

Cloning and Expression Analysis of Two Dehydrodolichyl Diphosphate Synthase Genes from *Tripterygium wilfordii*

Lin-Hui Gao^{a,b}, Ping Su^b, Yi-Feng Zhang^{a,b}, Li-Chan Tu^a, Yu-Jun Zhao^b, Tian-Yuan Hu^a, Jia-Wei Zhou^a, Bao-Wei Ma^a, Wei Gao^{a,c}, Lu-Qi Huang^b

^aSchool of Traditional Chinese Medicine, Capital Medical University, ^bState Key Laboratory of Breeding Base Dao-di Herbs, National Resource Center for Chinese Materia Medical, China Academy of Chinese Medical Sciences, ^cBeijing Key Laboratory of Traditional Chinese Medicine Collateral Disease Theory Research, Capital Medical University, Beijing, PR China

Abstract

Objective: To clone and investigate two dehydrodolichyl diphosphate synthase genes of *Tripterygium wilfordii* by bioinformatics and tissue expression analysis. **Materials and Methods:** According to the *T. wilfordii* transcriptome database, specific primers were designed to clone the *TwDHDDS1* and *TwDHDDS2* genes via PCR. Based on the cloned sequences, protein structure prediction, multiple sequence alignment and phylogenetic tree construction were performed. The expression levels of the genes in different tissues of *T. wilfordii* were measured by real-time quantitative PCR. **Results:** The *TwDHDDS1* gene encompassed a 873 bp open reading frame (ORF) and encoded a protein of 290 amino acids. The calculated molecular weight of the translated protein was about 33.46 kDa, and the theoretical isoelectric point (pI) was 8.67. The *TwDHDDS2* encompassed a 768 bp ORF, encoding a protein of 255 amino acids with a calculated molecular weight of about 21.19 kDa, and a theoretical isoelectric point (pI) of 7.72. Plant tissue expression analysis indicated that *TwDHDDS1* and *TwDHDDS2* both have relatively ubiquitous expression in all sampled organ tissues, but showed the highest transcription levels in the stems. **Conclusions:** The results of this study provide a basis for further functional studies of *TwDHDDS1* and *TwDHDDS2*. Most importantly, these genes are promising genetic targets for the regulation of the biosynthetic pathways of important bioactive terpenoids such as triptolide.

Keywords: Bioinformatic analysis, expression analysis, *Tripterygium wilfordii*, *TwDHDDS* gene

INTRODUCTION

Tripterygium wilfordii Hook F is a traditional Chinese medicinal herb that belongs to the family *Celastraceae*. The root is the main pharmacologically active part with a bitter taste and a cold essence according to the traditional Chinese medicine. It belongs to the four classics of the liver, heart, kidney, and stomach, and it is considered to have the functions of clearing the heat, detoxifying, removing wind and dehumidifying, relaxing tendons and activating blood circulation, eliminating swelling, relieving pain, and stopping itching.^[1] Modern pharmacological studies have shown that it has anticancer,^[2] anti-inflammatory, and immunoregulatory effects,^[3-6] and it shows promise in the treatment of rheumatoid arthritis.^[7] The main bioactive components are triptolide, celastrol, and other terpenoids.^[2-4,8] The current supply of these compounds is mainly dependent on extraction and isolation from the original plant. However, the content of active ingredients in the original plants is extremely low.^[9] The

extraction and isolation from the original plant is, therefore, far too inefficient to meet the clinical needs. In recent years, the exploration of key biosynthesis genes and utilization of the principles of synthetic biology to design and modify microbial

Address for correspondence: Prof. Wei Gao, School of Traditional Chinese Medicine, Capital Medical University, Beijing 100069, PR China.
E-mail: weigao@ccmu.edu.cn
Prof. Lu-Qi Huang, State Key Laboratory Breeding Base of Dao-di Herbs, National Resource Center for Chinese Materia Medical, China Academy of Chinese Medical Sciences, Beijing 100700, PR China.
E-mail: huangluqi01@126.com

