

Cloning of 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Gene from *Vanda Mimi Palmer* and Its Heterologous Expression in *Escherichia coli*

Seow Ling Teh¹, Janna Ong Abdullah¹, Parameswari Namasivayam² & Gwendoline Cheng Lian Ee³

¹ Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Selangor Darul Ehsan, Malaysia

² Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Selangor Darul Ehsan, Malaysia

³ Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, Selangor Darul Ehsan, Malaysia

Correspondence: Janna Ong Abdullah, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor Darul Ehsan, Malaysia. Tel: 60-389-466-697. E-mail: janna@biotech.upm.edu.my

Received: May 9, 2012 Accepted: June 14, 2012 Online Published: August 15, 2012

doi:10.5539/jps.v1n2p140

URL: <http://dx.doi.org/10.5539/jps.v1n2p140>

Abstract

Plant 3-hydroxy-3-methylglutaryl-CoA-reductase (HMGR) is involved in the conversion of HMG-CoA into mevalonate (MVA), which yields a biologically active isoprenoid precursor, isopentenyl pyrophosphate (IPP) unit. To date, heterologous expression of HMGR isolated from orchidaceae family has not been reported. The aims of this study were to isolate, clone, over-express and functionally characterize the cDNA encoding a 3-hydroxy-3-methylglutaryl-CoA-reductase of *Vanda Mimi Palmer* (*VMPHMGR*) in *Escherichia coli* strain BL21 (DE3) pLysS. The deduced *VMPHMGR* contains an open reading frame (ORF) of 1689 bp and generates a protein of 562 amino acids with a calculated molecular mass of 59780 Da and a predicted pI value of 6.63, which is 76% identical to other plant HMGRs. Expression analysis of *VMPHMGR* transcript by real-time RT-PCR showed that it was differentially regulated. Primers with appropriate restriction sites were used to amplify and facilitate in-frame cloning of the *VMPHMGR* into pET32(a). The expression of *VMPHMGR*, in *E. coli*, fused to N-terminal thioredoxin (Trx-Tag), S-Tag and His-Tag fusion proteins in pET32(a) yielded a partially soluble recombinant protein. This expressed *VMPHMGR* was subjected to functional enzymatic assay and GCMS analysis of the end products detected dehydromevalonic lactone and pantolactone, which were derivatives of mevalonate lactone.

Keywords: isoprenoid, mevalonate pathway, *Vanda Mimi Palmer*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, vandaceous orchid

1. Introduction

Plants have the capability to synthesize many volatile metabolites, either primary or secondary, with varied functions (Goff & Klee, 2006). Floral scent has a significant impact in plant reproduction as it attracts a variety of animal pollinators, especially insects. Most volatile compounds are derived from the three major biosynthesis pathways: phenylpropanoids, fatty acid derivatives and terpenoids (Pichersky, Noel, & Durareva, 2006). In plants, two distinct pathways, the methyl- β -erythritol 4-phosphate (MEP) and mevalonate (MVA) pathways are involved in the biosynthesis of isoprenoid compounds (Rohmer, Knani, Simonin, Sutter, & Sahn, 1993).

Detailed chemical analysis of volatile compositions and the identification of secondary metabolites related to scent production have led to the discovery of a number of novel flower scent-related genes including 3-hydroxy-3-methylglutaryl-coenzyme A reductase [HMGR] (Guterman et al., 2002; Knudsen & Gershenzon, 2006; Mohd-Hairul, Namasivayam, Gwendoline, & Janna, 2010) and terpene synthase (Dudavera et al., 2003). HMGR catalyzes the conversion of HMG-CoA into mevalonate (MVA) to yield a biologically active isoprenoid precursor, isopentenyl pyrophosphate (IPP) unit (Friesen & Rodwell, 2004; Jiang et al., 2006; Wang et al., 2007), which serves as the basis for the biosynthesis of molecules used in many metabolic processes such as steroid biosynthesis (Pitera, Paddon, Newman, & Keasling, 2007), terpenoid biosynthesis (Cheng et al., 2007) and

carotenoid biosynthesis (Tada et al., 1990). The isolation and molecular characterization of plant HMGRs have been performed in both monocots and dicots such as *Arabidopsis thaliana* (Dudareva et al., 2005), rice (Ha, Lee, Kim, & Hwang, 2001), *Camptotheca acuminata* (Maldonado-Mendoza, Vincent, & Nessler, 1997), *Ganoderma lucidum* (Shang et al., 2008), and hazel (Wang et al., 2007).

So far, HMGR gene has not been reported from vandaceous orchids or any orchid genera. Orchidaceae is one of the largest flowering plant families and evolutionary-wise, they are highly complex with varied floral structures. HMGR information gathered from this highly complex plant would enhance our understanding of this gene and its product, which is involved in the first rate-limiting step for isoprenoid biosynthesis. Therefore, in this paper we report our attempt in the isolation, cloning and functional characterization of the *VMPHMGR* protein in *Escherichia coli*. In our previous study, a *Vanda Mimi Palmer* expressed sequence tag database (VMPEST) (Teh, Chan, Janna, & Namasivayam, 2011) was generated to facilitate the identification of potential fragrance-related genes in this vandaceous orchid. From the VMPEST, a clone bearing high sequence homology to published HMGR was selected for this study. This finding of HMGR in orchid could provide an alternative resource for the enzyme and this might also increase the value of orchids besides being regarded as ornamental plants only. On top of that, it may prove interesting to elucidate potential compounds that might have synergetic effects with this enzyme *in vivo*.

2. Material and Methods

2.1 Sample Collection

Vanda Mimi Palmer potted plants were bought and maintained at the United Orchid Plantation nursery, Rawang, Selangor Darul Ehsan, Malaysia, under a 20-30% shade with temperature ranging between 25-30°C. The plants were brought and placed outside the laboratory building, which has almost similar conditions as the nursery, prior to samples collection (Chan, Janna, Namasivayam, & Maziah, 2009; Mohd-Hairul, 2010). The VMP floral samples for the RNA work were harvested according to their diameters: bud (0.8-1.4 cm), blooming (3.0-5.0 cm) and full-bloom (5.0-7.0 cm) as described by Chan et al. (2011). Various tissues (sepals, petals, lips, stems, stalks, columns, roots and leaves) were also collected and stored at -80°C until ready to be used for total RNA extraction.

2.2 Total RNA Extraction

The mortar, pestle and spatula used in the total RNA extraction were baked at 200°C for 2 to 3 hours prior to use to ensure removal of contaminating RNase. Total RNA were extracted from all frozen floral tissues, as mentioned earlier, using the standard CTAB protocols of Yu and Goh (2000).

2.3 Full Length cDNA Isolation of *VMPHMGR* from *Vanda Mimi Palmer*

A partial cDNA sequence that encodes a putative 3-hydroxy-3-methylglutaryl-CoA-reductase of *Vanda Mimi Palmer* (*VMPHMGR*) (GenBank accession no. GW392501) was selected from a *Vanda Mimi Palmer* floral EST database (Teh et al., 2011). This cDNA transcript lacks the 5'-region. The RACE cDNA Amplification Kit (Clontech, CA, USA) and Advantage 2 Polymerase Mix (Clontech, CA, USA) were used according to the manufacturers' instructions to isolate the full *VMPHMGR* cDNA sequence. A gene specific primer (5'-GGATTTCCCTCTTCCCTCGATCCAA-3'), and a universal primer mix (UPM) made up of a long primer: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' and a short primer: 5'-CTAATACGACTCACTATAGGGC-3', were used to isolate the 5'-end of the *VMPHMGR* transcript with a PCR cycling conditions of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 3 minutes, for a total of 25 cycles using a gradient thermal cycler (Eppendorf, Hamburg, Germany). The RACE PCR product was subsequently gel purified using a gel extraction kit (Qiagen, CA, USA) and cloned into the yT & A cloning vector (Yeastern Biotech, Taipei, Taiwan) prior to sequencing (Bioneer, Daejeon, Korea). The sequence obtained was analysed using the BLASTN, BLASTX, CAP CONTIG, Biology WorkBench 3.2 (<http://workbench.sdsc.edu/>), BioEdit version 7.0.9 software and ClustalW.

