Molecular cloning and functional identification of a cDNA encoding 4-hydroxy-3-methylbut-2-enyl diphosphate reductase from *Tripterygium wilfordii*

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**KEY WORDS**

*Tripterygium wilfordii*; Triptolide; 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase; Complementation; Gene expression

**Abstract**  The 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) is the last step key enzyme of the methylerythritol phosphate (MEP) pathway, synthesizing isopentenyl diphosphate and its allyl isomer dimethylallyl diphosphate, which is important for regulation of isoprenoid biosynthesis. Here the full-length cDNA of HDR, designated *TwHDR* (GenBank Accession No. KJ933412.1), was isolated from *Tripterygium wilfordii* for the first time. *TwHDR* has an open reading frame (ORF) of 1386 bp encoding 461 amino acids. *TwHDR* exhibits high homology with HDRs of other plants, with an N-terminal conserved domain and three conserved cysteine residues. *TwHDR* cDNA was cloned into an expression vector and transformed into an *Escherichia coli* *hdr* mutant. Since loss-of-function *E. coli* *hdr* mutant is lethal, the result showed that transformation of *TwHDR* cDNA rescued the *E. coli* *hdr* mutant. This complementation assay suggests that the *TwHDR* cDNA encodes a functional HDR enzyme. The expression of *TwHDR* was induced by methyl-jasmonate (MJ) in *T. wilfordii* suspension cells. The expression of *TwHDR* reached the highest level after 1 h of MJ treatment. These results indicate that we have identified a functional *TwHDR* enzyme, which may play a pivotal role in the biosynthesis of diterpenoid triptolide in *T. wilfordii*.

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1. Introduction

*Tripterygium wilfordii* Hook. F., also known as Lei Gong Teng or thunder god vine, is native to eastern and southern China. This vine-like plant belongs to the Celastraceae family, and has a long history of use in traditional Chinese medicine when treating autoimmune diseases and inflammatory dermatoses, such as psoriasis, erythema nodosum, rheumatoid arthritis, and systemic lupus erythematosus. The research for the medicinal value of *T. wilfordii* has found out that the plant possesses anti-HIV, anti-inflammatory, antitumor, and anti-Parkinsonian effects, which arouses great interest in the field of medicine. The major active compound responsible for its medicinal functions is believed to be triptolide. Currently, only limited information on the biosynthesis of triptolide is available.

Triptolide is a diterpene trioxepoxide derived from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). There are two independent pathways leading to the biosynthesis of both IPP and DMAPP localized in different cellular compartments which are the cytosolic mevalonic acid (MVA) pathway and the plastidic 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway. While the MVA pathway is responsible for synthesizing sesquiterpenes and triterpenes, the MEP pathway is in charge of the biosynthesis of monoterpenes, diterpenes, and tetraterpenes. As the last enzyme in the MEP pathway for isoprenoid biosynthesis, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) catalyzes (E)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) into a mixture of 5:1 IPP and DMAPP (Fig. 1). Silencing of HDR gene in *Nicotiana benthamiana* can make the isoprenoid-derived chlorophyll and carotenoid pigments decrease to less than 4% of the control plants. And overexpression of HDR gene contributes to increasing the production of isoprenoid-derived carotenoid and over-producing taxadiene up to 13-fold of the control group in transgenic *Arabidopsis*, proving its vital role in metabolic regulation of plastidial isoprenoid biosynthesis.

Because of the high toxicity, obtaining the effective components from *T. wilfordii* by traditional chemical methods is difficult spending much time and labor. And now the current studies regarding key enzymes of triptolide biosynthesis in *T. wilfordii* are few, and the production of triptolide still cannot be synthesized through biosynthesis methods. Based on the above issues, we present the cloning of full-length HDR cDNA of *T. wilfordii* (*TwHDR*) for the first time, proving it having the function of IspH and may acting as a role as a potential key enzyme for the biosynthesis of triptolide.