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strains has become a very promising alternative approach to acquire natural products.^[10-12]

The genes encoding key enzymes in the biosynthetic pathway of terpenoids in *T. wilfordii* have already been cloned and identified.^[13-20] The biosynthetic pathway consists of an upstream isoprenoid pathway, in which two common C₅ building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate, are formed through either the cytoplasmic mevalonate or the plastidic 2-C-methyl-D-erythritol-4-phosphate pathway,^[21] then prenyltransferase-catalyzed condensations of these two C₅ units, result in the formation of linear elongated prenyldiphosphates including the C₁₀ monoterpene precursor geranyl diphosphate, the C₃₀ triterpene (e.g., celastrol) precursor farnesyl diphosphate (FPP), and the C₂₀ diterpene (e.g., triptolide) precursor geranylgeranyl diphosphate. However, little is known about the terpenoid bypass metabolic pathway. Based on this knowledge, we identified two dehydrololichyl diphosphate synthase (DHDDS) genes from *T. wilfordii* by analyzing transcriptome data. DHDDS participates in the terpenoid bypass metabolic pathway, where it continuously condensates FPP with multiple IPP units to generate dehydrololichyl diphosphate (dedol-PP) which is the precursor of dolichol [Figure 1].^[22] Dolichol has a long chain composed of isopentenyl group and plays an important role in the process of glycosylation as a glycosyl carrier.

In this study, two *TwDHDDS* genes were cloned and investigated by bioinformatics and tissue expression analysis. The data enrich our knowledge of the terpenoid bypass metabolic pathway of *T. wilfordii* and provide genetic targets for the subsequent use of CRISPR-Cas9 gene editing technology to eliminate bypass genes or inhibit terpenoid bypass metabolic pathways to increase the metabolic flux toward the synthesis of valuable bioactive terpenoids such as triptolide.

MATERIALS AND METHODS

Plant material

Whole plants of *T. wilfordii* Hook F were obtained from the experimental fields of Fujian Agriculture and Forestry University (Fujian Province, China). Fresh leaves, stems, roots, and flowers were cleaned and stored at -80°C.

Isolation and purification of total RNA from different tissues of *Tripterygium wilfordii*

The total RNA of different tissues of *T. wilfordii* was extracted using the cetyltrimethylammonium bromide method^[23] and purified by RNA Purification Kit (TransGen Biotech, Beijing, China). The concentration of RNA was measured using a NanaDrop 2000 nucleic acid/protein quantifier (Thermo Scientific, USA).

Cloning and sequencing of *TwDHDDS* from *Tripterygium wilfordii*

The extracted RNA was converted to first-strand cDNA templates using the FastKing RT Kit (Tiangen Biotech, Beijing,

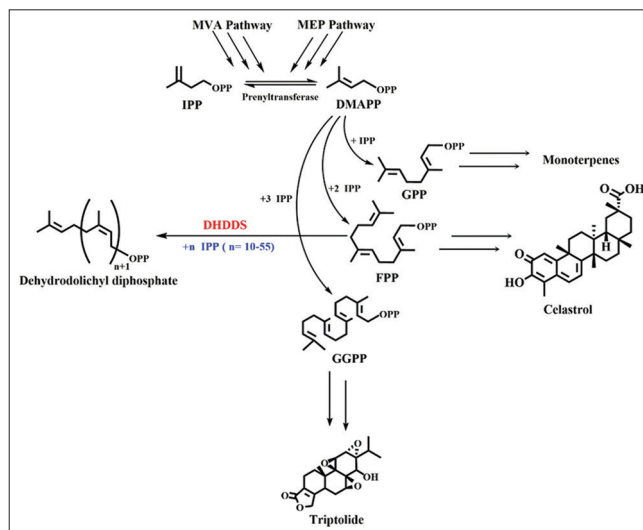


Figure 1: The biosynthesis pathway of dehydrololichyl diphosphate in *Tripterygium wilfordii*. MVA: Mevalonate, MEP: 2-C-methyl-D-erythritol-4-phosphate, OPP: Octaprenyl diphosphate, IPP: Isopentenyl diphosphate, DMAPP: Dimethylallyl diphosphate, GPP: Geranyl diphosphate, GGPP: Geranylgeranyl diphosphate, FPP: Farnesyl diphosphate, DHDDS: Dehydrololichyl diphosphate synthase

