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Cloning and expression analysis of 1-deoxy-D-xylulose-5-phosphate synthase gene from the medicinal plant *Conyza blinii* H.Lév.

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Abstract: *Conyza blinii* H.Lév. is a traditional Chinese medicinal plant that is distributed mainly in southwestern Sichuan and northern Yunnan. Its characteristic product is blinin, which has, among other properties, antigastric ulcer activity, and can serve as a quality-control standard for such medicine. The problem is that *C. blinii* only produces low yields of blinin. As a diterpene, blinin is likely formed by the methylerythritol phosphate pathway. While 1-deoxy-D-xylulose-5-phosphate synthase (DXS) is the first rate-limiting enzyme in diterpenoid biosynthesis, it is a switch in the pathway. The DXS gene was successfully cloned and characterized from *C. blinii* by homologous cloning and rapid-amplification of cDNA ends (RACE). It was designated *cbDXS* and contains a 2190-bp open reading frame encoding 730 amino acids (aa), including a 17-aa signal peptide and a 713-aa mature protein. Semiquantitative RT-PCR was used to determine the expression levels of *cbDXS* in different *C. blinii* tissues at the seedling stage. The corresponding blinin concentrations were also analyzed by high-performance liquid chromatography (HPLC). The *cbDXS* gene showed tissue specificity. Moreover, its expression levels were highly correlated to blinin concentrations. In summary, it is suggested that overexpression of this gene may increase flux toward blinin synthesis.

Key words: *Conyza blinii* H.Lév., blinin, DXS gene, RACE, semiquantitative RT-PCR, HPLC

1. Introduction

Conyza blinii H.Lév. is a member of the plant family Compositae. It is a traditional Chinese medicinal plant that is mainly distributed in southwestern Sichuan and northern Yunnan. It is used as a powerful drug for acute icteric hepatitis and chronic tracheitis. Its secondary metabolites include flavonoids, saponins, and blinin, among others. Of these, blinin is the characteristic product present in *C. blinii* and can be used as a quality-control standard in medicine (Wang et al., 2010). However, the plant produces only low yields of blinin, which are insufficient to meet the demand for pharmaceutical preparations. Improving blinin content is the key to improving the quality of *C. blinii*. Although knowledge of blinin biosynthesis and its pathway will provide a foundation for improving its content, there have been no studies on the blinin biosynthesis pathway.

Blinin is a new clerodane diterpene lactone. Terpenes can be biosynthesized via 2 pathways in plants: the mevalonate pathway or the methylerythritol phosphate (MEP) pathway (Ma et al., 2006). As a diterpene, blinin is likely formed by the latter (Figure 1). There are a number of key enzymes that regulate flux through the MEP pathway: 1-deoxy-D-xylulose-5-phosphate synthase (DXS) catalyzes the reaction of pyruvate with glyceraldehyde-3-phosphate, resulting in 1-deoxyxylulose-5-phosphate. This is the first step in the MEP pathway, and it has been documented in *Coleus forskohlii* (Engprasert et al., 2005) and *Elaeis guineensis* (Khemvong and Suvachittanont, 2005). DXS has been suggested as a regulatory enzyme in isoprenoid biosynthesis. In *Catharanthus roseus* hairy roots, the expression of DXS and geraniol-10-hydroxylase or anthranilate synthase increases accumulation of the terpenoid indole alkaloid (Peebles et al., 2011). In addition, overexpression of DXS increases anthraquinone production in transgenic cell-suspension cultures of *Morinda citrifolia* (Quevedo et al., 2010).

