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Cloning and Expression Analysis of the GrHDR Gene from *Gentiana rigescens* (Postprint)

Authors: Du Bo, Cai Chuantao, Zhang Ji

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Abstract

Gentiopicroside is the principal pharmacologically active constituent in the traditional Chinese medicine Gentiana, representing a derivative of terpenoid compounds. 1-Hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) constitutes a key enzyme in the terpenoid biosynthetic pathway. To investigate the relationship between the expression of the Gentiana rigescens HDR (GrHDR) gene and gentiopicroside content under varying light conditions, the GrHDR gene sequence was obtained from Gentiana rigescens leaf cDNA via PCR and TA cloning techniques. Bioinformatics and expression analyses were conducted on this sequence, while gentiopicroside content was quantified using high-performance liquid chromatography (HPLC) for comparative assessment with gene expression. The results demonstrated that the GrHDR gene (GenBank accession number: KJ917165.1) spans 1,398 bp, encoding 465 amino acids. The deduced GrHDR protein is hydrophilic and stable, with a relative molecular mass of 52,281.25 Da and a theoretical isoelectric point of 5.32. This protein belongs to the LYTB protein family, is potentially localized to chloroplasts, lacks a signal peptide, and its secondary structure comprises primarily -helix (45.16%), -turn (6.24%), random coil (33.98%), and extended chain (14.62%). The GrHDR protein sequence exhibits the highest similarity (95.71%) with the HDR protein from the congeneric species Gentiana macrophylla. Real-time quantitative PCR analysis revealed that GrHDR gene expression in Gentiana rigescens follows the pattern: root > leaf > stem, with substantial variations in expression across tissues under 10%, 30%, and 100% full-light conditions. HPLC analysis indicated that gentiopicroside content under different light conditions consistently follows the pattern: root > leaf > stem. Under 100% full-light conditions, gentiopicroside content in the medicinal root tissue reached 7.141%, approximately twice that observed under 30% and 10% full-light conditions; however, this result does not entirely correlate with the expression pattern of the GrHDR gene under identical light conditions. This study provides a reference for elucidating HDR gene function

and its relationship with gentiopicroside content.

Full Text

Preamble

Cloning and Expression Analysis of the GrHDR Gene in *Gentiana rigescens*

Bo Du[1,2], Chuantao Cai[1*], Ji Zhang[1,2,3]

[1]Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming 650221, China
[2]University of Chinese Academy of Sciences, Beijing 100049, China
[3]Medicinal Plants Research Institute, Yunnan Academy of Agricultural Sciences, Kunming 650200, China

Abstract: Gentiopicroside, a derivative of terpenoids, is the main medicinal ingredient in the traditional Chinese medicine "Long Dan" (Gentiana). 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) is a key enzyme in terpenoid biosynthesis. To explore the relationship between HDR (*GrHDR*) gene expression and gentiopicroside content under different light conditions, we used leaf cDNA from *Gentiana rigescens* as a template to obtain the *GrHDR* gene sequence via PCR and TA cloning. Bioinformatics and expression analyses were performed, and gentiopicroside content was determined by high-performance liquid chromatography (HPLC) for comparison with *GrHDR* expression levels. The results showed that the *GrHDR* gene (GenBank accession: KJ917165.1) is 1,398 bp in length, encoding 465 amino acids. The putative GrHDR protein is hydrophilic and stable, with a relative molecular mass of 52,281.25 Da and a theoretical isoelectric point of 5.32. The protein belongs to the LYTB family, likely localizes to chloroplasts, contains no signal peptide, and has a secondary structure composed primarily of -helix (45.16%), -turn (6.24%), random coil (33.98%), and extended chain (14.62%). The GrHDR protein sequence shows highest similarity (95.71%) to the HDR protein from the congeneric species *Gentiana macrophylla*. Real-time quantitative PCR revealed that *GrHDR* expression in *G. rigescens* follows the pattern: root > leaf > stem, with significant differences among tissues under 10%, 30%, and 100% full-light conditions. HPLC analysis showed that gentiopicroside content consistently followed root > leaf > stem across all light conditions. Under 100% full light, gentiopicroside content in the medicinal root reached 7.141%, approximately double that under 30% and 10% full-light conditions. However, this pattern did not completely align with *GrHDR* gene expression under the same light conditions. This study provides a reference for elucidating HDR gene function and its relationship with gentiopicroside content.

