

Full Length Research Paper

Molecular cloning and expression analysis of a leaf-specific expressing 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase gene from *Michelia chapensis* Dandy

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The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the conversion of HMG-CoA to mevalonate, which is the first committed step in the pathway for isoprenoid biosynthesis in plants. In this research, a full-length cDNA encoding HMGR, designated as *MichHMGR* (GenBank Accession No. DQ098012) was isolated from *Michelia chapensis* Dandy by rapid amplification of cDNA ends (RACE). The full-length cDNA of *MichHMGR* comprised 2229 base pairs (bp) with a 1671 bp open reading frame (ORF) encoding a 556-amino-acid polypeptide that contained two trans-membrane domains. The deduced protein designated as *MichHMGR* had an isoelectric point (pI) of 8.34 and a calculated molecular weight of about 59.3 kDa. Sequence comparison analysis showed that *MichHMGR* had highest homology to HMGR from *Morus alba*. As expected, phylogenetic tree analysis indicated that *MichHMGR* belonged to plant HMGR group. Southern blot analysis showed that *MichHMGR* belonged to a small gene family. Tissue expression pattern analysis showed that *MichHMGR* was only expressed in the leaves whereas no expression was found in the stems and roots. Reverse transcription-polymerase chain reaction (RT-PCR) analysis indicated that *MichHMGR* expression could be induced by exogenous methyl jasmonate and salicylic acid. The functional color complementation assay indicated that *MichHMGR* could accelerate the biosynthesis of carotenoids in the *Escherichia coli* transformant, demonstrating that *MichHMGR* played an influential role in isoprenoid biosynthesis.

Key words: Molecular cloning, *Michelia chapensis* Dandy, expression profiles, HMG-CoA reductase.

INTRODUCTION

In higher plants, there are at least two distinct biosynthetic pathways responsible for the synthesis of isopentenyl diphosphate (IPP) which is the universal precursor of all isoprenoids: The classical mevalonate (MVA) pathway in cytoplasm, and the recently unveiled 1-deoxy-D-xylulose 5-phosphate/2-C-methyl -D-erythritol 4-phosphate (DXP/MEP) pathway in plastid (Kai et al., 2006, 2011). The MVA pathway that mainly operates in the cytosolic and endoplasmic reticulum (ER) compartments, is responsible for biosynthesis of sesquiterpenes (C₁₅), triterpenes (C₃₀), and polyterpenes such as artemisine and rubber, whereas the DXP pathway

present in the plastids is involved in biosynthesis of isoprene (C₅), monoterpenes (C₁₀), diterpenes (C₂₀), tetraterpenes (C₄₀), and certain prenylated quinines (Ha et al., 2003). 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which catalyses the NADP-dependent synthesis of mevalonate from HMG-CoA, has been considered as the first step in the MVA pathway (Chappell, 1995; Ha et al., 2003). The plant HMGR is encoded by a gene family and regulated by light, growth regulators, wounding and treatment with pathogen or elicitors (Maldonado-Mendoza et al., 1997; Park et al., 1992). Loss of function of 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (HMG1) in *Arabidopsis* led to dwarfing, early senescence and male sterility, and reduced sterol levels, suggested HMG1 plays a critical role in isoprene biosynthesis (Suzuki et al., 2004). Recent, overexpression

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overexpression of HMGR gene in *Salvia miltiorrhiza* can significantly improve tanshinone production, indicating that HMGR was important in isoprene metabolism (Kai et al., 2011). *Michelia chapensis* Dandy is a famous ornamental timber plant belonging to the *Michelia* Linn. It is good to be a virescence tree species because of its characteristics such as great crown, fast growth, and cold resistance. Moreover, previous researches showed that *M. chapensis* contained lots of monoterpenoids, sesquiterpenoids and their oxy-derivatives, the volatile oils and extracts from leaves of *M. chapensis* had activities on antitumor, antibacterial and antioxidant (Liu et al., 2008a, Liu and Wang, 2008b). Recently, more and more sesquiterpene lactones (parthenolide, costunolide, magnograndiolide, michampanolide) were isolated from Magnolia plants (Abdelgaleil and Hashinaga, 2007; Feltenstein et al., 2004). Most compounds were proved that they have insecticidal activity, antitumor activity and other biological activities (Zhao, 2005).