In order to obtain the full *VMPHMGR* transcript sequence, a pair of gene specific primer was designed at the 5'-end of the forward (5'-GGGCAAGCAGTGGTATCAACGCAGA-3') and reverse (5'-GTCATCAAGTCGAAGCAGCTTTGGTC-3') primers. The PCR reaction was pre-denatured at 94°C for 3 minutes, denatured at 94°C for 45 seconds, annealed at 65°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. The PCR product was then purified, cloned into the yT&A vector (Yeastern Biotech, Taipei, Taiwan), and then transformed into competent *E. coli* strain *DH5α* cells. Plasmid DNA was isolated from the transformed cells and sequenced. Sequence analysis was performed using the BLASTX, ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>), BioEdit version 7.0.9 (Tom

Hall, Ibis Biosciences 1999), WoLF PSORT (<http://wolfsort.org/>), iPSORT (<http://ipsort.hgc.jp/>) and ExPASy (<http://www.expasy.ch/tools/>). MEGA 4.0 (<http://www.megasoftware.net/>) was used to infer the phylogenetic tree and estimate the relationship between the putative VMPHMGR and other plants' HMGRs.

2.4 Real-time Quantitative RT-PCR

The first-strand cDNA used in the quantitative real-time RT-PCR reaction was synthesized using a reverse transcription kit (Qiagen, CA, USA). Verification of differentially expressed genes was performed using a Bio-Rad iCycler (iCycler iQ5 Multicolor Real-Time PCR Detection System) and 2x SYBR Green I Hot-Start real-time PCR-Mix (GeneCraft, Cologne, Germany). Each cDNA sample was diluted 10 folds prior to use in the real-time quantitative RT-PCR reaction. The thermal cycling conditions were as follows: activation of DNA polymerase at 95°C for 1 minute, followed by 40 cycles of amplification at 95°C for 10 seconds, 59°C for 20 seconds, 72°C for 20 seconds. Four technical replicates were carried out for the reaction of each biological replicate. A master mix without the cDNA template was used as a negative control in the reaction. The relative gene expression level was calculated using the geometric mean method of Vandesompele et al. (2002) with actin (GenBank accession no. AF246716), cyclophilin (GenBank accession no. GW393531) and tubulin (GenBank accession no. GW393564) as reference transcripts (as described by Mohd-Hairul, 2010) for comparison of the *VMPHMGR* expression levels at different time points (2-hour intervals within a 24-hour cycle), at different developmental stages (buds, blooming and full-bloom flower), and in different types of tissues/organs (sepals, petals, lips, stems, stalks, columns, roots and leaves). The details of the primers used in the real-time RT-PCR experiment are shown in Table 1. Statistical significance of the relative expression levels of each gene transcript was assessed using an analysis of variance (ANOVA) at $p < 0.05$ (Karlen, McNair, Perseguers, Mazza, & Mermod, 2007).

Table 1. Primers used in real-time RT-PCR

Primer identity (amplicon size)	GenBank accession	Primer sequence
Actin (236bp)	AF246716	Forward: 5'-CAGTGTTTGGATTGGAGGTTTC-3' Reverse: 5'-CCAGCAGCAGTCAGGAAAA-3'
Tubulin (227bp)	GW393564	Forward: 5'-CTCCCGCATTGACCATAAAT-3' Reverse: 5'-GGAACCACACCCAAACTCTC-3'
Cyclophilin (200bp)	GW393531	Forward: 5'-TTGGATGTCGTGAAGGCAAT-3' Reverse: 5'-CAACACAAGAAGATAGCACAGCA-3'
Putative <i>VMPHMGR</i> (182bp)	GW392501	Forward: 5'-GAAAGGCGCAAACATGGAGTCT-3' Reverse: 5'-CAAGTGAAGCAGCTTTGGTCA-3'

Identities of the primers designed for *VMPHMGR* and the endogenous controls used in the real-time quantitative RT-PCR experiments are summarised in Table 1. The amplicon size for each primer set is indicated in parentheses.

2.5 Heterologous Expression of *VMPHMGR* in *Escherichia coli*

A mitochondrial targeting peptide was found in *VMPHMGR* using the iPSORT and SignalP 3.0 programme (Bendsten, Nielsen, Heijne, & Brunak, 2004). Hence, the first 30 amino acids were excluded in the cloning of *VMPHMGR* into the pET32(a) vector for expression analysis in *E. coli*. This contributed to a recombinant truncated protein with a calculated molecular weight of 70570 Da [including the Trx-Tag, S-Tag and His-Tag fusion proteins at the N-terminal]. The truncated *VMPHMGR* cDNA fragment was amplified with a forward primer: 5'-TAGAATTCTTCCGCCGCGCCGCAT-3' and reverse primer: 5'-GATCTCGAGTCAAGTGAAGCAGC-3', with *EcoRI* and *XhoI* restriction sites (underlined) engineered at the 5' end of the forward and reverse primers, respectively. The PCR amplification was carried out in a gradient thermal cycler (Eppendorf, Hamburg, Germany) using the KOD Hot Start DNA polymerase (Novagen, Darmstadt, Germany). The reaction mixture was first heated at 95°C for 2 minutes, amplified for 35 cycles at 95°C for 30 seconds with an annealing temperature of 65°C for 30 seconds and an extension at 72°C for 90 seconds. This was followed by a final extension at 72°C for 10 minutes. The PCR product was then digested with

EcoRI and *XhoI*, purified and ligated into the pET32(a) vector. Hereafter, the construct was designated as pET32(a)::VMPHMGR and was verified by colony PCR, restriction enzyme digestion and sequencing prior to heterologous expression. The transformation of pET32(a)::VMPHMGR construct into *E. coli* strain BL21(DE3) pLysS was carried out following the protocol provided in the Novagen BL21(DE3) pLysS kit (Merck, Darmstadt, Germany).

2.6 Recombinant Protein VMPHMGR Expression and Purification

Two millilitres of an overnight culture of *E. coli* harbouring the pET32(a)::VMPHMGR were inoculated into 50 ml of fresh Luria Bertani (LB) broth supplemented with 34 µg/ml chloramphenicol and 50 µg/ml ampicillin. The culture was grown with shaking at 200 rpm and 37°C to an OD₆₀₀ of approximately 0.5-0.7 prior to induction with 0.5 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG). This was followed by further incubation for 4 hours at 30°C with shaking at 220 rpm.

The cell culture was harvested by centrifugation at 4000 x g for 15 minutes at 4°C. Extraction of the targeted protein was carried out using the BugBuster Protein Extraction Reagent (Merck, Darmstadt, Germany). The wet weight of the bacterial cells was obtained and the BugBuster Reagent was added per gram of bacterial cells. Approximately 0.5 µl of Benzonase (Merck, Darmstadt, Germany) was added and the cells mixture was incubated at room temperature on a shaking platform at 60 rpm for 20 minutes. The insoluble proteins and cell fragments were precipitated by centrifugation at 8000 x g for 20 minutes at 4°C while the supernatant (soluble proteins) was further centrifuged at 8000 x g for 10 minutes at 4°C. The soluble crude VMPHMGR sample was concentrated by centrifugation at 10,000 x g for 45 minutes at 4°C using an Amicon Ultra-4 centrifugal filter unit of a 30 kDa protein exclusion size (Millipore, TechLink, Singapore).

