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Research Article

Sequence Analysis of New Isolated O-methyltransferase Transcript (VMPOMT) from *Vanda* Mimi Palmer

Barudin Mohd-Aiman, Ab. Rahim Mohd-Hairul*,Tan Suat Hian
Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, 26300 Gambang, Kuantan, Pahang, Malaysia.

***Corresponding author**
Ab. Rahim Mohd-Hairul
Email: mhairul@ump.edu.my hairul.bioteknpm@gmail.com

Abstract: *Vanda* Mimi Palmer is an orchid hybrid between *Vanda* Tan Chay Yan and *Vanda tessellata*. This orchid has won several international awards for its fragrance emission characteristic. To date, scientific studies on enzymes as well as their related genes and transcripts that are involved in floral scent production and biosynthesis are barely covered in fragrant orchids especially in vandaceous orchids. The aims of this study were to isolate full open reading frame (ORF) of O-methyltransferase transcript from *Vanda* Mimi Palmer (VMPOMT) and followed by sequence characterisation using bioinformatics tools. The size of successfully isolated full-length transcript of VMPOMT (Genbank accession no: KF278721) is 1,289bp, encoding for a polypeptide of 368 amino acid residues consisting of 1,104bp open reading frame (ORF) flanked by 2bp of 5'-untranslated region (UTR) and 182bp of 3'-UTR including a poly-A tail. Sequence analyses using bioinformatics tools have shown that VMPOMT has a conserved motif for O-methyltransferase group as found in OMT sequences from other plants that might be involved in methylation reactions in fragrance compounds biosynthesis in this orchid. Besides that, VMPOMT is predicted to be localised in cytoplasm instead of plastid due to the absence of chloroplast transit peptide in the sequence. Thus, sequence analyses on VMPOMT using several bioinformatics tools might contribute to the understanding of fragrance biosynthetic pathways in *Vanda* Mimi Palmer as well as in other fragrant orchids.
Keywords: *Vanda* Mimi Palmer; fragrant-orchid; fragrant-related transcript; O-methyltransferase, RACE-PCR, vandaceous orchids.

INTRODUCTION

Orchids belong to Orchidaceae family, the largest flowering plant family that consists of approximately 25,000 to 30,000 species comprises more than 800 genera [1]. Eventhough the actual number of orchid species is still far to be known clearly due to the continuous discovery of orchid species by orchid lovers and botanists, their number of species are estimated to be four times higher than mammals and twice of birds. To date, orchids have evolved from their ancestral characteristics due to selection pressure as well as their adaptation [2]. Orchid species have been hunted since mid-1700s by orchid hunters from all over the world due to their esthetic values as well as exotic characteristics that are mostly loved by orchid lovers. Due to the serious threat faced by wild orchid species, protection and conservation of the species has been seriously initiated by the Royal Botanic Garden in Kew, London. In addition, wild orchids covering estimated about 90% of orchid species from the entire world are categorised as an endangered species in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [3]. Thus, in these days, a lot of orchid hybrids have been produced by crossing orchids from the same genera (interspecific hybrids) as well as from different genera (inter-generic hybrids) for better characteristics including their colours, shapes as well as fragrance for commercialization purpose in floricultural industry.

Fragrant-orchids are commonly sold at much higher price compared to orchids without any fragrance. In nature, volatiles from orchids and other plants are mainly used by the plants to attract specific pollinators to help in their pollination whereby different types of pollinators are attracted to different types of fragrance [4]. It has been reported that orchid species including *Vanda tessellata*, *Phalaenopsis bellina*, *Platanthera chlorantha*, *Polystachya cultriformis* and *Zygopetalum crinitum* are among orchid species that produce and emit strong fragrances [5]. Unfortunately, extensive breeding works to produce attractive colours and shape of orchid flowers cause modern orchid hybrid to lose their fragrance characteristics because in previous traditional breeding, fragrance was not included as the main objective of the new hybrids. To date, some

orchid hybrids with fragrant characteristic are extensively cultivated in Southeast-Asian countries including *Vanda* Mimi Palmer, *Vanda* Small Boy Leong, *Vanda* Johanna Ljunggren and *Vandachostylis* Sri-Siam. The orchid hybrids are propagated through tissue culture approach, mainly in Thailand to be commercialised not only in Thailand and Malaysia but also all around the world [6].