Although HMGR genes have been isolated from many plant species such as *Camptotheca acuminata* (Maldonado-Mendoza et al., 1997), *Catharanthus roseus* (Maldonado-Mendoza et al., 1992), *Cucumis melo* (Kato-Emori et al., 2001), *Eucommia ulmoides* (Jiang et al., 2006), *Corylus avellana* (Wang et al., 2007), *Ginkgo biloba* (Shen et al., 2006) and *S. miltiorrhiza* (Liao et al., 2009). Until now, there is no report on the cloning and characterization of HMGR genes from *M. chapensis*. In the present study, we report the cloning, expression analysis and functional analysis of the HMGR gene from *M. chapensis*.

MATERIALS AND METHODS

Plant materials and growing condition

The young seedlings of *M. chapensis* Dandy were kindly provided by Professor Li Xiaochu at Jiangsu Academy of Forestry and grown in a greenhouse at 28°C with 16 h light and 8 h dark photoperiod.

Total RNA isolation

Total RNA from leaves, stems and roots of *M. chapensis* were extracted using the modification of cetyltrimethylammonium bromide (CTAB) method (Kai et al., 2006). In the experiment of investigating induction by methyl jasmonate (MeJA) and salicylic acid (SA) elicitor, The roots of seedlings were submerged into 10 mM MeJA and 100 mM SA solution, while the plant submerged into sterile water were used as control. The leaves were collected at 0, 6, 24, 48, 72 and 96 h after treatment for RNA extraction to analysis of *MichHMGR* expression profiles by semi-quantitative one-step reverse transcription-polymerase chain reaction (RT-PCR). The quality of RNA was checked by formaldehyde denatured gel electrophoresis, and the concentration determined through spectrophotometer. The RNA samples were stored at -70°C prior to rapid amplification of cDNA ends (RACE) and RT-PCR analysis.

Isolation of the full-length *MichHMGR* cDNA using RACE-PCR

Total RNA isolated from young leaves was used to synthesize the

first strand cDNA (3'-ready cDNA) by using SMARTTM RACE cDNA Amplification Kit (Clontech, USA) with the 3' RACE CDS Primer A provided by the kit. The 3' RACE was performed with the Universal Primer A Mix provided with the kit as the reverse primer, and one degenerate primer F2 (5'-GATGC[A/T/G/C]ATGGG[G/A]ATGAA[T/C]A TGG-3') as the forward primer that was designed and synthesized according to the conserved region of known plant HMGRs (Maldonado-Mendoza et al., 1992, 1997; Kato-Emori et al., 2001; Learned and Fink, 1989). Polymerase chain reaction (PCR) was conducted in a total volume of 50 μ l containing 2 μ l cDNA, 20 μ mol F2, 20 μ mol UPM, 10 μ mol dNTPs, 1 x Ex PCR buffer and 5U Ex *Taq* Polymerase, following the protocol: the cDNA was denatured at 94°C for 2 min followed by 30 cycles of amplification (94°C for 50 s, 58°C for 60 s, 72°C for 120 s) and by 10 min at 72°C. The amplified product was purified and cloned into pMD18-T vector (Takara, Dalian), and transformed into *Escherichia coli* DH5 α . Based on the color reaction using Xgal-IPTG System and PCR identification, the positive clones were picked out and sequenced.

