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Comprehensive Analysis of the *TIFY* Gene Family and Its Expression Profiles under Phytohormone Treatment and Abiotic Stresses in Roots of *Populus trichocarpa*

Hanzeng Wang [1], Xue Leng [1], Xuemei Xu [2] and Chenghao Li [1,*]

[1] State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, Harbin 150040, China; hzwang0@163.com (H.W.); lengxue19910514@163.com (X.L.)
[2] Library of Northeast Forestry University, Harbin 150040, China; xuemeixu1231@163.com
* Correspondence: chli@nefu.edu.cn

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Abstract: The *TIFY* gene family is specific to land plants, exerting immense influence on plant growth and development as well as responses to biotic and abiotic stresses. Here, we identify 25 *TIFY* genes in the poplar (*Populus trichocarpa*) genome. Phylogenetic tree analysis revealed these *PtrTIFY* genes were divided into four subfamilies within two groups. Promoter *cis*-element analysis indicated most *PtrTIFY* genes possess stress- and phytohormone-related *cis*-elements. Quantitative real-time reverse transcription polymerase chain reaction (qRT–PCR) analysis showed that *PtrTIFY* genes displayed different expression patterns in roots under abscisic acid, methyl jasmonate, and salicylic acid treatments, and drought, heat, and cold stresses. The protein interaction network indicated that members of the *PtrTIFY* family may interact with COI1, MYC2/3, and NINJA. Our results provide important information and new insights into the evolution and functions of *TIFY* genes in *P. trichocarpa*.

Keywords: *TIFY*; *Populus trichocarpa*; protein interaction network; phytohormone treatment; abiotic stress

1. Introduction

The *TIFY* gene family is specific to land plants and was first found in *Arabidopsis* and annotated as a transcription factor [1,2]. This gene family contains a core motif, TIF[F/Y]XG, previously known as ZIM (a zinc-finger protein expressed in inflorescence meristem) [1], and includes 20 members in *Rice*, 18 members in *Arabidopsis*, and 27 members in *Maize* [3–5]. Depending on the conservative domain, the proteins are divided into two groups: Group I containing the C2C2-GATA (CX2CX20CX2C) conservative domain, which is absent in Group II. Generally, the *TIFY* family can be divided into four subfamilies: *TIFY*, *JAZ*, *PPD*, and *ZML* [4]. The *TIFY* subfamily contains only the TIFY domain. The *JAZ* subfamily is named for its conserved Jas motif with 22 amino acids, which contains the TIFY domain and a C-terminal conserved domain, as well as the distinctive motif of SLX2FX2KRX2RX5PY [6,7]. The *PEAPOD* (*PPD*) subfamily was first found by map-based cloning method in *ppd* mutants, which has an exclusive N-terminal PPD domain of around 50 amino acids, a TIFY domain, and a Jas motif lacking the two conserved amino acids "PY" (SLX2FX2KRX2RX5). Finally, the ZML (ZIM-LIKE) subfamily contains a C-terminal GATA-type zinc-finger domain, a CCT (CONSTANS/CO-like/TOC1) domain, and a TIFY domain. The CCT domain is known to play an important role in light signal transduction and takes part in protein–protein interaction [4]. The *JAZ* (Jasmonate ZIM-domain) subfamily has been studied mostly as a key regulator of jasmonate signaling pathways [8]. The CCT domain is important

because it allows the proteins of the JAZ subfamily to interact with other proteins to regulate jasmonate signaling and take part in response to biotic and abiotic stresses in several plants [7]. For example, the F-box protein CORONATINE INSENSITIVE1 (COI1) acts as a hormone co-receptor and functions as a negative regulator of the jasmonate signaling pathway [8,9]. JAZ1 and JAZ9 can interact with COI1 in *Arabidopsis* and transgenic plants exhibited JA-insensitive phenotypes and increased resistance to pathogens [10]. Also, JA regulates the ethylene-stabilized transportation factors EIN3 and EIL1 by binding with the JAZ proteins to pathogen defense and development processes [11]. The R2R3 MYB-type transcription factors MYB21 and MYB24 can interact with JAZ1, JAZ8, and JAZ11 in yeast and plants to affect jasmonate-regulated stamen development in *Arabidopsis*. Recently, Ju et al. reported that the C-terminal region of TaJAZ1 interacts with TaABI5 to modulate seed germination and negatively modulate ABA response in wheat [12].

Functional analysis of the *TIFY* gene family has been carried out in several plant species. *PnJAZ1* increased tolerance to salt stress and decreased ABA sensitivity during seed germination and early development in *Pohlia nutans* [13]. As reported, *ZmJAZ14* was significantly induced by polyethylene glycol (PEG), MeJA, abscisic acid (ABA), and gibberellins (GAs). Overexpression of *ZmJAZ14* in Arabidopsis enhanced the plants' tolerance to JA and ABA treatments [14]. Additionally, AsJAZ1 can be involved in nodule development and nitrogen fixation by interacting with AsB2510 in *Astragalus sinicus* [15]. The PPD genes regulate tissue growth, modify lamina size, and restrict curvature of the leaf blade [16]. Removing PEAPOD results in increased leaf lamina size and altered shape of the silique [16]. In *Arabidopsis*, AtPPD2 interacts with AtLHP1 to directly or indirectly regulate several target genes to affect lateral organ development, while *Arabidopsis ppd2* shows greater leaf breadth than wild-type (WT) [17]. In *Maize*, *ZmTIFY4, 5, 8, 26,* and *28* respond to drought; additionally, *ZmTIFY1 19* and *28* show upregulation when treated by three kinds of pathogens [5]. In summary, different *TIFY* subfamily members have different functions during plant growth and development, as well as different responses to phytohormone treatment and abiotic stress.

