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Identification and Tissue-Specific Expression Analysis of *CYP720B* Subfamily Genes in Slash Pine and Loblolly Pine

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Abstract: Diterpene resin acids (DRAs) are major components of pine oleoresin that can effectively resist the invasion of insects and pathogenic microorganisms. The subfamily of cytochrome P450s, CYP720B, catalyzes diterpene products into DRAs. Identifying CYP720B subfamily members and revealing the characteristics of tissue-specific expression would help understand diterpene-rich structures and diverse types. Slash pine and loblolly pine are important pines that provide oleoresin products. In this study, we identified *CYP720B* candidate genes based on the *Pinus taeda* V2.0 genome and full-length transcriptome of slash pine by PacBio. A total of 17 genes in slash pine and 19 in loblolly pine were identified and classified into four main clades by phylogenetic analysis. An analysis of *cis*-acting elements showed that *CYP720B* genes were closely related to adversity resistance. The gene expression of these candidates in different tissues was quantified by real-time quantitative PCR (RT–qPCR) analysis. Most of the genes showed relatively higher expression levels in roots and stems than in the other tissues, corresponding with the results of DRA component detection by gas chromatography–mass spectrometry (GC–MS), which indicated that stems and roots might be important tissues in oleoresin biosynthesis. These results provide a valuable resource for a better understanding of the biological role of individual CYP720Bs in slash pine and loblolly pine.

Keywords: diterpene resin acids; CYP720B; expression patterns; biotic and abiotic stress

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1. Introduction

Diterpene resin acids (DRAs), which contain complex and variable diterpenoids, are important renewable resources of industrial biological products and have been widely exploited worldwide [1]. DRAs play a pivotal role in modern industrial applications and national economic development, such as in the production of solvents, flavors, fragrances, coatings, and resins [2–4]. DRAs, which are major components of pine oleoresin, play a critical role in conifer defense against insects and pathogens [5]. DRAs are distributed in resin canals, needles, stems, and roots. When herbivores or pathogenic microorganisms destroy these plant tissues, conifers produce a series of volatile mono- and sesquiterpene oleoresins to push insects out of the site of entry. The released DRAs can also clean and seal wounds [1]. Compared with mono- and sesquiterpenoids, detailed and thorough research on diterpenes for plants and insects is lacking. Many conifers produce tricyclic diterpene acids such as abietic, dehydroabietic, isopimaric, levopimaric, neoabietic, palustric, pimaric, and sandaracopimaric acids [1]. DRAs, originating from a common acyclic biosynthetic precursor (geranylgeranyl diphosphate, GGPP), are composed of 20-carbon bi- or tricyclic carboxylic acids of carbon skeletal types, commonly catalyzed by terpene synthases (TPSs) and cytochrome P450 enzymes (P450s) [6]. Their biosynthesis involves two steps: (1) diTPS catalyzes GGPP for multistep cyclization and rearrangement to produce various bicyclic

or tricyclic diterpenes; (2) carbon-18 in diterpenes is oxidized three times by P450s to form corresponding DRAs [7,8]. Diterpene synthase causes the structural diversity of the diterpene backbone, and P450s catalyze various oxidation and hydroxylation reactions in the primary and secondary metabolic processes of plants to produce various defensive DRAs [9,10].

The dominant catalyst for the biosynthesis of tricyclic DRAs is CYP720B from the CYP85 clan, which can accept various olefinic precursors (abietadiene and abietadiene-like, pimaradiene and pimaradiene-like, palustrastradiene, and dehydrabietadiene/abietatriene) or 13-hydroxy-8 (14)-abietene as substrates, which are unique to resinous conifers [11]. The CYP720B subfamily is known to be divided into four distinct clades (I–IV) [12]. The expression level of CYP720Bs can be increased after the conifers are injured, indicating that CYP720Bs are an important defense response gene in conifers [13]. For example, in *Picea abies* (L.) H.Karst., a large amount of DRAs accumulate and effectively prevent the invasion of *Ceratocystis polonica* after treatment with methyl jasmonate (MeJA), which is a lipophilic, linolenic-acid-derived plant hormone [14]. The expression levels of *CYP720B1* in *Pinus taeda* Linn. [15], *CYP720B4* in *Picea sitchensis* (Bong.) Carrière [16], and *CYP720B19* in *Pinus armandi* Franch. [17] are also significantly increased after treatment with MeJA. However, additional members of the CYP720B subfamily in conifers were recently described and remain to be functionally characterized [7,15,16].

Slash pine (*Pinus elliottii*. Engelm) and loblolly pine (*Pinus taeda*) have been widely cultivated in southern China since they were introduced from the southeastern United States in the last century [18]. With the advantages of fast growth, great strength, and high resin yield, slash pine and loblolly pine are widely used in industrial production, including the production of timber, paper, and oleoresin [19]. With the rapid development of domestic adhesives, coatings, inks, synthetic rubber, and other downstream industries, the global demand for resins continues to increase [20,21]. However, the discovery and verification of the gene function of the resin synthesis pathway are still at an early stage. At present, only the bifunctional multisubstrate *CYP720B1* (*PtCYP720B1*) has been found in loblolly pine. However, little is known about the diterpenoid profile of slash pine. The main objectives of the present study were to (1) identify *CYP720B* candidate genes based on slash pine transcriptome and loblolly pine genome sequences; (2) determine the gene expression patterns in different tissues, and (3) verify the content of diterpene resin acid in various tissues. These results will enrich our knowledge of *CYP720B* genes and strengthen our understanding of the slash pine and loblolly pine resin synthesis pathway.

2. Materials and Methods

2.1. Identification of CYP720B in Slash Pine and Loblolly Pine

The amino acid sequences of CYP720B subfamily members in conifers were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/sites/batchentrez, accessed on 15 August 2020). The accession numbers are listed in Table A1. The full-length transcriptome of slash pine [22] and loblolly pine v2.0 [23] assembly with annotations were used to identify the *CYP720B* candidate genes. A basic local alignment search tool (BLASTp) search (E-value < 1×10^{-50}) was performed between 29 previously characterized conifer CYP720Bs and the proteins corresponding to the full-length transcriptome of *Pinus elliotti* [22] and genome of *Pinus taeda* [23]. The conserved domain of *CYP720B* candidate genes were scanned in the Pfam database (http://pfam.xfam.org/, accessed on 15 August 2020) to confirm the integrity of the P450 domain (PF00067). The ClustalW program in MEGA X was used to remove the repetitive sequences. Moreover, the lengths of the amino acids, molecular weights (MWs), and isoelectric points (pIs) of CYP720B proteins were calculated using tools from ExPASy (http://www.expasy.ch/tools/pi_tool.html/, accessed on 15 August 2020).

2.2. Sequence Analysis of CYP720Bs

All identified *CYP720B* genes were divided into four clades according to previous studies [16]. Multiple alignments of CYP720B amino acids were performed using ClustalW. The phylogenetic trees were inferred by the neighbor-joining (NJ) method of MEGA X, with the following parameters: Poisson model, pairwise deletion, and 1000 bootstrap replications. In addition, the conserved CYP720B domains in proteins were identified using MEGA X. The gene structure analysis of loblolly pine *CYP720B* genes using the online program Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn/, accessed on 15 August 2020). The Multiple Expectation Maximization for Motif Elicitation (MEME) online program (http://meme-suite.org/tools/meme/, accessed on 15 August 2020) for protein sequence analysis was used to identify conserved motifs in the identified CYP720B proteins. The optimized parameters employed were the maximum number of motifs (10) and the optimum width of each motif (between 6 and 100 residues). A 2500 bp sequence upstream of the start codon of CYP720Bs was obtained and used to identify *cis*-acting elements using the PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 15 August 2020).

2.3. Plant Materials and Growth Conditions

The two-year-old slash pine and loblolly pine seedlings, which were selected with roughly the same growth, were grown in Hangzhou, Zhejiang Province (30°3′ N, 119°6′ E). The conditions of greenhouse were 75% relative humidity and 23 ± 2 °C temperature under a 16-h photoperiod with a photosynthetic flux density of approximately 50 ± 10 μmol m^{-2} s^{-1}.

