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***Full Length Article***

# The *CfAOS* and *CfAOC* Genes Related to Flower Fragrance Biosynthesis in *Cymbidium faberi* could Confer Drought Tolerance to Transgenic Tomatoes

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## Abstract

*Cymbidium faberi* is one of the longest cultivated oriental orchids. It has a strong flower fragrance, which is an economically important trait in oriental orchids. However, the molecular mechanism of biosynthesis and regulation of flower fragrance in *C. faberi* remains unclear. Methyl jasmonate (MeJA), one of the main components of flower fragrance in *C. faberi*, plays multiple roles, including insect attraction, as well as the mediation of anti-microbial and stress tolerance. Allene oxide synthase (AOS) and allene oxide cyclase (AOC) are crucial enzymes in the MeJA biosynthetic pathway. In this study, we cloned the *CfAOS* and *CfAOC* genes from *C. faberi* and overexpressed them individually in transgenic tomato plants. Whereas no increase in MeJA could be detected in the transformants, the expression level of the jasmonate-related *SlLOX* gene was significantly upregulated. Moreover, the expression levels of MeJA related genes, like *SlLOX*, *SlAOS*, *SlAOC*, *SlOPR3* and *SlJMT* in tomato transformants were increased in drought stress treatment. These results not only provide useful information for uncovering the biosynthesis mechanism of flower fragrance in *C. faberi*, but offer an example of unexpected wider applications in terms of stress resistance which can arise from basic research of flower fragrance. © 2018 Friends Science Publishers

**Keywords:** Floral scent; Methyl jasmonate; Functional identification; Stress tolerance; Orchid

## Introduction

Orchids represent the second largest market segment for cut flowers worldwide, and they possess great ornamental and economic value (Hossain *et al*., 2010). Oriental orchids are famous for their strong flower fragrance and quiet colors compared to tropical orchids. *Cymbidium faberi* is one of the oriental orchids longest in cultivation, which possesses the signature of strong floral scent. Flower scent is beneficial not only for insect pollination, but also confers anti- microbial, anti-herbivore or stress tolerance in many plants (Rodriguez-Saona *et al*., 2011). Along with the research on aroma components in *Clarkia breweri* (Dudareva *et al*., 1998), *Antirrhinum majus* (Dudareva *et al*., 2000), *Petunia hybrida* (Boatright *et al*., 2004), *Gymnadenia* (Huber *et al*., 2005) and *Rosa chinensis* (Flament *et al*., 1993), the chemical characterization of flower fragrance in *C. faberi* was also reported (Omata *et al*., 1990), but the related molecular mechanisms of biosynthesis and regulation remains unclear.

The main pathways involved in the biosynthesis of most flower scents are related to the biosynthesis of terpenoid, phenylpropanoid and fatty acid ester compounds (Croteau and Karp, 1991). It has been reported that the main components of flower fragrance in *C. faberi* are methyl jasmonate (MeJA) and methyl epijasmonate (Omata *et al*., 1990) which do not occur in tropical orchids such as *Phalaenopsis*, *Brassia* and *Epidendrum* (Peng, 2009). MeJA is a volatile compound with a pleasant smell resembling jasmine flowers. It is synthesized as part of the fatty acid ester synthetic pathway, which initiates with acetyl CoA, after which a fatty acid chain is synthesized and converted by phospholipase to form α-linolenic acid in the chloroplasts. Subsequently, the α-linolenic acid is converted into 12-oxo-phytodienoic acid (OPDA) by a series of enzymes, including lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC). The resulting OPDA is transferred from chloroplasts to the peroxisomes, where three cycles of β-oxidation yield jasmonic acid ((+)-7-iso-JA) as the final product (Zhang and Memelink, 2009). In some flowers, certain groups of the final product are modified by specific enzymes to increase the volatility of the resulting fragrance (Dudareva *et al*., 2004). For example, JA is converted into MeJA by jasmonic acid carboxyl methyltransferase (JMT) (Seo *et al*., 2001).

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AOS, the chloroplast-localized first branch enzyme in the JA metabolic pathway, is an unusual member of the cytochrome P450 family (Laudert and Weiler, 1998). The corresponding *AOS* genes have been cloned from a number of plants, including flax (Song *et al*., 1993), arabidopsis (Staswick, 1999), guayule (Pan *et al*., 1995), tobacco (Ziegler *et al*., 2001) and rice (Agrawal *et al*., 2002), and the crystal structures of AOS proteins from guayule and arabidopsis have also been determined (Chang *et al*., 2008; Lee *et al*., 2008). The metabolic reaction catalyzed by AOS is involved in plant development and various responses to the environment (Park *et al*., 2002). Overexpression of flax AOS in transgenic plants led to increased 13(*S*)-hydroperoxyoctadecatrienoic acid (13(*S*)-HPOT) metabolism and formation of 12-O-OPDA (Wang *et al*., 1999). The overexpression of AOS enhanced tolerance to insect attack in soybean and led to the activation of defense-related genes in tomato (Sivasankar *et al*., 2000; Wu *et al*., 2008). Inducible overexpression of a rice *AOS* gene increased the endogenous jasmonic acid level, pathogenesis-related gene expression, and host resistance to fungal infection (Mei *et al*., 2006).

