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Research Article

Molecular Cloning, Sequencing, and Characterization of a Putative Acetyl-CoA-C-acetyltransferase cDNA from a Highly Fragrant Orchid Hybrid *Vanda* Mimi Palmer

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Vanda Mimi Palmer, a hybrid of *Vanda* Tan Chay Yan and *Vanda tessellata* (Roxb.) Hk.*f*. ex G. Don, is cultivated as a potted ornamental plant mainly for its fragrance rather than its look. Plant acetyl-CoA-C-acetyltransferase (ACA) is involved in the condensation of two acetyl-CoAs to form acetoacetyl-CoA, which condenses with another acetyl-CoA to yield a crucial molecule, 3-hydroxyl-3-methylglutaryl-CoA, at the initial step of the mevalonate (MVA) pathway. An ACA gene from vandaceous orchid has never been reported. We describe the isolation and molecular characterization of an ACA-like gene from *V*. Mimi Palmer (designated as *VMPACA*) to facilitate a better understanding of the terpenoid biosynthesis pathway in orchids. The deduced *VMPACA* encodes a 376-amino-acid protein with a molecular weight of 39 kDa, which comprises an open reading frame of 1128 bp. It is flanked by 87 bp of 5′-untranslated region and 174 bp of 3′-untranslated region including a poly-A tail. Its protein sequence is 81% identical to other plant ACAs and contains a thiolase active site. The fluctuation expression pattern of *VMPACA* transcript by real-time RT-PCR showed that it is developmentally and temporally regulated with predominant expression in outer and lateral inner tepals compared to vegetative tissues.

1. Introduction

Orchidaceae belongs to the largest family of flowering plants, with approximately 880 genera worldwide. Among these, more than 120 genera are found naturally in Peninsular Malaysia [1, 2]. *Vanda* Mimi Palmer, a hybrid of *Vanda* Tan Chay Yan and *Vanda tessellata* (Roxb.) Hk.*f*. ex G. Don., is a famous ornamental potted plant cultivated in Malaysia selectively for its fragrance characteristic [3, 4]. Mohd-Hairul et al. [5] found that the floral scent of *V.* Mimi Palmer was predominated by terpenoid, benzenoid, and phenylpropanoid compounds using GC-MS analyses (Table 1).

Plant volatiles are synthesized from three major biosynthesis pathways including phenylpropanoids, fatty acids derivatives and terpenoids pathways. Mevalonic acid (MVA) and methyl-D-erythritol 4-phosphate (MEP) pathways are the two pathways responsible for the biosynthesis of isoprenoids in plants [6, 7].

Acetyl-CoA-C-acetyltransferase (ACA), classified as Thiolase II, is involved in catalysing the condensation of two units of acetyl-CoA (2C) to acetoacetyl-CoA (4C) in the mevalonate pathway. ACA is a crucial molecule involved in terpene biosynthesis, production of volatile benzenoids, and the biosyntheses of hormones and cholesterols [8–11]. To date, the isolation and molecular characterization of plant ACAs have been performed in *Clarkia breweri* [12], rose [13, 14], petunia [15] and *Perilla frutescens* [16]. And to our knowledge, there is no reported work of an ACA gene from vandaceous orchids.

Over the years, the numbers of fragrance-related cDNAs that have been isolated and characterized particularly in

Table 1: Volatile compounds emitted by fully open flower of *Vanda* Mimi Palmer with their relative retention times and spectral fragments. This table is adapted from Mohd-Hairul et al. [5].