China). Specific primers [Table 1] were designed based on sequences in the *T. wilfordii* transcriptome database.^[20] The 50 μ L reaction mixtures comprised 25 μ L Phusion HF MM (New England Biolabs, USA), 2.5 μ L (10 μ M) each of the forward and reverse primers, 1 μ L cDNA, and 19 μ L ddH₂O. The primers and polymerase chain reaction (PCR) conditions are listed in Table 1. The PCR product was purified and cloned into the *pEASY-T3* vector (TransGen Biotech) and transformed into *Escherichia coli* Trans 5 α competent cells (TransGen Biotech). Positive clones were selected and sequenced to obtain the *TwDHDDS* gene sequence. Primer synthesis and sequencing was conducted by Shanghai Majorbio Bio-pharm Technology Co., Ltd., PR China.

Bioinformatics analysis of *TwDHDDS*

The open reading frames (ORFs) were identified using ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder>) to obtain the protein sequences, which were compared with those from other species using NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the protein sequences of other species were downloaded. Multiple sequence alignments were performed using DNAMAN software is DNAMAN V6 software (Lynnon Biosoft, Quebec, QC, Canada) and the phylogenetic tree was constructed using molecular evolutionary genetics analysis (MEGA 6.0) software^[24] based on the results of the homology comparison. Interpro (<http://www.ebi.ac.uk/interpro>) was used to identify functional domains. The theoretical isoelectric point (pI) and molecular weight (Mw) were determined using the Compute pI/Mw tool (https://web.expasy.org/compute_pi/). The TargetP 1.1 server (<http://www.cbs.dtu.dk/services/TargetP/>) was used to analyze signal peptides. Subcellular localization was analyzed using Psort (<http://psort1.hgc.jp/form.html>). Transmembrane domains were analyzed using TMHMM server v2.0 ([16](http://</p>
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Table 1: Primers and polymerase chain reaction conditions

PCR type	Name	Primer sequence 5'-3'	PCR conditions
ORF-PCR	1-F	ATGGAGGAATTTAGTGGTGGTACCG	98°C 30 s, 40 cycles (98°C 10 s, 50°C 15 s, 72°C 20 s) 72°C 5 min, 4°C ∞
	1-R	TCATGACTGCTTCTTCTTTTGTCC	
	2-F	ATGGATTTTGAAGCTGAATTGCAG	
	2-R	TCAAAAACCATAATTTGGCGCACC	
qRT-PCR	RT1-F	AGCAGGATGAAAGGGAACAAC	Denature: 94°C 30 s PCR: 40 cycles (94°C 5 s, 60°C 30 s) Melting: 94°C 5 s, 60°C 30 s, 94°C Continuous cooling: 50°C 30 s
	RT1-R	CAAAGCACTGAAGGAGAATACAAG	
	RT2-F	TACACACTCTCCGTACCTGGC	
	RT2-R	GTTGCTGGTCCCTTAGCGATT	
	Ef1 α -F	CCAAGGGTGAAGCAAGGAGAGC	
	Ef1 α -R	CACTGGTGGTTTTGAGGCTGGTATCT	

PCR: Polymerase chain reaction, ORF: Open reading frame, qRT: Quantitative real-time, Ef1 α : Elongation factor 1 α

www.cbs.dtu.dk/services/TMHMM/). PredictProtein (<http://www.predictprotein.org>) was used to predict the secondary structure of the proteins. The 3D structure homology models were built using SWISS-MODEL (<https://www.swissmodel.expasy.org/interactive>).