Scientific researches on fragrant-orchids have been started since early 1990s on determination of their volatile components via analysis using Gas Chromatography-Mass Spectrometry (GC-MS) while molecular work on fragrance-related transcripts was only reported in 2006 by identification of several putative fragrance-related transcripts from *Phalaenopsis bellina* through establishment of Expressed Sequence Tags (ESTs) library and followed by characterisation of geranyl diphosphate synthase (GDPS) isolated from the library [7, 8]. Subsequently, some other fragrance-related transcripts, including alcohol acyltransferase (VMPAAT), phenylacetaldehyde synthase (VMPPAAS) and sesquiterpene synthase (VMPSTS) have been isolated by a research group from Malaysia on another orchid hybrid, *Vanda* Mimi Palmer that has won several international awards for its sweet-strong fragrance [9, 10]. Lately, the sesquiterpene synthase (VMPSTS) of *Vanda* Mimi Palmer has been reported to be functionally expressed in *Lactococcus lactis*, a gram positive bacteria that catalyses biosynthesis of multiple sesquiterpene compounds including germacrene D, coupaene and nerolidol [11].

However, understanding on mechanisms underlying the biosynthesis of floral fragrance in monocotyledonous plants, especially in orchids, is still in its infancy. Over the years, the numbers of fragrance-related cDNAs that have been isolated and characterised particularly in vandaceous orchids are only limited to this highly fragrant vandaceous orchid, *Vanda* Mimi Palmer. Among the identified and molecular characterised fragrance-related transcripts were 1-deoxy-D-xylulose 5-phosphate reductoisomerase (VMPDXR), Phenylacetaldehyde synthase (VMPPAAS), cytochrome P450 (VMPCyP450), alcohol acyltransferase (VMPAAT), acetyl-CoA-C-acetyltransferase (VMPACA), and sesquiterpene synthase (VMPSTS) [9, 10, 11, 12]. Recently, a sesquiterpene synthase from *Vanda* Mimi Palmer (VMPSTS) has been successfully cloned, expressed and functional characterised in *Lactococcus lactis*, a gram positive bacterium [11]. Besides that, Hsiao et al. [8] have characterised a geranyl diphosphate synthase (GDPS) from the scented orchid *Phalaenopsis bellina* that catalyses the formation geranyl diphosphate (GDP) which is the main precursors for the production of monoterpene compounds.

Thus, in this paper, we report the sequence of O-methyltransferase (VMPOMT) from *Vanda* Mimi Palmer that might be involved in fragrance biosynthesis. The findings from this study will contribute to the knowledge on fragrance biosynthesis pathway in fragrant orchids.

MATERIALS AND METHODS

Total RNA extraction

Fully open flowers of *Vanda* Mimi Palmer were detached at 10am from their mother plants prior to total RNA extraction. The detached flowers were quickly frozen in liquid nitrogen and stored at -80°C freezer for a temporary period of time, not more than a week. The flowers were ground with liquid nitrogen using mortar and pestle until fine powder formed. Then, cell lysis step was carried out at 65°C using CTAB RNA extraction buffer [12]. After that, a separation step was carried out by mixing vigorously the sample with an equal volume of chloroform:isomylalcohol (24:1) and followed by a centrifugation step at 12,857x*g* for 15 minutes at 4°C. The separation step was repeated twice and final top aqueous phase was subjected to 16 hours precipitation in 2M LiCl. Total RNA was then recovered by a centrifugation step at 12,857x*g* for 30 minutes at 4°C. Recovered total RNA was then washed with 80% (v/v) ethanol, air dried and dissolved in 200µl of autoclaved DEPC-treated water. The isolated total RNA was then stored at 4°C for further use.

Full ORF isolation of VMPOMT transcript

Partial-length transcript encoding O-methyltransferase was identified from the floral cDNA library of *Vanda* Mimi Palmer [12]. The 5'- and 3'-regions of the transcripts were isolated using a combination of the SMARTer RACE cDNA Amplification Kit (Clonetech, USA) and Advantage 2 polymerase mix (Clonetech, USA) with gene-specific primers and Universal Primer Mix (UPM). The open reading frame (ORF) of each transcript was isolated by PCR amplification using gene specific primers (Table 1) and 5'-RACE-Ready cDNA as template. The PCR reaction was performed by 31 cycles of denaturation at 95°C for 30 seconds; annealing at 65°C for 30 seconds, and extension at 72°C for 3 minutes in a Mastercycler Gradient (Eppendorf, Hamburg, Germany). The amplified PCR products were purified using GeneAll® ExpinTM PCR SV purification kit (GeneAll, Korea), cloned into pGEM-T-easy vector (Promega, USA) and sequenced using universal SP6 and T7 primers (Macrogen, Korea).

