

Cloning and Characterization of the Gene Encoding 3-hydroxy-3-Methylglutaryl-coenzyme A (HMG-CoA) Reductase from *Fritillaria Cirrhosa* D. Don

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ABSTRACT

The enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR; EC1.1.1.34) catalyzes the first committed step of isoprenoids biosynthesis in Mevalonate (MVA) pathway. Here we report for the first time the cloning and characterization of a full-length cDNA encoding HMGR from *Fritillaria cirrhosa* (FcHMGR), a bulbous medicinal plant. The full-length cDNA of FcHMGR was 2072 base pair (bp), containing a 1680-bp open reading frame. Bioinformatical analyses revealed that FcHMGR had HMG CoA-binding domains and two NADPH binding domains, which are required for HMGR activity. Quantitative real-time PCR (qRT-PCR) analysis revealed that FcHMGR expressed high in mature bulbs. A truncated version of FcHMGR protein lacking the N-terminal 249-bp GC rich area was expressed in *Escherichia coli*. The crude cell lysate containing the recombinant protein showed a better HMGR activity than the control and the relative enzyme activity was calculated to be 1.62 U/mg. The cloning, characterization and functional analysis of FcHMGR gene allowed us to further understand the role of FcHMGR involved in steroidal alkaloid biosynthetic pathway in *F. cirrhosa* at the molecular level.

Keywords: 3-Hydroxy-3-methylglutaryl-CoA reductases, *Fritillaria cirrhosa*, Molecular cloning, Expression pattern, Prokaryotic expression



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INTRODUCTION

Fritillaria cirrhosa D. Don (Family, Liliaceae) is a bulbous medicinal and perennial herbaceous plant, which is native to the southeastern margin of the Qinghai–Tibet Plateau region of China¹. It has been widely used in traditional Chinese medicine (TCM) for over 2,000 years as a curative therapy for chronic cough. Steroidal alkaloids are the main active secondary metabolites in *F. cirrhosa*. So far, at least twenty kinds of monomeric alkaloids, including peimine, imperialine, verticine, verticinone, and peimisine, have been isolated from this plant². Previous studies have demonstrated a wide array of pharmacological activities of these alkaloids, including anti-inflammatory and anti-cancer³. The content of alkaloids in *F. cirrhosa* were reported to be very low, limiting its medical application. Biotechnological strategies have been exploited to increase the yield of alkaloids from the cultured bulbs⁴ or via cell suspension cultures⁵. Overexpression of steroidal alkaloids biosynthesis-related genes combining with bulbs culturing is considered to be a practical way to stimulate the secondary metabolites production of *F. cirrhosa*.

The first key regulatory, rate-limiting enzyme in the mevalonate (MVA) pathway for the biosynthesis of phytosterol in plants is 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), which catalyzes the conversion of HMG-CoA to MVA⁷. The MVA pathway is the main route for the production of steroidal alkaloid backbones⁸. Because of its critical role in regulating sterol biosynthesis, HMGR has been studied as a rational candidate for phytosterol accumulation of plants. In *Nicotiana tabacum* L., the heterologous expression of *Withania somnifera* HMGR has been showed to be positively correlated with the accumulation of phytosteroids. Transgenic tobacco expressing an N-terminal truncated *Hevea brasiliensis* HMGR (t-HMGR) had up to 10-fold leaf phytosterol sterol levels. Moreover, it has been reported that characterization of a sterol-overproducing tobacco mutant showed a 3-fold increase in HMGR activity in leaf tissue comparing to the wild-type tissue⁹. These results indicate that the expression of HMGR gene is linked to the accumulation of phytosteroid in plants.

In this study, we cloned FcHMGR cDNA from *F. cirrhosa* bulbs, performed a bioinformatics analysis and studied its expression patterns. The functionality of the FcHMGR gene was determined in prokaryotic (*Escherichia coli*) expression model systems.

MATERIAL AND METHODS

Plant materials

The *F. cirrhosa* mature bulbs, roots, stems and leaves were collected from 3-year-old plants grown in the origin of the Kangding fold-thrust belt mountains (located at 30°3'44.9" N, 101°58'3.81" E, altitude 4300 m) in Sichuan Province, China, then snap frozen in liquid nitrogen and transferred to a -80 °C freezer till further use for comparative tissue specific expression profile.

Cloning and sequence analysis of the FcHMGR cDNA

Total RNA was extracted from the bulbs of *F. cirrhosa* using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of pooled RNA was carried out with oligodeoxythymidine using the PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The degenerate primers, HMGRF and HMGRR (Table1), were designed based on the conserved motifs of the

plant HMGR proteins. For 3' and 5' RACE, three universal primers [oligo(dT)18, AP1, and AP2] and three gene-specific primers (HMGR3', HMGR5'RT and HMGR5', Table 1) were used in the rapid amplification of cDNA ends (RACE)10. Based on the alignment of the 3' and 5' RACE fragments, primers (HMGR-ORFF and HMGR-ORFR) (Table 1) were designed to obtain the cDNA of FcHMGR gene. The products were cloned into pEASY-T vectors (TransGene) and sequenced. Sequences were examined for similarities with sequences in the GenBank database using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The amino acid sequences of cloned cDNA fragments were deduced and protein sequences were aligned using the program DNAMAN 6.0 (<http://www.lynnon.com>). The protein physical properties analysis were using the On-line Analysis System (http://web.expasy.org/compute_pi/). Phylogenetic relationship was analyzed by multiple alignments of HMGR proteins using the MEGA 4.1 program11. The protein tertiary structure was predicted by Swiss-model tool (<http://swissmodel.expasy.org/interactive>).

