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ORIGINAL PAPER

Molecular cloning and expression analysis of a new putative gene encoding 3-hydroxy-3-methylglutaryl-CoA synthase from *Salvia miltiorrhiza*

Lin Zhang · Xiangming Yan · Jing Wang · Shanshan Li · Pan Liao · Guoyin Kai

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Abstract A new putative gene encoding 3-hydroxy-3-methylglutaryl coenzyme A synthase (designated as *SmHMGS,* GenBank Accession No. FJ785326), which catalyses the condensation of acetyl-CoA and acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA as an early step in the mevalonic acid pathway, was isolated from young leaves of *Salvia miltiorrhiza* by rapid amplification of cDNA ends (RACE) for the first time. The full-length cDNA of the putative *SmHMGS* was 1,655 bp containing a 1,381 bp open reading frame (ORF) encoding a polypeptide of 460 amino acids. Comparative and bioinformatic analyses revealed that SmHMGS showed extensive homology with HMGSs from other plant species. Phylogenetic tree analysis indicated that SmHMGS belonged to the plant HMGS super family and had the closest relationship with HMGS from *Hevea brasiliensis.* Tissue expression pattern analysis revealed that the putative *SmHMGS* was constitutively expressed in all the tested tissues and strong in leaf, moderate in stem, weak in root, which was in contrast to *SmHMGR* reported before. The putative *SmHMGS* was found to be an elicitor-responsive gene, which could be induced by exogenous elicitors, including salicylic acid (SA) and methyl jasmonate (MJ). These results will help in understanding the role of *HMGS* in tanshinones biosynthesis in *S. miltiorrhiza.*

Keywords *Salvia miltiorrhiza* · Putative *SmHMGS* · Expression profile · Salicylic acid (SA) · Methyl jasmonate (MJ)

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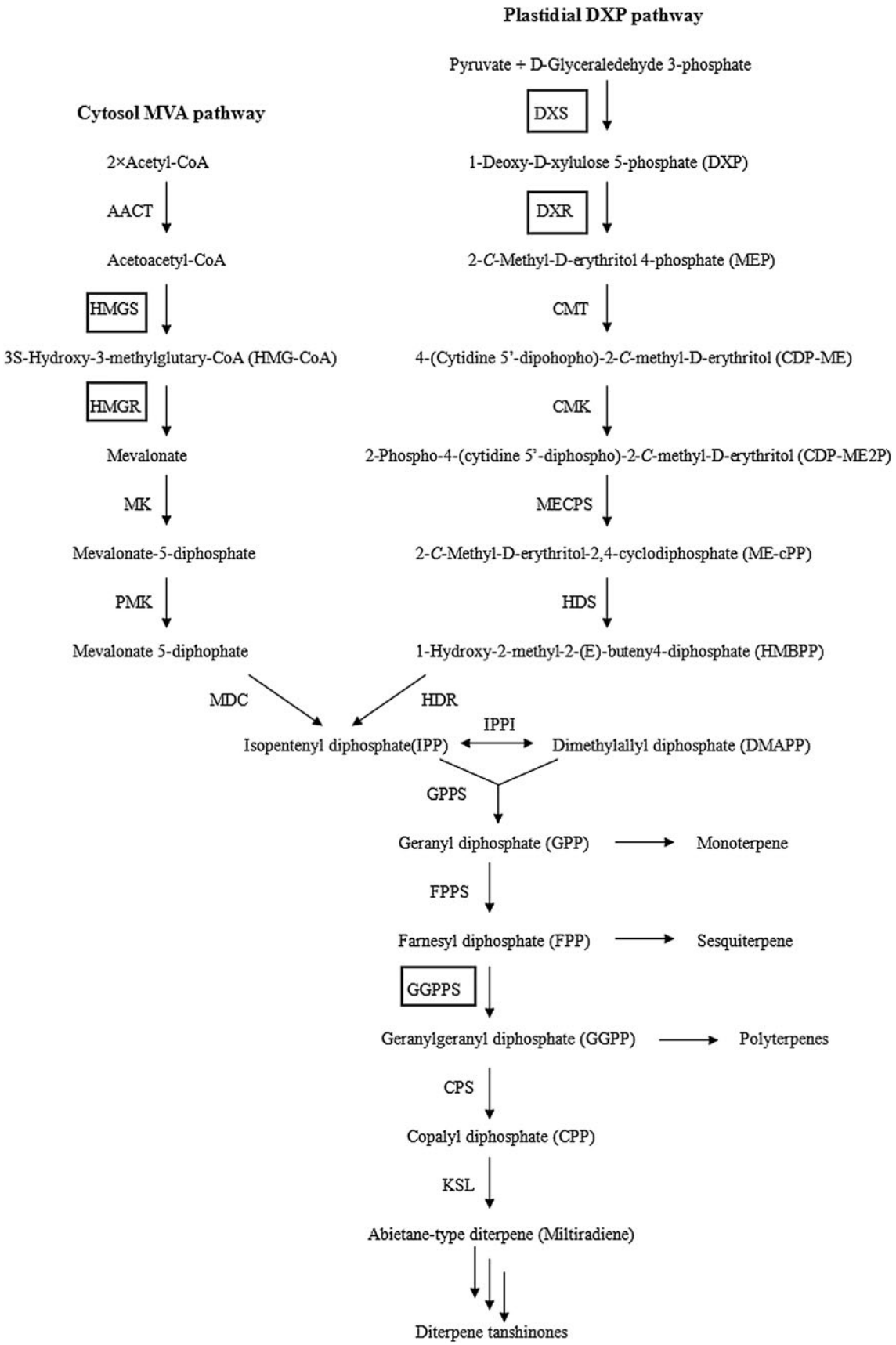
L. Zhang
Department of Pharmacy, Shaoxing People's Hospital, 312000 Shaoxing, People's Republic of China