Tissue expression analysis of *TwDHDDS*

The eukaryotic translation elongation factor 1 α (Ef1 α) gene was used as an endogenous control to normalize the expression level. The specific primers for quantitative real-time (qRT) PCR were designed according to the *TwDHDDS* sequence [Table 1]. The 10 μ L reaction mixes comprised 5 μ L qPCR SuperMix (TransGen Biotech), 0.2 μ L (10 μ M) each of the forward and reverse primers, 1 μ L cDNA, and 3.6 μ L ddH₂O, and the reactions were performed on a Roche LightCycler 480 Real-Time PCR System (Roche, Switzerland). PCR conditions are listed in Table 1. The expression of *TwDHDDS* was evaluated using the 2^{- $\Delta\Delta$ Ct} method^[25] with triplicate measurements based on three biological replicates. The error was estimated by evaluating the 2^{- $\Delta\Delta$ Ct} term using $\Delta\Delta$ Ct + standard deviation and $\Delta\Delta$ Ct - standard deviation.^[25]

RESULTS

Cloning and sequence homology analysis of *TwDHDDS1* and *TwDHDDS2*

The full-length cDNA sequences of *TwDHDDS1* and *TwDHDDS2* were obtained through RT-PCR with specific primers [Figure 2]. *TwDHDDS1* (GenBank accession number: MG787474) encodes a protein of 290 amino acids with high sequence identity (78%) with known DHDDS from *Corchorus olitorius* and *Gossypium raimondii*. *TwDHDDS2* (GenBank accession number: MG7874745) encodes a protein of 255 amino acids, which is 66% identical to the DHDDS from *Malus domestica* and *Pyrusx bretschneideri* [Figure 3 and Table 2]. The domain analysis conducted using InterPro showed that *TwDHDDS1* and *TwDHDDS2* share a conserved domain, in which *TwDHDDS1* has a conserved site of polyprenyltransferase activity.

Phylogenetic tree analysis of *TwDHDDS1* and *TwDHDDS2*

Sequence divergences were calculated using the Kimura two-parameter (K2P) distance model. A neighbor-joining

Table 2: The Basic Local Alignment Search Tool analysis of *TwDHDDS1* and *TwDHDDS2* protein sequences

Protein	Species	Identity (%)	Accession number
<i>TwDHDDS1</i>	<i>Corchorus olitorius</i>	78	OMP12223.1
	<i>Gossypium raimondii</i>	78	XP_012490689.1
	<i>Juglans regia</i>	77	XP_018827323.1
	<i>Prunus persica</i>	75	XP_007224278.1
	<i>Prunus avium</i>	75	XP_021814762.1
<i>TwDHDDS2</i>	<i>Malus domestica</i>	66	XP_008387378.1
	<i>Pyrusx bretschneideri</i>	66	XP_009375820.1
	<i>Theobroma cacao</i>	65	XP_017971582.1
	<i>Populus euphratica</i>	63	XP_011037556.1
	<i>Vitis vinifera</i>	63	XP_003631635.1

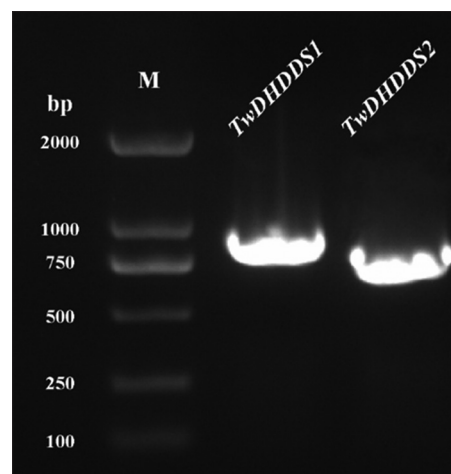


Figure 2: The polymerase chain reaction amplification results of *TwDHDDS1* and *TwDHDDS2* using specific primers. M: *Trans2K* DNA marker

tree was created using the MEGA software to evaluate the homology of the DHDDS sequences [Figure 4]. Bootstrapping was performed with 1000 replications and was reported as percentages. The results showed that *TwDHDDS1* shared more identity with the DHDDS from *C. olitorius*, *Durio zibethinus*, and *G. raimondii*, which clustered into one clade. By contrast, *TwDHDDS2* clustered close to the

