

Molecular cloning and characterization of *GuHMGR*, an HMG-CoA reductase gene from liquorice (*Glycyrrhiza uralensis*)

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Abstract A full length cDNA encoding HMGR (designated as *GuHMGR*) was isolated from liquorice (*Glycyrrhiza uralensis*) based on degenerated PCR and genome walking. The full length cDNA of *GuHMGR* was 2330 bp with a 1518-bp open reading frame (ORF) encoding a 505-aa polypeptide. Bioinformatics analysis indicated that there were two trans-membrane domains in *GuHMGR*. A molecular model of tertiary structure showed that *GuHMGR* is a novel HMGR with a similar spatial structure to other plant HMGRs. The deduced polypeptide of *GuHMGR* has an isoelectric point (pI) of 6.41 and a calculated molecular weight of about 54.7 kDa. Sequence comparison and phylogenetic tree analysis showed that *GuHMGR* had the highest homology with HMGRs from *Pisum sativum* and *Medicago truncatula*, indicating that *GuHMGR* belongs to the plant HMGR group. Expression analysis showed the similar amount of transcript level of *GuHMGR* in roots and leaves, suggesting that this gene was expressed constitutively in plants. Therefore, this novel *HMGR* gene would possibly provide a new strategy for studying the glycyrrhizin metabolism at the molecular level in the future.

Keywords cloning, liquorice, glycyrrhiza uralensis, genome walker, HMG-CoA reductase

Introduction

Liquorice (*Glycyrrhiza uralensis* Fisch.), also known as GANCAO or TIAN CAO in Chinese, is one of the many valuable medicinal plants for its great therapeutic effects. Usually, the roots and rhizomes of liquorice are used as sources of medicinal materials. Its major bioactive component is glycyrrhizin, which has the medicinal effects of anti-inflammation (Matsui et al., 2004) and hepatoprotection (Van Rossum et al., 1998; Shibata, 2000), etc. Glycyrrhizin also has antiviral effects, such as being anti-HIV (Ito et al., 1987) and against severe acute respiratory syndrome (SARS) associated coronavirus (Cinatl et al., 2003). In the meantime, glycyrrhizin is widely used in large quantities as a well-known natural sweetener and flavoring additive because of its sweet taste.

This many a day, glycyrrhiza plants used as medicinal materials were collected from wild resources. The ever-increasing demand of glycyrrhiza plants has resulted in a dramatic decline in the wild resource reserves and the meadow desertification by collecting the wild glycyrrhiza plants. In the past several decades, the glycyrrhiza plants have been cultivated as additional resources in order to increase the glycyrrhiza resources. However, the glycyrrhizin content is far lower from cultivated glycyrrhiza plants than from wild ones.

Glycyrrhizin belongs to triterpenoid saponin with a similar structure to terpenoid. All terpenoids are produced through a common biosynthetic pathway. Based on the previous studies (Hayashi et al., 2001; Jiang et al., 2006), the glycyrrhizin synthesis pathway of triterpenoid saponins is listed in Fig. 1. The series of enzyme catalyzed reactions suggested the glycyrrhizin biosynthesis pathway is a complex course. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), catalyzing the conversion of HMG-CoA to MVA, is considered as the first step in the MVA pathway in plants (Chappell, 1995). It is also considered a major rate-limiting

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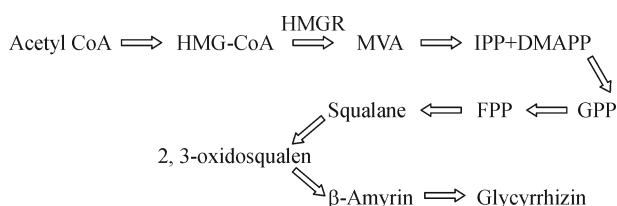


Figure 1 The glycyrrhizin biosynthesis pathway. HMG-CoA stands for 3-hydroxy-3-methylglutaryl coenzyme A, MVA for mevalonic acid, IPP for isopentenyl pyrophosphate, DMAPP for dimethylallyl pyrophosphate, GPP for geranyl pyrophosphate, and FPP for farnesyl pyrophosphate, respectively. HMGR, the enzyme that catalyze the second biochemical reaction from HMG-CoA to MVA, represents 3-hydroxy-3-methylglutaryl-CoA reductase.

step in the isoprenoid biosynthetic pathway (Bach, 1995).