X. Yan · J. Wang · S. Li · P. Liao · G. Kai (✉)
Laboratory of Plant Biotechnology, College of Life and Environment Sciences, Shanghai Normal University, 200234 Shanghai, People's Republic of China
e-mail: gykai@shnu.edu.cn

Introduction

Danshen, the dried root of *Salvia miltiorrhiza*, has been widely used in China and many other countries in the world for the treatment of cardiovascular and cerebrovascular diseases (Zhou et al. 2005). Being one of the two major active constituents of DanShen, tanshinones, such as tanshinone I, tanshinone IIA (TanIIA), tanshinone IIB and cryptotanshinone, etc. showed a variety of biological activities, including anti-ischemic, antioxidant, and anti-inflammation properties (Hu et al. 2005). For example, it is proved that tanshinone I has the anticancer effect on breast cancer cells (Nizamutdinova et al. 2008) and tanshinone IIA has been regarded as an effective anticancer and antioxidant (Li et al. 2008). Recent study also proved that cryptotanshinone showed neuroprotective effects by protecting primary cortical neurons from glutamate-induced neurotoxicity (Zhang et al. 2009). Therefore, it is necessary to improve the production of tanshinones by biotechnological method to meet the rapidly increasing clinical need.

Tanshinones, as a group of diterpenoids, are essentially biosynthesized from two C5 precursors: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Zhang et al. 2007). In higher plants, there are two different pathways for the synthesis of IPP (Scheme 1): the deoxyxylulose phosphate (DXP) pathway


Plastidial DXP pathway
Pyruvate + D-Glyceraldehyde 3-phosphate
DXS
Cytosol MVA pathway
2×Acetyl-CoA
1-Deoxy-D-xylulose 5-phosphate (DXP)
AACT
DXR
Acetoacetyl-CoA
2-C-Methyl-D-erythritol 4-phosphate (MEP)
HMGS
CMT
3S-Hydroxy-3-methylglutary-CoA (HMG-CoA)
4-(Cytidine 5'-dipohopho)-2-C-methyl-D-erythritol (CDP-ME)
HMGR
CMK
Mevalonate
2-Phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME2P)
MK
MECPS
Mevalonate-5-diphosphate
2-C-Methyl-D-erythritol-2,4-cyclodiphosphate (ME-cPP)
PMK
HDS
Mevalonate 5-diphophate
1-Hydroxy-2-methyl-2-(E)-buteny4-diphosphate (HMBPP)
MDC
HDR
IPPI
Isopentenyl diphosphate(IPP)
Dimethylallyl diphosphate (DMAPP)
GPPS
Geranyl diphosphate (GPP)
Monoterpene
FPPS
Farnesyl diphosphate (FPP)
Sesquiterpene
GGPPS
Geranylgeranyl diphosphate (GGPP)
Polyterpenes
CPS
Copalyl diphosphate (CPP)
KSL
Abietane-type diterpene (Miltiradiene)
Diterpene tanshinones

◀ **Scheme 1** Biosynthesis of tanshinon via the MVA pathway and the DXP pathway. The genes, encoding DXS, DXR, HMGS, HMGR and GGPPS of *Salvia miltiorrhiza* were cloned by our laboratory and are marked with *boxes*. *AACT* acetoacetyl-CoA thiolase, *CMK, 4-(cytidine 5′-diphospho)-2-cmethylerythritol kinase*, *CMT* 2-cmethylerythritol 4-phosphate cytidyl transferase, *CPS* copalyl diphosphate synthase, *DXR* deoxyxylulose 5-phosphate reductoisomerase, *DXS* deoxyxylulose 5-phosphate synthase, *FPPS* farnesyl diphosphate synthase, *GGPPS* geranylgeranyl diphosphate synthase, *GPPS* geranyl diphosphate synthase, *HDR* 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, *HDS* hydroxymethylbutenyl 4-diphosphate synthase, *HMGR* 3-hydroxy-3-methylglutaryl-CoA reductase, *HMGS* 3-hydroxy-3-methylglutaryl-CoA synthase, *IPPI* peppermint isopentenyl diphosphate isomerase, *KSL* kaurene synthase-like, *MDC* mevalonate 5-diphosphate decarboxylase, *MECPS* 2-C-methylerythritol 2,4-cyclodiphosphate synthase, *MK* mevalonate kinase, *PMK* phosphomevalonate kinase

in the plastids and the mevalonic acid (MVA) pathway in the cytosol (Lange et al. 2000; Eisenreich et al. 2001; Yan et al. 2009; Liao et al. 2009). There is some sort of crosstalk between the above two pathways to provide basic precursor for tanshinone biosynthesis (Ge and Wu 2005a, b).