First strand cDNA (5'-ready cDNA) synthesis in 5' RACE was performed according to the manual of the SMARTTM RACE cDNA Amplification Kit using the 5'-RACE CDS Primer provided by the kit. Based on the sequence of the 3' RACE product, the complementary reverse gene specific primer R2 (5'-CTCCTTGATGATTGCCTTGC-3') was designed and synthesized. The 5' RACE-PCR was carried out using primers R2 and UPM under the following condition: the template (the 5'-ready cDNA) was denatured at 94°C for 2 min followed by 30 cycles of amplification (94°C for 50 s, 56°C for 60 s and 72°C for 90 s) and by 10 min at 72°C. The PCR product was purified and cloned into the pMD18-T vector followed by sequencing. By assembling the sequences of the 3' RACE and 5' RACE products, the full-length cDNA sequence of *MichHMGR* was deduced, and subsequently amplified through PCR using a pair of primers F1 (5'-GCGGGGGGTTCTCTCTCC-3') and UPM followed by sequencing, which was repeated for three times.

Bioinformatic analysis

Bioinformatic-associated analyses of *MichHMGR* were performed using bioinformatic tools at websites (<http://www.ncbi.nlm.nih.gov>, <http://www.expasy.org>). HMGR-related amino acid sequence was aligned using the program Clustal W (1.82) under the default settings. The alignment output was used to generate a phylogenetic tree based on the neighbor-joining method, as implemented in the MEGA 4.0 software (Tamura et al., 2007). The Poisson correction metric was used together with the pairwise deletion option, and confidence of the tree branches was checked by bootstrap generated from 1,000 replicates.

Southern blot analysis

The genomic DNA (20 μ g/lane) was isolated from young leaves, digested overnight at 37°C with *Hind*III, *Eco*RI and *Xba*I respectively, which did not cut within the full-length cDNA region, electrophoresed in 0.8% agarose gel and then transferred to Hybond N⁺ membrane (Amersham Biosciences) according to the standard procedure. The 516 -bp probe was generated by PCR using the full-length sequence of *MichHMGR* as template with primer Michhmgr1 (5'-TGCGAGGCAATCATCAAG-3') and primer Michhmgr2 (5'-AGCAGCTTTGGATACATCT-3'), and then labeled by digoxigenin- dUTP using the random primer method (Holtke et al., 1995). After hybridization, the DNA blot was washed twice in 2x SSC, 0.1% SDS for 15 min at RT and 0.1x SSC, 0.1% SDS for 15 min at 65°C. Subsequently the hybridized signals were visualized by enzyme immunoassay.

Gene expression analysis by semi-quantitative RT-PCR

Semi-quantitative one-step RT-PCR (Takara, Dalian) was carried out to investigate the gene expression pattern. Total RNA was extracted from leaves, stems and roots, as well as leaves under different treatments mentioned above respectively. Aliquots of total RNA (1µg/sample) were used as templates. The forward primer HF1 (5'-GCGGGGGTCTCTCTCTC C-3') and reverse primer HR1 (5'-AAATGTTGTTCTCTCA CAAAT-3') were specific sequence of *MichHMGR*. The house-keeping gene (actin gene) using the specific primers actF (5'-GTGACAATGGAAGTGAATGG-3') and actR (5'-AGACGGAGGATAGCGTGAGG-3') designed according to the conserved regions of plant actin genes were performed as an internal control to estimate whether equal amounts of RNA among samples were used. Amplifications were performed under the following condition: 50°C for 60 min and 94°C for 2 min followed by 30 cycles of amplification (94°C for 30 s, 55°C for 30 s and 72°C for 90 s).

