Gene Cloning and Functional Characterization of Three 1-Deoxy-D-Xylulose 5-Phosphate Synthases in Simao Pine

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Pine oleoresin is an important industrial resource, used widely in pharmaceuticals, cosmetics, and insecticides. To reveal the function of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in oleoresin biosynthesis in Simao pine, three complete cDNAs of DXS genes were obtained, of lengths 2223 bp (PkDXS1), 2217 bp (PkDXS2), and 2142 bp (PkDXS3). Phylogenetic analysis showed that PkDXS1 belonged to DXS type 1, and both PkDXS2 and PkDXS3 belonged to DXS type 2. Functional complementation experiments indicated that the three PkDXS genes had DXS protein function. Real-time PCR detection showed that physical wounding slightly influenced the gene expression of PkDXS1 and strongly influenced the gene expression of PkDXS2 and PkDXS3. The gene expressions of PkDXS3 in high-oleoresin-yield individuals were higher than their gene expressions in low-oleoresin-yield individuals. This result implied that the gene expressions of DXS regulated the oleoresin yields in different individuals of Simao pine. These results will provide information to help reveal the mechanisms of high-oleoresin-yield of Simao pine in the future.

Keywords: P. kesiya var. langbianensis; DXS; Oleoresin; Gene expression; Gene function confirmation

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INTRODUCTION

Pine oleoresin possesses complex mixed terpenes, including volatile monoterpenes (C10) and sesquiterpenes (C15) (turpentine) and nonvolatile diterpenes (C20) (rosin). There are more than 40,000 different metabolites from pine oleoresin (Rodrigues-Corrêa *et al*. 2012). Pine oleoresin is a raw material widely used in industrial products: camphene, isopimaric acid, and isolongifolene, used in the pharmaceutical industry; carveol, α-terpineol, and β-caryophyllene, used in the cosmetics industry; α- and β-pinene and farnesene, used in insecticides; bisabolene, germacrene, and limonene, used in the food industry; and carveol, farnesene, and longifolene, used in the chemical industry (Rodrigues-Corrêa *et al*. 2013). Recently published research has shown that oleoresin can replace chemical petroleum products and will be a large-scale renewable resource (Tsanaktsidis *et al*. 2016).

Pine oleoresin can be synthesized by two separate pathways, one being methylerythritol 4-phosphate (MEP) and the other being mevalonate (MVA) pathways in conifers. Monoterpenes and diterpenes are biosynthesized by the MEP pathway, while the biosynthesis of sesquiterpenes is completed by the MVA pathway (Vranová *et al*. 2013). 1-deoxy-D-xylulose 5-phosphate synthase (DXS) is the first and key enzyme of the MEP pathway, which catalyzes a transketolase decarboxylation of pyruvate and glyceraldehyde

3-phosphate (GA-3P) and produces 1-deoxy-D-xylulose 5-phosphate (Henriquez *et al*. 2016). Several reports have shown that DXS gene expression influences the product of terpenes in plants (Enfissi *et al*. 2005; Kim *et al*. 2009; Peng *et al*. 2013; Xu *et al*. 2014).

Simao pine (*Pinus kesiya* Royle ex Gordon var. *langbianensis* (A. Chev.) Gaussen) is an important oleoresin resource tree species in Yunnan province, China. The annual output of pine oleoresin from Simao pine is 179,100,000 kg (Dong *et al*. 2009), and 90% of gum turpentine output is from Simao pine in Yunnan (Yin *et al*. 2005). The forestland area of Simao pine is 0.56 million hm^2 (Li *et al*. 2017). However, individual variation in oleoresin yield is wide, ranging from 3 kg to 140 kg annually (Xu *et al*. 2012). Although several DXS genes have been cloned and characterized, the function of DXS genes is unclear in Simao pine. Also, researchers have found that there are multiple DXS in one species. The function of the DXS existing in Simao pine is still unclear. To reveal the mechanisms of high oleoresin yield in Simao pine, three 1-deoxy-D-xylulose 5-phosphate synthase genes from Simao pine were cloned, and their functions were analyzed by quantitative polymerase chain reaction (qPCR) and heterologous expression.

