

*Full Length Research Paper*

# Molecular cloning, characterization and functional analysis of a 3-hydroxy-3-methylglutaryl coenzyme A reductase gene from *Jatropha curcas*

Juan Lin\*, Yuanjie Jin, Mingqi Zhou, Xin Zhou and Jinya Wang

State Key Laboratory of Genetic Engineering, School of Life Sciences, Morgan-Tan International Center for Life Sciences, Fudan-SJTU-Nottingham Plant Biotechnology R and D Center, Fudan University, Shanghai 200433, China.

Accepted 15 May, 2009

**3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC: 1.1.1.34) catalyzes the first committed step in mevalonic acid (MVA) pathway for biosynthesis of isoprenoids. The full-length cDNA encoding HMGR was isolated from *Jatropha curcas* for the first time (designated as JcHMGR), which contained a 1950 bp ORF encoding 584 amino acids. The JcHMGR genomic DNA sequence was also obtained, revealing JcHMGR had 4 exons and 3 introns. The deduced JcHMGR protein showed high identity to other plant HMGRs and contained 2 transmembrane domains and a catalytic domain. The potential significance of JcHMGR gene was also discussed.**

**Key words:** *Jatropha curcas*, RACE, 3-hydroxy-3-methylglutaryl coenzyme A reductase, color complementation assay.

## INTRODUCTION

*Jatropha curcas*, a member of the Euphorbia family, originated in central America. It has long been used around the world as a source of lamp oil and soap and also as a hedging plant (Lin et al., 2004). New research shows that the seeds of the plant are not only a source of biodiesel, but also contain several metabolites of pharmaceutical importance, such as phorbol esters (Fairless, 2007; Debnath and Bisen, 2008). Phorbol esters are the tetracyclic diterpenoids generally known for their tumor promoting activity (Liu et al., 2002). The phorbol esters contained in *J. curcas* were found to be toxin in different animal and insect (Sauerwein et al., 1993; Adam, 1974; Adam and Magzoub, 1975). *Jatropha* oil and phorbol esters exhibit insecticidal and molluscicidal activities over a wide range of organisms, suggesting their potential use in agriculture as biorational pesticides and as mollusc control agents (against water snails which transmit parasites, such as schistosomes or flukes)(Rug and Ruppel, 2000). This toxicity of phorbol esters limits the use of many nutritive plants and agricultural by-products to be

used as animal feed. Therefore, various chemical and physical treatments have been evaluated to extract or inactivate phorbol esters so that the seed meals rich in proteins could be used as feed resources (Goel et al., 2007). However, no much progress has been reported so far. With the exclusion of the phorbol esters possessed against the promastigote forms and antimycobacterial activity. These beneficial affects need the increasing the content of phorbol esters in the native *J. curcas* plant materials to be used as agriculture control (as biopesticide or insecticide)(Chan-Bacab and Peña-Rodríguez, 2001; Chumkaew et al., 2003). Therefore, to map the phorbol esters biosynthetic pathway at the level of molecular genetics may lead to high and low toxin *J. curcas* breeding to a bright path.

The structure of the phorbol esters is dependent on the tetracyclic diterpene carbon skeleton known as tigliane. In plants, it is well known that there are 2 distinct isoprenoids biosynthesis pathways, the plastidic 2C-methyl-D-erythritol 4-phosphate (MEP) pathway in the chloroplast and the cytosolic mevalonic acid (MVA) pathway in the cytosol. But the 2 pathways are not separated absolutely. In some extents, there are some forms of crosstalk between them. The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR; EC1.1.1.34) catalyzes the first committed

\*Corresponding author. E-mail: [linjuan@fudan.edu.cn](mailto:linjuan@fudan.edu.cn). Tel.: +86-21-65642425. Fax: +86-21-65642425.

**Table 1.** Primers used in the cloning and analysis of *JcHMGR* gene.

Primers	Sequence
FHMGR	5'-CC(G/A)ATGGCNCAC(C/T/G)ACNGA(A/G)GG(A/T/G)TG-3'
RHMGR	5'-AA(T/C)TGGAT(T/C)GAAGG(T/C/G)CG(T/G)GG(T/C)AA-3'
JcHMGRF1	5'-CAAGTAGATTTGCGAGGCTCCAAGGCGT-3'
JcHMGRF2	5'-TTCGGACAAGAAACCTGCTGCTGTAAAC-3'
JcHMGRR1	5'-TTGGAGCCTCGCAAATCTACTTGACCT-3'
JcHMGRR2	5'-GTCACTGATTCAAAAACGAACAACAGGCG-3'
JcHMGRfull-F	5'-ACGCGGGCCAACACTACTACTGTACGCCCTG-3'
JcHMGRfull-F1	5'-ATGGACCCCAACCGCCGCCAACAATA-3'
JcHMGRfull-R1	5'-TTATGATGCAACTCTCGATACATCTTTGC-3'
JcHMGR-Bg	5'-CGagatctATGGACCCCAACCGCCGCC-3'
JcHMGR-No	5'-TAGcgccgcTTATGATGCAACTCTCG-3'
3'-RACE CDS Primer	5'-AAGCAGTGGTATCAACGCAGAGTAC(T) <sub>30</sub> N-1N-3'
5'-RACE CDS Primer	5'-(T) <sub>25</sub> N-1N-3'
UPM	Long: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' Short: 5'-CTAATACGACTCACTATAGGGC-3'
NUP	5'-AAGCAGTGGTATCAACGCAGAGT-3'
SMART II A Oligo	5'-AAGCAGTGGTATCAACGCAGAGTA-3'
AP	5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTT-3'

step in MVA pathway for biosynthesis of isoprenoids. It is a key regulatory and potential rate-limiting enzyme, which provides the backbones from isoprenoids synthesized (Chappell, 1995). As diterpenes, phorbol esters are formed in the isoprenoid pathway. It has been demonstrated that the precursor of phorbol esters is biologically synthesized from both pathway. Therefore, HMGR probably acts as one of the key regulatory enzymes for phorbol esters biosynthesis in MVA pathway. However, up till now, there has been no report on the cloning of HMGR gene from *J. curcas*. In the present study, we report for the first time the molecular cloning and characterization of the HMGR gene from the *J. curcas* by RACE technique and validate its biological function in *Escherichia coli*, which will enable us to map and regulate an important step involved in phorbol esters biosynthetic pathway at the level of molecular genetics in the future.

