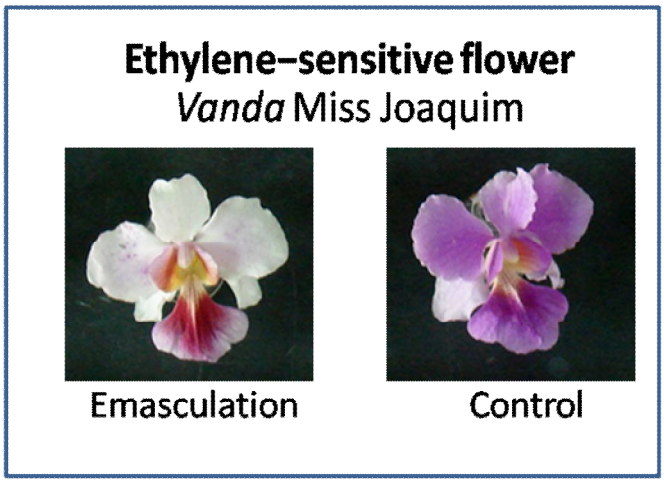

Figure 1. Flower of *Vanda* Miss Joaquim displays colour fading 24 hr after emasculation.

From the descriptions of the multi-domains in the 2OG-Fe(II) oxygenase superfamily, two highly conserved domains are disclosed in *Vanda* ACO by Blastp search (Figure 3). The DIOX_N domain of 2OG corresponding to morphine synthesis in opium poppy [31, 32] is located at the N terminal (residues 2–98). Furthermore, two potential leucine zipper domains for dimerisation of ACO proteins binding to the cell membrane [32] are predicted for leucine zipper I located at amino acid position 28–44 and for leucine zipper II located at residues 103–147 as shown in Figure 2. The second conserved domain, the Fe(II)-dependent oxygenase of the Fe(II) oxygenase superfamily (2OG-FeII_Oxy domain), is located at the residues 157–257, where an active site for a ferrous ion binding site (His181, Asp183 and His238) exists as the first template proposed in isopenicillin N synthase [32] and *Petunia* ACO crystal structure analysis [33].

The optimal orchid ACO sequence alignment shows a well conserved motif of the superfamily of Fe(II) ascorbate enzymes with a consensus His-Thr-Asp-Xaa-His-Arg sequence being the common Fe(II)-binding motif and shares a motif which is the putative co-substrate hydrogen binding site, Arg-Met-Ser, with an identical motif of ACO isolated from petunia [34]. The *Vanda* ACO amino acid sequences also contain 12 conserved residues (Pro7, Ala29, Gly34, His41, His181, Asp183, Leu199, Glu200, Gly222, His238, Arg248 and Ser250) of the ferrous ion and the ascorbate-requiring enzyme member of the Fe(II) oxygenase superfamily (Figures 2-4) corresponding to the ACO1 and ACO2 of papaya [35].