EXPERIMENTAL

Materials

Simao pines high in oleoresin yield and low in oleoresin yield were grown in a forest farm of Banpo Xiang, located in Jinggu county, Pu'er, Yunnan province, China. The V drop crossing method was used to measure resin yield (Miao *et al*. 2016). Both the high- and low-yield Simao pines came from seed breeding in 2006 and were 10 years of age. *E. coli* mutant EcAB4-2 was granted by Yanhong Xu of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China.

Methods

Transcriptome sequence and analysis

After comparing the oleoresin yields of individuals by the V drop crossing method, high-oleoresin-yield individual of Simao pine was selected. The bark of high-oleoresin-yield Simao pine was collected and immediately frozen in liquid N2 and stored at -80 °C until used. The RNA of Simao pine was extracted by a Qiagen Plant Mini RNA extraction kit. The transcriptome of high-oleoresin-yield Simao pine was *de novo* sequenced by BGI-Shenzhen, using an Illumina HiSeq 2000. To seek the putative DXS genes sequences in the transcriptome data, located Blast with known DXS protein was used to search all unigenes of transcriptome.

Cloning of PkDXS1, PkDXS2, and PKDXS3 genes

According to the transcriptome analysis, sequences of three DXS genes from the Simao pine transcriptome were obtained. The specific primers used for gene cloning were designed based on the sequences of the three DXS genes from transcriptome (Table 1). The RNeasy Plant Mini Kit (Qiagen) was used to isolate the RNA of Simao pine. Then, the cDNA was obtained by the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Beijing, China). The amplification of PCR products was performed by TransTaq DNA Polymerase High Fidelity. The PCR program consisted of 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 58 °C for 30 s, 72 °C for 3 min, and final extension at 72 °C for 10 min. The

PCR products were purified and cloned into the pEASY-T3 Cloning Vector (TransGene, Beijing, China). All positive clones were sequenced to ensure that the sequences were correct.

Table 1. Primers Used in This Study

Name	Sequence (5'-3')	Purpose
PkDXS1F	ATGGCAATTGCAAGCAGGGC	complete cDNA gene clone
PkDXS1R	TTATCGGTGCTTCAGAAGAG	
PkDXS2F	ATGGCATCACTGGGAGTGGT	
PkDXS2R	AGTGCTCTGC AGCTCCACTG	
PkDXS3F	ATGGCGATGGCATCGTCTGC	
PkDXS3R	TCAAGACATTACTTGCAGTG	
Tpkdxs1F	GGCACTTCATTATGTCTT	qPCR detection
Tpkdxs1R	GGCATCTTATCTCTTCTAC	
Tpkdxs2F	GCACTCCACTATGTATTCA	
Tpkdxs2R	ACCTCCTTCCTGTCAATA	
Tpkdxs3F	TACCAGATAGATACATTG	
Tpkdxs3R	GCATAACCTTATTGATAG	
EF1a-F	CAACAGACTTAACTTCAG	
EF1a-R	TTACAAGATTGGTGGTAT	

Bioinformatic analysis

Predictions of cell location and trans-membrane domains of proteins were made using TargetP (Emanuelsson *et al*. 2000) and ChloroP (Emanuelsson *et al*. 1999). To research conserved domains of DXS, the DNAman program (Lynnon Biosoft, Quebec, Canada) was used to create an alignment of five DXSs. To analyze the relationship between obtained DXS from Simao pine and other plant DXS, 39 plant DXS sequences were retrieved from GenBank. These DXS protein sequences were aligned with the ClustalW embedded in the MEGA 7.0.14 program. A phylogenetic tree was constructed using the minimum evolution method in MEGA 7.0.14 with 1000 replicates of bootstrap analysis.

Functional complementation

To confirm the functionality of the 3 DXS genes from Simao pine, the cDNA of PkDXS1, PkDXS2, and PkDXS3 was cloned into plasmid pEASY-Blunt E2. Expression vectors with PkDXS1, PkDXS2, and PkDXS3 were transformed into *E. coli* mutant EcAB4-2, which is defective in DXS activity (Perez-Gil *et al*. 2012). Transformants were selected on LB broth plates supplemented with the appropriate antibiotic (50 mg/mL ampicillin) and 1 mM mevalonate (MVA).