## MATERIALS AND METHODS

### Plant materials

The seeds of *Jatropha curcas* were collected from Panzhihua, Sichuan province, China. The seeds were grown in pots in the greenhouse at 27±1 °C with 16 h light and 8 h dark photoperiod. Leaves were collected and stored at -70 °C until use.

### Cloning of JcHMGR core cDNA fragment

RNA was isolated using CTAB-acidic phenolic method (Jiang and Zhang, 2003). The core cDNA fragment of JcHMGR gene was cloned according to the protocol of one step RNA PCR Kit (AMV)

(TaKaRa). The forward primer FHMGR and reverse primer RHMGR (Table 1) used for the cloning of JcHMGR core cDNA fragment were designed and synthesized according to the hmgr gene conserved regions of the Euphorbiaceae plant, such as *Hevea brasiliensis* and *Euphorbia peginensis* deposited in GenBank. PCR was carried out in a total volume of 50 µl containing 5 µl 10 × buffer, 10 µl MgCl<sub>2</sub>, 5 µl, 2.5 mmol dNTP Mix, 1 µl RNase inhibitor, 1 µl total RNA, 2 µl, 20 pmol primer FHMGR, 2 µl, 20 pmol primer RHMGR, 1 µl AMV Rtase XL, and 1 µl AMV-Optimized Taq polymerase. The amplification was performed in a GeneAmp PCR System 2400 for 30 min at 50 °C, 2 min at 94 °C and 30 cycles with 30 s at 94 °C, 30 s at 53 °C, 90 s at 72 °C. After the final cycle, the amplification was extended for 10 min at 72 °C.

### 5' RACE of JcHMGR

According to the protocol of the SMART<sup>TM</sup> RACE cDNA amplification kit (Clontech), about 100 ng of total RNA was reversely transcribed with primer 5'-CDS primer coupled with (dC) tailing and SMART II A oligo, which annealed to the tail of the RNA and served as an extended template for PowerScript RT. The reverse primer used for the cloning of partial coding sequence of JcHMGR was designed and synthesized according to the core cDNA fragment of JcHMGR gene obtained earlier. The first round PCR was performed with JcHMGRR1 as the reverse primer and universal primer A mix (UPM) as the forward primer (Table 1). PCR was carried out in a total volume of 50 µl containing 2.5 µl cDNA, 1 µl 10 pmol primer JcHMGRR1, 5 µl 10 pmol UPM, 41.5 µl master mix (34.5 µl PCR-grade water, 5 µl 10 × advantage 2 PCR buffer, 1 µl 10 mmol dNTP mix, 1 µl 50 × advantage 2 polymerase mix) under the following condition: the template was firstly denatured at 94 °C for 3 min and then subjected to 30 cycles of amplification (94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min) followed by 10 min at 72 °C. Subsequently, nested PCR was performed under normal PCR reaction conditions using JcHMGRR2 as reverse primer and the nested universal primer A (NUP) as forward primer (Table 1).

### 3' RACE of JcHMGR

According to the protocol of the SMART™ RACE cDNA amplification kit (Clontech), about 100 ng of total RNA was reversely transcribed with primer 3'-CDS primer (Table 1), provided within the kit. The gene-specific primer JcHMGRF1 was used as the forward primer and universal amplification primer (UAP) (Table 1) was used as the reverse primer to amplify the 3' end cDNA. PCR was performed in a total volume of 50  $\mu$ l containing 2  $\mu$ l cDNA, 2  $\mu$ l 1 pmol primers JcHMGRF1, 2  $\mu$ l 10 pmol primers UPM, 1  $\mu$ l 10 mmol dNTPs, 5  $\mu$ l 10  $\times$  cDNA reaction buffers and Taq polymerase. The amplification was performed in a GeneAmp PCR System 2400 for 3 min at 94°C followed by 35 cycles with 30 s at 94°C, 1 min at 48°C and 1 min at 72°C. After the final cycle, the amplification was extended for 10 min at 72°C. The gene-specific primer JcHMGRF2 was used as the forward primer and NUP (Table 1) was used as the reverse primer in the nested PCR, which was carried out in a total volume of 50  $\mu$ l containing 1  $\mu$ l of the first round 3' RACE-PCR product under the following condition: the template was denatured at 94°C for 3 min and then subjected to 35 cycles of amplification (94°C for 30 s, 57°C for 1 min, 72°C for 1 min) followed by 10 min at 72°C.

### Generation of the full-length cDNA of JcHMGR

By comparing and aligning the sequences of the core fragment, the 5' RACE and 3' RACE products, the full-length cDNA sequence of JcHMGR was deduced and subsequently amplified with primers JcHMGRfull-F and JcHMGRfull-R1 (Table 1), using 1 step PCR Kit (TaKaRa). RT-PCR was carried out in a total volume of 50  $\mu$ l containing 1  $\mu$ l total RNA, 1  $\mu$ l 10 pmol of JcHMGRfull-F and JcHMGRfull-R1 respectively, 1  $\mu$ l 10 mmol dNTPs, 5  $\mu$ l 10  $\times$  1 step RNA PCR buffer, 1  $\mu$ l RNase Inhibitor, 1  $\mu$ l AMV reverse transcriptase XL and 1  $\mu$ l AMV optimized Taq polymerase under the following conditions: 50°C for 30 min and 94°C for 2 min followed by 30 cycles of amplification (94°C for 30 s, 54°C for 30 s, 72°C for 1 min). After the final cycle, the amplification was extended for 10 min at 72°C.

### The genomic sequence amplification of JcHMGR

An aliquot of 1 g genomic DNA and a pair of PCR primers JcHMGRF1 and JcHMGRR1 (Table 1) were used for amplifying the genomic sequence of JcHMGR. The PCR was carried out in a total volume of 50  $\mu$ l reaction solution containing 5  $\mu$ l 10  $\times$  buffer (plus  $Mg^{2+}$ ), 1  $\mu$ l 10 mM each of dNTPs, 1  $\mu$ l 10 pM JcHMGRF1, 1  $\mu$ l 10 pM JcHMGRR1, 1  $\mu$ g genomic DNA and 2.5 units of Ex Taq DNA polymerase (Takara). Firstly, the template was denatured at 94°C for 3 min, followed by 35 cycles of amplification (1 min at 94°C, 1 min at 60°C, 3 min at 72°C) and extension at 72°C for 8 min. All the PCR products were purified using gel extraction mini kit (Watson, China), ligated to pMD18-T vectors (TaKaRa, China), transformed into *E. coli* strain DH5 $\alpha$  and then sequenced with DYEnamic direct dGTP sequencing Kit (Amersham Pharmacia, England) and a 373A DNA sequencer.