In addition to AOS, the enzyme responsible for the correct enantiomeric structure of JA, AOC, is of special importance. Numerous *AOC*s have been cloned from plants including arabidopsis (Stenzel *et al*., 2003a), tomato (Hause *et al*., 2000), wheat (Maucher *et al*., 2004) and rice (Riemann *et al*., 2013). Four *AOC*s were cloned from arabidopsis, containing a single intron, whereas only one copy of *AOC* was found in wheat, located on the 6H chromosome (Stenzel *et al*., 2003a; Maucher *et al*., 2004). The *CfAOC* gene comprises two introns and three exons. The subcellular localization of the corresponding protein is predicted to be confined to the chloroplasts (Zhou *et al*., 2015). Just like *AOS*s, most of the current research on *AOC*s is related to resistance and stress-tolerance of plants (Stenzel *et al*., 2003b; Stenzel *et al*., 2012).

*Cymbidium faberi* is a typical representative of oriental orchids, and MeJA was found to be one of the main fragrance components in many oriental orchids including *C. virescens*, *C. kanran*, *C. ensifolium*, as well as *C. faberi* itself (Dawipin) (Omata *et al*., 1990; Huang *et al*., 2015). In order to explore the molecular mechanism of MeJA formation, here we firstly measured the main components of flower scent in wild *C. faberi*. Subsequently we cloned the full-length coding sequence and genomic sequence of *CfAOS* from *C. faberi*, which is localized to the chloroplasts, has no introns, and is one of the most important enzymes in the MeJA biosynthetic pathway.

The qRT-PCR analysis showed that both *CfAOS* and *CfAOC* are highly expressed in blooming flowers, and that expression is lowest in roots. The ectopic expression of each of these two genes individually in tomato showed that the endogenous jasmonic acid metabolite was not influenced to a large extent, and only the expression level of lipoxygenase, one of the upstream enzymes in the MeJA biosynthetic pathway, was significantly elevated in the transgenic plants. Additionally, the expression levels of all the genes related to MeJA metabolism were increased compared to those from non-transgenic plants in drought stress treatment, while the overexpression of *CfAOS* in tomato could induce only slight variations of transcription levels upon wound treatment.

## Materials and Methods

### Plant Materials

Plantlets of *C. faberi* were collected from one wild population in Dangyang, Hubei province (30 ◦ 55’25’’N, 111 ◦ 51’24’’E), China. They were then transplanted and divided propagated in the greenhouse of Wuhan Institute of Bioengineering. The species identification of the plantlets was confirmed by taxonomist professor Yanqin Xu. Tissues from roots, leaves and flowers at various stages were harvested, immediately frozen in liquid nitrogen and stored at -80$^{o}$C for further analysis.

### Measurement of Flower Fragrance in *C. faberi*

For GC-MS analysis, headspace volatiles of fresh blooming flowers were collected in a sealed 20 mL container by solid-phase microextraction (SPME). Polydimethylsiloxane (PDMS) fibers (Supelco) were used for the absorption of volatiles for 40 min at room temperature. GC-MS analysis was performed by manual injection of volatiles into a gas chromatograph/mass spectrometer (Thermo Scientific, Trace DSQ II). Desorption was performed at 250℃ for 2 min and samples were separated on a DB5 column (30 m×0.25 mm×0.25 μm) (Agilent Technologies). The oven temperature program comprised 50$^{o}$C for 1 min, 5$^{o}$C min$^{-1}$ ramp until 120$^{o}$C, followed by an 8$^{o}$C min$^{-1}$ ramp until 200$^{o}$C, and a subsequent 12$^{o}$C min$^{-1}$ ramp until 250$^{o}$C, with a final hold at 250$^{o}$C for 7 min. The total run time was about 40 min. Data were analyzed using Xcalibur software. Components were putatively determined by comparison of the mass spectra to the NIST 2008 (U.S. National Institute of Standards and Technology) library. The relative content of each component was calculated with area normalization analysis. Each sample was measured at least twice, and only one set of results was shown.

### DNA Extraction, RNA Extraction and *CfAOS* Gene Cloning from *C. faberi*

Genomic DNA of *C. faberi* was isolated using the high-salt hypotonic method (Zhou *et al*., 2015). Total RNA was isolated from flowers of *C. faberi* according to the manufacturer’s instructions (TRIzol Reagent, Invitrogen) and subsequently reverse-transcribed into cDNA (PrimeScript$^{TM}$ II, TaKaRa) which was used as the template

to amplify the coding sequence of the *CfAOS* gene. Gene-specific primers used in full-length amplification of *CfAOS* gene were listed as following: AOS-F1 (GCTCTAGAATGAGCATCTCCAA, *Xba*I restriction site underlined) and AOS-R1 (CGGGATCCCTAGGCCCGAGCTTTAG, *Bam*HI restriction site underlined). PCR amplification was carried out in a thermal 35 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 1 min each. The same primers were used to amplify the genomic sequence of the *CfAOS* gene.

