Cloning and characterization of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) gene from *Paris fargesii* Franch

Yili Liang, XueMei Jiang, Qi Hu, Xiaoqi Li, Huaqun Yin, Diqiang Li, Yuguang Zhang and Xueduan Liu

*a* School of Mineral Processing and Bioengineering, Central South University, Changsha Hunan, China, 410083

*b* The Research Institute of Forest Ecology, Environment and Protection, Chinese academy of forest, Beijing, China, 100091

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3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) plays an important role in catalyzing the first committed step of isoprenoids biosynthesis in mevalonic acid (MVA) pathway. Here, we cloned a full-length transcript of *P. fargesii* HMGR. The full-length cDNA of *P. fargesii* HMGR (Pf-HMGR, GenBank accession no. JX508638) was 1,973 bp and contained a 1,728 bp ORF encoding 576 amino acids. Sequence analysis revealed that the deduced Pf-HMGR had high similarity with HMGRs from other plants, including *Ricinus communis* (77%), *Litchi chinensis* (76%), *Michelia chapensis* (75%) and *Panax quinquefolius* (72%). It had a calculated molecular mass of about 62.13 kDa and an isoelectric point (pI) of 8.47. It contained two transmembrane domains, two putative HMGR binding sites and two NADP(H)-binding sites. The predicted 3-D structure revealed that Pf-HMGR had a similar spatial structure with other plant HMGRs. Three catalytic regions, including L-domain, N-domain and S-domain were detected by structural modeling of HMGR. Tissue expression analysis revealed that Pf-HMGR was strongly expressed in roots and stems than in leaves. Taken together, our data laid a foundation for further investigation of HMGR’s functions and regulatory mechanisms in plants.

**Keywords:** *Paris fargesii* Franch, 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR), Cloning, RACE.

*Paris* belonging to the Liliaceae family contains 26 species globally, of which 21 are distributed in south west mainland of China. As an important Chinese herb for a long time, *Paris* has heat-clearing and detoxifying effects and is often used as the main crude material of the well-known Chinese medicine used in the hemostasis. Steroidal saponin (SAP), a major active ingredient derived from *Paris* spp. plays important role in the treatment of hemostasis, immunoregulation, cancer-resistance and cholesterol-lowering. Thus, due to its medicinal importance, industrial demand of SAP derived from *Paris* spp. has been expanding significantly in recent years.

Mevalonic acid (MVA) pathway that is responsible for biosynthesis of sesquiterpenes (C15), triterpenes (C30) and polyterpenes is considered as the main pathway to produce SAP, a secondary metabolite comprising 80% of the total compounds in *Mentha piperita*. The 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), which catalyzes the NAD(P) H-dependent reduction of 3-hydroxy-3-methylglutaryl CoA (HMGR-CoA) to mevalonate, is the first crucial enzyme in the MVA pathway of plants. Thus, in recent years, genes encoding HMGR have been cloned and characterized in several plants, such as *Catharanthus roseus*, *Camptotheca acuminata*, *Lycopersicon esculentum* and *Citrullus lanatus*. There is a strong similarity of the primary sequences of HMGR among plants, although its regulation remains unknown.

It is reported that the catalytic domain of HMGR has three domains: (i) N-Domain with small helical amino-terminal; (ii) L-Domain, the central and large domain with two motifs that bind to HMGR-CoA, including motifs (TTEGCLVA and EMPVGYVQIP) and motif (GTGVGGGT) that binds to NADP (H), and (iii) S-Domain, the small and helical domain that binds to NADP(H) with sequence (DAMGMNM). The functional complementation assay has shown that HMGR from *Euphorbia pekinensis* Rupr and *Clematis armandii* Franch could accelerate the biosynthesis of carotenoids, indicating that HMGR...
plays an important role in isoprenoid biosynthesis. In Arabidopsis thaliana, SUD1 encodes a putative E3 ubiquitin ligase that regulates HMGR activity without apparent changes in protein content.