The study of changes in gene expression in different cell types or the same cell types at different developmental stages or under different developmental conditions has become a research hotspot in biology (Liu et al., 2004). Worapan et al. (2010) and Zhang et al. (2007) analyzed the mRNA expression levels of genes from various organs of *Croton stellatopilosus* and *Triticum aestivum* Linn. Semiquantitative RT-PCR is an effective method for

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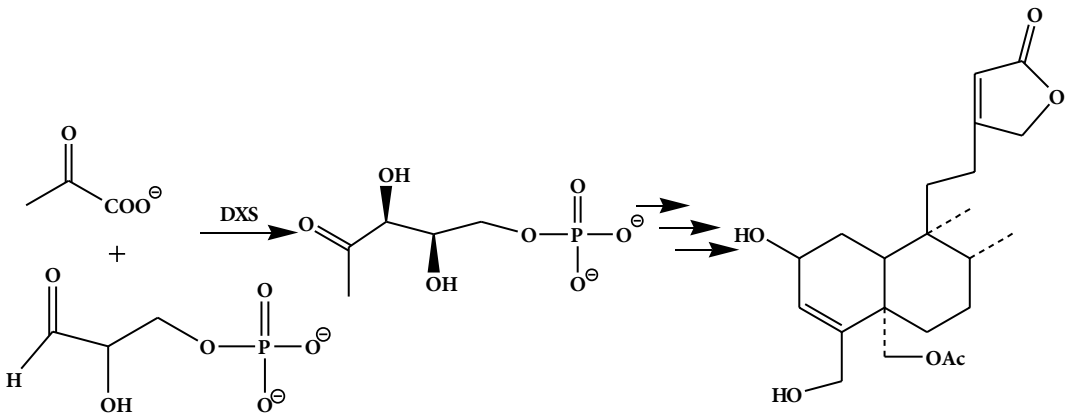

Figure 1. MEP pathway for blinin biosynthesis.

studying gene transcription levels (Cottrez et al., 1994) that is widely used due to its simple operation. Ding et al. (2010) used semiquantitative RT-PCR to investigate the effects of *Pseudoperonospora cubensis* Rostow, salicylic acid, and $CaCl_2$ on the expression of a resistance gene analog (RGA) in cucumber (*Cucumis sativus* L.) cultivar Dongnong 129, a variety that is resistant to downy mildew. Results showed that all of the treatments could increase the expression of RGA1 and RGA5. Using this method, Gao et al. (2006) determined the expression of the human bone morphogenetic protein-2 gene in different tissues of tobacco plants. The expression levels of a fusion protein in root and stem tissues were significantly higher than those in leaf tissues.

The aim of this work is to clone the DXS gene and analyze its expression. Rapid amplification of cDNA ends (RACE) technology was used to obtain the target gene. Semiquantitative RT-PCR was used to detect gene expression levels. Furthermore, blinin concentrations were analyzed by high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Plant material

The leaves, stems, and roots of *Conyza blinii* H.Lév. were obtained from plants growing in Panzhihua, a city located in southwestern Sichuan Province, in July 2012. All samples were identified by Prof Chun-Bang Ding of Sichuan Agricultural University. A voucher specimen (41945) was deposited in the Herbarium of the Department of Biology, Sichuan University, China.

2.2. Cloning of *DXS* cDNA

Total RNA was isolated from young leaves using the RNA Isolating Reagent Kit (TIANDZ). Total RNA (10 μg) was reverse-transcribed according to the manufacturer's protocol (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific) with oligo$(dT)_{18}$ primer (Thermo). After RNase H treatment, the resulting single-stranded cDNA mixtures were used as templates for the following PCR steps. According to the highly conserved amino acids, sets of degenerate primers were designed manually by Primer 5.0 software and used for core fragment amplification (Table) with the following PCR program: 94 °C for 4 min, then 30 cycles of 94 °C for 50 s, 50 °C for 40 s, and 72 °C for 50 s, with a final extension at 72 °C for 10 min. The PCR product was purified and cloned

Table. Primers used in this study.