Peak	Relative retention time (min)	Main spectrum fragments (m/z)	Compound name
Monoterpene			
1	8.636	36,41,53,67,79,93,105,121	Ocimene
2	9.147	41,43,59,81,93,112	Linalool oxide
3	9.592	41,43,69,71,93,107,121,136	Linalool
Sesquiterpene			
10	16.492	41,43,69,71,93,107,123,136,162	Nerolidol
Benzenoid			
4	9.702	51,77,105,136	Methylbenzoate
5	10.030	39,51,65,78,91,105,122	Benzyl acetate
Phenylpropanoid			
6	10.783	39,43,65,79,91,108,150	Phenylethanol
8	11.926	39,43,65,78,91,104	Phenylethyl acetate
Indole			
9	13.084	39,50,63,74,90,117	Indole
Formanilide			
7	12.260	39,52,65,76,93,161	Formanilide

vandaceous orchids are limited to the reports from our research group, working on this highly fragrant vandaceous orchid, *V*. Mimi Palmer. Among the identified fragrance-related transcripts reported were *V.* Mimi Palmer 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*VMPDXR*) [3], *V.* Mimi Palmer phenylacetaldehyde synthase (*VMPPAAS*) [17], *V.* Mimi Palmer alcohol acyltransferase (*VMPAAT*) and *V.* Mimi Palmer sesquiterpene synthase (*VMPSTS*) [18], *V.* Mimi Palmer 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (*VMPCMEK*) and *V.* Mimi Palmer cytochrome P450 (*VMPCyP450*) [1]. Recently, we reported the successful expression and functional characterization of the *VMPSTS* in *Lactococcus lactis* [19]. Besides the above mentioned fragrance-related transcripts, no other fragrance-related cDNAs have been reported from other orchids except *Phalaenopsis* [20].

We reported here a novel finding of an ACA-like gene from our previously constructed *V.* Mimi Palmer EST database (VMPEST), designated as *VMPACA* [4]. The isolated and molecular characterized full-length transcript will allow us to advance our study to understand better the molecular fluxes of terpenoids synthesis in an advanced plant like orchid.

2. Materials and Methods

2.1. Plant Materials and Samples Collection. *V.* Mimi Palmer plants used in this study were bought and maintained at the United Orchid Plantation in Rawang, Selangor, Malaysia under tropical climate with the temperature ranging between 25–30°C and 70–80% sunlight exposure (under shade) and prior to samples collection, the plants were brought and placed outside the laboratory building which has almost similar conditions to the nursery as described by Chan et al. [3]. The *V.* Mimi Palmer samples used for RNA work were collected based on tissue specificity, different time points (2-hour intervals within a 24-hour cycle), and different flower developmental stages as previously described by Chan et al. [18] and were stored at −80°C until ready to be used for total RNA extraction.

2.2. Total RNA Extraction. The standard CTAB protocols of Yu and Goh [21] were used in the total RNA extraction to extract all the frozen floral tissues as mentioned earlier. In order to remove RNase contamination, the mortar, pestle, and spatula were baked at 200°C.

2.3. Full Length cDNA Isolation and Sequence Analysis of VMPACA from Vanda Mimi Palmer. From the *V.* Mimi Palmer EST database (VMPEST) [4], a partial length cDNA sequence that encodes a putative acetyl-CoA-C-acetyltransferase of *V.* Mimi Palmer (*VMPACA*) (GenBank accession no. GW393499) was identified and selected for full length isolation. A universal primer mix (UPM) consisting of a long primer: 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′, a short primer: 5′-CTAATACGACTCACTATAGGGC-3′, and a gene-specific primer (5′-CATGCTCTCCATGCCACCACTCAC-3′) was used in the isolation of 5′-end of *VMPACA* transcript. Full-bloom flowers were used as the template for full length isolation. SMARTer RACE cDNA Amplification Kit (Clontech, CA, USA) and Advantage 2 Polymerase Mix (Clontech, CA, USA) were used according to the manufacturer's instructions in the full length cDNA isolation of *VMPACA* which lacks of 5′-end region. The PCR reaction was performed at 94°C for 30 seconds, annealed at 64.4°C for 30 seconds, and extended at 72°C for 3 minutes, for a total of 25 cycles using the Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany).