The studies about genes encoding HMGR in Paris are lacking, although the SAP of Paris has been characterized by a series of methods, including HPLC-ELSD. Here, we report the isolation and characterization of the full-length cDNA of HMGR from P. fargesii Franch for the first time.

**Materials and Methods**

**Plant material and growth conditions**

Paris fargesii plant (height 30 cm) was collected at the 1750 m altitude in Huping Mountain National Nature Reserve in Hunan, China. The plant growth was performed in TAISITE GZX400 illumination incubator with settings of: 14 h light/10 h darkness cycle, photosynthetic photon flux of 150 µmol m⁻² s⁻¹ with day temperature of 26°C and night temperature of 21°C, respectively.

**Sampling and RNA isolation**

The stem was culled from the seedlings of P. fargesii. After surface sterilization, 500 mg of tissue was rinsed and sterilized 3-times with water and then quickly put in a triturator with liquid nitrogen. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy Plant Mini Kit (Qiagen GmbH, 74104), following the manufacturer’s instructions. The RNA quality and quantity were determined by agarose gel electrophoresis and NanoDrop ND-1000 UV spectrophotometer (Nanodrop Inc, USA).

**Primer designing and internal conservative fragment cloning of Pf-HMGR**

A pair of degenerate primers, forward hmgr (5'-GATGC [A/C/G/T] ATGAGGAGCATGCTG-3') and reverse hmgr (5'-CCATCAT[T/A/C/G/T] ACAGCTCCATCAT-3') were designed and synthesized according to the conserved sequence of HMGR shared by other species. Reverse transcriptase PCR (RT-PCR) was carried out to amplify the conserved fragment of HMGR gene from P. fargesii. The amplified RT-PCR amplicon was subsequently cloned and sequenced. On the basis of sequence information of partial RT-PCR amplicon of isolated HMGR, gene-specific primers were designed for the cloning of full-length cDNA of Pf–HMGR by RACE method.

**3'-RACE and 5'-RACE of Pf-HMGR**

In accordance with the protocol of the SMARTer RACE cDNA amplification kit (Clontech, Japan), about 600 ng of total RNA was transcribed using 3'-CDS primer provided within the kit. Primer GSP1 (5'-GATGAAGACGGCGGACACGA-3'), universal primer UPM (5'-CTAATACGACTCACTATAGGGCAACGCATGTATCAACGCAGAGT–3') and 3' ready cDNA were used as template for the first round of 3'-RACE. Subsequently, primer NGSP1 (5'-CGCGGACACGATGTTGTGC-3') and nested universal NUP (5'-AAGCAGTGGTATCAACGCAGAGT–3'), the product of the first PCR were used for the nested PCR. The 5' end of the cDNA sequence was synthesized according to the protocol of the SMARTer RACE cDNA amplification kit (Clontech, Japan) using 5'-CDS primer and SMART II A oligo.

The first round PCR of 5'-RACE was performed with GSP2 (5'-CCGCAGCAGCCTTCGCTG-3') and universal primer UPM. The generated product was used as the template for the nested PCR with primers NGSP2 (5'-GCAGCAGCCTTCGCTGAGCA GTA-3') and universal primer NUP. The PCR product was purified and ligated into pGM-T vector and then sequenced. All the reactions were carried out in a volume of 50 mL as per manufacturer’s recommendation. The 3'-RACE and 5'-RACE products were purified and ligated into pGM-T vector and then transformed into Escherichia coli DH5α. On the basis of blue-white screening using Xgal-IPTG system and PCR identification, the recombinants were selected and sequenced.

**Cloning of Pf–HMGR full-length cDNA**

After aligning and assembling the sequences of conserved fragment, 3'-RACE and 5'-RACE products, the full-length cDNA sequence of Pf–HMGR gene was deduced and subsequently obtained by PCR using primer fPf–HMGR (5'-ATGGAGCAGGAACGACGT AGT-3') and rPf–HMGR (5'-GCAGTTGTGTCAC CGCAGATAC-3') with 5'-RACE-ready cDNA as template. The PCR products were purified and cloned into pGM-T vectors, followed by sequencing.