The 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS, EC 2.3.3.10, formerly EC 4.1.3.5) catalyses the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA in MVA pathway (Nagegowda et al. 2004; Kai et al. 2006). The HMG-CoA is then converted by HMG-CoA reductase (HMGR) to yield MVA which is subsequently converted to IPP, the universal precursor for the synthesis of isoprenoids, including tanshinones. Being a rate-limiting enzyme in MVA pathway, HMGR has been well documented and much is known about plant HMGR which is regulated by light, growth regulators, wounding and treatment with pathogen or elicitors (Alex and Bach 2000; Jiang et al. 2006; Kai et al. 2006; Liao et al. 2009), whereas limited information concerning HMGS genes is available. Recently, some plant HMGS genes have been isolated from plant species, such as *Arabidopsis thaliana* (Montamat et al. 1995), *Pinus sylvestris* (Wegener et al. 1997), *Brassica juncea* (Alex and Bach 2000), *Hevea brasiliensis* (Suwanmanee et al. 2002) and *Taxus* × *media* (Kai et al. 2006). However, not much is known about gene encoding HMGS in *S. miltiorrhiza* or their expression regulation mechanism.

As far as we know, some chemical elicitors, such as methyl jasmonate (MJ) have positive effect on accumulation of tanshinones (Ge and Wu 2005a; Wang et al. 2007; Yan et al. 2005). However, until now, there is limited information about the mRNA expression profile of *SmHMGS* either in different plant tissues or under various kinds of elicitor treatments. Several genes, such as *HMGR*, *DXR* and *GGPPS* involved in tanshinones biosynthetic pathway from *S. miltiorrhiza* have been isolated by our laboratory recently (Liao et al. 2009; Yan et al. 2009; Kai et al. 2010). It is also meaningful to explore expression profiles of *SmHMGS* and compare it with the above isolated genes involved in tanshinones biosynthesis.

In this paper, we report for the first time the cloning, characterization of the full-length putative *HMGS* cDNA of *S. miltiorrhiza* (designated as *SmHMGS*). The expression profiles of the putative *SmHMGS* in various tissues and under the induction by salicylic acid (SA) and MJ were also investigated, which will be useful to know more about the role of Sm*HMGS* in tanshinones biosynthesis and regulate this important step at the level of molecular genetics in the future.

Materials and methods

Materials

Salvia miltiorrhiza plants, collected from Henan province of China, were cultured in pots in the greenhouse of our laboratory under 25°C with 16-h light period (white fluorescent tubes: irradiance of 350 μmol m^{-2} s^{-1}) and relative air humidity of 50% (Yan et al. 2009; Liao et al. 2009). The pMD-18T vector and one-step RNA PCR Kit were purchased from TaKaRa Biotechnology Co., Ltd. Primers' synthesis and DNA sequencing were performed by Shanghai Sangon Biotechnological Company, China. All the other chemicals used were of analytical grade.

Plant treatment and total RNA isolation

RNA of the leaves from *S. miltiorrhiza* plants was extracted using the method reported previously (Yan et al. 2009; Liao et al. 2009; Kai et al. 2010). 4-week-old seedlings of *S. miltiorrhiza* were spayed with the solution of 50 μM MJ and 10 mg/L SA, respectively, with water as control for each treatment. MJ was first dissolved in a small volume of DMSO, and then diluted with distilled water to form final concentration 50 μM. SA (Salicylic acid, Shanghai Zhanyun Chemical Co., Ltd) was dissolved in distilled water to form the SA solution. 0.5 g leaves of seedlings treated with SA and MJ was harvested, respectively, at 0, 6, 12, 24, 48, 72, 96 h. The quality and concentration of the extracted RNA were checked and stored as described before (Kai et al. 2006).

Cloning of the putative *SmHMGS* full-length cDNA by RACE

Total RNA was used to synthesize the first-strand cDNA (5′-ready cDNA) in 5′-RACE, which was performed according to the manual of the SMART™ RACE cDNA

Amplification Kit (Clontech Laboratories Inc., USA) using the 5′-RACE CDS Primer (5′-$(T)_{25}N_{-1}N$-3′) provided by the kit. The complementary reverse degenerate primer R2 (5′-TGTACAAG(C/T)TT(G/A)TTGTA(A/T)GGAGA-3′) was designed and synthesized according to the conserved region of several plant *HMGSs* (Montamat et al. 1995; Wegener et al. 1997; Alex and Bach 2000; Suwanmanee et al. 2002; Kai et al. 2006) that had been reported. The 5′-RACE-PCR was carried out using primers R2 as the reverse primer and the universal primer A mix (UPM, long: 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′; short: 5′-CTAATACGACTCACTATAGGGC-3′) as the forward primer provided by the kit under the following condition: the template (the 5′-ready cDNA) was denatured at 94°C for 2 min followed by 35 cycles of amplification (94°C for 45 s, 58°C for 60 s and 72°C for 90 s) and by 10 min at 72°C. The PCR product was purified and cloned into the pMD-18T vector followed by sequencing.