**Bioinformatic Analysis of the *CfAOS* Gene**

The cDNA and gDNA sequences were compared to analyze the basic genomic structure of the *CfAOS* gene. The molecular mass and pI of the AOS protein were predicted based on ProtParam analysis (http://web.expasy.org/protparam/). The subcellular localization of AOS was predicted with CELLO v.2.5 (http://cello.life.nctu.edu.tw). The conserved domains of AOS were analyzed using the conserved domains database (CDD) from NCBI (http://www.ncbi.nlm.nih.gov/cdd). A phylogenetic tree of AOS sequences derived from different species was constructed based on 12 other AOS proteins downloaded from NCBI using DNAMAN in order to identify the homology relations of CfAOS.

**Analysis of the Spatial and Temporal Expression Patterns of the *CfAOS* and *CfAOC* Genes by qRT-PCR**

The expression of *CfAOS* and *CfAOC* genes in root, leaf, and flower tissues of *C. faberi* was determined using qRT-PCR. The flower samples were separated according to stage into early flowering, blooming and withering stage samples. The gene-specific primers for *CfAOS* and *CfAOC* were qAOS-F1 (CTTCCTCCCCGCCTTCCAC), qAOS-R1 (GCAAACCAGATGAGCCCACT), qAOC-F1 (GGTCTTGCTCCACCAAATCG) and qAOC-R1 (GCCACAGAAGGCTTCACAGG). The *β-actin* gene was used as internal control using the primers qActin-F (TTTATGAGGGTTATGCGCTTCC) and qActin-R (AACTACTGCAGAACGGGAAAT). SYBR green dye (Applied Biosystems, UK) was used for fluorescence signal determination and dissociation curves were applied to detect primer dimers and other non-specific by-products. The PCR procedure was performed according to default settings (ABI 7500, Applied Biosynthesis). Samples were analyzed in technical triplicates.

**Vector Construction**

The pC35ST binary vector was derived from pCambia2300, into which a double CaMV 35S promoter and a *nos* terminator were inserted into the multiple cloning sites (MCS). The full length *CfAOS* gene fragment digested with *Xba*I and *Bam*H was ligated with pC35ST to form pC35ST-CfAOS. The pC35ST-CfAOC vector was constructed using the same strategy. The constructed expression vectors were confirmed by sequencing (Sangon Biotech, Shanghai). Finally, the plasmids of pC35ST-CfAOS and pC35ST-CfAOC were transformed into *Agrobacterium tumefaciens* GV3101 using the liquid nitrogen freeze-thaw method (Holsters *et al*., 1978), after which positive clones were screened on LB agar plates containing 50 μg/mL kanamycin (Sigma, USA).

**Overexpression of *CfAOS* and *CfAOC* Genes in Tomato Plants**

Tomato (*Solanum lycopersicum* L.) variety HW0160 was used for genetic transformation by *Agrobacterium tumefaciens* GV3101 via cotyledon inoculation (Zhou *et al*., 2012). The transformed calli were screened with 50 μg/mL hygromycin (Sigma, USA) and the regenerated plants were rooted on the basic MS medium with 2 μg/mL IBA (Sangon Biotech, Shanghai). Genomic DNA of primary tomato transformants ($T_0$ generation) was isolated and confirmed using a PCR assay with specific primers derived from CfAOS, CfAOC and the hygromycin resistance cassette. Transgenic plants and non-transgenic controls were grown in soil at 22°C under cycles of 16 h light and 8 h darkness. The relevant phenotypic characteristics of transformants such as mRNA expression levels and MeJA concentrations were detected in all lines. The relative expression levels of genes related to MeJA biosynthesis in transgenic tomato plants were determined using qRT-PCR with specific primers. The primers listed in Table 1 were derived from NCBI GeneBank: *SlLoxD*: U37840.1; *SlAOS*: AJ271093.1; *SlAOC*: NM_001247090.1; *SlOPR3*: NM_001246944.2 and *SlJMT*: XM_004238227.2. GC-MS analysis of volatile organic compounds (VOC) in the flowers of transgenic tomatoes was conducted analogous to that in *C. faberi*, except that the temperature profile followed the standards recommended for VOC analysis of tomato (Baldassarre *et al*., 2015).

**Drought Stress and Wound Treatments of Transgenic Tomato Plants**

For the drought stress treatment, watering was withheld for three consecutive days in transgenic and non-transgenic plants at one-month of age. Wound treatment was carried out on young leaves by direct cutting with scissors. Samples were collected 2 h after wounding. Leaves of treated lines were collected for RNA isolation and a series of genes related to MeJA biosynthesis were investigated for the variation in relative expression levels. All experiments were done in triplicates.

**Statistical Analysis**

Data on the relative expression levels of genes detected with qRT-PCR represent the means ± SD and were analyzed by analysis of variance procedures using the SigmaPlot 12.5.