Populus trichocarpa serves as a model forest species and is widely used in forest tree genomics studies [18]. Previous works identified 24 *PtrTIFY* genes in the *P. trichocarpa* genome and analyzed their expression profiles under JA, MeJA, SA, salt, cold stresses, and pathogen infection in leaves [19,20]. In our study, besides these 24 genes, we found a new *TIFY* family member in the *P. trichocarpa* genome; because of the existence of the Jas and TIFY motif, we named it *PtrJAZ13*. Roots play an important role in woody plants' responses to phytohormone treatment and abiotic stress, but there are no reports to date that focus on the *TIFY* gene family in *P. trichocarpa* roots responding to such manipulations. We therefore used the roots of *P. trichocarpa* as experimental materials and employed quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) to analyze the expression profiles of all *TIFY* family genes under ABA, MeJA, and SA treatments plus drought, heat, and cold stresses. Our results provide new insights for future investigations into the roles of these candidate genes under hormone treatment and abiotic stress in *P. trichocarpa*.

2. Materials and Methods

2.1. Identification of TIFY Genes in P. trichocarpa

We used the protein family database Pfam (http://pfam.xfam.org/) [21] to search for the hidden Markov model (HMM) profile of the *TIFY* gene family (protein family ID:PF06200) based on an expected value (E-value) cutoff of 0.01 in HMMER 3.3 (http://hmmer.org/) to find *TIFY* genes in the *P. trichocarpa* genome. The genomic library, coding sequence (CDS), and protein database of *P. trichocarpa* were directly downloaded from the Phytozome v12.0 (https://phytozome.jgi.doe.gov) and NCBI (http://www.ncbi.nlm.nih.gov/). The Clustal X (version 2.0) and NCBI database were used to search the identification of the TIFY, ZML, PPD, and JAZ domains. We used the WoLF PSORT database to predict the subcellular localization of *PtrTIFY* genes. Aliphatic index, instability index,

and GRAVY (grand average of hydropathy) of *PtrTIFY* genes were identified for all *PtrTIFY* proteins by using ProtParam of Expasy tools (http://web.expasy.org/protparam) [22].

2.2. Phylogenetic Analysis

The ClustalX (version 2.0, http://www.clustal.org/clustal2/) [23] program and Bioedit 7.2 [24] software were used to performed multiple sequence alignment using the full-length protein sequence. In order to analyze the molecular features and phylogenetic relationships of the *TIFY* gene family in plants, MEGA 7.0 [25] was used to build an unrooted phylogenetic tree using the neighbor-joining (NJ) method, with a bootstrap test performed using 1000 replications and the poisson model. Gene clusters referring to the homologs within the three species (*P. trichocarpa*, *A. thaliana*, and *O. sativa*) which identified based on the NCBI database by BLAST and query cover >75% was regarded as homologous genes. The *PtrTIFY* gene exon/intron organization was determined using GSDS2.0 online software (http://gsds.cbi.pku.edu.cn) [26]. MEME 5.1.0 [27] was used to find motifs in *PuTIFY* genes using the default parameters and a conserved motif number of 15.

2.3. Promoter Cis-Element Analysis

The NCBI and Phytozome V12.0 (https://phytozome.jgi.doe.gov/pz/portal.html) databases were used to search for cis-regulating elements, which were limited the 2000 bp upstream of the ATG codon for each of the analyzed genes [28]. The online software Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [29] was used to predict and locate their *cis*-elements and analyze the functions of the *TIFY* gene family.

2.4. Plant Materials, Abiotic Stress, and Phytohormone Treatment

For plant samples, the clonally propagated *P. trichocarpa* (genotype Nisqually-1) was grown in woody plant medium (WPM) supplied with 20 g/L sucrose and 5.5 g agar. In vitro plants were cultured in 250 mL plastic containers and grown in a growth chamber with the 16 h light/8 h dark cycling at 23–25 °C with a light intensity of 46 μmol photons m^{-2} s^{-1} irradiation. Two-week-old in vitro plants were used for abiotic stress and phytohormone treatment. For abiotic stress, 7% PEG6000 simulated drought stress, 42 °C simulated heat stress, and 4 °C simulated cold stress. For phytohormone treatments, plants were exposed to a WPM medium containing 100 µM MeJA, 200 µM ABA, or 100 µM SA. Each treatment lasted for 0, 3, 6, 12, and 24 h. The 42 °C stress time points were at 0, 0.5, 1, and 3 h. Untreated in vitro plants at each time point served as control. Fresh roots undergoing each stress were frozen in liquid nitrogen immediately after being harvested and stored at −80°C for later use. Three independent biological replicates were performed for each treatment to ensure reliable data.