To date, a large number of plant genes for HMG-CoA reductase have been isolated and sequenced. The gene encoding HMGR has been cloned from some plants such as *Arabidopsis thaliana* (Caelles et al., 1989; Learned and Fink, 1989; Enjuto et al., 1994), potato (Park et al., 1992), tomato (Choi et al., 1992), *Catharanthus roseus* (Maldonado-Mendoza et al., 1992), *Camptotheca acuminata* (Maldonado-Mendoza et al., 1997), *Taxus media* (Liao et al., 2004), *Eucommia ulmoides* oliver (Jiang et al., 2006) and *Ginkgo biloba* (Shen et al., 2006). The *HMGR* gene cloning might provide the basis for increasing terpenoid production by genetic engineering. However, there is no report yet on the cloning of HMGR from liquorice species. In the present study, we isolated and characterized a novel gene encoding HMGR from *G. uralensis*, aiming at further exploration in the biochemical regulation mechanism of glycyrrhizin biosynthesis and improving the glycyrrhizin concentration in cultivated plants based on bioengineering approach in the future.

Materials and methods

Plant materials

The perennial rhizomes of *G. uralensis* were collected from Inner Mongolia Autonomous Region, China and transplanted in a greenhouse located in Agricultural University of Hebei, China. Routine cultivation was conducted for normal growth of *G. uralensis* plants. One month later, the fresh tissues of *G. uralensis* were sampled for genomic DNA extraction, gene cloning and gene expression analysis.

Methods

Total DNA and RNA isolation

All tissue materials including leaves and roots were obtained from *G. uralensis* plants and then triturated by mortar and pestle in liquid nitrogen. Total RNA was isolated with

cetyltrimethylammonium bromide (CTAB)-containing extraction buffer, essentially as described previously (Chang et al., 1993). Genomic DNA was extracted from leaves of *G. uralensis* using CTAB method with different ingredient concentrations. The RNA and DNA quality were determined by agarose gel electrophoresis and spectrophotometer analysis before use.

Cloning partial genomic sequence of *GuHMGR*

According to the conserved sequence of HMGR gene shared by other plant species, a pair of primers *GuHMGRF1* (5'-TTCTTCTCCGTCGCGTACTTTCT-3') and *GuHMGRR1* (5'-GCTAGATCTGTTGTATTTTCATGTG-3') were designed and synthesized. The predicted PCR product contained the terminal codon for the reverse primer in the position of 3' non-translated region. PCR was carried out in a total volume of 20 μ L reaction which contained 2 μ L 10 \times buffer (plus Mg^{2+}), 1.6 μ L 10 mM each of dNTPs, 0.4 μ L 10 μ M *GuHMGRF1*, 0.4 μ L 10 μ M *GuHMGRR1*, 1 μ L genomic DNA and 0.1 μ L Ex *Taq* DNA polymerase (TaKaRa, China). The thermal reactions were as follows: denaturation at 94°C for 3 min, followed by 40 cycles for 30 s at 94°C, 30 s at 53°C, and 2 min at 72°C, with a final extension at 72°C for 10 min. The amplified product was purified, ligated into pUCm-T vector and cloned into *E. coli* strain DH5a followed by sequencing.

Cloning of the full-length DNA sequence of *GuHMGR*

Based on the above partial DNA fragment of *GuHMGR*, genome walking was carried out to obtain the full gene sequence of *GuHMGR*. For that, in total, three 5' primers with reverse orientation were designed including *GuHMGRSP1* (5'-CTTTTGGTGGGAGCTTCGGGGAG-3'), *SP2* (5'-GATGAGGGAGACGATGGCGGC-3') and *SP3* (5'-GAGGA-GAAAGTACGCGACGGAGAA-3'). The genome walking procedure was performed as the manufacturer's recommendation by using the GenomeWalker™ Universal Kit (TaKaRa, China). The amplified products were purified, ligated into pUCm-T vector and cloned into *E. coli* strain DH5a followed by sequencing. The full-length DNA of *GuHMGR* was obtained by assembling these two sequences derived from PCR and genome walking.