Primer	Sequence (5'→3')
	Core fragment primers
DXSuF	ACACRCAGTTACKTTSGC
DXSuR	AATGCCAWTGCCTNTTGG
	5' and 3' RACE primers
3'-128	AGGGCTGGTTTGGTAGGAGCAGATGG
3'-209	GTGGTGATGGCTCCTTCTTGTGAGTCCG
5'-151	TCTGCTCCTACCAAACCAGCCCTGTCTAT
5'-339	TTATCTGGTGGGAGGAGTGAACCTATGC
5'-564	GCCTTTGCCTTTCTCGGTAACAATGTGGAC
	Recloning primers
DXSR	AAAATATATGGCTTCTTATAGTGC
DXSF	AGACTTTGGCATCATCTTTTCCCTG
	Semiquantitative RT-PCR primers
GAPDHsu	GTGGTGCCAAGAAAGTG
GAPDHsd	GCTAGAGGAGCAGGACA
DXSsu	AGGTTTAGCAACCGAAGG
DXSsd	TATCTGGTGGGAGGAGTGA

into the pMD19-T simple vector (TaKaRa), transformed into *Escherichia coli* strain DH5α, and sequenced. The core fragment was subsequently used to design the specific primers for cloning the full-length cDNA of *DXS* by RACE.

The 5' and 3' end cDNA fragments were amplified using the SMARTer RACE cDNA Amplification Kit (Clontech). The first-strand RACE-ready cDNA was prepared according to the kit and then used as a template for the second-strand RACE. The nested gene-specific primers designed for the 5' and 3' RACE are shown in the Table. The first cycle of amplification for RACE (both 5' RACE and 3' RACE) used the outside primers and UMP (universal primer A mix, provided in the kit) with the first-strand RACE-ready cDNA as templates. For the nested amplification, nested primers and NUP (nested universal primer A, provided in the kit) were used with the products of the previous amplification as templates. The PCR programs for 5' and 3' RACE were carried out under the conditions described in the kit's introduction. The first round consisted of 25 cycles of amplification (94 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min). The melting temperature of the next amplification round was increased by 2 °C and the number of cycles changed to 20. The nested PCR products were also purified and cloned into the pMD19-T simple vector, transformed into strain DH5α, and sequenced. The full-length cDNA sequence was obtained by assembling the core fragment, 3' RACE, and 5' RACE with DNAMAN software. The full-length cDNA sequence was then recloned using the gene-specific primers, which were designed according to the splicing sequence (Table). The sequence was then sequenced and analyzed by bioinformatic methods.

2.3. Bioinformatic analysis

The nucleotide sequence and the deduced amino acid (aa) sequence were analyzed and subjected to BLAST comparisons on the National Center for Biotechnology Information (NCBI) website. The SignalP 4.1 server was used to predict the signal peptide, and PSORT was used to predict its subcellular localization. The secondary structure was determined by the SOPMA server. MEGA 5.20 was used to construct the phylogenetic tree.

2.4. Extraction of blinin and determination of its content

Different tissues of *C. blinii* at the seedling stage were separately crushed in a grinder. A 1.5-g sample was then extracted with 45 mL of methanol by Soxhlet extraction. When the sample became colorless with a 50-mL volume, the solution was analyzed by HPLC. In addition, a blinin standard and methanol were used to produce standard solutions of different concentrations.

Blinin was analyzed on a Symmetry Shield C_{18} column with acetonitrile, methanol, and water (15:40:45, v/v) as the mobile phase for elution. The flow rate was 1.0 mL/min and the injection volume was 20 μL. Detection was performed under UV light (210 nm) at 25 °C. Three extraction samples were prepared for HPLC analysis, and each sample was injected 3 times.

2.5. Semiquantitative RT-PCR

Semiquantitative RT-PCR was used to determine the expression levels of *DXS* in the different types of tissues at the seedling stage. Total RNA was isolated from leaves, stems, and roots. The single-stranded cDNA was then reverse-transcribed with the PrimeScript RT Reagent Kit with gDNA Eraser (Thermo). The primer sets used to amplify *DXS* and the housekeeping gene *GAPDH* are shown in the Table. The PCRs were performed under optimal conditions. The PCR products were separated on a 2.0% agarose gel and stained with GoldView. Band intensities were measured using Quantity One software.