To analyze the expression profile of *CYP720B* and DRA content in different tissues, the two-year-old slash pine and loblolly pine seedlings were chosen for the young and mature needles, young and mature stems, and root samples (Figure A1). One sample was one biological replicate, three biological replicates were used for each species. All tissues were flash-frozen in liquid nitrogen and stored at −80 °C.

2.4. RNA Isolation and Gene Expression Analyses

Total RNA was isolated using the RNA Prep Pure Plant Plus Kit (TIANGEN BIOTECH company, Beijing, China). All tissues (50–100 mg) were ground into a fine powder in liquid nitrogen and processed to generate total RNA. The concentration of RNA was analyzed by agarose gel electrophoresis and then measured with an ultramicro UV spectrophotometer (Thermo Scientific NanoDrop 2000 and 2000c Spectrophotometers, Waltham, MA, USA). DNA-free RNA was used for the synthesis of the first strand of cDNA by using the PrimeScript™ RT Master Mix kit (Perfect Real Time, Takara Bio, RR036A, Dalian, China).

For the quantification of target transcripts, the housekeeping slash pine *UBI* gene and loblolly pine *18S* rRNA were used as internal controls [24,25]. For each biological replicate, RT–qPCR was performed with three technical replicates using TB Green Premix Ex Taq™ II (Takara Bio, RR820A, Dalian, China) on an ABI QuanStudio 7 Flex System (Life Technologies), following the manufacturer's recommendations. RT–qPCR was performed in a 20 μL reaction system containing 6 μL ddH_2O, 5 μL 2× TB Green Premix Ex Taq II, 0.8 μL forward primer (10 μM), 0.8 μL reverse primer (10 μM), and 2 μL of template cDNA (5 ng/μL). The PCR conditions were 30 s of preincubation at 95 °C, 40 cycles of 15 s at 95 °C, and 30 s at 60 °C. Melting curves for each amplicon were carefully examined, and technical duplication with the double peak was removed to confirm the specificity of the primer pair used. The RT–qPCR amplification data were analyzed using the $2^{-\Delta\Delta CT}$ method [26,27]. The relative expression of *CYP720B* genes in different tissues was compared with Tukey's test ($p < 0.05$).

Sequences of the primers used in this study are shown in detail in Table A2.

2.5. Extraction of DRAs

DRA was extracted according to the method of Robert et al. [28]: approximately 0.5 g of fresh plant tissues was ground into powder in liquid nitrogen, and then 4.5 mL of *tert*-

butyl methyl ether was added (Aladdin, B108115). The mixture was shaken repeatedly and soaked at room temperature overnight. The next day, 0.5 mL of extract was derivatized for diterpenoid analysis. For GC–MS analysis, DRAs extracted from samples were analyzed by GC–MS on a mass spectrometer (Agilent 5975B, Santa Clara, CA, USA) with a fused silica capillary column (DB-5MS) code with tetramethylammonium hydroxide-methanol (60 m × 0.25 mm internal diameter, 0.25 μM film thickness). The initial temperature of GC–MS was 60 °C for 2 min, and then was successively increased at 2 °C/min to 80 °C for 5 min, at 4 °C/min to 280 °C for 10 min, finally, at 20 °C/min to 290 °C for 2.5 min, and the temperature of the injection port was 260 °C. Volumes of 1.0 μL per sample were injected in pulsed splitless mode at 250 °C with a column flow of 1.8 mL/min helium and splitless pulse pressure. The carrier gas was high-purity helium (99.999%). The temperatures of the chromatography–mass spectrometer interface and the quadrupole were 230 °C and 150 °C, respectively. The ionization method was EI with an electron energy of 70 eV and a scanning mass range of 50–500 m/z. The content of DRAs in different tissues was compared with Tukey's test ($p < 0.05$).

3. Results

3.1. Identification and Phylogenetic Analysis of CYP720B in Slash Pine and Loblolly Pine

Based on the presence of apparently complete P450 domains, 17 *CYP720B* candidate genes were identified in slash pine and 19 were identified in loblolly pine. There were seven putative allelic variants (v, *c324680v1*, *c324680v2*, *c356010v1*, *c356010v2*, *c305557v1*, *c305557v2*, *c305557v3*) and four gene deletions (i, *c327173i1*, *c324680i1*, *c327174i1*, *c305557i1*) in the 17 genes of slash pine, and three pairs of allele mutations (*PITA_40733v1*(*PITA_40733*), *PITA_40733v2*(*PITA_00760*), *PITA_37500v1*(*PITA_37500*), *PITA_37500v2*(*PITA_23489*), *PITA_37500v3*(*PITA_39136*), *PITA_17539v1*(*PITA_17539*), *PITA_17539v2*(*PITA_01372*), *PITA_37500v4*(*PITA_16383*), *PITA_37500v5*(*PITA_00301*)) and one (*PITA_17539i1*(*PITA_37112*)) of gene fragments in the 19 candidate genes of loblolly pine. The slash pine *CYP720B* gene sequences found have been uploaded to the NCBI, and the corresponding accession numbers are OK484434-OK484449.

To analyze the relationship of the CYP720B subfamily among the four clades [5], an unrooted tree was constructed using the full-length amino acids of these *CYP720B* genes. Gene characteristics, including the length of the protein sequence (aa), the protein molecular weight (MolWt), and the isoelectric point (pI), were analyzed (Table 1). Among the 17 CYP720B proteins in slash pine, *c324680v2i2* was identified as the shortest protein, with 402 amino acids (aas), whereas the longest proteins were *c327173* and *c327174* (487 aas). The MolWt of the proteins ranged from 46,469.52 to 56,250.19 Da, and the pI ranged from 7.93 (*c324680v2*) to 9.57 (*c327173*). *PITA_43262* was identified as the smallest protein with 403 amino acids (aas), whereas the largest was *PITA_02864* (511 aas). The MolWt of the proteins ranged from 46,434.27 to 58,378.87 Da, and the pI ranged from 7.45 (*PITA_43262*) to 9.64 (*PITA_42322*) in loblolly pine.

Table 1. Basic information on the CYP720B subfamily of slash pine (*Pinus elliottii*) and loblolly pine (*Pinus taeda*).

Species	Gene ID	Protein Length (aas)	Molecular Weight (MolWt)	Isoelectric Point (pI)	Gene ID	Protein Length (aas)	Molecular Weight (MolWt)	Isoelectric Point (pI)
Pinus elliottii	*c330768*	479	55,417.44	9.5	*c323216*	477	55,019.62	8.23
	c161262	484	56,135.81	8.74	*c305297*	486	56,193.09	9.48
	c327173	487	56,246.2	9.57	*c327174*	487	56,250.19	9.36
	c327173i1	470	54,233.74	9.58	*c327174i1*	447	51,369.63	9.53
	c324680v1	456	52,894.31	9.08	*c356010v1*	470	54,039.35	8.63
	c324680v2i2	402	46,469.52	7.93	*c356010v2*	481	55,656.16	8.11
	c324680i1	464	53,577.96	8.46	*c305557v1*	486	56,469.38	9.29

Table 1. *Cont.*

Species	Gene ID	Protein Length (aas)	Molecular Weight (MolWt)	Isoelectric Point (pI)	Gene ID	Protein Length (aas)	Molecular Weight (MolWt)	Isoelectric Point (pI)
	c305557v2	486	56,499.45	9.15	*c305557v3*	486	56,489.41	9.15
	c305557i1	447	51,912.93	8.47				
Pinus taeda	*PITA_49896*	489	56,291.24	9.6	*PITA_14112*	497	57,223.98	8.56
	PITA_42322	431	49,609.36	9.64	*PITA_06980*	457	52,413.8	9.7
	PITA_11046	497	56,972.88	8.19	*PITA_02864*	511	58,378.87	9.04
	PITA_10468	478	54,899.42	7.89	*PITA_43262*	403	46,434.27	7.45
	PITA_22834	487	55,925.77	9.36	*PITA_17539v1*	482	55,576.02	7.88
	PITA_40733v1	487	56,629.66	9.29	*PITA_17539i1*	453	52,353.47	8.17
	PITA_40733v2	487	56,495.54	9.48	*PITA_17539v2*	482	55,646.18	8.07
	PITA_37500v1	487	56,041.91	9.51	*PITA_37500v4*	487	56,085.96	9.51
	PITA_37500v2	487	56,075.92	9.51	*PITA_37500v5*	487	56,013.85	9.51
	PITA_37500v3	487	56,055.93	9.51				