## Results

### The Measurement of Flower Fragrance Components in *C. faberi*

A total of 18 out of about 150 components in flower fragrance of *C. faberi* were successfully identified by SPME-GC/MS analysis. The identified compounds included esters, alkanes, terpenes, acids, ketones and aromatic compounds (Fig. 1). Esters were the main components of flower fragrance and their abundance reached 61.45% of the total. Among them methyl jasmonate was the most abundant VOC of the flowers (Table 2). Except for three main components, the relative concentrations of other contents showed little difference.

### Cloning and Bioinformatics Analysis of *CfAOS* in *C. faberi*

For PCR analysis, the predicted 1.5 kb *CfAOS* fragment was amplified from flowers of *C. faberi* (Fig. 2 lane 1). The sequencing results of the PCR products showed that the *CfAOS* gene was 1491 bp in length, encoding a protein 496 amino acids in length, with an expected molecular mass of 55.3 kD and pI of 8.74 based on the ProtParam analysis. A comparison with the genomic sequence of *CfAOS* suggested that the gene has no introns. According to Target P1.1 and CELLO v.2.5 analysis, *CfAOS* is probably located in the chloroplasts. The conserved domains of *CfAOS* were analyzed by CDD analysis on the NCBI website, and the protein was characterized as a member of the cytochrome P450 gene superfamily.

Phylogenetic tree analysis showed that the AOS proteins from *C. faberi* and the *C. ensifolium* were closest, whereas the sequences from other monocotyledon plants, such as *Oryza sativa*, *Zea mays*, *Hordeum vulgare* and *Triticum aestivum* were clustered closely together (Fig. 3), which is not surprising since they all belong to the grass family.

### The Transcription Levels of the *CfAOS* and *CfAOC* Genes were Significantly Increased at the Blooming Flower Stage

To demonstrate the expression patterns of the *CfAOS* and *CfAOC* genes in different organs and development stages of *C. faberi*, qRT-PCR was performed to determine the respective transcription levels of these genes (Fig. 4). The results showed that the expression level of the *CfAOS* and *CfAOC* genes was highest in flowers at the blooming stage, and decreased to the lowest level in the withering stage. The expression level of *CfAOS* was higher than that of *CfAOC* in vegetative organs, suggesting that the *CfAOS* and *CfAOC* genes may be directly involved in the biosynthesis of flower fragrance in *C. faberi*.

**Table 1:** Primers used in qRT-PCR derived from sequences related to the MeJA biosynthetic pathway in *Solanum lycopersicum*

| Name | Sequence (5'→3') |
|---|---|
| qTLOX-F | CGGCCCAAGTTTGCAGTG |
| qTLOX-R | CCAGATGAGGGTGGTGAGGA |
| qTAOS-F | TGGGTTGCCGGGTATTGG |
| qTAOS-R | ATGAATGGTCCCGGTGGC |
| qTAOC-F | TCGCCGTTCAGGGAGCGTA |
| qTAOC-R | GATGGCAGACCGGGGATC |
| qTOPR3-F | CCGATGACAAGGTGCAGAGC |
| qTOPR3-R | CCTGGCACATGAGGAAACCC |
| qTJMT-F | CGTTCACCGGAGATAGTCCC |
| qTJMT-R | GAAAGCGCATTTGCGAGA |

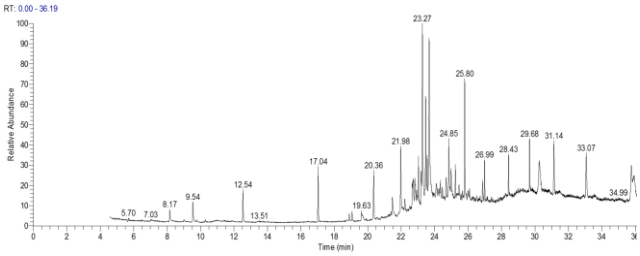


**Fig. 1:** Total ionic chromatogram of compounds in flower fragrance of *Cymbidium faberi*

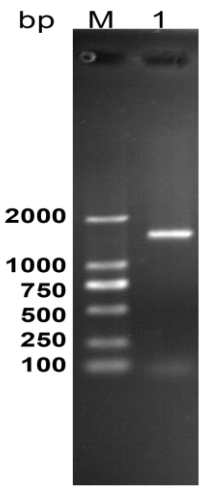


**Fig. 2:** The PCR amplification product of *CfAOS* gene in *Cymbidium faberi*. M: DL 2000 DNA Marker; lane 1: PCR amplification product of *CfAOS* gene

### Vector Construction and Molecular Characterization of Tomato Transformants

The pC35ST-CfAOS and pC35ST-CfAOC vectors containing the ORFs of *CfAOS* and *CfAOC* were double digested with *Xba*I/*Bam*HI, *Eco*RI/*Hin*dIII and *Eco*RI/*Pst*I, respectively, as shown in Fig. 5a and b. The digestion fragments of pC35ST-CfAOS were 10058 bp and 1497 bp with *Xba*I/*Bam*HI digestion, 8906 bp, 1721 bp and 928 bp