### Comparative and bioinformatic analyses

Comparative and bioinformatic analyses of JcHMGR were carried out online at the websites (<http://www.ncbi.nlm.nih.gov> and <http://cn.expasy.org>). The nucleotide sequence, deduced amino acid sequence and ORF (open reading frame) encoded by JcHMGR were analyzed and the sequence comparison was conducted through database search using BLAST program (<http://www.ncbi.nlm.nih.gov>). The chloroplast transit peptides (cTP) of JcHMGR

were predicted at the website (<http://www.cbs.dtu.dk/services/ChloroP/>). The homology-based 3-D structural modeling of JcHMGR was accomplished by swiss-modeling. WebLab ViewerLite was used for 3-D structure displaying (homology-based modeling by swiss-Model). The phylogenetic analysis of JcHMGR and HMGR from other species was aligned with CLUSTAL W (1.82) using default parameters. Phylogenetic tree was constructed using HMGR version 2.1 from CLUSTAL W alignments. The neighbor-joining method was used to construct the tree.

### Functional analysis of jcHMGR in *Escherichia coli*

*E. coli* strain Top 10F', plasmids pAC-BETA and pTrc-ATIPI (provided by Dr. Francis X. Cunningham, Department of Cell Biology and Molecular Genetics, University of Maryland, USA) were used to test the biological function of JcHMGR. A complementation strategy was used in this experiment. The plasmid, pAC-BETA, retains functional genes for geranylgeranyl pyrophosphate synthase (crtE), phytoene synthase (crtB), phytoene desaturase (crtL) and lycopene cyclase (crtY). It also retains a chloramphenicol resistance gene. Cells of *E. coli* containing this plasmid produce and accumulate  $\beta$ -carotene, resulting in yellow colonies (Cunningham et al., 1994; 1996). The plasmid, pTrc-ATIPI, retains an ampicillin resistance gene and an IPI gene whose product can accelerate the accumulation of  $\beta$ -carotene (Cunningham and Gantt, 2000). The plasmid pAC-BETA was introduced into *E. coli* Top 10F' to create a  $\beta$ -carotene accumulating strain of *E. coli*. The coding region of JcHMGR was amplified by PCR using primers JcHMGR-Bg and JcHMGR-No (Table 1). Both of the fragment and the plasmid pTrc-ATIPI were digested with BglII and NotI for 10 h. Subsequently, the coding region of JcHMGR was cloned into the expression vector pTrc to obtain the plasmid Trc-HMGR. The pTrc-HMGR was transformed into the Top 10F' containing pAC-BETA. Transformants were cultured on solid LB medium containing ampicillin (150 mg/l) and chloramphenicol (50 mg/l) at 28°C for 48 h. The color of the transformants can be used as a visible marker to test if JcHMGR can accelerate the accumulation of  $\beta$ -carotene.

## RESULTS AND DISCUSSION

### Cloning of the full-length cDNA of JcHMGR

By comparing and aligning the sequences of the core fragment, the 5' RACE and 3' RACE products, the full-length cDNA sequence of JcHMGR was deduced and subsequently amplified using 1 step PCR kit (TaKaRa) with primers JcHMGRfull-F and JcHMGRfull-R1 (Table 1). The full-length cDNA of JcHMGR was 1950 bp and contained an ORF of 1752 bp, flanked by stretches of 80 and 118 bp at the 5'- and 3'- untranslated regions respectively. The stop codon (tga) and one putative polyadenylation signal site (AATGAA) were recognized in the 3' untranslated region, which were followed by a short polyA tail. The ORF encoded a predicted polypeptide of 584 amino acid residues, with a predicted molecular mass of 62.4 kDa and pI of 7.63 (Figure 1).