3. Results

The gene that was successfully cloned and characterized from *C. blinii* was named *cbDXS*. Its GenBank accession number is KJ155788. PCR amplification and sequence assembly showed that *cbDXS* consists of 2479 bp. It contains a 2190-bp open reading frame encoding 730 aa, including a 17-aa signal peptide and a 713-aa mature protein. The entire coding region was compared with other plant *DXS* genes and revealed high levels of similarity (77.65%; Figure 2). According to the BLAST results, *cbDXS* had high similarity to *DXS* from *Tagetes erecta* (83%), *Solanum lycopersicum* (76%), and *Picea glauca* (74%). BLASTP comparisons suggested that the protein belongs to the transketolase and dehydrogenase E1 component family (Figure 3).

The protein had a calculated molecular mass of 78 kDa and an isoelectric point of 6.96. Its 2-dimensional structure was predicted by PBIL based on the SOPMA method, giving a composition of 38.68% alpha helices, 13.44% extended strands, 6.31% beta turns, and 41.56% random coils. PSORT suggested localization of cbDXS protein in the chloroplast. This coincides with an earlier report predicting that most of the DXS proteins belong to the chloroplast (Krushkal et al., 2003).

All of the tested seedling-stage tissues expressed *cbDXS*, with the gene showing strong tissue specificity (Figure 4). The highest levels of transcripts were detected in the leaves (72.67%) and the roots (28.00%); the lowest expression was found in the stems (17.67%). Blinin concentrations were analyzed by HPLC. The equation of the calibration curve was $y = 48.822x + 88.279$, with $R^2 = 0.9991$. The highest level of blinin was found in the leaves (0.961%). The blinin