The PCR products were gel-purified using QIAquick Gel Extraction Kit (Qiagen, CA, USA) and subsequently cloned into the yT&A vector using the protocol provided in the yT&A Cloning kit (Yeastern Biotech, Taipei, Taiwan). The ligation mixture consisted of 1X ligation buffer A, 1X ligation buffer B, yT&A cloning vector: PCR product (1 : 3), 0.2 unit of YEA T4 DNA ligase and topped up with distilled water to a final volume of 10 μL. The ligation mixture was mixed by pipetting gently and incubated overnight at 4°C. Two microliters of the ligation mixture was added to ice-thawed *Escherichia coli* strain *DH5α* competent cells. The mixture was incubated on ice for 20 minutes, then heat-shocked at 42°C for 90 seconds and immediately placed on ice for 1 minute to promote the binding of DNA to the cells membranes and the uptake of the DNA into the cells. Following that, 800 μL Luria-Bertani (LB) broth was added to the tube, with further incubation at 37°C for 45 minutes in a shaker at 250 rpm. Meanwhile, 0.1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) and 40 μg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal) was prepared and spread onto LB agar supplemented with 50 μg/mL ampicillin for blue/white colour screening for recombinants. The tube was then centrifuged at 14,100 g for 1 minute and 90% of the supernatant was removed. The cell pellets were resuspended in the remaining supernatant and plated on two LB agar plates each supplemented with 50 μg/mL ampicillin, IPTG, and X-gal. The plates were incubated overnight at 37°C. Colony PCR was done to verify the presence and size of inserts of targeted 5′-RACE PCR products prior to sequencing. The same PCR components as mentioned above except the primers M13F (−40) (5′-GTTTTCCCAGTCACGAC-3′) and M13R (−40) (5′-CAGGAAACAGCTATGAC-3′) were used. The putative transformants were sequenced by the Bioneer Inc. (Daejeon, Republic of Korea) using M13F (−40) and M13R (−40) primers and the sequences analyses were performed using several programs such as BLASTN, BLASTX, CAP CONTIG, Biology WorkBench 3.2 (http://workbench.sdsc.edu/), BioEdit version 7.0.9 softwares, and ClustalW.

In order to get the full length *VMPACA* into a plasmid construct, a pair of gene-specific forward (5′-GGGCAAGCAGTGGTATCAACG-3′) and reverse (5′-AGTCCAAGATGTTGCCATTGAGAT-3′) primers flanking the full length coding sequence of *VMPACA* was designed. The PCR reaction was performed using the following conditions: pre-denatured at 94°C for 3 minutes, denatured at 94°C for 45 seconds, annealed at 55.7°C for 40 seconds, extended at 72°C for 90 seconds for a total of 35 cycles, and a final extension at 72°C for 10 minutes. After purification of the PCR product, it was cloned into the yT&A cloning vector (Yeastern Biotech, Taipei, Taiwan), transformed into competent *E. coli* strain *DH5α* cells, and the plasmid DNAs of the transformed cells were isolated for later sequencing. These sequences were translated into amino acid sequences and analysed using BLASTX, multiple sequence alignment of proteins using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and BioEdit version 7.0.9 (Tom Hall, Ibis Biosciences 1999), protein subcellular localization prediction using WoLF PSORT (http://wolfpsort.org/), detection of N-terminal sorting signal using iPSORT (http://ipsort.hgc.jp/), and determination of the pI value and molecular weight of protein using ExPASy (http://www.expasy.ch/tools/). The sequence analyses indicated that the full coding sequence of the clone contained start and stop codons. The successfully isolated full length cDNA of *VMPACA* has been submitted to GenBank with the accession number JF911348. For better estimation of the relationships between putative VMACA and other plant ACAs, a phylogenetic tree was generated using neighbour-joining method from the MEGA 4.0 (http://www.megasoftware.net/).