The first-strand cDNA (3′-ready cDNA) using SMART™ RACE cDNA Amplification Kit (Clontech Laboratories Inc., USA) with the 3′-RACE CDS Primer A (5′-AAGCAGTGGTATCAACGCAGAGTAC $(T)_{30}N_{-1}N$-3′) provided by the kit. Base on 5′-RACE product, one specific primer F2 (5′-CGACCTTGCCAGTGAATATCCAG-3′) was synthesized as the forward primer, and the universal primer A mix (UPM) was used as the reverse primer to perform the 3′-RACE. Polymerase chain reaction (PCR) was conducted in a total volume of 50 μl containing 2 μl cDNA, 20 μmol of F2, 20 μmol of UPM, 10 μmol dNTPs, 1× Ex PCR buffer and 5U Ex *Taq* polymerase, following the protocol: the cDNA was denatured at 94°C for 3 min followed by 35 cycles of amplification (94°C for 30 s, 58°C for 30 s, 72°C for 60 s) and by 10 min at 72°C. The amplified product was purified and cloned into pMD-18T vector, and transformed into *E. coli* DH5α. Based on the color reaction using Xgal–IPTG System and PCR identification; the positive clones were picked out and sequenced by ABI 3730 Sequencer (Perkin–Elmer, USA).

After aligning and assembling the sequences of the 5′-RACE and 3′-RACE products, the full-length cDNA sequence of the putative *SmHMGS* gene was deduced, and subsequently amplified via PCR using a pair of primers F1 (5′-ACACCATTCAGCCACCCATTCTC-3′) and R1 (5′-CGATAAAGAAACTACAGATAAT-3′) with 3′-RACE-ready cDNA as template under the following condition: 5 min at 94°C followed by 35 cycles of amplification (45 s at 94°C, 60 s at 58°C, 100 s at 72°C) and by 10 min at 72°C. The PCR product was purified and cloned into the pMD-18T vector followed by sequencing. To avoid PCR errors, the PCR amplification and sequencing of the full-length cDNA of the putative *SmHMGS* was repeated for three times.

Bioinformatics analysis

Several websites and bioinformatics softwares were used for analyses of the putative *SmHMGS*. The nucleotide sequence, deduced amino acid sequence and open reading frame (ORF) were analyzed, and the sequence comparison was conducted through database search using BLAST program (NCBI, National Center for Biotechnology Services, http://www.ncbi.nlm.nih.gov). The putative *SmHMGS* and other *HMGSs* retrieved from GenBank were aligned with CLUSTAL W using default parameters. A phylogenetic tree was constructed using MEGA 2 (Kumar et al. 2001) combined with CLUSTAL W alignments.

Expression profile analysis

To investigate the expression pattern of the putative *SmHMGS* in different tissues (roots, stems and leaves) as well as its expression under elicitor treatments, semi-quantitative one-step reverse transcriptase (RT)-PCR was carried out according to the manufacturer's instruction (Takara, Japan). Aliquots of total RNA (0.5 μg) extracted from roots, stems and leaves of *S. miltiorrhiza*, were used as templates in one-step RT-PCR reaction with the forward primer SmHMGSKF (5′-*AGATCT*ATGGCCAAGAATGTCGGGATCCT-3′) and reverse primer SmHMGSKR (5′-*GGTCACC*TCAGTGGCCGTTCGCAACTGTGC-3′) specific to the encoding sequence of the putative *SmHMGS* using one-step RNA PCR kit. RT-PCR reactions for the house-keeping gene (*18S rRNA* gene) using the specific primers *18S*F (5′-CCAGGTCCAGACATAGTAAG-3′) and *18S*R (5′-GTACAAAGGGCAGGGACGTA-3′) designed according to the conserved regions of plant *18S rRNA* genes were performed as an internal control to estimate whether equal amounts of RNA among samples were used in semi-quantitative RT-PCR. Amplifications were performed under the following condition: 50°C for 60 min and 94°C for 2 min followed by 28 cycles of amplification (94°C for 45 s, 58°C for 60 s and 72°C for 1 min 30 s).

Results and discussion

Cloning of the full-length cDNA of the putative *SmHMGS*

According to the sequences of the conserved regions of *HMGSs* from other plant species, the degenerate primers R was designed and used to amplify about 900-bp 5′-end product of the putative *SmHMGS* with UPM. And a 5′-untranslated region (UTR) of 117 bp was found upstream from the first ATG codon in the amplified

sequence. Then, this 5′-RACE fragment was used to design a gene-specific primer for 3′-RACE of the putative *SmHMGS*. By 3′-RACE, about 950 bp PCR products was obtained in which a 3′-UTR of 142 bp was found downstream from the stop codon. Based on the sequences of the 5′ and 3′-RACE products, the full-length cDNA fragment was deduced and amplified by PCR using a pair of primers F1 and R1. The cloned full-length cDNA of the putative *SmHMGS* was 1,655 bp with 5′ and 3′-untranslated regions (UTR) and a poly A tail and contained a 1,383 bp ORF encoding a protein of 460 amino acids (Fig. 1). Using the software of Computer pI/Mw Tool at http://www.expasy.org, the calculated isoelectric point (*pI*) and molecular weight of the deduced SmHMGS were predicted to be 6.19 and 50.7 kDa, respectively.