Cloning of partial cDNA sequence of *GuHMGR*

According to the full length DNA sequence of *GuHMGR*, a pair of primers *GuHMGRF1* (5'-TTCTTCTCCGTCGCGTA-CTTTCT-3') and *GuHMGRR3* (5'-TTTTGATACCATGTT-CATCCCAT-3') were designed and synthesized. RT-PCR was carried out to amplify the conserved fragment of *HMGR* gene from *G. uralensis*. The PCR reaction solution and the PCR course were similar to that used for cloning of the partial genomic sequence of *GuHMGR*, but the template was the transcribed cDNA from roots instead of the genomic DNA. The amplified product was purified, ligated into pUCm-T

vector and cloned into *E. coli* strain DH5 α followed by sequencing from the forward strands.

Generation of the full-length cDNA of *GuHMGR*

The full length cDNA sequence of *GuHMGR* was deduced based on the comparison between the partial cDNA *GuHMGR*, full length genomic DNA of *GuHMGR*, and the full-length cDNA of *Pisum sativum* L. (GenBank accession No. AF303583).

Bioinformatics analyses

The homologous genes of *GuHMGR* were identified by BLAST search in which the full length cDNA sequence was the query. The obtained sequences were then analyzed using bioinformatics tool at websites (<http://www.ncbi.nlm.nih.gov/>, <http://www.expasy.org/>). The deduced *GuHMGR* and other known plant HMGRs retrieved from GenBank were aligned with DNASTar software.

Tissue expression pattern of *GuHMGR*

To investigate the *GuHMGR* expression pattern in different parts of *G. uralensis*, total RNA was extracted from taproots, lateral roots, old leaves and new leaves, respectively. Semiquantitative one-step RT-PCR was carried out according to the manufacturer's instructions (TaKaRa, China). An aliquot of 20 μ L reaction solution containing 2 μ L 10 \times buffer (without Mg²⁺), 1.2 μ L 25 mM each of MgCl₂, 1.6 μ L 10 mM each of dNTPs, 0.4 μ L 10 μ M *GuHMGRF1*, 0.4 μ L 10 μ M *GuHMGRR3*, 1 μ L cDNA and 0.15 μ L rTaq DNA polymerase (TaKaRa, China). The template was denatured at 94°C for 3 min, followed by 40 cycles of amplification (30 s at 94°C, 30 s at 53°C, 2 min at 72°C) and a final extension at 72°C for 10 min. The PCR results were determined by agarose gel electrophoresis.

Results

Cloning and characterization of the genomic DNA of *GuHMGR*

Using primers *GuHMGRF1* and *GuHMGRR1*, a band of 2633 bp was specially amplified (Fig. 2A). Sequence analysis showed that it was highly homologous to HMGRs in plant species. To obtain the full length of *GuHMGR* genomic sequence, genome walking was conducted in which three primers including SP1, SP2 and SP3 were then designed for the PCR amplification of the unknown 5' region. A product of 437 bp was specially amplified (Fig. 2B). The genomic DNA of *GuHMGR* was then obtained by assembling the sequences of the 2633 bp and 437 bp.

Comparison between the *GuHMGR* genomic sequence and the cDNA of *Pisum sativum* showed that the genomic DNA contained two introns. The lengths of three exons were 1206 bp, 181 bp and 943 bp, respectively. The lengths of the two introns were 87 bp and 245 bp, respectively.

Generation of the full-length cDNA of *GuHMGR* and characterization of the deduced *GuHMGR* protein

The full length cDNA of *GuHMGR* was obtained based on the comparison between the partial cDNA *GuHMGR*, full length genomic DNA of *GuHMGR*, and the full-length cDNA of *Pisum sativum* L. (GenBank accession No. AF303583). Sequencing results showed that the *GuHMGR* was 2330 bp in length and contained a 1518 bp ORF, encoding a protein of 505 amino acids (Fig. 3), with an isoelectric point of 6.41. The calculated molecular weight of *GuHMGR* was about 54.7 kDa, which is similar to previously reported plant HMGRs. The *GuHMGR* showed a homology with *Pisum sativum* HMGR (67.3), *Medicago truncatula* HMGR3 (65.3)