Functional complementation assay of *MichHMGR*

The plasmids pAC-BETA and pTrc-*AtIPI*, kindly provided by Francis X. Cunninham Jr at the Department of Cell Biology and Molecular Genetics, University of Maryland, USA, were used to investigate the biological function of *MichHMGR*. The pAC-BETA contains all functional genes of the β -carotene synthesis including crtE (GGPP synthase), crtB (phytoene synthase), crtI (phytoene desaturase) and crtY (lycopene cyclase) genes, and also retains a chloramphenicol resistance gene (Misawa et al., 1995; Cuningham et al., 1996). The pTrc-*AtIPI* retains an ampicillin resistance gene and *AtIPI* gene. The *E. coli* strain DH5 α was used as the host strain. The experiment was performed as follows: the coding region of *MichHMGR* was amplified by PCR using a pair of primers HF2 (5'-TATAGATCTATGGCCCCCATCAAG AG-3') and HR2 (5'-TCGAATTCTCAGGAAGCAGCTTTGG-3') containing *Bgl*II and *Eco*RV digesting sites. The PCR products were first fractionated on agarose gel and then purified and sequenced. After digesting both PCR fragment and pTrc-*AtIPI* with *Bgl*II and *Eco*RV, the coding region of *MichHMGR* was cloned into the empty vector pTrc to generate the plasmid pTrc-*MichHMGR* which was verified by sequencing. The pTrc-*AtIPI* was digested by *Pst*I and ligated by T4 DNA ligase as controls. The vectors pTrc-*MichHMGR* and pAC-BETA were co-transformed into the *E. coli* DH5 α . Meanwhile, the vector pTrc and pAC-BETA were also co-transformed into *E. coli* DH5 α as controls. Transformants were cultured on solid LB medium containing ampicillin (150 mg/L) and chloramphenicol (50 mg/L) at 28°C for 2 days. The color of the transformant was a visible marker to test the function of *MichHMGR*.

RESULTS

Cloning of the full-length cDNA of *MichHMGR*

Based on sequences of the conserved regions of HMGRs from other plant species, a degenerate primer (F2) was designed and used for the amplification of the 3' end of *MichHMGR*. A single fragment of about 798 bp was amplified using primers F2 and UPM, and a 3' untranslated region (UTR) of 503 bp was found downstream from the stop codon in the amplified sequence. The complementary reverse specific primer R2 was designed and used for the cloning of the 5'-end sequence of *MichHMGR* by 5'RACE based on the sequence of the

the 3' RACE fragment. A 1592 bp fragment was amplified in which a 5' UTR of 48 bp was found upstream of the first ATG codon. Based on the sequences of the 3' and 5' RACE products, the full-length cDNA fragment was deduced and amplified by PCR using a pair of primers F1 and UPM. The cloned full-length cDNA of *MichHMGR* was 2229 bp in length and contained a 1671 bp ORF encoding a protein of 556 amino acids residues.

Bioinformatics analysis of *MichHMGR*

The deduced *MichHMGR* protein was predicted to have a molecular mass of 59.3 kDa with a theoretical isoelectric point of 8.34. *MichHMGR* contained two HMG-CoA-binding motifs (EMPVG YVRLP and TTEGCLVA) and two NADP(H)-binding motifs (DAMGMNM and GTVGGG) (Figure 1), which is similar to other HMGRs reported previously (Jiang et al., 2006; Ruiz-Albert et al., 2002). Protein-protein BLAST showed that the predicted *MichHMGR* had high homology with other plant species HMGRs. The *MichHMGR* shared 75, 72, 70, 66 and 68% identities to MaHMGR, CmHMGR, EuHMGR, CrHMGR and GbHMGR respectively, and was 86, 82, 82, 79 and 81% similar to MaHMGR, CmHMGR, EuHMGR, CrHMGR and GbHMGR respectively. Analyzed by TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), *MichHMGR* was found to contain two transmembrane domains (one located between L₃₉ and M₆₁ and the other located between M₈₂ and V₁₀₄ along the polypeptide chain). Penetrating through most parts of the secondary structure, alpha helices and random coils are the most abundant structural elements of *MichHMGR*, while extended strands and beta turns were intermittently distributed in protein, suggesting *MichHMGR*'s key structure and functional sites of this type of enzyme was strongly conserved.

Phylogenetic tree was constructed based on HMGR sequences retrieved from GenBank to investigate the evolutionary relationships among the HMGRs of different species. HMGRs from plants, fungi, animals and insects were aligned for phylogenetic tree construction. As expected, the result showed that HMGRs were derived from an ancestor gene and evolved into four groups including plant, fungi, insect and animal HMGR groups. According to the phylogenetic tree, *MichHMGR* belonged to plant HMGR group, and is more ancient than other plant HMGRs (Figure 2).

