

Molecular Cloning and Characterization of Two Key Enzymes involved in the Diterpenoid Biosynthesis Pathway of *Isodon rubescens*

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Abstract

Isodon rubescens, an important medical plant, contains various terpenoids. This plant's active compounds are primarily oridonin with antitumor properties. As the precursor for oridonin biosynthesis, are synthesized by MEP pathway. On the basis of our earlier studies, we isolated and cloned two important genes catalyzing diterpenoid biosynthesis in the MEP pathway. 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase are the fifth enzymes and the last step key enzyme for the methylerythritol phosphate (MEP) pathway, respectively, which is important for the regulation of isoprenoid biosynthesis. Sequence analysis revealed that DclspF (accession no. KT948057) was 966 bp, contains a gene open reading frame (ORF) of 708 bp belonging to the MECDP-synthase superfamily and DclspH (accession no. KT948058) contains a 1389 bp ORF encoding a predicted 462 amino acid polypeptides as a member of the lytB_ispH superfamily. The deduced DclspF and DclspH amino acid sequences shared high similarity with DclspF and DclspH of other plant respectively, each of them exhibiting an N-terminal transit peptide and conserved amino acid sites. Quantitative real-time PCR analysis showed that the expression of DclspF was considerably higher in leaves, the lowest in callus. These results indicate that we have identified functional DclspF and DclspH enzymes, which may play a pivotal role in the biosynthesis of diterpenoid in *I. rubescens*.

Keywords: *Isodon rubescens*; Oridonin; 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase

Introduction

I. rubescens, a plant rich in diterpenoid, a well-known traditional Chinese medicine also known as donglingcao, bingingcao, and poxuedan, is a small shrub of Lamiaceae family. This plant has been an important herb in China for the treatment of various diseases, such as pharyngitis, acute tonsillitis and chronic hepatitis [1]. In addition, this herb can relieve the symptoms and prolong the life of esophageal cancer, cardiac cancer, liver cancer, breast cancer, and rectal cancer [2]. These medicinal properties of *I. rubescens* have been correlated with the presence of diterpenoid which is present in the leaves and stems of *I. rubescens*, especially the leaves [3,4].

To date, numerous studies have shown that oridonin are the main active compounds in *I. rubescens*. It not only has obvious inhibitory effect on tumor cells, but also has obvious anti-mutation and antioxidant effect [5]. So, it has become a research hot spot for medical scholars at home and abroad. But numerous studies about it was focused on pharmacological activities [6,7]. Very limited information is available on the biosynthetic pathways of oridonin. Now the current studies regarding key enzymes of it biosynthesis in *I. rubescens* are few, and the production of oridonin still cannot be synthesized through biosynthesis methods. It is reported that the biosynthetic pathway of terpenoids is mainly from mevalonate (MVA) and methylerythritol phosphate (MEP) [8,9]. The MVA pathway is responsible for synthesizing sesquiterpenes, triterpenes, the monoterpenes, while diterpenes, tetraterpenes were mainly from MEP pathway. Similar to other higher plants, the *I. rubescens* synthesizes diterpenoids through the MEP pathway. So far, many findings have provided us with a relatively complete and convincing outline of MEP pathway. All enzymes involved in the MEP pathway have been identified in plants and bacterial, such as DXP synthase, DXP reductoisomerase (DXR or IspC), CDP-ME synthase (CMS or IspD), CMK or IspE, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase

(MCS or IspF), HMBPP synthase (HDS or IspG), HMBPP reductase (HDR or IspH), which produce the IPP and DMAPP universal basic blocks of isoprenoid compounds from the precursors pyruvate and D-glyceraldehyde 3-phosphate [10].

As MEP pathway plays important role in oridonin biosynthesis, a systemic analysis of the biosynthesis genes in *I. rubescens* was necessary. The availability of high-throughput transcriptomics has enabled a paradigm shift in pathway discovery and can be efficiently applied to non-model species [11,12]. In our previous study, we got a full understanding of the *I. rubescens* transcriptome, and found that many unigenes were annotated as genes encoding the terpenoid skeleton synthesis [13]. Based on theses, among the seven enzymic transformations expected to function in *I. rubescens*, DXP reductoisomerase (DXS), DXP reductoisomerase (DXR or IspC) which is transcribed both in *I. rubescens* leaves, stems, and roots have been cloned by us [14,15]. The other five enzymes yet to be described in *I. rubescens*. Among the five enzymes of MEP pathway, IspF (MDS) is critical since it catalyzes the first cyclization step to convert 4-(cytidine 5'diphospho)-2C-methyl-D-erythritol-2-phosphate (CDP-ME2P) into 2C-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP) [16]. 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR), also known as isoprenoid synthesis H (IspH), lysis-tolerant B (LytB), or HMBPP reductase, is key enzyme for the last step of the methylerythritol phosphate (MEP) pathway, catalyzing the reductive dehydroxylation of HMBPP to isopentenyl diphosphate

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Received June 16, 2017; Accepted June 22, 2017; Published June 26, 2017

Citation: Su X, Yin L, Chen S (2017) Molecular Cloning and Characterization of Two Key Enzymes involved in the Diterpenoid Biosynthesis Pathway of *Isodon rubescens*. J Anal Bioanal Tech 8: 369. doi: [10.4172/2155-9872.1000369](https://doi.org/10.4172/2155-9872.1000369)

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(IPP) and dimethylallyl diphosphate (DMAPP). The overexpression and suppression of AaHDR, protein level in plastids differentially affect artemisinin and other terpenoid biosynthesis in *Artemisia annua* L [17]. However, fewer reports have showed effects of IspF and IspH expression manipulation on *I. rubescens* metabolisms. In this study, we report cloning and characterization of full-length cDNAs of IspF and IspH genes and the expression pattern of IspF and IspH in various tissues including roots, stems and leaves of *I. rubescens* was also studied. This is the first report about the isolation, characterization of IspF and IspH gene from the *I. rubescens*. Our work about cloning and identification of these genes will help us know more about the biosynthesis pathway of diterpenoid especially oridonin in *I. rubescens*. Our research could provide important information for further regulation of oridonin biosynthesis in *I. rubescens* and lay the foundation for elucidating the biological mechanism of oridonin in *I. rubescens*.