Subsequently, the recombinant VMPHMGR protein was purified using the His SpinTrap kit (GE Healthcare, Uppsala, Sweden). The column was equilibrated with five column volumes (5 CVs, 3 ml in total) of binding buffer (1X phosphate buffer stock solution and 20 mM imidazole, pH 7.4). Approximately 600 µl (1 CV) of binding buffer were added, followed by centrifugation for 1 minute at 1000 x g at 4°C. After that, 600 µl of the crude protein was added (each application) and centrifuged. The bound protein was washed with 10 CVs of the binding buffer, followed by elution twice with the elution buffer (100 µl each of 1X phosphate buffer and 200 mM imidazole). Step-wise purification was performed on the induced soluble fraction of VMPHMGR with imidazole at 100, 200, 300, 400 and 500 mM. The mixture of proteins was separated based on their molecular weights using a 0.75-mm thickness sodium dodecyl sulphate-polyacrylamide gel. The detection and verification on the expressed recombinant HMGR protein was carried out using the his-tag monoclonal antibody and HRP Chemiluminescent Detection kit (Amresco, OH, USA).

2.7 Functional Enzymatic Assay

Gas chromatography-mass spectrophotometry (GC-MS) was applied to detect the enzymatic end products from the HMGR samples (crude induced protein, crude un-induced protein, and his-tag purified protein) and crude extract from BL21(DE3)pLysS:pET32(a) [without insert, as a negative control]. The enzymatic assay was carried out using the protocols adapted from Chappell, Wolf, Proulx, Ceullar, and Saunders (1995) and Pitera et al. (2007) with some modifications. A GC glass vial containing 4 mg of protein, 100 mM KH₂PO₄ (pH7.0), 3 mM NADPH (Merck, Darmstadt, Germany) and 20 µM HMG-CoA (Fluka, MO, USA) was incubated at 30°C with gentle shaking at 220 rpm for 2 hours. The reaction mixture was then added with 500 mM HCl to convert the mevalonate produced to mevalonic acid lactone, followed by an additional incubation at 30°C for 15 minutes with shaking at 220 rpm. An equal amount of pentane was then added to the vial and vortexed for 5 minutes. Finally, the pentane extract was transferred to a fresh GC glass vial before analysis. The pentane extract of each sample was analyzed using the GCMS-QP2010 Plus system (Shimadzu, Tokyo, Japan). The GC column used was a fused silica capillary column (SGE BPX-5, 30 M x 0.25 mm x 0.25 µm) with helium (53.6 kPa) as a carrier gas at a constant flow rate of 1 ml per min. A splitless injection mode with an injection volume of 0.1 µl was used. The GC column oven temperature profiles were as follows: 3 minutes at 50°C, followed by increments of 10°C/minute to 250°C, and a final temperature at 250°C for 10 minutes. The scan range of the mass spectrum was recorded for the m/z values of 50 to 450. The retention time and mass spectrum of mevalonate lactone (as standard) were obtained by comparing with the National Institute of Standards and Technology (NIST 2008) and Wiley mass spectral libraries (Scientific Instrument Services, NJ, USA). The single-ion peaks generated from each protein sample were integrated and compared with the peaks generated using the standard, mevalonate lactone (Sigma-Aldrich, Steinheim, Germany).

3. Results and Discussion

3.1 Sequence Analysis of Full Length VMPHMGR cDNA Transcript

The full length cDNA of *VMPHMGR* comprises of 1920 bp with an open reading frame (ORF) of 1689 bp encoding a 562 amino acids protein. It is flanked by 123 bp of 5'-untranslated region and 108 bp of 3'-untranslated region including a poly-A tail. The deduced protein has a calculated molecular weight of 59780 Da (BioEdit version 7.0.9) and a predicted isoelectric point (pI) of 6.63 (<http://ipsort.hgc.jp/>). This sequence was submitted to the GenBank with the accession number JF519820. *VMPHMGR* is predicted to have a mitochondrial targeting peptide for the first 30 amino acids using the iPSORT and SignalP 3.0 programme (Bendsten et al., 2004). We consequently excluded the first 30 amino acids in the cloning of *VMPHMGR* into the pET32(a) for expression analysis in *E. coli* as described in this paper. Studies by other researchers have demonstrated that deletion of the N-terminal domain of HMGR still produced an active protein. Truncation of the N-terminal transmembrane domain of *Saccharomyces cerevisiae* Hmg1p was found to produce an active cytosolic truncated protein (Donald, Hampton, & Fritz, 1997; Jackson, Hart-Wells, & Matsuda, 2003). When this truncated gene was expressed in *Neurospora crassa*, it was found to be functional (Wang & Keasling, 2002).

The Blast Search and multiple sequence alignment analysis with CLUSTAL W (<http://align.genome.jp/>) revealed that *VMPHMGR* was identical to the HMGR of many other plant species, such as *Oryza sativa* Indica (76%; GenBank accession no. Q9XHL5), *Zea mays* (75%; GenBank accession no. CAA70440), *Morus alba* (74%; GenBank accession no. AAD03789.1), *Ricinus communis* (73%; GenBank accession no. EEF52919.1) and *Salvia miltiorrhiza* (72%; GenBank accession no. ACD37361), suggesting that *VMPHMGR* belongs to the HMGR family. The N-terminal end of *VMPHMGR* was quite distinct in length and amino acid composition while the C-terminal catalytic domain was highly identical to other plant species (Figure 1). Kevei et al. (2007) reported that the availability of this catalytic motif aids the NADPH-dependent reduction of HMG-CoA to mevalonate.

Phylogenetic analysis suggested a divergence of the known HMGRs into two classes: Class I enzymes of eukaryotes and some archaea, and Class II enzymes of prokaryotes (Friesen & Rodwell, 2004). The structural comparison between both classes was earlier reviewed by Istvan (2001). The catalytic domains among the eukaryotes are said to be highly conserved. *VMPHMGR* has two conserved HMG-CoA binding motifs [EMPVGYVQLP (aa₂₃₂-aa₂₄₁) and TTEGCLVA (aa₂₆₁-aa₂₆₈)] and two NADPH binding motifs [DAMGMNM (aa₃₅₇-aa₃₆₃) and GTVGGGT (aa₅₀₆-aa₅₁₂)] (Figure 1). These motifs are also found in other plants' HMGRs (Jiang et al., 2006; Wang et al., 2007).

