Cloning and Expression Analysis of the PvDXS Gene from *Prunella vulgaris* (Postprint)

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Abstract

Based on transcriptome sequencing of Prunella vulgaris, specific primers were designed to obtain the full-length nucleotide sequence of this gene via reverse transcription PCR. Bioinformatics analysis was subsequently performed, and qRT-PCR was employed to analyze the expression levels of PvDXS in different tissues of Prunella vulgaris and under induction by various exogenous substances. The cloned PvDXS gene contained an open reading frame of 2181 bp, encoding 726 amino acids with a theoretical molecular weight of 78,040.47 Da and an isoelectric point of 6.75. The PvDXS protein possesses both Transketolase_C and Transket_pyr domains. Phylogenetic analysis revealed that PvDXS is closely related to DXS proteins from Salvia miltiorrhiza and Catharanthus roseus (SmDXS2 and CrDXS2), suggesting that PvDXS belongs to the type II DXS protein family. qRT-PCR analysis demonstrated that PvDXS expression was higher in leaves compared to fruit spikes and stems. Following treatment of fruit spikes with seven exogenous substances for 24 h, GA3 treatment increased PvDXS expression, whereas the other six treatments decreased its expression, with CaCl2, SNP, and SA treatments causing significant reductions. The PvDXS gene exhibited substantial differential expression across tissues and was responsive to exogenous substance induction. These findings establish a foundation for further investigation of the PvDXR gene' s function and regulatory mechanisms in the terpenoid biosynthetic pathway of Prunella vulgaris.

Full Text

Preamble

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Cloning and Expression Analysis of the PvDXS Gene from *Prunella vulgaris*

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Abstract: Based on transcriptome sequencing data of *Prunella vulgaris*, specific primers were designed to obtain the full-length nucleotide sequence of the PvDXS gene via reverse transcription PCR. Bioinformatics analysis was performed, and qRT-PCR was used to analyze PvDXS expression levels in different tissues and under various exogenous substance treatments. The cloned PvDXS gene contained an open reading frame of 2,181 bp, encoding 726 amino acids with a theoretical molecular weight of 78,040.47 Da and an isoelectric point of 6.75. The PvDXS protein possessed both Transketolase_C and Transket_pyr domains. Phylogenetic tree analysis revealed that PvDXS was closely related to DXS proteins from *Salvia miltiorrhiza* (SmDXS2) and *Catharanthus roseus* (CrDXS2), suggesting that PvDXS belongs to the class II DXS protein family. qRT-PCR analysis showed that PvDXS expression was higher in leaves than in fruit spikes and stems. After treatment of fruit spikes with seven exogenous substances for 24 h, gene expression increased under GA3 treatment but decreased under the other six treatments, with significant reductions observed following CaCl2, SNP, and SA treatments. These findings demonstrate that PvDXS exhibits tissue-specific expression patterns and is responsive to exogenous induction, providing a foundation for further investigation of PvDXS function and regulatory mechanisms in terpenoid biosynthesis pathways in *P. vulgaris*.

Keywords: *Prunella vulgaris*; 1-deoxy-D-xylulose 5-phosphate synthase gene; gene cloning; expression analysis

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Introduction

DXS is a transketolase that catalyzes the non-mevalonate pathway (DXP pathway). Under the action of thiamine pyrophosphate (TPP), this enzyme decarboxylates pyruvate and condenses it with glyceraldehyde-3-phosphate to form DXP [**?**, **?**]. Research indicates that DXS is localized to plant plastid thylakoids [**?**, **?**]. The enzyme contains three functional domains: a TPP-binding domain, a transketolase domain, and a pyrimidine-binding domain, along with a plastid transit peptide at its N-terminus [**?**, **?**]. Querol et al. (2001) reported a high-performance liquid chromatography method for measuring DXS enzyme activity in acidic media. As a crucial rate-limiting enzyme in terpenoid biosynthesis, cloning the DXS gene is essential for studying terpenoid biosynthetic pathways. While DXS gene expression, regulation, and genetic transformation

have been investigated in various plants [**?**, **?**], few studies have focused on the DXS gene in *Prunella vulgaris*.

Prunella vulgaris is a Lamiaceae herb whose dried fruit spikes are used medicinally for clearing liver heat, improving vision, and reducing swelling. Terpenoids represent important bioactive components in this species. Sun et al. (2014) found that the tissue expression pattern of DXS in *Conyza blinii* positively correlated with the accumulation pattern of the diterpenoid blinin, suggesting that DXS overexpression might enhance diterpenoid synthesis. Gong et al. (2006) reported that the DXS gene in *Ginkgo biloba* was regulated by exogenous substances such as MeJA and ASA. Building upon our previous transcriptome database of *P. vulgaris*, this study cloned the DXS gene using RT-PCR and conducted bioinformatics analysis, tissue expression profiling, and induction expression analysis to elucidate its role in terpenoid metabolism.