Experimental

Materials and methods

Plant materials: The plant was identified as *I. rubescens*, a perennial herb of Labiate, by Prof. S.Q. Chen. Samples were collected from the aseptic seedlings of *I. rubescens* and cultured in the plant tissue culture room at Henan University of traditional Chinese Medicine, China, at 25 ± 2°C with a 16-h light/8-h dark photoperiod provided by cool-white fluorescent lamps. Roots, stems and leaves were collected separately, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis.

Cloning of full-length cDNA of DcIspF and DcIspH: Total RNA was extracted from the leaves of *I. rubescens* using the RNeasy Plant Mini Kit (Kangwei, Beijing, China) following the manufacturer's procedure. After the RNA integrity was examined by 1% agarose gel electrophoresis, the first strand of cDNA was synthesized by reverse transcription using the cDNA first strand reverse transcription Kit (Fermentas) according to the instructions. Primers of the DcIspF and DcIspH were designed and synthesized based on the transcriptome sequence of *I. rubescens*. Premier 5.0 software was used to design specific primers to amplify the two sequences: DcIspF, Primer_F (GCAAGTTCAAGTTAGCAGATTGG), Primer_R (CAGTCACTTCCAAACCAGCA); DcIspH, Primer_F (AGCGTCGC TTGAGTAGACTTC); PCR was conducted in a total volume of 25 µL Primer_R (CAAATGCAATATTTACCATGA); PCR was conducted in a total volume of 50 µL containing 5 µL Reaction buffer, 3 µL Mg²⁺, 4 µL dNTP, 3 µL cDNA, 2 µL-F, 2 µL-R, 0.25 µL Taq DNA Polymerase and 10.75 µL ddH₂O, performed as follows: 95°C for 5 min; 35 cycles at 94°C for 45 s, 55°C for 1 min, and 72°C for 1.5 min with a final extension step of 72°C for 7 min. PCR products were TA cloned into the pMD19TVector (Takara, Japan), transformed into *Escherichia coli* DH5α and then sequenced.

Bioinformatic analysis: Amino-acid sequence alignment was performed using MUSCLE (Multiple Sequence Comparison by Log-Expectation) in Jalview [18]. The neighbor joining trees and bootstrapping analysis were calculated using the Molecular Evolutionary Genetics Analysis (MEGA) 6.0 program. Homologues of genes were

identified using the BLAST at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/Blast>), and the open reading frame (ORF) was analyzed. The protein sequence of the gene sequence was deduced. ExPASy software was used to analyze the physical and chemical properties. Phosphorylation sites were predicted by NetPhos 2.0. Secondary structure prediction was performed with SOPMA. Homology based structural modeling and the three-dimensional (3-D) modeling was accomplished by Swiss-Model at their website.

Expression analysis and quantitative real-time PCR: Quantitative real-time (qRT)-PCR was used to investigate the transcript accumulation levels in various tissues. Using GAPDH as a reference, the quantitative RT-PCR reaction contained 10 µL 2 × SYBR Green PCR Master Mix (QIAGEN), 2 µL reverse transcription reaction product, 0.4 µL of 10 µmol/L forward and reverse primers, and water to 20 µL. The reaction was performed in a CFX96™ Touch Real-Time PCR Detection System (BioRad, CA, USA), and the reaction conditions were 95°C for 5 min, and then 40 cycles of 95°C for 10 seconds, 60°C for 30 s and 72°C for 30 s. The primers were described in Table 1. Three replicates of each cDNA sample were performed for quantitative RT-PCR analysis.

Results

Molecular cloning of the full-length cDNA of DcIspF

Sequence analysis showed that the full-length cDNA (from the ATG initiation codon to the TGA stop codon) which were designated DcIspF, was 966 bp (accession no. KT948057), and contains a gene open reading frame (ORF) of 708 bp located in the 191-898 bp area encoding a protein of 235 amino acids with a molecular weight 24.8 kDa and a theoretical isoelectric point of 8.36. These parameters are very similar to other plants reported earlier [19,20]. The instability index of DcIspF was 40.97, indicating that it was an unstable protein. The ProtParam software was used to predict the total average hydrophilicity of DcIspF protein, which was 0.061, so it can be concluded that the DcIspF protein is a hydrophobic protein (Table 2).

TMHMM Server.v.2.0 was used to predict the transmembrane domain of the DcIspF protein. The results showed that there was no transmembrane domain. In addition, Analysis using the computer program Signal P indicated that there was no signal peptide in DcIspF protein sequence. Protein subcellular localization typically reflects its biological function. To explore the subcellular location of DcIspF protein, we performed subcellular localization prediction using ProtComp9.0 (<http://www.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc>), which indicated DcIspF protein localized in the chloroplast, which is consistent with the cellular localization of the MEP pathway.