Sequence analysis of the putative SmHMGS

Sequence comparison by performing Blast P Search (http://www.ncbi.nih.gov) showed that SmHMGS had higher homology with other HMGSs, such as *Hevea brasiliensis* HMGS2 (Sirinupong and Suwanmanee 2005), *Camptotheca acuminate* HMGS, *Medicago truncatula* HMGS, *Arabidopsis thaliana* HMGS (Montamat et al. 1995) and *Brassica juncea* HMGS2 (Alex and Bach 2000; Nagegowda et al. 2004) from plant species indicating that SmHMGS belonged to plant HMGS superfamily. On the protein level, SmHMGS was 83, 83, 80, 80 and 79% identical to MtHMGS, HbHMGS2, BjHMGS and AtHMGS (Fig. 2). From the results of multiple alignment of SmHMGS with other HMGSs (Thompson et al. 1994, 1997), it is also revealed that SmHGMS contained the active site peptide (amino acids G^{107}–A^{125}) for HMGS activity, which is essential to the physiological and biochemical function of HMGS, and showed conservation across other species (Fig. 2, marked in black box) (Kai et al. 2006).

With the CLUSTAL W and MEGA 2 program, a phylogenetic tree was constructed based on the deduced amino acid sequences of SmHMGS and other HMGSs from different organisms, including mammalians, fungi and plant to investigate the evolutionary relationships among different HMGSs. The results revealed that HMGSs were derived from a common ancestor and evolved into four groups and SmHMGS belongs to the plant group and has close relationship with HMGS from *H. brasiliensis* (Fig. 3).

Expression analysis of the putative *SmHMGS* in different tissues of *S. miltiorrhiza*

The roots and stems of *S. miltiorrhiza* plants are usually used as the major parts for traditional Chinese medicine, as tanshinone content in the parts is much higher than other parts, such as leaves (Li et al. 2001). Hence, it is also very interesting to explore the expression pattern of the putative *SmHMGS* in these parts. Total RNA was isolated from different tissues, including leaves, roots and stems, and subjected to one-step RT-PCR analysis using the primers SmHMGSKF and SmHMGSKR. The results showed that the putative *SmHMGS* was differentially expressed in various tissues and was found to be highest in leaf followed by stem and root (Fig. 4), which was similar to *SmDXR* and *SmGGPPS* expression (Yan et al. 2009; Kai et al. 2010), whereas was contrary to *SmHMGR*. *SmHMGR* showed the highest expression in roots, while weak expression was detected in leafs (Liao et al. 2009). Although these four genes (*SmHMGS, SmDXR, SmGGPPS* and *SmHMGR*) showed the highest expression level either in leaves or roots, they are still all constitutively expressed gene.

Expression analysis of the putative *SmHMGS* under induction of SA and MeJA elicitor

Salicylic acid is a phenolic phytohormone and a key signal molecule with plays role in plant responses to different abiotic stresses, such as UV radiation and ozone exposure (Rao and Davis 1999; Senaratna et al. 2000; Huang et al. 2008; Lu et al. 2009). It also plays an important role in plant growth and development, photosynthesis, transpiration, ion uptake and transport (Hayat and Ahmad 2007).

The putative *SmHMGS* expression under the SA treatment was first analyzed. The results showed that the expression of the putative *SmHMGS* in leaves varied along with the treatment time (Fig. 5a). The expression of the putative *SmHMGS* was strongly induced by SA, reaching the first peak 12 h after treatment and considerably decreased 24 h, and later it is significantly increased 48 h followed by gradual decrease after 72 h, but still can be detected at 96 h. The results were not similar to BjHMGS expression tendency upon SA treatment (Alex and Bach 2000), which may be due to difference of species and concentrations of elicitor. Our results revealed that the putative *SmHMGS* was induced by SA at least at transcriptional level. In the comparison of *SmHMGR* (Liao et al. 2009), the expression of the putative *SmHMGS* is much higher than *SmHMGR* by comparing the expression of 18s rRNA. What is impressive is that the four genes including *SmHMGS*, *SmHMGR*, *SmDXR* and *SmGGPPS* showed the highest expression level at 48 h after SA induction treatment (Liao et al. 2009; Yan et al. 2009; Kai et al. 2010).

Methyl jasmonate was also proved as a signal molecule of altered gene expression in various plant responses to biotic and abiotic stresses and also as one kind of elicitor triggering the pathway of secondary metabolism in plant