### Comparative and bioinformatic analyses of JcHMGR

The HMGR genes have been cloned and characterized

```

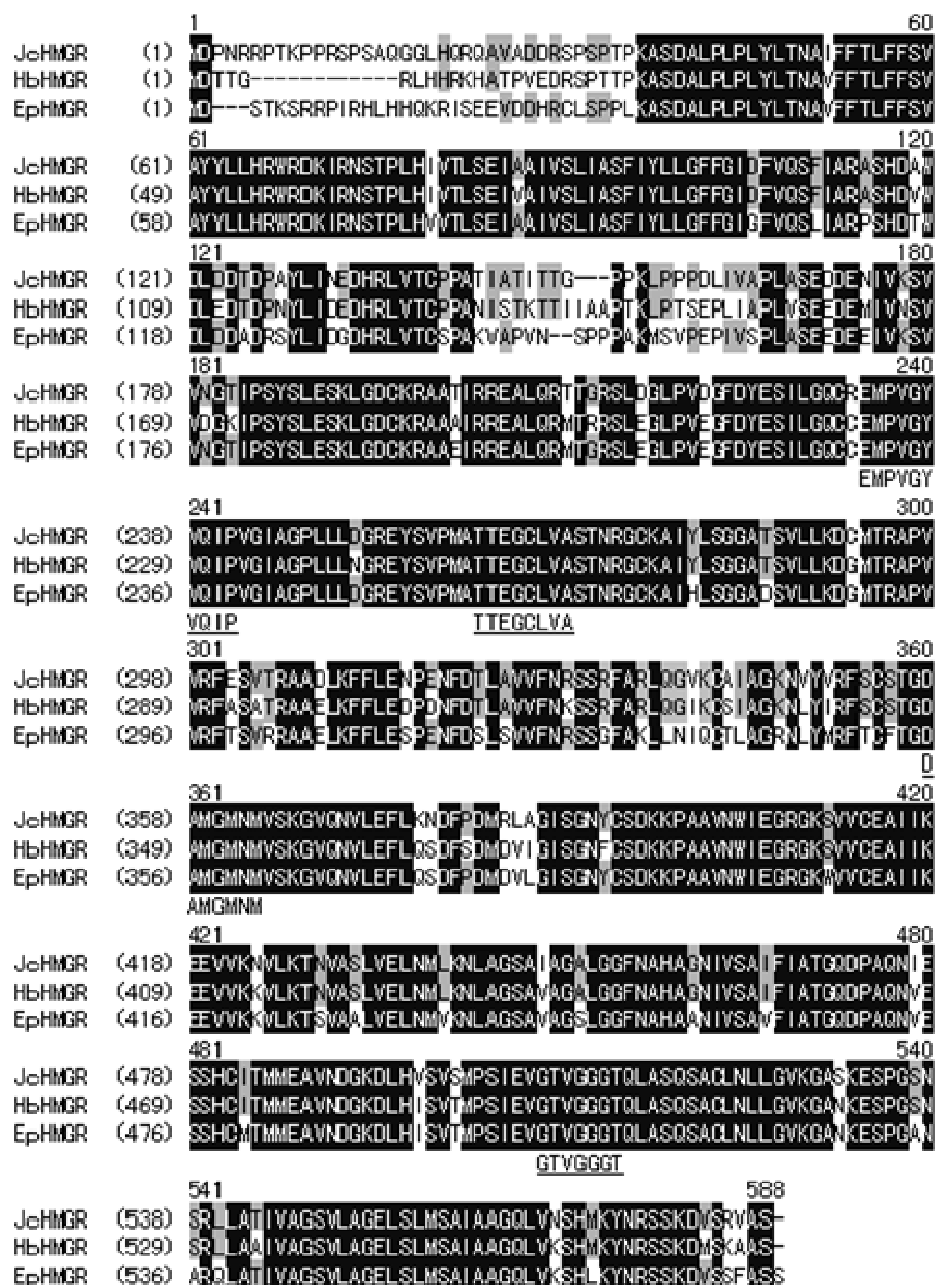
1  ACGCGGGCAACTACTACTGTACGGCCCTGTGGAAATAAACTACTCTCTCCCTGTTTCTAT 60
61  TTTTTCGGCGAATATTTTAAATGGAOCCCAACCGCGCCCACTAAACCACCGGTTCC 120
      M D P N R R P T K P P R S P
121  CCTCCGCGCAAGGTGGCTCCACCAAGCGCAAGCTGTAGCGGACGACCGCTCTCCATCTC 180
      S A Q G G L H Q R Q A V A D D R S P S P
181  CCACTCCGAAAGCGTGGAGCGCCCTTCCGCTGCCACTGTATCTACTAATGCAATTTTCT 240
      T P K A S D A L P L P L Y L T N A I F F
241  TCACGCTGTTCTTCTCGGTGGCATATTAACCTCTCCACCGGTGGGTGACAAGATCCGCA 300
      T L F F S V A Y Y L L H R W R D K I R N
301  ACTCTACTCCCTCCACATCGTTAOCCTCTCTGAGATTGGCGCATTGCTCCCTCATTG 360
      S T P L H I V T L S E I A A I V S L I A
361  CTTCCITCATTTATCTCCTCGATTCTTTGGAATCGATTTTGTCAATCATTTATTCAC 420
      S F I Y L L G F F G I D F V Q S F I A R
421  CGCCCTCCCATGATCGGTGGACCTCGACATACCGATCCCGCTACCTTATCAAGGAA 480
      A S H D A W D L D D T D P A Y L I N E D
481  ATCACCGTCTCGTACGGTCCCTCCCAACTATCGCTACCAITACACCGGACCTCCCA 540
      H R L V T C P P A T I A T I T T G P P K
541  AATTGGCGCTCCGATCTCATAGTCCACCTTTACCTCCGAGGATGACGAAACATCG 600
      L P P P D L I V A P L A S E D D E N I V
601  TCAATCCGTCGTAAGTGAAGCATTCTTCTACTTCTCTGGAATCAAACTCCGCGATT 660
      K S V V N G T I P S Y S L E S K L G D C
661  GCAAACGACCGCGCAACAAITCGACCGCGCTTTGCAAGCAGCAGCAAGGTCGCTGG 720
      K R A A T I R R E A L Q R T T G R S L D
721  ATGCTTACCGATCGATCGGTGGTGGATTACGAGTCAATCTAGCGGACTGCTGTGAATCC 780
      G L P V D G F D Y E S I L G Q C C E M P
781  CAGTCCGATACGTCAGATCCAGTAGGAAITCGCGGACCGCTATTGCTAGCGGACCGG 840
      V G Y V Q I P V G I A G P L L L D G R E
841  AGTACTCTGTTCCGATGGGACTACCGGCGTGTGGTGGCGAGCCACCAATAGCGGT 900
      Y S V P M A T T E G C L V A S T N R G C
901  GTAAGCGGATCTACTTGTACGGTGGCGCTACCAGCGTGTGTGAAGATTGCATGACAA 960
      K A I Y L S G G A T S V L L K D C M T R
961  GACCGCTGTTGTTGGTTTGAATCAGTCAAGACCTGAGATTGAAGTCTTTTTGG 1020
      A P V V R F E S V T R A A D L K F F L E
1021  AGAATCCGTAATAATTTGACACCTTGGCGGTTGCTTTTACAGTCCGGTCTTTTatagg 1080
      N P E N F D T L A V V F N R
1081  at t t c t g a a a a c g c t g t t c t a t t a a t g t a a t g t t t t g t t c t t c t g t g a t g t g a t 1140
1141  agat at t t agacat c t t a c c a a g a a c t t g t a g a t a t g t t t t g g t g t g a g g a t t g a a 1200
1201  g g a a a a a a g g g g a a a a t a a a a c t g a t t t g g a t c c g a a a c a t g t i a g t c a a g t g a a t t g a 1260
1261  a a c g a t t t g g a t c c t t a a t a t t g a g t c a a g t a g a t t t c c a g c c t c c a a g c g t a a 1320
      S S R F A R L Q G V K
1321  ATGTCTATTCCCGCAAGCATGTTTATGTAAATTTAGTTCGACACTGGTGTATGCAAT 1380
      C A I A G K N V Y V R F S C S T G D A M
1381  GGGGATGAACATGGTTCCAAAGGGGTCAAAACGTTCTTGAATTCCTTAAGATGATTT 1440
      G M N M V S K G V Q N V L E F L K N D F
1441  CCCTGACATCGCTCTTCTGTCATCTCAGTgaat t c c t t a t t a t c t c t a a g t t t a t 1500
