Cloning and analysis of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase genes HsHDR1 and HsHDR2 in Huperzia serrate

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Abstract We cloned and analyzed the two genes of the 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) gene family from Huperzia serrate. The two transcripts coding HDR, named HsHDR1 and HsHDR2, were discovered in the transcriptome dataset of H. serrate and were cloned by reverse transcription-polymerase chain reaction (RT-PCR). The physicochemical properties, protein domains, protein secondary structure, and 3D structure of the putative HsHDR1 and HsHDR2 proteins were analyzed. The full-length cDNA of the HsHDR1 gene contained 1431 bp encoding a putative protein with 476 amino acids, whereas the HsHDR2 gene contained 1428 bp encoding a putative protein of 475 amino acids. These two proteins contained the conserved domain of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (PF02401), but without the transmembrane region and signal peptide. The most abundant expression of HsHDR1 and HsHDR2 was detected in H. serrate roots, followed by the stems and leaves. Our results provide a foundation for exploring the function of HsHDR1 and HsHDR2 in terpenoid and sterol biosynthesis in Huperziaceae plants.

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Abbreviations: CAS, cycloartenol synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DMAPP, dimethylallyl diphosphate; FPS, farnesyl pyrophosphate synthase; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase; HMBPP, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate; IPP, isopentenyl diphosphate; MVA, mevalonic acid; MEP, 2-methyl-1-cyclopentenyl-4-phosphate; ORF, open reading frame; SQS, squalene synthase; SE, squalene epoxidase

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1. Introduction

Huperzia serrata (Thunb.) Trev., belonging to the Huperziaceae genus, is also known as “Jin bu huan”, “Liu guo nu”, and “She zu cao”\(^1\). H. serrata grows world-wide and is used for the treatment of contusion, strain, swelling, pneumonia, and pulmonary abscess in traditional Chinese medicine (TCM). A number of bioactive natural products have been isolated from this plant. For example, huperzine A, which is a lycodine alkaloid, is a highly efficient and selective central acetylcholinesterase inhibitor\(^1\). Besides alkaloids, terpenoids (e.g., serratane) have also been isolated from H. serrata. Serratane has a variety of biological functions, including the inhibition of secreted aspartyl proteinase (SAP)\(^1\) and antitumor function\(^1\). Although several types of terpenoids have been isolated from H. serrata, the biosynthetic pathway of terpenoids in this plant has not been elucidated. A handful of genes, which may play roles in the biosynthesis of terpenoids, have been cloned from H. serrata, such as cycloartenol synthase (CAS)\(^5\), 1-deoxy-x-olulose-5-phosphate reductoisomerase (DXR)\(^5\), and squalene synthase (SQS)\(^1\).

Terpenoids are the largest group of natural products with diverse molecular structures\(^6\) and play multiple roles in the growth and developmental regulation of higher plants. In addition, the valuable chemical properties and bioactivities of terpenoids contribute to a wide range of applications in commercial products, such as food flavorings, pharmaceuticals, and cosmetics. Terpenoids are biosynthesized from the cytoplasmic mevalonic acid (MVA) pathway and the plastid-localized 2-methyl-D-erythritol-4-phosphate (HMBPP) to produce IPP\(^12\). 1-Hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR), the last enzyme involved in the MEP pathway, catalyzes 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (DMAPP) via several enzymatic steps. IPP and DMAPP can produce different kinds of terpenoids after backbone extension and modification reactions\(^10\), \(^11\). 1-Hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR), the last enzyme involved in the MEP pathway, catalyzes 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBPP) to produce IPP\(^12\), HDR genes have been cloned and analyzed from Arabidopsis thaliana (L.) Heynh\(^13\), Gingko biloba L.\(^14\), Camptotheca acuminata Decne.\(^15\), Hevea brasiliensis (Willd. ex A. Juss.) Muell. Arg.\(^16\) and Eucommia ulmoides Oliver.\(^17\). However, HDR genes have not been cloned and analyzed from H. serrata.

