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# Identification and expression analysis of a 3-hydroxy-3-methylglutaryl coenzyme A reductase gene from American ginseng

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#### Abstract

3-Hydroxy-3-methylgutary coenzyme A reductase (HMGR, EC 1.1.1.34) catalyzes the NAD(P)H-dependent reduction of HMG-CoA to mevalonate, the first committed step in the isoprenoid pathway, which produces the largest group of contemporary natural products. We report the cloning and characterization of a full-length cDNA that encodes HMGR (designated as PqHMGR) from a 4-year-old *Panax quinquefolius* root, a ginsenoside-producing plant. The full-length cDNA of PqHMGR is 2327 bp, with a 1770 bp open reading frame that encodes a protein containing 589 amino-acids. Bioinformatics analyses revealed that the deduced PqHMGR is a new HMGR with a spatial structure similar to that of *Homo sapiens* with a sequence identity of 57.7%. PqHMGR shows high homology to plant HMGRs, particularly with considerable similarity to the HMG-CoA reductase of *Camptotheca acuminata*, suggesting that this enzyme may have an important function in terpenoid biosynthesis in *P. quinquefolius*. Expression analysis by real-time quantitative PCR indicates that PqHMGR is differentially expressed among tissues, with a strong expression in the leaf and evolutionary case of HMGR in plants is presented in this study. The results enable further elucidation of the genes involved in ginsenoside biosynthesis in a tissue-specific manner.

**Keywords:** 3-Hydroxy-3-methylgutary coenzyme A reductase; RACE; mevalonate biosynthesis; *Panax quinquefolius*; real-time quantitative PCR.

**Abbreviations:** DMAPP - Dimethylallyl diphosphate; IPP - Isopentenyl diphosphate; ORF - Open reading frame; RACE - Rapid amplification of cDNA ends.

#### Introduction

In Asia, the roots of Panax quinquefolius (Xiyangshen or American ginseng) have served as a source of health-promoting nutrients and as tonic herbs for approximately 300 years. Ginseng products are referred to as "adaptogens" that purportedly increase resistance to physical stress and build up vitality (Kiefer and Pantuso, 2003). The pharmacological properties of this herb are attributed primarily to ginsenosides, a group of triterpenoids that are found in the extracts of ginseng roots (Attele et al., 1999). Nearly 30 types of ginsenosides have been discovered (Ma et al., 2006), some of which exhibit anti-cancer, anti-oxidant, and anti-metastatic properties (Peralta et al., 2009; Rasheed et al., 2008; Lee et al., 2009; Qiu et al., 2009). Among ginsenosides, Rb1, Rb2, Rc, Rd, Re, and Rg1 are the main compounds. Despite their pharmacological potential, ginseng roots have low ginsenoside yields. Remarkable progress has recently been observed in the molecular regulation of the biosynthesis of plant secondary metabolites. The taxadiene production in yeast has increased by 40-fold via a combined strategy that involves the heterogeneous expression of enzymes in the early stage of Taxol biosynthesis, codon optimization, and introduction of regulatory elements to inhibit competitive pathways (Engels et al., 2008). Ro et al. (2006) first reported the construction of a biosynthetic pathway of artemisinin precursor. The reconstruction of a biosynthetic pathway of secondary metabolites through biotechnology is considered a promising approach to stimulating secondary metabolite production. Triterpenoids are produced from 2,3-oxidosqualene, which is

synthesized from isopentenyl diphosphate and its allylic isomer dimethylallyl diphosphate via the mevalonate (MVA) pathway (Haralampidis et al., 2002). In plants, MVA is the general precursor of various identified isoprenoids, such as sterols, plant growth regulators, and terpenoids (Wani et al., 1971; Schepmann et al., 2001). 3-Hydroxy-3-methylgutary coenzyme A reductase (HMGR), located in the endoplasmic reticulum, catalyzes the conversion of HMG-CoA to MVA. This reaction is a rate-limiting step in isoprenoid biosynthesis. Recently, we have successfully initiated large-scale expressed sequence tag (EST) projects to explore the pathway of ginsenosides, and have discovered new enzymes involved in ginsenoside backbone synthesis (Sun et al., 2010; Wu et al., 2010). These results serve as guidance for the scientific community in exploring the molecular genetics and functional genomics of P. quinquefolius. In this work, we cloned a full-length cDNA of PqHMGR from the ETSs of a 4-year-old P. quinquefolius root. We also report the cloning and sequence analysis of a gene that encodes HMGR from this commercially important medicinal plant to better elucidate the role of HMGR in triterpene saponin biosynthesis. The expression profile and molecular evolution of PqHMGR gene are also described.

#### **Results and discussion**

# Molecular cloning of full-length PqHMGR cDNA

Two sequences, Cl1267 and Cl128, were identified in the ESTs that were obtained from the cDNA libraries of *P. quinquefolius*.

Table 1. Pairwise comparisons of the encoded amino acid sequences of selected HMG-CoA reductase genes.

