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Cloning and characterization of a novel 3-hydroxy-3-methylglutaryl coenzyme A reductase gene from *Salvia miltiorrhiza* involved in diterpenoid tanshinone accumulation

Zhubo Dai[a], Guanghong Cui[a], Shu-Feng Zhou[b,*], Xianan Zhang[a], Luqi Huang[a,**]

[a] Institute of Chinese Materia Medica, Chinese Academy of Chinese Medical Science, Beijing 100700, China
[b] School of Health Sciences and Health Innovations and Research Institute (HIRi), RMIT University, Plenty Road, Bundoora, Victoria 3083, Australia

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ABSTRACT

The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the conversion of HMG-CoA to mevalonate (MVA), which is a rate-limiting step in the isoprenoid biosynthesis via the MVA pathway. In this study, the full-length cDNA encoding HMGR (designated as SmHMGR2, GenBank accession no. FJ747636) was isolated from *Salvia miltiorrhiza* by rapid amplification of cDNA ends (RACE). The cloned gene was then transformed into the hairy root of *S. miltiorrhiza*, and the enzyme activity and production of diterpenoid tanshinones and squalene were monitored. The full-length cDNA of *SmHMGR2* comprises 1959 bp, with a 1653-bp open reading frame encoding a 550-amino-acid protein. Molecular modeling showed that SmHMGR2 is a new HMGR with a spatial structure similar to other plant HMGRs. SmHMGR2 contains two HMG-CoA-binding motifs and two NADP(H)-binding motifs. The SmHMGR2 catalytic domain can form a homodimer. The deduced protein has an isoelectric point of 6.28 and a calculated molecular weight of approximately 58.67 kDa. Sequence comparison analysis showed that SmHMGR2 had the highest homology to HMGR from *Atractylodes lancea*. As expected, a phylogenetic tree analysis indicates that SmHMGR2 belongs to plant HMGR group. Tissue expression pattern analysis shows that SmHMGR2 is strongly expressed in the leaves, stem, and roots. Functional complementation of SmHMGR2 in HMGR-deficient mutant yeast JRY2394 demonstrates that SmHMGR2 mediates the MVA biosynthesis in yeasts. Overexpression of SmHMGR2 increased enzyme activity and enhanced the production of tanshinones and squalene in cultured hairy roots of *S. miltiorrhiza*. Our DNA gel blot analysis has confirmed the presence and integration of the associated *SmHMGR2* gene. SmHMGR2 is a novel and important enzyme involved in the biosynthesis of diterpenoid tanshinones in *S. miltiorrhiza*.

Introduction

Tanshinones are abietane-type norditerpenoid quinones found in a commonly used Chinese medicinal herb *Salvia miltiorrhiza* Bunge., a member of the Lamiaceae family (Zhou et al., 2005; Yuan et al., 2009). This group of diterpenoids mainly includes dihydrotanshinone I, tanshinone I, tanshinone IIA and cryptotanshinone (Fig. 1) (Zhou et al., 2005). These compounds have exhibited diverse pharmacological activities, including antibacterial, antioxidant, antiinflammatory, cytotoxic, neuroprotective, cardioprotective, antiplatelet, and antitumor effects (Zhou et al., 2005; Yuan et al., 2009). *S. miltiorrhiza* is mainly used in the clinical treatment of coronary heart diseases and stroke in China and other Asian countries. However, the content of tanshinones in *S. miltiorrhiza* is low. Biotechnology strategies have been applied to efficiently increase the yield of tanshinones from the cultured hairy roots (Zhang et al., 2004; Wu et al., 2009; Yuan et al., 2009) or via cell suspension cultures (Chen et al., 1997), and by transgenic *S. miltiorrhiza* (Lee et al., 2008). Overexpression of important biosynthesis-related genes in combination with traditional hairy root culturing is considered a practical means to stimulate secondary metabolite production.

Diterpenoids are generated from geranylgeranyl diphosphate, which is synthesized from isopentenyl diphosphate and its allylic isomer dimethylallyldiphosphate via two distinct pathways, the classic mevalonate (MVA) pathway in the cytosol and the non-MVA, 1-deoxy-D-xylulose 5-phosphate (DXP) route occurring in

Abbreviations: ANOVA, analysis of variance; DXP, 1-deoxy-D-xylulose 5-phosphate; GFP, green fluorescence protein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MeJA, methyl-jasmonate; MS, Murashige and Skoog; MVA, mevalonic acid; ORF, open reading frame; RACE, rapid amplification of cDNA end; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; UTR, untranslated region.

* Corresponding author. Tel.: +61 3 9925 7794; fax: +61 3 9925 7178.
** Corresponding author. Tel.: +86 10 6401 4411; fax: +86 10 6401 3996.
E-mail addresses: shufeng.zhou@rmit.edu.au (S.-F. Zhou), huangluqi@263.net (L. Huang).

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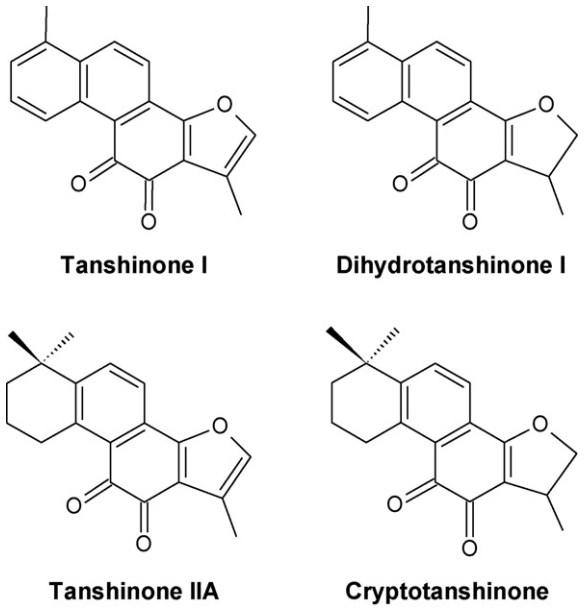