Southern blot analysis

Southern hybridization carried out under varying stringency was performed to investigate the genomic organization of the *MichHMGR* gene in *M. chapensis*, genomic DNA was completely digested with *Hind*III, *Eco*RI and *Xba*I, which were not present in the *MichHMGR* sequence. The results of hybridization

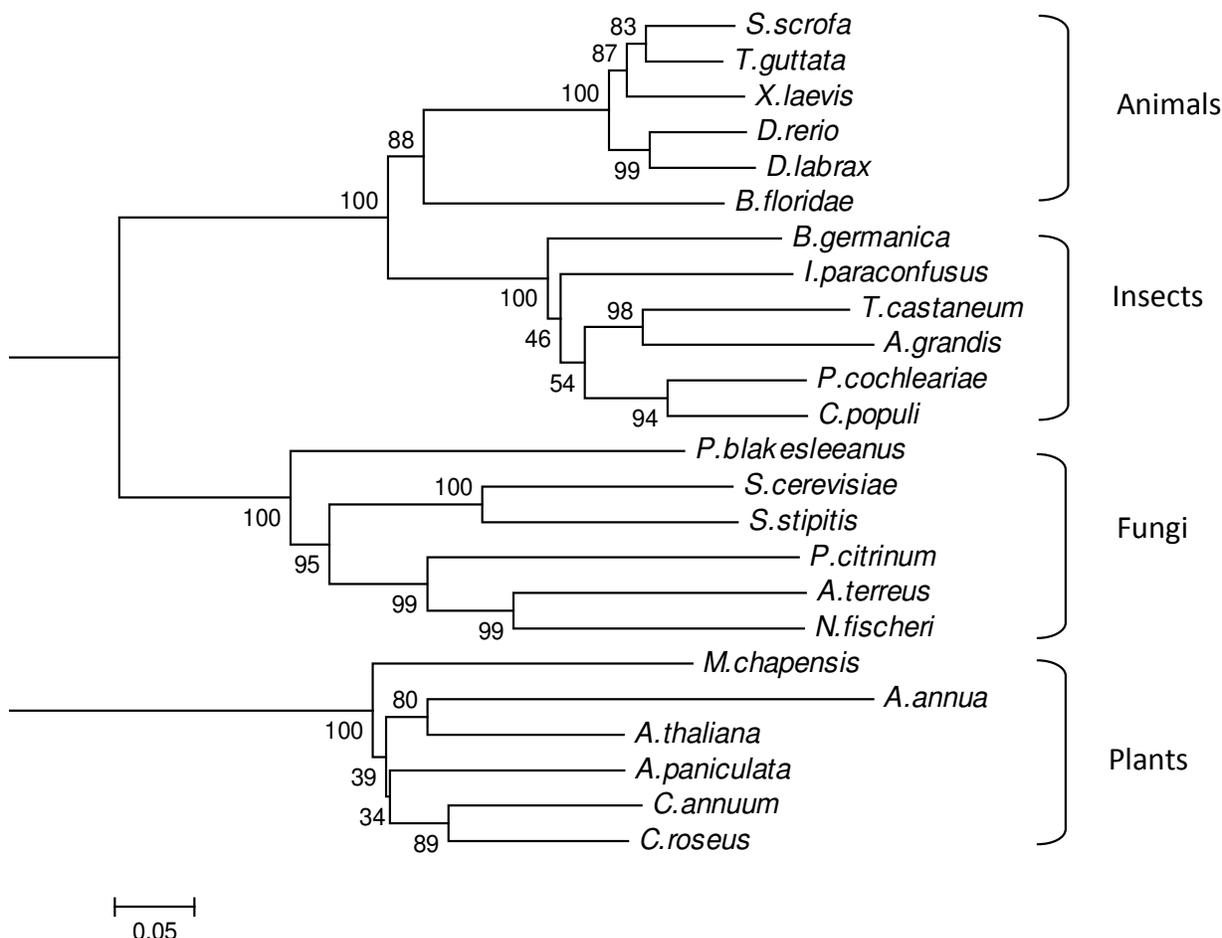


Figure 2. A phylogenetic tree of HMGRs from various organisms using the MEGA 4.0 program. Accession numbers: *Michelia chapensis*, AAY87014; *Artemisia annua*, AAD47596; *Andrographis paniculata*, AF389879_1; *Arabidopsis thaliana*, NP_177775; *Capsicum annuum*, AF110383_1; *Catharanthus roseus*, AAT52222; *Sus scrofa*, NP_001116460; *Taeniopygia guttata*, XP_002187727; *Xenopus laevis*, AAI70008; *Danio rerio*, NP_001119931; *Dicentrarchus labrax*, AAR02862; *Branchiostoma floridae*, XP_002205034; *Saccharomyces cerevisiae*, EDN59347; *Pichia stipitis*, ABN67745; *Aspergillus terreus*, AAD34556; *Phycomyces blakesleeenus*, CAB97179; *Neosartorya fischeri*, XP_001264646; *Penicillium citrinum*, BAC20567; *Tribolium castaneum*, XP_973160; *Anthonomus grandis*, AF162705_1; *Phaedon cochleariae*, ABO37160; *Chrysomela populi*, ABO37162; *Ips paraconfusus*, AAD20975; *Blattella germanica*, CAA49628.

revealed that the hybridization pattern of *MichHMGR* was simple for several enzyme combinations, showed at least four bands in each lane (Figure 3), indicating that *MichHMGR* belonged to a small gene family in *M. chapensis*.

Expression pattern analysis

To investigate *MichHMGR* expression pattern in various tissues of *M. chapensis*, the semi-quantitative one-step RT-PCR analysis were performed using total RNA isolated from different tissues including leaves, stems and roots. The result demonstrated that the transcript of *MichHMGR* occurred only in leaves and no transcript

accumulation of *MichHMGR* was detected in stems and roots (Figure 4). These indicated that the expression of *MichHMGR* was leaf-specific and the result was consistent with the *McFPS* expression in *M. chapensis* (Yin et al., 2011).

One-step RT-PCR analysis was carried out to investigate the changes of *MichHMGR* expression upon SA and MeJA treatment. The results revealed that when subjected to MeJA, *MichHMGR* transcript level reached the highest level after 72 h of the treatment. When elicited by SA, the *MichHMGR* transcript level increased rapidly and reached the highest level after 48 h of the treatment and then gradually decreased, still maintaining the higher level than the control even after 96 h (Figure 5). The results revealed that *MichHMGR* was