2.4. Real-Time RT-PCR Analysis of VMPACA Transcript. First strand cDNA of *VMPACA* was reverse transcribed from 1 μg of each RNA sample using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, USA) according to the manufacturer's protocol. Real-time RT-PCR was performed using a Bio-Rad iCycler (iCycler iQ5 Multicolor Real-Time PCR Detection System). A master mix of each reaction consists of 2X SYBR Green I Hot-Start real-time PCR-Mix (GeneCraft, Cologne, Germany), 10X diluted cDNA template with a pair of gene-specific primers (forward primer: 5′-TTCTGTTGTTGCTGTGGCCAAT-3′ and reverse primer: 5′-CAAACCCCAGCAGCTCCATATT-3′). The following thermal cycling conditions were used: 95°C 1 minute, 40 cycles at 95°C for 10 seconds, 59°C for 20 seconds followed by 72°C for 20 seconds, and 81 cycles at 55–95°C for 10 seconds for melting curve analysis to ensure the amplification of only the desired amplicons. All samples were run in four replicates where the mean value was considered and a negative control (master mix without template) was included in each reaction. Geometric mean method of Vandesompele et al. [22] was applied in the normalization of relative gene expression of the transcript in different types of tissue, at different time points and at different developmental stages, with actin (GenBank accession no. AF246716), cyclophilin (GenBank accession no. GW393531), and tubulin (GenBank accession no. GW393564) as reference genes. The primers' details of the three endogenous controls used in the normalization steps are shown in Table 2. The statistically significant relative expression level was addressed by an analysis of variance (ANOVA) as described by Karlen et al. [23] and the significant differences between the means were analysed using *post hoc* tests at $P < 0.05$.

3. Results and Discussion

3.1. Sequence Analysis of Full Length VMPACA cDNA Transcript. Full length *VMPACA* cDNA transcript comprises of 1389 bp with an open reading frame (ORF) of 1128 bp, encoding a 376-amino-acid protein with a calculated molecular weight of about 39 kDa and a pI value of 5.87. It is flanked by 87 bp 5′-untranslated region and 174 bp 3′-untranslated region including a poly-A tail. Analysis of VMPACA using iPSORT and SignalP 3.0 programme [24] predicted it does not have any mitochondrial targeting peptide or chloroplast transit peptide.