2.5. RNA Isolation and Real-Time Quantitative PCR

Highly purified RNA was extracted using the CTAB method [30]. The TransScript® First-Strand cDNA Synthesis SuperMix (TransGen) was used to obtain the cDNA, with the following reaction system: 500 ng of total RNA, 1 µL of anchored oligo$(dT)_{18}$, 10 µL of 2 × ES reaction mix, 1 µL of EasyScript®RT/RI enzyme mix and appropriate ddH_2O to make the total volume to 20 µL. The cDNA was diluted 10-fold (cDNA:nuclease free water = 1:10) for further qRT–PCR analysis. Gene-specific primers were designed using Primer Premier 6.25, and NCBI primer-BLAST was used to check their specificity. TransStart®Top Green qPCR Super Mix (TransGen Biotech, Beijing, China) was employed to carry out the qRT–PCR, with the following reaction system: 10 µL of 2 × TransStart® Top Green qPCR Super Mix, 7 µL of double-distilled H_2O, 1 µL of diluted template, and 2 µL of forward primer and reverse primer. qTOWER 3G Cycler and qPCR software (Analytik Jena, Germany) were used as a work program and the $2^{-\Delta\Delta CT}$ method [31] was used to perform the relative gene expression level analysis. The *PtrActin* was used as the internal reference gene. Triplicate independent technical and biological replications were used in the analysis. The primers for qRT–PCR are listed in Table S1.

2.6. Protein Interaction Network Analysis

The online database STRING v11.0 (https://string-db.org) [32] was used to predict the *PtrTIFY* protein interaction network according to the corresponding homologs between *P. trichocarpa* and *Arabidopsis*. Each amino acid sequence of the *PtrTIFY* genes was used to generate the protein interaction network. In the STRING database, a variety of active interaction sources were provided for the user to select the evidence of interaction [32]. In this study, active interaction sources were selected as "experiments" and "co-expression" with a minimum required interaction score of 0.4. Cytoscape 3.7.2 software [33] was used to visualize and analyze the interactions.

2.7. Statistical Analysis

SPSS software (version 20, IBM, Chicago, USA) was used to analyze data using one-way analysis of variance (ANOVA) followed by Tukey's test to assess significant differences between the control and each treatment. Significance was defined as * $p < 0.05$, ** $p < 0.01$.

3. Results

3.1. Identification of TIFY Genes in P. trichocarpa

In order to identify all assumptive *TIFY* genes in *P. trichocarpa*, the HMM profile of the TIFY domain (protein ID:PF06200) was used as a query to compare with the *P. trichocarpa* genome. In the end, a total of 25 candidate *TIFY* gene members were obtained. Wang et al. found 24 *PtrTIFY* genes in the *P. trichocarpa* genome; in our study, besides the founded 24 *PtrTIFY* genes, we found another *PtrTIFY* gene (*PtrJAZ13*). According to distinct domain, we named TIFY proteins as *PtrTIFY* (1–2), *PtrJAZ* (1–13), *PtrZML* (1–8), and *PtrPPD* (1–2). WoLF PSORT was used to predict the subcellular location of each candidate TIFY protein in *P. trichocarpa*. The results showed that *PtrJAZ6* and *PtrJAZ10* were predicted to be in the cytoplasm and chloroplasts, respectively; *PtrTIFY* (*PtrTIFY1*, -2) and *PtrPPD* (*PtrPPD1*, -2) subfamily members were predicted to be in the nucleus; *PtrZML2*, *-3*, *-4*, *-6* and *-7* were predicted to be in the nucleus, *PtrZML1* and *PtrZML5* were predicted to be in the cytoplasm, and *PtrZML8* was predicted to be in chloroplasts. The *TIFY* gene family has all ten different kinds of TIFY formation in *P. trichocarpa*. *PtrTIFY1*, *-2*, two *PtrJAZ* subfamily members (*PtrJAZ6*, *-12*), and *PtrPPD1*, *-2* shared the TIFY motifs with TIFYGG and TIFYCG, respectively. *PtrJAZ1*, *-4*, *-9*, *-13* contained the TIFYNG motif and *PtrJAZ2*, *-3*, *-5*, *-7*, *-8*, *-10*, *-11* shared the TIFYAG conserved motif. Eight *PtrZML* subfamily members own six different kinds of TIFY formation, including TLSFEG, TLTFRG, TLSFEG, TLSFQG, TIAFEG, TLTFQG. The GRAVY ranged from −0.225 to −0.893, the instability index ranged from 29.88 to 81.91, and the aliphatic index ranged from 48.96 to 85.07. *PtrJAZ13* had the highest stability index; *PtrZML7* possessed the minimum GRAVY (grand average of hydropathy), instability index, and aliphatic index; and *PtrZML5* contained the maximum aliphatic index. Detailed information about *PtrTIFY* genes is given in Table 1.

Table 1. Basic information of the *TIFY* family in *P. trichocarpa*.