1. Materials and Methods

1.1 Materials and Reagents

Prunella vulgaris samples were collected from a GAP cultivation base in Queshan County, Henan Province, and identified by Professor Chengming Dong of Henan University of Traditional Chinese Medicine as *Prunella vulgaris* L. (PVL).

Total RNA extraction kit (Beijing CoWin Biotech Co., Ltd.), reverse transcription kit (Thermo Fisher Scientific), qPCR kit (QIAGEN), DNA marker (TaKaRa), PCR product purification kit (Shanghai Sangon Biotech), PCR instrument (Bio-Rad C1000 Touch Thermal Cycler), and real-time PCR system (Applied Biosystems Step One Plus) were used.

1.2 Total RNA Extraction and First-Strand cDNA Synthesis

Total RNA was extracted from different tissues of *P. vulgaris* using the CoWin plant RNA extraction kit, and RNA integrity was verified by 1% agarose gel electrophoresis. First-strand cDNA synthesis was performed according to the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit protocol and stored at -20°C.

1.3 Full-Length cDNA Cloning

Based on the *P. vulgaris* transcriptome database, a pair of specific primers (DXR-F, DXR-R) was designed from the untranslated regions flanking the DXR gene sequence (Table 1). Using leaf cDNA as template, PCR amplification was performed in a 20 L reaction containing 2.0 L cDNA, 10.0 L 2× Es Taq mix, 1.0 L each of 10.0 mol · L ^{1} forward and reverse primers, and 6.0 L ddH O. The thermal cycling program was: 95°C for 1 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1.5 min; and final extension at 72°C for 5 min. PCR products were detected by 1% agarose gel electrophoresis, and target bands were

excised, purified, ligated into the pMD19-T vector, and transformed. Positive clones identified by blue-white screening and colony PCR were sequenced by Sangon Biotech. The constitutively expressed *actin* gene (KJ010818) served as an internal reference.

Table 1 Primer sequences and applications

Gene naming	Primer sequence (5' to 3')	Application
DXS-F	5' -ATGTCATCGTCTTGTGGAGTTATC-3'	Gene cloning
DXS-R	5' -ATGTTCGTCTTGTGGAGTT-3'	Gene cloning
qDXS-F	5' -CTTGCTCAAGGCTCCAACGG-3'	Real-time PCR
qDXS-R	5' -GCCGGATTCCTCGACTCCAA-3'	Real-time PCR
qactin-F	5' -GACCAGCTCTGCTGTGGAGA-3'	Fluorescence quantitative reference gene
qactin-R	5' -ATGGCTGGAAGAGGACCTCAG-3'	Fluorescence quantitative reference gene

1.4 Bioinformatics Analysis

Online tools were used for bioinformatics analysis of the PvDXS gene and encoded protein. ORF Finder (http://www.bioinformatics.org/sms2/orf_find.html) was used to analyze the open reading frame. DNAMAN software was employed to translate the amino acid sequence and align homologous proteins. ExPASy Proteomics Server Protparam (http://www.expasy.ch/tools/protparam.html) analyzed physicochemical properties. SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) predicted signal peptides. SMART (http://smart.emb-heidelberg.de/) analyzed protein functional domains. NPSA server (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_sopma.pl) predicted secondary structure. Swiss-Model (http://swissmodel.expasy.org/interactive) predicted tertiary structure. BaCelIo (http://gpcr.biocomp.unibo.it/bacello/) and ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) predicted subcellular localization and chloroplast transit peptide cleavage sites. MEGA5.1 software constructed a phylogenetic tree using amino acid sequences of the target gene and homologs.

1.5 Tissue-Specific and Induced Expression Analysis of PvDXS

During the vigorous growth period of *P. vulgaris*, fruit spikes of uniform growth status were treated by spraying with: 50 μmol · L ^{1} methyl jasmonate (MeJA), 17.14 μmol · L ^{1} indole-3-acetic acid (IAA), 100 μmol · L ^{1} ethephon (ETH), 100

μmol · L ¹ sodium nitroprusside (SNP), 1 mmol · L ¹ anhydrous calcium chloride (CaCl), 10 μmol · L ¹ salicylic acid (SA), or 2.88 μmol · L ¹ gibberellin (GA). Untreated fruit spikes (0 h) served as controls, and samples were collected 24 h after treatment.