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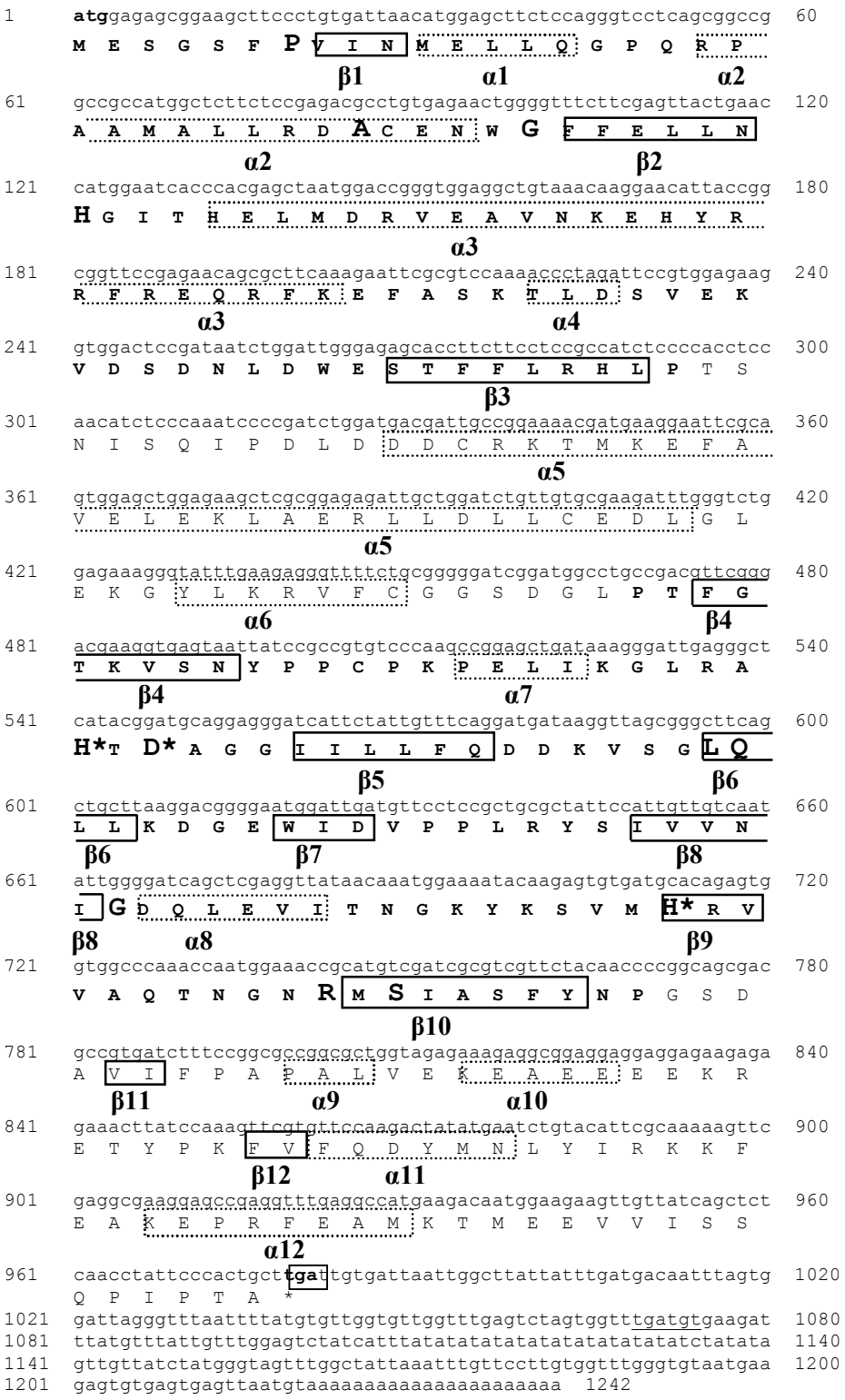

Figure 2. Nucleotide and deduced amino acid sequence of the ACO of *Vanda* Miss Joaquim. Twelve α-helices (solid line boxes) and β-sheets (dashed line boxes) are generated by Accelrys DS Visualize version 2.0.1.7347. The 12 amino acid residues conserved across all members of this enzyme which require ascorbate are indicated by bold letters together with an asterisk marking the Fe^{+2} binding residues.

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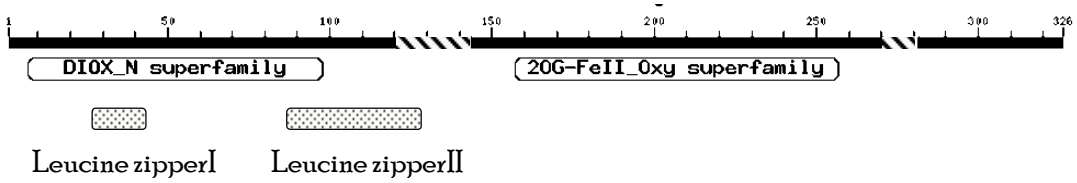

Figure 3. Diagram of three predicted conserved domains. The black bar represents a polypeptide chain with two hatched areas for spacers. The boxes indicate four conserved domains of the non-haem dioxygenase (DIOX_N) superfamily, Fe(II)-dependent oxygenase (2OG-FeII_Oxy) superfamily and two leucine zippers that are placed along the polypeptide.