Our research group found a small HsHDR gene family (including two members of HsHDR1 and HsHDR2) from the transcriptome data of H. serrata\(^18\), \(^19\). In this study, we obtained the full-length cDNA sequence and analyzed the putative proteins encoded by these two genes using bioinformatic approaches. The results of our study provided the basic information to identify the function of HsHDR1 and HsHDR2 and to study the molecular mechanism of terpenoid biosynthesis in H. serrata.

2. Materials and methods

2.1. Plant materials

Whole H. serrata plants were sampled in Bawangling Nature Reserve of Hainan province (at the altitude of 1320 m, 109°10'E, 19°7'N). The plants were rinsed with clean water for 5–8 times and dried on filter paper, and then frozen in liquid nitrogen immediately after separation into roots, stems and leaves. The different parts were preserved at −80°C for further use\(^18\), \(^19\).

2.2. RNA isolation and cDNA synthesis

H. serrata roots, stems and leaves, 0.5 g each, were separated and ground into powder in liquid nitrogen. Total RNA was extracted from these tissues using an RNAprep pure Plant Kit provided by Tiangen Biotech Co., Ltd. (Beijing, China). The concentration of the total RNA was measured by NanoDrop 2000 (Thermo, USA).

First-strand cDNA was synthesized by reverse transcriptase using a PrimScript\(^\text{TM}^7\) first-strand cDNA synthesis kit (Takara, Japan) after the digestion of DNA using DNase (Takara, Japan). The cDNA of H. serrata roots, stems and leaves was used for quantitative RT-PCR (qRT-PCR) to detect the relative expression levels of HsHDR1 and HsHDR2 in different parts. The mixed cDNA from different parts was used for gene cloning.

2.3. Gene amplification and sequencing

The HsHDR1 and HsHDR2 genes were cloned by an RT-PCR method using the cDNA as a template according to the following amplification system: cDNA, 1.0 μL; Pyrobest DNA polymerase, 0.2 μL; each of upstream and downstream primers (10 μmol/L), 0.5 μL; dNTP (10 mmol/L) 0.7 μL; and 10 × Pyrobest buffer, 3.0 μL. Doubly distilled H₂O (ddH₂O) was added to reach a final volume of 30.0 μL. The PCR reactions were carried out at 94°C (pre-denaturation) for 3 min, followed by 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min for 30 cycles. The duration of the 72°C elongation step

| Item of analysis | Online tool | Website |
|------------------|-------------|---------|
| Physicochemical property | ExPASy proteomics | http://web.expasy.org/protparam/ |
| Domain | Pfam | http://pfam.xfam.org/ |
| Secondary structure | Network protein Sequence analysis | http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopm.html |
| Three-dimensional structure mode | SWISS-MODEL | http://swissmodel.expasy.org/ |
| Secretary protein | Signal P. 0 Server Target p. 1 server | http://www.cbs.dtu.dk/services/SignalP/ http://www.cbs.dtu.dk/services/TargetP/ |
| Subcellular localization signal | WOLF PSORT Predict Protein | http://www.genscript.com/psort/wolf_psort.html https://https://www.predictprotein.org/ |
| Transmembrane regions | HMMP TO TMHMM Server v. 2.0 | http://www.enzim.hu/hmmtop/html/adv_submit.html http://www.cbs.dtu.dk/services/TMHMM/ |
was 7 min, and the holding temperature was 10 °C. The integrity of the PCR products was determined using 1% agarose electrophoresis, and the amplified target fragment was retrieved by gel extraction. The PCR products were ligated into the pMD19-T vector. The recombinant vector was transformed into competent cells of *Escherichia coli* DH5α. The positive clones were sequenced using the Sanger method by Malorbio Bio-pharm Technology Co., Ltd. (Shanghai, China).