Quantitative real-time PCR (qRT-PCR) analysis

To investigate the function of PkDXS1-2, twig and needle of high- and low-oleoresin-yield Simao pine were collected and frozen in liquid nitrogen for RNA extraction. For the physical wounding induction experiments, the V drop crossing method was used to induce the drainage of oleoresin in bark. The bark samples of high- and low-oleoresin-yield Simao pine were collected and frozen in liquid nitrogen after physical wounding of 0 h, 6 h, and 12 h. The RNA of different samples was extracted using an RNeasy Plant Mini Kit (Qiagen). The cDNA synthesis was performed with 1 μg total RNA

using a reverse transcriptase kit (TaKaRa Super RT Kit) according to the manufacturer's instructions. SYBR Green (Invitrogen) was used for detection of PCR products with specific primers (Table 1). The optional parameters for 25 μL reaction system were as below: 2×SYBR Green master mix 12.5 μL, upstream and downstream primer (10 μM/L) 0.5 μL, template (cDNA) 1 μL, and ddH_2O 10.5 μL. PCR reactions were performed using a PCR thermal cycler (ABI 7300; Applied Biosystems, Foster City, CA, USA). The reaction system was used: denaturation program (95 ℃ for 10 min), amplification and quantification program repeated 45 times (95 ºC for 15 s, 57 ºC for 10 s, and 72 ºC for 15 s with a single fluorescence measurement), melting curve program (60 ºC to 95 ºC with a heating rate of 0.1 ºC/s and a continuous fluorescence measurement), and finally a cooling step to 40 ºC. The elongation factor 1-alpha (EF1a) gene was used as the internal control for normalization of gene expression. At least two independent biological replicates and three technical replicates of each biological replicate for each sample were analyzed by qPCR to ensure reproducibility and reliability. The comparative C_t value method to calculate relative gene expression quantity F, where $F=2^{-\Delta\Delta Ct}$ and $\Delta\Delta C_t$ = (C_t value of the test target gene group - C_t value of the test group reference gene) - (C_t value of the control group target gene - C_t value of the control group reference gene).

RESULTS AND DISCUSSION

Transcriptome *de Novo* Assembly

An Illumina HiSeq 2000 was used to generate RNAseq data for the high oleoresin yield Simao pine bark transcriptome. Finally, 5,828,966,460 bases were from 64,766,294 clean reads, and 59,636 unigenes were identified from assembled transcripts. The mean unigene size was 713 bp, and mean N50 was 1089 bp.

Gene Cloning of Complete cDNAs

For unigene annotation, sequence similarity searches were conducted against the NCBI non-redundant protein, Swiss-Prot protein, gene ontology (GO), and KEGG Orthology (KO) databases using the BLASTX algorithm. Then, 9 putative DXS unigenes were obtained by search annotation file of known DXS from NCBI and local BLAST search transcriptome assemble file (Table 2).

Table 2. The 9 Putative DXS Unigenes from Transcriptome Assemble File

Gene ID	Length (bp)	Raw fragments	FPKM
Unigene3555	954	34	1.4579
Unigene3217	2045	706	14.1223
Unigene1482	2607	1248	24.2294
Unigene19459	2537	4472	147.8854
Unigene21512	217	373	70.3141
Unigene23720	761	353	18.9751
Unigene24867	2888	58304	825.8369
Unigene25133	468	125	10.9259
Unigene280	772	227	12.0282

After open reading fragment analysis and bioinformatic analysis, there were three

complete cDNAs of DXS in the transcriptome of Simao pine. According to the sequence of DXS from the transcriptome, specific primers with start and stop codons were designed. Three DXS genes with complete open reading frames was cloned into pEASY-T3 Vector, named PkDXS1, PkDXS2, and PkDXS3. The results of sequencing showed that PkDXS1, PkDXS2, and PkDXS3 had 2223 bp, 2217 bp, and 2142 bp, respectively.