Name	Accession Number	Subcellular Location	TIFY Motif	GRAVY	Instability Index	Aliphatic Index
PtrTIFY1	POPTR_0006s26370	nucleus	TIFYGG	−0.686	52.89	57.67
PtrTIFY2	POPTR_0018s01160	nucleus	TIFYGG	−0.642	49.32	55.05
PtrPPD1	POPTR_0002s04920	nucleus	TIFYCG	−0.662	59.33	73.36
PtrPPD2	POPTR_0005s23600	nucleus	TIFYCG	−0.743	60.38	68.33
PtrZML1	POPTR_0002s11140	cytoplasm	TLSFEG	−0.702	48.49	69.33
PtrZML2	POPTR_0002s11150	nucleus	TLTFRG	−0.682	44.88	65.52
PtrZML3	POPTR_0005s19550	nucleus	TLSFEG	−0.626	43.65	67.84
PtrZML4	POPTR_005G152800	nucleus	TLTFRG	−0.698	44.14	61.94
PtrZML5	POPTR_007G116500	cytoplasm	TLSFQG	−0.239	30.39	85.07
PtrZML6	POPTR_0007s03120	nucleus	TIAFEG	−0.852	43.09	58.1
PtrZML7	POPTR_0010s25810	nucleus	TLTFQG	−0.893	29.88	48.96
PtrZML8	POPTR_0017s06970	chloroplast	TIAFEG	−0.753	38.35	62.7
PtrJAZ1	POPTR_0001s13240	nucleus	TIFYNG	−0.563	55.46	69.74
PtrJAZ2	POPTR_0001s16640	nucleus	TIFYAG	−0.603	50.76	66.47
PtrJAZ3	POPTR_0003s06670	nucleus	TIFYAG	−0.635	50.12	63.75
PtrJAZ4	POPTR_0003s16350	nucleus	TIFYNG	−0.567	59.08	72.34
PtrJAZ5	POPTR_0006s14160	nucleus	TIFYAG	−0.417	60.09	65.18
PtrJAZ6	POPTR_0006s23390	cytoplasm	TIFYGG	−0.167	56.63	78.26
PtrJAZ7	POPTR_0008s13290	nucleus	TIFYAG	−0.271	49.13	68.32
PtrJAZ8	POPTR_0010s11850	nucleus	TIFYAG	−0.225	59.27	68.89
PtrJAZ9	POPTR_0011s02260	nucleus	TIFYNG	−0.799	73.46	78.52
PtrJAZ10	POPTR_0012s04220	chloroplast	TIFYAG	−0.329	53.25	68.37
PtrJAZ11	POPTR_0015s04880	nucleus	TIFYAG	−0.296	52.6	67.78
PtrJAZ12	POPTR_0018s08300	nucleus	TIFYGG	−0.559	56.38	62.53
PtrJAZ13	POPTR_006G023300	nucleus	TIFYNG	−0.782	81.91	71.67

3.2. Phylogenetic Analysis of TIFY Genes

To gain insight into the evolutionary relationship between TIFY proteins in *P. trichocarpa*, the 25 full-length TIFY protein sequences from *P. trichocarpa*, 17 proteins from *Rice*, and 18 proteins from *Arabidopsis* were used to build the neighbor-joining phylogenetic tree. MEGA 7.0 software was used to visualize the results. As shown in Figure 1, nine clades were identified in the phylogenetic tree, including JAZ I to V, PPD, TIFY I, II, and ZML. Eight PtrTIFY proteins (PtrZML1, -2, -3, -4, -5, -6, -7, -8) were clustered together in Group I, which contained a GATA zinc-finger domain and a CCT motif based on distinct domain. The remaining 17 PtrTIFY proteins were clustered in Group II. This group contained all PtrJAZ, PtrTIFY, and PtrPPD subfamily members. The *PtrJAZ* subfamily contains the largest numbers of members, which were distributed in three of the four JAZ clades, omitting JAZII. *PtrJAZ13* was in the JAZIV clade and belongs to Group II. From the genetic relationship, *PtrJAZ2*, *-3*, *-5*, *-6*, *-7*, *-8*, *-10*, *-11*, *PtrZML1*, *-3*, PtrTIFY*1*, *-2* and *PtrPPD1*, *-2* were clustered with TIFY proteins in *Arabidopsis*. This phenomenon indicates that PtrTIFY proteins are more closely related to those of *Arabidopsis* than to those of *Rice*.