Table 1. Primers used for PCR in this study

Primers	Sequences (5' - 3')	Usage
HMGRF	THYTNGGNCARTGYTGYGARATGCC	Cloning of <i>FcHMGR</i> cDNA
HMGRR	CDATCCARTTNACNGCNGGYTT	Cloning of <i>FcHMGR</i> cDNA
AP1	GTCAACGATACGCTACGTAACG	Anchor
AP2	TACGTAACGGCATGACAGTG	Anchor
HMGR3'	TCTGTGACCATGCCATCTA	3' RACE
HMGR5'RT	GTACTCGCAACCAAACACCCCT	5' RACE
HMGR5'	TTCGATTCGAGTATATATG	5' RACE
HMGR-ORFF	ATGGACATCCGCCGCCG	Cloning of <i>FcHMGR</i> ORF
HMGR-ORFR	TCAAGAGCCAGCCTTAGACATATCT	Cloning of <i>FcHMGR</i> ORF
HMGRqF	GCTCCAAGGTATTCAATGCTCA	Quantitative Real-time PCR
HMGRqR	CGATGACTGCCTCACATACCA	Quantitative Real-time PCR
18SqF	TACGACTCTCGGCAACGGA	Quantitative Real-time PCR
18SqR	CAAAGGGGCAATGGGAACA	Quantitative Real-time PCR
Nt-HMGR-GEXF	CGCGGATCCATGATCTACCTCATCAGCTTCTTC	Construction of the pGEX vector
Nt-HMGR-GEXR	CGGAATTCTCAAGAGCCAGCCTTAGACATATCT	Construction of the pGEX vector

Expression analysis of FcHMGR by real-time quantitative PCR

Total RNA was extracted using the same method as described above. Approximately 0.5 µg of the total RNA from each pool from bulbs, roots, stems and leaves were reverse transcribed into cDNA with oligodeoxythymidine as primer in a volume of 10 µL following the PrimeScript™ RT Reagent Kit protocol. The mixture was diluted to 100 µL with sterile water and the synthesized DNA was used as template in quantitative real-time PCR (qRT-PCR). Gene-specific primers (HMGRqF and HMGRqR (Table 1) were designed to amplify the specific cDNA fragment of FcHMGR and the expected size of the RT-PCR product is 248 bp. The 18S gene (GeneBank accession no. AY616727.1) of *Fritillaria cirrhosa* was used as the internal

reference. All qRT-PCRs were performed using SYBR® Premix Ex Taq™II (TaKaRa) and a Bio-Rad CFX96 Real-time PCR machine. The PCR protocol was 95 °C for 10 s initially, followed by 40 cycles, each comprising 95 °C for 10 s, 58 °C for 20 s and 72 °C for 20 s, then 3 min at 72 °C. At least three independent replicates of each experiment were performed.

Expression of N-terminal truncated FcHMGR (FcNt-HMGR) in E.coli BL21

Primers Nt-HMGR-GEXF and Nt-HMGR-GEXR (Table 1) were designed and synthesized to amplify the coding sequence of N-terminal truncated FcHMGR which lacking a 249-bp GC enrichment area. The resulting fragment was digested with BamH I/EcoR I and ligated into the corresponding restriction sites of the expression vector pGEX6p-1. The recombinant plasmid pGEX-FcNt-HMGR was transformed into E.coli strain BL21 (DE3) for protein expression.

Three colonies of E.coli strain BL21 cells harboring the recombinant plasmid pGEX-FcNt-HMGR were inoculated at 37°C in LB medium containing ampicillin (100 mg l⁻¹), with shaking (220 rpm) until the cell cultures at OD600 value of 0.6. Then the protein expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.25 mM and the cultures were grown for an additional 4 h. Over-expressed proteins were detected by analyzing total protein on 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by coomassie brilliant blue R250 staining.

Determination of the FcNt-HMGR relative enzyme activity

Cells containing FcNt-HMGR were induced at 37°C for 4 h and harvested by centrifugation at 5,000 g for 15 min and immediately frozen at -80°C. Frozen cell pellets were thawed and suspended in protein binding buffer (30 mM Tris-HCl, pH7.5, 5% glycerol, 100 mM NaCl, 1 mM PMSF). The cells were then lysed by sonication on ice. Cellular debris was removed by centrifugation at 4°C, 20,000 g for 30 min. Supernatant containing FcNt-HMGR was subjected to enzyme activity assay according to the method of Li et al with minor modifications¹². Enzymatic activities were assayed in 200 µl of reaction mixture consisting of 50 mM Tris-HCl buffer (pH 7.0), 0.075 mM NAD⁺, 4 mM DTT, and crude cell lysate. The substrate 3-Hydroxy-3-methylglutaryl coenzyme A (Sigma) was added to the reaction mixtures after preincubation for 10 min at 37 °C to start the reactions. Enzyme activities were determined according to the absorbance at 340 nm (reflecting the consumption of NAD⁺). One unit of the activity was defined as 1 µmol/min of NAD⁺ consumption. Each determination was carried out at least three times. The supernatant of the culture without IPTG induction was used as a negative control. Protein concentration was measured by bicinchoninic acid (BCA) method¹³ using a protein assay kit (Thermo, USA). All assays were repeated at least three times.