2.4. Bioinformatic analysis

The physicochemical properties, protein domains, secondary structures, secretory proteins, subcellular localization signals, transmembrane regions, and 3D structure models of the putative proteins of HsHDR1 and HsHDR2 were predicted and analyzed using online bioinformatics tools, as listed in Table 1. To strengthen the conclusions, more than one bioinformatics tool was used in the prediction of secretory proteins, subcellular localization signals, and transmembrane regions for these two putative proteins. The sequence alignment of amino acids was performed by using the BLASTX program, which is available on the NCBI website. The amino acid sequences of HsHDR1 and HsHDR2 were aligned, and the phylogenetic tree was constructed with neighbor-joining criteria by the MEGA 6.0 software.

2.5. qRT-PCR analysis

The total volume of the qRT-PCR assay was 25 μL, including 12.5 μL 2 × SYBR Premix Ex Taq™ (Takara, Japan), 1 μL cDNA template, and 1 μL each of forward and reverse primers (1 μmol/L), with ddH2O added to final volume. The target fragment was amplified using the following program: 95 °C for 30 s and 40 cycles in two steps (95 °C for 5 s and 60 °C for 34 s). The reference gene is glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The expression level of HsHDR2 in the leaf was used as the control. The primer sequences are listed in Table 2. The qRT-PCR was performed by the 7500 Real Time PCR System (Applied Biosystems, USA).

2.6. Statistical analysis

All data obtained from the qRT-PCR were calculated to the value of 2^{−ΔΔCt} and differences were evaluated statistically using a *t*-test in SPSS 20.0 software (IBM, Chicago, USA). The relative expression levels of each gene in different tissues were calculated to the 2^{−ΔΔCt} of the mean and the standard error (SE) from the three replications. Statistical significance was set with a *P* value less than 0.01.

3. Results

3.1. Cloning of HsHDR1 and HsHDR2 from *H. serrate*

After searching the *H. serrate* transcriptome data[^18][^19], we found two transcripts encoding complete open reading frames (ORF) in each sequence, which were annotated as HDR genes, named HsHDR1 and HsHDR2, respectively. Specific primers for cloning the two genes were designed according to the two ORF sequences (Table 2). The two genes were cloned with the full lengths of 1431 bp and 1428 bp for HsHDR1 and HsHDR2, respectively. The two cDNA sequences were submitted to the Genbank database with the accession numbers of KP343686 (HsHDR1) and KP343687 (HsHDR2). The amplification of HsHDR1 and HsHDR2 was evident upon electrophoresis, as shown in Fig. 1, suggesting the specific amplification of these two gene cDNAs from *H. serrate*.

![Figure 1 Amplification of cDNA sequence of HsHDR1 and HsHDR2](image-url)
3.2. Prediction and analysis of the putative proteins encoded by HsHDR1 and HsHDR2

3.2.1. Analysis of the physicochemical properties
We analyzed the physicochemical properties of the putative proteins encoded by HsHDR1 and HsHDR2 using the ProtParam of ExPASy Proteomics Server. The instability coefficients of the two putative HsHDR1 and HsHDR2 proteins were 26.84 and 25.26, respectively, indicating that these proteins were stable (Table 3). The aliphatic indices for the two proteins were 84.14 (HsHDR1) and 82.06 (HsHDR2), and the grand averages of hydropathicity were −0.330 (HsHDR1) and −0.363 (HsHDR2) (Table 3). The other detailed results of the physicochemical properties for these two proteins, including the formula, molecular weight, isoelectric point, positively charged residues (Arg+Lys), and negatively charged residues (Asp+Glu), were summarized and are shown in Table 3. These results indicate that most of the physicochemical properties of the two members of HsHDR gene family are different, suggesting different functions for these two genes.

3.2.2. Prediction of secondary structure and structural domains
The secondary structures of these two putative proteins were predicted using the Network Protein Sequence Analysis software. The percentage of α-helix, β-sheet, and random coil was 44.12%, 10.29%, and 24.58% respectively in HsHDR1 (Fig. 2A). The α-helix, β-sheet, and random coil represented 35.58%, 10.74%, and 29.47% respectively in HsHDR2 (Fig. 2B).