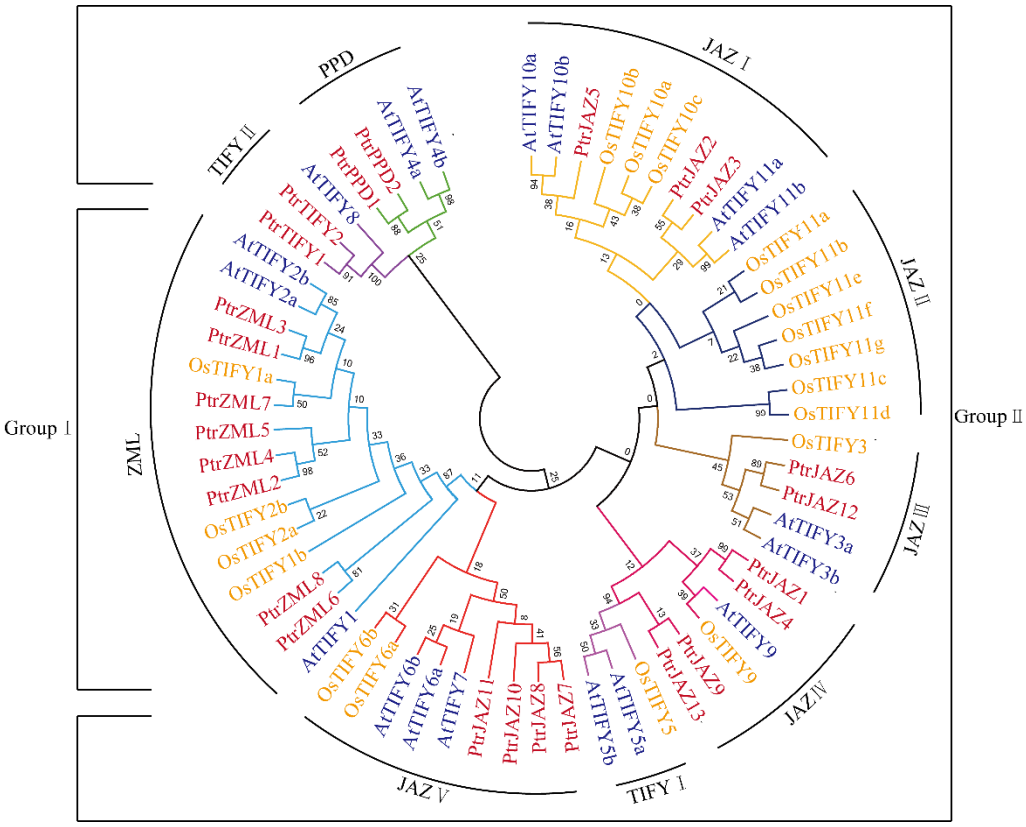

Figure 1. Phylogenetic analysis of the TIFY family members from *P. trichocarpa*, *O. sativa*, and *Arabidopsis*. The phylogenetic trees were constructed using the neighbor-joining (NJ) method of MEGA7 with 1000 bootstrap replicates. For Group I and Group II, Group I contained the C2C2-GATA (CX2CX20CX2C) conservative domain, which was absent in Group II. Each clade was represented by different color lines. The naming approach of each clade was following a previous study [34]. The explanation of group I and II of Figure 1 was added in the Figure 1 legend which was highlighted in red font and yellow background

3.3. Phylogenetic Analysis, Gene Structure, and Conserved Motifs of TIFY Genes in P. trichocarpa

In order to analyze the phylogenetic relationships among the *TIFY* genes in *P. trichocarpa*, an unrooted phylogenic tree was constructed from alignments of the full-length *PtrTIFY* protein sequences. As shown in Figure 2A, *PtrTIFY* genes were clustered into five sections, including two parts of *PtrJAZ*. Based on the phylogenetic analysis, we identified 11 sister pairs, all of which had strong bootstrap support (>90%). To gain further insights into the structural diversity of *PtrTIFY* genes, we analyzed the exon/intron organization in the full-length cDNAs with their corresponding genomic DNA sequences of individual *PtrTIFY* genes in *P. trichocarpa* (Figure 2B). Most *PtrTIFY* subfamily members shared similar exon/intron lengths and numbers, especially the sister pair genes within the same subfamily. For example, both *PtrTIFY1* and *PtrTIFY2* contained five introns of similar lengths, while *PtrJAZ13* and its homologous gene *PtrJAZ9* possessed only one intron. Distinct motifs were found in *PtrTIFY* genes by the MEME website. All *PtrTIFY* genes contained the conserved TIFY motif. As expected, the most closely related genes contained similar motifs, indicating functional diversity among TIFY proteins in the subgroup. In total, we found 15 motifs within 25 *PtrTIFY* genes, including Motif 1 representing the TIFY motif, Motif 2 representing the JAZ motif, and Motif 13 representing the PPD motif. *PtrJAZ9* and *PtrJAZ13* contained the minimum motif in number (Motifs 1 and 2). Interestingly, *PtrJAZ8* possessed one more Motif 6 than *PtrJAZ7* (Figure 2C). Detailed motif information is shown in Table S2.