Conservation of Sequence Similarity of Six Orchid ACOs

The full lengths of six orchid ACOs isolated from *Dendrobium* hybrid cultivar, *Doritaenopsis* sp., *Cattleya bicolor*, *Cymbidium* hybrid cultivar, *Phalaenopsis* hybrid cultivar and *Vanda* Miss Joaquim are 324–326 residues. These are 5–7 residues longer than the *Petunia* ACO (GenBank ID: Q08506; PDB ID: 1W9Y_A), an out-group candidate. Multiple alignments of the *Vanda* ACO-amino acid sequence with six published ACOs reveal highly conserved sequences with 91–95% identity among three orchids (*Doritaenopsis, Phalaenopsis* and *Vanda*) (Figure 4). On the other hand, the *Dendrobium*, *Cattleya* and *Cymbidium* ACOs show only 87–88% identity with the *Vanda* ACO sequence. However, the *Vanda* ACO shares only 69% identity with the *Petunia* ACO. The alignments of these seven ACOs demonstrate that the identities of the amino acid sequences are 94.5, 94 and 96% on average when the whole proteins, DIOX_N domain (113 amino acids) and 2OG-Fe(II)_Oxy domain (101 amino acids) of three orchid ACOs of *Doritaenopsis, Phalaenopsis* and *Vanda*, respectively, are clustered in the same subgroup.

Surprisingly, the flowers of these three orchid species are highly sensitive to endogenous ethylene after the pollinia have been dislodged. The ACOs isolated from less ethylene-sensitive species such as *Dendrobium*, *Cattleya* and *Cymbidium* are appropriately clustered in the second subgroup with less homology and slight divergence at identity levels of 87.33% (whole protein), 85.33% (DIOX_N domain) and 95% (2OG-Fe(II)_Oxy domain) on average. However, the amino acids of *Vanda* ACO share only 68%, 50% and 89% identity with *Petunia* ACO, DIOX_N domain and 2OG-Fe(II)_Oxy domain respectively.

The highly conserved sequences in the 2OG-Fe(II)_Oxy domain consist of the active site for Fe^{2+} binding (His181, Asp183 and His238) and the corresponding site for ascorbate binding (Arg248 and Ser250) as described by Tang and colleagues [34]. These sequences are clearly evident in the six orchid ACOs with the phylogenetic trees generated by either the complete or partial (domain) protein sequences displaying the six distinctive orchid ACOs in a similar topology to the two major subgroups of the ACOs isolated from ethylene-sensitive species (*Vanda, Phalaenopsis*, and *Doritaenopsis*) and less ethylene-sensitive species (*Cymbidium*, *Dendrobium* and *Cattleya*) (Figure 5). Interestingly, in this study the clustering groups of orchid ACOs classified by amino acid similarity correlate well with the characteristics of orchid flowers in terms of ethylene sensitivity, senescence phenomenon and ethylene production.