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1                                                                   acaccattcagccacccattctctctc
28 tacctctctgattcctgagctctgcgactactgtgtgtgctgtttaactgatccgctatttccttcatttcgtcaactcaagaagtcgag
118 atggccaagaatgtcgggatcctcgccatggaaatctacttccctcccacttgcatccagcaggaggtattggaagctcacgatggagca
    M  A  K  N  V  G  I  L  A  M  E  I  Y  F  P  P  T  C  I  Q  Q  E  V  L  E  A  H  D  G  A
208 agcaaagggaagtacacaattgggcttggccaagattgcatggcattttgttcggaggttgaagatgtcatttcgatgagcatgacagcg
    S  K  G  K  Y  T  I  G  L  G  Q  D  C  M  A  F  C  S  E  V  E  D  V  I  S  M  S  M  T  A
298 gttacttcgcttctagggaagtacaatgttgatccgaagcagattggacgtcttgaagttggaagtgagaccgtactcgacaagagcaaa
    V  T  S  L  L  G  K  Y  N  V  D  P  K  Q  I  G  R  L  E  V  G  S  E  T  V  L  D  K  S  K
388 tccattaagacatttctgatgccgatcttcgagaaatgtggcaatattgacatcgaaggtgttgactcaagcaatgcttgctatggtggg
    S  I  K  T  F  L  M  P  I  F  E  K  C  G  N  I  D  I  E  G  V  D  S  S  N  A  C  Y  G  G
478 actgcagcactatttaactgtgtcaattgggtggaaagtagttcttgggatggaagatatgggcttgttgtctgcacagacagtgcggta
    T  A  A  L  F  N  C  V  N  W  V  E  S  S  S  W  D  G  R  Y  G  L  V  V  C  T  D  S  A  V
568 tatgccgagggaccagctagacctactggtggggctgcagctattgccatgctaataggaccaaatgcacccattgcttttgaaagcaag
    Y  A  E  G  P  A  R  P  T  G  G  A  A  A  I  A  M  L  I  G  P  N  A  P  I  A  F  E  S  K
658 cttagggcgaatcacatggctcatgtttatgatttttacaagcccgaccttgccagtgaatatccagttgttgatggcaagctttctcag
    L  R  A  N  H  M  A  H  V  Y  D  F  Y  K  P  D  L  A  S  E  Y  P  V  V  D  G  K  L  S  Q
748 acttgttgccttacggcactggatgcttgttacaaaggcttctgccaaaagtttgagaagcaggagggcaagcagttctcgatcttggat
    T  C  C  L  T  A  L  D  A  C  Y  K  G  F  C  Q  K  F  E  K  Q  E  G  K  Q  F  S  I  L  D
838 gccgactactttgtatttcattctccatacaacaagcttgtacagaaaagcttctctagattgttgttcaatgacttttcgagaaatgcc
    A  D  Y  F  V  F  H  S  P  Y  N  K  L  V  Q  K  S  F  S  R  L  L  F  N  D  F  S  R  N  A
928 agctccatcgatgaggctgctaaagaaaagctggcaccattttcatcattaagcaacgaggaaagctaccaaagtcgtgatcttgagaag
    S  S  I  D  E  A  A  K  E  K  L  A  P  F  S  S  L  S  N  E  E  S  Y  Q  S  R  D  L  E  K
1018 gcatctcaacaagtcgctaaacctttctttgataccaaggtgcaaccatctactctcgtcccaaaacaagtgggaaacatgtatactgca
    A  S  Q  Q  V  A  K  P  F  F  D  T  K  V  Q  P  S  T  L  V  P  K  Q  V  G  N  M  Y  T  A
1108 tcgctctatgctgcatttgcttccctcatccacaacaagagtagcactttggctgggcagagggtcatattgttttcctacggcagtggt
    S  L  Y  A  A  F  A  S  L  I  H  N  K  S  S  T  L  A  G  Q  R  V  I  L  F  S  Y  G  S  G
1198 ctgtcagccatcatgttctctcttcgtttcactgagggagaacatcctttcagcctgtccaacattgcatctgttatgaatgtttcagag
    L  S  A  I  M  F  S  L  R  F  T  E  G  E  H  P  F  S  L  S  N  I  A  S  V  M  N  V  S  E
1288 aagttgaagtcaaggcacgagttcccaccagagaaattcgtcgaactgatgcagctcatggagcacagatatggaggcaaagacttcatc
    K  L  K  S  R  H  E  F  P  P  E  K  F  V  E  L  M  Q  L  M  E  H  R  Y  G  G  K  D  F  I
1378 acaagcaaggactgcagccttcttgcaccaggtacatactatctcactgaagtcgactcccaataccgaagattctatgccaagaaggcc
    T  S  K  D  C  S  L  L  A  P  G  T  Y  Y  L  T  E  V  D  S  Q  Y  R  R  F  Y  A  K  K  A
1468 attgcgaatggcacagttgcgaacggccac tgaagatgtgatatgccataaagttggctcttcttctgtctagttgattcagcacataga
    I  A  N  G  T  V  A  N  G  H  *
1558 aaaataatctaatctattttcattgtttgatacaagcatttgagttggtttgacagatgaattattatctgtagtttctttatcgaaaaa
1648 aaaaaaaa
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Fig. 1 The full-length cDNA sequence and deduced amino acid sequence o*f S. miltiorrhiza* 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase (the putative *SmHMGS*). The start codon (ATG) was *bolded* and the stop codon (TGA) was *underlined italically*. The conserved motifs were *underlined*