Keywords: *Gentiana rigescens*, *GrHDR* gene, sequence analysis, tissue expression analysis, gentiopicroside content

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Author Introduction: Bo Du (1994-), female (Bai ethnicity), from Dali, Yunnan, Master' s degree, research focus on medicinal plant cultivation, (E-mail) dubo@xtbg.ac.cn.
*Corresponding Author: Chuantao Cai, Researcher, Doctoral Supervisor, research focus on medicinal plant cultivation, (E-mail) caict@xtbg.ac.cn.

Introduction

Gentiana rigescens Franch. ex Hemsl., also known as "Jian Long Dan," is a perennial herb in the Gentianaceae family and one of the source plants for the traditional Chinese medicinal herb Gentiana. It is mainly distributed in forests, thickets, and meadows of Yunnan, Sichuan, Guangxi, and Guizhou provinces. The dried roots and rhizomes are used medicinally, with the main active ingredient being the iridoid gentiopicroside, which is used to clear heat, dry dampness, and purge liver-gallbladder fire. In recent years, due to indiscriminate harvesting, wild resources of *G. rigescens* have declined sharply, and it has been listed as a nationally protected wild medicinal species (Level III) by the Endangered Species Scientific Commission of China. Research on artificial cultivation practices to increase active ingredient content could alleviate pressure on wild resources. To achieve this, it is essential to understand the biosynthetic pathway and regulatory mechanisms of gentiopicroside and identify factors affecting its content.

Gentiopicroside belongs to the secoiridoid class of compounds, whose synthesis occurs in three stages. The first stage involves synthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) via the plastidial methylerythritol phosphate (MEP) pathway and the cytosolic mevalonate (MVA) pathway. IPP and DMAPP are essential precursors for terpenoid biosynthesis. The second stage converts IPP and DMAPP into secologanin through a series of reactions. The third stage involves complex enzymatic modifications of the terpenoid carbon skeleton to form stable terpenoid compounds. This study cloned the final key enzyme of the first-stage MEP pathway: 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR), which catalyzes the conversion of HMBPP (4-hydroxy-3-methylbut-2-enyl diphosphate) to IPP or DMAPP. The *HDR* gene has been isolated from various plants including *Arabidopsis*, *Huperzia serrata*, *Oncidium* orchid, *Gentiana macrophylla*, and *Artemisia annua*. Its expression has been linked to carotenoid production in *Arabidopsis* seedlings, ginkgolide content in ginkgo, and camptothecin synthesis in *Camptotheca acuminata*, making it an ideal target for manipulating terpenoid metabolism. However, no studies on the *HDR* gene have been reported in *G. rigescens*, where gentiopicroside is the

main active component.

Light is closely related to the growth, development, and secondary metabolite accumulation in medicinal plants. Studies have shown that shading is a major factor affecting secondary metabolites in *Bletilla striata*, with 60% shade being optimal, while approximately 70% shade benefits active ingredient accumulation in *Paris polyphylla*. However, the effects of shading on gene expression and gentiopicroside content in *G. rigescens* remain unreported. This study designed specific primers based on *HDR* gene sequences from the *G. rigescens* transcriptome, amplified the *GrHDR* gene from young leaves of one-year-old plants via RT-PCR, and performed TA cloning, sequencing, and sequence analysis. Expression analysis was conducted under three light levels using quantitative PCR, and gentiopicroside content was measured by HPLC to provide a basis for understanding the metabolic process of gentiopicroside synthesis and lay a foundation for investigating HDR function and its relationship with gentiopicroside.

1.1 Experimental Materials

Gentiana rigescens plants were cultivated in Henitang Township, Yongde County, Lincang City, Yunnan Province (altitude 1,822.3 m, 99°25 46 E, 23°54 13 N). Fresh one-year-old plants were used for gene cloning, sampled on May 9, 2019 (sown on May 8, 2018). For tissue-specific expression analysis, potted plants under different shading treatments (shading nets deployed on August 31, 2018, with three treatments: full light, single-layer shade, and double-layer shade) were sampled on September 1, 2019. During sampling, middle portions of roots, stems, and leaves were collected, immediately frozen in liquid nitrogen, and stored at -80°C. Remaining root, stem, and leaf materials from these plants were used for gentiopicroside content determination.

1.2.1 Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from young leaves using the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, batch: AIF2395A) according to the manufacturer' s instructions. cDNA was synthesized using the RT6 cDNA Synthesis Kit (TSINGKE) following the protocol provided.