Sequence analysis of VMPOMT transcript

The full sequence of VMPOMT transcript was subjected to BLASTX analysis for identification of putative function of the transcript by comparing with its homologous protein sequences from other organisms that are available at the NCBI GenBank database [13]. The transcript sequence was further translated into amino acid sequence in selected frame using BioEdit

software [14]. The translated amino acid sequence of VMPOMT was then subjected to BLASTP analysis at the NCBI GenBank database [13] for identification of putative function of the protein by comparing with available protein sequences in the database. After that, homologous protein sequences that show high identity with VMPOMT were retrieved from the database and subjected to Clustal W Multiple alignment using BioEdit software for identification of conserved regions. In addition, the deduced amino acid sequence of VMPOMT with all the retrieved protein sequences were subjected to phylogenetic tree construction using MEGA 6.0 software [15] to determine the relationship of VMPOMT sequence with its homologous sequences from other plants. Besides that, VMPOMT sequence was subjected to prediction of protein molecular weight, isoelectric charge, subcellular localisation as well as motifs identification. The analyses were carried out using multiple online bioinformatics servers including ScanProsite [16], ProP 1.0 server [17], TMHMM server 2.0 [18], SignalP 4.1 server [19], ChloroP 1.1 server [20], TargetP 1.1 server [21], iPSORT server [22], Net NES 1.1 server [23], Net Phos 2.0 server [24] and Net PhosK 1.0 server [25].

RESULTS AND DISCUSSION

The full-length VMPOMT transcript (Genbank accession no: KF278721) comprises 1,289bp (Figure 1), encoding for a polypeptide of 368 amino acid residues consisting of 1,104bp open reading frame (ORF) flanked by 2bp of 5'-untranslated region (UTR) and 182bp of 3'-UTR including a poly-A tail. The predicted molecular weight of this protein is 41.6kD with an isoelectric point (pI) of 5.74. The BLASTP analysis (NCBI) shows that the deduced amino acid sequence of VMPOMT is 42-47% homologous to OMT sequences from other plants such as *Rosa* hybrid cultivar (AAM23004.1), *Rosa chinensis* (AEC13057.1), *Rosa hugonis* (CAJ65638.1), *Rosa gallica* (CAJ65624.1) and *Rosa canina* (CAJ65610.1).

VMPOMT has a conserved motif for O-methyltransferase group as found in OMT sequences from other plants (Figure 2). O-methyltransferase (OMT) is the enzyme which catalyses the transfer of a methyl group from S-adenosyl-L-Met (SAM) to a hydroxyl functionality [26]. The methylation process can occur on both small and macro molecules for various functional and regulatory purposes. O-methylation in plants helps in various functions like the synthesis of lignin, can tolerate to stress condition and give resistance to plant diseases [27]. So far, OMTs in plants are well characterised, but the role of OMTs in microbes remained to be understood eventhough certain bacterial and fungal strains have been reported to be involved in methylation of other compounds for biosynthesis of antibiotic and aflatoxins [28]. Besides that, OMTs are closely related to other plant methyltransferases that have substrates range from isoflavones to phenylpropenes. In previous studies on OMTs, the activity of OMTs is varied due to the fact that methylation function can be different depending on different type of substrate, especially for several phenolic substrates, including 3,5-dihydroxytoluene (orcinol), 3-methoxy,5-hydroxytoluene (orcinol monomethyl ether), 1-methoxy,2-hydroxy benezene (guaiacol) and eugenol [29]. Generally, O-methyltransferases are involved in catalysing the last two steps of the biosynthetic pathway leading to the phenolic methyl ether 3,5-dimethoxytoluene (DMT) as previously reported by the study on OMTs on orcinol, as an important substrate for this OMT enzymes in rose varieties [30]. The activity of OMTs, mainly for OOMT can be increased directly related to membranes during petal development of roses, suggesting that the scent biosynthesis pathway catalyzed by these enzymes (OMTs) might be directly linked to the cells' secretory machinery.

The phylogenetic tree of VMPOMT (Figure 3) shows that VMPOMT is clustered together with OMT proteins from *Setaria italica* (XP_004975403.1) and *Rosa chinensis* (AEC13057.1). From the phylogenetic tree, VMPOMT has shown a direct phylogenetic linkage with both OMTs from *Setaria italica* and *Rosa chinensis*. Eventhough, OMT from *Vanda* Mimi Palmer has different order, family and subfamily in scientific classification with both organisms, but they are originated from the same kingdom, *Plantae* and grouped in angiosperms. Besides that, analysis on VMPOMT protein sequence using ScanProsite [16], a bioinformatics online tools has shown the presence of a phosphatidylinositol-specific phospholipase x-box domain (aa102-139) as listed in Table 2 and highlighted in Figure 2. For this condition, VMPOMT might have homologous amino acid sequences with other amino acid sequences from the previous studies on different substrates, reacting with the methylation of this type of enzyme, O-methyltransferase. Besides that, sequence analysis using ScanProsite online tool [16] has detected the presence of four weak motifs such as casein kinase II phosphorylation site, N-myristoylation site, protein kinase c phosphorylation site and N-glycosylation site as listed in Table 2 and highlighted in Figure 2.