Bioinformatic Analysis of DXS from Simao Pine

The basic protein information of PkDXS1, PkDXS2, and PkDXS3 was predicted by the ExPASy Proteomics Server online software ProtParam. The PkDXS1 protein had 740 amino acids with a calculated molecular weight of 79.3 kDa and a pI value of 8.54; the formula of PkDXS1 was $C_{3541}H_{5668}N_{980}O_{1035}S_{27}$. The PkDXS2 protein had 738 amino acids with a calculated molecular weight of 79.1 kDa and a pI value of 8.22, and the formula of PkDXS2 was $C_{3514}H_{5609}N_{987}O_{1032}S_{30}$. The PkDXS3 protein had 713 amino acids with a calculated molecular weight of 76.7 kDa and a pI value of 6.40; the formula of PkDXS3 was $C_{3395}H_{5388}N_{948}O_{1019}S_{31}$. The ChloroP 1.1 Prediction Server was used to predict their chloroplast transit peptides. The results showed that PkDXS1, PkDXS2, and PkDXS3 had chloroplast transit peptides at the N terminus of the 48-, 58-, and 50- amino acid sequences, respectively. Alignment analysis by DNAman showed that PkDXS1, PkDXS2, and PkDXS3 had the thiamine pyrophosphate (TPP)-binding domain. All of them had the highly conserved sequence GDG as the start of the TPP-binding domain and the highly conserved sequence LNDN as the end of the TPP-binding domain. The residues in the active site of binding the glyceraldehyde 3-phosphate (GAP) molecule (Sangari *et al*. 2010) also existed in PkDXS proteins 1 to 3, such as histidine (PkDXS1, 134 PkDXS2, 135, PkDXS3, 109) and tyrosine (PkDXS1, 112; PkDXS2, 108, PkDXS3) (Fig. 1). The amino acid sequences of the three DXS from Simao pine and other DXS were used to generate multiple alignments and phylogenetic trees (Fig. 2). The phylogenetic tree showed that they could be divided into three main clades. Three DXS from Simao pine *langbianensis* belong to two main clades. PkDXS1 was grouped into the DXS1 clade and has the highest homology with *Pinus densiflora* DXS1. PkDXS2, PkDXS3, and another gymnosperm DXS type 2 were grouped into the DXS2 clade.

Table 3. Information of Three DXS from Simao Pine

Gene	ORF Length	AA number	molecular weight	pI	Formula
PkDXS1	2223 bp	740	79.3 kDa	8.54	$C_{3541}H_{5668}N_{980}O_{1035}S_{27}$
PkDXS2	2217 bp	738	79.1 kDa	8.22	$C_{3514}H_{5609}N_{987}O_{1032}S_{30}$
PkDXS3	2142 bp	713	76.7 kDa	6.40	$C_{3395}H_{5388}N_{948}O_{1019}S_{31}$

Fig. 1. Alignment of amino acid sequences for DXS. PaDXS: *Picea abies* DXS (ABS50519); PdDXS: *Pinus densiflora* DXS (ACC54554). The alignments were performed with DNAman. Amino acid residues conserved among all sequences are marked with an asterisk; variability between two amino acid residues is marked with a dot. The chloroplast transit peptides are marked with red. The transketolase consensus thiamine pyrophosphatase (TPP)-binding domain is marked with yellow. Residues in the active site of binding the glyceraldehyde 3-phosphate (GAP) molecule are in bold.