Organisms	Identity with <sup>a</sup>								
	С.	PqHMGR	<i>S</i> .	С.	Gbiloba	Т.	Н.	Р.	<i>G</i> .
	acuminata		miltiorrhiza	avellana		media	sapiens	jadinii	lucidum
C. acuminata		0.177	0.204	0.218	0.466	0.490	1.075	1.074	1.178
PqHMGR	0.838		0.180	0.206	0.435	0.454	1.045	1.034	1.154
S. miltiorrhiza	0.819	0.835		0.220	0.406	0.439	0.927	0.979	0.958
C. avellana	0.804	0.815	0.809		0.463	0.490	0.992	1.043	1.092
G. biloba	0.644	0.661	0.679	0.645		0.263	0.997	1.125	1.086
T. media	0.641	0.659	0.675	0.646	0.776		1.041	1.216	1.176
H. sapiens	0.430	0.444	0.470	0.463	0.424	0.437		1.447	1.504
P. jadinii	0.422	0.447	0.465	0.441	0.418	0.396	0.345		1.300
G. lucidum	0.399	0.401	0.455	0.423	0.406	0.391	0.327	0.395	

<sup>a</sup> Tabulated below the diagonal are the similarity between the amino acid sequences. Shown above the diagonal are the estimated numbers of amino acid substitutions per site as calculated using the "Dayhoff PAM Matrix" option in the program PRODST of the PHYLIP 3.67 package.

91	TCTCTCTCCCCATAGTTGCCAACCTCCATAAAAATGGACGTTCGCCGGCGATCAGTCACTAAAACACTCACGGCCGGC
1	M D V R R R S V T K T L T A G E P L
181	AATCACAAAAATCAACACCCCCTCTCTCTCAAAAGCCTCTGATGCTCTTCCTCCCCATTGTATCTCACCAATGGCTTGTTCTTCACCATG
19	K S Q N Q H S S S L K A S D A L P L P L Y L T N G L F F T M
271	TCTTCTCAGTCATGTATTTCCTTCTCCATCGGTGGAGGAGGAGGAGAAGATTAGGAATTCTGTCCCTCTCCATGTGGTGACCCTTTCAGAATTG
49	F F S V M Y F L L H R W R E K I R N S V P L H V V T L S E L
361	CCGCCTTGGTTTGTTGGTTGCGTCTGTAATTTACCTCCTTGGATTTTTGGGATTGGATTTGTCCAGTCACTCATCAGGCCCTCGCCT
19	A A L V L L V A S V I Y L L G F F G I G F V Q S L I R P S P
151	ATAGTTGGGACATTCTCGAAGATGACAATGCCATTCTCGAGGAAGATAGCCGTTGCGAACCATGCGCAGCGGCTATTGATTG
109	D S N D I L E D D N A I L E E D S R C E P C A A A I D C S L
541	$\tt CTCCTAATCTCAAGATTGTCCGCATGGTTCCCCCAAAAGCATAAATCAGCTTTTGCGGATGTGGTGGAGGAACAGAAACAATCTGCATCCCCCCAAAACAATCTGCATCAGCTTTTGCGGATGTGGTGGAGGAACAAACA$
139	P P N L K I V R M V P Q K H K S A F A D V V E E Q K Q S A S
631	CAACCATCATAGACGAAGACGAGGAGATCATTAACGCAGTAGTGGCGGGGGACCACTCCTTCGTACTCCCTCGAGTCAAAACTCGGGGAC
169	A T I I D E D E E I I N A V V A G T T P S Y S L E S K L G D
721	GCCTGAAAGCAGCTGCCATCAGACGCGAAGCATTGCAGAGAATTACTGGAAAGTCACTGGACGGGCTGCCGTTGGATGGTTTTGATTAC
199	C L K A A A I R R E A L Q R I T G K S L D G L P L D G F D Y
811	AGTCCATTTTGGGACAATGCTGCGAGATGCCGGTGGGGTACGTAC
229	E S I L G Q C C E M P V G Y V Q I P V G I A G P L L L N E T
901	AGTATTCGGTGCCAATGGCGACCACAGAAGGGTGCCTGGTGGCGAGTACTAATCGGGGTTGTAAAGCTATCTAT
259	EYSVPMATTEGCLVASTNRGCKAIYASGGA
991	CCAGTGTATTGTTAAGGGATGGCATGACTAGGGCCCCTGTGGTTAGGTTTGGTAGTGCCAAGAGGGCGGCAGAATTGAAATTCTTTTTG
289	T S V L L R D G M T R A P V V R F G S A K R A A E L K F F L
1081	AGGATCCCATGAATTTTGAGACTCTTGCCCTTGTCTTTAACAAATCAAGCAGGTTTGGAAGATTACAAGGAATTAAATGTGCAATAGCA
319	E D P M N F E T L A L V F N K S S R F G R L Q G I K C A I A
1171	GAAAGAACTTATACATGAGATTTACCTGCAGCACTGGGGATGCAATGGGAATGAAT
349	G K N L Y M R F T C S T G D A M G M N M V S K G V Q N V L D
1261	TCCTTCAGAACGATTTTCCTGACATGGACGTCATGGGTATTTCTGGAAATTATTGCTCTGATAAGAAACCAGCAGCAGCAGTGAACTGGAT
379	F L Q N D F P D M D V M G I S G N Y C S D K K P A A V N W I
1351	AGGGGCGTGGTAAATCGGTTGTCTGTGAGGCAATTATAAAGGAGGAGGTAGTGAAGAAGGTGTTGAAGACTAATGTGGCAGCCTTGGTG
409	EGRGKSVVCEAIIKEEVVKKVLKTNVAALV
1441	AGCTTAATATGCTTAAAAATCTTGCAGGCTCAGCAGTGGCTGGC
139	E L N M L K N L A G S A V A G A L G G F N A H A S N I V S A
1531	TGTATATAGCCACCGGCCAGGACCCGGCCCAAAATATTGAGAGTTCTCACTGTATCACTATGATGGAAGCAGTTAATGATGGCAAGGAC
469	VYIATGODPAONIESSHCITMMEAVNDGKD
1621	TTCATATATCTGTCACCATGCCTTCCATTGAGGTGGGGACAGTTGGAGGTGGGACCCAACTAGCATCTCAGTCAG
199	LHISVTMPSIEVGTVGGGTOLASOSACLNL
1711	TTGGAGTGAAGGGTGCCAGCAAGGAGTCACCCAGGGTCAAACTCCAGGCTCTTGGCCAGCATTGTAGCTGGTTCAGTCCTTGCTGGTGAG
529	L G V K G A S K E S P G S N S R L L A S I V A G S V L A G E
1801	TTTCCCTTATGTCTGCACTAGCTGCAGGGCAACTTGTAAAGAGCCACATGAAATTCAATAGATCTAGCAAAGATGTCTCCCAAGCTCTCT
559	L S L M S A L A A G O L V K S H M K F N R S S K D V S K L S
1891	CCTAATTACAAAAATAGCCACCAAGAAACATTTTCAATGGAGGAGGAGGATAGATGGTGGTGTGGTATTCTTTTTCATAAATATCAAT
589	S *
1981	TCTTGTGGGGGGGCCAATGCAATGCAATGGTTGTGAAGTTGGGGGGGG
2071	gggttgtgttttgtataatgcctgccacttgggtgtttccgtaactaagaaggggttgccaataaacaatgaagctctcttctctctc
2161	CTCTCTCATGTATGTAGTTTCTCATTAGTTTGTAGTAATTTATTAGGAAGAAGGCTTCTCTTGATTGGGTATTGTGATGGGTTCTCT