3.2. Multiple Sequence Alignment, Phylogenetic Analysis, and Classification of CYP720B

The phylogenetic relationship of the CYP720B proteins was examined by multiple sequence alignment of their full-length amino acid sequence. According to the division principle of Hamberger et al. [16], the identified CYP720B members were divided into four clades from I to IV (Figure 1): 21 in clade I, 1 in clade II, 12 in clade III, and 2 in clade IV. As shown in Table 2, the CYP720B protein sequences were all found to have a C-terminus containing highly conserved sequences (FxxGxxxCxG). This heme-binding region is highly conserved among P450 enzymes and regarded as the fingerprint of P450s [29]. The proline-rich (P/G)PPGPx(G/P)xP motif [30] was found to have a single amino acid variation in *PITA_22834* and *PITA_17539v1*. (A/G)Gx(D/E)T was thought to bind to an oxygen molecule in the I helix [31]; however, there was a certain amino acid variation in clade IV, which became "AG-QT," and *PITA_42322* lost this domain. "ExxR" is highly conserved within the K helix [32], which is coordinated as the fifth thiolate ligand to P450 heme iron; *PITA_42322* lost this domain in clade II.

Table 2. The amino acid sequence of the conserved regions of CYP720B.

Clade	Gene ID	(P/I)PGSx(G/P)xP	AGx(D/E)T	ExxR	PxRx	FxxGxxxCxG
Clade I	*c161262*	PPGSRGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	c305297	PPGSRGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	c305557v1	PPGSSGWP	AGHET	ETHR	PWRW	FGGGLRLCPG
	c305557i1	PPGSSGWP	AGHET	ETHR	PWRW	FGGGLRLCPG
	c305557v2	PPGSSGWP	AGHET	ETHR	PWRW	FGGGLRLCPG
	c305557v3	PPGSSGWP	AGHET	ETHR	PWRW	FGGGLRLCPG
	c327173	PPGSTGLP	AGHET	ETLR	PWRW	FGGGARLCPG
	c327173i1	PPGSTGLP	AGHET	ETLR	PWRW	FGGGARLCPG
	c327174	PPGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	c327174i1	PPGSTGWP	AGHET	–LR	PWRW	FGGGARLCPG
	PITA_06980	PPGSTGWP	AGHET	ETLR	PWRW	FGGGPRLCPG
	PITA_14112	PPGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	PITA_22834	PAGSRGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	PITA_49896	PPGSTGLP	AGHET	ETLR	PWRW	FGGGARLCPG
	PITA_37500v1	PPGSRGWP	AGHET	ETLR	PWRW	FGSGARLCPG
	PITA_37500v2	PPGSRGWP	AGHET	ETLR	PWRW	FGSGARFCPG
	PITA_37500v3	PPGSRGWP	AGHET	ETLR	PWRW	FGSGARLCPG
	PITA_37500v4	PPGSRGWP	AGHET	ETLR	PWRW	FGSGARLCPG
	PITA_37500v5	PPGSRGWP	AGHET	ETLR	PWRW	FGSGARLCPG
	PITA_40733v1	PPGSSGWP	AGHET	ETHR	PWRW	FGGGLRLCPG
	PITA_40733v2	PPGSSGWP	AGHET	ETHR	PWRW	FGGGLRLCPG

Table 2. *Cont.*

Clade	Gene ID	(P/I)PGSx(G/P)xP	AGx(D/E)T	ExxR	PxRx	FxxGxxxCxG
Clade III	*c323216*	PPGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	c324680v1	PPGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	c324680i1	PPGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	c324680v2i2	PPGSTGWP	——	ETLR	PWRW	FGGGARLCPG
	c356010v1	PPGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	c356010v2	PPGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	PITA_10468	PPGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	PITA_11046	PPGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	PITA_43262	PPGSTGWP	——	ETLR	PWRW	FGEGARLCPG
	PITA_17539v1	PTGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
	PITA_17539i1	PPGSTGWP	AGHET	EILR	PWRW	FGGGARLCPG
	PITA_17539v2	PTGSTGWP	AGHET	ETLR	PWRW	FGGGARLCPG
Clade II	*PITA_42322*	PPGSTGWP	—-T	—-	PSRW	FGGGARLCPG
Clade IV	*c330768*	PPGSTGWP	AG-QT	ETLR	PWRW	FGAGARLCPG
	PITA_02864	PPGSTGWP	AG-QT	ETLR	PWRW	FGAGARLCPG

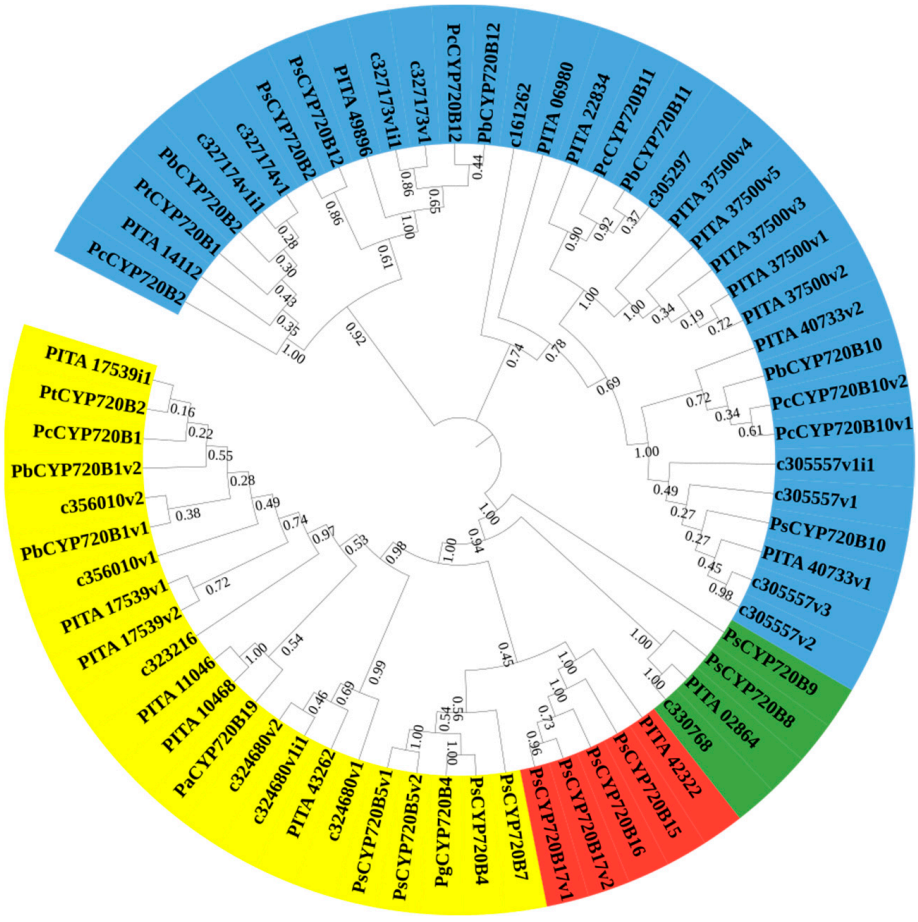

Figure 1. Phylogenetic relationships of the conifer-specific *CYP720B* genes. Note: v, putative allelic variant; i, insertion and deletion of fragments. The numbers in the figure represent the bootstraps value and the bootstraps value range from 0 to 1. The genes with blue backgrounds belong to clade I, green backgrounds belong to clade IV, red backgrounds belong to clade II, and yellow backgrounds belong to clade III.

3.3. Gene Structure and Motif Composition of CYP720B

The exon–intron organizations of all the identified *CYP720B* genes were examined to gain more insight into the evolution of the CYP720B subfamily in loblolly pine. As shown in Figure 2, genes within the same clade usually have similar structures; for instance, all *CYP720B* genes possessed 9 exons in clades I and IV. *PITA_43222* had 10 exons in clade II, which was different from the others. *PITA_17539i1* and *PITA_43262*, which were in clade III, had 8 exons. A schematic diagram of the structure of all CYP720B proteins was constructed from the MEME motif analysis results. As shown in Figure 3, the similar motif arrangements among CYP720B proteins indicated that the structure of the protein was conserved within an identical clade. However, the functions of most of these conserved motifs remain to be elucidated.