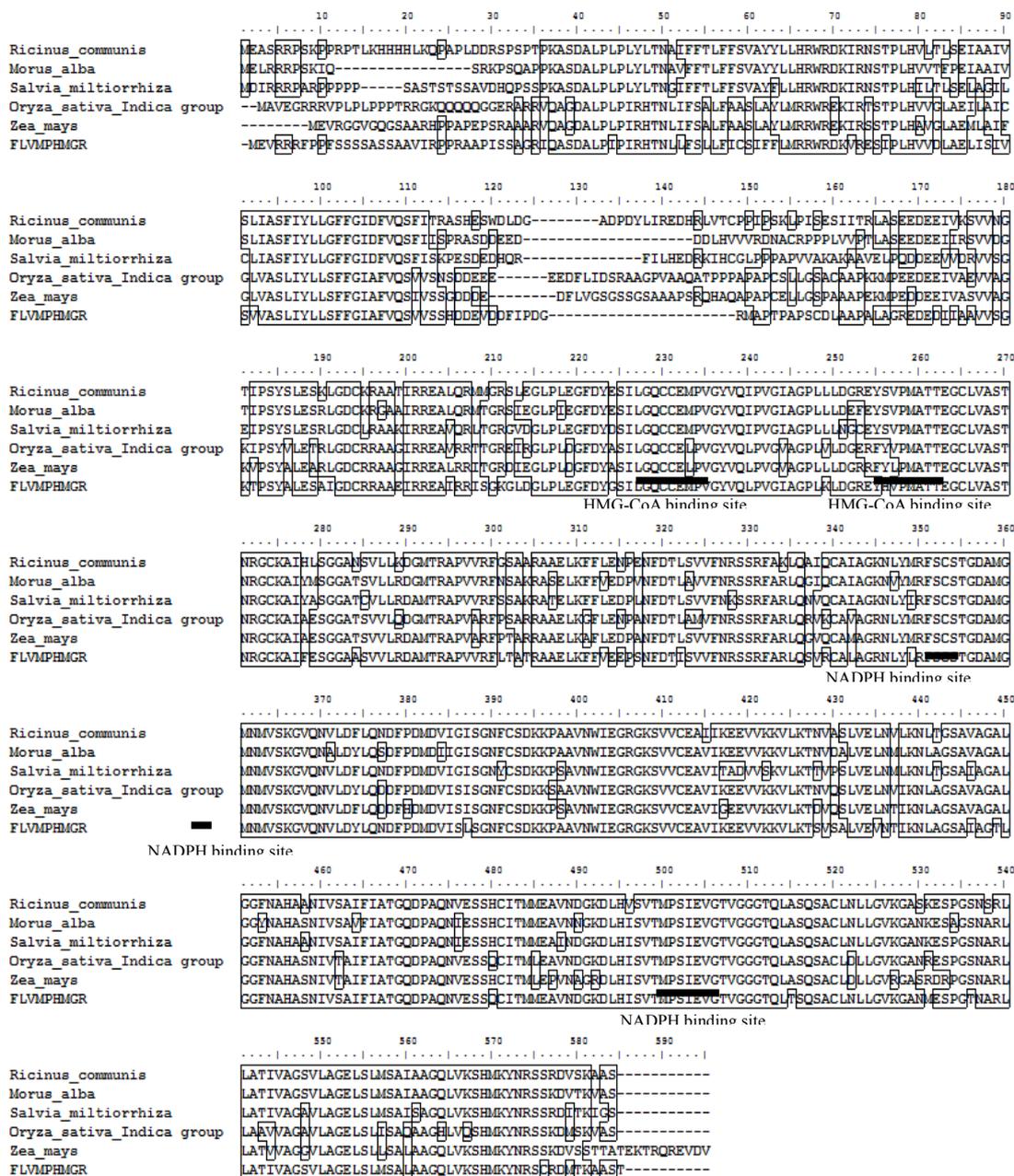


Figure 1. Multiple alignment of the deduced amino acid sequence of VMPHMGR (putative 3-hydroxy-3-methylglutaryl-CoA-reductase) and other closely related protein sequences from *Ricinus communis* (GenBank accession no. EEF52919.1), *Morus alba* (GenBank accession no. AAD03789.1), *Salvia miltiorrhiza* (GenBank accession no. ACD37361), *Oryza sativa Indica* group (GenBank accession no. Q9XHL5) and *Zea mays* (GenBank accession no. CAA70440).

The HMG-CoA binding sites and NADPH binding motifs are underlined

From the TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) prediction analysis, two trans-membrane regions were found in the polypeptide chain of VMPHMGR [one was located between Pro₄₂ (P) and Met₆₄ (M), while the other was positioned between Leu₈₅ (L) and Val₁₀₇ (V)]. Secondary structure analysis using SOPMA (Geourjon & Deleage, 1995) shows that the putative VMPHMGR peptide is rich in alpha helices (46.98%) and random coils (36.30%) while extended strands (12.10%) and beta turns (4.63%) are distributed intermittently. These structural elements patterns are similar to the HMGR reported previously (Jiang et al., 2006).

From the phylogenetic tree constructed (Figure 2) to compare *VMPHMGR* evolutionary relationship with other

reported plant *HMGRs*, it seems that all the plants *HMGRs* share a common ancestry and *VMPHMGR* is clustered closely to the *HMGR* from *Oryza sativa*. This is not surprising considering that both are monocot plants. Although *Zea mays* shows a common conserved catalytic region, it deviates at the N terminus [the membrane spanning region] (Nelson, Doerner, Zhu, & Lamb, 1994) and the C terminus (Figure 1). The divergence is obvious between *Zea mays* and *VMPHMGR* whereby *VMPHMGR* sequence contains several gaps at the N terminus.

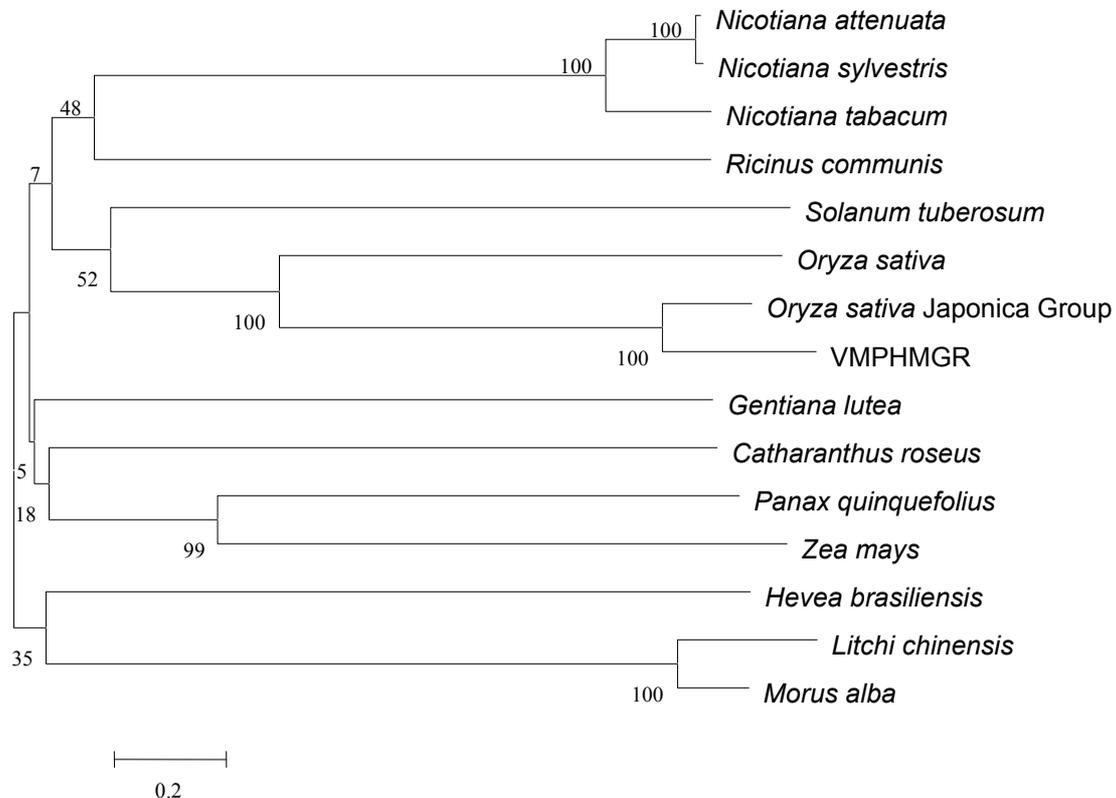


Figure 2. Phylogenetic relationships between *HMGRs* from various plant sources by MEGA 4.0. The bootstrap neighbour-joining method was used to construct the tree. The phylogenetic tree analysis of *HMGR* involved those from *Zea mays* (GenBank accession no. CAA70440), *Oryza sativa* Japonica group (GenBank accession no. BAD10066.1), *Oryza sativa* (GenBank accession no. AAD38873.1), *Nicotiana tabacum* (GenBank accession no. AAB87727.1), *Morus alba* (GenBank accession no. AAD03789.1), *Ricinus communis* (GenBank accession no. EEF52919.1), *Solanum tuberosum* (GenBank accession no. AAA93498.1), *Hevea brasiliensis* (GenBank accession no. AAQ63055.1), *Nicotiana attenuata* (GenBank accession no. AAQ85554.1), *Nicotiana sylvestris* (GenBank accession no. CAA45181.1), *Gentiana lutea* (GenBank accession no. BAE92730.1), *Litchi chinensis* (GenBank accession no. ABF56518.1), *Catharanthus roseus* (GenBank accession no. AAA33108.1) and *Panax quinquefolius* (GenBank accession no. ACV65036.1).