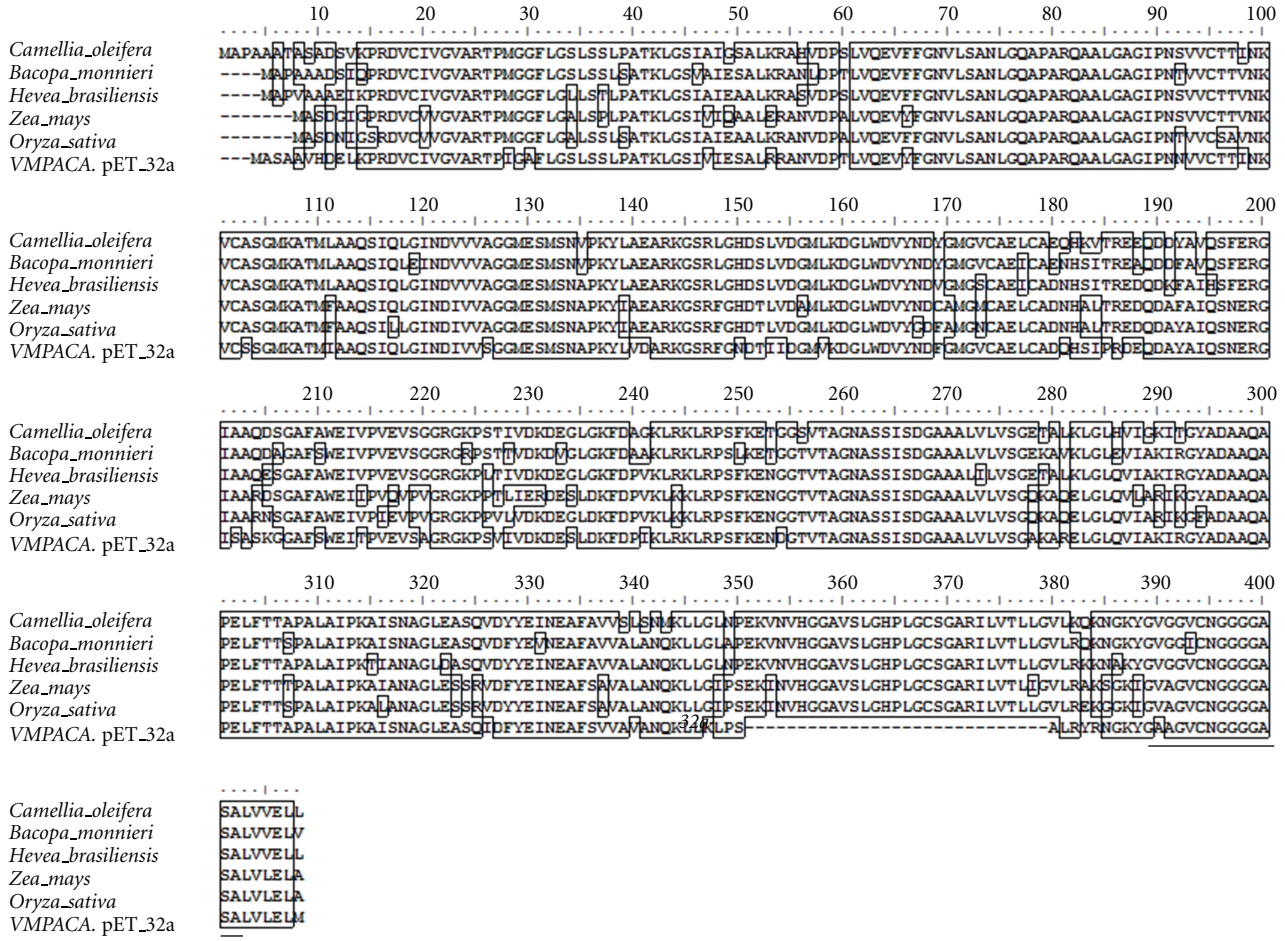

Figure 1: Amino acid sequence alignment of VMPACA (putative acetyl-CoA-C-acetyltransferase) and other closely related protein sequences from *Camellia oleifera* (GenBank accession no. ADD10719), *Hevea brosiliensis* (GenBank accession no. BAF98276), *Zea mays* (GenBank accession no. ACG32450), *Bacopa monnieri* (GenBank accession no. ACU87560), and *Oryza sativa* Japonica group (GenBank accession no. BAD22334). Thiolase II active site (GVAGVCNGGGGASA) is underlined.

Table 2: Endogenous controls and the primers sets used in real-time RT-PCR. Actin, tubulin, and cyclophilin were used as endogenous controls in the real-time RT-PCR analysis.

Primers identity/amplicon size	GenBank accession	Primers sequences
Actin (236 bp)	AF246716	Forward: 5′-CAGTGTTTGGATTGGAGGTTC-3′
		Reverse: 5′- CCAGCAGCAGTCAGGAAAA-3′
Tubulin (227 bp)	GW393564	Forward: 5′- CTCCCGCATTGACCATAAAT-3′
		Reverse: 5′-GGAACCACACCCAAACTCTC-3′
Cyclophilin (200 bp)	GW393531	Forward: 5′-TTGGATGTCGTGAAGGCAAT-3′
		Reverse: 5′-CAACACAAGAAGATAGCACAGCA-3′

Protein sequence comparison using ClustalW and Blastx indicates that VMPACA has 81% protein sequence identity to the ACAs encoded by *Camellia oleifera* and *Hevea brosiliensis*, followed by *Zea mays* and *Bacopa monnieri* at 80% identity, and *Oryza sativa* Japonica group at 79% (Figure 1).