Prediction of phosphorylation sites of DcIspF protein of *I. rubescens* is beneficial to further understanding its function. Potential phosphorylation sites using the NetPhos2.0 were also investigated. The results showed that 12 phosphorylation sites were detected on the DcIspF protein. 8, 9, 10, 21, 74, 113, 145 and 178 phosphorylation sites for serine; 4,20 and 27 phosphorylation sites for threonine, and 128 phosphorylation sites for tyrosine were found.

Primer usage	Gene	Forward (5'→3')	Reverse (5'→3')
Gene cloning	IspF	GCAAGTTCAAGTTAGCAGATTGG	CAGTCACTTCCAAACCAGCA
	IspH	AGCGTCGCTTGAGTAGACTTC	CAAATGCAATATTTACCATGA
qRT-PCR	IspF	ATGATGCTGCTCCTTTCCAC	AAGCTCACTCCGATGGTGAT
	IspH	AGAAGGTGCCTCGTCTTCAG	CTCCTCCTACGCCCAAAC
	GAPDH	AAACGCCTAACTTCGCATCT	CCCGACTGTCCCCTGTAATCA

Table 1: Primers used in this study.

Gene Name	ORF (bp)	Length (aa)	MW (kDa)	PI	Instability Index	The total average hydrophilicity
DclspF	708	235	24.8	8.36	40.97	0.061
DclspH	1389	462	52.1	5.82	37.36	-0.455

Table 2: Bioinformatics analysis of DclspF and DclspH.

InterProScan software predicted that the encoded protein was DclspF with one conservative functional domain (IPR003526) from position 38 to 195, named as 2-C- methyl erythritol -2,4- pyrophosphate synthetase, which is related to terpenoid biosynthesis. The presence and the length of putative domains were confirmed by the Superfamily protein database as well. The results of sequence alignment and BLASTP search in GenBank database (<http://www.ncbi.nih.gov>) showed DclspF had high identity and similarity with other IspF proteins of plants, sharing 89% identity with *Salvia miltiorrhiza* (AEZ55667.1; AFQ95411.1) and *Arabidopsis thaliana* (NP_850971.1, 87% identity with *Erythranthe guttata* (XP_012854324.1), 78% identity with *Nicotiana sylvestris* (XP_009758081.1), *Nicotiana tomentosiformis* (XP_009590055.1), and *Nicotiana tabacum* (XP_016512172.1). Moreover, DclspF also exhibited 68-77% identity with other plants. It only presented a 32-49% identity with microbial IspF protein, which indicated that the protein sequence is conserved in plants.

As shown in Figure 1, conserved active site residues D86, H88 and H134 which putatively contribute to coordinate tetrahedrally with a Zn²⁺ ion was also conserved in *I. rubescens*, in addition, the conserved residues E234 (Mg²⁺ or Mn²⁺-binding site) and R236, H97, H241 which conforms the top of the cavity, are highly conserved in plants including the *I. rubescens* [21]. D230 appears to be a highly conserved feature among the plant MCS (IspF) enzymes was also found in *I. rubescens*, H226, S231 were also conserved in *I. rubescens*. Both of them to be critical for IspF of *I. rubescens* which may play important roles highly conserved regions were detected in the active site residues. These findings suggest that DclspF shares the same active site motif with *A. thaliana*. means that the cloned DclspF from *I. rubescens* is a new member of IspF gene family in higher plants. In addition, Sequence analysis also indicated the amino acid sequence of *Escherichia coli* is more than 76 amino acid residues in N-terminal compared to *I. rubescens*. So, it can be speculating the transit peptide structure existed in this region. Chloro P program predicted that the deduced DclspF has the N-terminal chloroplast transit peptide consisting of 55 residues, similarly to *Ginkgo biloba* (GbMECS) [19].

To evaluate the evolutionary relationship of DclspF, a phylogenetic tree was constructed according to the deduced amino acid sequences of DclspF and other IspF from different organisms (Figure 2). The tree clearly showed that the IspF from *Plasmodium falciparum* 3D7 as a different branch from the branches of plants, bacteria, *Microcystis aeruginosa*, and *Thermotoga maritima* MSB8. All the IspF selected from the plants clustered together, DclspF exhibited the highest homology with IspF from *Salvia miltiorrhiza*, which is involved in terpenoid biosynthesis in *Salvia miltiorrhiza* [20].

The second-level structure of DclspF was analyzed in line with SOPMA [22] (Figure 3a). The results showed that the DclspF peptide contains 29.79% Alpha helix, 15.32% extended strand, 10.64% beta turn, and 44.26% random coils. The random coil and Alpha helix are the most abundant structural elements in the DclspF protein of *I. rubescens*, which has high similarity with the known IspF or MECS structures. The three-dimensional protein structures of DclspF and were modeled using Swiss-Model (2pmp.1.A, Seqidentity: 91.19%) (Figure 3b).

Molecular cloning of the full-length cDNA of DclspH

Specific primers were designed according to the sequence of the IspF gene in the transcriptome database of *I. rubescens*, named as DclspH. Using the RT-PCR technique, we also isolated a DclspH gene cDNA (1445 bp, from the ATG initiation codon to the TAA stop codon; accession no KT948058), containing a 1389 bp ORF located in the 28-1416 bp, encoding a predicted 462 amino acid polypeptide with a calculated molecular mass of 52.1 kDa and an isoelectric point of 5.82, similar to the HDS protein from *Tripterygium wilfordii* [23]. The instability coefficient was 37.36, indicating it was a stable protein. The total average hydrophilicity was -0.455, which suggested that the DclspH protein was a hydrophilic protein (Table 1).