1.2.2 TA Cloning of the *GrHDR* Gene

Specific primers were designed based on the *GrHDR* gene sequence from the *G. rigescens* transcriptome . PCR amplification was performed using cDNA as template in a 50 L reaction containing 25 L 2× High-Fidelity Master Mix, 2 L each of forward and reverse primers, 2 L cDNA template, and ddH O to volume.

The PCR program consisted of initial denaturation at 98°C for 2 min; 35 cycles of 98°C for 10 s, 60°C for 15 s, 72°C for 20 s; and final extension at 72°C for 2 min. PCR products were detected by 1% (w/v) agarose gel electrophoresis, purified, and ligated into the pClone007 Simple vector (TSINGKE, batch: TSV-007S). After transformation into *E. coli* DH5 competent cells (TSINGKE, batch: TSV-A07) and overnight culture, positive clones were identified by PCR and sequenced.

Table 1 Primers for *GrHDR* gene amplification

Primer name	Primer sequence (5’ -3’)
HDR-CDS-F	ATGGCAATCTCTTTGCAATTCG
HDR-CDS-R	TTATGCCAGTTGCAAGGCTTC

1.2.3 Bioinformatics Analysis of *GrHDR*

The *GrHDR* gene was translated into protein sequence using the NCBI ORFfinder tool. Various online bioinformatics tools were employed to predict and analyze the physicochemical properties, domains, secondary structure, signal peptides, transit peptides, subcellular localization signals, and three-dimensional structure models of the GrHDR protein. Sequence alignment was performed using DNAMAN 5.2.2, and a phylogenetic tree was constructed using MEGA 6.0. The tools used are listed in .

Table 2 Tools for bioinformatics analysis

Analysis type	Software/Website
ORF prediction	ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/)
Protein physicochemical properties	Protparam (https://web.expasy.org/protparam/)
Secondary structure prediction	SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html)
Domain prediction	Pfam (http://pfam.xfam.org/search)
Signal peptide prediction	SignalP (http://www.cbs.dtu.dk/services/SignalP/)
Transit peptide prediction	ChloroP (http://www.cbs.dtu.dk/services/ChloroP/)
Subcellular localization	Predict Protein, Softberry (https://www.predictprotein.org/, http://www.softberry.com/berry.phtml?topic=protcomppl&group=progr
Tertiary structure prediction	Swissmodel (https://swissmodel.expasy.org/interactive)

Analysis type	Software/Website
Transmembrane domain prediction	TMHMM (http://www.cbs.dtu.dk/services/TMHMM/)

1.2.4 Tissue Expression Analysis of *GrHDR*

Total RNA was extracted from roots, stems, and leaves of one-year-old *G. rigescens*, reverse-transcribed into cDNA for qPCR template. The *GrGAPDH* gene was used as an internal reference . Amplification was performed using 2× T5 Fast qPCR Mix (SYBR Green I, TSINGKE, batch: TSE202) with the following conditions: pre-denaturation (95°C, 1 min); cycling (95°C, 10 s; 60°C, 5 s; 72°C, 10 s); melting curve analysis (95°C, 15 s; 60°C, 1 min; 95°C, 15 s). Fluorescence signals were collected at 72°C during the cycling stage. Relative expression was calculated using the 2^(-ΔΔCT) method and plotted using GraphPad Prism 5.0.

Table 3 Primer sequences for *GrHDR* and reference genes

Primer name	Primer sequence (5' -3')
HDR-F	CGTTGCCGGAGTCGAAGATA
HDR-R	CGAGTCAACACTCACAGACGA
GAPDH-F	TGTTCGGCGTTAGAAACCCA
GAPDH-R	CTTCGCACCTCCCTTGATGT

1.2.5 Tissue Expression Under Different Light Conditions

Light intensities under full light, single-layer, and double-layer shade nets were measured using a digital lux meter (GM1040, Shenzhen Jumoyuan Technology) as (107,248 ± 7,938) lx, (31,196 ± 3,406) lx, and (10,217 ± 804) lx, respectively, representing 100%, 30%, and 10% full-light conditions. After one year of treatment, roots, stems, and leaves were collected for quantitative PCR analysis of *GrHDR* expression using the methods and reference gene described in Section 1.2.4.

1.3.1 Reagents and Instruments

Gentiopicroside standard (batch: S2220010) was purchased from Shanghai Anpu Experimental Technology. Methanol was HPLC grade; other reagents were analytical grade. The Agilent Technologies 1290 Infinity II HPLC system and

AS10200 ultrasonic cleaner (Tianjin Autoscience Instrument) were used for analysis.