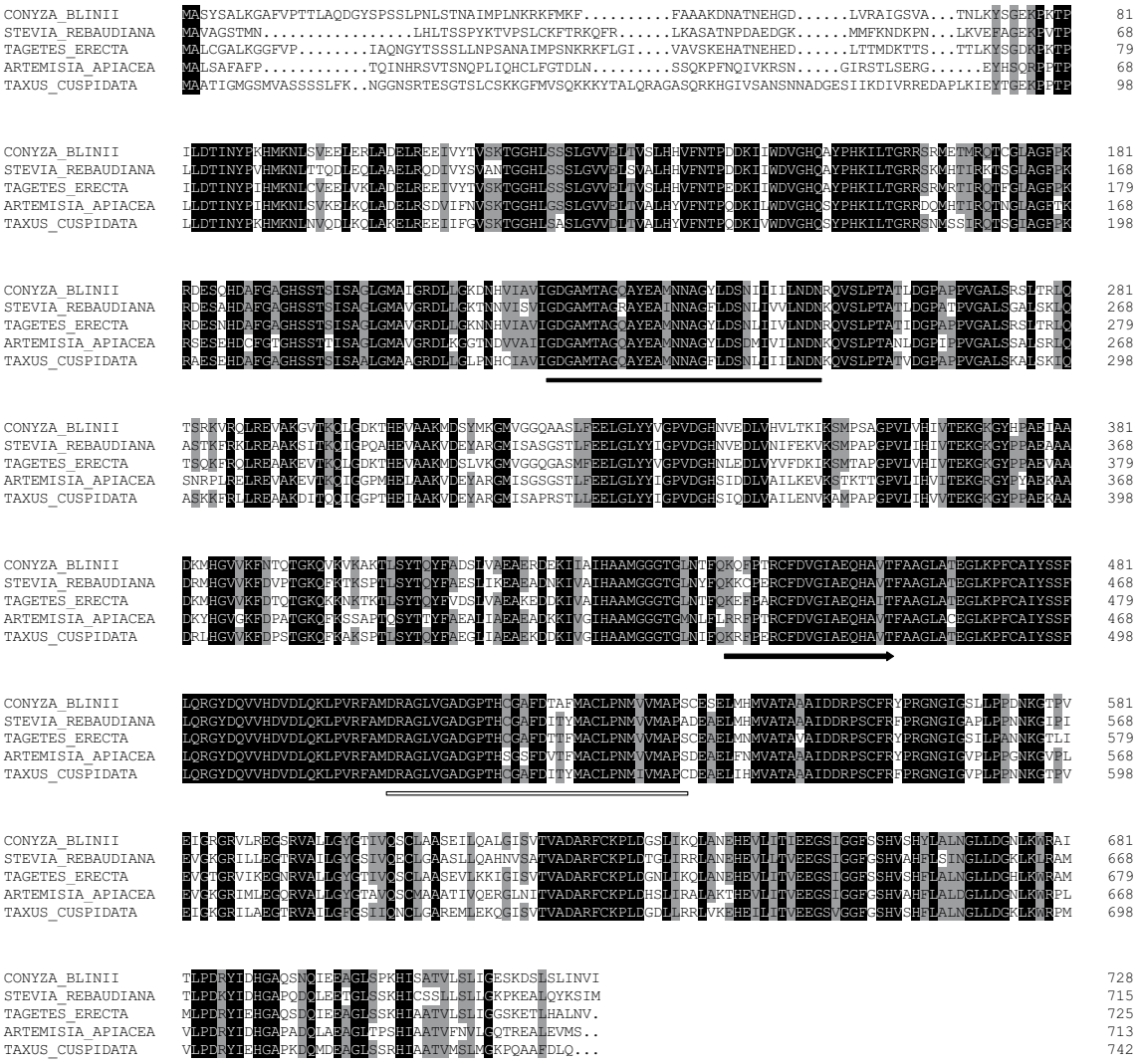

Figure 2. Alignment of the deduced amino acid sequences of cbDXS and related DXS proteins from *Stevia rebaudiana*, *Tagetes erecta*, *Artemisia apiacea*, and *Taxus cuspidata*. Residues shaded in black are identical residues that are conserved in the 5 sequences; residues shaded in gray are identical in 4 of the sequences shown. The thick horizontal line indicates the region corresponding to the putative thiamine–diphosphate binding site, and the pyrimidine-binding domain is marked by a double horizontal line. The transketolase motif is indicated by a long arrow.

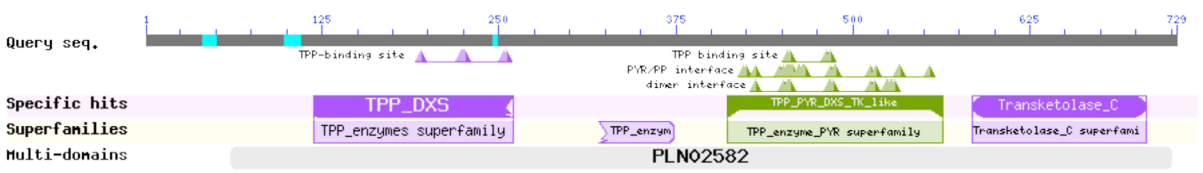

Figure 3. The result of protein comparisons by BLASTP.

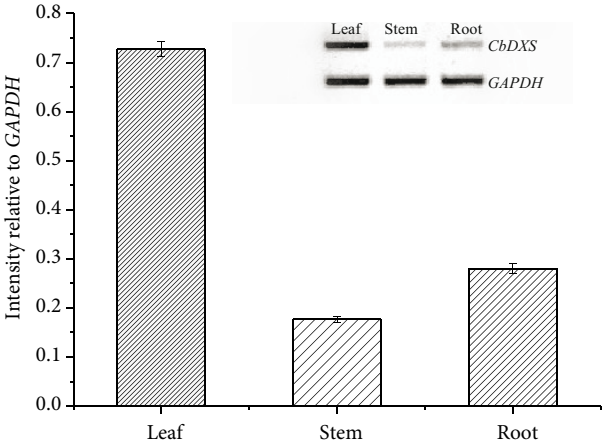

Figure 4. Expression levels of *cbDXS* in different tissues of *C. blinii* at the seedling stage determined by semiquantitative RT-PCR. Intensities are relative to *GAPDH*. Each tissue was analyzed in 3 replicates, and each band intensity was determined 3 times. Standard deviation was calculated by SPSS software.