The conserved domains of HsHDR1 and HsHDR2 were predicted by Pfam (http://pfam.xfam.org/). A conserved domain named 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (PF02401) was present in both proteins. Fig. 3 shows that this conserved domain is located between the amino acids from 123 (V) to 464 (V) in HsHDR1 and from 122 (V) to 463 (V) in HsHDR2 (Fig. 3). The alignment of HsHDR1 and HsHDR2 also shows that the N-terminal sequences are not conserved between these two proteins (Fig. 3).

3.2.3. Construction of 3D structure model
The 3D structures of the putative HsHDR1 and HsHDR2 proteins were predicted using the SWISS-MODEL, using the Plasmodium falciparum HDR (PfHDR) protein (4n7b.1.A) as the template. Fig. 4 shows the different 3D structure models of HsHDR1 (Fig. 4A) and HsHDR2 (Fig. 4B). The sequence identity of HsHDR1 with PfHDR and HsHDR2 with PfHDR was 28.87% and 28.52%, respectively.

3.2.4. Prediction of signal peptide and transmembrane domain
We analyzed the signal peptides and transmembrane domains in HsHDR1 and HsHDR2 by using the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and HMMTOP, respectively. No signal peptides and transmembrane domains were predicted in these two putative proteins, which indicated that HsHDR1 and HsHDR2 were not secretory proteins and might be located in the cytoplasm of cells. In addition, the TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP/) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) were also used to predict and analyze the signal peptides and transmembrane domains, respectively. No signal peptides or transmembrane domains were predicted in these proteins by these programs.

3.2.5. Prediction of subcellular localization
The subcellular localization of HsHDR1 and HsHDR2 was predicted using the WOLF PSORT (http://www.genscript.com/psort/wolf.psort.html). The coefficients in the different subcellular parts for these two proteins were identical, such as the coefficient in the chloroplast was 5.0 (chlo: 5.0), 4.0 in the cytoplasm (cyto: 4.0), 2.0 in the vacuole (vacu: 2.0), and 1.0 in the nucleus (nuc: 1.0). Furthermore, we also used the PredictProtein (https://www.predictprotein.org/) to analyze the subcellular localization of these two proteins. The results also indicated that the two HsHDRs were most likely located in the chloroplast of H. serrate cells.

3.3. Alignment of amino acids for phylogenetic analysis
We submitted the amino acid sequences of HsHDR1 and HsHDR2 to the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for homologous sequences. Both HsHDR1 and HsHDR2 showed the highest sequence homology to the HDR protein of Huperzia. carinata (Desv.ex Poiret) Trev (HcHDR). The sequence homology of HsHDR1 and HcHDR was 95%, and the homology of HsHDR2 and HcHDR was 86%, whereas the sequence homology of HsHDR1 and HsHDR2 was 84% (Fig. 3).

A phylogenetic tree was constructed based on the alignment of 15 HDR protein sequences of different species. These HDR proteins included the HDR from Campotheca acuminata Decne, Morus notabilis Schneid, Siraitia grosvenorii (Swingle) C.Jeffrey ex Lu et Z.Y.Zhang, Aquilaria sinensis (Lour.) Spreng, Artemisia

| Protein characteristic | HsHDR1 | HsHDR2 |
|------------------------|--------|--------|
| The number of amino acids | 476 | 475 |
| Formula | C_{2339}H_{3722}N_{628}O_{729}S_{16} | C_{2339}H_{3722}N_{628}O_{731}S_{17} |
| Molecular weight | 53073.2 Da | 53073.2 Da |
| Isoelectric point (pI) | 5.62 | 5.76 |
| Positively charged residues (Arg+Lys) | 56 | 58 |
| Negatively charged residues (Asp+Glu) | 67 | 66 |
| Instability index | 26.84 | 25.26 |
| Aliphatic index | 84.14 | 82.06 |
| Grand average of hydropathicity | −0.330 | −0.363 |
**Figure 2** Secondary structure prediction of HsHDR1 and HsHDR2 using SWISS-MODEL. $\alpha$-Helix is represented in blue; $\beta$-sheet is represented in green; random coil is represented in yellow. (A) The secondary structure of HsHDR1; (B) The secondary structure of HsHDR2.