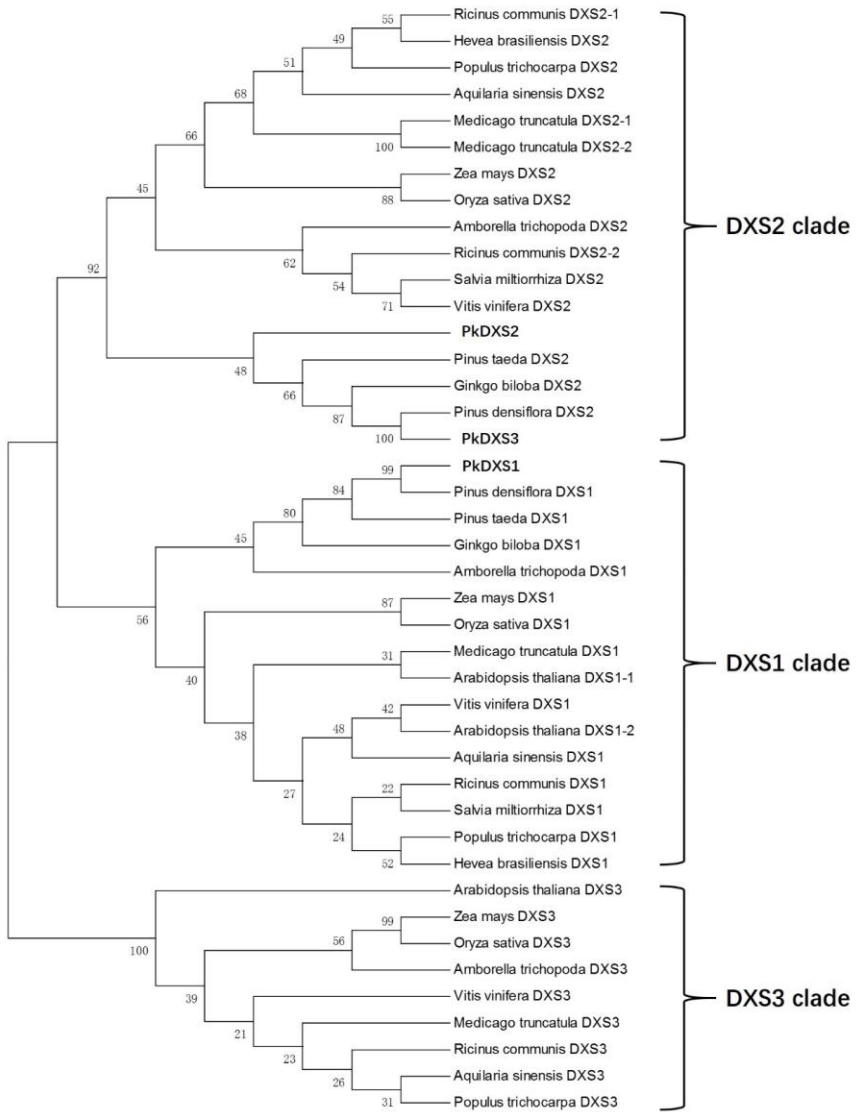

Fig. 2. Phylogenetic relationships of DXS from Simao pine with other plant DXS. The deduced DXS protein was aligned with fungal DXS sequences retrieved from GenBank. *Amborella trichopoda* DXS1 (XP_006855370.1); *A. trichopoda* DXS2 (XP_020523522.1); *A. trichopoda* DXS3 (XP_020529570.1); *Aquilaria sinensis* DXS1 (AFU75321.1); *A. sinensis* DXS2 (AHI62962.1); *A. sinensis* DXS3 (AFU75320); *Arabidopsis thaliana* DXS1-1 (NP_850620); *A. thaliana* DXS1-2 (NP_193291); *A. thaliana* DXS3 (NP_001078570); *Ginkgo biloba* DXS1 (AAS89341.1); *G. biloba* DXS2 (AAR95699.1); *Hevea brasiliensis* DXS1 (AAS94123.1); *H. brasiliensis* DXS2 (ABF18929.1); *Medicago truncatula* DXS1 (XP_003594033.1); *M. truncatula* DXS2-1 (XP_013446073.1); *M. truncatula* DXS2-2 (XP_003628891.2); *M. truncatula* DXS3 (XP_013461894.1); *Oryza sativa japonica* DXS1 (XP_015640505.1); *O. sativa japonica* DXS2 (XP_015647944.1); *O. sativa Japonica* DXS3 (XP_015642490.1); *Pinus densiflora* DXS1 (ACC54557.1); *P. densiflora* DXS2 (ACC54554); *P. taeda* DXS1 (ACJ67021); *P. taeda* DXS2 (ACJ67020.1); *Populus trichocarpa* DXS1 (XP_002312717.1); *P. trichocarpa* DXS2 (XP_002303416.1); *P. trichocarpa* DXS3 (XP_002308644.1); *Ricinus communis* DXS1 (XP_015573388.1); *R. communis* DXS2-1 (XP_002532384.1); *R. communis* DXS2-2 (XP_002533688); *R. communis* DXS3 (XP_002514364); *Salvia miltiorrhiza* DXS1 (ACF21004.1); *S. miltiorrhiza* DXS2 (ACQ66107.1); *Vitis vinifera* DXS1 (XP_002277919.1); *V. vinifera* DXS2 (XP_002277919.1); *V. vinifera* DXS3 (XP_002277919.1); *Zea mays* DXS1 (NP_001157805.1); *Z. mays* DXS2 (NP_001295426.1); *Z. mays* DXS3 (NP_001170088.1). DXS of Simao pine are marked in bold.