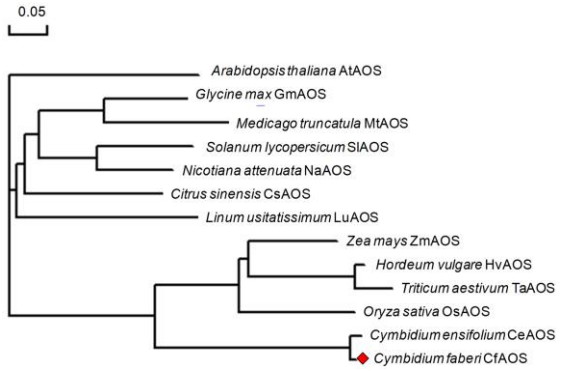


**Fig. 3:** Phylogenetic analysis of selected plant AOS proteins

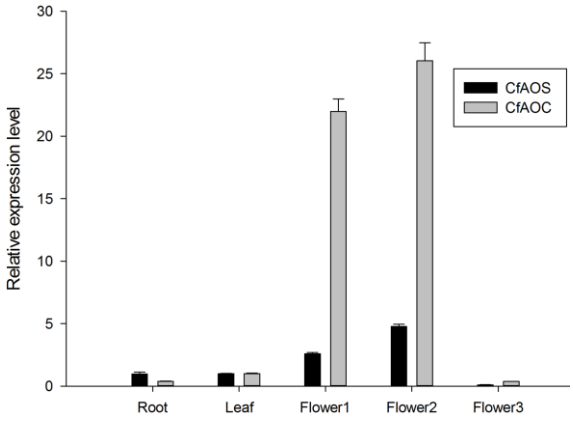


**Fig. 4:** The spatial-temporal specific expression analysis of *CfAOS* and *CfAOC* genes in *Cymbidium faberi* by qRT-PCR. Flower 1: flower bud stage; Flower 2: blooming stage; Flower 3: withered stage. Data on relative expression levels are the means ± SD (n=3)

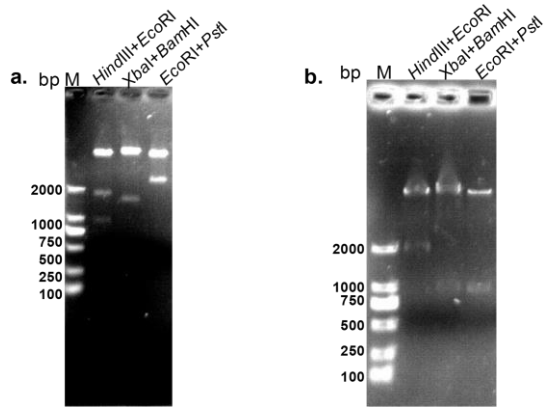


**Fig. 5:** Restriction digestion analysis of pC35ST-CfAOS (a) and pC35ST-CfAOC (b) recombinant plasmids. M: DL 2000 DNA Marker

with *Eco*RI/*Hin*dIII, 8915 bp and 2640 bp with *Eco*RI/*Pst*I digestion. The corresponding digestion results of pC35ST-CfAOC were 10058 bp/777 bp; 8907 bp/1928 bp and 8915 bp/981 bp/939 bp, respectively which is consistent with the restriction digestion maps of the pC35ST-CfAOS and pC35ST-CfAOC vectors (Fig. 6a, b).

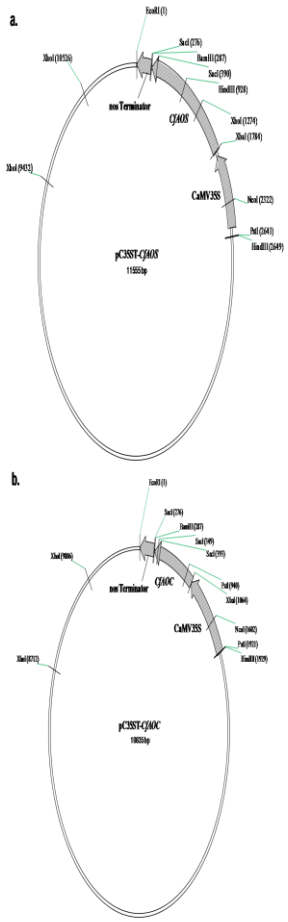


**Fig. 6:** Vector map of pC35ST-CfAOS (a) and pC35ST-CfAOC (b) with restriction sites and related elements

Fifteen independent transformants each were obtained from inoculations with *Agrobacterium tumefaciens* strains harboring the pC35ST-CfAOS and pC35ST-CfAOC vectors, respectively. All of the transformants were phenotypically indistinguishable from wild-type plants (WT).

The ectopic expression of *CfAOS* and *CfAOC* genes in tomato transformants was detected with qRT-PCR in lines AOS1-15 and AOC1-15. The transcription level of *CfAOS* in line AOS3 increased 4.3-fold and the transcription levels of *CfAOC* in lines AOC1-3 increased 21.4-, 9.8- and 16.45-fold compared to control, respectively (Fig. 7). Consequently, we have chosen a single pC35ST-CfAOS plant (AOS3) and three pC35ST-CfAOC plants (AOC1-3) with the highest relative expression levels for further study.