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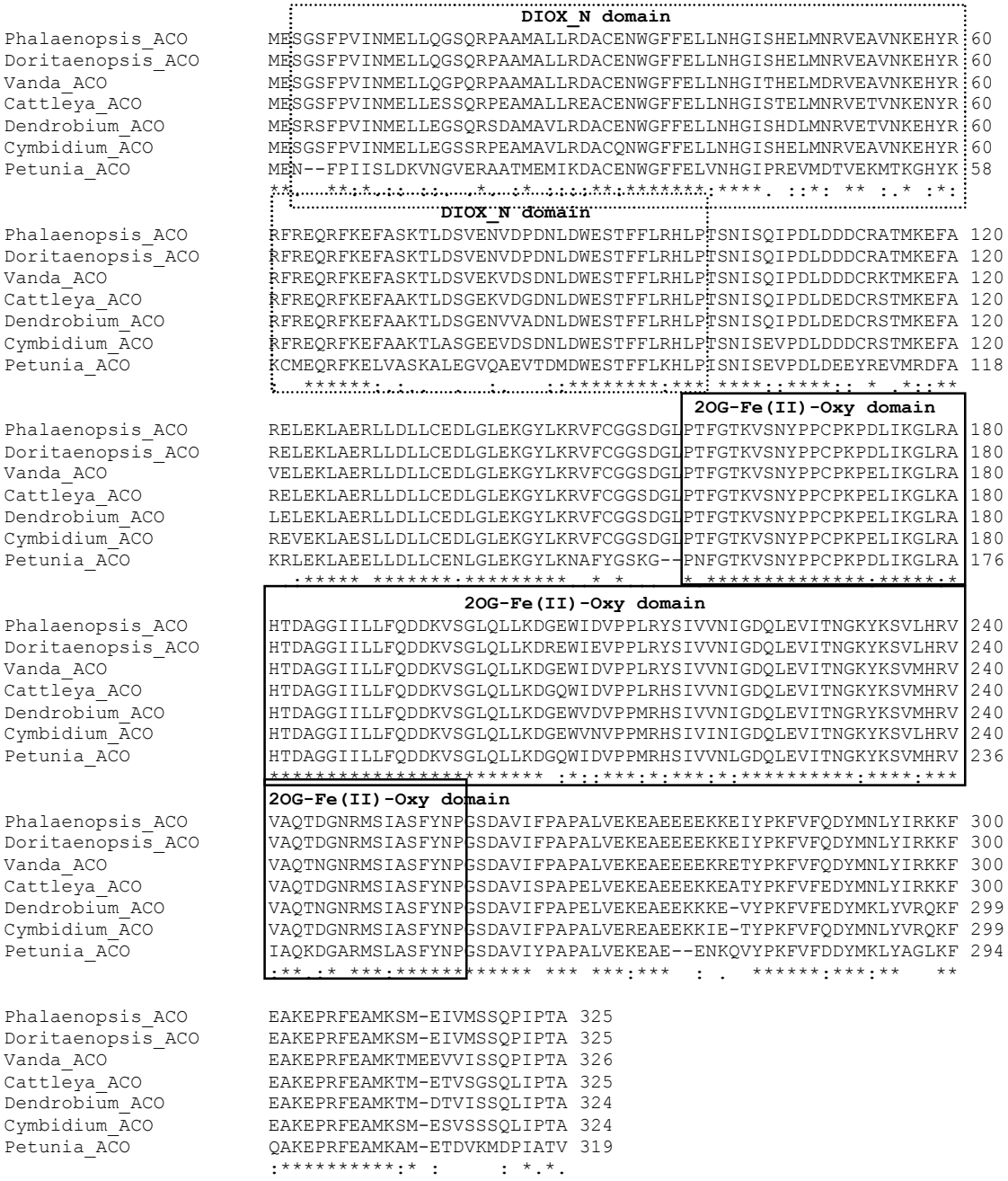

Figure 4. Amino acid alignment of six orchid ACO sequences assessed from the GenBank ID: ABK32881 (*Dendrobium*), AAA21611 (*Doritaenopsis*), AAT02192 (*Cattleya*), BAF36562 (*Cymbidium*), AAR00506 (*Phalaenopsis*) and ACS34759 (*Vanda*). Two conserved domains of DIOX_N and three conserved domains of 2OG- Fe(II)_Oxy are boxed by dotted lines and solid lines respectively.