Subcellular localisation analyses using TMHMM 2.0 [18], SignalP 4.1 [19], ChloroP 1.1 [20], TargetP 1.1 [21] and iPSORT [22] servers have shown that the deduced amino acid sequence of VMPOMT protein is predicted to have no signal peptide cleavage site, propeptide cleavage site (Arginine/Lysine), transmembrane helices, secretory pathway signal peptide, chloroplast transit peptide, N-terminal presequences as well as mitochondrial targeting peptide due to the score of less than 0.5 (Table 3). From the analyses, VMPOMT protein is predicted to be localised in cytosol and not being transported from cytosol to plastid or from plastid to cytosol since it is predicted to have no chloroplast transit peptide that responsible to transport the protein from plastid to cytosol. In addition,

VMPOMT gene might definitely located in nuclear DNA instead of plastid DNA like fragrance-related genes in terpenoid pathway that specifically involve in biosynthesis of monoterpene compounds such as linalool, ocimene, terpeneol and β-pinene [31].

Meanwhile for nuclear transport signal analysis using NetNES 1.1 server has shown the presence of leucine-rich amino acid residue at position 43 while isoleucine-rich amino acid residue at position 45 and 67 and thus VMPOMT is predicted to be transported from nucleus cell to cytoplasm through nuclear pore in the presence of nuclear export signal as highlighted in Figure 2 and described in both Figure 4 and Table 4 [23]. VMPOMT protein sequences are predicted to have a leucine and isoleucine-rich motif which show the structural motif, comprised of repeating a few amino acid stretches that are abnormally rich in the hydrophobic amino acid leucine and isoleucine residues at position of 43, 45 and 67, respectively. Frequently, this kind of amino acid repeats are normally involved in the formation of protein-protein interactions [32].

In addition, VMPOMT is predicted by the NetPhos 2.0 server [24] to have 9 serine sites, 7 threonine sites and only one tyrosine site (Figure 5). Thus, VMPOMT might have different function in other signalling process regarding to different amino acid residues on its amino acid sequences, not only limited for its specific function of methylation to other specific substrates as stated in various studies that have been done so far [33]. We postulate that VMPOMT revealed the presence of putative multiple phosphorylation sites as shown in other OMTs protein sequences like cathecol-OMTs [34]. This putative OMT sequence from *Vanda* Mimi Palmer might be regulated by reversible phosphorylation of particular serine and threonine residues. In addition, conserved domain of phosphorylation sites are also found in other OMTs reported from other plants, eventhough their location of residues are different which might be reflected by their slightly different functional mechanism of enzymatic activities with their respective substrates [35]. Interestingly, information on protein kinase C specific phosphorylation site that is predicted to be present at aa_{42-44} (refer Figure 2 and Table 2) in VMPOMT protein sequence might be important to understand its exact role in protein post-translational modification [25]. However, phosphorylation of protein is a very complex biological process *in vivo* [36]. Thus, the result of predicted potential site (aa_{42-44}) of kinase specific phosphorylation on VMPOMT protein sequence is not enough to determine the exact phosphorylation status without a proper *in vitro* experimental work. Further *in vitro* studies need to be done to get better understanding on how the *in vivo* environment of VMPOMT protein look like in kinase-specific phosphorylation prediction.

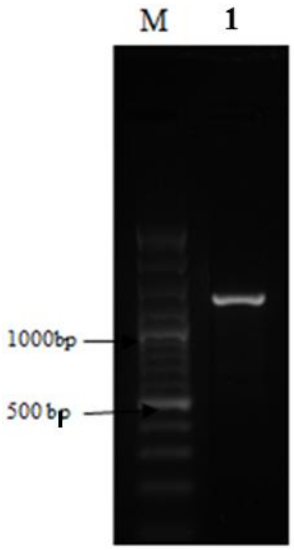

Fig-1: PCR product of open reading frame (ORF) of fragrance-related transcript, VMPOMT. The PCR product was electrophoresed on 1.2 % (w/v) agarose gel. Lane M: 100 bp DNA marker (Vivantis, Malaysia); Lane 1: *Vanda* Mimi Palmer O-methyltransferase (VMPOMT).