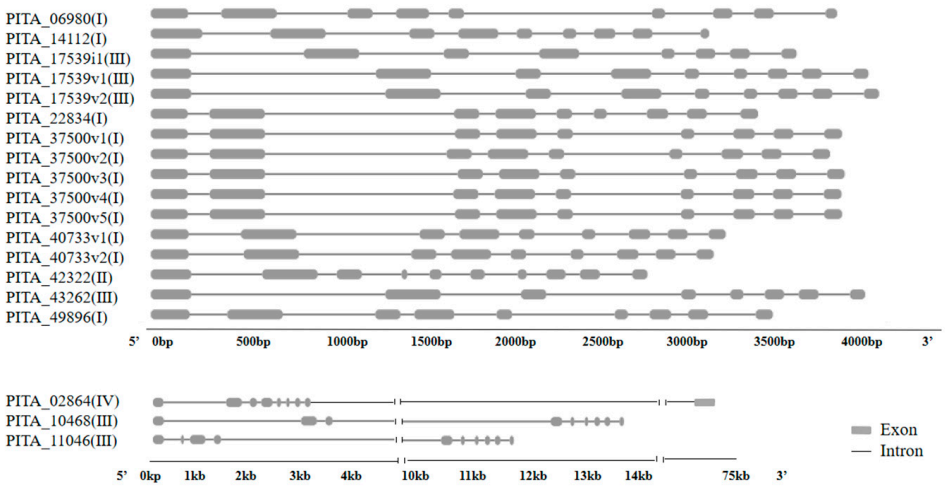

Figure 2. Exon-intron structure of loblolly pine *CYP720B* genes. The gray boxes indicate exons; black lines indicate introns, and protein lengths from 5′ to 3′ can be estimated using the scale at the bottom. The long introns in the middle of the last three protein (*PITA_02864, PITA_10468, PITA_11046*) sequences were broken by parallel bars.

In summary, the conserved motif compositions and similar gene structures of the CYP720B members, as well as the phylogenetic analysis results, could strongly support the reliability of the clade classifications.

3.4. Upstream Cis-Acting Elements of Loblolly Pine CYP720B

Since CYP720Bs are involved in the biosynthesis of defense-related terpenoids induced by insects, pathogens, wounds, or MeJA [33,34], we analyzed the upstream genomic regions of CYP720Bs for putative *cis*-acting elements associated with plant defense responses. Our analysis of upstream sequences for *cis*-acting elements covered 2500 bp upstream of the ATG start codon for CYP720Bs to understand the possible biological functions and regulatory characteristics of CYP720Bs. Putative *cis*-acting elements were identified by a similarity search of the PlantCARE database [35]. The results showed that the promoters of 19 genes of loblolly pine CYP720Bs contained 9 types and 125 *cis*-acting elements (Figure 4). A large category was plant hormone response elements, such as the gibberellin response element (GARE-motif, P-box, TATC-box), auxin response element (TGA-element, AuxRE), salicylic acid inducing element (TCA-element), MeJA response element (CGTCA-motif, TGACG-motif), ABA response element (ABRE), and flavonoid response element (MBSI); a large category was abiotic stress response elements, such as MYB drought-inducible binding site (MBS), disease-resistance and stress-inducing element (TC-rich repeats), and low-temperature response element (LTR).

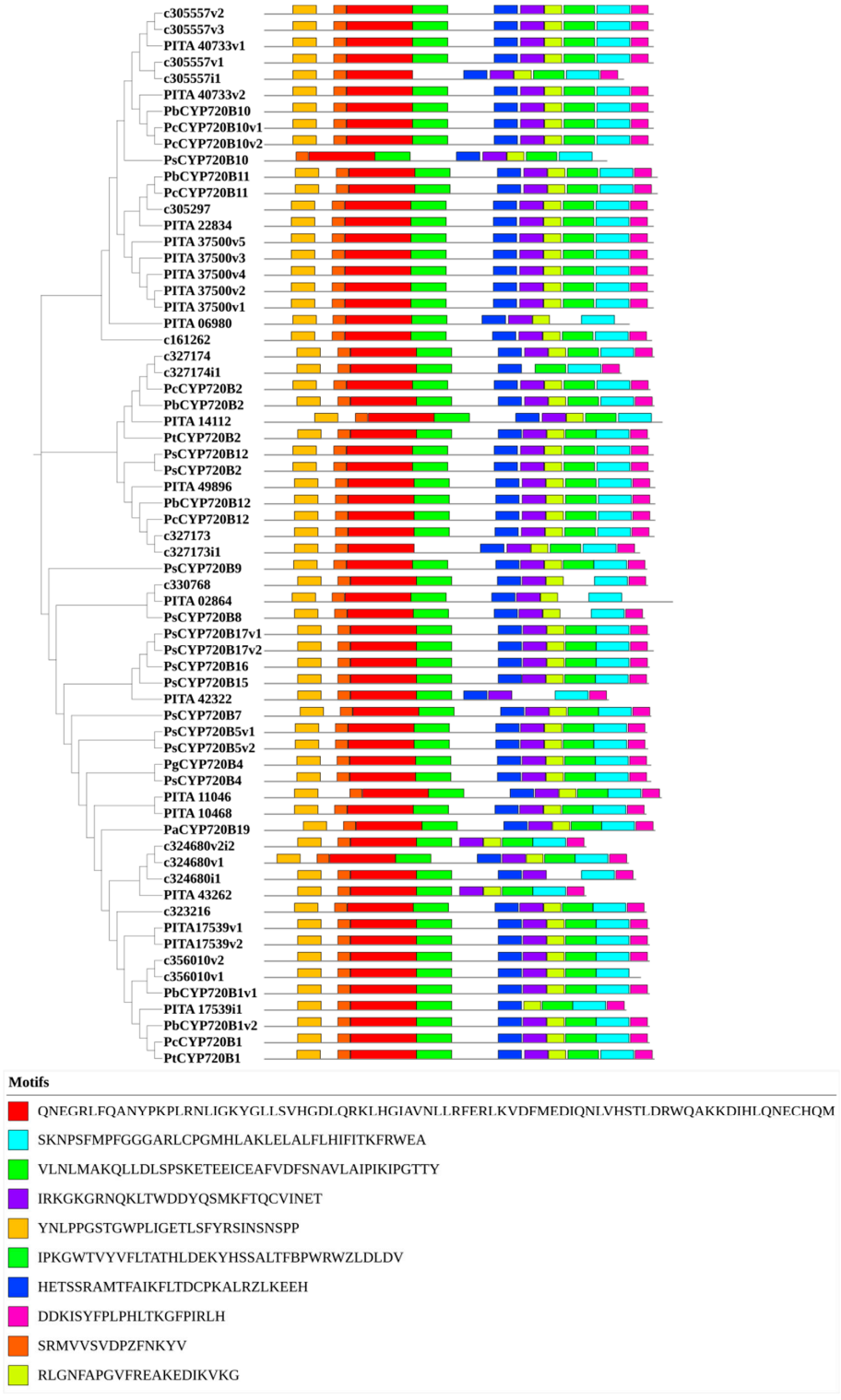

Figure 3. Phylogenetic relationships and motif compositions of CYP720B proteins of slash pine and loblolly pine identified in this study and previously characterized CYP720B in conifers. The gene names beginning with *c* and *PITA* stand for the *CYP720B* genes of slash pine and loblolly pine identified in this study, respectively. The detailed information of other *CYP720B* genes identified in previous studies was shown in Table A1. Left panel: An unrooted phylogenetic tree constructed using MEGA X by the neighbor-joining method. The proteins were clustered into four main clades. Right panel: distribution of conserved motifs in the CYP720B proteins. The different-colored boxes represent different motifs and their position in each CYP720B protein sequence.