**Table 2:** Main compounds and their relative contents in the flower fragrance of *C. faberi*

| Retention time (min) | Compound name | Molecular formula | Relative content (%) |
|---|---|---|---|
| 9.54 | (E)-3,7-dimethyl-1,3,6-octatriene | $C_{10}H_{16}$ | 1.11 |
| 17.03 | dodecamethyl-cyclohexasiloxane | $C_{12}H_{36}O_6Si_6$ | 2.54 |
| 21.48 | [S-(Z)]-3,7,11-trimethyl-1,6,10-dodecatrien-3-ol | $C_{15}H_{26}O$ | 1.2 |
| 21.98 | Hexadecane | $C_{16}H_{34}$ | 3.16 |
| 22.69 | Decanoic acid decyl ester | $C_{20}H_{40}O_2$ | 3.31 |
| 23.03 | hexadecamethyl-cyclooctasiloxane | $C_{16}H_{48}O_8Si_8$ | 2.06 |
| 23.19 | 2-methyl-à-phenyl-1,1-cyclopropanedimethanol | $C_{12}H_{16}O_2$ | 1.21 |
| 23.27 | Methyl jasmonate | $C_{13}H_{20}O_3$ | 9.62 |
| 23.47 | Heptadecane | $C_{17}H_{36}$ | 4.43 |
| 23.55 | 2,6,10-trimethyl-dodecane | $C_{15}H_{32}$ | 2.17 |
| 23.68 | Methyl epijasmonate | $C_{13}H_{18}O_3$ | 8.25 |
| 24.69 | p,p'-Benzylidenebis(N,N-dimethylaniline) | $C_{23}H_{26}N_2$ | 1.09 |
| 24.85 | Octadecane | $C_{18}H_{38}$ | 2.96 |
| 24.99 | 2,6,10,14-tetramethyl-hexadecane | $C_{20}H_{42}$ | 1.37 |
| 26.87 | Dibutyl phthalate | $C_{16}H_{22}O_4$ | 1.15 |
| 28.62 | 5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-enal | $C_{20}H_{32}O$ | 1.18 |
| 29.06 | Friedelan-3-one | $C_{30}H_{50}O$ | 1.45 |
| 30.26 | Hexa decamethyl octasiloxane | $C_{16}H_{50}O_7Si_8$ | 3.34 |

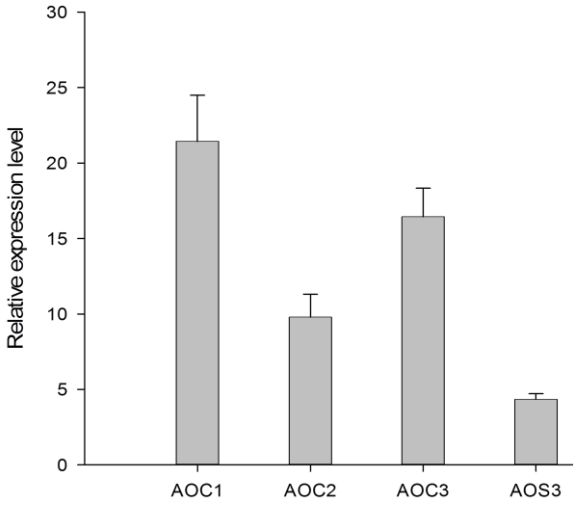


**Fig. 7:** The transcription levels of *CfAOS* and *CfAOC* genes in AOS and AOC tomato transformants. Data are the means ± SD (n=3)

**Overexpression of *CfAOS* and *CfAOC* in Tomato Plants did not Improve the Endogenous MeJA in Tomato Flower Fragrance but Significantly Increased the Transcription Level of the *SlLOX* Gene**

The fresh flowers of transformants and WT plants were collected and their VOC profiles analyzed using SPME-GC/MS. No MeJA was detected in the flower fragrance of any of the transformants (data not shown). The main component of tomato flower scent was 3-carene, the same as that in the wounded leaves and flowers of the transformants. The transcription levels of genes involved in MeJA biosynthesis in tomato, including *SlLOX*, *SlAOS*, *SlAOC*, *SlOPR3* and *SlJMT* were measured by qRT-PCR. Interestingly, the expression level of the upstream gene *SlLOX* was significantly up-regulated and reached 1.46-, 4.04- and 8.69-fold compared to the control in AOC1, AOC2 and AOC3, respectively. The expression levels of other genes, especially endogenous *SlAOC* and downstream genes, were not altered in *CfAOC* transformants (Fig. 8). In AOS3, the transcript levels of *SlLOX*, *SlAOS*, *SlAOC* and *SlJMT* were 2.43-, 3.48-, 1.61- and 3.84-fold higher than the control, but that of *SlOPR3* was not altered (Fig. 8). These results suggested that ectopic expression of *CfAOS* and *CfAOC* indeed influenced the MeJA biosynthesis in the tomato plants, but not significantly enough to make the transformants synthesize the final product of MeJA in flower fragrance.