RESULTS

Cloning and sequence analysis of FcHMGR

The full-length cDNA of the FcHMGR gene had a size of 2072 bp with 5' and 3' – untranslated regions (UTRs) and a polyA tail (Figure 1). The open reading frame (ORF) was flanked by a 60-bp 5-UTR and a 332-bp 3-UTR. An ORF search showed that FcHMGR contained a 1680 bp ORF encoding a protein with 559 amino acids (Figure 1, 2). The calculated molecular weight of the encoded protein was

approximately 59.2 kDa, with a theoretical isoelectric point of 7.48. Using the amino acid sequence as query, the sequence similarities database search (NCBI) showed high homology to several HMGR-like proteins including the Phoenix dactylifera HMGR (80% identity), Elaeis guineensis HMGR(80% identity), Brachypodium distachyon HMGR(80% identity), Sorghum bicolor HMGR(78% identity) and Dendrobium huoshanense HMGR(76% identity). DNAMAN 6.0 software was used to carry on the homologous comparison and the results are shown in Figure3, we can see from the figure that the sequence of amino acids of the higher plant HMGR reaches low homology from 150 amino acids downstream of the N-terminal, followed by a highly conserved region, which contains two HMG CoA - binding domains (EMP (V/I) GY (V/I) QIP, TTEGCLVA) and two NADPH binding domain (DAMGMNM). A comparative 3D modeling of FcHMGR catalytic domain was performed using Swiss-Model with the HMGR (PDB no. 1dqa.1.A; sequence identity is 58.39%) with Homo sapiens HMGR protein as the template. The space layout of HMGR function area is folded into a "V" shape. The L structure domain in the middle possesses two functions: HMG-CoA binding motif and NADPH binding motif (Figure3, 4B). Phylogenetic analysis showed that the evolutionary tree clustering has two kinds, monocots and dicotyledonous. FcHMGR was closely related to the Dendrobium huoshanense HMGR, which is in the same sort of angiosperm and monocotyledon, clusters (Figure4A).

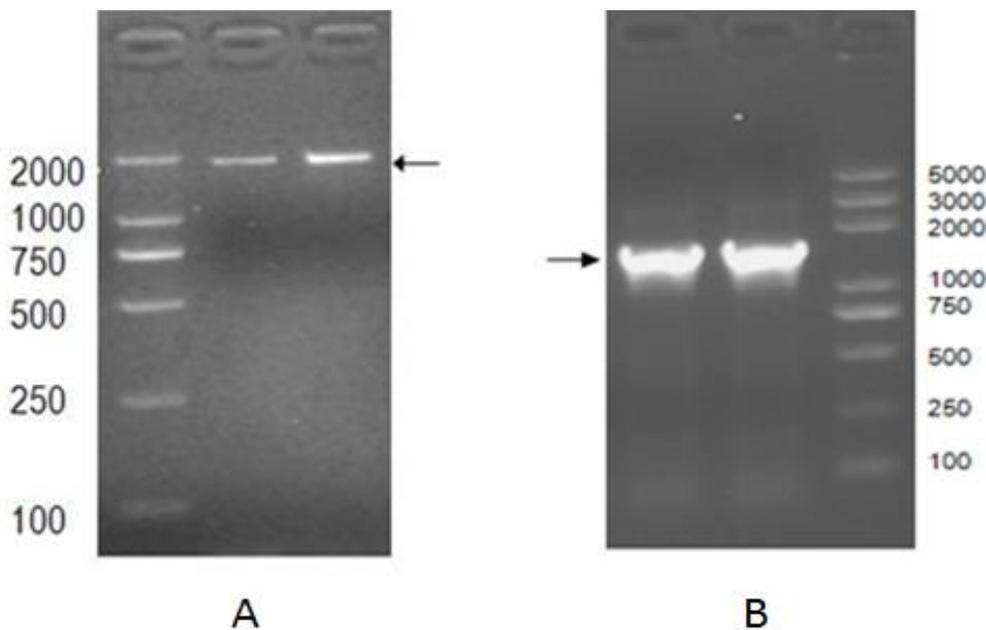


Figure 1 Amplification of *FcHMGR* full-length cDNA (A) and ORF (B).

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GATTCCACACACCTTTTCGCGGCGGGAGGAAGGCGGGCGGGTTCTCTCCCTCCCG 60
A70ACATCCGCGCCGAAACCTCCACCCGCGCCCTCCGCGGGCGGGCGCCGCTC 120
M D I R R R K P S T R P P P A A A P P L
CCCTCCGCGCATCAAAGCTCTGACGCTCTCCCTCTCCCATCCGCCACCAACCTTCTC 180
P S R I Q A S D A L P L P I R H T N L L
TTCCTCGCCCTCTTCGCGGCTCCCTCGTCTCTCATGCGCGGTGGCGGAAAAGTTC 240
F S A L F A A S L V F L M R R W R E K V
CGGCTCTCCGCCCTCCACGTCATCGGCTCGCCGACATGGCCCATGATCTCGCTC 300
R V S A P L H V I G L A D M A A M I C L
GTCGCTCCCTCATCTACCTCATCAGCTTCTTCGGGATCGCCCTCAGTCCGCTCGTC 360
V A S L I Y L I S F F G I A Y V Q S V V
TCCTCACACGAGGATGACGACGACTTCTCTCCCTCCGCGGCGAGCCGCCCGCGG 420
S S H E D D D D F L L P S P A Q P A P P
GCCGCGCGACTCAATGCCCTCTCCGCTCGGACAAGAATCAATCTCAGTACCGGCT 480
A A P T Q C P L L R S D K E S I S V P A
GTCGATGCGAGTGATGAAGACATAATCGCTCAGTGGTTCGGTAAAACCTTCATAT 540
V D A S D E D I I A S V V S G K T P S Y
ATACTCGAATCGAAGCTGGGCGACTGCAGGCGGGCCGTTCGATCCGCGGTGAGCGCTG 600
5' RACE
I L E S K L G D C R R A V A I R R E A L
CGGCGATGACCGCGCCGATGGAGGGGCTGCCAATTGAGGGTTTACTATGATCG 660
R R M T G R P M E G L P I E G F D Y A S
ATCTGGGGCAGTGTCTGAGTTGCCGATTGGGTATGTTACGCTCCCGTGGGGATTGCT 720
I L G Q C C E L P I G Y V Q L P V G I A
GGGCCACTGGCCCTGGATGGGAGGAGTTCTATGTCCGATGGCGACACTGAGGGGTGT 780
G P L A L D G R E F Y V P M A T T E G C
TTGGTTGCGAGTACAAACAGGGGTTGTAAGCTATATTTGAGTCCGCGGGGCGATGAGT 840
3' RACE
L V A S T N R G C K A I L E S G G A M S
GTGGTGTGAAGATGGGATGACCGGGCGCGGGGTGAGGTTTTCGACGGCGATGAGG 900
V V L K D G M T R A P A V R F S T A M R
GCCGCTGAGCTCAAGTCTCTTTCGAGGAGCCAGAGAATTGATACACTGGCTGTGTC 960
A A E L K F F L E E P E N F D T L A V V
TTCAATAGTTCGAGCAGATTTGCAAGGCTCCAAGGTATTCAATGCTCACTCGCAGGGAGG 1020
F N R S S R F A R L Q G I Q C S L A G R