2. Materials and methods

2.1. Plant material

*T. wilfordii* cell suspensions were cultured in Murashige and Skoog (MS) medium containing 30 g/L sucrose and 8 g/L agar with 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L kinetin (KT), and 0.5 mg/L indole-3-butyric acid (IBA). All suspension cell cultures were maintained at 25 ± 1 °C with shaking by orbital shaker (DZ-100, Suzhou experimental equipment Co., Ltd., Suzhou, China) at 120 rpm in the dark.

2.2. RNA isolation

The 10-day-old *T. wilfordii* suspension cells were treated with MJ for 0, 1, 4, 12, 24, 48 and 72 h at a final concentration of 50 μmol/L.
Figure 2  Amino acid sequence alignment of TwHDR with other plant HDRs and bacterial IspHs. Tw, *Tripterygium wilfordii*; As, *Aquilaria sinensi*; At, *Arabidopsis thaliana*; Sm, *Salvia miltiorrhiza*; Nt, *Nicotiana tabacum*; Ca, *Camptotheca acuminate*; Hb, *Hevea brasiliensis*; Sy, *Synechocystis* sp. PCC 6803; Aa, *Aquifex aeolicus*; Rc, *Rhodobacter capsulatus*; Ec, *Escherichia coli*. The NCD among the plants and cyanobacteria is indicated at the top of the alignment. Arrowheads 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.
Capsulatus (ADE87147), Aquifex aeolicus (O67625), and E. coli (NP_414570) were aligned with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) and DNAMAN Version 9 (Fig. 2).

2.5. Phylogenetic analysis and homology modeling of Arabidopsis HDR

TwHDR and other HDRs downloaded from GenBank were aligned, and the phylogenetic tree was constructed by the neighbor-joining method using MEGA 7.0. The 3-dimensional (3D) structural modeling was predicted by Swiss-Model.

2.6. Functional expression of TwHDR in E. coli hdr mutant

The E. coli hdr mutant was maintained on LB medium containing 50 μg/mL kanamycin (Kan) and 0.2% (w/v) arabinose (Ara)\(^{17}\). Primers 5ʹ-CCTTGGATCCATGGCGATATCTC-3ʹ and 5ʹ-CCTTGGTACCTTTACGCTAAATTGCAAG-3ʹ were used to amplify the full-length cDNA of TwHDR by PCR. The PCR products were digested with BamHI and KpnI, and ligated to the pQE-30 expression vector (Qiagen, Valencia, CA, USA) which was cut by the same restriction enzymes. The resulting construct pQE-TwHDR was transformed into E. coli hdr mutant competent cells and selected on LB plates containing 50 μg/mL Kan, 50 μg/mL ampicillin (Amp), and 0.2% (w/v) Ara. The presence of pQE-TwHDR plasmid in surviving colonies was verified. Transformants containing pQE-TwHDR plasmids were grown on LB plates containing 50 μg/mL Kan, 50 μg/mL Amp, 0.2% (w/v) glucose (Glc) and 0.5 mmol/L IPTG to test if the TwHDR protein could complement the E. coli hdr mutant. As a control, the empty pQE-30 vector was transformed into the E. coli mutant and selected on LB plates containing 50 μg/mL Kan, 50 μg/mL Amp and 0.2% Ara.

3. Results

3.1. Cloning of full-length cDNA of TwHDR and sequence analysis of TwHDR from T. wilfordii

The full-length cDNA of TwHDR is 1456 bp containing a 1386 bp ORF (GenBank Accession No. KJ933412.1). The gene encodes a 461-amino-acid protein with a molecular weight of 52.1 kDa and a theoretical isoelectric point of 5.60.