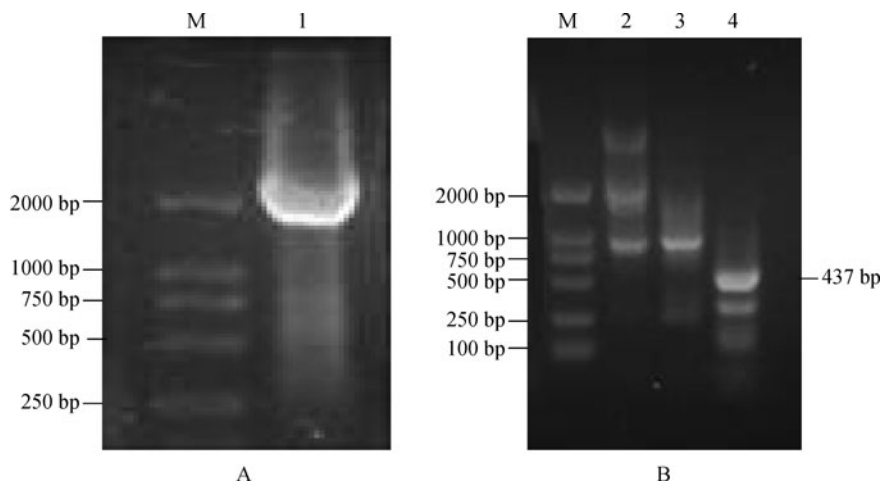


Figure 2 The PCR amplified pattern of the *GuHMGR* gene from *Glycyrrhiza uralensis* Fisch. A is 2633 bp conservative sequence of the *GuHMGR* gene using the primer pair (*GuHMGRF1*, *GuHMGRR1*); B is Genome walking products of the *GuHMGR* gene. M is Molecular weight marker DL2000; 1–4 represent 2633 bp conservative sequence of the *GuHMGR* gene, the first round PCR product of genome walking, the second round PCR product of genome walking, and the third round PCR product of genome walking respectively.