TTCAATAGTTCGAGCAGATTTGCAAGGCTCCAAGGTATTCAATGCTCACTCGCAGGGAGG 1020
F N R S S R F A R L Q G I Q C S L A G R
AATCTACATGAGATTCTGCTGTAGCACAGGTGATGCCATGGGGATGAATATGGTGTC 1080
N L Y M R F C C S T G D A M G M N M V S
AAGGGAGTGCAGAATGTGTGGATTATCTCAGAAGCATTTCGGGATATGGATGTGGTC 1140
K G V Q N V L D Y L Q N D F P D M D V V
AGCATCTCAGGGAATTTCTGTTCCGACAAGAAGCTGCTGCTGAACTGGATCGAAGG 1200
S I S G N F C S D K K P A A V N W I E G
AGAGGCAATCAGTGGTATGTGAGGAGTATCGGGGAAGATGGTGAAGAAGTCTG 1260
R G K S V V C E A V I G E D V V K K V L
AAGACCAGTCCGCTCTGTTAGAGCTGAACATGATCAAGAATCTCGCTGGCTCAGCT 1320
K T T V P A L V E L N M I K N L A G S A
GTCGCGGTGCTTGGGGGATTCAACGCCACCCAGCAACATCGTGTCTGCCCTCTC 1380
V A G A L G G F N A H A S N I V S A V F
ATTGCCACTGGCAAGATCCCGCCAGAAGCTCGAGAGCTCTCACTGATCACCATGAT 1440
I A T G Q D P A Q N V E S S H C I T M M
GAGGCTGTAATGGCGGAAAGATCTTCATGCTCTGTGACCATGCCATCTATTGAGGT 1500
3' RACE
E A V N G G K D L H V S V T M P S I E V
GGTACAGTTGGGGTGGGACCCAGTGGCGGCTCAGGCTGTGTTGGATCTCTCGGT 1560
G T V G G G T Q L A A Q A A C L D L L G
GTGAAGGCGCAAGCATGGAGTACCTGGAGCCAATGGAGGCTCCTGGCTACTATCATA 1620
V K G A S M E S P G A N A R L L A T I I
GCTGGCCCGTTTTAGCCGGGAGCTCTCTCATGTCCGCCCTTGGGCTGGTCAGCTC 1680
A G A V L A G E L S L M S A L A A G Q L
GTGAAGCCACATGAAGTATAACAGGTCAGCAAAGATATGCTAAGGCTGGCTCTTGA 1740
V K S H M K Y N R S S K D M S K A G S *
GATGCTCCCTCGTTCGACGGTATAATTAATTTGTTTGTGTTTGAAGGCAAT 1800
CGAGGAAGTAGTAATAATGGACGATGAAGTCTGATGTTGAGTGTCTCCCTAGATGTGT 1860
TCTTATGCTTAACCATGTGATCAACAGACTGTCATGTTGTTTACTTTTCATAGGACAT 1920
GAGAAGCACATGAGGAATCCTCTGGATCTGGTTTCTGAGTGTGATTTTGTATCT 1980
CTATGTCAGTCTCAAATGATATTATTCTCTGTGCTTGGACAAATGAATAAATACTAT 2040
CTATGTGTCTGTCCGTTTGCCTAAAAAAA 2072

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Figure 2 Nucleotide and the deduced amino acid sequence of *FcHMGR* from *F. cirrhosa*.

(The N-terminal truncated region is underlined; The 3' and 5' RACE primers were marked with forward and reverse solid arrows, respectively. Asterisk denotes translation terminator)