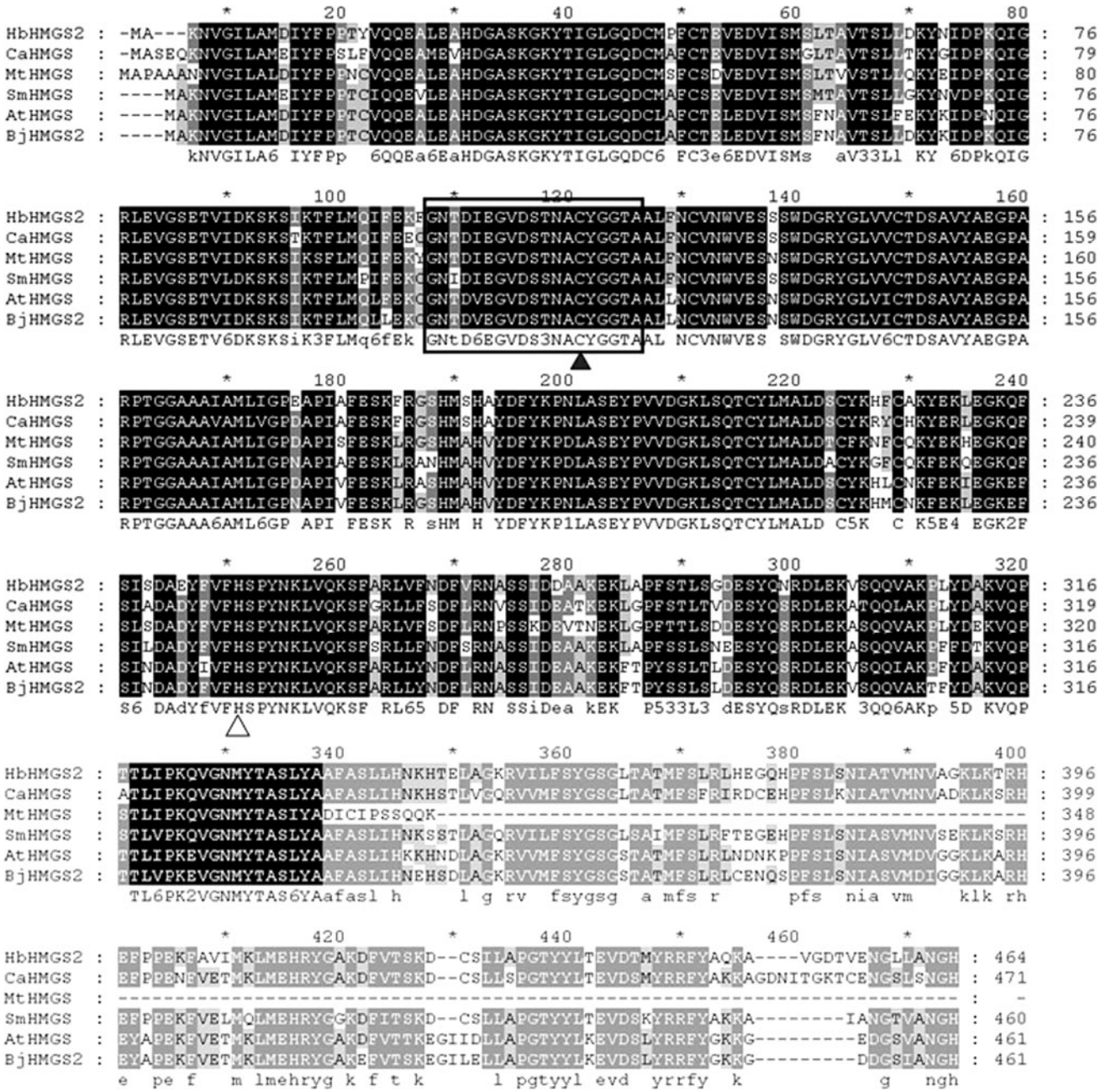

Fig. 2 Multiple alignment of SmHMGS with the following other plant HMGSs: *Hevea brasiliensis* HMGS2 (BAF98279.1); *Camptotheca acuminate* HMGS (ACD87446.1), *Arabidopsis thaliana* HMGS (NP_192919.1), *Brassica juncea* HMGS (AAG32922.1), *Medicago truncatula* HMGS (ABE73758.1). *Black boxes* indicate identical residues; *gray boxes* indicate identical residues for at least three of the sequences

cells (Creelman and Mullet 1997; Wasternack and Parthier 1997; Pauwels et al. 2008). Exogenously applied JA or MJ are capable of inducing the synthesis of defense proteins and secondary defense metabolites in a wide range of plant species (Huang et al. 2008). MJ treatment can effectively induce tanshinones biosynthesis in *Salvia miltiorrhiza* hairy root indicating that there was positive correlation with tanshinones biosynthesis upon MJ treatment (Wang et al. 2007). The *HMGR* expression of *S. miltiorrhiza* was induced under the treatment of MJ in earlier study from our laboratory (Liao et al. 2009). Hence, it is interesting to uncover the expression profiles of the putative *SmHMGS* gene involved in tanshinone biosynthesis under MJ treatments, which will be meaningful to compare whether the expression profile of the putative *SmHMGS* is identical with *HMGR* of *S. miltiorrhiza.*