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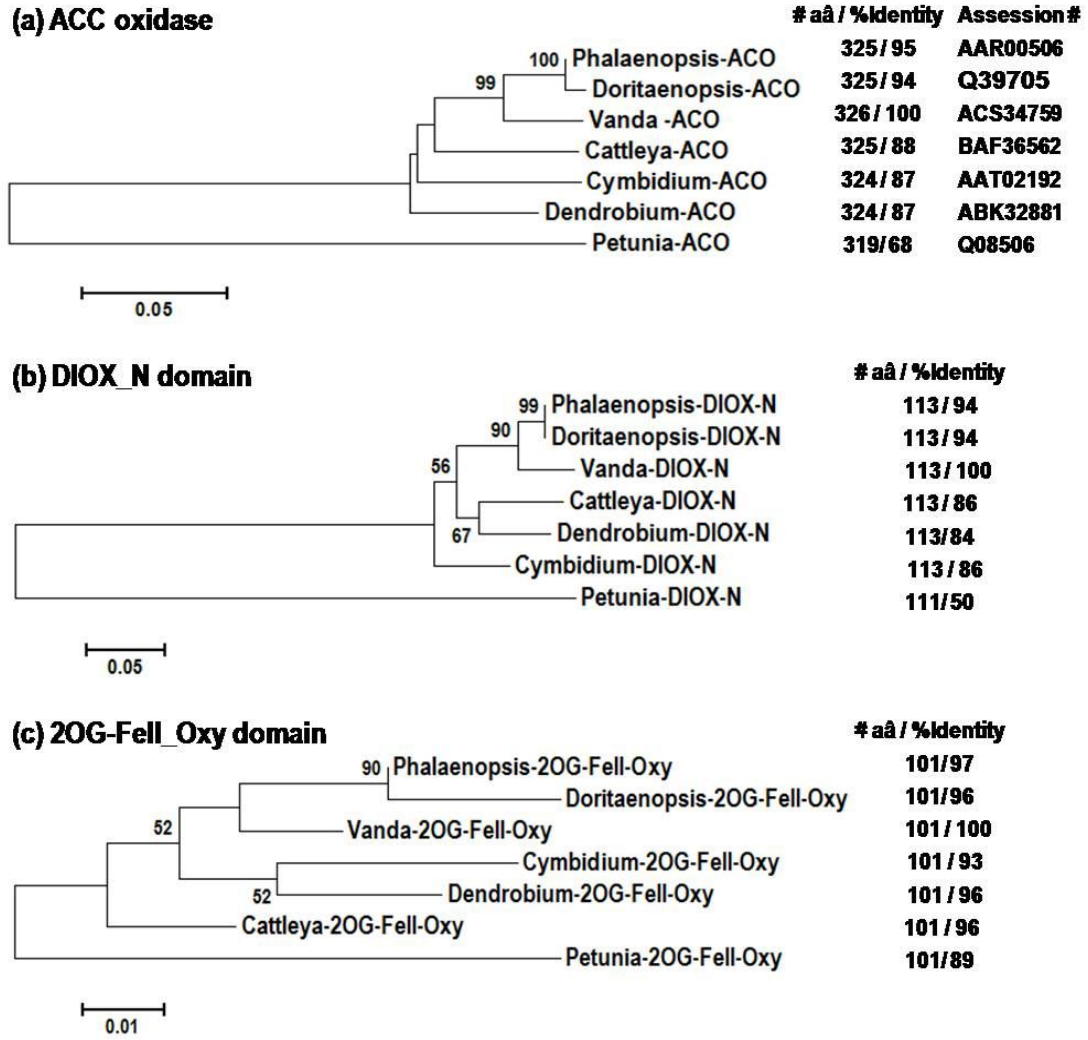

Figure 5. Neighbour-joining phylogenetic relationship of *Vanda* ACO to other orchid ACOs. The ACO proteins used in the construction are retrieved from the database as complete proteins (a), DIOX_N domain (b), and 2OG-Fe(II)_Oxy domain (c). The clustering is performed with 1,000 replicates for bootstrapping analysis using MEGA5.1 computer software, and only bootstrap values higher than 50% are indicated. The term # aa is defined as the number of amino acids.

Homologous Regions of *Vanda* and *Petunia* ACO 3D Structures

To identify the homologous regions of *Vanda* ACO structure in 3D-structure template, the initial 3D structure of *Vanda* ACO was analysed by SWISS-MODEL using *Petunia* ACO structure (PDB ID: 1W9Y_A) [33] as the template model. Both *Vanda* and *Petunia* ACO 3D structures constructed by an Accelrys DS Visualiser are comparable in folding structures with 12 α-helices complexes with 12 β-sheets, where 2 α-helices (α7 and α8) and 6 β-sheets (β4–β10) contribute to most of the 2OG-FeII_Oxy domain. By using a pairwise amino acid sequence comparison of these two proteins, highly conserved sequences are found in all α-helix and β-sheet regions. In particular, the most conserved sequence regions are located in the 2OG-Fe(II)_Oxy domain between 157–257 residues (according to *Vanda* sequences), where the active site of the Fe^{2+} binding site (His181, Asp183 and His238) is located. However, the regions in some linkage sequences between α3–α4, α4–β3, α6–β4 and α10–β12 all contain gaps which correspond to the different conformations of the 3D structures of these sequence-variable regions (Figures 6a-6b).