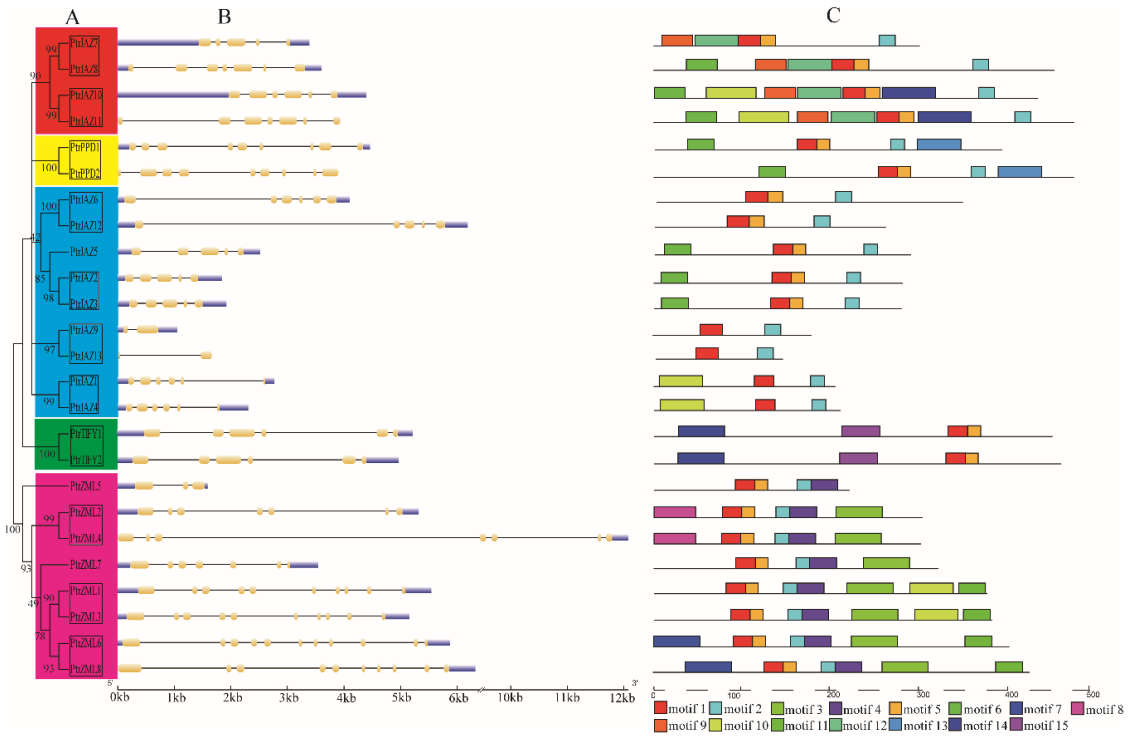

Figure 2. Phylogenetic relationships and gene structure of *TIFYs* in *P. trichocarpa*. (**A**) Multiple alignment of full-length amino acid sequences of *PtrTIFY* genes was carried out with ClustalX 2.0. The phylogenetic tree was constructed using neighbor-joining method with MEGA7.0. The sister pairs was marked with black-box. Red and blue boxes represent the *JAZ* subfamily, yellow box represents the *PPD* subfamily, green box represents the *TIFY* subfamily, and pink box represents the *ZML* subfamily. (**B**) Exon/intron structures of the *PtrTIFY* gene family. (**C**) The conserved motifs were obtained from the MEME website. Fifteen different kinds of conserved motifs were marked with different colors.

3.4. Promoter Cis-Element Analysis

Promoter *cis*-elements play pivotal roles in the transcriptional regulation of genes when plants are under biotic and abiotic conditions. Phytohormones, including ABA, JA, and SA, apart from their functions during growth and development, play important roles in various signal transduction pathways during responses to environmental stresses [35]. Hence, we decided to identify putative *cis*-acting regulatory DNA elements in *PtrTIFY* genes, based on their promoter sequences (2000 bp upstream of the initiation codon). Six different kinds of *cis*-elements relating to phytohormones and environmental stress signals were identified. Most *PtrTIFY* genes possessed the AREB *cis*-elements (cis-acting elements involved in abscisic acid responsiveness). It is worth noting that *PtrJAZ8* contained nine AREB *cis*-elements in different positions of its promoter. *PtrJAZ13*, *PtrZML2*, *-3*, *-5*, and *PtrTIFY2* contained only one AREB *cis*-element. *PtrJAZ4*, *PtrPPD2,* and *PtrZML4* lack the AREB *cis*-element. The remaining *PtrTIFY* genes contained 2–7 AREB *cis*-elements. Thirteen *PtrTIFY* genes contained the CGTCA/TGACG *cis*-element (*cis*-acting element involved in MeJA-responsiveness). *PtrJAZ1*, *-6*, *-7*, *-10*, *-12*, *PtrPPD1*, *-2*, *PtrZML1*, *-2*, *-4*, *-7*, *-8*, and *PtrTIFY2* contained both CGTCA and TGACG *cis*-elements in their promotors. Eight *PtrJAZ* genes, one *PtrPPD* gene, three *PtrZML* genes, and one *PtrTIFY* gene possessed a TCA-element that is involved in SA responsiveness. GAAnnTTC is the typical *cis*-element of heat shock elements (HSE) [36]. In the *PtrTIFY* gene family, *PtrJAZ1*, *-2*, *-3*, *-8*, *-10*, *-13* contained HSE in their promotors, with *PtrJAZ10* possessing the largest number of HSEs. *PtrPPD2*, *PtrZML2*, *6* as well as *PtrTIFY1* and *PtrTIFY2* contained 1–3 HSEs (Figure 3). In summary, each *PtrTIFY* member contained at least one biotic or phytohormone responsive *cis*-element in its promotor. Detailed promoter *cis*-element information is listed in Supplementary Table S3.