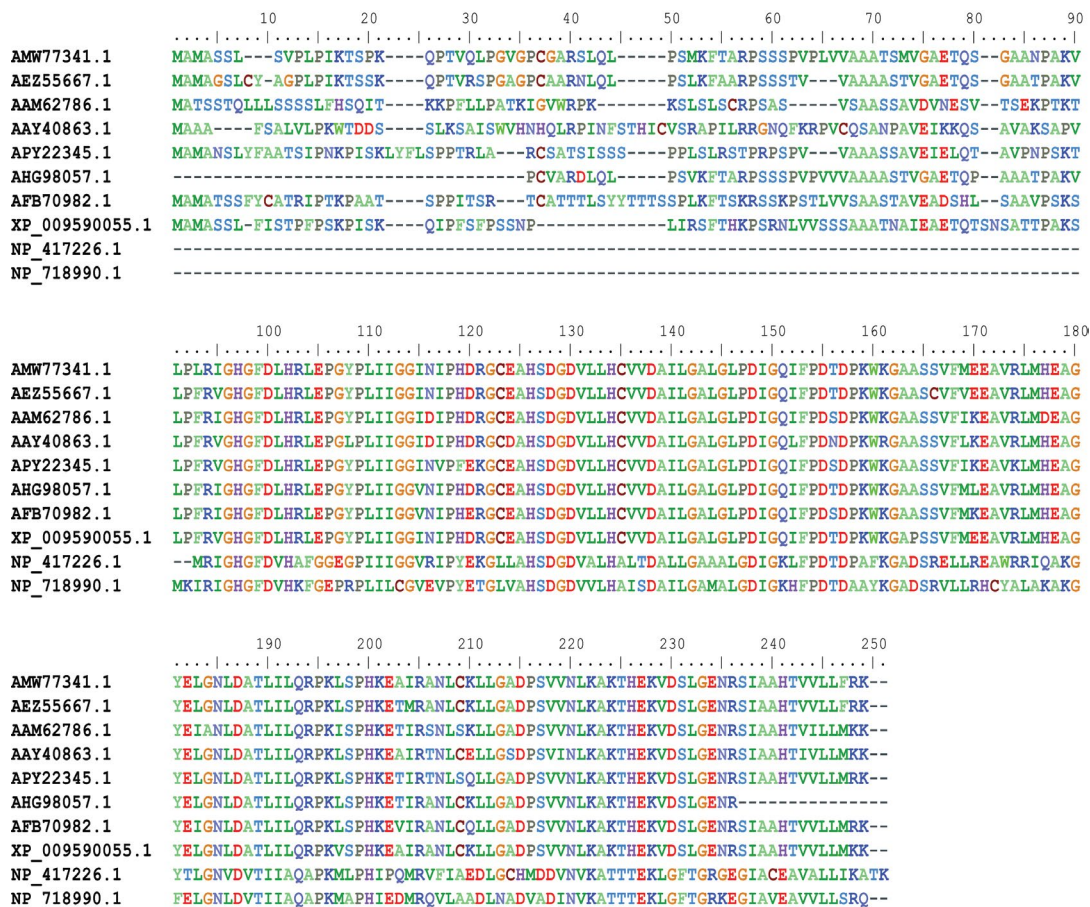
TMHMM Server.v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) was also used to predict the transmembrane domain of DclspH protein. The results showed the DclspH protein has no transmembrane structure and no signal peptide was detected in DclspH protein sequence using the online Signal P programme, which indicated that it was not a secreted protein. Potential phosphorylation suggesting sites for amino acid residues was also predicted using the NetPhos2.0 with a defined threshold of 0.5. In total, there were 22 amino acid phosphorylation sites of the DclspH protein polypeptide chain, including 13 serine, at positions 11, 30, 50, 65, 84, 198, 245, 276, 290, 313, 397, 434, and 437, and 6 threonine, at positions 12, 108, 207, 344, 429, and 438, and 3 tyrosine at positions 102, 261, and 357.

NCBI predicted that the encoded protein of DclspH was a multi domain protein belonging to the lytB_ishP (4-hydroxy-3-methylbut-2-enyl diphosphate reductase) family. The protein family has an iron sulfur cluster structure.

BLAST results indicated that the DclspH protein has high homology with many plant DclspH proteins, with identity from 73%-88%, such as *Lavandula angustifolia* (AGQ04157.1) 88%, *Salvia miltiorrhiza* (AEZ55670.1) 87%, *Sesamum indicum* (XP_011092767.1) 81%, *Arachis ipaensis* XP_016187047.1) 76% and *Tarenaya hassleriana* 73%. The identity of 22%-28% of microbial protein was low.

In the multiple sequence alignment of DclspH proteins from other species, we found all of the plant IspH or HDRs all contain an extra N-terminal conserved domain (NCD) compared with the bacterial IspH, that is essential for their function [24,25] (Figure 4). Beyond the NCD, DclspH had an extended N-terminal sequence with 110-112 lengths, which suggests that there may be a transport peptide sequence in this region, and it may serve as transit peptides to target *I. rubescens* DclspH to the chloroplast. Chloro P 1.1 Server revealed the DclspH protein transporter peptide with a length of 83 amino acid residues. We also find three conserved cysteine residues (C118, C 209, C346) in DclspH, which are involved in iron-sulfur cluster formation. It is likely that the *I. rubescens* DclspH is also an iron-sulfur protein. Similar to the reaction mechanism of *A. thaliana* and *E. coli* IspH [26,27]. The tyrosine-68, G105 residue in the amino-terminal which was critical or important for *Arabidopsis* HDR was also conserved in *I. rubescens*. The conserved residue E239, T308, S375, H148, H237, E238, T240, N374 are related to substrate binding or catalysis was also found in *I. rubescens* (Figure 4).