      P D M R L A G I S G
1501  at g t t g g t a t t c t t t c a c t t c c a c t g c c a a a t t c c t t g g a a a t t t t g a a t t g g a t g c t g c a a 1560
1561  t t c t g g c t a t g t t t t c a t t g a c g c t t a a a c g a a t g t c t a a a t a a a a a c a t a t a c 1620
1621  g t a t g t t t c c t c a c t t c a a t t a t a a c c t c a g t a t t c a g t t t t a a a a a c t t a c c c g 1680
1681  t c t g g c g t c a t t a t c a a t t t a t c a c a a t c a t t g c a t t a t c t c a t t t c t t a g t t t t a t g 1740
1741  t t t a t c c g a g t a t g t t t c t g t t a g g t t g a t a a t t a t t t t t g c a c a a t a c t c t t g 1800
1801  t g g t t c a a g c a t a g c t a a a a t t g c a g a t g a c a t a t g t t t a g t t c c t t t g t a c a g g 1860
1861  a g a c a c a a g a g t g t g t t c t g t c a a t t t t g g c t c a t t t t t c a t g c t t t c a t a t t g t c 1920
1921  t t g a a a a c a t c a t g a a a c t c a g t t c a t t g a t c a a a t a t g t g a a a g c g t t t g a a a g t t t 1980
1981  t c g t t c a c t c a t t t t c t t a c t t g a a a t a c t a a a t a g t c a t t t t g a t g c t t t t c a a a t 2040
2041  t t c t g g a t g t c g a g t g c t t t a c t g t a g a a a t t a t t g a g g a g t a g g t g t t t c c t t g g 2100
2101  t t g c a g t a t c t t t c t g t c a t g g a t t a t g a t t a t a t t t t t c t c a a t t g c c t a g g a a 2160
      N
2161  ATTATTGTTCGGACAAAGAAOCTGCTGCTGTAACCTGGATTGAGCGAAGGGCCAAATCAG 2220
      Y C S D K K P A A V N W I E G R G K S V
2221  TTGTTTGTGAGGCAATTATCAAGCAAGCGTGGTGAANAATGATTAANAACAATGTTG 2280
      V C E A I I K E E V V K N V L K T N V A
2281  CTTCCCTAGTGGACTCAACATGCTAAAAATCTGCTGGTCTCCTATTGCTGTGCTC 2340
      S L V E L N M L K N L A G S A I A G A L
2341  TGGGTGGGTTAATGCTCATGCAGGAACATTGCTCTGCAATCTTTATTGCAACTGGCC 2400
      G G F N A H A G N I V S A I F I A T G Q
2401  AGGATCCAGCACAGATATTGAGAGTCTCACTGCATTACGATGATGGAAGCTGITAATG 2460
      D P A Q N I E S S H C I T M M E A V N D
2461  ATGAAAGGATCTCCATGCTCAGTGTCAATGCCCTCCATTGAGgt t a t t g a c t t g a a c 2520
      G K D L H V S V S M P S I E
2521  c t t t c a t t a a t c a t g g t t a c c a t a g c t t g c a g t g t t a t c a g t a a t t g a g g a t a a t g 2580
2581  t a a t t a a c t a t c t t t g t c t g c a g t c c g g t a c a g t t g a g c a g a a c t c a a c t t g c a 2640
      V G T V G G G T Q L A
2641  TCTCAGTCTGCTGTCTAAATTTGCTTGGGGTGAAGGGGGCAAGCAAGAGTCAACAGGA 2700
      S Q S A C L N L L G V K G A S K E S P G
2701  TCAACTCAAGGCTCCTTCCACCATAGTAGCTGGTTCAGTTTTCCCGGTCAGCTGTCT 2760
      S N S R L L A T I V A G S V L A G E L S
2761  CTGATGCTGCCATTGCACCTGGCACCTGTGCACAGCCACATGAATACATAGATCC 2820
      L M S A I A A G Q L V N S H M K Y N R S
2821  AGCAAAAGATGTATCGAGGTGCATCATAAGTGGAAAGAGGTGGTCCACGCAATGAAA 2880
      S K D V S R V A S
2881  GGGTTTGAATAAAGAGTGGAGGACTGGTGTGGGAGGTAAAAAGACAAATCGGG 2940
2941  ATCAGACAGAAAAAAAAAAAAAAA 2965