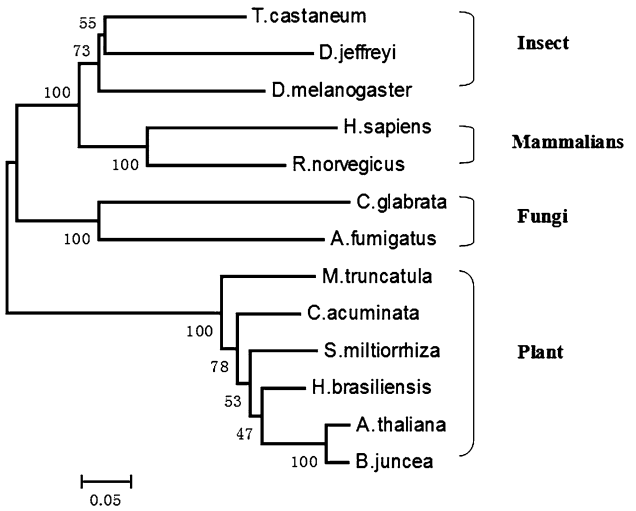

Fig. 3 Phylogenetic tree analysis of the putative SmHMGS and other HMGSs. *T. castaneum* (XP_973437), *D. jeffreyi* (AAF89580), *D. melanogaster* (NP_524711), *H. sapiens* (CAA58593), *R. norvegicus* (CAA36852), *C. glabrata* (XP_446972), *A. fumigatus* (XP_747519), *M. truncatula* (ABE73758), *C. acuminata* (ACD87446), *H. brasiliensis* (BAF98279), *A. thaliana* (NP_192919), *B. juncea* (AAG32922)

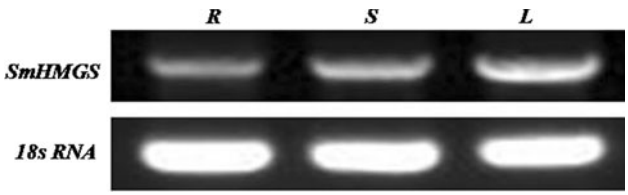

Fig. 4 Expression pattern analysis of the putative *SmHMGS* in different tissues of *S. miltiorrhiza*. Total RNA (0.5 μg/sample) from roots, stems and leaves, respectively, was subjected to one-step RT-PCR amplification with 28 cycles (*upper panel*). *18S rRNA* gene was used as the control to show the normalization of the templates in PCR reactions (*lower panel*). The experiment was performed in triplicate, representative results are shown. *R*, *S* and *L* indicate roots, stems and leaves, respectively

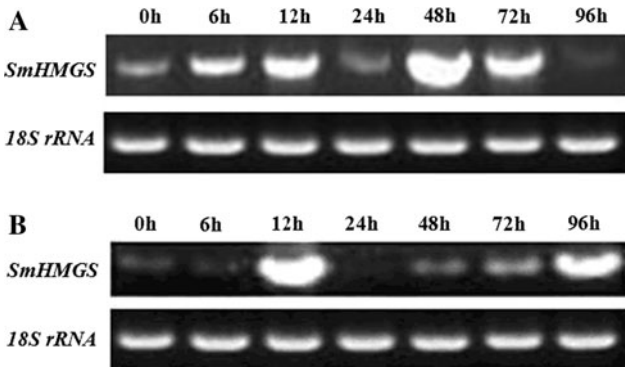

Fig.. 5 Expression profile of the putative *SmHMGS* upon 10 mg/L SA (**a**) and 50 μmol MJ (**b**) induction treatment. RT-PCR analysis was performed using total RNA isolated from treated tissues at different time points (0, 6, 12, 24, 48, 72 and 96 h). The *18S rRNA* gene was used as the control. The experiment was performed in triplicate and representative results are shown

The results showed that the putative *SmHMGS* expression was effectively induced by MJ (Fig. 5b). The putative *SmHMGS* transcript level reached the highest level after 12 h of the treatment and then gradually decreased, but reached the second peak at 96 h, which was different from BjHMGS expression profile upon MJ treatment (Alex and Bach 2000). Our results revealed that the putative *SmHMGS* was MJ elicitor-responsive and could be effectively elicited at least at transcription level, coinciding with their induction effects for improving the tanshinones production as reported previously (Ge and Wu 2005b; Wang et al. 2007).

In addition, the expression of *SmHMGR* can also be induced by MJ, but with weaker level in the leaf than the putative *SmHMGS*. *SmHMGR* expression reached the highest level after 48 h of the MJ treatment in roots and stems, and after 96 h of the MJ treatment in leaf (Liao et al. 2009). However, the expression of *SmDXR* and *SmGGPPS* was inhibited by MJ treatment in leaf (Yan et al. 2009; Kai et al. 2010), which was different from the putative *SmHMGS*.

In this paper, we have successfully cloned and characterized a new putative gene encoding HMGS involved in the biosynthesis of tanshinones from *S. miltiorrhiza* and multiple alignments showed that the deduced SmHMGS had high identity to other plant HMGSs, and contained all conserved substrate-binding motifs of HMGSs. Detailed expression analysis of the putative *SmHMGS* were performed and the expression profiles revealed in different tissues and under different elicitor treatments, such as SA and MJ were compared for the first time. The characterization and expression profile of the putative *SmHMGS* is instrumental to elucidate the tanshinone biosynthesis at molecular level and the regulatory mechanisms involved in the MVA pathway.

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