The phylogenetic tree was constructed according to the deduced amino acid sequences of DclspH and other IspH or HDRs from different organisms. As Figure 5 showed, all the IspHs or HDRs selected from the plants clustered together, and the IspHs from bacteria *Escherichia coli* str. K-12 substr. MG1655, *Shewanella oneidensis* MR-1 and *Deinococcus radiodurans* clustered as a different branch. The DclspH close to IspH or HDRs from *Lavandula angustifolia* and *Salvia miltiorrhiza*.



AMW77341.1, *Isodon rubescens*; AEZ55667.1 *Salvia miltiorrhiza*; AAM62786.1, *Arabidopsis thaliana*; AAY40863.1, *Ginkgo biloba*; APY22345.1, *Hedera helix*; AHG98057.1, *Plectranthus barbatus*; AFB70982.1, *Mitragyna speciosa*; XP_009590055.1, *Nicotiana tomentosiformis*; NP_417226.1, *Escherichia coli* str. K-12 substr. MG1655; NP_718990.1, *Shewanella oneidensis* MR-1 Active site residues are marked by dark triangles and residues of the molecular cavity are marked by dark diamonds.

Figure 1: Amino acid sequence alignment of DcIspF with other organisms DcIspF.

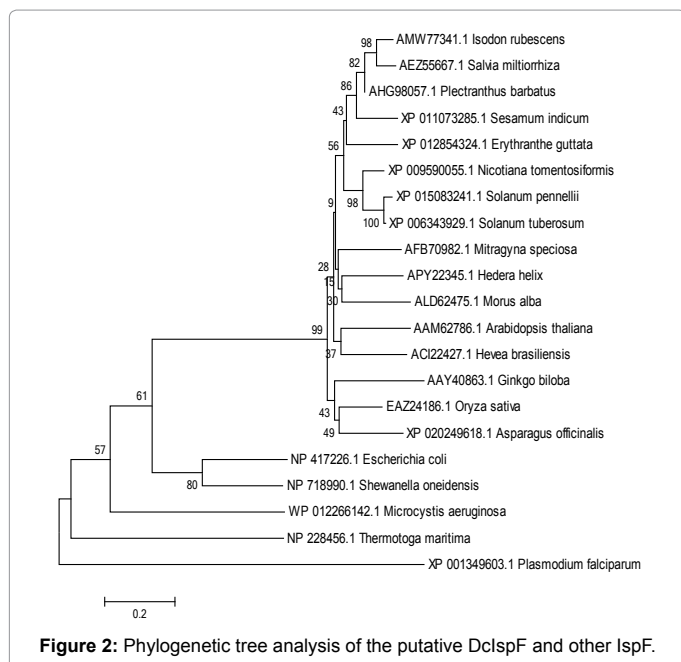


Figure 2: Phylogenetic tree analysis of the putative DcIspF and other IspF.

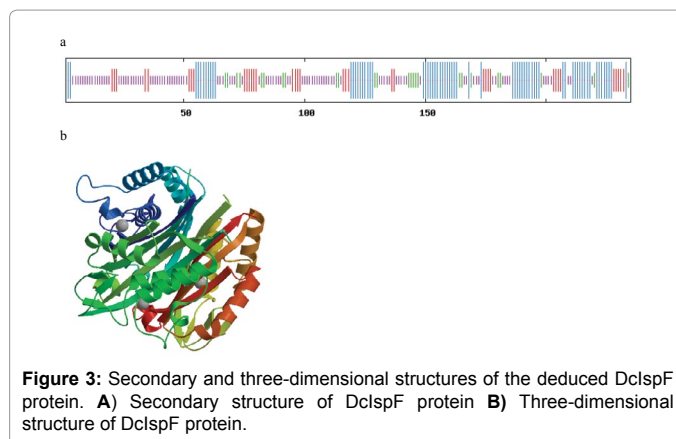
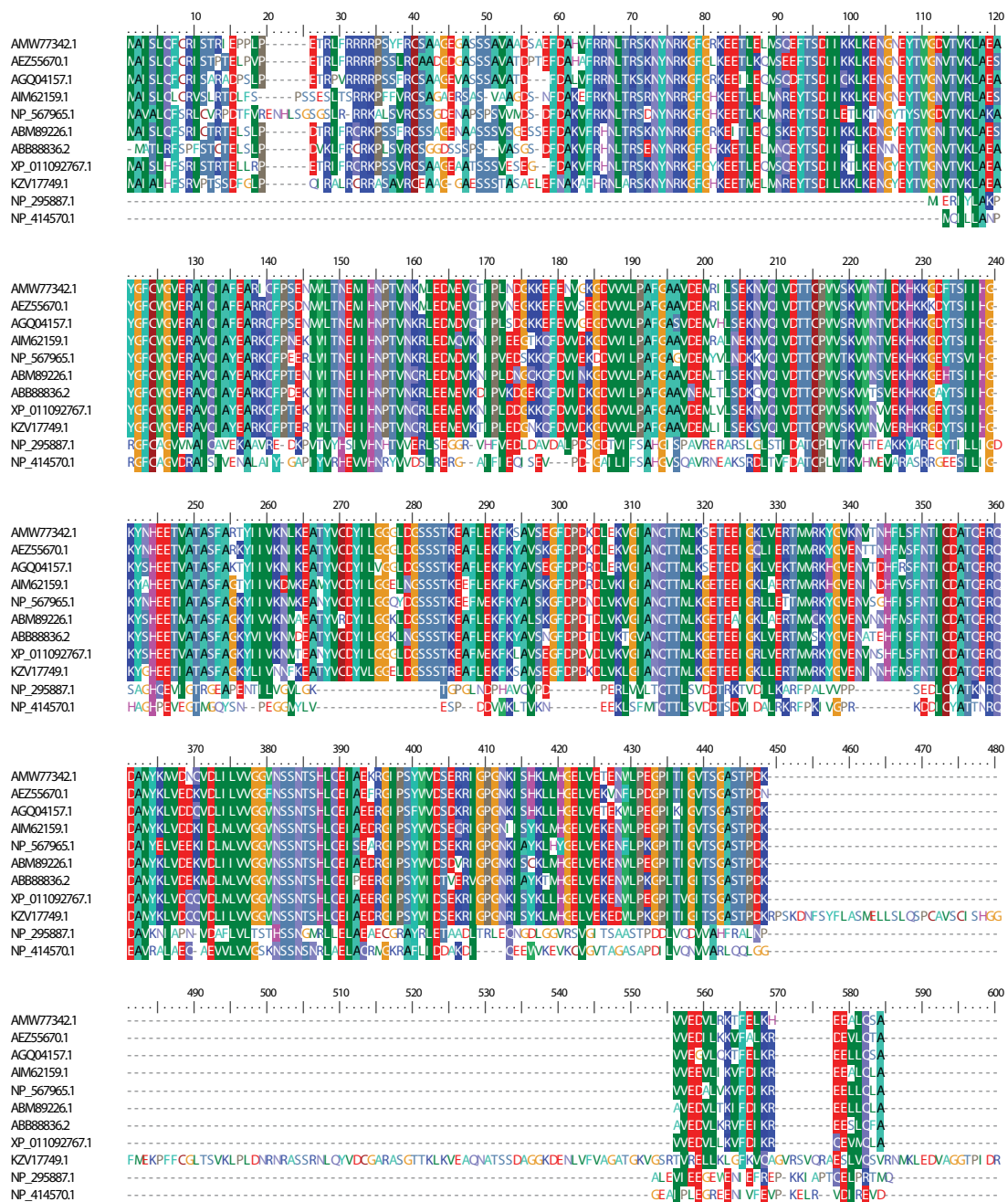