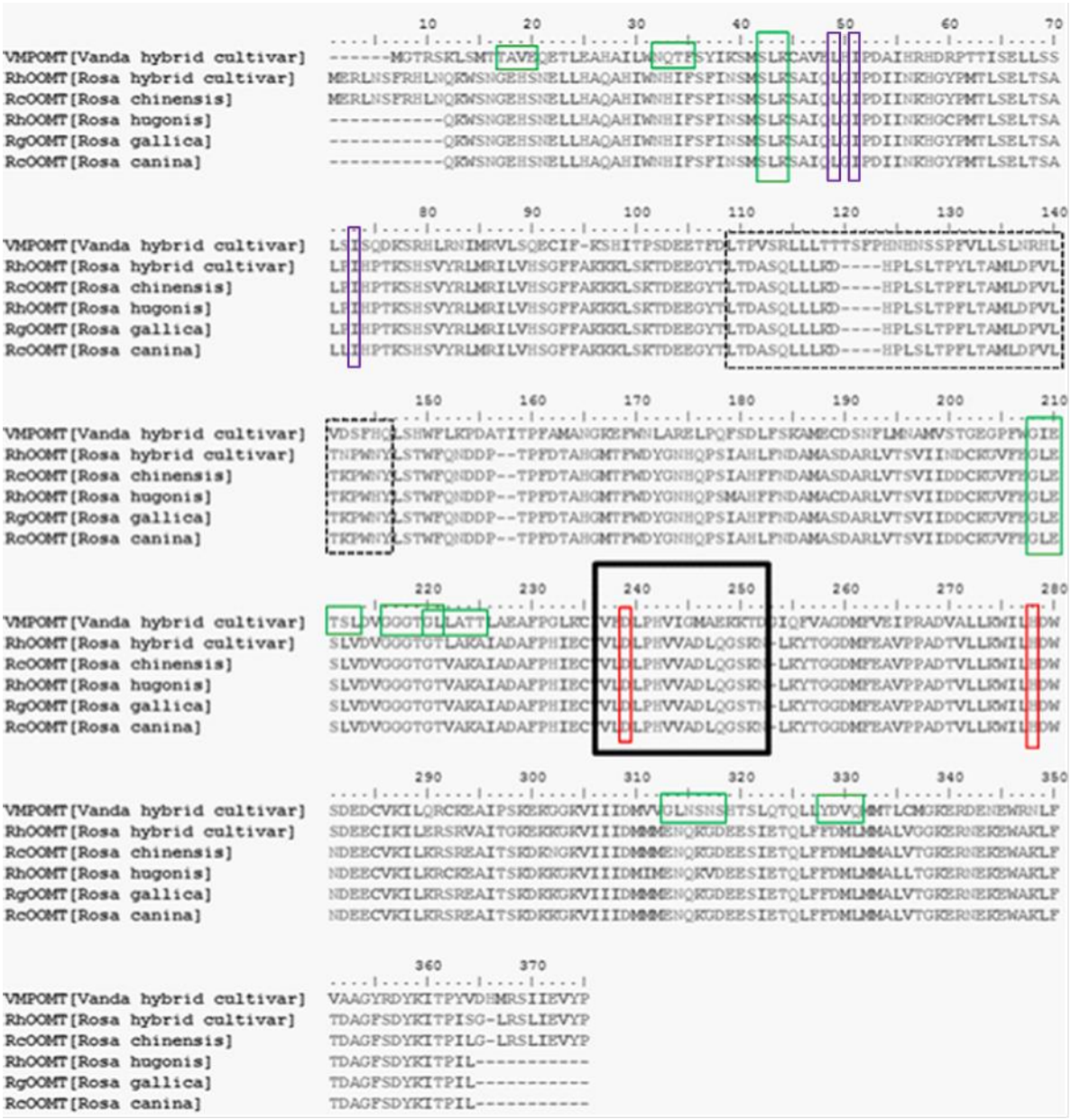

Fig-2: **Alignment of VMPOMT with other closely related protein sequences retrieved from the NCBI GeneBank database. The VMPOMT amino acids sequence is aligned with orcinol O-methyltransferase of *Rosa* hybrid cultivar (AAM23004.1), orcinol O-methyltransferase-like protein of *Rosa chinensis* (AEC13057.1), putative orcinol O-methyltransferase of *Rosa hugonis* (CAJ65638.1), putative orcinol O-methyltransferase of *Rosa gallica* (CAJ65624.1) and putative orcinol O-methyltransferase of *Rosa canina* (CAJ65610.1). O-methyltransferase attachement site is highlight in black box (VLDLPHVVAGLQGSKN) shows a conserved motif in the deduced protein sequence of VMPOMT. Note: S-adenosyl-L-methionine binding site at aa_{239} (red box); active site (proton acceptor) at aa_{278} (red box); Phosphatidylinositol-specific phospholipase X-box domain ($aa_{109-146}$) (dotted-line black box); Casein kinase II phosphorylation site (aa_{17-20}) (green box); N-glycosylation site (aa_{32-35}) (green box); Protein kinase C phosphorylation site (aa_{42-44}) (green box); N-myristoylation site ($aa_{208-213}$, $aa_{216-221}$, $aa_{220-225}$, $aa_{313-318}$) (green boxes); Tyrosine Phosphorylation binding site ($aa_{328-331}$) (green box); Predicted leucine amino acid repeats (aa_{43}) (purple box); Predicted isoleucine amino acid repeats (aa_{45}, aa_{67}) (purple box).**