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1   GAGCAGAAAGTACGCGACGGAGAAGATGAGGGACACGATGGCGCGATGTGGGAGCGGGAGGAAAAGTAGTGGGTCCGAGCAGCAGT
90  GGTGGAGAGTCCGTGTGTGTCGCGTGTGTACCCCTTTGCAGTTACACACACTCCGTCCAGTCCCAGTCACTCACTCTATATGGAAAGTT
180 GGAGGGATCACCATTCAATTCATCTCCACCATCCATCATCAATTCACATTCCCTCCCTTTTTCTCTCTAGCACCACCATACCTCC
270 GACACCTTCGCTGCCACTAATACCATTCTCCCTCACTGTCATGAGCTCCGACATCGTCCCCATCCCCGGCGGTGGCAAAAAGCCTCC
      M E L R H R S P S P P U A K A S
360 GAGGCCTCCCACTCCCATCCGTCTCACCACGCAATTCCTTCTCCCTCTTCTCTCCGTCGGTACTTTCTCCTCCACCGTGGGGC
      D A L P L P I R L T N A F F F S L F F S U A Y F L L H R W R
450 GAGAAGATCCGAACCTCCACCCCTCTCCACGTCGTCACGCTCTCCGAGATCGCCGCCATCGTCTCCCTCACTCCGCTCCGTCATCTACCTC
      E K I R T S T P L H U U T L S E I A A I U S L I A S U I Y L
540 ATGGCCTTCTTCGGCATTGGCTTCATCTCCACCCGTTCCGCGCCTCAGCGCGCTCCCGACGACGACGATCGAAGCTGACATCATC
      M A F F G I G F I L H P F A A S R A U P D D D E Y E A D I I
630 AAAGACGGCGCTCCCTGCCTCGTCGGCTGCTCCCGAAGCTCCACCAAAAGTCGTGGACGAAAACACCCGGCACCAGGATCGACTCTG
      K D A G P C L U G C S P K L P P K U U D E K P P A P A S T L
720 TCCCGGACGACGAGGAGGTAGTCAAGGCGGTGGTGTGGGGTCGATTCGCTCGTACTCGTGGATCAAAACTCGTGACTGCCGACGG
      S R D D E E U V U K A U U S G S I P S Y S L E S K L G D C R R
810 GCGCGCGGATTCGGCAGGCGGTGGAGAGGTCACCGGAGTCCGTCGAGGGTTACCGGTTGAAGGATTCGATTACGAGTCGATA
      A A A I R H E A U E R U T G R S L E G L P U E G F D Y E S I
900 TTGGTCAGTGTGAGATGCCGATAGGGTTGTTTCAGATCCCGTAGGGTGGCAGGGCCATTGCTTCTCGACGGGAAGGATACACA
      L G Q C C E M P I G F U Q I P U G U A G P L L L D G K E Y T
990 GTTCCATGGCAACCCAGGGGTTGTTGGTGGCCAGCACCAGAGGCTGCAAGGCCATTTACGTTCCGGCGGTGCCCTCCCTCGTG
      U P M A T T E G C L U A S T N R G C K A I Y U S G G A S S U
1080 CTCCTCCGTGACGGCATGACCAGGCCACGCTCGTCAGGTTCCGGTCCGCCAAGAGAGCCCTCACTTGAAGTCTATCTAGAAGCTCT
      L L R D G M T R A H U U R F G S A K R A S Q L K F Y L E D P
1170 CTCAATTTGATTCCTCGCTGCTCACTTTCAACAAGTCAAGCAGGTTTCCGAGATTGCAGAGTATTAAAGCTAATATGGCAGGGAAGAT
      L N F D S L A U T F N K S S R F A R L Q S I K A N M A G K N
1260 TTGTATATTAGATTCACTGTCAGCAGTGGGATGCCATGGGATGAACATGGTATCCAAAGGTGCCAAAACGTACTTGATTCCTTCAC
      L Y I R F S C S T G D A M G M N M U S K G U Q N U L D F L H
1350 AATGATTTACCTGACATGGATGTCATTGGGATATCTGGGATTTCTGTTCCGACAAGAAGCAGCGCGGTTGATTGGATTGAAGGAGG
      N D L P D M D U I G I S G N F C S D K K A A A U N W I E G R
1440 GGAAGTCGGTGGTGGCAGGCTGTGATTAGGAAGAGGTTGTTGAAGAAGTGTGAAGAGCAGCCTGGAGGCCGTTGGTTGAGCTCAAC
      G K S U U C E A U I K E E U U K K U L K S S U E A L U E L N
1530 ATGCTCAAGAATCTTACTGGCTGCGCGTGGTGGCTCCCTTGGTGGATTCACAGCCCATGCCAGCAATATTGTTTGGCATTACTTG
      M L K N L T G S A U A G A L G G F N A H A S N I U S A I Y L
1620 GCCACTGGTCAGGATCCAGCTCAGAAATGGGAGCTCTCACTGTATCACTATGATGGAGCTGTCAACGATGGCAAGGACCTTCACATC
      A T G Q D P A Q N U E S S H C I T M H E A U N D G K D L H I
1710 TCCGCTACTATCCCTCCATTGAGGTACAAGTACAACACAATTACCTACTGTTTTTTTTTTTTTATCAATTGGAGGCCCGAAGACCAA
      S U T M P S I E U Q U Q H N Y L L U F F F F I N W R P E D Q
1800 TTATTACCTACTGTTAATTCCTCCCTAATTCGTTATTATTGTTACTACTGTATCTAATTTGATTTTCAAGAATGTTAGTGGCCACA
      L L P T C L I P P
1890 AAAATTTTATTAGTAAAATGCGAGCTGTAGTACCTTTCCGTACGAGTCTTGATGTGATTTATTGAGCTATATTATTGGTGGGACCGT
1980 GATAACAGATCCGGTGTGTTGGTCCCTAGCTATAGCATAGCATAGCATAGCATAGCATGTTGTACATATACAAGCTTTATCCCGAGGTG
2070 AGATAGAAAACAGACTCTGGTGCAGATATCCCTATCCCTCCCTGGGAATGAGCTAGTCCCTTTCTATATAATTCATCTCTGTTCCAT
2160 TACATGCATCATGTTCTCTCGGCTCTGATAGAAATCTCAATTATATAGTAGCTAGCAGACAAAGTTGCTCAATTTCTAGTTGGACC
2250 CCGACTCGTCTGTTAACAACCTACCCCTGTGCTTTAATTTGTTAAGACTTAAGATCCCATAGATTACTACATGGGT

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Figure 3 The full-length cDNA sequence and deduced amino acid sequence of *G. uralensis* 3-hydroxy-3-methylglutaryl1-CoA reductase (*GuHMGR*). The start codon (ATG) and the stop codon (TAA) are boxed.

and HMGR2 (64.1) and HMGR1 (63.3), and *Litchi chinensis* HMGR (64.1) (Fig. 4).