Fritillariae CirrhosaeMDIRRRKPS..TRPPAAAFPLPSRIQSDALPLFIRHNLFSALFAASL	49
Phoenix_dactyliferaMDVRRFPQSAANPEDASS.VGGRIQASDALPLFIRHNLFSALFAASL	51
Elaeis_guineensisMDVRRFPKSAASPEVASVGGAAQVQASDALPLFIRHNLFSALFAASL	52
Musa_acuminata_subspMEVRRRLPSRPSVAPSPFRDGGACLPSSSPAVPRLOASDALPLFIRHNLFSALFVGSIL	63
Sorghum_bicolorMEVRCGVVQGGQSAAGHRRHPPAAAP.....RVQAGDALPLFIRHNLFSALFAASL	57
Brachypodium_distachyonMEVQRQVAPVVAAGGAARRSAGAGATR.....ALQAGDALPLFIRHNLFSALFAASL	57
Dendrobium_huoshanenseMEFRRRALSKPHSLRTAGAFFAKSGSSTRVQASDALPLFIRHNLFSALFVMSYI	56
Zea_maysMEVRCGVVQGGQSAARHPAPFPSPRAAA.....RVQAGDALPLFIRHNLFSALFAASL	53
Setaria_italicaMAMEVRRRLPPHGGAPAGHPRRRPSAAGAEGERVQAGDALPLFIRHNLFSALFAASL	63
Oryza_sativaMEVRRRAPLPPFPFG.....RVQAGDALPLFIRHNLFSALFAASL	41
Nicotiana_tabacumMDVRRSEKAPYPTKEFAAGEKPLPKHQQQQNSLLIASDALPLFLYLNGLFTMFSVM	63
Arabidopsis	MKKKQAGPQQTCGFVSKTCLISPSHLRHLTSSLLSLPERLPPMDLRRRPPKPVVNNDTGFSRSYQTRISDDHRRRTTIAPFPKSDALPLFLYLNGLFTMFSVA	115
Fritillariae Cirrhosae	VFLMRRWRKRVVSAFLVIGLADMAVICLIVASLTIISFFGIAYGVVSSHEDDDDFLLPSPAPAPAAPTQCPLLRSD.....K.....	133
Phoenix_dactylifera	VFLMRRWRKRVVSSMLVHVVGLTELLIVGLVASLTIISFFGITPVQSVSHHEDDFLLTPAPTAASPAAPAPAPAAA.PCPLLCDGSP.....	144
Elaeis_guineensis	VFLMRRWRKRVVSTPLVHVVGLSELLIVGLVASLTIISFFGITPVQSVSHHEDDFLLTHAPAAAPQPRPSRGNPAPARPRLLCDAAP.....	146
Musa_acuminata_subsp	VFLMRRWRKRVVCSPTLHLLGLSELLIVGLVASLTIISFFGITPVQSVSHHEDDFLLSGAAPSAPSAPICPLLSTDG.....TI.....	149
Sorghum_bicolor	AYLMRRWRKRVVSSSTPLVHVVGLAEMLIFGLVASLTIISFFGITPVQSVSSDDDFLLVGGSGAPATAAAPSRSRQQAPACALLGSP.....A	150
Brachypodium_distachyon	AYLMRRWRKRVVSSSTPLVHVVGLTELLIVGLVASLTIISFFGITPVQSVSSDDEDFLLVGSAG.....APAPAQGLLGS.A.....	137
Dendrobium_huoshanense	YYLMCRWRKRVVSTGAPLHLLTLDIPAVIGLIVASLTIISFFGITPVQSVSHYDDEDFLLSSDQAPCPCLTCSKSP.....	135
Zea_mays	AYLMRRWRKRVVSSSTPLHVVGLAEMLIFGLVASLTIISFFGITPVQSVSSDDEDFLLVGGSGAPASAAAPSR.QHAQAPACALLGSP.....	143
Setaria_italica	YYLMCRWRKRVVSTGAPLHVVGLAEMLIFGLVASLTIISFFGITPVQSVSSDDEDFLLIDSRRAAAPPPP.....QQPAPTICLLGNP.....	151
Oryza_sativa	AYLMRRWRKRVVSSSTPLHVVGLAEMLIFGLVASLTIISFFGITPVQSVSSDDEDFLLVFGARGSSAAAAPRAPSPSPACALLGSP.....H	134
Nicotiana_tabacum	YYLHRRWRKRVVSTPLHVVTFSELVAVSLVSLVTLGGFGLVQVGSFVSRNNDSDVDEDEDFLLLEEDSRRGPATLGGCTAVPPPAPRVVPPVPPAKVAAMSEK	178
Arabidopsis	YYLHRRWRKRVVSTPLHVVTFSELVAVSLVSLVTLGGFGLVQVGSFVSRASGDWADLADTIDEDHRLVTCSPPTIVSVAKLPNPEP.....	207
Fritillariae Cirrhosae	ESISVPAVDASDEEIIASVSGKTPSILESKLGDCCRAAGIRREALRRTGRPEGLFIEGFDVASTLGCQCELPVIGVQLPVGIAGPLLDGREFVPMATTEGCLVASTNRG	248
Phoenix_dactylifera	APGKMPAVTEDEEIIFVSVAGKTPSILESKLGDCCRAAGIRREALRRTGRSLEGLPLDGFVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	259
Elaeis_guineensis	SPGKMPAVTEDEEIIFVSVAGKTPSILESKLGDCCRAAGIRREALRRTGRSLEGLPLDGFVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	261
Musa_acuminata_subsp	SSQRNLEITADDEIVSSVSGKTPSILESKLGDCCRAAGIRREALRRTGRILEGLPLDGLVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	264
Sorghum_bicolor	AAAHEKMPDEDEEIVASVAGKTPSILETRLDCCRAAGIRREALRRTGRILEGLPLDGFVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	265
Brachypodium_distachyon	.DAVTEKMPDEDEEIVASVSGKTPSILETRLDCCRAAGIRREALRRTGRILEGLPLDGFVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	251
Dendrobium_huoshanense	..LAESLFGDDEEIVASVAGKTPSILEAKLGDCCRAAGIRREALRRTGRILEGLPLDGFVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	248
Zea_mays	.AAAFKMPDEDEEIVASVAGKTPSILEARLDCCRAAGIRREALRRTGRILEGLPLDGFVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	257
Setaria_italica	.AAAFKMPDEDEEIVASVAGKTPSILETRLDCCRAAGIRREALRRTGRILEGLPLDGFVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	265
Oryza_sativa	DDAARPEDEDEEIVSSVAGKTPSILETRLDCCRAAGIRREALRRTGRILEGLPLDGFVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	249
Nicotiana_tabacum	APLVTAAEEDDEEIKSVGKTPSILESKLGDCCRAAGIRREALRRTGRILEGLPLDGFVIGAILGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	293
Arabidopsis	..IVTESLPEKDEEIVKSIDVIPSYSLESLKGDCCRAASIRREALRRTGRSMEGLPLDGFVIGESTLGCQCELPVIGVQLPVGIAGPLLDGDRQVYPMATTEGCLVASTNRG	320
HMG-CoA binding domains		