3.2 Expression Analysis of Putative *VMPHMGR*

3.2.1 Developmentally Regulated Expression of Putative *VMPHMGR*

In order to understand the molecular profile of *VMPHMGR*, real-time RT-PCR was performed. The putative *VMPHMGR* has a significantly ($p < 0.05$) high level of expression at the early developmental stage (bud) and lower expressions at both the blooming and full-bloom stages (Figure 3 A). The low expressions at the blooming stage imply that *HMGR* is crucial during early flower development when the flower bud is enlarging and engaging to bloom. These results corresponded well with observations made in other studies (Ha et al., 2003; Narita & Gruissem, 1989) that *HMGR* is under transcriptional control during development. *Zea mays* *HMGR* also showed a developmentally regulated pattern during seed development and germination (Moore & Oishi, 1993). Such dominant role of *HMGR* is noted with it being a key regulatory enzyme in the isoprenoid biosynthesis pathway, crucial in the biosynthesis of mevalonate (MVA) as starting material for the production of

various isoprenoids (Dudareva et al., 2005). Maldonado-Mendoza et al. (1997) highlighted that HMGR might be supplying the terpenoid intermediates for the formation of indole alkaloids during seeds germination. Jelesko, Jenkins, Rodriguez-Concepcion, and Grisse (1999) reported that the accumulation of HMGR in tomato was observed only during the early growth phases, and they postulated that HMGR played crucial roles during cell division and growth. Likewise, Narvaez, Canto Canche, Perez, and Madrid (2001) discovered that the highest expression profile of *Bixa orellana* HMGR was in the immature seed compared to the mature seed.

3.2.2 Expression of VMPHMGR in Different Floral Parts and Vegetative Tissues

The *VMPHMGR* transcript was expressed at significantly ($p < 0.05$) low levels in all tissues compared to the bud (Figure 3 B). This may be attributed to the overall low abundance of *VMPHMGR* which thus diluted its expression level in all parts of the plant. The end product of plant *HMGRs* (mevalonate) was reported to be beneficial in the physiological responses of plants and in the biosynthesis of carotenoids and sterols for cell membrane maintenance (Pitera et al., 2007). A differential expression profile was also noted for both *Arabidopsis thaliana* *HMGRs* (Enjuto et al., 1994). In contrast, *Eucommia ulmoides* *HMGR* was found to be a housekeeping gene (Jiang et al., 2006). All the reported studies mentioned above and the differential expression pattern of *VMPHMGR* imply that *VMPHMGR* might have discrete functions in regulating the mevalonate pathway.

3.2.3 Expression of VMPHMGR at Different Time-Points

The real-time RT-PCR performed on the full-bloom flower using a pair of *VMPHMGR*-specific primer showed that the expression of *VMPHMGR* transcript was significantly ($p < 0.05$) highest in the dark with an unusual peaking of approximately 1.3-fold at 12 a.m., following which slightly lower levels were exhibited throughout the whole morning (Figure 3C) before plunging further at noon. Its expression was low or undetectable in the afternoon and evening (12 noon to 10 p.m.). Enjuto et al. (1994) observed high level of *HMGR* in *Arabidopsis thaliana* seedlings placed in the dark. Similar expression profile was also observed for maize *HMGR* activity during seedlings germination in the dark (Moore & Oishi, 1993). From the *VMPHMGR* result, however, it is inconclusive to maintain that the expression level of *VMPHMGR* is regulated by light since the VMP samples were taken from plants grown under a non-controlled environmental conditions. Besides light, temperature is said to play a significant role in *HMGR* expression. Low temperature has been shown to increase the expression of *HMGR* in *Picrorhiza kurrooa* (Kawoosa et al., 2010). The slightly higher expression levels in the morning (Figure 3 C) could have been attributed to a slightly cooler temperature. Nevertheless, further experiments with controlled growth conditions, such as placing the plants in a growth chamber, need to be conducted in order to ascertain the expression pattern of *VMPHMGR* transcript.

3.3 Cloning and Expression of Recombinant VMPHMGR in *E. coli*

Cloning of the coding region of *VMPHMGR* into pET32(a) for expression in *E. coli* BL21(DE3)pLysS induced with 0.5 mM IPTG for 4 hours at 30°C yielded a protein of 70570 Da (Figures 4 A and 4 B). It was noted that the over-expressed protein was present only in the induced his-tag purified or whole cell lysates of soluble and insoluble fractions. Unfortunately, the 70570 Da band for the whole cell lysates of the induced soluble fractions (Figure 4A, Lane 6) could not be discerned clearly on the SDS-PAGE but was visible in the Western blot (Figure 4B, Lane 3). We attributed this to the higher sensitivity of the Western blot and also the interference of too many cellular proteins in the whole cell lysate sample loaded on the gel. As expected, the whole cell lysate of un-induced soluble fraction did not show the 70570 Da band. Likewise, this band did not appear in the pET32a vector control lane (Figure 4A, Lane 9) implying that the expressed recombinant protein was from the constructed vector itself and not from the *E. coli* expression system.

3.4 Functional Characterization of a 3-hydroxy-3-methylglutaryl-CoA-reductase of *Vanda Mimi* Palmer (*VMPHMGR*)

The potential products produced from the catalytic activities of *VMPHMGR* samples comprising of his-tag purified induced protein sample, crude induced and un-induced soluble proteins, and whole cell extract from the host *E. coli* BL21(DE3)pLysS harbouring an empty pET32(a) vector as control, were all subjected to GC-MS.