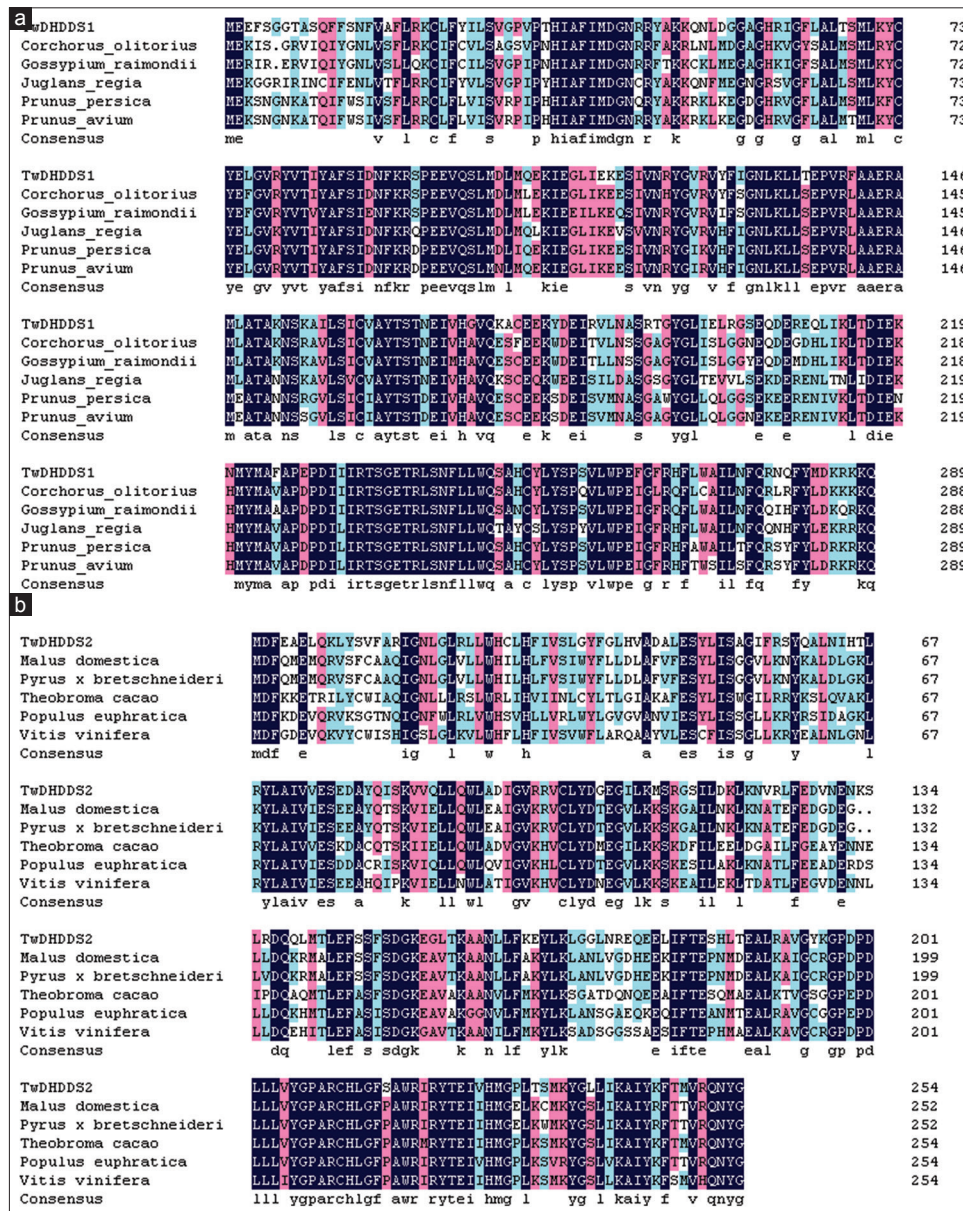


Figure 3: Sequence alignment of *TwDHDDS1* (a) and *TwDHDDS2* (b) with homologous proteins

dehydrodolichyl diphosphate synthase complex subunit NUS1 sequences from *Carica papaya*, *Vitis vinifera*, and *Populus euphratica*, indicating that *TwDHDDS2* and dehydrodolichyl diphosphate synthase complex subunit NUS1 have a closer genetic relationship.