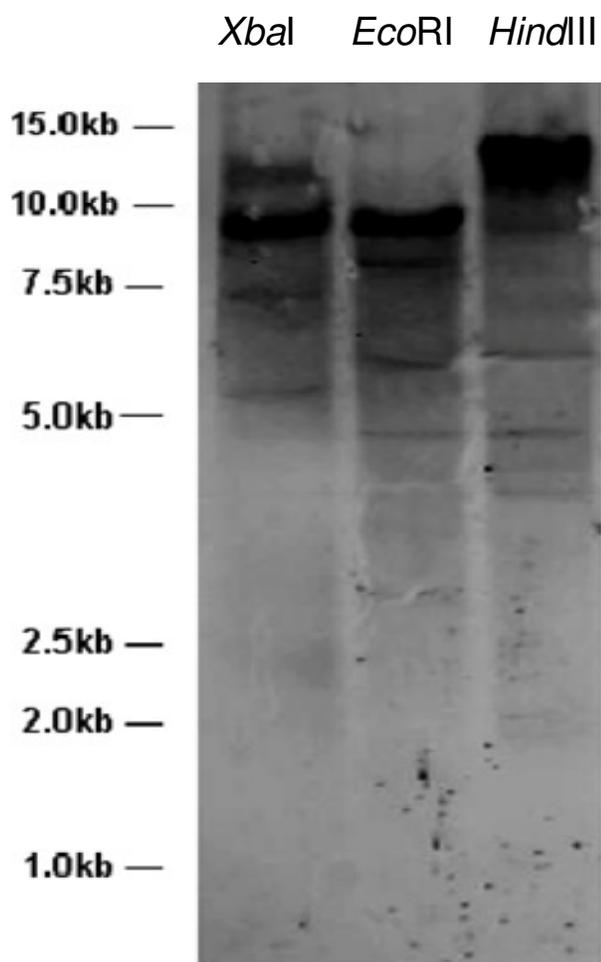


Figure 3. Southern blot analysis. Genomic DNA of *M. chapensis* was digested with *Hind*III, *Eco*RI and *Xba*I respectively, followed by hybridization with the biotin-labeled *MichHMGR* fragment as the probe.

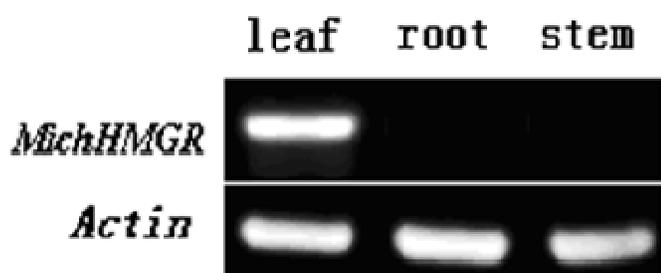


Figure 4. Expression pattern of *MichHMGR* in different *M. chapensis* tissues. Total RNA (1 μ g/sample) isolated from leaves, roots and stems respectively was subjected to one-step RT-PCR amplification (upper panel). *Actin* gene was used as the control to show the normalization of the templates in PCR reactions.

elicitor-responsive and could be effectively elicited at least at transcription level.

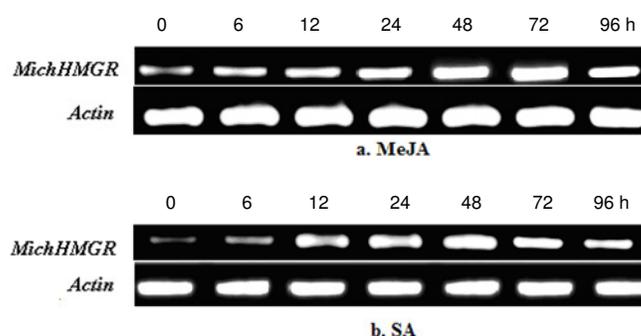


Figure 5. Induced gene expression of *MichHMGR*. Total RNA (1 μ g/sample) isolated from leaves of *M. chapensis* infected by 10 mM MeJA (a) and 100 mM SA (b) at different time point, was subjected to one-step RT-PCR amplification. *Actin* gene was used as the control to show the normalization of the templates in PCR reactions.

Functional complementation assay

E. coli introduced with foreign carotenogenic gene clusters had the ability to produce carotenoids and accumulate carotenoid pigments, while overexpression of enzymes within the carotenoid pathway can increase the carotenoid accumulation (Misawa et al., 1995), which could be as a visible marker for the functional testing of gene products (Wang et al., 2007; Yao et al., 2008; Yan et al 2009; Kai et al., 2010). In our study, we used the *E. coli* strain DH5 α to test if *MichHMGR* encoded the anticipated functional enzyme. As a result, *E. coli* DH5 α containing pTrc-*MichHMGR* and pAC-BETA accumulated significantly higher orange- yellow β -carotene than the control with vector pTrc and pAC-BETA (Figure 6), demonstrating that *MichHMGR* could accelerate the accumulation of β -carotene. Therefore, the functioning of the *MichHMGR* has been confirmed by the change in the color of *E. coli* cultures.