Figure 3: Secondary and three-dimensional structures of the deduced DcIspF protein. **A)** Secondary structure of DcIspF protein **B)** Three-dimensional structure of DcIspF protein.

The NCD among the plants and bacteria is indicated at the top of the alignment. Triangles indicate the critical Cys residues that are involved in iron-sulfur cluster formation. Round dots indicate the conserved amino acids near the substrate-binding site.

The second-level structure of DcIspH were also analyzed in line with SOPMA [22]. The analysis of the DcIspH protein showed 48.05%



AMW77342.1, *Isodon rubescens*; AEZ55670.1, *Salvia miltiorrhiza*; AGQ04157.1, *Lavandula angustifolia*; AIM62159.1, *Tripterygium wilfordii*; NP_567965.1, *Arabidopsis thaliana*; ABM89226.1, *Picrorhiza kurrooa*; ABB88836.2, *Stevia rebaudiana*; XP_011092767.1, *Sesamum indicum*; KZV17749.1, *Dorcoceras hygrometricum*; NP_295887.1, *Deinococcus radiodurans* R1; NP_414570.1, *Escherichia coli* str. K-12 substr. MG1655.

Figure 4: Amino acid sequence alignment of DclSpH with another organism IspH.

alpha helix, 14.94% extended strand, 11.04% beta turn, and 25.97% random coil (Figure 6a). In the DclSpH peptide, alpha helices and random coils are highly abundant along the secondary structures of the peptide while extended strands and beta turns are intermittently distributed in the protein. The three-dimensional protein structures of DclSpH were modeled using Swiss-Model (template: 3dnf.1.A, Seq identity: 31.25%) (<http://swissmodel.expasy.org/workspace/>). The result showed that the DclSpH protein showed an itrefoil-like structure, similar to most plant IspH (HDR) proteins (Figure 6b).

Analysis of gene expression in different tissues

We measured the transcript accumulation of the DclSpF and DclSpH by real-time PCR in various organs (roots, stems, leaves, and callus). As shown in Figure 7 the expression of the genes differs in all tissues examined. The transcript of DclSpF was most abundant in leaves, which was 1.24-fold, 1.82-fold, and 7.75-fold higher in comparison to roots, stems, and callus, respectively. However, the highest transcript level of DclSpH was found in the roots. The second highest transcript