Bioinformatic analysis of GuHMGR

Sequence comparisons by performing Blast Search in GenBank database (<http://www.ncbi.nih.gov>) and multiple alignment analysis revealed that *GuHMGR* had a high homology with many other plant HMGRs, such as *Pisum sativum* HMGR (PsHMGR), *Hevea brasiliensis* HMGR (HbHMGR), *Medicago truncatula* HMGR1-4 (MHHMGR), etc. The results suggested that *GuHMGR* belongs to the plant HMGR superfamily. Using TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0>), two transmembrane regions were predicted with one located between Pro 20 and Leu 42 and the other located between Ile 63 and Ile 85 along the polypeptide chain. A likely signal peptide cleavage site between Ala 28 and Phe 29 was found using SignalP3.0

(<http://www.cbs.dtu.dk/services/signalP/>). To better characterize the *GuHMGR* protein, comparative 3-D modeling was performed by SWISS-MODEL (Schwede et al., 2003) with the HMGR from human as the template, the crystal structure of which has been elucidated (Fig. 5).

Tissue expression analysis

To investigate *GuHMGR* expression pattern in different tissues of *G. uralensis*, the one-step RT-PCR analysis was performed using same amount of total RNA isolated from different tissues including old leaves, new leaves, the taproots and lateral roots as the templates and the HMGRF1 and HMGRR3 as the primers. The result showed that *GuHMGR* is expressed in all four tested tissues at similar transcribed levels, suggesting that this gene is expressed in a constitutive pattern in plants (Fig. 6).