Relative expression levels of PvDXS in fruit spikes, stems, leaves, and fruit spikes treated with seven exogenous substances were determined by quantitative real-time PCR (qPCR). The 20 μL qPCR reaction contained 10.0 μL 2× SYBR Green PCR Master Mix, 0.1 μL QN Rox Reference Dye, 0.4 μL each of forward and reverse primers, 2.0 μL template cDNA, and 7.1 μL RNase-Free Water. The thermal program was: 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 56°C for 20 s, then 95°C for 1 s and 60°C for 20 s. The *actin* gene (KJ010818) served as an internal reference with specific primers (Table 1). Melting curve analysis was performed after amplification, and relative expression levels were calculated using the 2^(-ΔΔCt) method.

2. Results and Analysis

2.1 Cloning of PvDXS Gene

Based on *P. vulgaris* transcriptome sequencing data, primers were designed and PCR amplification using reverse-transcribed cDNA as template yielded a fragment of approximately 2,000 bp. ORF Finder analysis of the cloned and sequenced product revealed a complete open reading frame of 2,181 bp, designated as PvDXS.

Note: 1. PCR amplification product of PvDXS gene; M. DL2000 DNA marker.

Figure 1 [Figure 1: see original paper] Cloning of DXS gene from *P. vulgaris*

2.2 Bioinformatics Analysis

2.2.1 Physicochemical Properties ExPASy Proteomics Server Protparam analysis predicted that the encoded protein contains 726 amino acids with a theoretical molecular weight of 78,040.47 Da and an isoelectric point (pI) of 6.75. The total atom count is 11,002, with atomic composition C H N O S . The protein contains 77 negatively charged residues (Asp + Glu) and 73 positively charged residues (Arg + Lys), indicating an acidic protein. Alanine (Ala) is the most abundant amino acid (9.5% of total), followed by glycine (Gly) (9.2%), while cysteine (Cys) is the least abundant (1.2%). Hydrophobicity prediction revealed the lowest score of -2.522 at position 183 and the highest score of 2.332 at position 710, with fewer hydrophobic than hydrophilic amino acids, suggesting PvDXS is a hydrophilic protein (Figure 2 [Figure 2: see original paper]).

Figure 2 Prediction of hydrophobicity of PvDXS protein

2.2.2 Subcellular Localization and Functional Domains Subcellular localization prediction indicated that PvDXS is localized in chloroplasts. SMART analysis revealed that PvDXS contains Transketolase_C and Transket_pyr domains located at positions 583–706 and 404–569, respectively. The protein also contains a conserved IPP-binding domain (DXP_synthase_N) similar to transketolase and the E1 subunit of pyruvate dehydrogenase (E1_dh family) at positions 82–367 and 109–261 [**?**, **?**].

Figure 3 [Figure 3: see original paper] PvDXS functional domain prediction

2.2.3 Secondary and Tertiary Structure Prediction NPSA server analysis of the PvDXS-encoded protein predicted a mixed structure comprising 36.91% -helix, 19.7% extended strand, 10.33% -turn, and 33.06% random coil. Swiss-Model prediction showed that PvDXS shares 49.11% similarity with *E. coli* DXS protein (2o1s.1.A).

Note: Blue, -helix; Red, extended strand; Green, -turn; Orange, random coil.

Figure 4 [Figure 4: see original paper] Prediction of secondary structure of PvDXS protein

Figure 5 [Figure 5: see original paper] PvDXS protein tertiary structure prediction

2.2.4 Phylogenetic Tree Construction and Multiple Sequence Alignment Using BLAST, DXS protein sequences were retrieved from NCBI for *Arabidopsis thaliana* (AtDXS), *Salvia miltiorrhiza* (SmDXS), *Catharanthus roseus* (CrDXS), *Hevea brasiliensis* (HbDXS), *Medicago truncatula* (MtDXS), *Zea mays* (ZmDXS), *Aquilaria sinensis* (AsDXS), *Vitis vinifera* (VvDXS), *Sorghum bicolor* (SbDXS), *Alpinia officinarum* (AoDXS), *Ginkgo biloba* (GbDXS), *Physcomitrella patens* (PpDXS), *Pyropia yezoensis* (PvDXS), *Elaeis guineensis* (EgDXS), *Pinus densiflora* (PdDXS), *Pinus taeda* (PtDXS), *Pinus kesiya* var. *langbianensis* (PkDXS), *Bifidobacterium longum* subsp. *longum* BBMN68 (BlDXS), and *Escherichia coli* (coliDXS). A phylogenetic tree was constructed using MEGA5.1 with the neighbor-joining (N-J) method, and multiple sequence alignment was performed using DNAMAN.