1.3.2 Sample Preparation

Plant materials were dried at 50°C, ground, and passed through a 40-mesh sieve. Sample powder (0.25 g) was weighed into a 50 mL conical flask, mixed with 10 mL methanol, and re-weighed. After ultrasonic extraction (300 W, 40 kHz) at 40°C for 30 min, samples were cooled and re-weighed, with methanol added to compensate for weight loss. The solution was filtered through a 0.22 m membrane for HPLC analysis.

1.3.3 HPLC Conditions for Gentiopicroside Detection

An InertSustain C18 column (250 mm × 4.6 mm, 5 m) was used at 30°C with detection at 241 nm, flow rate of 1 mL · min ^{1}, and injection volume of 20 L. Mobile phases were methanol (A) and 0.1% formic acid (B) with gradient elution: 0–15 min, 7–35% A; 15–32 min, 35–90% A; 36–42 min, 10–93% B.

2.1 Cloning of *GrHDR* ORF Sequence

Total RNA extracted from young leaves had a concentration of 612.4 g · mL ^{1} with an A / ratio of 1.82, indicating high purity. Using leaf cDNA as template, specific primers amplified a fragment of approximately 1,500 bp [Figure 1: see original paper]. TA cloning and sequencing confirmed the *GrHDR* ORF sequence (GenBank: KJ917165.1) as 1,398 bp encoding 465 amino acids.

Figure 1 Gel electrophoresis of *GrHDR* gene from *Gentiana rigescens*. M: DNA marker DL2000; 1: PCR product.

2.2.1 Physicochemical Properties of GrHDR Protein

Protparam analysis revealed an instability index of 30.34, indicating a stable protein. The molecular mass was 52,281.25 Da, theoretical pI 5.32, aliphatic index 77.98, and grand average of hydropathicity -0.427, confirming a hydrophilic protein .

Table 4 Predicted physicochemical properties of GrHDR protein

Property	Value
Number of amino acids	465

Property	Value
Formula	C H N O S
Molecular weight	52,281.25 Da
Isoelectric point (pI)	5.32
Positively charged residues (Arg+Lys)	49
Negatively charged residues (Asp+Glu)	62
Instability index	30.34
Aliphatic index	77.98
Grand average of hydropathicity	-0.427

2.2.2 Structural Prediction of GrHDR Protein

SOPMA prediction showed the secondary structure composition as -helix (45.16%), -turn (6.24%), random coil (33.98%), and extended chain (14.62%) [Figure 2: see original paper]. Domain analysis using Pfam indicated membership in the LYTB (HDR) protein family. SignalP 5.0 predicted a signal peptide probability of 0.0006, confirming a non-secreted protein. ChloroP 1.1 Server predicted a 35-amino-acid transit peptide. TMHMM 2.0 detected no transmembrane helices. Subcellular localization prediction by PredictProtein (71% confidence) and Softberry ProtComp 9.0 (score 9.3) both indicated chloroplast localization.

Figure 2 Secondary structure prediction of GrHDR protein. Blue: -helix; green: -turn; yellow: random coil; red: extended chain.

2.2.3 Tertiary Structure Prediction

SWISS-MODEL predicted the tertiary structure using *Aquifex aeolicus* 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (SMTL ID: 3dnf.1) as template for residues 80–297 with 36.86% sequence identity. The global model quality estimate (GMQE) was 0.42, indicating moderate reliability [Figure 3: see original paper].

Figure 3 Predicted tertiary structure model of GrHDR protein.

2.3 Multiple Sequence Alignment and Phylogenetic Analysis

BLASTp analysis showed highest similarity (95.71%) with *Gentiana macrophylla* GmHDR, followed by *Rauvolfia verticillata* RvHDR (84.09%), and high similarity with *Catharanthus roseus*, *Camptotheca acuminata*, *Osmanthus fragrans*, *Olea europaea* var. *sylvestris*, *Erythranthe guttata*, and *Picrorhiza kurrooa*.

Multiple sequence alignment is shown in [Figure 4: see original paper]. Phylogenetic analysis using MEGA 6.0 placed GrHDR and GmHDR on the same branch, indicating the closest relationship [Figure 5: see original paper].

Figure 4 Multiple sequence alignment of GrHDR with other plant HDR proteins. Black: 100% similarity; red: 75-100% similarity; white: <75% similarity.