Fritillariae Cirrhosae	CKAILESQGANVYIKDGMTRAPVRFSTARRAELHFLLEPENFDLAVFNRRSFRARLGGIQSLRGNLIMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISIS	363
Phoenix_dactylifera	CKAIAESGGASVYLRDMTRAPVRFSLARRAELHFLLEPNSFETLSLVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	374
Elaeis_guineensis	CKAIAESGGASVYLRDMTRAPVRFSLARRAELHFLLEPNSFETLSLVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	376
Musa_acuminata_subsp	CKAIAESGGASVYLRDMTRAPVRFSLARRAELHFLLEPNDNFEILEVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	379
Sorghum_bicolor	CKAIAESGGASVYLRDMTRAPVRFSLARRAELHFLLEPNDNFEILEVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	380
Brachypodium_distachyon	CKAIAESGGASVYLRDMTRAPVRFSLARRAELHFLLEPNDNFEILEVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	366
Dendrobium_huoshanense	CKAIALSDGAVCVLKDGMTRAPVRFSLARRAELHFLLEPNDNFEILEVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	363
Zea_mays	CKAIAESGGASVYLRDMTRAPVRFSLARRAELHFLLEPNDNFEILEVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	372
Setaria_italica	CKAIAESGGASVYLRDMTRAPVRFSLARRAELHFLLEPNDNFEILEVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	380
Oryza_sativa	CKAIAESGGASVYLRDMTRAPVRFSLARRAELHFLLEPNDNFEILEVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	364
Nicotiana_tabacum	CKAIYASGGASVYLRDMTRAPVRFSLARRAELHFLLEPNDNFEILEVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	408
Arabidopsis	CKAMYISGGASVYLRDMTRAPVRFSLARRAELHFLLEPNDNFEILEVFNRRSFRARLGGIQALAGNLMRFSGSTGDAMGMNYSKGVQNVLDYQDDFFDMVVISI	435
NADPH binding domains		
Fritillariae Cirrhosae	...GNFCSDKKPAAYNIIEGRGKSVVCEAVIKEDVKKVVKLTINPALVELNMIKNLGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	475
Phoenix_dactylifera	SLSGNFCSDKKPAAYNIIEGRGKSVVCEAVIKEDVKKVVKLTINPALVELNMIKNLGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEALNDGKDLHISVTM	489
Elaeis_guineensis	...GNFCSDKKPAAYNIIEGRGKSVVCEAVIKEDVKKVVKLTINPALVELNMIKNLGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	488
Musa_acuminata_subsp	...GNFCSDKKPAAYNIIEGRGKSVVCEAVIKEDVKKVVKLTINPALVELNMIKNLGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	491
Sorghum_bicolor	...GNFCSDKKPAAYNIIEGRGKSVVCEAVIKEDVKKVVKLTINQSLVELNITKNLAGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	492
Brachypodium_distachyon	...GNFCSDKKPAAYNIIEGRGKSVVCEAVINEEIKRVVKLTINQSLVELNITKNLAGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	478
Dendrobium_huoshanense	...GNFCSDKKPAAYNIIEGRGKSVVCEAVIKEDVKKVVKLTINQSLVELNITKNLAGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	475
Zea_mays	...GNFCSDKKPAAYNIIEGRGKSVVCEAVIGEEVKKVVKLTINQSLVELNITKNLAGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	484
Setaria_italica	...GNFCSDKKPAAYNIIEGRGKSVVCEAVIKEDVKKVVKLTINQSLVELNITKNLAGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	492
Oryza_sativa	...GNFCSDKKPAAYNIIEGRGKSVVCEAVIKEDVKKVVKLTINQSLVELNITKNLAGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	476
Nicotiana_tabacum	...GNFCSDKKPAAYNIIEGRGKSVVCEAVITTEVKKVVKLTINQSLVELNITKNLAGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	520
Arabidopsis	...GNFCSDKKPAAYNIIEGRGKSVVCEAVIKGEINVKVVKLTINQSLVELNITKNLAGSAVAGALGGFNHASNINSAVFIATGQDPAQVNESSHCITNMEAVNDGKDLHISVTM	547
Fritillariae Cirrhosae	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	559
Phoenix_dactylifera	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	573
Elaeis_guineensis	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	571
Musa_acuminata_subsp	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	575
Sorghum_bicolor	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	576
Brachypodium_distachyon	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	565
Dendrobium_huoshanense	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	578
Zea_mays	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	582
Setaria_italica	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	584
Oryza_sativa	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	560
Nicotiana_tabacum	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	604
Arabidopsis	PSIEVGTGGGTQLASQAACLDLLGVKGSLESPGANARLLATVAGAVLAGELSLSLSAAGQLVSKHMYNRSKDKMSKAGS.....	635