**Overexpression of *CfAOS* and *CfAOC* Elevated the Transcription Levels of MeJA Related Genes in Tomato Transformants While Inducing only Slight Variations upon Wound Treatment**

After drought stress treatment, the transformants were slightly affected whereas the WT plants severely wilted (Fig. 9). The treated leaves were collected and the transcription levels of genes relevant for MeJA biosynthesis were measured by qRT-PCR. The transcription levels of *SlLOX*, *SlAOS*, *SlAOC*, *SlOPR3* and *SlJMT* in AOC2 were increased to 3.18-, 1.84-, 1.24-, 1.28- and 4.25-fold, respectively. The transcription levels of the same genes were 2.80-, 2.27-, 1.88-, 1.81- and 4.83-fold higher in the AOS3 (Fig. 10). For the wound treatment, the transcription levels of *SlLOX*, *SlAOS*, *SlAOC* and *SlOPR3* in AOC2 were increased to 2.41-, 1.90-, 1.84- and 4.35-fold, respectively (Fig. 11). On the other hand, the expression level of *SlJMT* was down-regulated and the transcription levels of related genes were not altered significantly in AOS3 following wound treatment.

## Discussion

The genome sequence of the tropical orchid *Phalaenopsis equestris* provided invaluable information for deep genetic investigation of orchids (Cai *et al*., 2015).

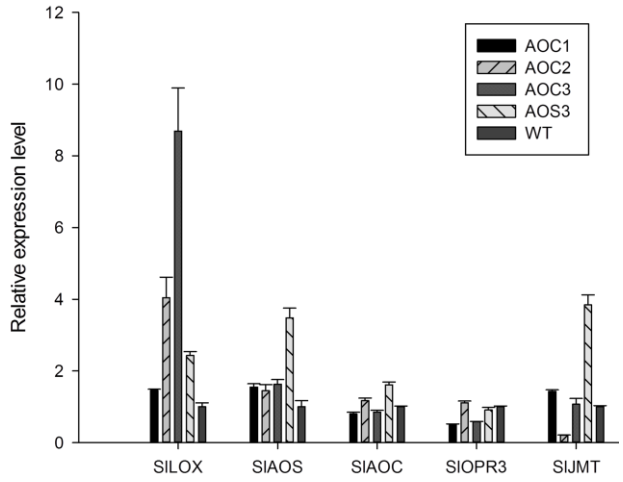


**Fig. 8:** The transcription levels of genes related to MeJA biosynthesis in transgenic AOC1, AOC2, AOC3, AOS3 and wild-type (WT) tomato plants. Data are the means ± SD (n=3). MeJA, methyl jasmonate


**Fig. 9:** The transgenic tomato plants overexpressed *CfAOS* and *CfAOC* genes showed more resistant to drought stress. A Wild-type (WT) and two transgenic plants (AOS3 and AOC2) at one-month of age were not watered for three consecutive days to induce drought stress. These plants are representative for all the plants that were tested for each treatment

However, the large differences between tropical orchids and oriental orchids hindered the development of intensive research in oriental orchids. This study aims to lay the foundations for understanding the biosynthetic mechanism of flower fragrance in oriental orchid *C. faberi*. AOS and AOC are two of the most important enzymes in the MeJA biosynthesis pathway, which is responsible for the biosynthesis of the main component of flower fragrance in *C. faberi*. However, the function of *CfAOS* and *CfAOC* has not been comprehensively characterized to date. In this study, *CfAOS* was cloned from *C. faberi*, and its gene structure revealed the absence of introns. *CfAOS* and *CfAOC* were independently ectopically expressed into tomato plants to determine their functions in MeJA biosynthesis.

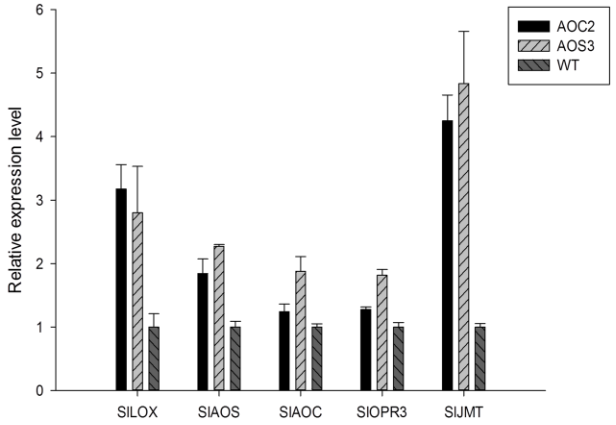


**Fig. 10:** The transcription levels of genes related to MeJA biosynthesis in transgenic AOC2, AOS3 and wild-type (WT) tomato plants upon drought stress. Data are the means ± SD (n=3). MeJA, methyl jasmonate