Fig 1. Full-length cDNA and deduced amino acid sequence of PqHMGR. The initial codon is underlined and the stop codon is represented by an asterisk. The sequence was submitted to GenBank with the Accession Number FJ755158.

The sequences show high similarity to the HMGRs in Hevea brasiliensis (E-value at 4.00E-23) and Solanum tuberosum (E-value at 1.00E-28), respectively. Gene-specific primers were synthesized from the core fragments and used to generate the 5'-end and 3'-end DNA fragments. By nested PCR, we obtained the expected products from the core sequence Cl128. The cDNA ends with approximately 1.5 and 1 kb fragments were amplified by 5' RACE and 3' RACE, respectively. After the obtained sequences and the core fragment were assembled, we deduced and amplified the full-length cDNA of PqHMGR (GenBank Accession No.: FJ755158). The full-length cDNA of PqHMGR is 2327 bp with a 1770 bp open reading frame (ORF; Fig. 1). Sequence alignment revealed that Cl1267 paired well with the 3'-terminal of this full-length cDNA. This gene, which encodes a polypeptide of 589 amino acids is linked with 125 bp of a 5'-untranslated region and 398 bp of a 3'-untranslated region with a polyA tail of 33 bp. The predicted HMGR protein has a calculated molecular mass of 63 kDa and a theoretical pI of 6.3. The prediction of transmembrane helices by TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) revealed two transmembrane regions in the PqHMGR polypeptide. One is located between  $A_{35}$  (P) and  $A_{57}$  (L), and the other is located between  $A_{78}$  (L) and  $A_{100}$  (V) along the polypeptide chain. The structure of plant HMGRs markedly differs from that of animals and fungi, in which membrane-spanning domains contain seven or eight transmembrane segments; by contrast, higher plants usually have only two membrane-spanning domains (Roitelman et al., 1992; Learned and Fink, 1989). The secondary structure prediction of PqHMGR was performed using the SOPMA server (Combet et al., 2000). On the basis of hierarchical neural network analysis, we determined that PqHMGR protein comprises 52.46% α-helix, 28.52% random coil, 14.26% extended strand, and 4.75% β-turns. The α-helix and random coil are the most abundant structural elements,