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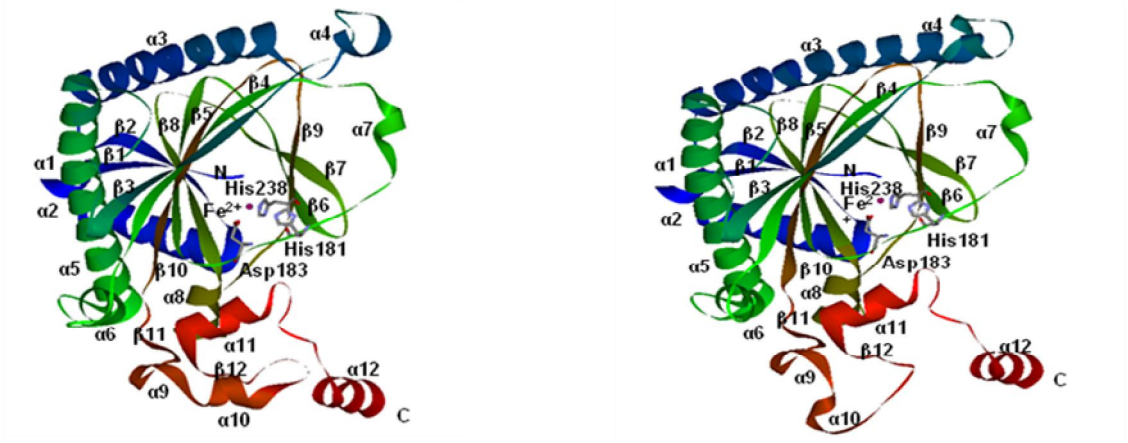
(a) ***Vanda*** ***Petunia***



(b)

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                 β1  α1          α2              β2              α3
Vanda-ACO        MESGSFPVINMELLQGPQRPAAMALLRDACENWGFFELLNHGITHELMDRVEAVNKEHYR 60
Petunia-ACO      MEN--FPIISLDKVNGVERAATMEMIKDACENWGFFELVNHGIPREVMDTVEKMTKGHYK 58
                 **.  **:*.:: ::* :*.*:* :::************:****.:*:** ** :.* **:

                 α3          α4                  β3                      α5
Vanda-ACO        RFREQRFKEFAS-KTLDSVEKVDSDNLDWESTFFLRHLPTSNISQIPDLDDDCRKTMKEF 119
Petunia-ACO      KCMEQRFKELVASKALEGVQAEVTD-MDWESTFFLKHLPISNISEVPDLDEEYREVMRDF 117
                 :  ******:.: *:*:.*:   :* :*********:*** ****::****:: *:.*::*

                       α5              α6              β4          α7
Vanda-ACO        AVELEKLAERLLDLLCEDLGLEKGYLKRVFGGGSDGLPTFGTKVSNYPPCPKPELIKGLR 179
Petunia-ACO      AKRLEKLAEELLDLLCENLGLEKGYLKNAFYGSKG--PNFGTKVSNYPPCPKPDLIKGLR 175
                 * .*******.********:*********..* *...  *.***************:*******

                       β5          β6      β7          β8      α8          β9
Vanda-ACO        AHTDAGGIILLFQDDKVSGLQLLKDGEWIDVPPLRYSIVVNIGDQLEVITNGKYKSVMHR 239
Petunia-ACO      AHTDAGGIILLFQDDKVSGLQLLKDGQWIDVPPMRHSIVVNIGDQLEVITNGKYKSVMHR 235
                 ************************:*******:*:*****:******************