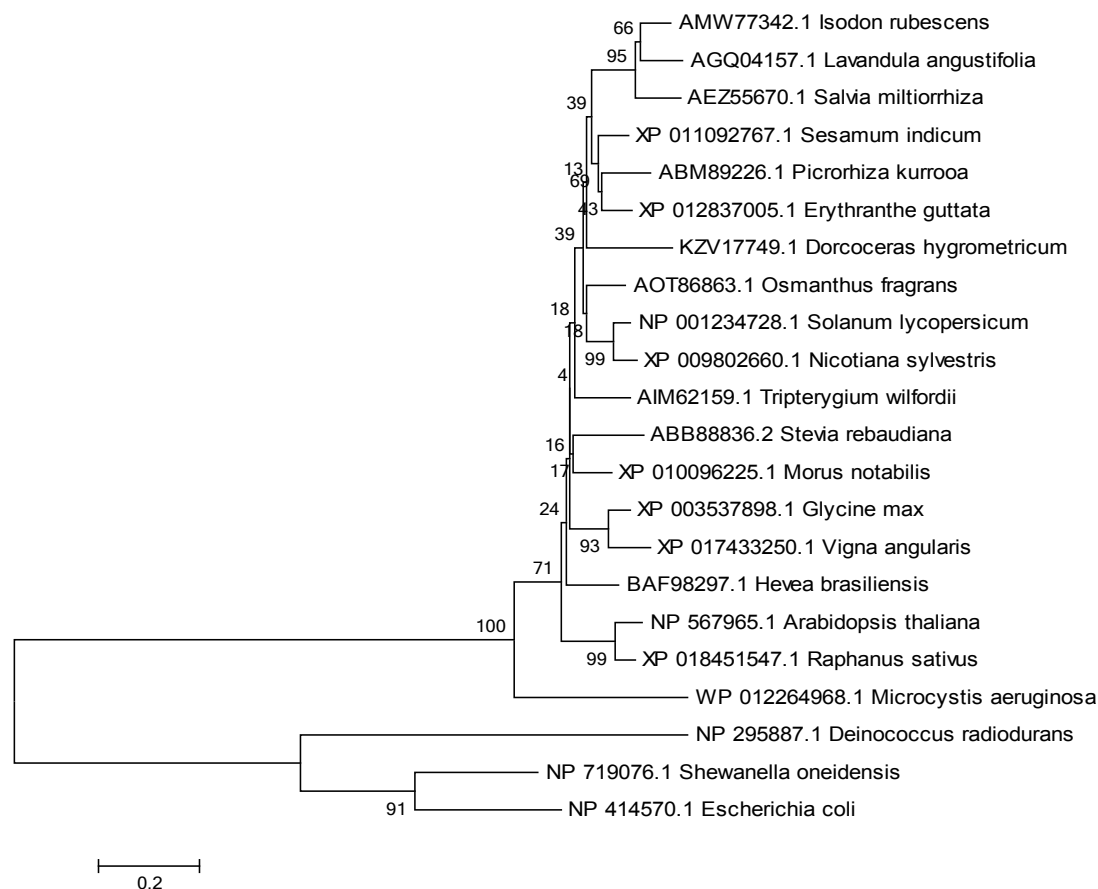


Figure 5: Phylogenetic tree analysis of the putative DcIspH and other IspH or HDRs.

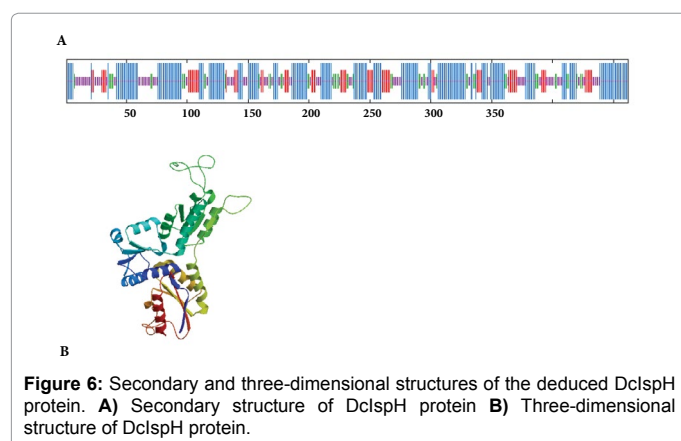


Figure 6: Secondary and three-dimensional structures of the deduced DcIspH protein. A) Secondary structure of DcIspH protein B) Three-dimensional structure of DcIspH protein.

level was found in the leaves, which was 200-fold and 192-fold higher in comparison to callus, respectively. It is interesting that both the genes encoding DcIspF and DcIspH differ in their expression pattern in different tissues, and they were least expressed in callus.

Discussion

Oridonin, an important member of diterpene of *I. rubescens*, were used as indicators for the quality of this medicinal plant according to Chinese National Pharmacopeia [28]. Many studies have demonstrated that oridonin plays an important role in anti-tumor tumor of *I.*

rubescens. Though oridonin has been extensively studied, very little information is available on genes encoding for the enzymes of the pathway involved in biosynthesis of it. The research on the structure and function of key enzyme in synthetic pathway is the main method to regulate the synthesis of the oridonin. As the precursor for oridonin biosynthesis, are synthesized by MEP pathway. DXS, the first enzyme of this pathway, DXR is the first committed step for terpenoid biosynthesis, 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate synthase (IspF), the fifth enzyme, IspH or HDR, the last enzyme considered to be the rate limiting enzyme in the MEP pathway. Several studies support that each of them has an important role for terpene biosynthesis [29-32]. In our previous study, we report the DXS and DXR from *I. rubescens*. In present study, we have cloned cDNA encoding IspF and IspH from *I. rubescens*.

2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase is the fifth enzyme in the MEP pathway, which catalytic CDP-MEP generates ME-Cpp [33]. The bioinformatics analysis results showed that the DcIspF protein has a typical plant IspF motif and domain, meaning that the cloned IspF is a new member of IspF gene family in higher plants. The structure and mechanisms of *Arabidopsis thaliana* IspF have been characterized [25]. The amino acid sequence alignment of DcIspF showed highly conserved residues such as D86, H88, and H134 and other conserved active sites. These results suggest that the *I. rubescens* IspF proteins may have similar enzymatic mechanisms in catalyzing the formation of ME-cPP.