C.ACUMINATA E.ULMDIDES N.TABACUM FQHMGR P.TRICHOCARPA S.TUBEROSUM	MO.VRREPTKSLEPAKT.AAAGEPLKPHHQNESSLKASDALPLPLYLINGVEFTLEFSVAYFLLERWREXIRTSTTELHIVTVSEL MO.LRERPFKFAATNGRNHELHAQGISSSPAIDCSFSFIFKASDALPLPLYLINGIEFTLEFSVAYFLLERWREXIRTSTFLEVIJLSEL MO.VRREPVKPFYPSKDOVSAGESLKPHKQTQQVSSFKASDALPLPLYLINGLEFTMFFSVAYFLLVRWREXIRNSTFLEVIJLSEL MO.VRREVVKTLTAGEPLKSQNCHSSSLKASDALPLPLYLINGLEFTMFFSVAYFLLHRWREXIRNSTFLEVIJLSEL MEGTRRRSTTTTKPFRKLTDHDESSALTGPKASDALPLPLYLINGLEFTMFFSVAYFLLVRWREXIRNSTPLEVIJLSEL MO.VRREPVKPLYT.SKD.ASAGEPLKQQEVSSPKASDALPLPLYLINGLEFTMFFSVAYFLLVRWREXIRNSTPLEVIJLSEL	83 89 86 78 80 81				
C.ACUMINATA E.ULMOIDES N.TABACUM PQHMGR P.TRICHOCARPA S.TUBEROSUM	ARLISLIRSVIYLEGFGIDFVGSFITRASHDSRDVEYIN.DDRFILEEDSRCPCARIDR.SLVPPQVAAVAPPKPKVV ARIVSLIRSFIYLEGFGIDFVGSFIARASHDPMDVDDDERFILEEDRRGPCARIDC.LVGPVAPSISDVRKLM VMVSLIRSVIYLEGFGIGFVGSFVSBSNSSSDVEDEN.TEGFILEEDSRGPCARITLGCNVPPPSRADUVPMVPQDPAKVALVA ARLVLVRSVIYLEGFGGIGFVGSI.RSPSBSNDIEDNRAILEEDSRGPCARIDC.SLPM.LKVVRMVPQVRKSAFADVV VAIFAFVASFIYNLGFGGIGFVGSILRSPSBVAAEDDDEDEEDLLKENARKVPGGALDCTAPPQK.LAVVSPPLKFKVV LAVVSLIRSVIYLEGFGGIGFVGSVSBSNSSSDVEDEN.AEQLITEEDSRGPCARVTLGCVVPPPVRKIAFMVPQDPAKVALSQT	162 164 175 160 162 170				
C.ACUMINATA E.UIMOIDES N.TABACUM PQHNCR P.TRICHOCARPA S.TUBEROSUM	HMG-CoA-binding mot ECPVPVIAP, 7SEEDEELIKSVVAGTIPSYSLESRUGO (KRAAAIRREALQRUTGKSLAGLPLOGFDYESILGOO ENAVGYVOPA/GIA DPP, APLPSVEDEOMVKSV LSGTVPSYSLESRUGO (KRAAAIRREALQRUTGKSLSGLPLOGFDYESILGOO ENAVGYVOPA/GIA EKRAPIITPA/SEDDEELIGSVVQKTPSYSLESRUGO (IAAAAIRREALQRUTGKSLSGLPLOGFDYESILGOO ENAVGYVOIP/GIA EELTP. FPTIMEEDEELINSVVAGTIPSYSLESRUGO (IAAAAIRREALQRUTGKSLSGLPLOGFDYESILGOO ENAVGYVOIP/GIA EELTP. FPTIMEEDEELINSVVAGTIPSYSLESRUGO (IAAAAIRREALQRUTGKSLSGLPLOGFDYESILGOO ENAVGYVOIP/GIA EEKRSPIIMPALSEDEELINSVVAGTIPSYSLESRUGO (IAAAAIRREALQRUTGKSLSGLPLOGFDYESILGOO ENAVGYVOIP/GIA	tif [ 251 250 265 250 250 250 260				
C.ACUMINATA E.ULMOIDES N.TABACUM PQHMGR P.TRICHOCARPA S.TUBEROSUM	HMG-CoA-binding motif II GFLLLDGREYLVFMUTTEOCLVAETINGCKALYABOGATSVILRDOMTRAPVVREGTARMSAELKEFLEDERNEDILAVVFNKSSREGRE GFLLLNGEYSVFMUTTEOCLVAETINGCKALYABOGATSULLRDOMTRAPVVREGTARMSAELKEFLEDERNETILSMVFNKSSREARL GFLLLDGREYSVFMUTTEOCLVAETINGCKALFABOGATSVILRDOMTRAPVVREGTARMSAELKEFVEDELNEETLSJVFNKSSREARL GFLLLDGREYSVFMUTTEOCLVAETINGCKALFABOGATSVILRDOMTRAPVVREGTARMSAELKEFVEDELNEETLSJVFNKSSREARL GFLLLDGREYSVFMUTTEOCLVAETINGCKALFABOGATSVILRDOMTRAPVVREGTARMSAELKEFVEDENNEETLALVFNKSSREARL GFLLLDGREYSVFMUTTEOCLVAETINGCKALFABOGATSVILRDOMTRAPVVREGTARMSAELKEFVEDENNEETLALVFNKSSREARL GFLLLDGREYSVFMUTTEOCLVAETINGCKALFABOGATSVILRDOMTRAPVVREGTARMSAELKEFVEDENNEETLALVFNKSSREARL	341 340 355 340 340 350				
C.ACUMINATA E.ULMOIDES N.TABACUM PQHMGR P.TRICHOCARPA S.TUBEROSUM	NADP(H)-binding motif I CGTHCALAGONLYMFESCET CRAMMINE KGVCNVLDFLCNDESCMOVIGIBONFCSDKKEAAVMIEGRIGKSVVCESIIKEEVVPRVL CGTCCSIAGKNLYMFFTCSTDRAMMINE SKSVCNVLDFLCNDFFCMOVIGIBONFCSDKKEAAVMIEGRIGKSVVCEATISEDIVRVL CGTCCATAGKNLYMFTCSTDRAMMINE SKSVCNVLDFLCNDFFCMOVIGIBONFCSDKKEAAVMIEGRIGKSVVCEATISEDIVRVL CGTCCATAGKNLYMFFTCSTDRAMMINE SKSVCNVLDFLCNDFFCMOVIGISONYCSDKKEAAVMIEGRIGKSVVCEATISEDVKKVL CGTCCATAGKNLYMFFSCETSTRAMMINE SKSVCNVLDFLCNDFFCMOVIGISONYCSDKKEAAVMIEGRIGKSVVCEATISEDVKKVL CGTCCATAGKNLYMFFSCETSTRAMMINE SKSVCNVLDFLCKDFFCMOVIGISONYCSDKKEAAVMIEGRIGKSVVCEATISEDVKKVL	431 430 445 430 430 490				
C.ACUMINATA	NADP(H)-binding motif II KT: WASEVEERMERKETGER-AGALGERWHÄSNEVSEVYINTGCEPACH/ESSHCITTE-EAVECE/DER/SUSMPSIER, IMOGOTICEA 520					
E.AUMOIDES N.TABACUM FQHUGR P.TRICHOCARPA S.TUBEROSUM	NTTVPALVELAMLENLAGSVVAGALGEFNAHANTVSXVFINTGOPAQNIESSHOTMAEAVNOCKOLH SVTMPSIEVGTVGGTQLA NTEVASLVELAVLENITGSAMAGALGGENAHASNIVSAVYLATGOPAQNIESSHOTMAEAVNOCKOLH SVTMPSIEVGTVGGTQLA NTWAALVELAMLENLAGSVAGALGGENAHASNIVSAVYLATGOPAQNIESSHOTMAEAVNOCKOLH SVTMPSIEVGTVGGTQLA NTWAALVELAMLENITGSAMAGALGGENAASNIVSAVYLATGOPAQNIESSHOTMAEAVNOCKOLH SVTMPSIEVGTVGGTQLA NTWAALVELAMLENITGSAMAGALGGENAHASNIVSAVYLATGOPAQNIESSHOTMAEAVNOCKOLH SVTMPSIEVGTVGGTQLA	520 535 520 520 530				
C.ACUMINATA E.ULMOIDES N.TABACUM PQHMGR P.TRICHOCARPA S.TUBEROSUM	BOSACLINLLOVKGASKESFOSNSFLLATIVAGSVLAGELEIMSA IZAQQU/KSHNRYNRESKEITKVSS SOSACLINLLOVKGANKESPOSNSFLLAATVAGSVLAGELSIMSA IZAQQU/NSHNRYNRESKEVTS SOSACLINLLOVKGANREZAGSNSFLLAATVAGSVLAGELSIMSA ISAQQU/KSHNRYNRESKEVTKISS SOSACLINLLOVKGASKESPOSNSFLLASTVAGSVLAGELSIMSA IZAQQU/KSHNRFNRS SKEVSKLSS SOSACLINLLOVKGASKESPOSNSFLLASTVAGSVLAGELSIMSA IZAQQU/KSHNRFNRS KEVSKUSS SOSACLINLLOVKGANREZAGSNAFLLASTVAGSVLAGELSIMSA IZAQQU/KSHNRFNRS KEVSKUSS	589 589 604 589 589 589 596				

