Plant Physiology and Biochemistry 70 (2013) 21–32

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Cloning, molecular characterization and functional analysis of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) gene for diterpenoid tanshinone biosynthesis in *Salvia miltiorrhiza* Bge. f. alba

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ARTICLE INFO

Article history:
Received 20 March 2013
Accepted 13 May 2013
Available online 22 May 2013

Keywords:
Salvia miltiorrhiza Bge. f. alba
*SmHDR*1
Expression profiling
Tanshinones
Hairy root

ABSTRACT

The enzyme 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) is a terminal-acting enzyme in the plastid MEP pathway, which produce isoprenoid precursors. The full-length cDNA of *HDR*, designated *SmHDR*1 (Genbank Accession No. JX516088), was isolated for the first time from *Salvia miltiorrhiza* Bge. f. alba. *SmHDR*1 contains a 1389-bp open reading frame encoding 463 amino acids. The deduced SmHDR1 protein, which shows high identity to HDRs of other plant species, is predicted to possess a chloroplast transit peptide at the N-terminus and four conserved cysteine residues. Transcription pattern analysis revealed that *SmHDR*1 has high levels of transcription in leaves and low levels of transcription in roots and stems. The expression of *SmHDR*1 was induced by 0.1 mM methyl-jasmonate (MeJA) and salicylic acid (SA), but not by 0.1 mM abscisic acid (ABA), in the hairy roots of *S. miltiorrhiza* Bge. f. alba. Complementation of SmHDR1 in the *Escherichia coli HDR* mutant MG1655 *ara* < > *ispH* demonstrated the function of this enzyme. A functional color assay in *E. coli* showed that SmHDR1 accelerates the biosynthesis of β-carotene, indicating that *SmHDR*1 encodes a functional protein. Overexpression of *SmHDR*1 enhanced the production of tanshinones in cultured hairy roots of *S. miltiorrhiza* Bge. f. alba. These results indicate that SmHDR1 is a novel and important enzyme involved in the biosynthesis of diterpenoid tanshinones in *S. miltiorrhiza* Bge. f. alba.

1. Introduction

Salvia miltiorrhiza Bunge, also known in China as Dan Shen, is a well-known and very important traditional Chinese medicinal herb that is used for the treatment of various cardiovascular diseases, menstrual disorders, blood circulation disturbances and inflammation [1]. As its Chinese name suggests, this herb is characterized by the abundance of red pigment, which is formed by a group of lipophilic diterpene quinone derivatives generically known as tanshinones, e.g., tanshinone I, IIA and IIB, isotanshinone I and II and cryptotanshinone. Tanshinones, the major bioactive constituents of Dan Shen, have a number of pharmacological activities including antibacterial, antioxidant and antineoplastic activity [1]. Tanshinone IIA is widely used to treat various cardiovascular and cerebrovascular diseases and is regarded as an effective anticancer agent and an antioxidant [2]. The diterpenoid tanshinones are considered to be unique to *S. miltiorrhiza* and have received the most attention. Terpenoids (isoprenoids) represent the largest group of natural products with diverse molecular structures. Terpenoids play multiple roles in the growth and development of

Abbreviations: CTAB, cetyltrimethylammonium bromide; DMAPP, dimethylallyl diphosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GGPP, geranylgeranyl diphosphate; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HPLC, high performance liquid chromatography; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; MVA, mevalonic acid; MCA, metabolic control analysis; MeJA, methyl-jasmonate; PDC, pyruvate decarboxylase; PSY, phytoene synthase; SA, salicylic acid; ABA, abscisic acid.

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http://dx.doi.org/10.1016/j.plaphy.2013.05.010

higher plants. In addition, terpenoids have unique and valuable chemical properties and bioactivities and are used in a wide range of commercial products such as food flavorings, pharmaceuticals and cosmetics.

White-flowered *S. miltiorrhiza* Bge. f. alba, which is grown only in Shandong province in China, is a variety of *S. miltiorrhiza* Bunge. The morphological difference between *S. miltiorrhiza* Bge. f. alba and *S. miltiorrhiza* Bunge is the color of the flowers: *S. miltiorrhiza* Bunge has purple flowers, while *S. miltiorrhiza* Bge. f. alba has white flowers. In addition to this difference, *S. miltiorrhiza* Bge. f. alba also has unique pharmacological effects and is used for the treatment of thromboangiitis obliterans [3]. The content of caffeic acid-derived phenolic acids in *S. miltiorrhiza* Bge. f. alba is approximately two times higher than that in *S. miltiorrhiza* Bunge [4]. In addition, Qi et al. reported that the content of trace elements such as Fe, Mg and Mn in *S. miltiorrhiza* Bge. f. alba is higher in *S. miltiorrhiza* Bge. f. alba than in *S. miltiorrhiza* Bunge [3]. While the contents of tanshinones, the major bioactive constituents of Dan Shen, are similar in *S. miltiorrhiza* Bge. f. alba and in *S. miltiorrhiza* Bunge [5]. Jiao et al. [4] found that *S. miltiorrhiza* Bge. f. alba root preparations inhibit proliferation and induce apoptosis of human gastric cancer cells. *S. miltiorrhiza* Bge. f. alba also significantly increases cerebral blood flow, reduces neuronal apoptosis and promotes neuronal regeneration in rats with cerebral ischemia/reperfusion impairment [5]. These results indicate that the white-flowered *S. miltiorrhiza* Bge. f. alba has important pharmaceutical values. Recently, we successfully constructed a full-length cDNA library from white-flowered *S. miltiorrhiza* Bge. f. alba roots [3], performed cloning and functional analysis of the SmPDC gene [6] and successfully induced hairy roots from the leaves of *S. miltiorrhiza* Bge. f. alba using *Agrobacterium rhizogenes* ACCC10060 [7].