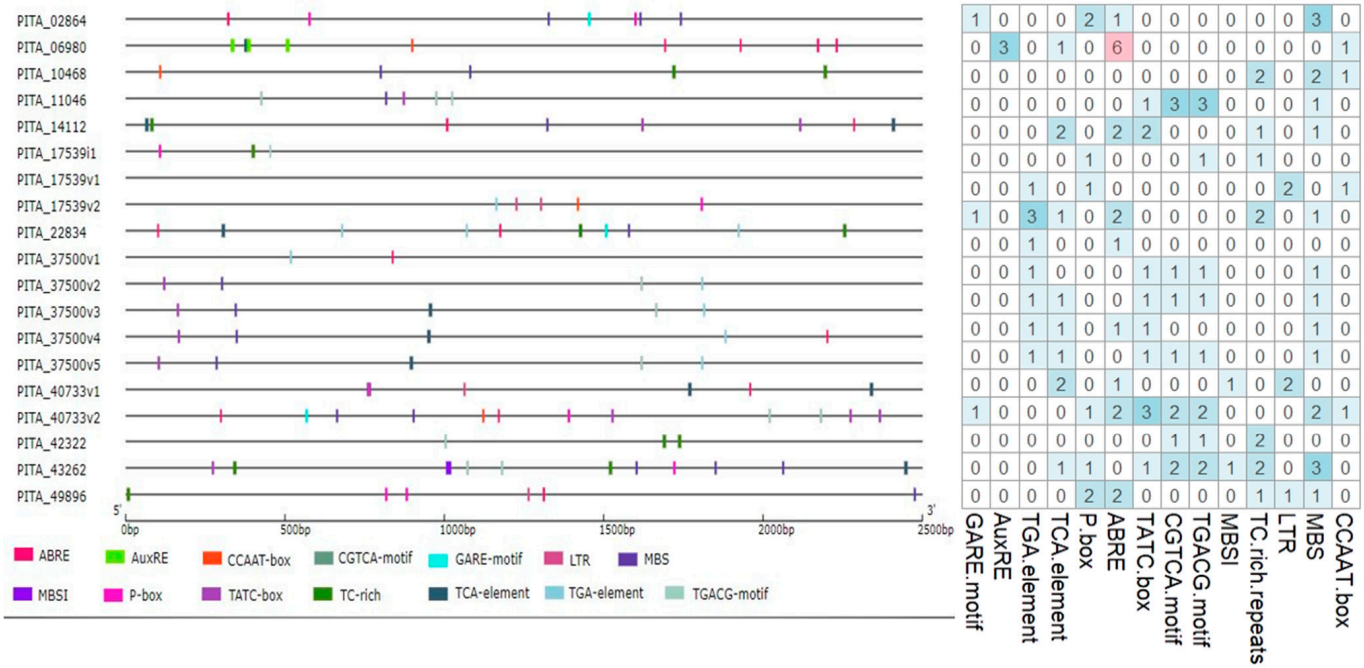

Figure 4. Analysis of *cis*-acting elements in the promoter region of the loblolly pine *CYP720B* genes. The left picture shows the position of the *cis*-acting in the upstream 2500 bp sequence, the right picture shows the number of each *cis*-acting element, and the number in the boxes indicates the number of *cis*-acting elements, The more the number, the darker the color, red means the maximum number. GARE-motif, P-box, TATC-box: gibberellin-responsive element; TGA-motif, AuxRE: auxin-responsive element; TCA-element: salicylic acid element; ABRE: abscisic acid responsiveness element; CGTCA-motif, TGACG-motif: MeJA responsiveness element; MBSI: MYB binding site involved in gene regulation of flavonoid biosynthesis; TC-rich: defense and stress element; LTR: low-temperature responsiveness element; MBS: MYB binding site participating in drought induction; CCAAT-box: MYBHv1 binding site.

In addition, the frequency of the MeJA-response element CGTCA motif was greatest among all *cis*-acting elements. A total of 23 MeJA responsiveness elements (CGTCA motifs and TGACG motifs) were located in 18 CYP720B upstream sequences. The second most frequent was in response to gibberellin element (GARE-motif, P-box, TATC-box) content, with a total of 22 distributed in 13 gene promoters, such as *PITA_40733v2*, *PITA_02864*, and *PITA_43262*. The lowest number was observed for MSBI-responsive flavonoid elements at only 2. The above results indicate that the different subfamily genes of the CYP720B subfamily have different abilities to respond to plant hormones and abiotic stresses and that the overall response to plant hormones is more obvious.

3.5. Revealing the Difference in the Expression of CYP720Bs in Different Tissues

To assess the correlations of *CYP720B* genes with oleoresin accumulation in slash pine and loblolly pine tissues, we removed allelic variant genes and fragment insertion or deletion genes and finally performed comparative and quantitative transcript analysis using RT–qPCR for the 9 slash pine *CYP720B*s and 12 loblolly pine *CYP720B*s across a range of tissues: young and mature needles, young and mature shoots, and roots. Transcript profiles are shown in Figure 5. For three genes, *PITA_11046*, *PITA_06980*, and *PITA_02864*, transcript levels were very low across all samples tested; we speculated that these three genes are not expressed during the seedling stage of loblolly pine.

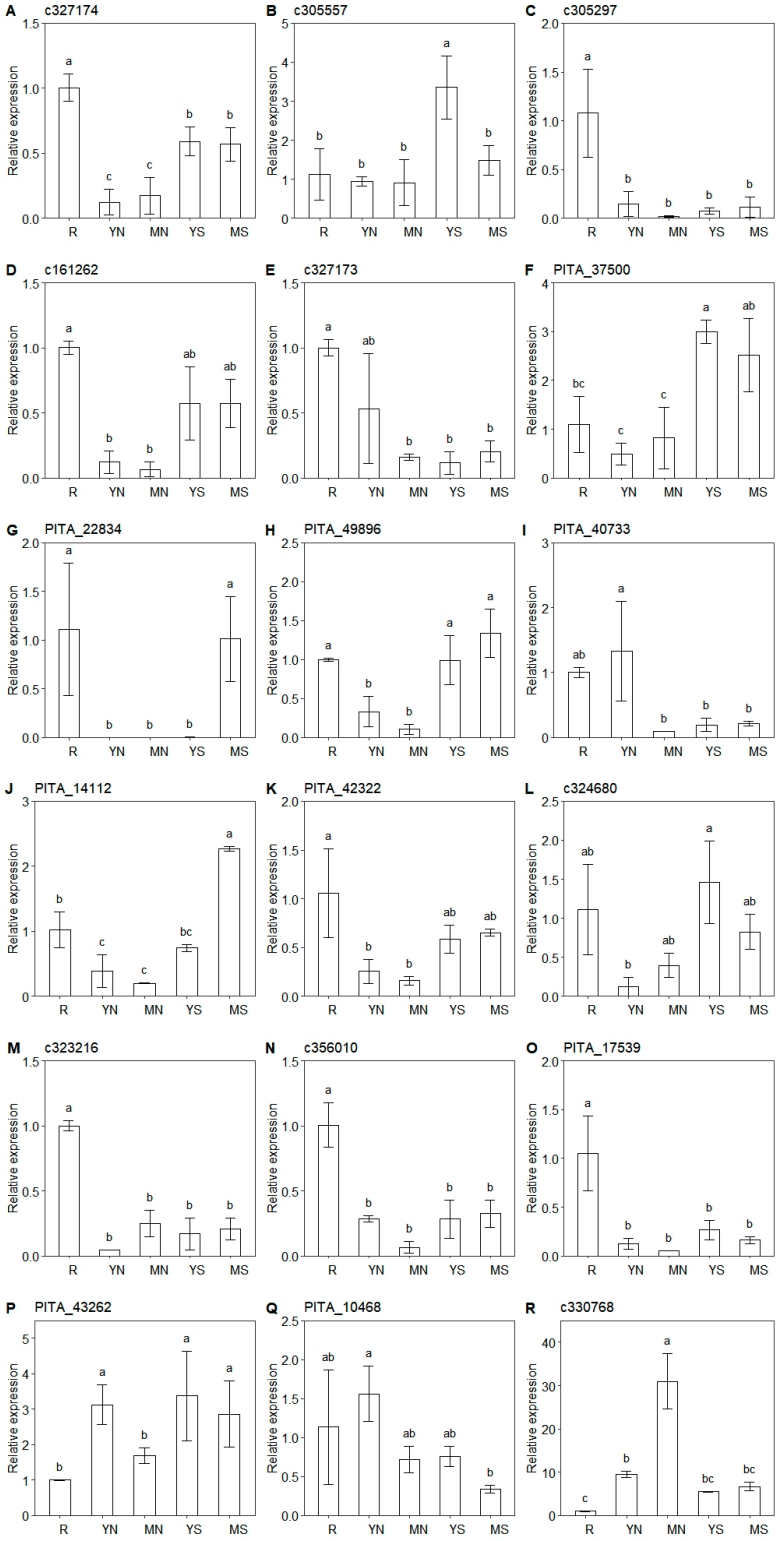

Figure 5. Expression analysis of 18 *CYP720B* genes by RT–qPCR. The gene names beginning with *c* and *PITA* stand for the *CYP720B* genes of slash pine and loblolly pine. *UBI* and *18S* rRNA were selected as the reference gene. Data were normalized and vertical bars indicate the standard deviation of biological replicates ($n = 3$). Statistically significant differences are indicated with different letters (Tukey's test, $p < 0.05$). (**A**–**J**) belonged to clade I, (**K**) belonged to clade II, (**L**–**Q**) belonged to clade III, and (**R**) belonged to clade IV. Transcript quantification was measured in young needles (YN), mature needles (MN), young stems (YS), mature stems (MN), and roots (R).