Fig 2. Alignment of amino acid sequences of selected HMG-CoA reductases. The identical residues are shown in black against a gray background. The HMG-CoA-binding motifs and NADP(H)-binding motifs are indicated by black boxes.



Fig 3. The proposed 3D structure of PqHMGR established by homology-based modeling. For clarity, only one monomer is shown. The polypeptide chain is shown in ribbons and the HMG-CoA binding motifs and NADP(H)-binding motifs are represented by balls and sticks.

whereas the extended strand and  $\beta$ -turn are intermittently distributed in the polypeptide.

# Sequence comparison of PqHMGR

A BLAST search of the protein database at GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the polypeptide sequence of P. quinquefolius (PqHMGR) has 69%-81% identity and 88%-79% positives with HMGRs from other organisms. A multi-alignment analysis using Clustal X demonstrates that PqHMGR exhibits high amino acid sequence similarity to other plants, such as Camptotheca acuminata (80.7%; Accession No.: AAB69727), Solanum tuberosum (78.7%; Accession No.: P48020), Nicotiana tabacum (78.6%; Accession No.: AAB87727), Eucommia ulmoides (75.9%; Accession No.: AAV54051) and Populus trichocarpa (77%; Accession No.: XP\_002313569; Fig. 2). The results showed that PqHMGR belongs to the HMGR superfamily. The comparison of the amino acid sequences of HMGRs from different organisms revealed two HMG-CoA-binding motifs (EMPVGYVQIP and TTEGCLVA) and two NADP(H)-binding motifs (DAMGMNM and GTVGGG). In the first HMG-CoA binding site, the diversities among plant species may contribute to substrate selection. These substrate-binding motifs are functionally important and highly conserved in plant HMGR proteins. The similarity levels are much higher toward the C-terminal sequences than toward the N-terminal sequences among different enzymes. Studies have also shown that the N-terminal hydrophobic region is unnecessary for catalytic activity and are more diverse in terms of amino acid composition and length (Basson et al., 1988; Ruiz-Albert et al., 2002). Sequence comparison among HMGRs revealed two distinct classes: the eukaryotic HMGRs (class I) and prokaryotic HMGRs (class II) (Bochar et al., 1999). Class I HMGRs contain N-terminal membrane domains involved in the sterol-regulated degradation of the HMGR protein, whereas class II HMGRs lack a membrane domain.