Tanshinones are in the labdane-related class of diterpenoids, whose biosynthesis is uniquely initiated by a sequential pair of cyclization reactions. The characteristic fused bicyclic hydrocarbon structure is formed from the universal diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP, **5**) in an initial carbon–carbon double-bond protonation-initiated reaction catalyzed by class II diterpene cyclases [8]. Geranylgeranyl diphosphate synthase (GGPPS) catalyzes the consecutive condensation of a dimethylallyl diphosphate (DMAPP) with three molecules of IPP to form 20-carbon geranylgeranyl diphosphate (GGPP), the key precursor for the biosynthesis of carotenoids and diterpenes such as tanshinones [9]. Despite their structural diversity, terpenoids are all derived from two common precursors, IPP (isopentenyl diphosphate) and DMAPP (dimethylallyl diphosphate) [1,10]. In higher plants, IPP and DMAPP are synthesized through two distinct pathways in separate cellular compartments, including the mevalonate (MVA [mevalonic acid]) pathway, which occurs in the cytosol, and the MEP (2-C-methyl-D-erythritol 4-phosphate) pathway, which occurs in the plastids [1]. The MEP pathway is responsible for the biosynthesis of monoterpenes, certain sesquiterpenes and photosynthesis-related disoprenoids. However, there is crosstalk between the two pathways for isoprenoid biosynthesis in some plants such as *Arabidopsis thaliana*. The enzyme 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) simultaneously synthesizes IPP and DMAPP in the last step of the pathway. HDR is key enzyme in the biosynthesis of precursors of isoprenoids [11]. Overexpression studies using genes from cyanobacteria (*Synechocystis*) and plants (*Adonis aestivalis*) showed that the HDR enzyme is a limiting factor for isoprenoid biosynthesis in *Escherichia coli* [12]. Studies on overexpression of tomato *HDR* cDNA in *Arabidopsis* plants led to the conclusion that plant HDR protein plays a key role in controlling the biosynthesis of plastid isoprenoids [13]. The Arabidopsis HDR is involved in the plastid nonmevalonate pathway of isoprenoid biosynthesis [14].

Recently, we successfully applied a genomics approach to explore 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 [8]. In the current study, we cloned a full-length cDNA of *SmHDR*1 from the roots of *S. miltiorrhiza* Bge. f .alba. The role of *SmHDR*1 in isoprenoid biosynthesis was identified using functional complementation to examine for the growth of the *E. coli HDR* mutant MG1655 *ara* < > *ispH* in response to complementation by *SmHDR*1. To further evaluate the contribution of SmHDR1 to diterpenoid tanshinone biosynthesis in *S. miltiorrhiza* Bge. f. alba, we produced a gene construct containing *SmHDR*1 driven by the constitutive cauliflower mosaic virus 35S promoter and transformed this construct into hairy roots cultures of *S. miltiorrhiza* Bge. f. alba. The tanshinone content was monitored in transgenic hairy roots of *S. miltiorrhiza* Bge. f. alba. This work will help further our understanding of important steps in the tanshinone biosynthesis pathway and may enable the metabolic engineering of *S. miltiorrhiza* Bge. f. alba to improve tanshinones production in the near future.

2. Results

2.1. Cloning the full-length cDNA of SmHDR1 from S. miltiorrhiza Bge. f. alba and bioinformatic analysis

The construction of cDNA expression libraries is an important technique in molecular biology. By sequencing clones of a cDNA library, researchers can analyze both known and novel genes. By randomly sequencing positive clones from a previously constructed cDNA library, we obtained the full-length sequence of *HDR* from *S. miltiorrhiza* Bge. f. alba. Using the ORF Finder program from NCBI, we found that the full-length cDNA of *HDR* from the cDNA library of *S. miltiorrhiza* Bge. f. alba (designated *SmHDR*1, GenBank Accession No. JX516088) was 1500 bp in length, with an open reading frame (ORF) of 1389 bp, encoding a putative 463 amino acid protein with a molecular weight of 51.86 kDa and a theoretical isoelectric point of 5.81. All these dates show that a new full-length *HDR* gene had been cloned.

2.2. Comparative and bioinformatic analysis of SmHDR1

Sequence BLAST (http://www.ncbi.nlm.nih.gov/BLAST) results showed that *SmHDR*1 belongs to the LYTB family. We compared the amino acid sequence of SmHDR1 with that of other proteins by performing a Blast search against the GenBank database. This search revealed that SmHDR1 has high homology with many other HDRs, such as *Picrorhiza kurrooa* HDR (PkHDR; 81% homology), *Camptotheca acuminata* HDR (CaHDR; 81%), *Catharanthus roseus* HDR (CrHDR; 79%), *A. aestivalis var. palaestina* HDR (AavpHDR; 79%), *Hevea brasiliensis* HDR (HbHDR; 78%) and *A. thaliana* HDR (AtHDR; 74%), suggesting that SmHDR1 belongs to the plant HDR superfamily (Fig. 1). SmHDR1 was predicted to have a secondary structure that is similar to that of AtHDR from *Arabidopsis*. All of these results suggest that *SmHDR*1 encodes a functional HDR protein.

Multiple alignments of SmHDR1 with HDRs from other plants and bacteria indicated that plant and *Synechocystis* HDRs are the most similar (Fig. 1). Nevertheless, *Synechocystis* and *E. coli* HDR proteins, to some extent, lack an N-terminal extension that is present in plant HDRs, which varies significantly among different species (Fig. 1). Furthermore, four conserved cysteine residues found in SmHDR1 are present in all plant HDRs; these residues might participate in the coordination of the iron-sulfur bridge thought to be involved in catalysis [15]. The position of one of these cysteine residues is not conserved in the *E. coli* protein and in that of