BLAST result indicated that TwHDR has high homology with many plant HDRs, such as Aquilaria sinensis HDR (AsHDR, 85%), Camptotheca acuminata HDR (CaHDR, 83%), H. brasiliensis HDR (HbHDR, 82%), S. miltiorrhiza HDR (SmHDR, 78%) and A. thaliana HDR (AtHDR, 77%). According to the functional

Figure 3 Phylogenetic tree analysis of the putative TwHDR and other HDRs constructed by the neighbor-joining method (A) and the 3D structure of TwHDR (B).
domain analysis, TwHDR has the IspH/LYTb domain. The sequence alignment showed that all of E. coli, A. aeolicus and Rhodobacter capsulatus IspHs lacked of a stretch of 53 amino acids in the N-terminus to the cyanobacterial HDR (Fig. 2). And these amino acids are highly conserved in cyanobacteria, T. wilfordii and other plants. And beyond the N-terminal conserved domain (NCD), the plant HDR had an extended N-terminal sequence, which was not highly conserved, and it may serve as transit peptides to target plant HDRs.

The T. wilfordii IspH domain (amino acid residues 106–461, encompassing the bacterial IspH) shares approximately 21.67% identity with the E. coli protein. Many amino acid residues found to be critical for E. coli and A. aeolicus IspHs18–22 were also conserved in cyanobacteria and plants including T. wilfordii, which may play important roles as iron-sulfur cluster formation and substrate binding. Three conserved cysteine residues of the conserved residues found in TwHDR are present in all HDRs, which may participate in the coordination of the iron-sulfur bridge which might be involved in the catalysis23 (Fig. 2). And these three cysteine residues have been proved by E. coli complementation assays that they are essential for Arabidopsis HDR function24.

3.2. Phylogenetic analysis and homology modeling for TwHDR

The phylogenetic tree was constructed according to the deduced amino acid sequences of TwHDR and other HDRs from different hosts (Fig. 3A). The tree revealed that TwHDR exhibited the highest homology with HDR from A. sinensis. All the HDRs selected from the plants clustered together, and the HDRs from eumycophyta clustered as a different branch from the branch of plants and cyanobacteria. The HDRs from bacteria Salmonella enterica, E. coli and Shigella flexneri clustered as a different branch from the branch of plants and cyanobacteria. 3D modeling of TwHDR was built by the Swiss-Model used the amino acids 102–453 (template: 3dnfB, Seq identity: 29.96%, Fig. 3B).

3.3. T. wilfordii HDR complements the E. coli hdr mutant

To further test whether the T. wilfordii and E. coli HDR proteins are functionally conserved, we performed a complementation assay with a lethal E. coli mutant detective in the HDR gene (strain MG1655). In E. coli ispH mutant strain MG1655 ara < >

Figure 4 Complementation of E. coli hdr mutant strain MG1655 ara < > HDR.

4. Discussion

HDR enzyme catalyzes the last step in IPP biosynthesis, playing a key role in terpenoid biosynthesis. Although HDR gene has been cloned from many plants, such as A. thaliana25, Ginkgo biloba26 and Salvia miltiorrhiza Bge. G. alba27, there is no report on cloning and characterization of the T. wilfordii gene encoding HDR. In this study, we examined the biosynthesis pathway of terpenoid in T. wilfordii by cloning the HDR gene for the first time. TwHDR was transformed into a proper E. coli mutant strain to verify its function. Furthermore, we also examined the effects of MJ on the expression of TwHDR.

Figure 5 Expression level of TwHDR in suspension cells after methyl-jasmonate (MJ) treatment. CK, the control group; MJ, the MJ-induced group.

ispH, the endogenous ispH gene was replaced by a kanamycin-resistant cassette and a single copy of ispH was present on the chromosome under the control of the P BAD promoter7. Since HDR gene is essential for survival, the E. coli hdr mutant could only grow in the medium containing Ara but not in the medium containing Glc (Fig. 4, left). Upon transformation with the constructed vector harboring the TwHDR gene (pQE-TwHDR), the lethal phenotype of the mutant strain was rescued and cells could grow in medium with Glc. The opposite was observed for cells transformed with the empty pQE-30 vector (Fig. 4 right). Therefore, the enzymatic mechanism involved in the synthesis of the isoprenoid precursors between TwHDR and E. coli HDR might be similar.