```

**Figure 1.** The full-length cDNA sequence, intron sequence and the deduced amino acid sequence of *J. curcas* 3-hydroxy-3-methylglutaryl-CoA reductase (hmgr). The cDNA sequence is indicated in capital letter and the introns are indicated in lowercase. The start codon (ATG) and the stop codon (TAA) are underlined.

from some plant species such as *H. brasiliensis* (Sando et al., 2008), *E. pekinensis* (Cao et al., 2007), *Arabidopsis thaliana* (Caelles et al., 1989; Learned and Fink, 1989), *Lycopericon esculentum* (Park et al., 1992), *Solanum tuberosum* (Choi et al., 1992), *Cucumis melo* (Kato-Emori et al., 2001). Sequence alignment using vector NTI suite 8.0 and BLAST research showed that the predicted JcHMGR from *J. curcas* had highly similarity with other plant HMGR in a range of 58%-87%, while the similarity with other non-plant eukaryotic and prokaryotic homologues is considerably lower. In plant, the JcHMGR showed above 80% with Euphorbiaceae plant and below 80% with other plant HMGRs. The JcHMGR showed 87% and 83% identities to those of *H. brasiliensis* (P29057) and *E.*

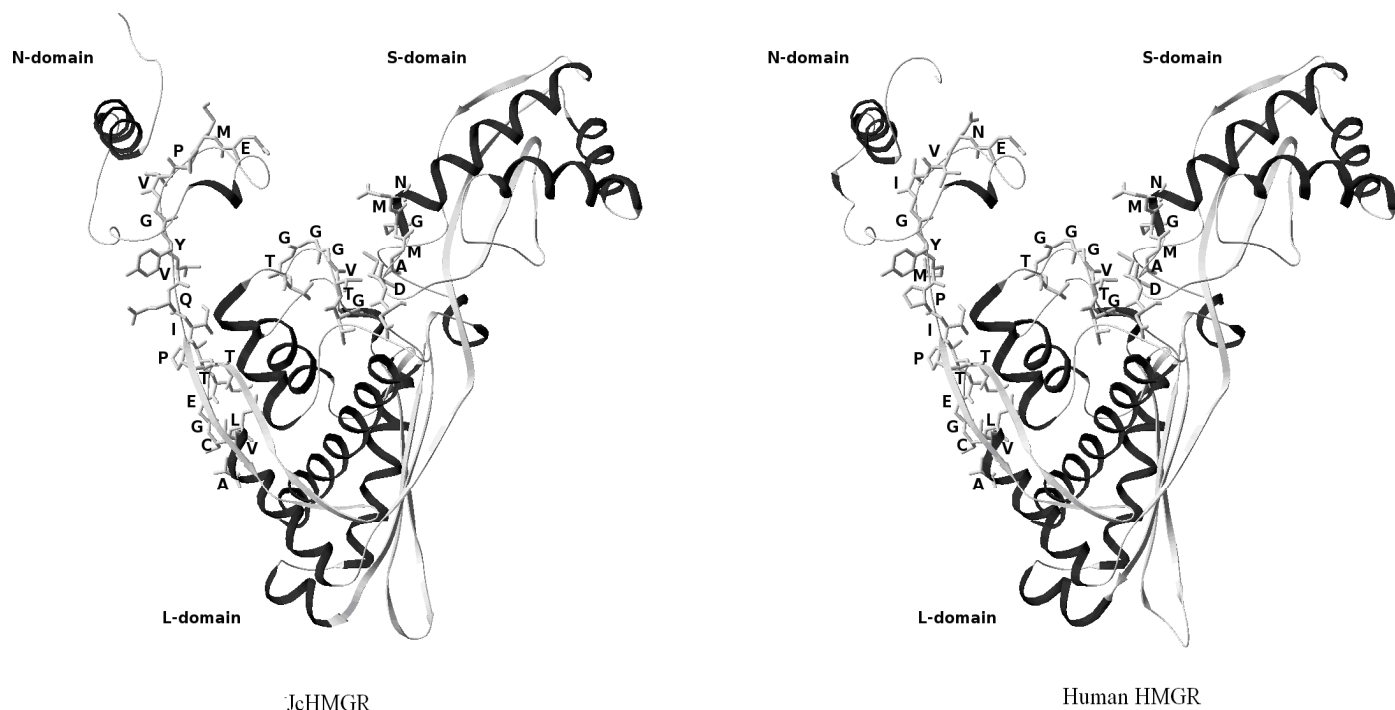
*pekinensis* (ABK56831), respectively (Figure 2). The results of BlastP also showed that the predicted JcHMGR belonged to the HMG-CoA reductase superfamily, a single domain family. Signallp1.1 analysis (<http://www.cbs.dtu.dk/services/SignalP/>) showed JcHMGR had no signal peptide that synthesized in the cytoplasm. 2 transmembrane region of JcHMGR were identified by TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) analysis, one was located between Pro43 and Leu-65 and the other was located between Ile86 and Val108 along the polypeptide chain. 2-dimensional structural prediction of JcHMGR was performed by the SOPMA server ([http://bip.weizmann.ac.il/bio\\_tools/fag.html](http://bip.weizmann.ac.il/bio_tools/fag.html)). Based on the hierarchical neural network analysis, JcHM-



**Figure 2.** Alignment of JcHMGR with other HMGR protein from Euphorbiaceae plant. Conserved regions are underlined with a solid line. Conserved amino acid residues in all the sequences used in this alignment are black boxed, while similar amino acids are in gray boxes. Alignments were performed with the Vector NTI 8.0 program.

GR protein was composed of 47.43%  $\alpha$ -helix, 13.53% extended strand, 5.48%  $\beta$ -turn and 33.56% random coil. The crystal structure of the human HMGR has been published (Istvan et al., 2000). The homology-based 3-D structural modeling of JcHMGR was analyzed by Swiss-modeling on the basis of human HMGR crystal structure and displayed by WebLab ViewerLite. According to the obtained result, the catalytic region of JcHMGR consisted of 3 domains: (i) the small helical N-terminal N-domain (ii)

the large, central L-domain harboring two HMG-CoA binding motifs EMPIGFLQIP and TTEGCLVA) and a NADP(H)-binding motifs (GTVGGGT) whose architecture resembled a prism with an  $\alpha$ -helix forming the central structural element (iii) the small helical S-domain harboring a NADP(H)-binding motif characterized by the sequence DAMGMN (Figure 3). All the bioinformatics analysis results strongly suggested that JcHMGR should be a functional plant HMGR protein involved in the biosyn-



**Figure 3.** The 3-D structures of JcHMGR (left) and human HMGR (right). JcHMGR had three domains: (1) the small, helical N-terminal N-domain; (2) the large, central L-domain harboring two HMG-CoA binding motifs (EMPIGFLQIP and TTEGCLVA) and a NADP(H)-binding motifs (GTVGGGT) whose architecture resembled a prism with an  $\alpha$ -helix forming the central structural element; (3) the small helical S-domain harboring a NADP(H)-binding motif characterized by the sequence DAMGMN. The  $\alpha$ -helix is shown in helix-shaped, the sheet in wide ribbon-shaped and the random coil also in line-shaped. The substrate-binding motifs are in grey and marked with letters.

thesis of mevalonate.

### Cloning genomic DNA sequence of *JcHMGR*

The PCR for genomic sequence resulted in a clear band of 2949 bp, which was 1015 bp longer than that of the coding sequence. The comparison with the cDNA showed that the genomic DNA and cDNA matched base to base except that the genomic DNA contained 3 introns. The splicing of introns was correlated to the GU-AG splicing rule. The lengths of 4 exons were 983, 182, 347 and 240 bp respectively (Figure 1). The lengths of the 3 introns were 224, 688 and 103 bp respectively. Similarly, it was found that most of the HMGR genes from plant species were composed of 4 exons and 3 introns, such as *Gossypium hirsutum* (Loguercio et al., 1999) and *L. esculentum* (Park et al., 1992) and *Ginkgo biloba* (Shen et al., 2006). But the lengths of the first exon and fourth exon from cotton, tomato and *G. biloba* were different with those of *J. curcas*.