For *CYP720B*s of clade I, *c305557*, *PITA_37500*, *PITA_49896*, and *PITA_14112* showed high transcript levels in stem tissues, while *PITA_40733* was preferentially expressed in young needles. Transcript levels of *c327173*, *c305297*, *c161262*, c327174, and *PITA_22834* were higher in roots. For *CYP720B*s of clade III, *c324680* and *PITA_43262* showed high levels of transcripts in stem tissues, and *PITA_10468* was preferentially expressed in young needles. The transcript levels of c323216, c356010, and PITA_17539 were higher in roots. For *CYP720B*s of clades II and IV, *PITA_42322* was most highly expressed in the roots, and the transcript levels of *c330768* were higher in mature needles.

From the above RT–qPCR results, *CYP720B*s were highly expressed in roots and stems. We speculated that the content of diterpene products catalyzed by CYP720B in roots and stems was higher than that of the needles. To verify our conjecture, we detected the content of DRAs in different tissues using GC–MS. GC–MS analysis of organic solvent extracts identified DRAs as the most abundant diterpenoids across all tested tissue types in both slash pine and loblolly pine. The contents of abietic acid, dehydroabietic acid, and isopimaric acid in slash pine were significantly higher than those of the other four DARs; the content of levopimaric acid in loblolly pine was the highest, followed by dehydroabietic acid, abietic acid, isopimaric acid, neoabietic acid, pimaric acid, and sandaracopimaric acid in loblolly pine (Figure 6).

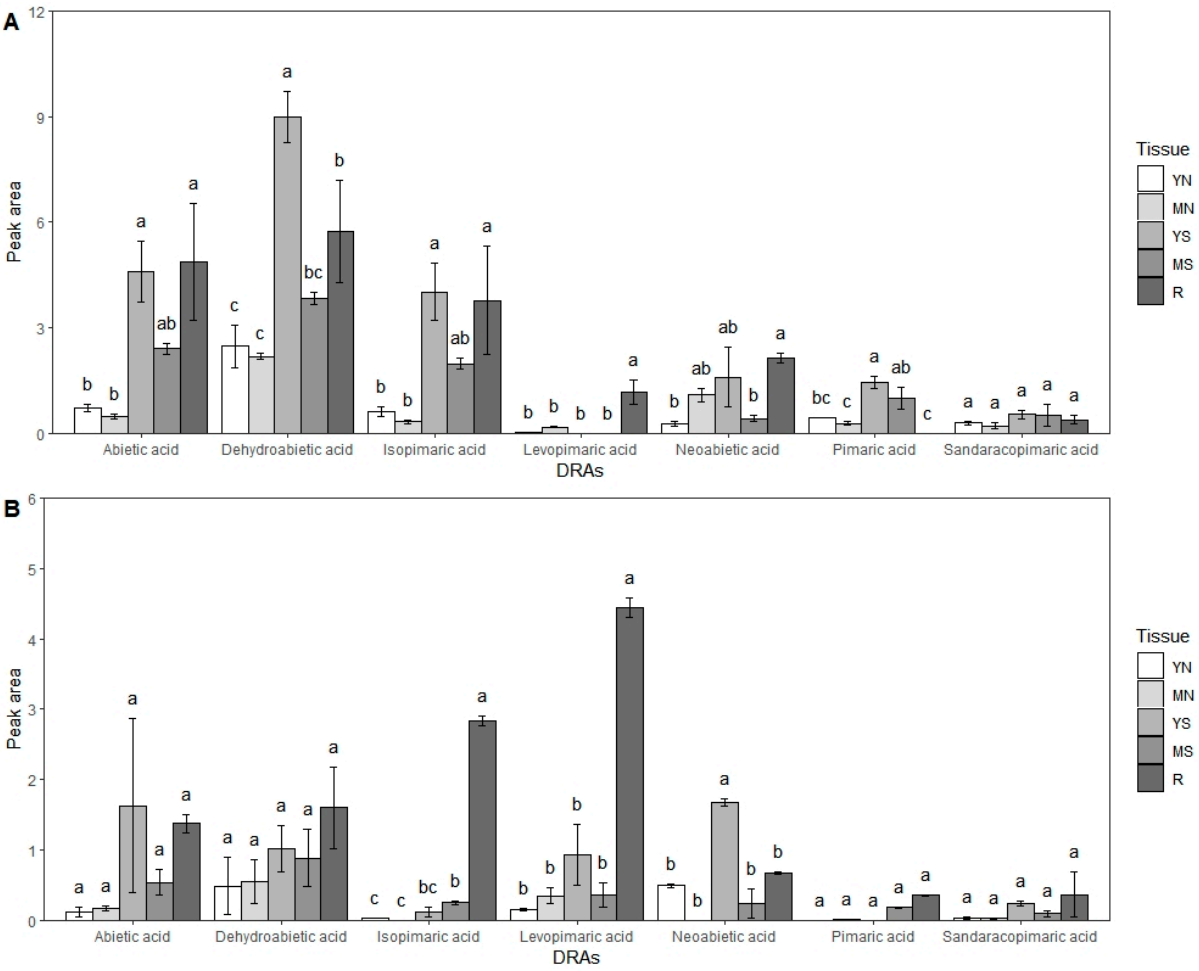

Figure 6. DRAs content in different tissues. (**A**) DRA content of tissues in slash pine; (**B**) DRA content in different tissues in loblolly pine. Error bars represent standard deviation values of biological replicates (n = 3). Statistically significant differences are indicated with different letters (Tukey's test, $p < 0.05$). YN represents young needles, MN represents mature needles, YS represents young stems, MN represents mature stems, and R represents roots.

4. Discussion

The development of sequencing technologies provides a good reference to identify genes associated with important biological pathways. The first genome assembly of loblolly pine is built from short Illumina reads in 2014 [33], and an improved assembly is published in 2016 using long-read sequencing technology [23]. In this study, 19 *CYP720B* candidate genes were identified based on the *Pinus teada* v2.0 genome assembly, which is located in 19 different scaffolds. We could not infer whether some candidate genes were located on the same chromosome because the genome was not assembled at the chromosome level. The large size and complexity of the Pinus genome hinder whole-genome sequencing and assembly for other pine species without reference genome sequences. RNA long read sequencing technology would also provide a good reference in genetic studies. Diao et al. [22] 2019 sequenced the long transcripts of five mixed tissues in slash pine using third-generation sequencing technology, which were further corrected by short read sequencing of more than 200 slash pine individuals (unpublished). This is a very good data resource for identifying *CYP720B* genes in slash pine. In this study, 17 *CYP720B* genes were identified in slash pine. Mining *CYP720B* candidate genes would be helpful for further exploring the mechanism of DRA diversity.

4.1. Characterization of CYP720B

The conserved structural domains of the slash pine and loblolly pine CYP720B proteins were assessed in this study. Multiple sequence comparisons of the identified CYP720B protein with *Picea sitchensis*, *Pinus banksiana*, and *Pinus contorta* protein revealed that the C-terminus of the CYP720B protein sequences contains a highly conserved heme-binding domain, FxxGxxxCxG. The heme-binding domain consists of a highly conserved cysteine residue that forms a thiolate bond with the iron in the ferroheme of the catalytic site, thus constituting the key protein–oxygen structure and the active site [11]. There was also a conserved domain in the C-terminus, the PxRx motif, which is found in the structure of many P450s. This reflects that this family is relatively conserved in long-term evolution, which is consistent with the results of studies on the *CYP720B* gene family of *Picea sitchensis*, *Pinus banksiana*, and *Pinus contorta* [7]. In addition, by analyzing the gene structure of loblolly pine *CYP720B*, it was shown that the gene structure of this family was conserved during the evolution process.