3.4. Expression of TwHDR in the suspension cells

As shown in Fig. 5, quantitative real-time PCR revealed the TwHDR expression which was induced by 50 μmol/L MJ in suspension cell cultures. The relative expression level of TwHDR in the MJ-induced group peaked at 1 h after the MeJA treatment (9.98 fold of that at the beginning time). After 1 h, the expression level decreased to 3.89 fold at 4 h than at 0 h. From 4 to 24 h, the expression level gradually increased, and it reached up to 6.11 fold at 24 h. And after 24 h it fell down to 10% at 72 h of that at 0 h. At the same time, in the control group, the expression level of TwHDR also reached its peak at 1 h. And then it progressively decreased to 13% at 72 h of that at 0 h with only a small increase between 4 and 12 h.

**Figure 5** Expression level of *TwHDR* in suspension cells after methyl-jasmonate (MJ) treatment. CK, the control group; MJ, the MJ-induced group.
Although TwHDR only shares about 21.67% identity with the E. coli protein, it was still able to rescue the lethal phenotype of the E. coli hdr mutant (as shown in Fig. 4). The E. coli IspH protein is a reductase that possesses a dioxygen-sensitive [4Fe-4S] cluster\(^3\). The result of amino acid sequence alignment has demonstrated that there may be involved in iron-sulfur cluster formation were conserved in E. coli and all plant HDRs including TwHDR (Fig. 2). These results indicate that TwHDR might participate in the coordination of the iron-sulfur bridge. This complementation assay demonstrated that TwHDR encodes an active HDR enzyme, with similar enzymatic mechanism in the biosynthesis of IPP and DMAPP.

The expression of TwHDR in suspension cells was examined after 1, 4, 12, 24, 48 and 72 h of MJ treatment. The relative expression of TwHDR peaked at 1 h. This result indicated that a short-term MJ excitation could activate secondary metabolism MEP pathway and stimulate the plant stress defense system. About the small increase of TwHDR expression level between 12 and 24 h, we still cannot find the exact reason. But in the study of wound to jasmonates content in A. sinensis, we found the same trend\(^3\). In that study, the jasmonates peaked at 1 h and then decreased, after 6 h, it increased again and went to the second highest content at 24 h, and then fell again. We speculate that this variation trend may be one way that plant cultures make response to the elicitation, but more study is needed to explain its mechanism. Our results prove that TwHDR is an important enzyme in terpenoid biosynthesis pathway, which may be a good target for engineering active terpenoids in T. wilfordii.

Co-expression of a HDR from tomato and a taxadiene synthase from Taxus baccata in transgenic A. thaliana led to a 13-fold increase in the amount of taxadiene produced\(^1\). Therefore, it will be an interesting and effective way to improve triptolide content by genetic engineering. The cloning and identification of key enzyme genes in the biosynthesis of active compounds from medicinal plants is important for the analysis of synthesis pathways. Now, more and more enzyme genes in triptolide biosynthesis pathway have been cloned and identified, such as TwDXS\(^2\), TwDXR\(^5\), TwFPS\(^6\), TwHMGS\(^3\) and TwGGPPS\(^5\). Our work about cloning and identification of TwHDR helps know more about the biosynthesis pathway of terpenoids in T. wilfordii. As the biosynthesis pathway of triptolide is still unknown and the transgenic regeneration system of T. wilfordii remains unsolved, further studies on HDR and the isolation of relevant genes involved in the biosynthesis of terpenoids are still needed, which may provide insights into the production of triptolide in T. wilfordii.

5. Conclusions

We analyzed the function of TwHDR after successfully cloned and characterized the full-length TwHDR cDNA from T. wilfordii for the first time. The combination of cloning, identification, and functional analysis data of TwHDR will offer us more insights into the role of HDR in the MEP pathway and facilitate prospects of triptolide biosynthesis at the molecular level.

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