The phylogenetic tree showed that PvDXS is closely related to DXS from *S. miltiorrhiza* and *C. roseus* (SmDXS2, CrDXS2), suggesting it belongs to the class II DXS protein family, which is involved in secondary metabolite terpenoid biosynthesis [**?**, **?**]. The high similarity between PvDXS and SmDXS indicates high conservation within the Lamiaceae family. Multiple alignment revealed that PvDXS shares 73.76% sequence identity with HbDXS and contains a thiamine pyrophosphate-binding site (GDG(X) E(X) A(X) NDN) and a transketolase domain (DRAGX PXD). The protein localizes to chloroplasts with a 56-amino-acid plastid transit peptide at the N-terminus (Figure 7 [Figure 7: see original paper]).

Note: Accession numbers precede gene names.

Figure 6 [Figure 6: see original paper] Phylogenetic tree of PvDXS

Note: Solid frame indicates thiamine diphosphate binding site; dotted frame indicates transketolase domain; arrow indicates plastid transport peptide cleavage site.

Figure 7 [Figure 7: see original paper] Multiple alignment of PvDXS amino acid sequences

2.3 Tissue-Specific and Exogenous Substance-Induced Expression

qRT-PCR analysis of PvDXS expression in fruit spikes, leaves, and stems of *P. vulgaris* (with fruit spikes as control) revealed expression levels ranging from 0.671 to 753.296. PvDXS was highly expressed in leaves, moderately in fruit spikes, and lowest in stems, with leaf expression 753.2-fold higher than in fruit spikes, consistent with our previous transcriptome sequencing results.

Note: qPCR, verified relative gene expression; RPKM, transcriptome sequencing result.

Figure 8 [Figure 8: see original paper] Analysis of expression pattern of PvDXS gene

Figure 9 [Figure 9: see original paper] PvDXS expression after treatment with exogenous substances

Analysis of PvDXS expression trends in fruit spikes after treatment with seven exogenous substances showed that GA3 treatment significantly increased expression to 32.8-fold of the control. The other six treatments reduced expression to 0.005–0.260-fold, with CaCl , SNP, and SA showing the strongest inhibitory effects, followed by IAA, MeJA, and ETH treatments.

3. Discussion

Terpenoids are widespread plant secondary metabolites including steroids, quinones, carotenoids, and phytohormones that participate in various physiological processes such as photosynthesis and respiration [**?**, **?**]. DXS plays a key role in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway of terpenoid biosynthesis by catalyzing the condensation of pyruvate and glyceraldehyde-3-phosphate to form DXP [**?**, **?**]. Zhou et al. (2016) found that overexpressing SmDXS1 and SmDXS2 in *Salvia miltiorrhiza* hairy roots significantly increased tanshinone content. Sharma et al. (2015) reported that MeJA treatment (5 $\mathrm{mol \cdot L^{1}}$ for 24 h) increased andrographolide production 5.25-fold in *Andrographis paniculata* cell suspension cultures, correlating positively with DXS expression levels. Henriquez et al. (2016) observed increased chlorophyll and carotenoid levels in *Arabidopsis* leaves overexpressing potato StDXS1. These findings demonstrate that DXS is an important regulatory enzyme in terpenoid metabolism and that its expression can alter terpenoid content.

Studies have shown that DXS expression is tissue-specific, with stronger expression typically in green tissues such as leaves and stems and weaker expression in other organs [**?**, **?**]. Our qPCR analysis of PvDXS expression in leaves, stems, and fruit spikes of *P. vulgaris* revealed differential expression across tissues, with the highest level in leaves (753.2-fold higher than in fruit spikes). Based on sequence evolution, DXS genes are classified into three types: DXS I, DXS II, and DXS III [**?**, **?**]. Phylogenetic analysis showed that PvDXS is closely related to class II DXS proteins from *S. miltiorrhiza* and *C. roseus* (SmDXS2, CrDXS2), suggesting PvDXS belongs to the class II DXS family that encodes enzymes for specialized secondary metabolite biosynthesis, indicating its involvement in bioactive terpenoid production in *P. vulgaris*. Numerous studies have demonstrated positive correlations between DXS expression and secondary metabolite content. For example, Munoz-Bertomeu et al. (2016) reported that overexpressing *Arabidopsis* DXS in lavender significantly increased essential oil content in leaves and flowers. In our study, GA3 treatment dramatically increased PvDXS expression (32.8-fold), while the other six exogenous substances inhibited expression. Gong et al. (2006) found that ginkgolide synthesis correlated positively with GbDXS expression after MeJA and ASA treatment. Murcia et al. (2016) observed increased sugars and terpenoids in grapes after GA3 spraying, suggesting GA3 may enhance secondary metabolites such as terpenoids in *P. vulgaris*. Additionally, Sun et al. (2014) found that JsDXS expression in jasmine was regulated by circadian rhythms. The inhibitory effects of the other six exogenous substances on PvDXS may be related to such regulatory mechanisms. This study expands the repertoire of DXS genes in the MEP pathway and establishes a foundation for future functional studies.

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