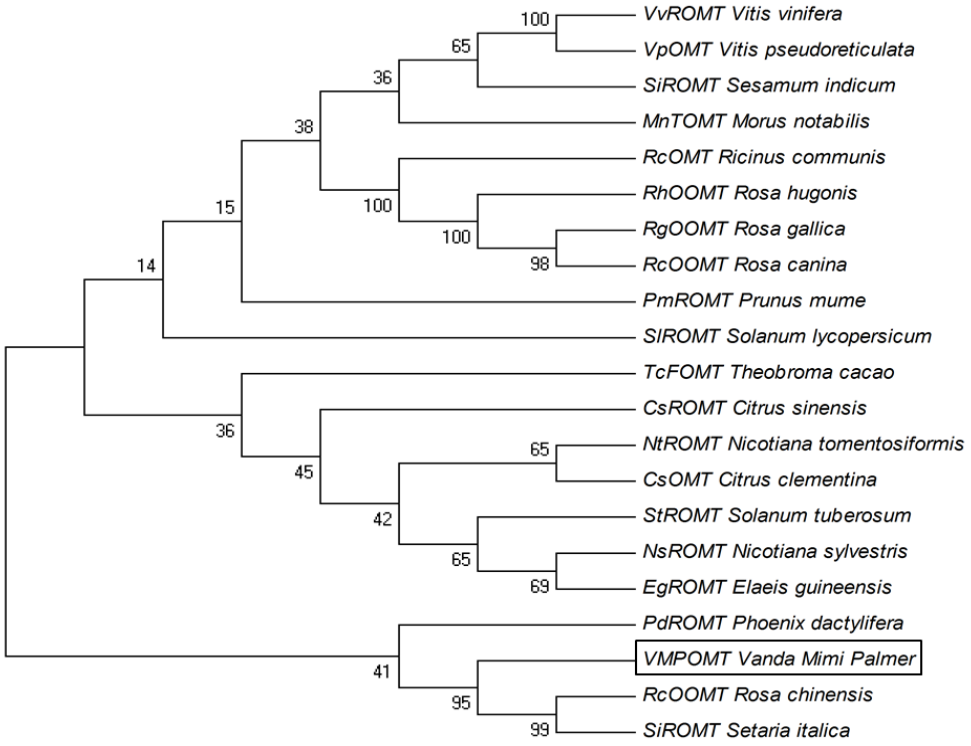

Fig-3: Phylogenetic tree of VMPOMT with homologous proteins. The phylogenetic tree is constructed by MEGA software version 6.0 using amino acid sequence of VMPOMT (as boxed) with the homologous proteins available in GenBank database (NCBI) including trans-resveratrol di-O-methyltransferase-like isoform X1 of *Phoenix dactylifera* (XP_008783119.1), trans-resveratrol di-O-methyltransferase of *Vitis vinifera* (XP_002278294.1), O-methyltransferase of *Vitis pseudoreticulata* (AEF97347.1), trans-resveratrol di-O-methyltransferase-like of *Nicotiana tomentosiformis* (XP_009602136.1), trans-resveratrol di-O-methyltransferase-like of *Nicotiana sylvestris* (XP_009780941.1), trans-resveratrol di-O-methyltransferase-like of *Elaeis guineensis* (XP_010916033.1), trans-resveratrol di-O-methyltransferase-like of *Solanum lycopersicum* (XP_004235335.1), trans-resveratrol di-O-methyltransferase-like of *Solanum tuberosum* (XP_006366298.1), trans-resveratrol di-O-methyltransferase-like of *Prunus mume* (XP_008229109.1), trans-resveratrol di-O-methyltransferase-like of *Citrus