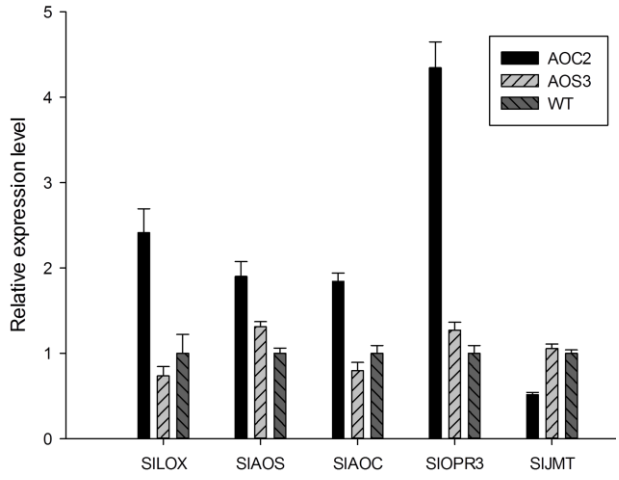


**Fig. 11:** The transcription levels of genes related to MeJA biosynthesis in transgenic AOC2, AOS3 and wild-type (WT) tomato plants treated with mechanical wounding. Data are the means ± SD (n=3). MeJA, methyl jasmonate

Whereas no emission of MeJA was observed in the flower fragrance of the tomato transformants in our study, it was possibly due to an insufficiency in *CfAOS* and *CfAOC*-mediated activation of downstream genes in the MeJA biosynthetic pathway. Among these, especially the activity of jasmonic acid carboxyl methyltransferase (JMT) may be limited, which catalyzes the last rate-limiting step in the pathway. JA occurs ubiquitously in all plant species. It has been reported that the expression of a flax *AOS* cDNA leads to the increased endogenous JA levels in transgenic tomato plants, but not to a corresponding activation of JA-responding genes (Harms *et al*., 1995). Interestingly, overexpression of the flax *AOS* did not increase JA levels in healthy, undamaged leaf tissues but did increase it in wounded tissues of transgenic tobacco (Wang *et al*., 1999).

An equivalent result was reported for the overexpression of the *AtAOS* gene in Arabidopsis (Laudert *et al*., 2000). However, in our study no increased MeJA levels were detected in wounded transgenic tomato leaves (data not shown). In a separate study, the expression of *AOC* in transgenic tomato increased the quantity and activity of AOC protein. However, the JA level and the transcription levels of *OPDA* and *PIN2* were not altered (Stenzel *et al*., 2003b). It has ever been reported that constitutive overexpression of AOC in tomato plants elevated the levels of some jasmonates and octadecanoids in flower organs but not in leaves (Miersch *et al*., 2004). On the other hand, the AOC gene in wheat enhances salinity tolerance via jasmonate signaling (Zhao *et al*., 2014). It is possible that the jasmonate metabolic network is regulated in a different way in monocotyledonous and dicotyledonous plants, which would explain these disparities but require further research.

When tomato transformants were treated with drought stress, genes related to the MeJA pathway displayed significant fluctuations in their expression levels as shown in Fig. 10. It inferred that the overexpression of *CfAOS* and *CfAOC* genes may elevate the drought tolerance in transgenic tomatoes, which need more verification. It has been reported that tomatoes may use genetically distinct signaling pathways for the regulation of different wound responsive genes (Howe *et al*., 2000; Froehlich *et al*., 2001). Upon wounding treatment, the transcript levels of all genes related to MeJA biosynthesis in transgenic tomatoes performed slight variation except that of *SlJMT* gene as shown in Fig. 11, which was decreased compared to that in non-transformed control. JA, as an important hormone, can be methylated into MeJA. Meanwhile, it also can be conjugated to isoleucine to form JA-isoleucine (JA-Ile), which is a bioactive molecule regulating a broad array of plant defense responses. JA-Ile brings together the F-box protein CORONATINE INSENSITIVE 1 ($SCF^{COI1}$) complex and a JA ZIM-domain (JAZ) protein to activate jasmonate signaling pathway (Goossens *et al*. 2016). It is possible that the metabolic flux of jasmonate pathway may branch into JA-Ile upon wounding leading to the reduced MeJA, so the expression of *SlJMT* gene is accompanied decreased with it.

Importantly, many transcription factors such as WRKY, MYB and bHLH involved in the JA metabolic biosynthesis and signal transduction have been reported in rice (Zhang *et al*., 2009; Peng *et al*., 2012), tea (Gohain *et al*., 2012), arabidopsis (Niu *et al*., 2011) and tomato (Yan *et al*., 2013), which provides new insights and leads for uncovering the molecular mechanism of MeJA regulation and biosynthesis in *C. faberi*.

The traditional breeding of oriental *Cymbidium* cultivars is limited to transplanting and domestication of wild species. However, due to years of overexploitation, the wild resources of *Cymbidium* have been dramatically reduced. It is therefore imperative to establish new breeding methods. Our study aims to lay the foundation for genetic breeding of new oriental orchids, especially concerning the development of new cultivars with improved flower fragrance. Therefore further and intensive research should be conducted to reveal not only the biosynthesis of flower fragrance in oriental orchids, but also ways in which orchid genes may be used to improve drought tolerance of plants - an offshoot of research into flower fragrances.

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