Upstream transcription factors regulate their expression levels by binding to *cis*-acting elements in the promoters of target genes [34]. The promoters of *CYP720B* subfamily genes in slash pine and loblolly pine contain many *cis*-acting elements that respond to hormones, indicating that they may play a certain role in hormone response plant growth, development, and senescence. In our research, most of the CYP720B subfamily members contained low temperature, drought, defense, or adversity response elements, indicating that the *CYP720B* subfamily genes in pine may play an important role in resistance to low temperature, drought resistance, and other abiotic stresses. Previous articles could provide evidence, and the results show that *CYP720B1* and *CYP720B4* are involved in biotic and abiotic stress responses in conifers such as insects, pathogens, wounds, or MeJA [16,28]. The analysis of these *cis*-acting elements provides a valuable reference for future studies of the transcriptional regulation of conifer defense genes [35,36].

4.2. Function of CYP720B

There is considerable evidence that *CYP720B* genes play significant roles in the chemical defense system of plants and in conferring tolerance to biotic and abiotic stresses, including pests, pathogens, and wounding [9]. For example, *PsCYP720B4* was recently reported to produce miltiradienic acid when coexpressed with TPS from *Tripterygium wilfordii* [37]. In previous studies on the protein function of the *CYP720B* gene subfamily, only *CYP720B1* and *CYP720B4* in clade III and *CYP720B2* and *CYP720B12* in clade I have been reported [35]. However, it is not clear whether clade II and IV CYP720Bs contribute to DRA biosynthesis or if they play a role in other biosynthesis pathways. *PtCYP720B1*, the first

identified member from *Pinus teada*, has been verified to have activity as a C-18 oxidase and can accept various olefinic precursors, such as abietadiene, abietadienol, abietadienal, levopimaradienol, dehydroabietadienol, and dehydroabietadienal [15]. The amino acid sequence identity among *PITA_13579*, *c323216*, and *c356010* identified in this study and the previously identified *PtCYP720B1* was 96%, 95%, and 99%, respectively. Thus, we speculated that the functions of *PITA_13579*, *c323216* and *c356010* might be similar to that of *PtCYP720B1*. We found that these three genes showed higher expression patterns in roots in both slash pine and loblolly pine. *PsCYP720B4* from *Picea sitchensis*, also a C18 oxidase, is able to oxidize a total of 24 substrates and appears to be highly active in the biosynthesis of dehydroabietic acid [16]. However, we did not identify any genes clustered with *PsCYP720B4* and *PgCYP720B4* in the phylogenetic tree, as in the previous *CYP720B* gene subfamily analysis in lodgepole pine (*Pinus contorta*) and jack pine (*Pinus banksiana*), indicating possibly different DRA biosynthesis mechanisms between *Picea* and *Pinus. CYP720B2* and *CYP720B12* of *Pinus contorta*, *Picea sitchensis*, and *Pinus banksiana* use 13-hydroxy-8(14)-abietene as a substrate to produce 3-hydroxy-8(14)-abietic acid, which is unstable and dehydrates to form abietic acid, neoabietic acid, levopimaric acid, and palustric acid [7]. In this study, the amino acid sequence identity between *c327174*, *PITA_14112*, and *PtCYP720B2* was 99% and 99%, respectively, and the amino acid sequence identity between *c327173*, *PITA_49896*, and *PbCYP720B12* was 99% and 99%, respectively. We speculated that these candidate genes might contain similar functions to *CYP720B2* and *CYP720B12* in slash pine and loblolly pine. The *CYP720B2*- and *CYP720B12*-like genes showed different tissue expression patterns in slash pine and loblolly pine, which might indicate that *CYP720B* candidate genes with the same function might perform differently in different species.

The function of some *CYP720B* candidate members has still not been proven. Mining the function of *CYP720B* candidate genes is very meaningful for revealing the DRA biosynthesis mechanism in the future.

4.3. Expression Specificity of CYP720B

Diterpene acid is the most abundant substance in resin and can be produced by all tissues of pines [9]. In this study, we found that the DRA profile detected by GC–MS was quite different among tissues, which was consistent with previous studies [5,38]. The content of DRAs might be related to *CYP720B* gene expression levels. The GC–MS profile of DRA profiles was quite different in slash pine and loblolly pine. Both slash pine and loblolly *CYP720B1*-like genes (*PITA_13579*, *c323216*, and *c356010*) showed higher expression patterns in roots. However, *CYP720B2*-like (*c327174*, *PITA_14112*) and *CYP720B12*-like genes (*c327173*, *PITA_49896*) showed different expression patterns in the two species, which might lead to different product amounts. The content of each DRA in root and stem generally showed higher in root and stem than needles corresponding with the gene expression levels of CYP720B members in slash pine and loblolly pine. High expression levels of *CYP720B* in roots were also revealed in *Picea sitchensis*, which also showed that some CYP720B members showed higher expression levels in roots than in stem tissues and that some CYP720B members did not show significant differences between stem tissues and roots [16].

The high DRA content in roots indicated that the DRAs in roots might play a critical role in the lifespan of pine. DRA redistribution of roots in *Pinus pinaster* might play a critical role in protecting the root response to drought stress [39]. However, few other studies have introduced the roles of DRAs in the roots of pine. Recent studies have shown that plant terpenes play an important role in belowground interactions [40]. The diterpenes synthesized in the root of *Arabidopsis thaliana* and rice have been revealed to participate in the defense against belowground herbivory [41] and plant–plant allelopathy [42], respectively. Whether and how DRAs synthesized in the roots of pine participate in belowground interactions are poorly known and worth studying in the future.

5. Conclusions

This study conducted an analysis of *CYP720B* genes in slash pine and loblolly pine. A total of 17 genes in slash pine and 19 genes in loblolly pine were identified in this study. These candidate genes were classified into four main clades in the phylogenetic analysis with previously identified CYP720B in conifers. The exon–intron structures analysis of loblolly pine CYP720B reveals the genes within the same clade usually have similar gene structures. A total of 125 *cis*-acting elements from nine types were identified in the promoter regions of 19 loblolly pine CYP720Bs. The *cis*-acting elements analysis showed that *CYP720B* genes were closely related to adversity resistance. The *CYP720B* genes expression profiles quantified by RT-qPCR and DRA profiles detected by GC–MS were different among different tissues. Most of the *CYP720B* genes showed relatively higher expression levels in roots and stems than in the other tissues. This corresponds with the results of DRA component detection by GC–MS, indicating the stems and roots might be important tissues in oleoresin biosynthesis. Phylogenetic and gene expression analysis would concededly be helpful to better understand the potential functions of *CYP720B* genes and their possible role in terpenoid synthesis and abiotic or biotic stress. This study provides valuable resources for a better understanding of the biological role of individual *CYP720B* genes in slash pine and loblolly pine.