Fig. 1. Chemical structures of tanshinone I, dihydrotanshinone I, tanshinone IIA, and cryptotanshinone.

cellular plastids (Buhaescu and Izzedine, 2007; Hunter, 2007). Generally, the main MVA derived isoprenoid end-products are certain sesquiterpenes, sterols and the side chain of mitochondrial ubiquinones, whereas monoterpenes, certain sesquiterpenes and photosynthesis-related isoprenoids, are derived from the DXP pathway (Lichtenthaler, 1999). However, there is a crosstalk between the two pathways for isoprenoid biosynthesis in some plants such as *Arabidopsis thaliana* (Laule et al., 2003).

The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the conversion of HMG-CoA to MVA, which is a rate-limiting enzyme in isoprenoid biosynthesis via the MVA pathway (Buhaescu and Izzedine, 2007; Hunter, 2007). HMGR is known to be regulated by a number of external and internal factors. Recently, we have successfully applied a genomics approach to exploring the pathways of tanshinone biosynthesis in *S. miltiorrhiza* and found that labdadienyl/copalyl diphosphate synthase and kaurene synthase-like may be involved in tanshinone biosynthesis (Gao et al., 2009). Mevinolin, an HMGR inhibitor, reduced tanshinone accumulation in the hairy roots of *S. miltiorrhiza*, while the activity of HMGR was stimulated by Ag^+ exposure (Ge and Wu, 2005). In this work, we cloned a full-length cDNA of *SmHMGR2* from the hairy roots of *S. miltiorrhiza*. Its role in isoprenoid biosynthesis was identified by the method of functional complementation for the growth of the yeast strain JRY2394 by *SmHMGR2*. To further evaluate the contribution of *SmHMGR2* to diterpenoid tanshinone biosynthesis in *S. miltiorrhiza*, *SmHMGR2* was driven by the constitutive cauliflower mosaic virus 35S promoter, and transformed into the hairy roots of *S. miltiorrhiza*. The content of squalene and tanshinones was monitored in hairy roots of *S. miltiorrhiza*.

Materials and methods

Plant materials culture

The mature seeds of *Salvia miltiorrhiza* Bunge. were surface-sterilized by 0.1% mercuric chloride (Sigma–Aldrich, St. Louis, MO, USA) and cultured on solid, hormone-free MS basal medium (Murashige and Skoog, 1962). The MS medium contained 30 g/L sucrose and 8 g/L agar without ammonium nitrate for germination. Cultures were maintained at 25 °C under a 16 h light/8 h dark photoperiod with light provided by cool white fluorescent lamps at an intensity of 25 $\mu mol\, m^{-2}\, s^{-1}$.

DNA and RNA isolation

Roots, stems and leaves were collected from mature *S. miltiorrhiza*. The 20-d-old hairy roots were treated with MeJA for 0, 12, 24, 48, 72 and 96 h at a final concentration of 100 mM, and roots were harvested for RNA isolation. The hairy root lines were collected 30 d after inoculation. Total RNA was extracted from the tissues by Trizol method (Invitrogen, Carlsbad, CA, USA). Genomic DNA was isolated using the modified cetyl-trimethylammonium bromide method (Del Sal et al., 1989).

Cloning of SmHMGR2 full-length cDNA by rapid amplification of cDNA ends (RACE)

5′-RACE was performed according to the manual of the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA, USA). The 5′-RACE PCR was carried out using the 5′-RACE primer and universal primer (UPM, Universal Primer A Mix). The PCR product was purified and cloned into pMD19-T vectors followed by sequencing. After aligning and assembling the sequences, the full-length cDNA sequence of the *SmHMGR2* gene was deduced, and subsequently amplified by PCR using a pair of primers. The genome sequence of the *SmHMGR2* was confirmed by PCR with the genome DNA as a template.

Bioinformatics analysis

The nucleotide sequence, deduced amino acid sequence and open reading frame (ORF) were analyzed using DNAstar, and the sequence comparison was conducted through a database search using BLAST. SmHMGR2 and other HMGRs retrieved from GenBank were aligned using Clustal W. A phylogenetic tree was constructed using neighbor-joining method. Transmembrane domain was analyzed by TMHMM 2.0 and homology based structural modeling was accomplished by Swiss-Model (http://www.expasy.org/).

Construction of the Escherichia coli–Saccharomyces cerevisiae shuttle vector pRS406-SmHMGR2

The entire *SmHMGR2* cDNA was amplified by PCR using forward and reverse primers. The PCR product was digested with EcoRI and NotI, gel-purified, and ligated into the same restriction sites within the pRS406 vector (Sikorski and Hieter, 1989). Positive clones were confirmed by PCR and subsequent sequencing analysis for the presence of the *SmHMGR2* gene and used to be transformed into the *S. cerevisiae*.

Construction of the SmHMGR2 binary expression vector pH7WG2D-SmHMGR2

The *SmHMGR2* gene was PCR-amplified and the resultant PCR products were purified with the DNA purification kit (Sangon, Shanghai, China). We sequentially subcloned the PCR product into a donor vector (pDONR221) and created an entry vector. The recombination reaction between the entry and destination vectors (pH7WG2D,1) was performed in LR Clonase™ II enzyme mix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The *E. coli* DH5α was transformed with the product of LR reaction using the heat shock transformation method. The destination vector pH7WG2D,1 with the ccdB (control of cell death) gene knockout was used as the control vector (pH7WG2D-Control) (Coleman et al., 1991).