Function Confirmation of DXS by Complementation

To confirm the function of the PkDXS proteins, their cDNA was ligated into pEASY-Blunt E2 expression vectors. The PkDXS expression vectors were transferred into *E. coli* strain EcAB4-2, which is defective in the DXS gene and requires MVA for growth (Floss *et al*. 2008; Perez-Gil *et al*. 2012). Complementation experiments showed that *E. coli* strain EcAB4-2 with PkDXS (1 to 3) expression vectors could grow on the selection plate without MVA, while EcAB4-2 containing the empty vector did not grow on the same selection plate (Fig. 3). This result indicated that these three genes (PkDXS1, PkDXS2, and PkDXS3) encode functional DXS proteins.

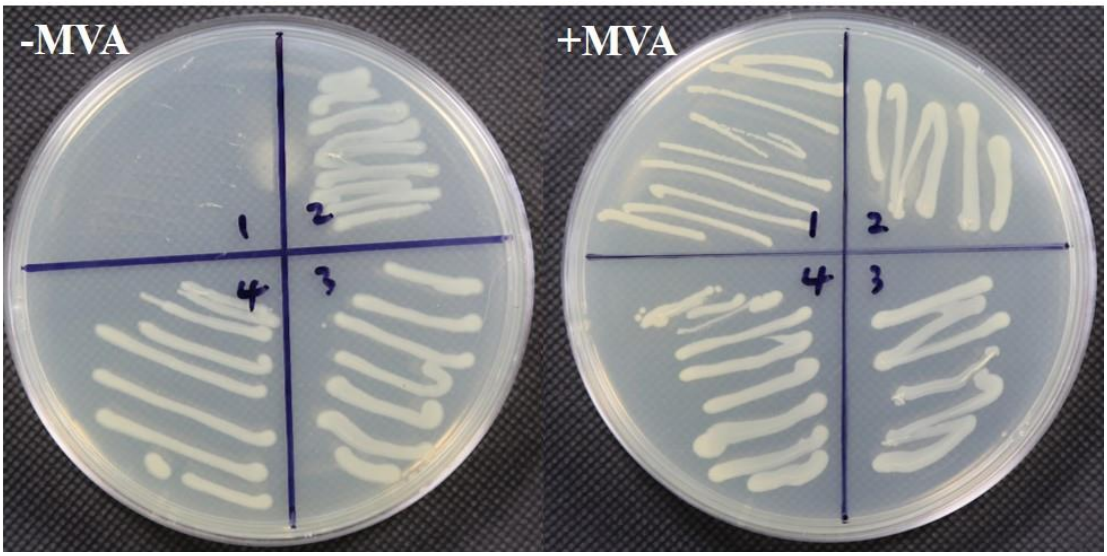

Fig. 3. Heterologous expression of PkDXS genes in *E. coli* strain EcAB4-2: *E. coli* strain EcAB4-2 with empty vector (negative control) (1); *E. coli* strain EcAB4-2 with PkDXS1, PkDXS2, and PkDXS3 expression vectors, respectively (2 to 4). Growth on media lacking mevalonate (-MVA) indicates an active DXS gene.