                 β9          β10         β11     α9      α10         β12 α11
Vanda-ACO        VVAQTNGNRMSIASFYNPGSDAVIFPAPAIVEKEAEEEEKRETYPKFVFQDYMNLYIRKK 299
Petunia-ACO      VIAQKDGARMSLASFYNPGSDAVIYPAPAIVEKEAEE--NKQVYPKFVFDDYMKLYAGLK 293
                 *:**.:* ***:************:***********  :::.******:***:**   *

                    α12
Vanda-ACO        FEAKEPRFEAMKTMEEVVISSQPIPTA 326
Petunia-ACO      FQAKEPRFEAMKAMETDVK-MDPIATV 319
                 *:**********:**  *   :**.*.|
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Figure 6. (a) Comparison of 3D structures of *Vanda* ACO and *Petunia* ACO (1W9Y_A) as constructed by the Accelrys DS Visualiser programme. Both the β-sheets and α-helices are numbered. The iron binding site of the 2OG-Fe(II) oxygenase superfamily consisting of three amino acid residues (His181, Asp183 and His238) together with the Fe^{2+} ion is shown. (b) Twelve α-helices (solid boxes) and β-sheets (dotted boxes) are marked and three Fe^{+2} binding residues, His181 (H), Asp183 (D) and His238 (H), are indicated by bold letters.

Vanda ACO has a 3D structural character similar to the *Petunia* template reported by Zhang and colleagues [33], regardless of the 68% sequence identity over 326 residues. The predicted folding in the core conformation region of the iron binding site is conserved and protected by two enclosing chains of α-helices, which serve as the backbone (with the N-terminal region consisting of α-1 to α-5) and the lid (with the C-terminal region consisting of α-7 to α-11). However, the variable regions between the α3 and α4 helices only create longer extended α3 helices of the *Petunia* backbone. In addition, *Petunia* flowers are more likely to be classified as less sensitive to ethylene, which is contrary to the flowers of *Vanda* Miss Joaquim [4, 36].

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Expression Pattern of ACO Gene in *Vanda* Miss Joaquim

In evaluating the *ACO* mRNA transcript levels in *Vanda* orchid tissues by qPCR, the *ACO* gene (clone pACOJ9) isolated from emasculated fully-open flowers was determined to be more highly expressed in the reproductive tissues (perianth, lip and column) than in the vegetative tissues (root and leaf). In particular, the abundance of *ACO* transcripts found in the column tissue of the fully-open flower (67.7 $\times 10^3$ copies/µg total RNA) was significantly greater (at 7-, 4-, 3.5- and 2.5-fold) compared to that in the root (9.8 $\times 10^3$ copies/µg total RNA), perianth (16.4$\times 10^3$ copies/µg total RNA), leaf (17.6 $\times 10^3$ copies/µg total RNA) and lip tissues (26.3$\times 10^3$ copies/µg total RNA) (Figure 7). However, comparable expression levels of the *ACO* transcripts were found in both leaf and perianth samples. Similar results were obtained from triplicate measurements. The mean and standard deviation of the copy number of genes were calculated throughout and the differences were considered significant at the P <0.05 level. The results suggest that the *Vanda* ACO clone gene obtained in this study corresponds to the flower emasculation of senescing flowers.