Physicochemical properties and three-dimensional structure prediction of *TwDHDDS1* and *TwDHDDS2*

The calculated Mw of *TwDHDDS1* was 33.46 kDa, and the predicted pI was 8.67. *TwDHDDS1* is a secretory protein with a signal peptide, predicted to be localized to microbody. The results of transmembrane domain analysis showed that the amino acids 18–44 and 61–83 were located in the transmembrane region. The results of the secondary structure prediction showed a predominantly helix and loop structure, with 54.5% α -helices and 35.2% loops and 10.3% β -strands.

Using the structure 2 vg3.1.B as a template for homology modeling, a 3D structure was generated [Figure 5a], with 27.87% identity. The calculated Mw of *TwDHDDS2* was 29.19 kDa, and the predicted pI was 7.72. *TwDHDDS2* is also a secretory protein with a signal peptide and was also predicted to be localized to microbody. However, transmembrane domain analysis showed that the *TwDHDDS2* protein does not have a transmembrane region. The results of secondary structure prediction showed a predominantly α -helical structure, with 71% α -helices, 20.4% loops, and 8.6% β -strands. Using 3wyl. 1.A as a template for homology modeling, 3D structure was generated [Figure 5b], with 18.97% identity.

Tissue expression analysis of *TwDHDDS1* and *TwDHDDS2*

To investigate the physiological roles of *TwDHDDS1* and *TwDHDDS2* in *T. wilfordii*, we performed qRT-PCR to

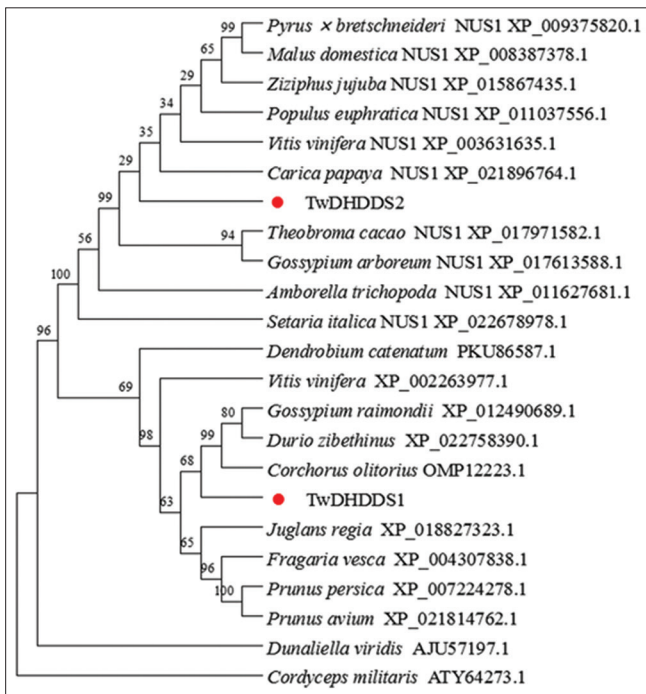


Figure 4: Phylogenetic tree of dehydrodolichyl diphosphate synthase sequences from different species