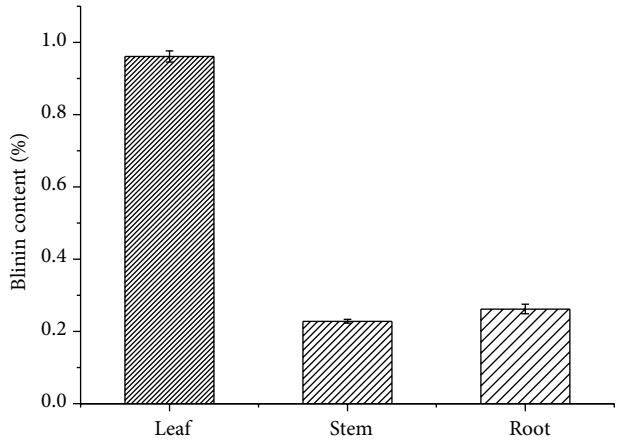

Figure 5. Blinin concentrations in different tissues of *C. blinii* at the seedling stage determined by HPLC. Each tissue was analyzed in 3 replicates. Standard deviation was calculated by SPSS software.

level was low in the roots and in the stems (0.262% and 0.228%, respectively) (Figure 5). The data showed that the expression levels of *cbDXS* and blinin concentrations were highly correlated.

4. Discussion

DXS plays an important role in the secondary metabolic pathway. It is a switch that regulates flux through the MEP pathway. It has been reported that the overexpression of *DXS* in *Arabidopsis* led to an increase in terpenoid concentrations (Estévez et al., 2001). Overexpression of DXS in transgenic cell-suspension cultures of *Morinda citrifolia* increases anthraquinone production (Quevedo et al., 2010). These results suggest that the overexpression of DXS can increase secondary metabolic concentrations; however, cloning and analysis of the DXS gene are important prerequisites. If we know nothing about the gene we cannot overexpress it. In this study we successfully cloned *cbDXS* from *C. blinii* and analyzed its expression to provide a basis for further study.

DXS has been identified and characterized from many plants (Bouvier et al., 1998; Lange et al., 1998; Lee et al., 2007). However, nothing is known about *DXS* from *C. blinii*. We successfully cloned *cbDXS* from this plant. It contained a 2190-bp open reading frame encoding 730 aa. This result is in line with a previous study suggesting that plant DXS protein is approximately 691 to 738 aa long (Jin et al., 2007). Cluster analysis showed a close genetic relationship between cbDXS and the DXS protein from *Tagetes erecta* (Figure 6) and a distant genetic relationship with the DXS protein from *Artemisia annua*. Plant DXS can be classified into 2 clusters: DXS1 and DXS2 (Walter et al., 2002). The cbDXS and DXS from *Tagetes erecta* belong to the DXS2 family, while the DXS from *Artemisia annua* belongs to DXS1 family, explaining our phylogenetic results.

The highest expression level of *cbDXS* was determined in the leaves, and the lowest level in the stems. The high expression in the leaves might be due to the increased demand for pigments for chloroplast formation and growth (Mayrhofer et al., 2005). Blinin is a new clerodane diterpenoid lactone. Similar to gibberellin, it may be produced mainly in tender leaves and root tips. Photosynthesis occurs in the leaf, and its metabolism is therefore more vigorous than the root's. Consequently, leaf blinin concentration was higher than root concentration.

The expression studies indicate correlation between levels of accumulated *cbDXS* mRNA in given tissues and the blinin concentrations. The *cbDXS* expression is highly correlated to blinin concentration. The results support the view that the *cbDXS* gene is involved in blinin biosynthesis. Overexpression of *cbDXS* is an attractive goal for metabolic engineering aimed at increasing blinin biosynthesis.