DISCUSSION

In this paper, we provide the first cloning, expression and functional characterization of a cDNA encoding HMGR from *M. chapensis*. The deduced *MichHMGR* protein showed high identity to HMGR proteins from other plant species through multiple alignments. Bioinformatics analysis of *MichHMGR* showed that its structure and functional sites were strongly conserved when compared with other plant HMGRs. To elucidate the evolutionary relationships among the HMGRs of different species, a phylogenetic tree was created. The result showed that *MichHMGR* belonged to the plant HMGR group, and is more ancient than other plant HMGRs. All of these results suggested that *MichHMGR* could encode a functional HMGR protein.

Southern blot analysis showed that there were at least four bands per lane. It is not surprising to detect more

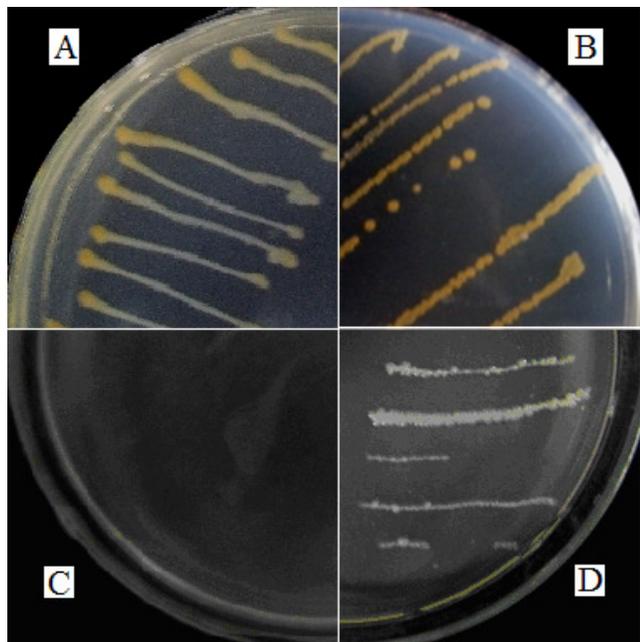


Figure 6. Functional demonstration of *MichHMGR* in *E. coli*. A: The *E. coli* strain DH5 α transformed with pTrc and pAC-BETA; B: The *E. coli* strain DH5 α transformed with pAC-BETA and pTrc-*MichHMGR*; C: Black control; D: The *E. coli* strain DH5 α .

than one isoforms of HMGR in plants as it has been reported that HMGR is encoded by more genes in tomato (Denbow et al., 1996), mulberry (Jain et al., 2000) and Hazel (Wang et al., 2007). Based on the results in southern blot analysis, it is likely that *MichHMGR* belongs to a small gene family in *M. chapensis*.

Expression analysis of *MichHMGR* genes in various tissues of *M. chapensis* showed that the transcript of *MichHMGR* occurred only in leaves and no transcript accumulation of *MichHMGR* was detected in stems and roots. In order to assume that the *MichHMGR* transcript level in stems and roots was not too low to be detected, we increased the RNA template used up to 2 μ g/sample, and increased the PCR cycle numbers up to 40 cycles. However, no transcript accumulation of *MichHMGR* was detected in stems and roots, too (data not shown). These results implied that *MichHMGR* might express in a tissue-specific manner and the expression of *MichHMGR* correlated with the sesquiterpenes biosynthesis and reflected the higher sesquiterpenes contents in the leaves.

MeJA and SA can stimulate plant secondary metabolism (Gutierrez-carbajal et al., 2010). HMGR catalyzes the first step in the biosynthesis of isoprenoids by converting 3-hydroxy-3-methylglutaryl-CoA to mevalonate. SA treatment increased artemisinin concentration in *A. annua* by positive regulation of HMGR and other genes involved in artemisinin biosynthesis (Pu et al., 2009). *SmHMGR* is elicitor-responsive gene against SA and MeJA (Liao et al., 2009). It was interesting to

know whether *MichHMGR* was also induced by MeJA and SA. Our results provided direct evidence that *MichHMGR* is also a MeJA and SA elicitor-responsive gene and can be effectively elicited at least at transcription level. Further investigations are required to characterize the *MichHMGR* enzyme activity and fully understand its regulatory role in sesquiterpenoids biosynthesis of *M. chapensis* at the molecular level.

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