Figure 3 Multiple alignment of FcHMGR proteins from several species. (The HMG-CoA binding domains and NADPH binding domains were marked with solid line and dotted line, respectively. The highly conserved regions of the alignments have been shaded)

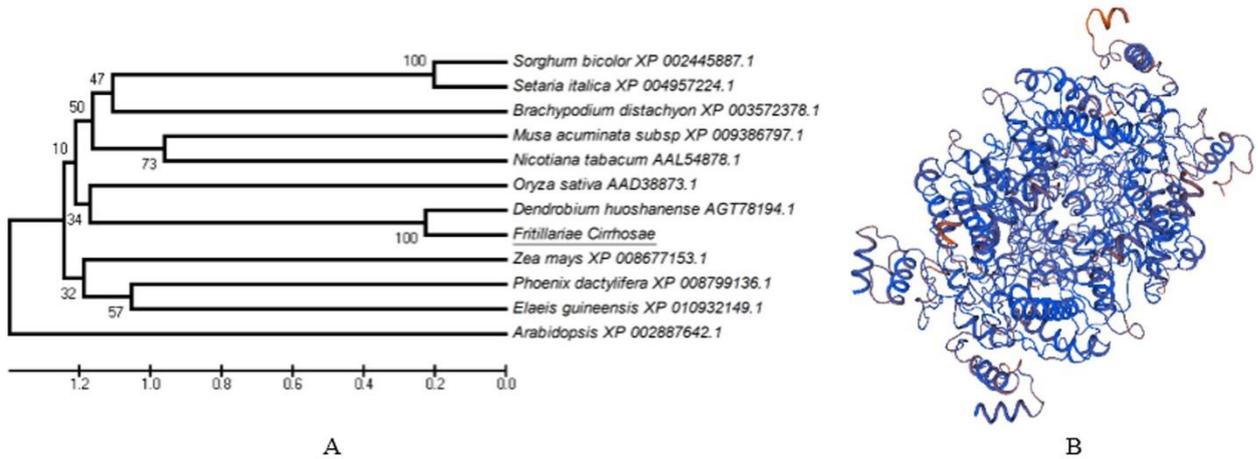


Figure 4 FcHMGR protein phylogenetic and tertiary structure analysis.

(A) Phylogenetic tree of FcHMGR protein sequences from different species.

(B) FcHMGR protein tertiary structure.

Transcription profile of *FcHMGR*

To better understand the expression of FcHMGR, we further analyzed the expression patterns of FcHMGR in bulbs, roots, stems and leaves of 3-year-old *F. cirrhosa* seedlings. The transcription level of FcHMGR was determined by qRT-PCR. Results indicated that FcHMGR were expressed in all examined tissues, with the highest expression in the bulb. The expression level of FcHMGR in leaf were 0.6 fold lower than that in bulb (Figure 5). This result indicated that expression of FcHMGR in vegetative tissues was non-tissue-specific.

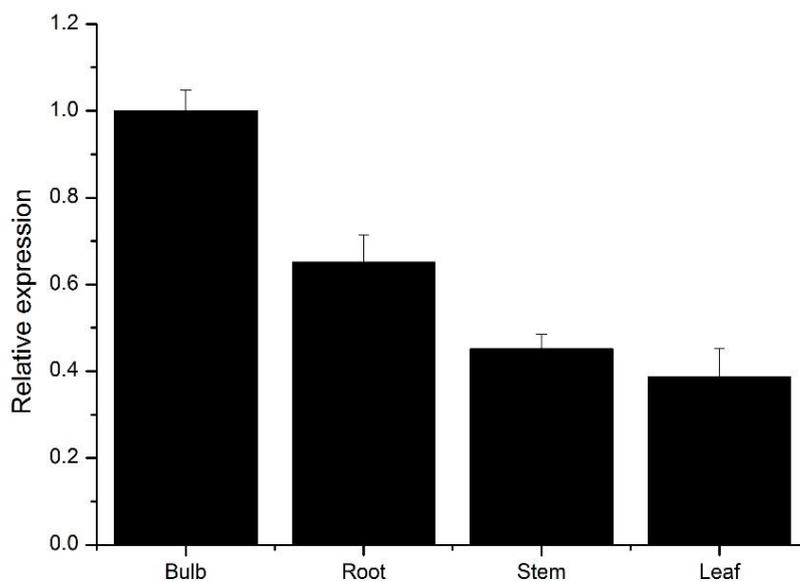


Figure 5 Spatial expression patterns of *FcHMGR* gene measured by qRT-PCR.

Expression and enzyme activity assay of the recombinant FcNt-HMGR in E.coli BL21

We previously found that the expression of the full length recombinant FcHMGR in E.coli was not detectable (data not shown), presumably due to the GC-rich region near the N-terminal of the sequence. We sub-cloned an N-terminal 249-bp truncated FcHMGR (FcNt-HMGR) into pGEX-6p-1 for recombinant protein expression. The N-terminal 83 amino acids of FcHMGR showed low homology with plant HMGR families and had no predicted enzyme activity domains. Subsequent prokaryotic expression assays proved that this recombinant FcNt-HMGR can be successfully expressed as soluble protein in E.coli BL21 strain under IPTG induction, which was not present in the extracts obtained from non-induced E. coli cells (Figure 6A). Because of the GST-tag fusion, molecular masses of the FcNt-HMGR proteins was 75 kDa.

To determine whether the recombinant FcNt-HMGR protein was a functional HMGR protein, the enzyme activity of FcNt-HMGR was measured according to the absorbance attenuation of NADPH at 340 nm. As shown in Figure 6B, when the supernatant containing FcNt-HMGR was added into the reaction solution, the NADPH is rapidly consumed, resulting in a sharp decline of absorbance at 340 nm. The absorbance of the control (non-induced FcNt-HMGR E. coli cells) showed no dramatic change. This indicated that the heterologous expressed FcNt-HMGR protein had a better reductase activity than the control and the relative enzyme activity was calculated to be 1.62 U/mg.