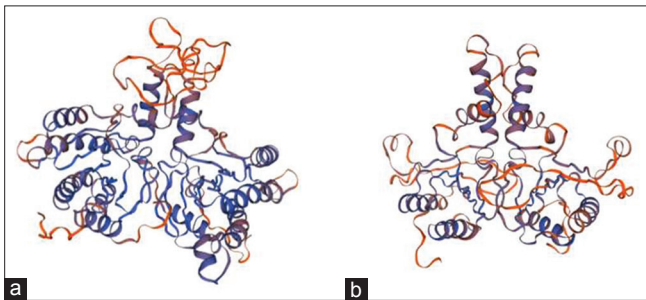


Figure 5: Homology models of the three-dimensional structures of the two *TwDHDDS* proteins from this study. (a) *TwDHDDS1* (b) *TwDHDDS2*

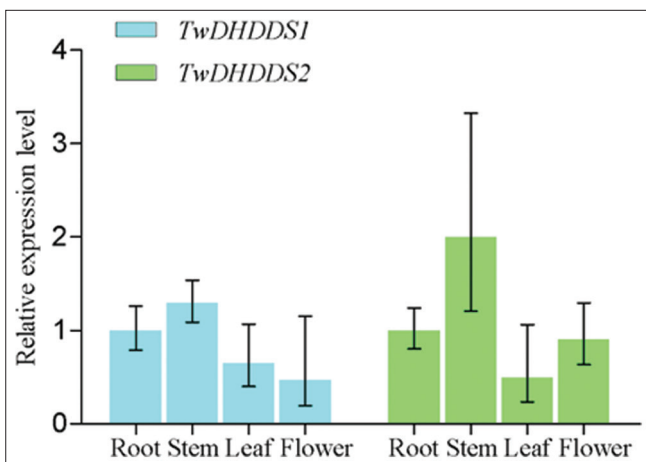


Figure 6: The transcript levels of *TwDHDDS1* and *TwDHDDS2* in different tissues of *Tripterygium wilfordii*

evaluate the corresponding mRNA levels in various organs including root, stem, leaf, and flower. The results showed

that *TwDHDDS1* and *TwDHDDS2* were expressed practically ubiquitously in all sampled organ tissues, with the highest transcription levels in stems [Figure 6].

DISCUSSION

DHDDS are conserved and widespread in a variety of organisms including yeasts, bacteria, animals, and plants.^[26-29] They belong to the isopentenyl transferase family and can be subdivided into different classes according to the structural features of their respective substrates and products such as cis or trans structure and the number of carbon atoms. The tissue expression analysis revealed that both *TwDHDDS1* and *TwDHDDS2* were expressed practically ubiquitously in all sampled organ tissues, and showed the highest transcription levels in stems which was different from the expression pattern of *Arabidopsis thaliana DHDDS* gene.^[26] These results suggested that stem tissues need an more abundant supply of dedol-PP to sustain an active synthesis of dolichol. The results of BLAST and phylogenetic tree analysis showed that *TwDHDDS1* had a close genetic relationship with dehydrodolichyl diphosphate synthases from other species, while *TwDHDDS2* and the dehydrodolichyl diphosphate synthase NUS1 subunit had a closer genetic relationship. Hence, this study provides the basis for further functional investigations of *TwDHDDS1* and *TwDHDDS2*.

FPP and IPP are important precursors of monoterpenes, sesquiterpenes, diterpenes (e.g., triptolide), and triterpenes (e.g., celastrol).^[30,31] *DHDDS* can continuously condense FPP and multiple IPP units to produce dehydrodolichyl diphosphate and the biosynthesis of dolichol will competitively consume these precursors.^[31] The genes encoding key enzymes in the upstream biosynthetic pathways of triptolide and celastrol have already been cloned and identified and the accumulation of triptolide and celastrol can be directly increased by overexpressing the key enzymes involved in the upstream pathway through genetic manipulation. Here, two *TwDHDDS* genes involved in the dolichol synthetic pathway were cloned and studied, enriching our knowledge of the *T. wilfordii* terpenoid bypass pathway. The blockage of this synthetic pathway by CRISPR-Cas9-based genome editing can provide a new strategy to improve the production of important bioactive terpenoids.

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Conflicts of interest

There are no conflicts of interest.

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