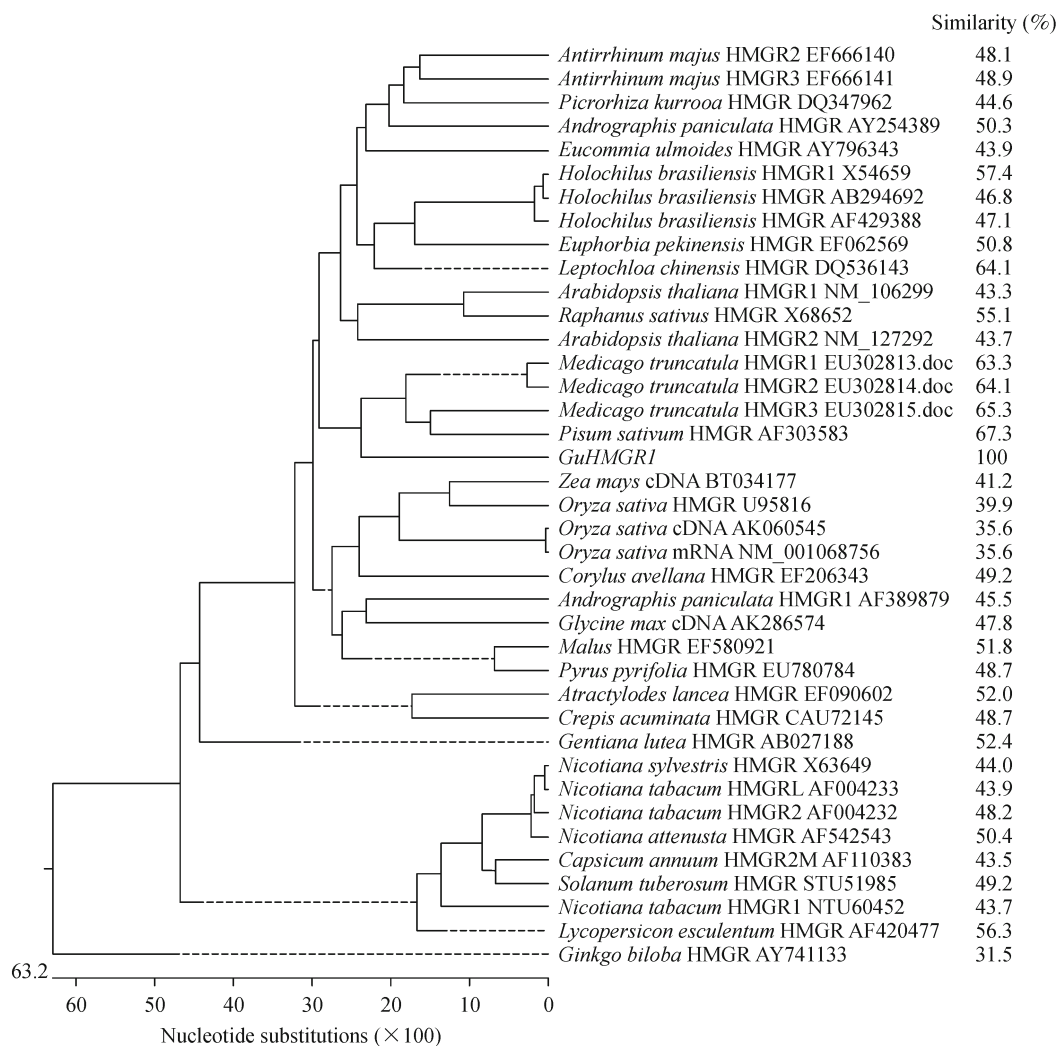


Figure 4 Relationships between the primary amino acid sequences of HMGRs in plants.



Figure 5 The 3-D model of *GuHMGR*.

Discussion

HMGR was studied initially in mammals because of its significant role in regulating the cholesterol biosynthesis (Goldstein and Brown, 1990). Subsequently, the HMGRs

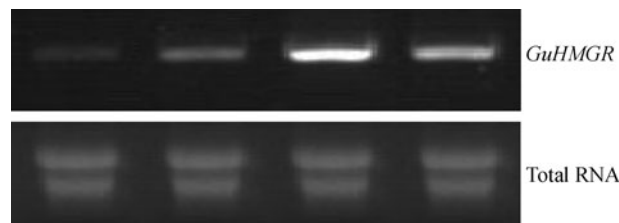


Figure 6 Expression profile of *GuHMGR* gene in new leaf, old leaf, taproot and lateral root.

have been widely studied in plant species. In tomato, a *HMG1::GUS* reporter gene fusion was used to analyze the regulation of *HMG1* gene expression which suggested that the role of *HMG1* was to supply the MVA demand associated with cell division and growth (Jelesko et al., 1999).

Till now, only few glycyrrhizin biosynthetic genes have been successfully isolated from glycyrrhiza plants. An oxidosqualene cyclase cDNA encoding β -amyrin synthase, was isolated from cultured cells of *Glycyrrhiza glabra*

(Hayashi et al., 2001). Two cDNAs of squalene synthase were isolated from *G. glabra* (Hayashi et al., 1999). Molecular cloning of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *G. uralensis* provides a useful tool to elucidate the glycyrrhizin biosyntheses in glycyrrhiza plants. Further experiments are required to study the combined function of the glycyrrhiza gene in plant for glycyrrhizin biosyntheses.

In this paper, the full length genomic DNA sequence and cDNA of *GuHMGR* gene were cloned from *G. uralensis*. The deduced polypeptide of *GuHMGRs* share high identities with other plant *HMGRs*, such as *HMGR* from *Pisum sativum* and *Medicago truncatula*. This suggested that *GuHMGR*, the *HMGR* gene derived from glycyrrhiza plants possibly plays an important role in the biosynthesis in glycyrrhizin.

Usually root and rhizome are used as the sources of medicinal materials. But some reports suggest that other organs of the glycyrrhiza plants also contain the medicinal content such as glycyrrhizin. Hence, it is of great value to determine whether the *GuHMGR* expression is correlated with the glycyrrhizin content in different tissues of *G. uralensis*. In this study, the one-step RT-PCR analysis was performed using total RNA from different tissues, including old leaves, new leaves, the taproots and lateral roots with the primers *HMGRF1* and *HMGRR3*. The results showed that *GuHMGR* was expressed in all four tissues, which suggested that the *GuHMGR* may be a constitutively expressed gene. However, the expression in roots was higher than that in leaves, corresponding with the fact that the roots are usually used as medicinal material and more glycyrrhizin is accumulated in roots than leaves. Further study will be undertaken to identify the functions of *GuHMGR* in improving glycyrrhizin biosynthesis based on bioengineering approach in the future.

Acknowledgements

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