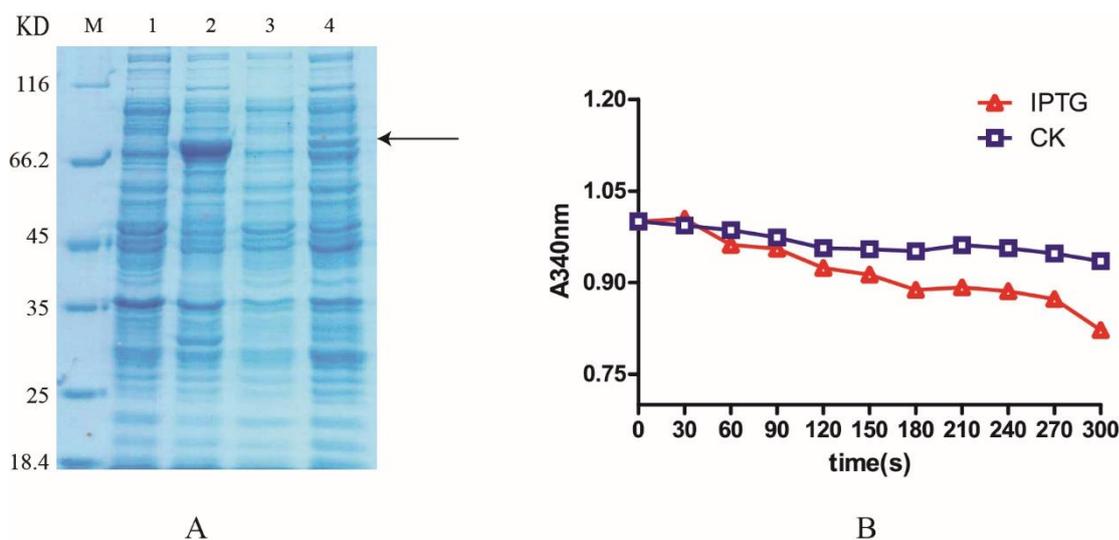


Figure 6 Recombinant FcNt-HMGR protein expression in BL21DE3 cells (A) and enzyme activity assay (B) (A) SDS-PAGE (12%) analysis of FcNt-HMGR over-expression in E.coli BL21. Lane M, protein marker (Beyotime p0061); lane 1, without IPTG induction; lane 2, IPTG induction (arrow mark is 75 kDa); lane 3, the supernatant of lysate without IPTG induction; lane 4, the supernatant of lysate with IPTG induction. (B) The enzyme activity assay by measuring the absorbance attenuation of NADPH at 340 nm. CK, non-induced recombinant FcNt-HMGR BL21 cells; IPTG, induced recombinant FcNt-HMGR BL21 cells.

DISCUSSION

HMGR are generally conserved in land plants, playing an important role in the regulation of secondary metabolism of the phytosteroid^{7,14}. In this study, we isolated a gene, FcHMGR, encoding a HMGR-like protein from *F. cirrhosa*. Homologous and phylogenetic analysis indicated that the FcHMGR protein has the typical polypeptide sites which are required for HMGR activity, including two HMG CoA - binding

domains (EMP (V/I) GY (V/I) QIP, TTEGCLVA) and two NADPH binding domains (DAMGMNM). Eti et al. found that the glutamic acid of TTEGCLVA plays a crucial role in the HMGR catalytic process¹⁵. Homology modeling demonstrated that the main body of the space layout of HMGR function area was folded into a "V" shape. The structure domains of L in the middle part possess the HMG-CoA binding motif and NADPH binding motif¹⁶. Combining with the tertiary structure prediction analysis (Figure 3,4B), these results suggested that FcHMGR is a functional protein that plays a role in the biosynthetic pathway of phytosteroid.

Transcription analysis is a useful tool for deciphering gene functions. The bulbs of *F. cirrhosa* are used for medicinal purposes¹⁷. qRT-PCR showed that the basal FcHMGR mRNA levels was high in mature bulbs (Figure 5), consistent with its high level of phytochemicals¹⁸, suggesting a positive correlation between the expression of this FcHMGR-like genes and accumulation of phytochemicals in *F. cirrhosa* bulbs.

We exploited the prokaryotic expression system to further evaluate the enzymatic activity of the protein encoded by this gene. Initially, the full protein of FcHMGR could not be expressed in *E. coli*, which was most likely related the GC enrichment region at the N-terminal of the gene. So, we deleted 249-bp from this region of FcHMGR to verify whether this change will affect the recombinant protein expression and function. Subsequent experiments showed this N-terminal truncation was in favor of Fc-HMGR expression. The recombinant protein showed reductase activity according to the result from out in vitro assay (Figure 6). This result also suggested that the function of Fc-HMGR depends on its conserved structure. The low homology region, including the N-terminal 249-bp truncated area in this study, caused no change of the protein function.

CONCLUSION

In conclusion, based on the results presented in this study, the correlation between phytochemicals content and FcHMGR gene expression level in *F. cirrhosa* bulbs have been established to some extent, which needs to be further verified in studies with larger samples. Some functional information was also obtained according to the deduced amino acid sequence of the FcHMGR enzyme. We also evaluated the functional validity of FcHMGR in the in vitro enzymatic reaction, but few correspond to its molecular mechanisms responsible for substrate specificity still need to be clarified, CRISPR/Cas9 mutagenesis technology will be a useful method for such a study¹⁹, which may be conducted in the future. On the basis of this study, the HMGR enzyme of *F. cirrhosa* could be a good candidate for the engineering of the *F. cirrhosa* phytochemicals synthesis pathway.

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