There are two types of thiolase based on their catalytic activities: Thiolase I (acetyl-CoA C-acyltransferase) and Thiolase II (acetyl-CoA C-acetyltransferase). Thiolase I is also known as a degradative thiolase while Thiolase II is known as a synthetic thiolase [9]. Based on the motif predicted by PROSITE program (http://expasy.org/prosite/), a thiolase II active site (GAAGVCNGGGGASA) is located in the ORF of VMPACA sequence (Figure 1). It has a significant similarity with other plants' ACA. However, this putative VMPACA lacks a portion of the conserved domain (NVHGGAVSIGHPIGCSG) at the C-terminal end. Dudareva et al. [12] also found a limited sequence similarity at the C-terminus of BEAT, an acetyl-CoA:benzylalcohol acetyltransferase from *Clarkia breweri*. Further studies are required to confirm the catalytic activities of *VMPACA*

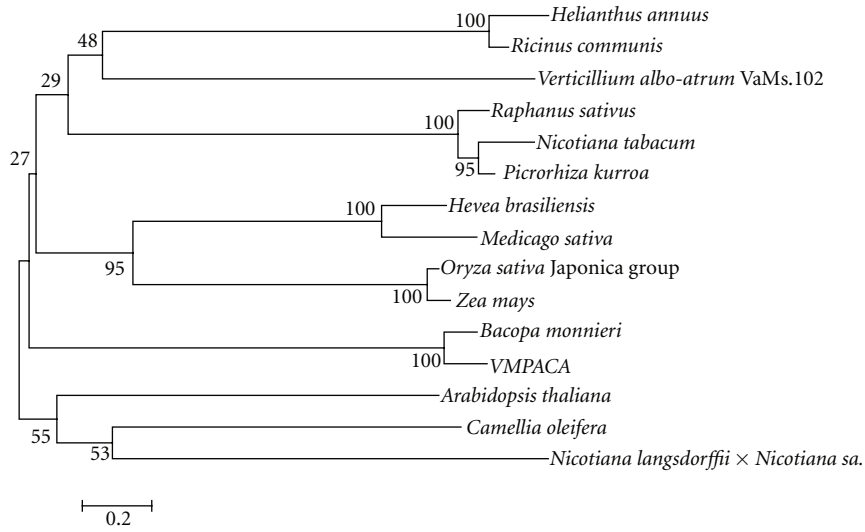

Figure 2: Phylogenetic tree of VMPACA with homologues proteins by using MEGA 4.0. The bootstrap neighbor-joining method was used to construct the tree. The phylogenetic tree analysis of ACA has included *Zea mays* (GenBank accession no. ACG32450.1), *Ricinus communis* (GenBank accession no. EEF39476.1), *Oryza sativa* Japonica Group (GenBank accession no. BAD22334.1), *Nicotiana tabacum* (GenBank accession no. AAU95619.1), *Hevea brasiliensis* (GenBank accession no. AAL18924.1), *Helianthus annus* (GenBank accession no. ACX46381.1), *Arabidopsis thaliana* (GenBank accession no. AAM67058.1), *Bacopa monnieri* (GenBank accession no. ACU87560.2), *Picrorhiza kurroa* (GenBank accession no. ABC74567.1), *Camellia oleifera* (GenBank accession no. ADD10719.1), *Medicago sativa* (GenBank accession no. ACX47470.1), *Raphanus sativus* (GenBank accession no. CAA55006.1), *Nicotiana langsdorffii x Nicotiana sanderae* (GenBank accession no. ABV02024.1), and *Verticillium albo-atrum* VaMs.102 (GenBank accession no. EEY17628.1).

and dissimilarities or redundancies in *VMPACA* sequence compared to other plants' ACA.

A phylogenetic tree was constructed using MEGA 4.0 software to estimate the relationships between the putative VMPACA and other plant ACAs. The phylogenetic tree analysis suggested that VMPACA is highly similar to ACA from *Bacopa monnieri* (Figure 2). These two ACAs might originate from a common evolutionary ancestry and it is plausible VMPACA is involved in the mevalonate biosynthesis pathway. However, both ACAs might belong to different thiolase groups with the production of different catalytic end products.