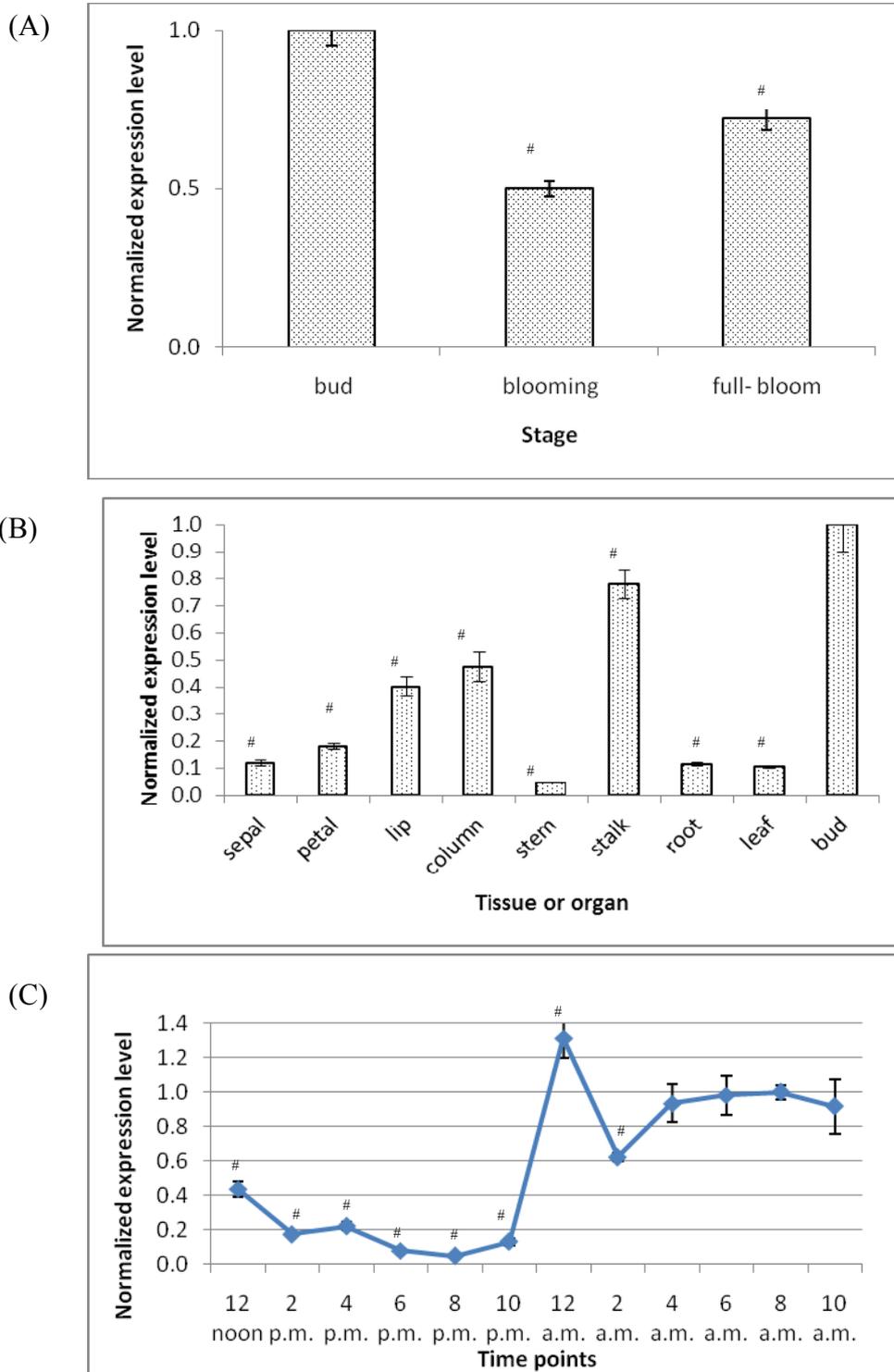


Figure 3. Expression profile of putative *VMPHMGR* transcript. Real-time RT-PCR was performed: (A) at different flower developmental stages, (B) in different tissues of VMP and (C) expression in full-bloom flower at different time-points for 2-hour intervals within 24-hour respectively. Error bars represent standard deviations on the normalized ratios. # indicates significant differences with $p < 0.05$

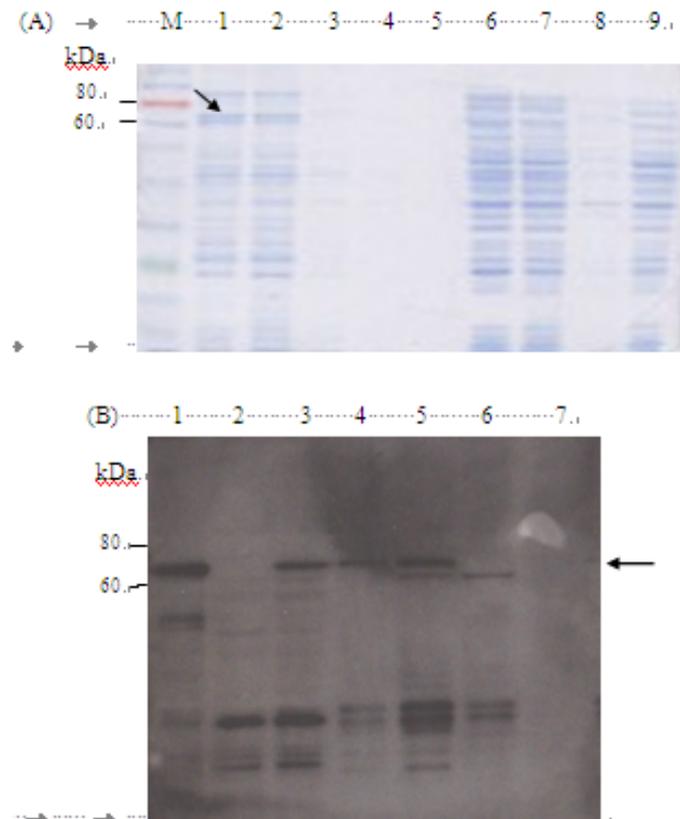


Figure 4. Expression of the putative *VMPHMGR* in *Escherichia coli* strain BL21(DE3)pLysS (A) *VMPHMGR* expression induced with 0.5 mM IPTG at 30°C, 220 rpm for 4 hours. The crude and his-tag purified proteins of each fraction were separated on SDS-PAGE with Coomassie brilliant blue staining. Lane M, molecular weights standard (ColorPlus Prestained Protein Ladder, Broad Range, NEB); Lanes 1-5, his-tag purified induced soluble fraction of *VMPHMGR* eluted with different imidazole concentrations (100 mM to 500 mM respectively); Lane 6, whole cell lysate of induced soluble *VMPHMGR*; Lane 7, whole cell lysate of un-induced soluble fraction; Lane 8, whole cell lysate of induced insoluble *VMPHMGR*; Lane 9, empty pET32(a) vector (without insert). (B) Immunoblot of protein extracts. Lane 1, whole cell lysate of induced insoluble *VMPHMGR*; Lane 2, whole cell lysate of un-induced soluble fraction; Lane 3, whole cell lysate of induced soluble *VMPHMGR*; Lanes 4-6, his-tag purified induced soluble fraction of *VMPHMGR* eluted with different imidazole concentrations (300 mM to 100 mM in decreasing order); Lane 7, empty pET32(a) vector (without insert). Arrows indicate over-expressed *VMPHMGR* of the expected size of 70570 Da. analyses.

Briefly, all the above mentioned protein lysate samples were acidified with 500 mM HCl to convert the HMG-CoA to mevalonic acid lactone through the reaction of HMGR. The reaction was performed based on the principle that if HMGR is present and functional in the bacterial lysates, it will be able to catalyze the conversion of HMG-CoA to mevalonate, followed by acidification to mevalonate lactone. A crude un-induced soluble *VMPHMGR* sample was included as a control for detecting potential leakage expression from the constructed plasmid. The end products produced from each of the above enzymatic reactions were subjected to GC-MS analysis.

The GC-MS single ion chromatogram showed two components from the above reaction (Figure 5 A). Dehydromevalonic lactone and pantolactone were detected at the retention times of 11.50 minutes and 13.20 minutes, respectively. By comparing the mass spectra of each component produced [Figures 5 B (i) and 5 C (i)] with the values at the NIST 2008 and Wiley mass spectral libraries, dehydromevalonic lactone was identified as the main spectrum fragments of m/z 54, 67, 82 and 112 while pantolactone as the main spectrum fragments of m/z 53, 58, 71, 85, 103, 115 and 131 [Figure 5 B(ii) and C(ii), respectively]. This shows that the recombinant *VMPHMGR* was functional and active in catalyzing the conversion of HMG-CoA to derivatives of mevalonate lactone.

Lactone was not detected from the crude un-induced soluble protein sample [Figure 5 D (i) and E (i)] as seen from the comparison made between the mass spectra of each component produced with the values at the NIST 2008 and Wiley libraries [Figures 5 D (ii) and E (ii)], indicating no leakage expression from the construct as the BL21(DE3)pLysS system avoids un-induced expression. In addition, no lactone was detected in the control lysate prepared from the bacterial cells harbouring the empty plasmid (without the *VMPHMGR* insert).