### Homology model of the PqHMGR protein

The 3D structure of PqHMGR was predicted by the Swiss-Model; sequence homology-based structural modeling with human HMGR (PDB No.: 1DQ8; Fig. 3) was carried out. The molecular modeling results revealed that PqHMGR forms a homodimer and that each monomer consists of three domains: (1) the central L-domain that harbors two HMG-CoA binding (EMPVGYVQIP motifs and TTEGCLVA) and NADP(H)-binding motif (GTVGGG); (2) the small S-domain that contains an NADP(H)-binding motif (DAMGMNM); and (3) the N-domain. A 3D model with high similarity to human HMGR would be helpful in elucidating the enzyme kinetics of PqHMGR.

# Analysis of the molecular evolution of PqHMGR gene

To clarify the evolutional relationships of plant HMGRs, we calculated the sequence homologies between some known plant HMGR clones (Table 1) and constructed a phylogenetic tree that is based on HMGRs from different organisms, including plants, animals, fungi, and bacteria (Fig. 4). The results showed that HMGRs originate from the same ancestral lineage. The plant HMGR clones form one big cluster in the phylogenetic tree, indicating that the plants have acquired HMGR gene before they diverged into individual species during the course of evolution. In contrast to archaebacteria, fungi, and animals, the plant group diverged at a later period. Among the plant

group, T. media and Ginkgo biloba diverged earlier than did other plant species, which is consistent with the evolutional position of gymnosperms and angiosperms. On the basis of the phylogenetic analysis, we deduced that PqHMGR belongs to the plant group and representes the most recent diverged lineage within that group. The phylogenetic tree also shows that PqHMGR, together with C. acuminata, forms one branch of the HMGR cluster with S. miltiorrhiza. Furthermore, PqHMGR shows high similarity (83.8%-83.5%) to the HMGRs of C. acuminata and S. miltiorrhiza. The HMGR of C. acuminata is known as the MVA source of the terpenoid moiety that is associated with an anti-cancer monoterpenoid indole alkaloid. Pairwise comparisons of the encoded amino acid sequences revealed that similarities (64.1%-83.8%) within a class are significantly higher than those between classes (32.7% to 47%). Compared with gymnosperms (64.1%-64.4%), PqHMGR shows higher similarity (80.4%-83.8%) to angiosperms. This result suggests that PqHMGR is a more evolved clone in the plant HMGR gene families.

### Expression of HMGR in different parts of P. quinquefolius

The sensitive quantitative real-time PCR was performed using total RNA isolated from various tissues of American ginseng. The expression of PqHMGR could be detected in all of the tissues tested. However, it is highly expressed in the leaves and abundantly expressed in the flowers and roots. In the stems, however, PqHMGR is least expressed (Fig. 5). The results are consistent with the findings of a previous study (Qu et al., 2009), in which the total contents of 12 ginsenosides in different parts of American ginseng follow the order leaf > root-hair > rhizome > root > stem. This result indicates a tissue-specific expression and regulation of this gene, which correlates with ginsenoside synthesis. Thus, the expression levels of HMGR likely exhibit a positive correlation with the content of triterpene saponins, such as ginsenosides, in P. quinquefolius. Our results also suggest that leaves are crucial to terpenoid biosynthesis in P. quinquefolius. In Traditional Chinese Medicine, only the roots of ginseng are used for medical purposes, whereas other parts (e.g., leaves and stems) are discarded. Leaves are a promising available source of ginsenosides, as supported by scientific evidence. The analysis of the spatial expression of PqHMGR provides useful information regarding the association between the protein and tissue-specific functions. HMGR dominates the branch-point of primary and secondary metabolism. Thus, identifying the expression and regulation of HMGR involved in ginsenoside biosynthesis is also of interest. Terpenoids are derived from the repeated condensation of isoprenoids, among which mevalonic acid function as a precursor. Within plant cells, the concentration of MVA is strictly controlled by the activity of HMGR. HMGR is one of the most highly regulated enzymes that have been identified (Goldstein and Brown, 1990). The activity of HMGR is regulated by the concentration of the products of the MVA pathway (Nakanishi et al., 1988). Although PqHMGR transcripts have been detected in various tissues, they are particularly abundant in leaves than in any other parts. This finding provides evidence for the comprehensive use of aerial resources.

# Materials and methods

### Plant materials and sample preparation

*P. quinquefolius* L. was collected from the planting base of Huai-rou County, Beijing, China (116°62′ E, 40°32′ N). Fresh



**Fig 4.** Phylogenetic tree of HMGRs that was generated from various organisms using the Clustal X program by the neighbor-joining method. The numbers above the nodal branches are the bootstrap values performed with 1,000 replicates and those > 50% are shown at the branches. The symbol in front of each species indicates their respective taxonomic groups, indicated as follows:  $\triangle$  or  $\diamondsuit$ : Plants;  $\blacksquare$ : Animals;  $\blacksquare$ : fungi;  $\bigcirc$ : bacteria.



Fig 5. Real-time PCR results for HMGR gene expression among different tissues of *Panax quinquefolius* 

roots, leaves, and stems were cut off and washed with running water. The roots were quickly cut into 2-3 mm thick slices. All the tissues were immediately frozen in liquid nitrogen and stored at -80 °C prior to total RNA extraction.