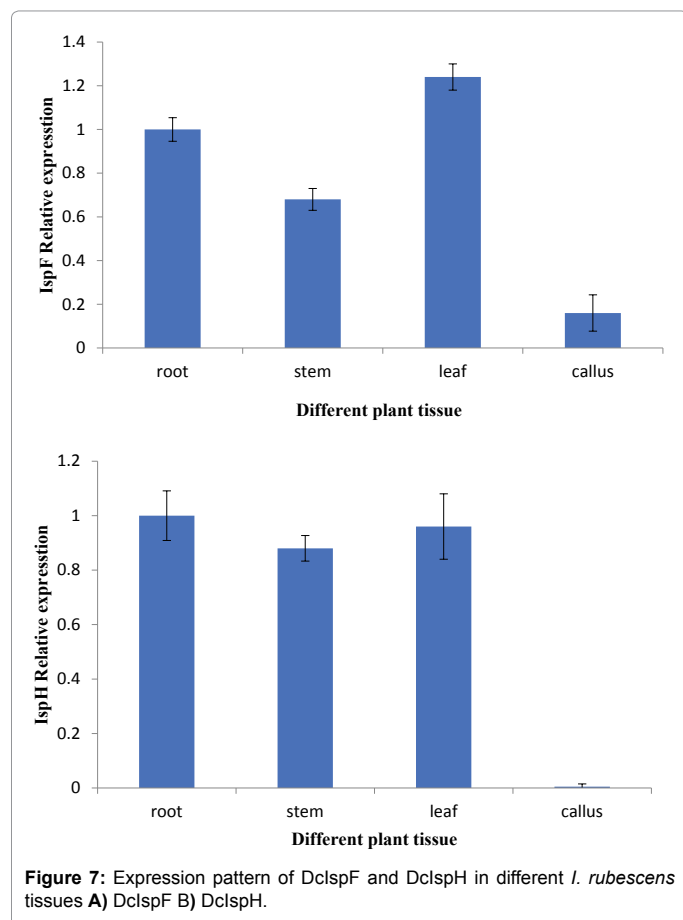


Figure 7: Expression pattern of DcIspF and DcIspH in different *I. rubescens* tissues A) DcIspF B) DcIspH.

As the last enzyme in the MEP pathway for isoprenoid biosynthesis, IspH or HDR protein is considered to be the rate limiting enzyme in the MEP pathway and plays a key role in the regulation of the MEP pathway [34]. Many studies showed that most of the plant IspH (HDR) contains a chloroplast transit peptide. In our study, we found that the DcIspH (HDR) gene is located in chloroplasts with a length of 83 amino acid residue transit peptide in the N-terminus, which was important for the protein transport from the cytoplasm to the chloroplast. This is consistent with the localization of IspH (HDR) proteins in other plants and is also consistent with the cellular localization of the MEP pathway, which suggests that the DcIspH protein is involved in the terpenoid synthesis of the MEP pathway in *I. rubescens*. Previous studies indicated all of the plant IspH contains an extra N-terminal conserved domain (NCD), was also conserved in *I. rubescens*, which is highly conserved in plants and may protect the enzyme from a high concentration of oxygen during photosynthesis [24]. In addition, many amino acid residues observed to be critical for *E. coli* and *Arabidopsis thaliana*, such as three conserved cysteine residues that may be involved in substrate binding were also conserved in *I. rubescens*. These results indicate that DcIspH might participate in the coordination of the iron-sulfur bridge and encode an active IspH (HDR) enzyme, with a similar enzymatic mechanism to the biosynthesis of IPP and DMAPP.

In our study, Expression analysis in different tissues showed higher level of DcIspF and DcIspH transcripts in leaf, which is reflected with the reported higher rates of oridonin biosynthesis in leaves than in stem and callus [35,36]. Our previous study showed that oridonin accumulation and chlorophyll content showed a significant positive correlation [36]. So, it can be inferred that the biosynthesis of oridonin

was higher in leaf may be due to the products of photosynthesis, and complete utilization of reaction products for the synthesis of isoprenoids in addition to high transcription levels. While both of the DcIspF and DcIspH transcription showed higher level in roots, may be because their absence in chloroplast resulting in a lack of photosynthetic products, which leads to no accumulation of oridonin. Lower oridonin accumulation may be due to low but detectable expression of DcIspF and DcIspH transcripts and chlorophyll content in callus. Similar observations for expression of other genes in MEP path way have been observed by us [14,15]. This indicates that callus may be the relatively less active tissue for isoprenoid biosynthesis utilizing substrate from MEP pathway, but leaves are the prime site for oridonin.

As we have little knowledge regarding the gene function and the regulation of the IspF and IspH gene or its enzyme activity in *I. rubescens*, the diterpenoid biosynthesis pathway of *I. rubescens* is still unknown and the transgenic regeneration system of *I. rubescens* remains unsolved, further studies are needed to construct gene over expression or knockout transgenic lines to study the diterpenoid synthesis effect, which may provide insights into the production of oridonin in *I. rubescens*.

Conclusion

In our study, two important genes, DcIspF and DcIspH, involved in isoprenoid biosynthesis from *I. rubescens*, have been characterized for the first time. Alignment of multiple sequences and construction of phylogenetic trees determined the classification of these two proteins. These findings might shed light on the mechanism of oridonin biosynthesis in *I. rubescens* and provide molecular wealth for biotechnological improvement of this medicinal plant.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81173486).

Author Contributions

Suiqing Chen conceived and designed the study, Lei Yin performed the experiments, Xiuhong Su wrote the paper and Suiqing Chen reviewed and edited the manuscript. All authors read and approved the manuscript.

Conflicts of Interest

The authors in this paper declare that they have no conflict of interest.

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Citation: Su X, Yin L, Chen S (2017) Molecular Cloning and Characterization of Two Key Enzymes involved in the Diterpenoid Biosynthesis Pathway of *Isodon rubescens*. *J Anal Bioanal Tech* 8: 369. doi: [10.4172/2155-9872.1000369](https://doi.org/10.4172/2155-9872.1000369)