Different Tissue Expression Profile of DXS in Simao Pine

To detect the gene expressions of the PkDXS genes in different tissues and different individuals (high- and low-oleoresin-yield), bark of high- and low-oleoresin-yield individuals after physical wounding of 0 h, 6 h, and 12 h, and the pine needle and twig of high- and low-oleoresin-yield individuals were collected and immediately frozen in liquid nitrogen. RNA was isolated from all samples and converted to cDNA. SYBR Green was used for qPCR detection of the three PkDXS genes. The results showed that gene expression of PkDXS1 is lower than that of PkDXS2 and PkDXS3 in all of the tissues, and the physical wounding slightly influenced gene expression of PkDXS1. PkDXS2 and PkDXS3 showed highest expression in the pine needle of high- and low-oleoresin-yield individuals. Physical-wounding-induced gene expression of DXS also was observed. The qPCR detection showed that gene expression of PkDXS2 and PkDXS3 was maximized after physical wounding of 6 h. However, in the wounded bark and pine needle, the gene expressions of PkDXS3 in high-oleoresin-yield individuals were higher than their gene expressions in low-oleoresin yield-individuals (Fig 4). This result implied that the gene expression of DXS regulated the oleoresin yield in different individuals of Simao pine.

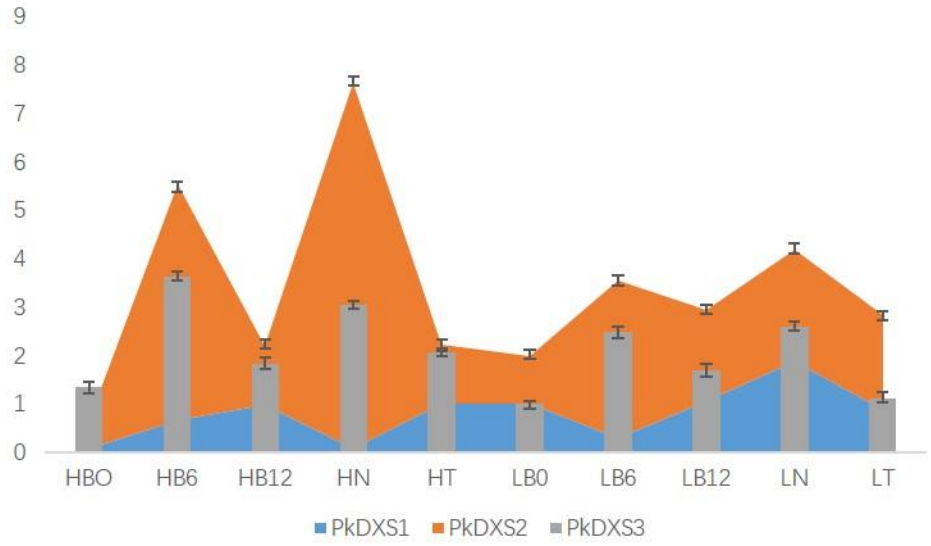

Fig. 4. Gene expression profile of PkDXS genes in different tissues: HB0, HB6, and HB12: bark of high-oleoresin-yield individuals after physical wounding of 0 h, 6 h, and 12 h; HN: pine needle of high-oleoresin-yield individuals; HT: twig of high-oleoresin-yield individuals; LB0, LB6, and LB12: bark of low-oleoresin-yield individuals after physical wounding of 0 h, 6 h, and 12 h; LN: pine needle of low-oleoresin-yield individuals; LT: twig of low-oleoresin-yield individuals. The gene expression detected by qPCR and the elongation factor 1-alpha was used as the internal control. Error bars indicate standard deviations of three biological replicates. The vertical coordinates are gene relative expression.

Discussion

Pine forests play important ecological functions. They are sinks of atmospheric carbon and provide renewable sources of numerous useful products, including wood, cellulose, and non-wood products, such as pine oleoresin (Rodrigues-Corrêa *et al*. 2012). Pine oleoresin is an abundant source of useful terpenes, which are used in the pharmaceutical, cosmetic, and food industries, as well as in the chemical industry in the manufacturing of various products, including paint, varnishes, adhesives, insecticides, and disinfectants (Rodrigues-Corrêa *et al*. 2013). Furthermore, pine oleoresin is a viable alternative to replace chemical petroleum products (Lieutier *et al*. 2004). Therefore, there have been many scientific studies on the oleoresin, including oleoresin tapping techniques (Wang *et al*. 2006; Füller *et al*. 2016; Rodríguez-García *et al*. 2016), the defense function of oleoresin (Trapp and Croteau 2001; Wainhouse *et al*. 2009), and influencing factors of oleoresin yield improvement (Rodríguez-García *et al*. 2015). Recently, there have been several reports focused on molecular mechanisms of oleoresin (Kim *et al*. 2009; Westbrook *et al*. 2013; Zhang *et al*. 2016). However, the mechanisms of high oleoresin yield in pine remain unclear.