3.2. Analysis of Putative VMPACA Transcript Levels Using Real-Time RT-PCR

3.2.1. Developmentally Regulated Expression of VMPACA. So far, no work corresponding to acetyl-CoA-C-acetyltransferase of vandaceous orchids has been reported. To characterize *VMPACA* transcript accumulation, RNA was extracted from various developing flowers (Figure 3(a)) and subjected to real-time RT-PCR analyses. The expression of *VMPACA* transcripts appeared to be developmentally regulated during flowering. Low level of *VMPACA* expression was detected at an early stage of flower development (bud stage). However, at later stage of development (blooming and full-bloom), a significant ($P < 0.05$) increase in *VMPACA* transcript expression was observed, that is, around 3.5-fold. From previous studies, flower buds were identified to be having the least fragrance emitted or barely detectable [17, 25]. The results suggest that the volatile compounds produced from these transcripts are important in attracting pollinators during full-bloom stage, as had been observed in petunia flowers [10, 26]. Acetyl-CoA:benzylalcohol acetyltransferase (*BEAT*) of *Clarkia breweri* [12] showed the same expression profile where its expression level peaked during anthesis. Besides, alcohol acetyltransferase of *Rosa hybrid* (*RhAAT*) was reported to have maximum expression in the open flower stage [14]. This implies that open flower might be the main stage for the expression of *VMPACA*.

3.2.2. Expression of VMPACA Transcript in Different Floral Tissues. Expression of genes encoding scent-biosynthesis enzymes was previously reported to be spatially regulated during flower development as different fragrance-related transcripts were shown to have different tissue-specific expression patterns whereby some were upregulated solely in the reproductive parts and some in the vegetative parts [27, 28]. In order to investigate the expression pattern of *VMPACA* in different tissues, real-time RT-PCR was also carried out on samples collected from different tissues/organs of *V*. Mimi Palmer. Putative *VMPACA* is predominantly expressed in outer tepal (4.5-fold), followed by lateral inner tepal (3-fold) and lip (2.5-fold) (Figure 3(b)). However, *VMPACA* level is observed to be significantly low ($P < 0.05$) in other tissues, including stem, stalk, root, and leaf. This expression is similar to the other two previously characterized floral-related genes in *V*. Mimi Palmer: phenylacetaldehyde synthase (*VMPPAAS*) involved in the catalysis of phenylalanine decarboxylation [17] and 1-deoxy-D-xylulose 5-phosphate reductoisomerase *(VMPDXR*) which