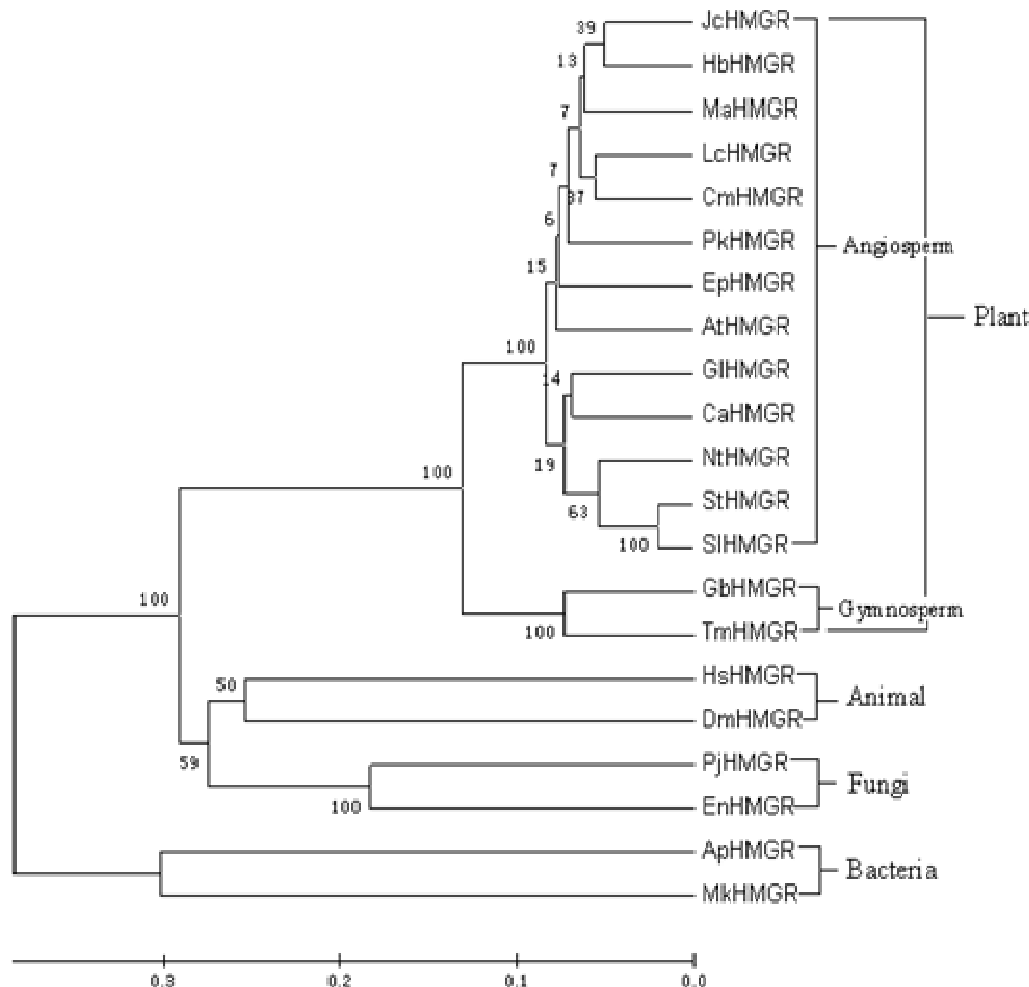
### Molecular evolution analysis

JcHMGR was the first gene cloned from *J. curcas* plants.

Therefore it would be interesting to investigate its evolutionary position among the phylogenetic tree of various HMGRs. The phylogenetic tree of HMGR was constructed from different organisms including plants, animal, fungi and bacteria using MEGA version 2.1 from CLUSTAL W alignments. According to the phylogenetic tree, JcHMGR had higher identity with plant HMGRs than animal, fungi and bacteria HMGRs (Figure 4), which was closely related to HMGR from *H. brasiliensis*. Apparently, all the HMGRs are derived from a common ancestor in evolution, suggesting that they share a common evolutionary origin. All the analysis results strongly suggest that JcHMGR is a plant HMGR protein involved in the mevalonate-independent biosynthesis.

### Biological function analysis of JcHMGR in *E. coli*

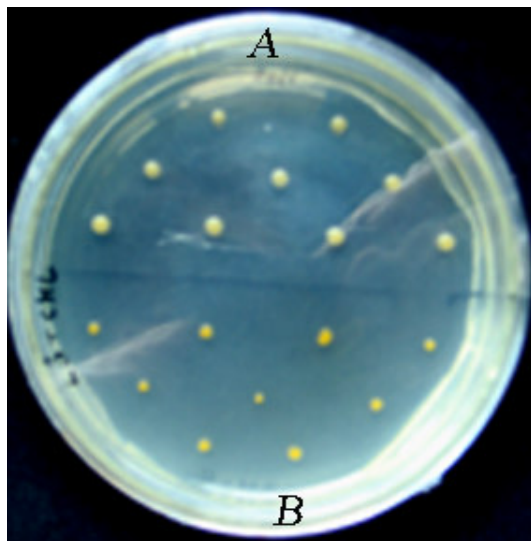
In the color complementation assay, we used the *E. coli* strain top 10F to test if JcHMGR encoded the anticipated functional enzyme. *E. coli* could not synthesize carotenoids because of lacking of carotenogenic genes. However, *E. coli* introduced with foreign carotenogenic genes clusters had the ability to produce carotenoids and transformed bacteria, owing to accumulating carotenoid pigments, could act as a visible marker for providing an easily screenable phenotype (Matthews and Wurtzel,



**Figure 4.** Phylogenetic analysis of HMGR from *J. curcas* and other plants, bacteria, fungi and animal species by MEGA version 2.1 from CLUSTAL W alignments. The neighbor-joining method was used to construct the tree. The HMGR used in phylogenetic tree analysis were those from *Hevea brasiliensis* (GenBank accession No. P29057), *Morus alba* (GenBank accession No. AAD03789), *Litchi chinensis* (GenBank accession No. DQ536143), *Cucumis melo* (GenBank accession No. BAA36291), *Picrorhiza kurroa* (GenBank accession No. ABC74565), *Euphorbia pekinensis* (GenBank accession No. ABK56831), *Arabidopsis thaliana* (GenBank accession No. NM\_106299), *Gentiana lutea* (GenBank accession No. BAE92730), *Camptotheca acuminata* (GenBank accession No. CAU72145), *Nicotiana tabacum* (GenBank accession No. AAB87727), *Solanum tuberosum* (GenBank accession No. AAB52551), *Solanum lycopersicum* (GenBank accession No. P48022), *Ginkgo biloba* (GenBank accession No. AY741133), *Taxus x media* (GenBank accession No. AY277740), *Homo sapiens* (GenBank accession No. NP\_000850), *Drosophila melanogaster* (GenBank accession No. P14773), *Pichia jadinii* (GenBank accession No. O74164), *Emericella nidulans* (GenBank accession No. AAO85434), *Aeropyrum pernix* (GenBank accession No. Q9YAS4), *Methanopyrus kandleri* (GenBank accession No. NP\_613640).

2000). As a result, the Top 10F' containing pAC-BETA can manufacture and accumulate  $\beta$ -carotene and form yellow colonies. When pTrc-HMGR containing the coding region of JcHMGR was transformed into this  $\beta$ -carotene accumulating *E. coli* Top 10F', the color of the bacteria turned from yellow to orange-yellow, indicating that JcHMGR could accelerate the accumulation of  $\beta$ -carotene (Figure 2). Therefore, the functioning of the JcHMGR has been confirmed by the change in the color of *E. coli*

cultures. Color complementation assay demonstrated that JcHM-GR helped to accelerate the accumulation of  $\beta$ -carotene. Since both  $\beta$ -carotene and phorbol esters, a kind of diterpenes, are mainly biosynthesized through MVA pathway (Bick and Lange, 2003), it can be expected that JcHMGR helps to accelerate the accumulation of phorbol esters. Therefore, JcHMGR may be a target gene in metabolic engineering to improve the contents of phorbol esters.