1-deoxy-D-xylulose 5-phosphate synthase (DXS) is the first key enzyme of the MEP pathway involved with monoterpenes and diterpenes (Cordoba *et al*. 2009; Xiang *et al*. 2012). Multiple DXS genes have been found in *Aquilaria sinensis* (Xu *et al*. 2014), *Ginkgo biloba* (Kim *et al*. 2006), *Medicago truncatula* (Floss *et al*. 2008), *Oryza sativa* (Kim *et al*. 2005), *Picea abies* (Phillips *et al*. 2007), *Pinus densiflora* (Kim *et al*. 2009), and *Zea mays* (Cordoba *et al*. 2011). Phylogenetic analysis shows that the DXS genes in plants can be grouped into three independent clades (Cordoba *et al*. 2011; Xu *et al*. 2014). According to the function of DXS, the DXS can be divided into two types (Cordoba *et al*.

2009). DXS1 type is thought to be involved in photosynthetic processes (Cordoba *et al*. 2011), while DXS2 type is thought to be involved in biotic or abiotic resistance defenses and produce special isoprenoid compounds (Kim *et al*. 2006, 2009). In this study, phylogenetic analysis showed that three DXS genes from Simao pine were grouped into two clades. PkDXS1 belonged to the DXS1 clade, which is likely involved in photosynthetic processes, while PkDXS2 and PkDXS3 belonged to the DXS2 clade, which may be involved in pine oleoresin to resist biotic or abiotic injury. In *O. sativa* and *P. abies*, there are also three different DXS genes, two of which cluster into the same clade (Kim *et al*. 2005; Phillips *et al*. 2007). The gene expression profile also supported the results of phylogenetic analysis. Physical wounding strongly stimulated the gene expression of PkDXS2 and PkDXS3, with slight influence on the gene expression of PkDXS1.

To understand the function of DXS in high-oleoresin-yield individuals, the gene expressions of three DXS genes in high- and low-oleoresin-yield individuals were analyzed. The qPCR results showed that the gene expressions of PkDXS2 in the wounded bark and pine needle of high-oleoresin-yield individuals were obviously higher than in low-oleoresin-yield individuals. This result implies that the baseline and induced gene expression of PkDXS2 in high-oleoresin-yield individuals are higher than in low-oleoresin-yield individuals. PkDXS2 can be used as molecular markers to differentiate high- and low-oleoresin-yield individuals. Especially, the strengths of gene expressions of PkDXS2 in pine needle coincide with the oleoresin yield. In future work, more individuals of Simao pine will be studied to develop molecular markers for high-oleoresin-yield molecular breeding of Simao pine.

CONCLUSIONS

1. Three 1-deoxy-D-xylulose 5-phosphate synthases (PkDXS1, PkDXS2, and PkDXS3) were cloned from Simao pine, and their functions were confirmed by functional complementation experiments. This result means that there are three DXS genes at least.
2. Real-time PCR detection showed that the three DXS from Simao pine had different functions in pine oleoresin biosynthesis. The gene expressions of PkDXS2 and PkDXS3 regulated the oleoresin yield in different individuals of Simao pine.

ACKNOWLEDGMENTS

The authors are grateful for the support of the China Project of State Special Research Funds of Forestry Sector for Public Welfare, Grant No. 201304105, and the Yunnan Academy of Forestry Innovation Fund Project, Grant No. QN2018-01.

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Article submitted: March 7, 2018; Peer review completed: June 14, 2018; Revised version received: June 19, 2018; Accepted: July 3, 2018; Published: July 6, 2018.
DOI: 10.15376/biores.13.3.6370-6382