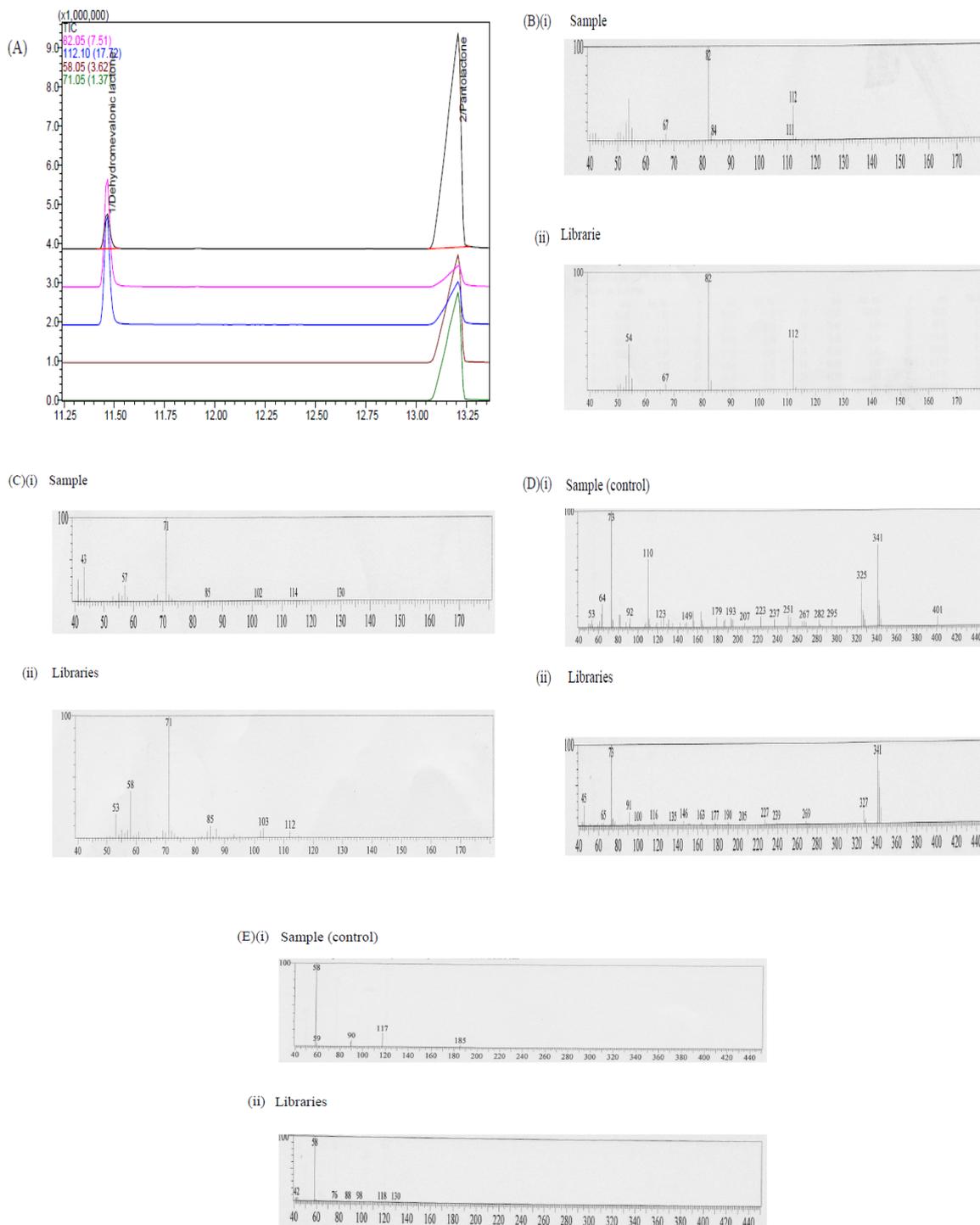


Figure 5. GC-MS analyses of products formed from the enzymatic activity of VMPHMGR. (A) Single ion chromatogram formed from the crude induced soluble VMPHMGR. Peaks with different colours indicate the

mass spectra of corresponding products at respective retention times. (B) (i) and (C) (i) are mass spectra of corresponding products produced from crude induced soluble VMPHMGR while (B) (ii) and (C) (ii) are the highest hit with NIST 2008 and Wiley libraries. (D) (i) and (E) (i) are mass spectra of corresponding products produced from crude un-induced soluble VMPHMGR (control) while (D) (ii) and (E) (ii) showed the chromatograms of the highest match with NIST 2008 and Wiley libraries.

4. Conclusion

This is the first report on the over-expression and biochemical characterization of a truncated recombinant HMGR protein, from a vandaceous orchid, produced in the *E. coli* system. VMPHMGR was functionally expressed in *E. coli* and GC-MS analysis showed its catalytic activities resulted in the production of dehydromevalonic lactone and pantolactone. Such successful molecular and biochemical results would lend a better understanding of fragrance production in vandaceous orchids. Current work in our laboratory is focusing on increasing the solubility and purity of the recombinant protein for use in a wider range of biochemical and catalytic studies of this fragrance-related enzyme.

Acknowledgements

We would like to thank the Ministry of Higher Education Malaysia and Universiti Putra Malaysia for financial support through grants, Fundamental Research Grant Scheme (01-11-08-6470FR and 02-12-10-1002FR) and Research University Grant Scheme (05-04-08-0551RU), respectively. We thank Ms Adelene Song for helpful comments and advices in the functional enzymatic analysis.

References

- Bendsten, J. D., Nielsen, H., Heijne, G., & Brunak, S. (2004). Improved prediction of signal peptides-SignalP 3.0. *Journal of Molecular Biology*, 340(4), 783-795. <http://dx.doi.org/10.1016/j.jmb.2004.05.028>
- Chan, W. S., Janna, O. A., Namasivayam, P., & Maziah, M. (2009). Molecular characterization of a new 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) transcript from *Vanda Mimi* Palmer. *Scientia Horticulturae*, 121(3), 378-382. <http://dx.doi.org/10.1016/j.scienta.2009.02.015>
- Chan, W. S., Janna, O. A., & Namasivayam, P. (2011). Isolation, cloning and characterization of fragrance-related transcripts from *Vanda Mimi* Palmer. *Scientia Horticulturae*, 127(3), 388-397. <http://dx.doi.org/10.1016/j.scienta.2010.09.024>
- Chappell, J., Wolf, F., Proulx, J., Ceullar, R., & Saunders, C. (1995). Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants? *Plant Physiology*, 109 (4), 1337-1343.
- Cheng, A.-X., Lou, Y. G., Mao, Y. B., Lu, S., Wang, L. J., & Chen, X. Y. (2007). Plant terpenoids: biosynthesis and ecological functions. *Journal of Integrative Plant Biology*, 49(2), 179-186. <http://dx.doi.org/10.1111/j.1744-7909.2007.00395.x>
- Donald, K. A., Hampton, R. Y., & Fritz, I. B. (1997). Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, 6 (9), 3341-3344.
- Dudavera, N., Martin, D., Kish, C. M., Kolosova, N., Gorenstein, N., Faldt, J., ... Bohlmann, J. (2003). (*E*)- β -Ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: Function and expression of three synthase genes of a new terpene synthase subfamily. *The Plant Cell*, 15, 1227-1241. <http://dx.doi.org/10.1105/tpc.011015>
- Dudareva, N., Andersson, S., Orlova, I., Gatto, N., Reichelt, M., Rhodes, D., Boland, W., & Gershenzon, J. (2005). The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. *PNAS*, 102(3), 933-938. <http://dx.doi.org/10.1073/pnas.0407360102>
- Enjuto, M., Balcells, L., Campos, N., Caelles, C., Arro, M., & Boronat, A. (1994). *Arabidopsis thaliana* contains two differentially expressed 3-hydroxy-3-methylglutaryl-CoA reductase genes, which encode microsomal forms of the enzyme. *Plant Biology*, 91 (3), 927-931.
- Friesen, J. A., & Rodwell, V. W. (2004). Protein family review: the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductases. *Genome Biology*, 5(11), 248-254. <http://dx.doi.org/10.1186/gb-2004-5-11-248>
- Geourjon, C., & Deleage, G. (1995). SOPMA: significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments. *CABIOS*, 11(6), 681-684.