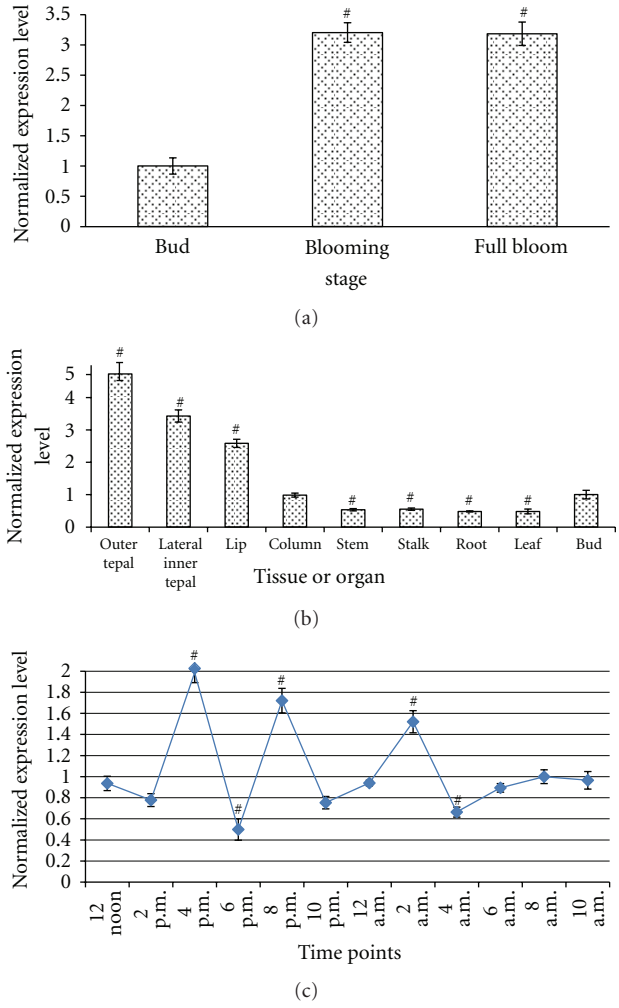

Figure 3: Expression profile of putative *VMPACA* transcript. Real-time RT-PCR of *VMPACA* transcript was performed: (a) at different flower developmental stages, (b) in different tissues of *V*. Mimi Palmer, and (c) expression in full-bloom flower at different time points for 2-hour intervals within 24 hours, respectively. Error bars represent standard deviations on the normalized ratio. #indicates the significant differences with $P < 0.05$.

converts 1-deoxy-D-xylulose 5-phosphate (*DXP*) to 2-C-methyl-D-erythritol 4-phosphate (*MEP*) leading to the formation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [3]. Acetyl-CoA:benzylalcohol acetyltransferase (*BEAT*) and benzoyl-CoA:benzylalcohol benzoyl transferase (*BEBT*) of *Clarkia breweri* which belong to the family BAHD of acyltransferase were found to have the highest expression levels in petal [12, 29]. Moreover, the 3-ketoacyl-CoA thiolase (*PhKAT1*) and acetyl-CoA:coniferyl alcohol acetyltransferase of *Petunia hybrid* (*PhCFAT*) also showed upregulated expression similarly restricted to the petal [10, 15]. It is not uncommon to find petal as the primary site for fragrance emissions in many other plants too such as *Clarkia breweri* [30], snapdragon flowers [31, 32], and petunia [15]. In our histological and scanning electron microscopy work, we found high number of trichomes distributed on the outer and lateral inner tepals of *V*. Mimi Palmer compared to other floral parts (unpublished data). Trichomes have been reported to be the potential sites for volatiles and scent emission [33–35].

3.2.3. Expression of VMPACA Transcript at Different Time Points. Analysis of the expression level of putative *VMPACA* transcript was also further investigated in full-bloom flower at different time points for 2-hour intervals within 24 hours. It showed significantly ($P < 0.05$) highest expression level at 4 p.m. (2-fold), followed by 8 p.m. (1.8-fold) and 2 a.m. (1.5-fold) (Figure 3(c)). Overall, the results indicated that the expression levels of *VMPACA* were affected by the time of the day. Expression pattern of *VMPACA* transcript is comparable to the other two fragrance-related transcripts reported in *V*. Mimi Palmer (*VMPPAAS* and *VMPDXR*) where the highest expression was observed during the dark [3, 17]. An almost similar expression pattern was reported from benzoyl CoA: benzyl alcohol/phenylethanol benzoyltransferase of *Petunia hybrid* (*PhBPBT*) [36]. The current data is still preliminary to suggest that *VMPACA* might be expressed late in the afternoon or approaching night time for the synthesis of isoprenoids in the mevalonate pathway for subsequent emission of sesquiterpenes during the day. More experimental data need to be gathered such as prolonging the duration of the experiment to encompass external time signals of continuous darkness or light exposure.

4. Conclusion

A full length *ACA*-like gene, designated as *VMPACA*, was successfully isolated from a vandaceous orchid, *V*. Mimi Palmer, and molecular characterized. The protein sequence of this *ACA*-like gene is quite similar to other reported plant *ACAs* except it lacks a short-conserved domain at the C-terminal. The fluctuation expression pattern of *VMPACA* transcript by real-time RT-PCR showed that it is developmentally and temporally regulated with predominant expression in outer and lateral inner tepals. The data obtained in this work currently serves as the foundation for future functional studies of *VMPACA* to provide better understanding of fragrance biosynthesis in vandaceous orchids.

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