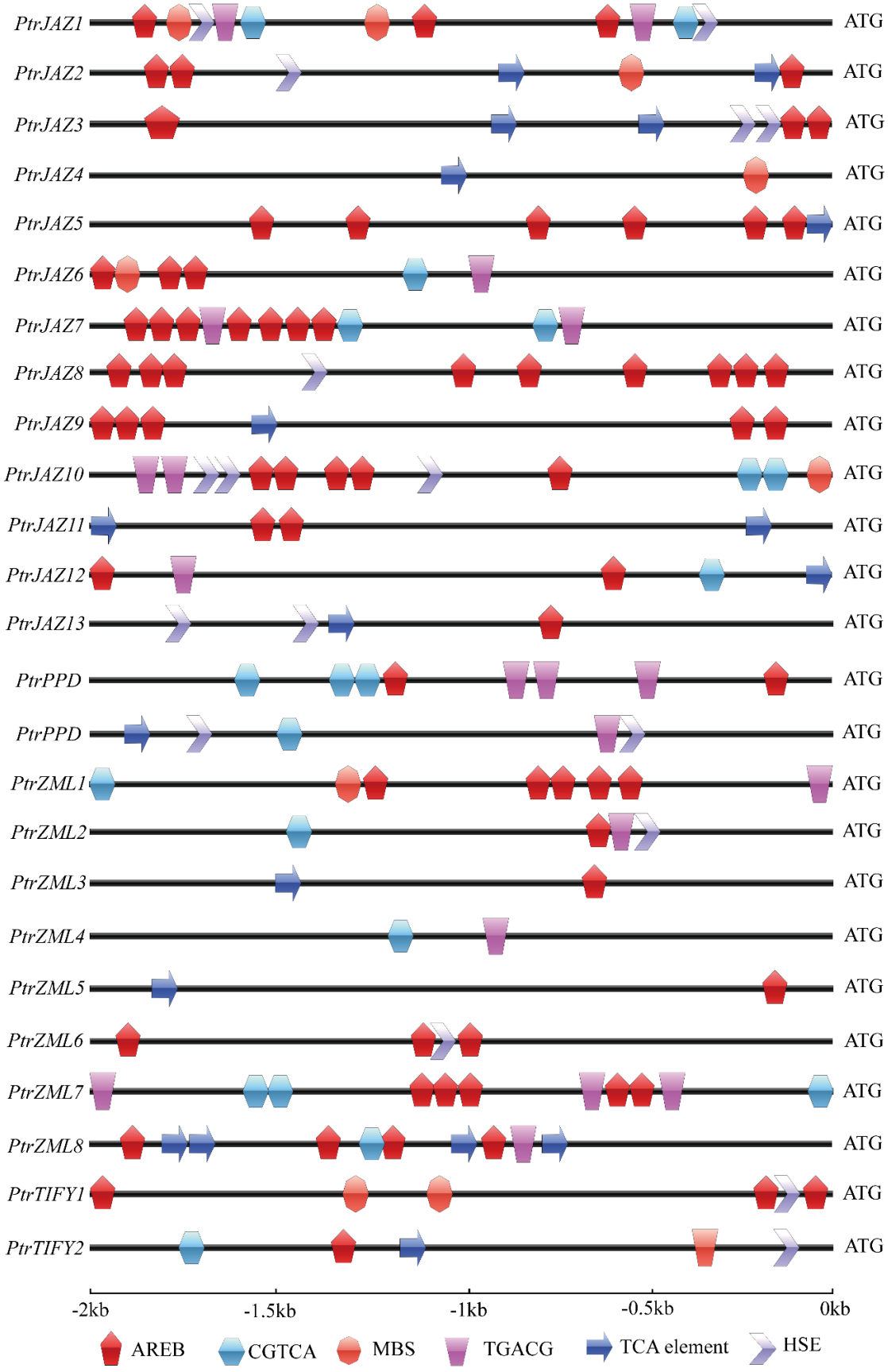

Figure 3. Abiotic stress and phytohormone response elements in *PtrTIFY* gene promoters.

3.5. Expression Pattern of PtrTIFY Genes under Drought, Heat, Cold Stress

To understand how *PtrTIFY* genes take part in drought stress response, we analyzed the expression of *PtrTIFY* genes in roots under drought at 3, 6, 12, and 24 h using qRT–PCR. Our data showed that all *PtrJAZ* subfamily members and *PtrZML2, 3* as well as *PtrZML2* and *PtrZML3* were induced in roots by drought stress. It is noteworthy that *PtrJAZ10* was significantly upregulated at all time points. *PtrPPD1* and *PtrPPD2* as well as *PtrZML5* and *PtrZML8* did not change significantly compared to untreated controls. Finally, *PtrZML4, PtrZML7* and *PtrTIFY2* were downregulated at all time points (Figure 4).

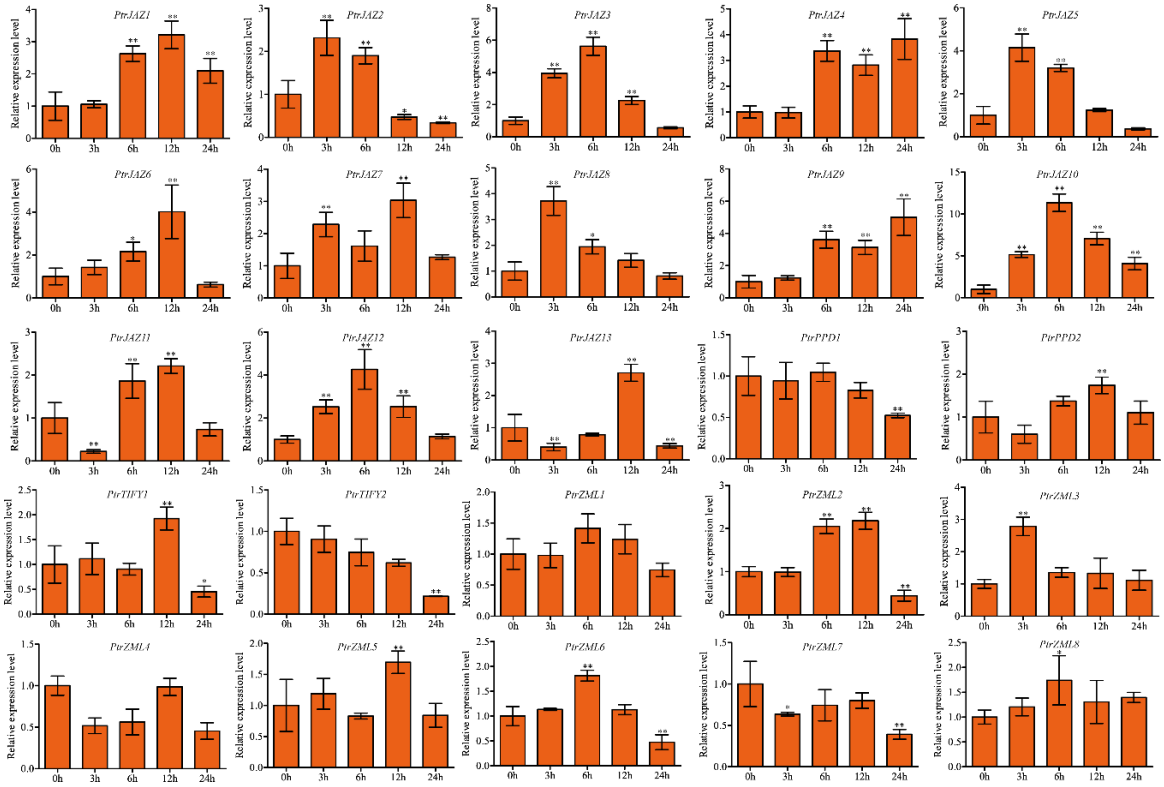

Figure 4. Expression analysis of *PtrTIFY* genes in root under drought stress by qRT–PCR. Error bars represent the standard deviations from three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* $p < 0.05$, ** $p < 0.01$).