**Figure 3** Amino acid sequence alignment of HsHDR1 and HsHDR2. The upper one is HsHDR1, and the lower one is HsHDR2. Dark blue represents the same amino acid residue between HsHDR1 and HsHDR2, and light blue represents an amino acid difference. The red line indicates the conserved domain.

**Figure 4** 3D model for HsHDR1 and HsHDR2. (A) The 3D structure of HsHDR1; (B) the 3D structure of HsHDR2.
annua L., Tanacetum parthenium (L.) Sch. Bip., Salvia miltiorrhiza Bunge, Picrorhiza kurrooa Royle ex Benth, Adonis aestivalis var. palaestina, Hevea brasiliensis (Willd. ex A. Juss.) Muell. Arg, Arabidopsis thaliana (L.) Heynh, Aegilops tauschii Coss, Ginkgo biloba L., Larix kaempferi (Lamb.) Carr and Huperzia carinata (Desv.ex Poir) Trev. The phylogenetic analysis suggested that both HsHDR1 and HsHDR2 had the closest relationships with the HcHDR, all of which were located in the gymnosperm group (Fig. 5). This result is consistent with the plant category for H. serrate.

3.4. Analysis of gene expression patterns of HsHDR1 and HsHDR2

To detect the expression profiles of HsHDR1 and HsHDR2, we used a qRT-PCR approach to quantify the mRNA abundance of HsHDR1 and HsHDR2 in the roots, stems, and leaves of H. serrate. The results suggested that both HsHDR1 and HsHDR2 were expressed highly in the roots of H. serrate, followed by the stems and leaves. HsHDR1 showed significantly higher expression levels than that of HsHDR2, especially in the roots and the stems (Fig. 6), indicating HsHDR1 may play more important roles in terpenoid biosynthesis in H. serrate.

4. Discussion

In this study, we cloned the HsHDR1 and HsHDR2 genes from H. serrate and analyzed the corresponding proteins encoded by the two genes using bioinformatics methods. According to the phylogenetic analysis, HsHDR1 was most closely related to HcHDR, but not to HsHDR2. Additionally, the expression level of HsHDR1 in different tissues was higher than that of HsHDR2, especially in the roots and the stems (Fig. 6), indicating HsHDR1 may play more important roles in terpenoid biosynthesis in H. serrate.

secondary metabolites. HDR is the last enzyme of the MEP pathway that catalyzes the conversion of HMBPP to IPP. Bioinformatic analysis by WOLFPSORT suggested that HsHDR1 and HsHDR2 were more likely located in the plastid. The predicted subcellular location of these two proteins is consistent with the location of the MEP pathway, providing support to the putative functions of HsHDR1 and HsHDR2 in terpenoid biosynthesis.

The expression patterns of HsHDR1 and HsHDR2 showed that both HsHDR1 and HsHDR2 were more highly expressed in the roots than in stems and leaves, and this finding was similar to the expression patterns of other terpenoid biosynthesis-related genes in H. serrate, such as HsCAS and HsSQS1, which contributes to the prediction that the biosynthesis of terpenoids might be active in the roots of H. serrate.

In summary, based on the cloning of the full length cDNA sequence of the two members (HsHDR1 and HsHDR2) of HDR family in H. serrate, we detected and compared the expression patterns of these two genes, and predicted and analyzed the two putative proteins using bioinformatics tools. These results of our study will pave the way to further identify the functions of HsHDR1 and HsHDR2, and their role in terpenoid biosynthesis in H. serrate.
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