# Cloning of full-length cDNA of PqHMGR by rapid amplification of cDNA ends

Total RNA was isolated from the leaves of *P. quinquefolius* using the RNeasy plant kit according to the manufacturer's instructions (BioTeke, China). RNA concentration was measured using a GeneQuant100 spectrophotometer (GE Healthcare, UK). RNA quality was tested on ethidium

bromide-stained agarose gels. A SMART<sup>TM</sup> RACE cDNA Amplification Kit (Clontech, USA) was used to isolate the 5'and 3'-ends of PqHMGR cDNA. First-strand 5'-RACE-ready and 3'-RACE-ready cDNA samples from P. quinquefolius were prepared. The 5'-RACE and 3'-RACE nested PCR primers were prepared as follows: PqHMGR5ou (5'-CTGCCACA-TTAGTCTTCAACACCT-3'), PqHMGR5in (5'-CATTGGC-ACCGAATACTCCGTCTC-3'), PqHMGR3ou (5'-GGCG-AACCCCTCAAATCACAAA-3'), and PqHMGR3in (5'-CCTCGCCTGATAGTTGGGACATTC-3'). The universal primers were provided by the manufacturer. For the PCR amplification of the 5'- and 3'-ends of PqHMGR cDNA, we used an Advantage<sup>TM</sup> 2 PCR Kit (Clontech, USA). The PCR products were purified and subcloned into pGEM T-easy vectors and then sequenced. After the obtained sequences were aligned and assembled, the full-length cDNA of PqHMGR was deduced and subsequently amplified by PCR using a pair of primers. The ORF of PqHMGR was predicted using Lasergene 7

# Bioinformatics analysis, phylogenetic construction, and similarity comparison

The nucleotide sequence and amino acid sequence were analyzed using Lasergene 7. The sequence comparison was conducted through a database search by BLAST. The transmembrane domain was analyzed by TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The amino acid sequences of HMGRs from different organisms were aligned with that of PqHMGR using the program Clustal X (Thompson et al., 1997). The GenBank/EMBL/DDBJ accession numbers of the sequences used are as follows: AAB69726 (C. acuminata), AAC15475 (T. erecta), AAK95406 (M. domestica), AAO85434 (A. nidulans), AAP14352 (A. paniculata), AAQ82685 (T. media), AAU08214 (H. brasiliensis), AAU87798 (S. miltiorrhiza), AAU89123 (G. biloba), ABC74565 (Pricrorhiza kurrooa), ABK56831 (E. pekinensis), ABK88909 (A. lancea), ABV25902 (A. majus), ABY20976 (Medicago truncatula), ACE80254 (Pyrus pyrifolia), BAA36291 (C. melo), BAE92730 (G. lutea), CAE00496 (Rhizomucor miehei), NP\_000850 (H. sapiens), NP\_177775 (A. thaliana), NP\_613640 (M. kandleri), NP\_989816 (G. gallus), O74164 (Pichia jadinii), and Q9YAS4 (Aeropyrum pernix). A phylogenetic tree was constructed using the minimum evolution method with MEGA 4. The evolutionary distances were computed using the Poisson correction method, and the robustness of the tree topology was calculated from bootstrap analysis with 1000 resamplings of the sequences. Pairwise comparison of the encoded amino acid sequences of selected HMGR genes was accomplished by DNAMAN. A 3D structure of P. quinquefolius HMGR was constructed with Swiss-Model by a homology-based modeling according to the ternary complex structure of human counterparts (Kiefer et al., 2009). WebLab ViewerLite was used to create the 3D structure display. The program Protdist of Phylip 3.67 package (http://cmgm.stanford.edu/phylip/) was used to compute a distance matrix under the PAM matrices of Margaret Dayhoff (Retief, 2000; Kosiol and Goldman, 2005). The program was also used to compute the similarity between the amino acid sequences.

#### Gene expression analysis by quantitative real-time PCR

PrimeScript<sup>TM</sup> 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) was used to synthesize single-strand cDNA template. GAPDH was amplified from *P. quinquefolius* as internal controls. The primers were designed on the basis of the core fragments of GAPDH as follows: PqGAPDHF\_RT (5'-CAAAGACTGGA-

GAGGTGGAAGAG-3') and PqGAPDHR\_RT (5'-TGCAGGT-AGCACTTTACCAACAG-3'). For the quantification of PqHMGR gene transcripts in various tissues, Power SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystem, USA) was used. The primers of quantitative real-time PCR were designed using Primer Express (Applied Biosystems, USA) as follows: PaHmgr O5f (5'-CACTCCTCTTCTCTCAAAGCCTC-3'), and PqHmgr\_Q5r (5'-AATCCAATCCCAAAAAATCCA-3'), PqGAPDHF\_RT 5'-CAAAGACTGGAGAGGTGGAAGAG-3') and PqGAPDHR\_RT (5'-TGCAGGTAGCACTTTACCAAC-AG-3'). The reaction was performed on an IQ5 Multicolor Real-Time PCR Detection System (BIO-RAD, USA) using SYBR Green detection with a reaction mixture (20 µL) that contains 1× Power SYBR Green PCR Master mix, 0.2 µM each of forward and reverse primers, and 1 ng/µL of template cDNA. PCR amplification was performed under the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative level of mRNA was normalized to the mRNA expression of GAPDH. Gene expression was evaluated by the  $2^{-\Delta\Delta Ct}$  method, where  $\Delta Ct$  is the difference in the threshold cycle value (Ct) (Livak and Schmittgen, 2001).

#### Conclusion

*PqHMGR* gene represents a key contributing member to the biosynthesis of ginsenosides in *P. quinquefolius*. The cloning, characterization, and expression analysis of PqHMGR facilitates the elucidation of triterpenoid biosynthesis and may contribute to the higher accumulation of ginsenosides in *Panax* species.