**Bioinformatics analysis**

The obtained sequences of Pf–HMGR were analyzed using several online web services and
software. Comparative and bioinformatics analyses of HMGR proteins were performed online at two websites NCBI (http://www.ncbi.nih.gov) and EXPASY (http://expasy.org/tools). The deduced Pf-HMGR and other known plant HMGRs retrieved from GenBank were aligned with CLUSTAL W (1.82). The transmembrane helixes of Pf-HMGR were analyzed with TMHMM v2.0 server (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Finally, the 3-D structure was simulated by Insight II software package developed by Accelrys Software Inc., running on the Dell Precision 470 workstation with Red Hat Linux system.

Tissue expression analysis

To study the expression pattern of Pf-HMGR in different tissues (leaves, stems and roots) of P. fargesii, semi-quantitative one-step RT-PCR was performed according to the protocol of one-step RNA PCR kit (Takara, Japan). Total RNA was obtained from leaves, stems and roots, respectively and used as templates in one-step RT-PCR reaction with Pf-HMGR-1 (5'-TATCTCTGCTCCGTCAAGCA-3') and Pf-HMGR-2 (5'-ATCGCATCGTAGTCGAAACC-3') as primers. Meanwhile, the 18S rRNA gene of P. fargesii was amplified by the specific primers 18S F (5'-ACCTGGTTGATCCTGCCAGT-3') and 18S R (5'-TCACCTACGGAAACCTTGT-3') as an internal control in RT-PCR. Amplifications were carried out by transcribing the template at 50°C for 50 min and denaturing at 94°C for 3 min, followed by 30 cycles of amplification. The RT-PCR products were fractionated on 1.5% agarose gel stained with ethidium bromide.

Results and Discussion

Generation of full-length cDNA of Pf-HMGR gene

The genes encoding HMGRs are indispensable for saponin biosynthesis. Using a pair of degenerated primers, a conserved fragment of 404 bp was amplified from the total RNA of from P. fargesii. Blastn result revealed that the sequence had high similarity with HMGR genes of other plant species, such as Michelia chapensi (DQ098012.1, 81%), Linum usitatissimum L. (FJ667605.1, 80%) and Ricinus communis L. (XM_002510686.1, 78%).

Based on the specific fragment, three pairs of gene-specific primers were then designed and synthesized for the 3'-RACE and 5'-RACE. By using the above-mentioned methods, the full-length cDNA of Pf-HMGR was then deduced, amplified and subsequently confirmed by sequencing. The full-length Pf-HMGR clone (GenBank accession no JX508638) was 1,973 bp, with 3’ untranslated regions, polyA tail and a 1728 bp ORF encoding a deduced protein of 576 amino acids (Fig. 1).

Characterization of deduced Pf HMGR protein

ExPASy server analysis showed the inferred protein had a theoretical isoelectric point (pI) of 6.64 and calculated molecular mass of about 61 kDa (http://www.expasy.ch/cgi-bin/pi_tool). The predicted protein contained all kinds of amino acids with different proportions. Leu (10.76%) was the most abundant amino acid in the deduced Pf-HMGR, followed by Ala (10.1%), Val (9.6%), Ser (8.9%) and Gly (8.5%). 49% of the total amino acids were negatively charged and 47% were positively charged.

Sequence aligning and protein-protein BLAST demonstrated that the deduced Pf-HMGR amino acid sequence was highly homologous to many other HMGR sequences from higher plants, including R. communis (77%), Litchi chinensis (76%), M. chapensis (75%) and Panax quinquefolius (72%), indicating that Pf-HMGR belonged to the HMGR family. The amino acids sequence of Pf-HMGR was quite diverse in both length and composition in the N-terminus, when compared with other plant HMGRs, especially at two locations, amino acid 1–59 and 130–190. The high homology region appeared to be centered on the NADP (H) and HMG-CoA binding sites. Three amino acid sites in the first HMG-CoA binding site (EMPVGYV QMP) were diverse among plant species, while the amino acids composed of the second HMG-CoA binding site (TTEGCLVA) and the two NADP(H) binding sites (DAMGMNM and GTVGGG) were the same in all the aligned plant HMGRs. In the first HMG-CoA binding site, the diversities among plant species might contribute to the substrate selectivity, evolutionary significance and enzyme kinetics, which are worthy of further investigation. In addition, conserved regions, including one protein kinase C phosphorylation site, two casein kinase II phosphorylation sites and five N-glycosylation sites were predicted by prosite motif search (Fig. 2), indicating that activity of the protein may be regulated by phosphorylation.