Aside from being a characteristic compound of *C. blinii*, blinin also has antiulcer activity (Su et al., 2007). Moreover, it has been reported that clerodane diterpenoids may prevent insects from feeding, and they shows antitumor activity (Xu et al., 1998). Studies show the medical value of blinin. In this manuscript, a basic study was performed of its biosynthetic pathway. However, systematic research into the blinin pathway is still lacking. Further work

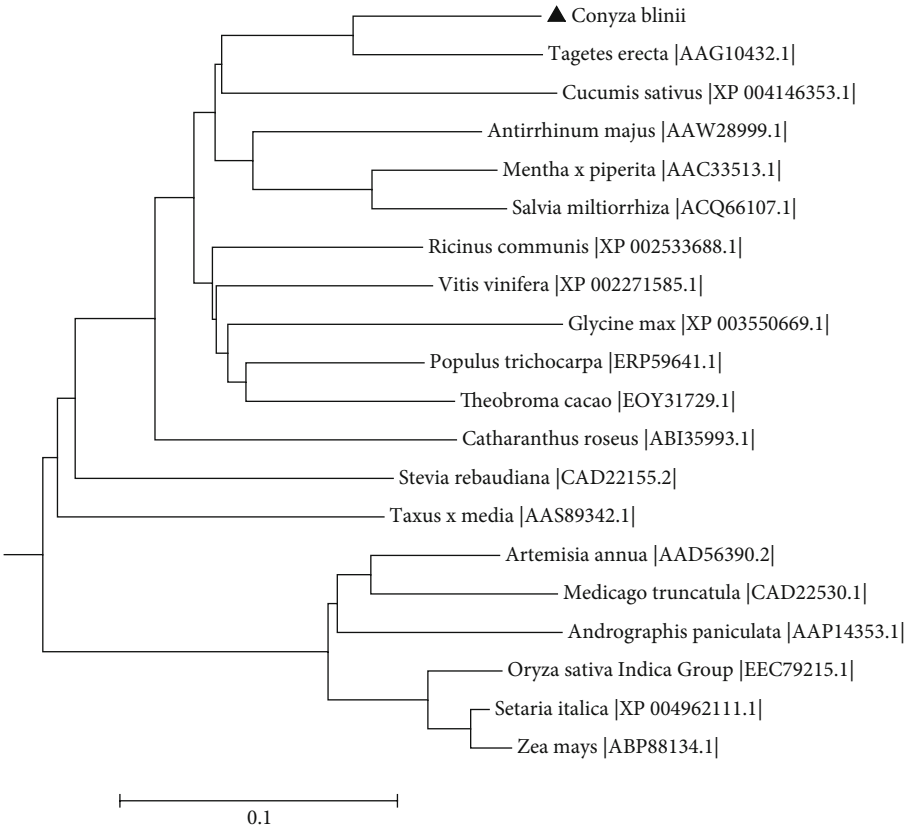

Figure 6. Phylogenetic analysis of DXS proteins from different plant species. Sequence analysis was performed using MEGA 5.2. The neighbor-joining method was used to create the tree. Accession numbers are written after the plant names.

should be invested in cloning other genes of this pathway, expressing those genes in plants, and understanding their functions with the aim of enhancing blinin concentrations and thereby enabling development of its potential.

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References

Bouvier F, d'Harlingue A, Suire C, Backhaus RA, Camara B (1998). Dedicated roles of plastid transketolases during the early onset of isoprenoid biogenesis in pepper fruits. Plant Physiol 117: 1423–1431.

Cottrez F, Auriault C, Capron A, Groux H (1994). Quantitative PCR: validation of the use of a multispecific internal control. Nucleic Acids Res 22: 2712–2713.

Ding GH, Xu CM, Yu H, Zhou XY, Qin ZW (2010). Expression analysis of the resistance gene analog in cucumber by semi-quantitative RT-PCR. Acta Bot Boreal-Occident Sin 30: 659–664.

Engprasert S, Taura F, Shoyama Y (2005). Molecular cloning, expression and characterization of recombinant 1-deoxy-D-xylulose-5-phosphate reductoisomerase from *Coleus forskohlii* Briq. Plant Sci 169: 287–294.

Estévez JM, Cantero A, Reindl A, Reichler S, León P (2001). 1-Deoxy-D-xylulose-5- phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J Biol Chem 276: 22901–22909.

Gao Y, Suo GL, Han J, He ZQ, Yao W, Dai JW (2006). Expression of human BMP-2 gene in different tissues of tobacco plants. Acta Genet Sin 33: 56–62.