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#### References

- Attele AS, Wu JA, Yuan CS (1999) Ginseng pharmacology: Multiple constituents and multiple actions. Biochem Pharmacol. 58 (11):1685–1693
- Basson ME, Thorsness M, Finer-Moore J, Stroud RM, Rine J (1988) Structural and functional conservation between yeast and human 3-hydroxy-3-methylglutaryl coenzyme A reductases, the rate-limiting enzyme of sterol biosynthesis. Mol Cell Biol. 8 (9):3797–3808
- Bochar DA, Stauffacher CV, Rodwell VW (1999) Sequence comparisons reveal two classes of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mol Genet Metab. 66 (2):122–127
- Combet C, Blanchet C, Geourjon C, Deleage G (2000) NPS@: Network protein sequence analysis. Trends Biochem Sci. 25 (3):147–150
- Kiefer D, Pantuso T. (2003) *Panax ginseng*. Am Fam Physician. 68 (8):1539–1542
- Engels B, Dahm P, Jennewein S (2008) Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab Eng. 10 (3-4):201–206
- Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. Nature. 343 (6257):425–430
- Haralampidis K, Trojanowska M, Osbourn AE (2002) Biosynthesis of triterpenoid saponins in plants. Adv Biochem Eng Biot 75:31–49
- Kiefer F, Arnold K, Kunzli M, Bordoli L, Schwede T (2009) The SWISS-MODEL Repository and associated resources. Nucleic Acids Res. 37 (Database issue):D387–392

- Kosiol C, Goldman N (2005) Different versions of the Dayhoff rate matrix. Mol Biol Evol. 22 (2):193–199
- Learned RM, Fink GR (1989) 3-Hydroxy-3-methylglutaryl-coenzyme A reductase from *Arabidopsis thaliana* is structurally distinct from the yeast and animal enzymes. P NATL ACAD SCI USA. 86 (8):2779–2783
- Lee SY, Kim GT, Roh SH, Song JS, Kim HJ, Hong SS, Kwon SW, Park JH (2009) Proteomic analysis of the anti-cancer effect of 20S-ginsenoside Rg3 in human colon cancer cell lines. Biosci Biotech Bioch. 73 (4):811–816
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using realtime quantitative PCR and the 2-deltadelta CT method. Methods. 25:402–408
- Ma X, Xiao H, Liang X (2006) Identification of Ginsenosides in *Panax quinquefolium* by LC-MS. Chromatographia. 64 (1):31–36
- Nakanishi M, Goldstein JL, Brown MS (1988) Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. J Biol Chem. 263 (18):8929–8937
- Peralta EA, Murphy LL, Minnis J, Louis S, Dunnington GL (2009) American Ginseng inhibits induced COX-2 and NFKB activation in breast cancer cells. J Surg Res. 157 (2):261–267
- Qiu YK, Dou DQ, Cai LP, Jiang HP, Kang TG, Yang BY, Kuang HX, Li MZ (2009) Dammarane-type saponins from *Panax quinquefolium* and their inhibition activity on human breast cancer MCF-7 cells. Fitoterapia. 80 (4):219–222
- Qu C, Bai Y, Jin X, Wang Y, Zhang K, You J, Zhang H (2009) Study on ginsenosides in different parts and ages of *Panax quinquefolius* L. Food Chemistry. 115 (1):340-346
- Rasheed N, Tyagi E, Ahmad A, Siripurapu KB, Lahiri S, Shukla R, Palit G (2008) Involvement of monoamines and proinflammatory cytokines in mediating the anti-stress effects of *Panax quinquefolium*. J Ethnopharmacol. 117 (2):257–262
- Retief JD (2000) Phylogenetic analysis using PHYLIP. In: Misener, S. and Krawetz, S. A.(ed) Bioinformatics methods and protocols. Humana Press Inc, Totowa, New Jersey
- Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature. 440 (7086):940–943
- Roitelman J, Olender EH, Bar-Nun S, Dunn WA, Jr., Simoni RD (1992) Immunological evidence for eight spans in the membrane domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase: Implications for enzyme degradation in the endoplasmic reticulum. J Cell Biol. 117 (5):959–973
- Ruiz-Albert J, Cerda-Olmedo E, Corrochano LM (2002) Genes for mevalonate biosynthesis in Phycomyces. Mol Genet Genomics. 266 (5):768–777
- Schepmann HG, Pang J, Matsuda SP (2001) Cloning and characterization of *Ginkgo biloba* levopimaradiene synthase which catalyzes the first committed step in ginkgolide biosynthesis. Arch Biochem Biophys. 392 (2):263–269
- Sun C, Li Y, Wu Q, Luo H, Sun Y, Song J, Lui EM, Chen S (2010) De novo sequencing and analysis of the American ginseng root transcriptome using a GS FLX Titanium platform to discover putative genes involved in ginsenoside biosynthesis. BMC Genomics. 11:262
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL\_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25 (24):4876–4882

- Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J Am Chem Soc. 93 (9):2325–2327
- Wu Q, Song J, Sun Y, Suo F, Li C, Luo H, Liu Y, Li Y, Zhang X, Yao H, Li X, Hu S, Sun C (2010) Transcript profiles of *Panax quinquefolius* from flower, leaf and root bring new insights into genes related to ginsenosides biosynthesis and transcriptional regulation. Physiol Plantarum. 138 (2):134–149