sinensis* (XP_006469069.1), hypothetical protein of *Citrus clementina* (XP_006446728.1), O-methyltransferase, putative of *Ricinus communis* (XP_002525159.1), orcinol O-methyltransferase of Rosa hybrid cultivar (AAM23004.1), orcinol O-methyltransferase-like protein of *Rosa chinensis* (AEC13057.1), Flavonoid o-methyltransferase related of *Theobroma cacao* (XP_007043718.1), putative orcinol O-methyltransferase of *Rosa hugonis* (CAJ65638.1), trans-resveratrol di-O-methyltransferase-like of *Sesamum indicum* (XP_011091580.1), Tabersonine 16-O-methyltransferase of *Morus notabilis* (XP_010093978.1), 5-pentadecatrienyl resorcinol O-methyltransferase-like of *Setaria italica* (XP_004975403.1), putative orcinol O-methyltransferase of *Rosa gallica* (CAJ65624.1) and putative orcinol O-methyltransferase of *Rosa canina* (CAJ65610.1).

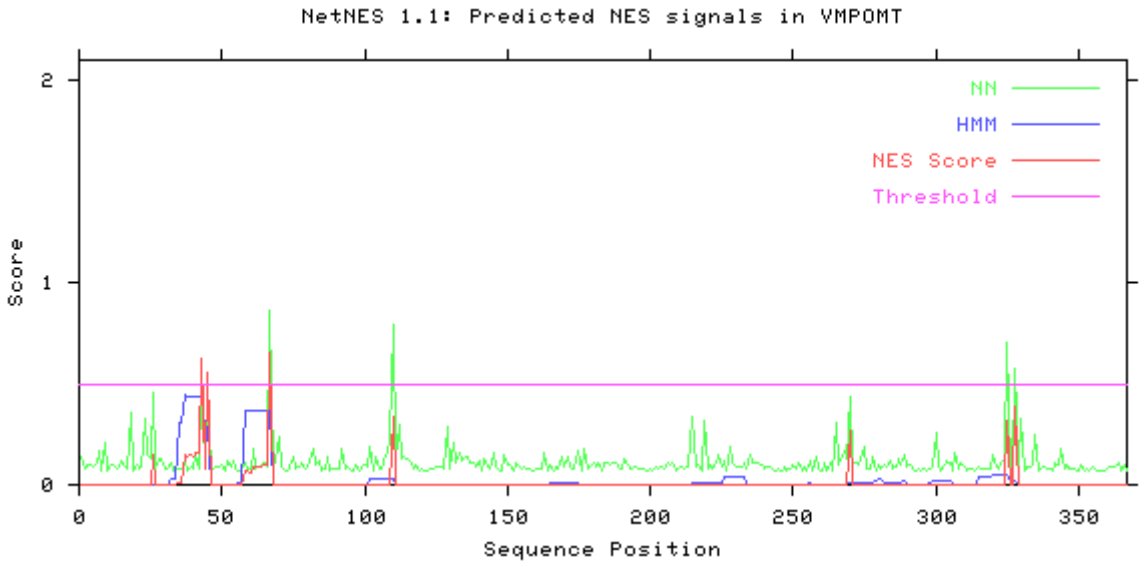

Fig- 4: Prediction of leucine and isoleucine amino acid repeats on VMPOMT protein sequences using NetNES 1.1 Server. NES score (red line) was used to determine the predicted leucine and isoleucine amino acid repeats that exceeded the threshold line (pink line).