Figure 5 Phylogenetic tree of GrHDR and other plant HDR proteins.

2.4 Tissue-Specific Expression of *GrHDR* in *G. rigescens*

Quantitative RT-PCR analysis of roots, stems, and leaves showed highest *GrHDR* expression in roots, followed by leaves and stems [Figure 6: see original paper].

Figure 6 Relative expression of *GrHDR* in root, stem, and leaf. Expression in stem set as 1.

2.5 Tissue Expression Under Different Light Conditions

GrHDR expression varied significantly among tissues under different light conditions. Under 100% full light, expression was leaf > stem > root (leaf expression 2.59× root). Under 30% full light, expression was root > leaf > stem (leaf expression 0.70× root). Under 10% full light, expression was leaf > root > stem (leaf expression 2.75× root). Notably, root expression was highest under 30% full light [Figure 7: see original paper].

Figure 7 *GrHDR* expression under different light conditions.

2.6 Gentiopicroside Content Analysis

Gentiopicroside showed good linearity (r = 0.9993) in the range of 0.001-0.500 $mg \cdot mL^{1}$ with regression equation y = 24,960x + 28.671. Calculated content (mass fraction) is shown in [Figure 8: see original paper]. Most root and leaf samples exceeded 1.5% gentiopicroside, meeting pharmacopeia standards. Across all light conditions, content followed root > leaf > stem, consistent with secoiridoid accumulation patterns. Under 100% full light, root gentiopicroside reached 7.141%, 2.25× and 2.26× higher than under 30% and 10% full light, respectively.

Figure 8 Gentiopicroside content in different tissues of *G. rigescens* under different light conditions.

Discussion

Flux through the MEP pathway determines final product yield, while enzyme reaction rates control pathway flux. Although all MEP pathway genes have been identified in bacteria and plants, extensive work is needed to analyze their roles in controlling intermediate flux. The final MEP step, catalyzed by HDR (formerly LytB or IspH), converts HMBPP to IPP/DMAPP and critically regulates terpenoid synthesis. HDR overexpression can drive IPP/DMAPP synthesis at a 5:1-6:1 ratio for artemisinin production. HDR expression varies significantly among tissues in *Camptotheca acuminata* and *Dendrobium officinale*, paralleling differences in terpenoid content.

In this study, we cloned *GrHDR* from *G. rigescens* and analyzed its encoded protein. The 1,398 bp ORF encodes a 465-amino-acid LYTB family member localized to chloroplasts, consistent with the plastidial MEP pathway. GrHDR shows highest similarity to *G. macrophylla* HDR, with phylogenetic analysis confirming their close relationship.

Shading affects plant gene expression and secondary metabolite accumulation. For example, caffeine content in tea shows a decreasing-then-increasing trend under different shading, while key caffeine biosynthesis genes like *TCS1* show the opposite expression pattern. We examined *GrHDR* expression and gentiopicroside content under three shading levels. *GrHDR* was expressed in all tissues, with expression patterns varying under different light conditions and not completely matching gentiopicroside accumulation patterns. The material in [Figure 6: see original paper] was from 30% full-light conditions, showing root > leaf > stem expression, consistent with [Figure 7: see original paper] under the same light level, though September samples ([Figure 7: see original paper]) showed higher expression than May samples ([Figure 6: see original paper]), suggesting light duration affects expression. Further studies could examine expression changes across growth years. Gentiopicroside accumulation consistently showed root > leaf > stem, but root content was lower under shading, suggesting *G. rigescens* seedlings may not require shading during cultivation.

The relationship between *GrHDR* expression and gentiopicroside content is complex. Under 100% full light, one-year-old plants accumulated gentiopicroside as root > leaf > stem, while *GrHDR* expression was leaf > stem > root. This may reflect gentiopicroside synthesis in leaves, followed by transport to roots for storage. Since gentiopicroside biosynthesis involves multiple genes, *GrHDR* expression alone cannot definitively explain content variation.

This study provides sequence and expression analysis of *GrHDR*, contributing to molecular understanding of gentiopicroside biosynthesis, offering candidate genes for metabolic engineering, and enriching our knowledge of *GrHDR* expression patterns. The examination of *GrHDR* expression and gentiopicroside content under different light conditions provides guidance for *G. rigescens* cultivation. Future work should investigate gene function through overexpression and complementation studies to further clarify the relationship between *GrHDR*

expression and gentiopicroside content.

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