TMHMM analysis showed that predicted protein encoded by Pf-HMGR exists two transmembrane
helices, amino acid residues are located at 34-56 and 77-99 in the N-terminal, respectively (Fig. 3A). They were observed to be located in the cytoplasmic membrane based on analysis by the PSORTb v2.0 server (http://www.psort.org/psortb/index.html). We also carried out membrane-spanning regions prediction by using TMPRED software, which revealed the possible existence of four membrane-spanning regions (Fig. 3B), located at 42-60, 86-109, 431-457 and 531-554, respectively. The hydrophobicity profile indicated the N-terminal region of the protein included transmembrane segments separated by hydrophilic loops which may be used to anchor the enzyme to the plasma membrane, as in other HMG-CoA reductases. It is well-known that the membrane domains of plant HMGRs contain two membranes panning helices; however, the exact number in Pf-HMGR requires further experiments for validation.
Prediction of structure for Pf-HMGR protein product

When searched in PDB with the sequence of the Pf-HMGR, a structure of catalytic portion of human HMG-CoA reductase (PDB code 1DQ8) had the best sequence alignment score (with 1,118 total score and 56% identities), thus it was selected as the template to build the molecular model of the Pf-HMGR. The Pf-HMGR was constructed and simulated by Insight II software through a series of procedures. The obtained molecular model was finally assessed by Profile 3D and ProStat programs and indicated to be reliable.

The result of molecular modeling (Fig. 4) showed that Pf-HMGR had a spatial architecture that was very similar to HMGR from human and other plants. Pf-HMGR consisted of 43.1% α-helix, 14.0% extended strand and 42.9% random coil and contained three domains: (i) the large, central L-domain harboring two HMG-CoA binding motifs and a NADP(H)-binding motif, (ii) the small helical N-terminal domain, and (iii) the smallest S-domain harboring a NADP(H)-binding motif. These results implied that Pf-HMGR could encode a functional HMGR protein and the 3D structure of target enzyme was, therefore, required to construct the integral active pocket of Pf-HMGR. It is reported that the interface between NADPH and HMG-CoA pockets is the catalytic region of HMGR and several residues in this region can participate in substrate catalysis.
The knowledge of binding ligand in the active site of target enzyme needs to be further clearly elucidated.

Expression analysis of Pf-HMGR in different tissues

HMGR has been deemed to function in the initial step of the MVA pathway and indirectly influence the biosynthesis of SAP. The root of *P. fargesii* is often used as the main material for traditional Chinese medicine as the major medicinal active ingredient SAP is mostly found in the root. In the present study, RT-PCR assay revealed that Pf-HMGR could be detected in three tissues, including roots, stems and leaves, but expression was higher in roots and stems than in leaves (Fig. 5), indicating that Pf-HMGR expressed constitutively in all plant tissues in order to synthesize the sterol which is necessary for the growth. Earlier, it is also found that the transcript of HMGR in *Ginkgo biloba* is present specifically in roots and the SAP biosynthesis takes place in the roots, but subsequently transported to the leaves.

In conclusion, we cloned and characterized the full-length transcript of *P. fargesii* HMGR by RACE and RT-PCR for the first time, which will be helpful in understanding the SAP biosynthesis at the genetic level. Further investigation is in progress to confirm the correlation between SAP biosynthesis and Pf-HMGR gene expression.

Fig. 5—Expression pattern of Pf-HMGR in different *P. fargesii* tissues [Total RNA from roots, leaves and stems, respectively was subjected to one-step RT-PCR amplification (upper panel). 18S rRNA gene was used as the control to show the normalization of the templates in PCR reactions (lower panel)]

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References