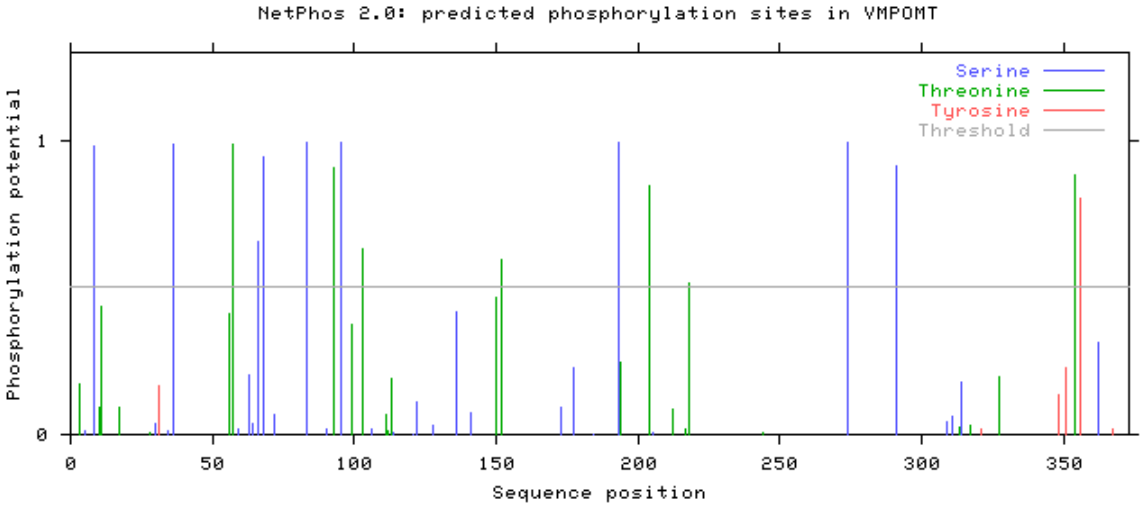

Fig-5: Predicted phosphorylation sites on VMPOMT protein sequences using NetPhos 2.0 server. The lines exceeded the phosphorylation potential after the threshold line (grey line) was selected to be predicted phosphorylation sites. Note: Serine residue (blue color) has nine sites; Threonine residue (green color) has seven sites; Tyrosine residue (red color) has only one site.

Table-1: Gene specific primers used in 5'-region and full open reading frame (ORF) isolation of *Vanda* Mimi Palmer O-methyltransferase (VMPOMT) transcript.

PCR product	Primer Sequences	Annealing Temperature
VMPOMT ORF	VMPOOMT ORF Forward: 5'-CATGACGACTGCCGTGGAACAAGAAACCC-3' VMPOOMT ORF Reverse: 5'-GCTACATTCACAATGCTCAGCCATAGC-3'	65ºC
VMPOMT 5'-region	5'-GAGACAGGACGCGCATGATGCTGCGAAGG-3'	65ºC

Table 2: Predicted binding sites on aligned VMPOMT protein sequences with other OMT protein sequences annotated from NCBI GenBank database using BLASTP program.

No.	Name of binding sites	Amino acid location
1.	O-methyltransferase attachment site	$aa_{237-252}$
2.	S-adenosyl-L-methionine binding site	aa_{239}
3.	Proton acceptor (active site)	aa_{278}
4.	Phosphatidylinositol-specific phospholipase X-box domain	$aa_{109-146}$
5.	Casein kinase II phosphorylation site	aa_{17-20}
6.	N-glycosylation site	aa_{32-35}
7.	Protein kinase C phosphorylation site	aa_{42-44}
8.	N-myristoylation site	$aa_{208-213}$, $aa_{216-221}$, $aa_{220-225}$, $aa_{313-318}$
9.	Tyrosine Phosphorylation binding sites	$aa_{328-331}$

Table 3: Predicted results of VMPOMT protein sequences using bioinformatic tools.

Bioinformatics Analysis Tools	Predicted results			
ChloroP 1.1 Server	*Score*	*cTP*	*CS-score*	*cTP-length*
	0.431	-	4.143	33
TargetP 1.1 Server	*cTP*	*mTP*	*Sp*	*Others*
	0.066	0.133	0.050	0.797

Note: cTP: Chloroplast transit peptide; CS: Cleavage site; mTP: Mitochondrial targeting peptide; SP: Signal peptide. (-) means cTP (chloroplast transit peptide) is not available in VMPOMT protein sequence.

Table 4: Predicted result of leucine and isoleucine amino acid repeats on VMPOMT protein sequence using NetNES 1.1 Server.

Sequence	Position	Residue	ANN	HMM	NES	Predicted
VMPOMT	aa_{43}	Leucine	0.477	0.441	0.627	Yes
	aa_{45}	Isoleucine	0.454	0.289	0.557	Yes
	aa_{67}	Isoleucine	0.858	0.367	0.651	Yes

Note: ANN: Artificial neural network; HMM: Hidden Markov models; NES: Nuclear export signals. 'Yes' means the predicted leucine and isoleucine repeats were present in VMPOMT protein sequences.

CONCLUSION

Full-length transcript sequence of VMPOMT was successfully isolated from a vandaceous orchid, *Vanda* Mimi Palmer, and molecular characterised by sequence analyses using multiple bioinformatics tools. The data obtained from this work might serve as the foundation for future functional studies to provide better understanding on fragrance biosynthesis mechanisms in vandaceous orchids.

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