**Figure 5.** Functional demonstration of JcHMGR activity, using *E. coli* strain Top 10F'. *E. coli* cells were transformed with pAC-BETA (A) and pAC-BETA and pTrc-HMGR (B).

## ACKNOWLEDGMENTS

This research was supported by the Natural Science Foundation of China (30771745), Universities Dr. Subjects of Natural Science Research Fund of China (New Teachers) (20070246154) and National Science Fund for Fostering Talents in Basic Science, Nation Science Education Base at Fudan University (J0630643).

## REFERENCES

- Adam SE (1974). Toxic effects of *Jatropha curcas* in mice. *Toxicology*, 2: 67-76.
- Adam SE, Magzoub M (1975). Toxicity of *Jatropha curcas* for goats. *Toxicology*, 4: 347-354.
- Bick JA, Lange BM (2003). Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. *Arch. Biochem. Biophys.* 415: 146-154
- Caelles C, Ferrer A, Balcells L, Hegardt FG, Boronat A (1989). Isolation and structural characterization of a cDNA encoding *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Plant Mol. Biol.* 13: 627-638.
- Cao XY, Jiang JH, Liu Q, Dai CC, Chen FM (2007). Cloning and analysis of a cDNA encoding key enzyme gene (hmgr) of the MVA pathway in medicinal plant *Euphorbia pекinensis*. *J. Wuhan Botanical Res.* 25: 123-126.
- Chan-Bacab MJ, Peña-Rodríguez LM (2001). Plant natural products with leishmanicidal activity. *Nat. Prod. Rep.* 18:674-688.
- Chappell J (1995). Biochemistry and molecular biology of the isoprenoid biosynthesis pathway in plants. *Ann. Rev. Plant Physiol. Plant Mol.* 46:521-547.
- Choi D, Ward BL, Bostock RM (1992). Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. *Plant Cell*, 4: 1333-1344.
- Chumkaew P, Karalai C, Ponglimanont C, Chantrapromma K (2003). Antimycobacterial activity of phorbol esters from the fruits of *Sapium indicum*. *J. Nat. Prod.* 66: 540-543.
- Cunningham FX, Pogson B, Sun ZR, McDonald KA, DellaPenna D, Gantt E (1996). Functional analysis of the beta and epsilon lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell*, 8: 1613-1626.
- Cunningham FX, Sun ZR, Chamovitz D, Hirschberg J, Gantt E (1994). Molecular structure and enzymatic function of lycopene cyclase from the *Cyanobacterium synechococcus* Sp Strain Pcc7942. *Plant Cell*, 6: 1107-1121.
- Cunningham FX, Gantt E (2000). Identification of multigene families encoding isopentenyl diphosphate isomerase in plants by heterologous complementation in *Escherichia coli*. *Plant Cell Physiol.* 41: 119-123.
- Debnath M, Bisen PS (2008). *Jatropha curcas* L., a multipurpose stress resistant plant with a potential for ethnomedicine and renewable energy. *Curr. Pharm. Biotechnol.* 9: 288-306.
- Fairless D (2007). Biofuel: the little shrub that could-maybe. *Nature*, 449: 652-655.
- Goel G, Makkar HP, Francis G, Becker K (2007). Phorbol esters: structure, biological activity, and toxicity in animals. *Int. J. Toxicol.* 26: 279-288.
- Istvan ES, Palnitkar M, Buchanan SK, Deisenhofer J (2000). Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. *EMBO J.* 19: 819-830.
- Jiang JX, Zhang TZ (2003) Extraction of total RNA in cotton tissue with CTAB-acidic phenolic method. *Cotton Sci.* 15: 166-167.
- Kato-Emori S, Higashi K, Hosoya K, Kobayashi T, Ezura H (2001). Cloning and characterization of the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase in melon (*Cucumis melo* L. reticulatus). *Mol. Gen. Genet.* 265: 135-142.
- Learned RM, Fink GR (1989). 3-Hydroxy-3-methylglutarylcoenzyme A reductase from *Arabidopsis thaliana* is structurally distinct from the yeast and animal enzymes. *Proc. Natl. Acad. Sci. USA*, 86: 2779-2783.
- Lin J, Zhou XW, Tang KX, Chen F (2004). A survey of the studies on the resources of *Jatropha curcas*. *J. Trop. Subtrop. Bot.* 12: 285-290.
- Liu LG, Meng JC, Wu SX, Li XY, Zhao XC, Tan RX (2002). New macrocyclic diterpenoids from *Euphorbia esula*. *Planta Med.* 68: 244-248.
- Loguercio LL, Scott HC, Trolinder NL, Wilkins TA (1999). HMG-CoA reductase gene family in cotton (*Gossypium hirsutum* L.), unique structural features and differential expression of hmg2 potentially associated with synthesis of specific isoprenoids in developing embryos. *Plant Cell Physiol.* 40: 750-761.
- Matthews PD, Wurtzel ET (2000). Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase. *Appl. Microbiol. Biotechnol.* 53: 396-400.
- Park H, Denbow CJ, Cramer CL (1992). Structure and nucleotide sequence of tomato HMG2 encoding 3-hydroxy-3-methyl-glutaryl coenzyme A reductase. *Plant Mol. Biol.* 20: 327-331.
- Rug M, Ruppel A (2000). Toxic activities of the plant *Jatropha curcas* against intermediate snail hosts and larvae of schistosomes. *Trop Med. Int. Health*, 5: 423-430.
- Sando T, Takaoka C, Mukai Y, Okumoto H, Kuzuyama T, Yamashita A, Hattori M, Ogasawara N, Fukusaki E, Kobayashi A (2008). Cloning and characterization of mevalonate pathway genes in a natural rubber producing plant, *Hevea brasiliensis*. *Biosci. Biotechnol. Biochem.* 72: 2049-2060.
- Sauerwein M, Sporer F, Wink M (1993). Insect-toxicity of phorbol esters from *Jatropha curcas* seed oil. *Planta Med.* 59: p. 686.
- Shen G, Pang Y, Wu W, Liao Z, Zhao L, Sun X, Tang K (2006). Cloning and characterization of a root-specific expressing gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase from *Ginkgo biloba*. *Mol. Biol. Rep.* 33: 117-127.