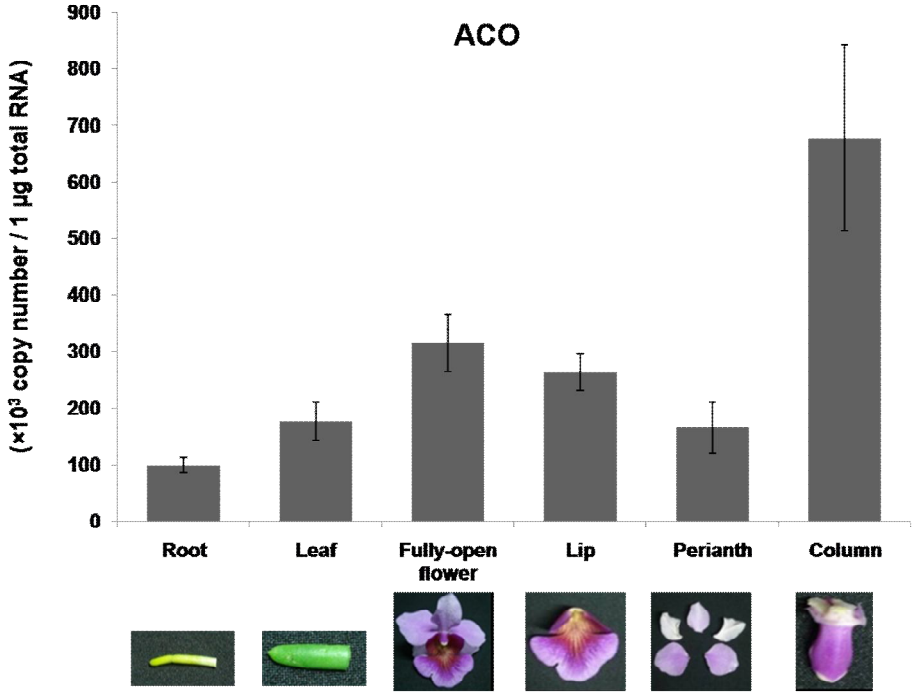

Figure 7. Expression analysis of *ACO* transcripts in various tissues of *Vanda* Miss Joaquim evaluated by quantitative real-time PCR with 5.8 *rRNA* used as internal control

Although found in most tissues of *Vanda* Miss Joaquim, the most abundant *ACO* transcript was detected early in the column tissue of the fully-open flower, followed by the lip and then much less in the perianth, leaf and root. This ACO gene expression pattern is commonly found in *Phalaenopsis* cv SM9180 flowers, which are ethylene sensitive [12]. After pollination, the stigma of the orchid column complex tissue is an initial source of ethylene production in flowers, after which it becomes a senescing organ and plays a significant role in the induction of the senescence programme and ethylene production in these organs [12, 15]. The *ACO* transcripts and activity in the column of *Phalaenopsis* cv SM9180 rapidly increase to very high levels 6 hr after pollination but to a slower extent in the perianth, where their presence is first observed at 12 hr [15]. Ethylene

is mainly produced in the orchid column, which acts as the central organ and then translocates the signal, either ethylene or ACC, to other parts of the floral organs to induce ethylene biosynthesis, in particular the sepal and petal where ACC is oxidised to ethylene [15, 37].

Meanwhile, after removal of the pollinia from the column without pollination, the endogenous ethylene biosynthesis is rapidly produced, resulting in colour fading of *Vanda* Miss Joaquim (Figure 1). It can be suggested that the *Vanda* ACO encoding gene in ethylene biosynthesis is regulated by pollinia in *Vanda* Miss Joaquim. In addition, the high level of *ACO* in the column of senescing *Vanda* Miss Joaquim is likely to be ready to convert ACC to ethylene after emasculation. The *Vanda ACO* gene therefore has a potential to be used as a molecular key for ethylene responsiveness by emasculation induced in the ethylene-sensitive flowers, which is consistent with *Phalaenopsis ACO* but contrary to the constitutive *ACO* expression in carnation and petunia styles as well as *Dendrobium* column tips [38, 39]. The dramatically increasing abundance of *ACO* transcripts in the gynoecium and labellum of *Phalaenopsis* flower is coordinately regulated by emasculation, auxin and ethylene after self-pollination [15, 37].

CONCLUSIONS

The cDNA *ACO* sequence isolated from highly ethylene-sensitive flowers of *Vanda* Miss Joaquim shares 94.5% on average of the sequences of *Phalaenopsis* and *Doritaenopsis* ACOs, whose flowers are sensitive to the induction of endogenous ethylene through the removal of pollinia from the stigma of column tissue, where most of the *ACO* transcript accumulates. The most variable regions (85.33% on average) are located in the DIOX_N domain while most sequences in the 2OG-Fe(II)_Oxy domain are conserved (95.14% on average). The variable regions verified by the 3D-structure comparison between the structures of *Vanda* ACO and *Petunia* ACO (1W9Y_A) are located in some linkage sequences. The ACO sequence analysis of the orchid *Vanda* Miss Joaquim has provided much useful information for the identification of a potential target sequence to suppress *ACO* transcripts and thus reduce ethylene production in *Vanda* orchid flowers.

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