Author Contributions: Conceptualization, Y.Z. and S.D.; methodology, Y.Z. and S.D.; formal analysis, Y.Z.; investigation, Q.L., X.D., S.D. and Y.Z.; resources, S.D. and Q.L.; data curation, S.D. and Y.Z.; writing—original draft preparation, Y.Z.; writing—review and editing, S.D. and Y.Z.; supervision, Q.L. and J.J.; project administration, S.D. and Q.L.; funding acquisition, S.D. and Q.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The loblolly pine v2.0 assembly with annotations were analyzed in this study. This data can be found here: https://treegenesdb.org/jbrowse?page=1, accessed on 15 August 2021.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

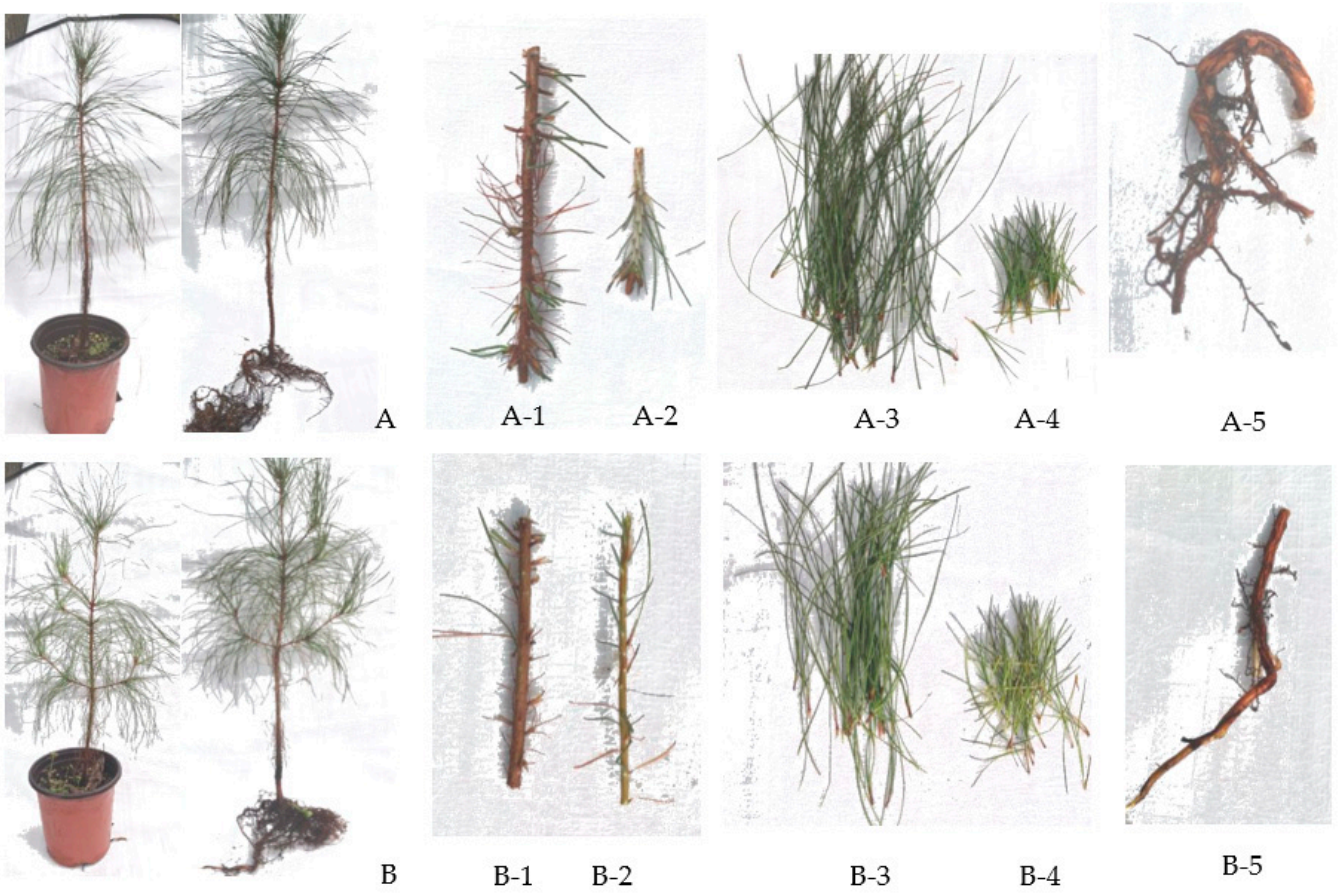

Figure A1. The pictures of slash pine and loblolly pine. (**A**) stands for slash pine, (**A1**–**A5**) represent mature and young stems, mature and young needles, and roots; (**B**) stands for loblolly pine, (**B1**–**B5**) represent mature and young stems, mature and young needles, and roots.

Appendix B

Table A1. Information on 29 homologous proteins.

Homologous Protein	Accession Number	Species	Homologous Protein	Accession Number	Species
PsCYP720B12	HM245397	*Picea sitchensis*	*PbCYP720B1v1*	KJ845665	*Pinus banksiana*
PsCYP720B15	HM245398	*Picea sitchensis*	*PbCYP720B1v2*	KJ845666	*Pinus banksiana*
PsCYP720B16	HM245399	*Picea sitchensis*	*PbCYP720B2*	KJ845667	*Pinus banksiana*
PsCYP720B17v1	HM245400	*Picea sitchensis*	*PbCYP720B10*	KJ845668	*Pinus banksiana*
PsCYP720B17v2	HM245401	*Picea sitchensis*	*PbCYP720B11*	KJ845669	*Pinus banksiana*
PsCYP720B2	HM245402	*Picea sitchensis*	*PbCYP720B12*	KJ845670	*Pinus banksiana*
PsCYP720B4	HM245403	*Picea sitchensis*	*PcCYP720B1*	KJ845671	*Pinus contorta*
PsCYP720B5v1	HM245404	*Picea sitchensis*	*PcCYP720B2*	KJ845672	*Pinus contorta*
PsCYP720B5v2	HM245405	*Picea sitchensis*	*PcCYP720B10v1*	KJ845673	*Pinus contorta*
PsCYP720B7	HM245406	*Picea sitchensis*	*PcCYP720B10v2*	KJ845674	*Pinus contorta*
PsCYP720B8	HM245407	*Picea sitchensis*	*PcCYP720B11*	KJ845675	*Pinus contorta*
PsCYP720B10	HM245408	*Picea sitchensis*	*PcCYP720B12*	KJ845676	*Pinus contorta*
PsCYP720B9	HM245410	*Picea sitchensis*	*PaCYP720B19*	KJ624415	*Pinus armandi*
PtCYP720B2	Q50EK5	*Pinus taeda*	*PgCYP720B4*	FJ609175	*Pinus glauca*
PtCYP720B1	Q50EK6	*Pinus taeda*			

Table A2. Primers used for RT–qPCR.

Gene_ID	F′(to 3′)	R′(to 5′)
PITA_02864	ATCCAGAGTTGAGTGCACCA	AGAACGAGGGAAGGCTCTTT
PITA_06980	GGCAGGCTGTTTCAATCCAA	AACGTTCTGTATGCCCTCCA
PITA_10468	ATTATCGCTCCATGACCAGC	AGAACCTCCCCTCGTTTTGT
PITA_11046	TTATTCGGAAGCCCAGCAGT	AGCCTCTCAAATCCCAGCAA
PITA_40733	ATGGCTGGTGTTCTTCGTCT	TTGGCGTGGTTGAGAAATGG
PITA_42322	AGCCTCTCAAACCTCAGCAA	AGCAGATCCCCAGTTCAACA
PITA_49896	ACAAACATGTCCTGCAGCAC	AGCCTCTCGAACCTCAACAA
PITA_37500	TTCTGCGCTCACATTTGACC	ATGAAGAAAGAGGGCCAGCT
PITA_14112	ACCTCTCGAACCTCAGCAAA	TTGTGTCCGTGGATCCAGAA
PITA_22834	TGTCCTCCATGAAGTGCACA	GGCAGGCTGTTTCAATCCAA
PITA_17539	TGTCGTCGATGAATTGCCTG	TTTACAGGTGGTGGAATGCC
PITA_43262	ACCGCTATTCCATGGAGCTT	TCAGCAGATCCCCAGTTCAA
c356010	TTACAGGTGGTGGAATGCC	TGTCGTCGATGAATTGCCTG
c323216	CTAATCGAGAGGTACATCTGCC	TTGAACTGGGGATCTGCTGA
c327174	AGTGCTGGGCTTCTTATTGC	CGTGGTTGAGCAGTGGAATT
c161262	TTCGTCTGTTTCGTTCTGGC	CTTGAATGAATCGGCGTGGT
c327173	AAGCATCCACAAGTTGTCCG	GTCGTGCTCAGCCTTCAATT
c305297	CCATTTCTCAACCACGCCAA	ATTGAACCAGCTTGCCTTCG
c305557	AAACACATCGTCTGGCCAAC	AACGCCATGGGTCAAACTTG
c330768	AGAACGAGGGAAGGCTCTTT	ATCCAGAGTTGAGTGCACCA
c324680	AAGCTCCATGGAATAGCGGT	GAGCAGTTGTTTGGCCATCA

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