Jin R, Zhu CQ, Xu CJ (2007). I-V deoxy xylulose-phosphate synthase (DXS) et gene encoding. Chinese J Cell Biol 29: 706–712 (article in Chinese).

Khemvong S, Suvachittanont W (2005). Molecular cloning and expression of a cDNA encoding 1-deoxy-D-xylulose-5-phosphate synthase from oil palm *Elaeis guineensis* Jacq. Plant Sci 169: 571–578.

Krushkal J, Pistilli M, Ferrell KM, Souret FF, Weathers PJ (2003). Computational analysis of the evolution of the structure and function of 1-deoxy-D-xylulose-5-phosphate synthase, a key regulator of the mevalonate-independent pathway in plants. Gene 313: 127–138.

Lange BM, Wildung MR, McCaskill D, Croteau R (1998). A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. P Natl Acad Sci U S A 95: 2100–2104.

Lee JK, Oh DK, Kim SY (2007). Cloning and characterization of the dxs gene, encoding 1-deoxy-D-xylulose-5-phosphate synthase from *Agrobacterium tumefaciens*, and its overexpression in *Agrobacterium tumefaciens*. J Biotechnol 128: 555–566.

Liu ZC, Zhao J, Liu MJ (2004). An introduction to mRNA differential display technique. Mol Plant Breeding 2: 895–900.

Ma L, Ding P, Yang GX, He GY (2006). Advances on the plant terpenoid isoprenoid biosynthetic pathway and its key enzymes. Biotechnol Bull 1: 22–30.

Mayrhofer S, Teuber M, Zimmer I, Louis S, Fischbach RJ, Schnitzler JP (2005). Diurnal and seasonal variation of isoprene biosynthesis-related genes in grey poplar leaves. Plant Physiol 139: 474–484.

Peebles CA, Sander GW, Hughes EH, Peacock R, Shanks JV, San KY (2011). The expression of 1-deoxy-D-xylulose synthase and geraniol-10-hydroxylase or anthranilate synthase increases terpenoid indole alkaloid accumulation in *Catharanthus roseus* hairy roots. Metab Eng 13: 234–240.

Quevedo C, Perassolo M, Alechine E, Corach D, Giulietti AM, Talou JR (2010). Increasing anthraquinone production by overexpression of 1-deoxy-D-xylulose-5- phosphate synthase in transgenic cell suspension cultures of *Morinda citrifolia*. Biotechnol Lett 32: 997–1003.

Su YF, Chen L, Lu Y, Chai X, Li M, Guo DA (2007). Chemical constituents and their antiulcerogenic studies on whole herb of *Conyza blinii*. Chinese Traditional and Herbal Drugs 38: 332–334.

Walter MH, Hans J, Strack D (2002). Two distantly related genes encoding 1-deoxy-d-xylulose-5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. Plant J 31: 243–254.

Wang R, Li WY, Xu H, Zhao SM, Hu YC, Li YM, Wang ZT (2010). M. qualitas medicinae Gentian. Journal of Chinese Medicinal Materials 33: 884–886 (article in Chinese).

Worapan S, Juraithip W, Tanawan S, Tossaton C, Yutaka E, Wanchai DE (2010). Cloning and expression of 1-deoxy-D-xylulose5-phosphate synthase cDNA from *Croton stellatopilosus* and expression of 2C-methyl-D-erythritol4-phosphate synthase and geranylgeranyl diphosphate synthase, key enzymes of plaunotol biosynthesis. J Plant Physiol 167: 292–300.

Xu JH, Shang ZZ, Yang SH, Chen HM, Min ZD (1998). Et actio est ratio agendi, ratione studiorum Cornelius diterpenoid insect aspernabilis. Acta Entomol Sin 41: 366–370 (article in Chinese).

Zhang XJ, Zhang LS, Yang Y (2007). Analysis of wheat dehydrin like gene during water stress condition by semi-quantitative RT-PCR. Acta Bot Boreal-Occident Sin 27: 2158–2162.