- Goff, S., & Klee, H. (2006). Plant volatile compounds: sensory cues for health and nutritional value? *Science*, *311*(5762), 815-819. <http://dx.doi.org/10.1126/science.1112614>
- Guterman, I., Shalit, M., Menda, N., Piestun, D., Dafny, Y., Shalev, G., ... Weiss, D. (2002). Rose scent: genomics approach to discovering novel floral fragrance-related genes. *The Plant Cell*, *14*(10), 2325-2338. <http://dx.doi.org/10.1105/tpc.005207>
- Ha, S. H., Lee, S. W., Kim, Y. M., & Hwang, Y. S. (2001). Molecular characterization of *Hmg2* gene encoding a 3-hydroxy-3-methylglutaryl-CoA reductase in rice. *Molecules and Cells*, *11*(3), 295-302.
- Ha, S. H., Kim, J. B., Hwang, Y. S., & Lee, S. W. (2003). Molecular characterization of three 3-hydroxy-3-methylglutaryl-CoA reductase genes including pathogen-induced *Hmg2* from pepper (*Capsicum annuum*). *Biochimica et Biophysica Acta (BBA)*, *1625*(3), 253-260. [http://dx.doi.org/10.1016/S0167-4781\(02\)00624-3](http://dx.doi.org/10.1016/S0167-4781(02)00624-3)
- Istvan, E. S. (2001). Bacterial and mammalian HMG-CoA reductases: related enzymes with distinct architectures. *Current Opinion in Structural Biology*, *11*(6), 746-751. [http://dx.doi.org/10.1016/S0959-440X\(01\)00276-7](http://dx.doi.org/10.1016/S0959-440X(01)00276-7)
- Jackson, B. E., Hart-Wells, E. A., & Matsuda, S. P. T. (2003). Metabolic engineering to produce sesquiterpenes in yeast. *Organic Letters*, *5*(10), 1629-1632.
- Jelesko, J., Jenkins, S., Rodriguez-Concepcion, M., & Grissem, W. (1999). Regulation of tomato HMG1 during cell proliferation and growth. *Planta*, *208*(3), 310-318. <http://dx.doi.org/10.1007/s004250050564>
- Jiang, J. H., Kai, G. Y., Cao, X. Y., Chen, F. M., He, D. N., & Liu, Q. (2006). Molecular cloning of a HMG-CoA reductase gene from *Eucommia ulmoides* oliver. *Bioscience Reports*, *26*(2), 171-181. <http://dx.doi.org/10.1007/s10540-006-9010-3>
- Karlen, Y., McNair, A., Perseguers, S., Mazza, C., & Mermod, N. (2007). Statistical significance of quantitative PCR. *BMC Bioinformatics*, *8*, 131-147. <http://dx.doi.org/10.1186/1471-2105-8-131>
- Kawoosa, T., Singh, H., Kumar, A., Sharma, S. K., Devi, K., Dutt, S., ... Kumar, S. (2010). Light and temperature regulated terpene biosynthesis: hepatoprotective monoterpene picroside accumulation in *Picrorhiza kurrooa*. *Functional and Integrative Genomics*, *10*, 393-404. <http://dx.doi.org/10.1007/s10142-009-0152-9>
- Kevei, Z., Loughon, G., Mergaert, P., Horvath, G. V., Kereszt, A., Jayaraman, D., ... Ane, J. M. (2007). 3-hydroxy-3-methylglutaryl coenzyme A reductase I interacts with NORK and is crucial for nodulation in *Medicago truncatula*. *The Plant Cell*, *19*(12), 3974-3989. <http://dx.doi.org/10.1105/tpc.107.053975>
- Knudsen, J., & Gershenzon, J. (2006). The chemical diversity of floral scent. In: Dudareva N, Pichersky E. (eds) *Biology of floral scent*. CRC Press, Florida, pp. 27-52.
- Maldonado-Mendoza, I. E., Vincent, R. M., & Nessler, C. L. (1997). Molecular characterization of three differentially expressed members of the *Camptotheca acuminata* 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) gene family. *Plant Molecular Biology*, *34*(5), 781-790. <http://dx.doi.org/10.1023/A:1005866813347>
- Mohd Hairul, A. R. (2010). *Chemical composition of floral volatiles and expression of scent-related genes in Vanda Mimi Palmer*. Master dissertation, Universiti Putra Malaysia, Selangor Darul Ehsan, Malaysia.
- Mohd Hairul, A. R., Namasivayam, P., Gwendoline, E. C. L., & Janna O. A. (2010). Terpenoid, benzenoid and phenylpropanoid compounds in the floral scent of *Vanda Mimi Palmer*. *Journal of Plant Biology*, *53*(5), 358-366. <http://dx.doi.org/10.1007/s12374-010-9123-x>
- Moore, K. B., & Oishi, K. K. (1993). Characterization of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity during maize seed development, germination and seedling emergence. *Plant Physiology*, *101*(2), 485-491.
- Narita, J. O., & Grissem, W. (1989). Tomato hydroxymethylglutaryl-CoA reductase is required early in fruit development but not during ripening. *The Plant Cell*, *1*(2), 181-190.
- Narvaez, J. A., Canto, C. B. B., Perez, P. F., & Madrid, R. R. (2001). Differential expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) during flower and fruit development of *Bixa orellana*. *Journal of Plant Physiology*, *158*(11), 1471-1477. <http://dx.doi.org/10.1078/0176-1617-00614>
- Nelson, A. J., Doerner, P. W., Zhu, Q., & Lamb, C. J. (1994). Isolation of a monocot 3-hydroxy-3-methylglutaryl coenzyme A reductase gene that is elicitor-inducible. *Plant Molecular Biology*, *25*, 401-412.

- Pichersky, E., Noel, J., & Durareva N. (2006). Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science*, 311(5762), 808-811. <http://dx.doi.org/10.1126/science.1118510>
- Pitera, D., Paddon, C., Newman, J., & Keasling, J. (2007). Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. *Metabolic Engineering*, 9(2), 193-207. <http://dx.doi.org/10.1016/j.ymben.2006.11.002>
- Rohmer, M., Knani, M., Simonin, P., Sutter, B., & Sahn, H. (1993). Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochemistry Journal*, 295(2), 517-524.
- Shang, C. H., Zhu, F., Li, N., Ou-Yang, X., Shi, L., Zhao, M. W., & Li, Y. X. (2008). Cloning and characterization of a gene encoding HMG-CoA reductase from *Ganoderma lucidum* and its functional identification in yeast. *Bioscience, Biotechnology and Biochemistry*, 72(5), 1333-1339. <http://dx.doi.org/10.1271/bbb.80011>
- Tada, M., Tsubouchi, M., Matsuo, K., Takimoto, H., Kimura, Y., & Takagi, S. (1990). Mechanism of photoregulated carotenogenesis in *Rhodotorula minuta* VIII. Effect of mevinolin on photoinduced carotenogenesis. *Plant Cell Physiology*, 31(3), 319-323.
- Teh, S. L., Chan, W. S., Janna, O. A., & Namasivayam, P. (2011). Development of expressed sequence tag resources for *Vanda Mimi* Palmer and data mining for EST-SSR. *Molecular Biology Report*, 38(6), 3903-3909. <http://dx.doi.org/10.1007/s11033-010-0506-3>
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3(7), 1-12. <http://dx.doi.org/10.1186/gb-2002-3-7-research0034>
- Wang, G. Y., & Keasling, J. D. (2002). Amplification of HMG-CoA reductase production enhances carotenoid accumulation in *Neurospora crassa*. *Metabolic Engineering*, 4(3), 193-201. <http://dx.doi.org/10.1006/mben.2002.0225>
- Wang, Y. C., Guo, B. H., Zhang, F., Yao, H. Y., Miao, Z. Q., & Tang, K. X. (2007). Molecular cloning and functional analysis of the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase from hazel (*Corylus avellana* L. Gasaway). *Journal of Biochemistry and Molecular Biology*, 40(6), 861-869.
- Yu, H., & Goh, C. J. (2000). Identification and characterization of three orchid MADS Box genes of the API/APL9 subfamily during floral transition. *Plant Physiology*, 123, 1325-1336. <http://dx.doi.org/10.1104/pp.123.4.1325>