We also analyzed the expression pattern of the *PtrTIFY* genes in the roots under 42 °C at 0.5, 1, and 3 h. The results showed all *PtrJAZ* subfamily genes were induced by heat stress except for *PtrJAZ2* and *PtrJAZ13*, which were suppressed at all time points. *PtrJAZ4* was upregulated about 17-fold at 1 h. Interestingly, *PtrJAZ6* was downregulated at the 0.5 h time point and was rapidly upregulated at 1 and 3 h. *PtrJAZ12* responded rapidly to heat stress, with the relative expression level being upregulated 10-fold compared with normal and then gradually declining to 5-fold at 3 h. *PtrPPD2* and *PtrTIFY1* were upregulated at the 1-h time point. *PtrZML3* was highly upregulated at all the time points. Finally, *PtrPPD1*, *PtrZML1*, and *PtrZML6* showed no change in expression under heat stress at any time point ($p > 0.05$; Figure 5).

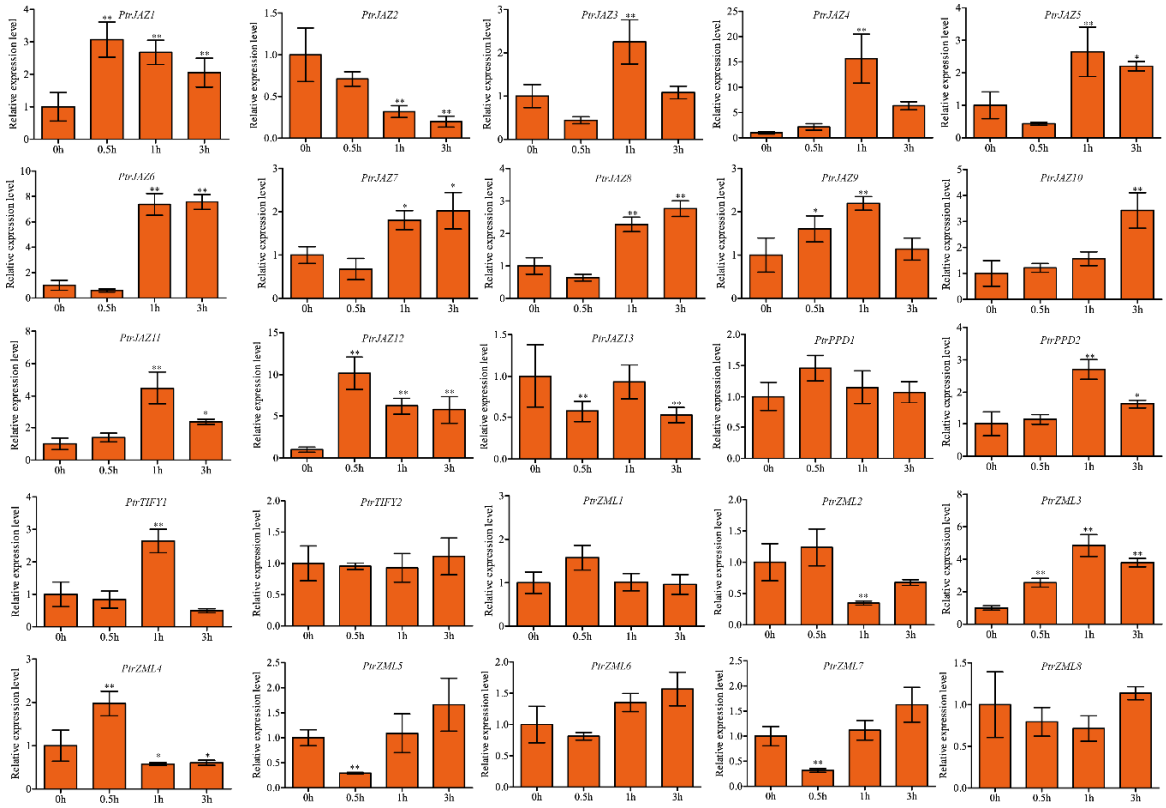

Figure 5. Expression analysis of *PtrTIFY* genes in root under heat stress by qRT–PCR. Error bars represent the standard deviations from three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* $p < 0.05$, ** $p < 0.01$).

For cold stress, eleven *PtrJAZ* subfamily members were induced by cold stress in roots. *PtrJAZ6* was downregulated at 12 h and *PtrJAZ11* was significantly downregulated during the early response period and was elevated back to a level comparable to that of untreated control at 12 and 24 h. *PtrJAZ9* and *PtrJAZ12* were dramatically upregulated at all time points, *PtrJAZ9* and *PtrJAZ12* were upregulated about 11-fold at 24 h and 19-fold at 3 h, respectively. One *PtrPPD* gene and half of the *PtrZML* subfamily genes were strongly induced by cold stress (Figure 6).

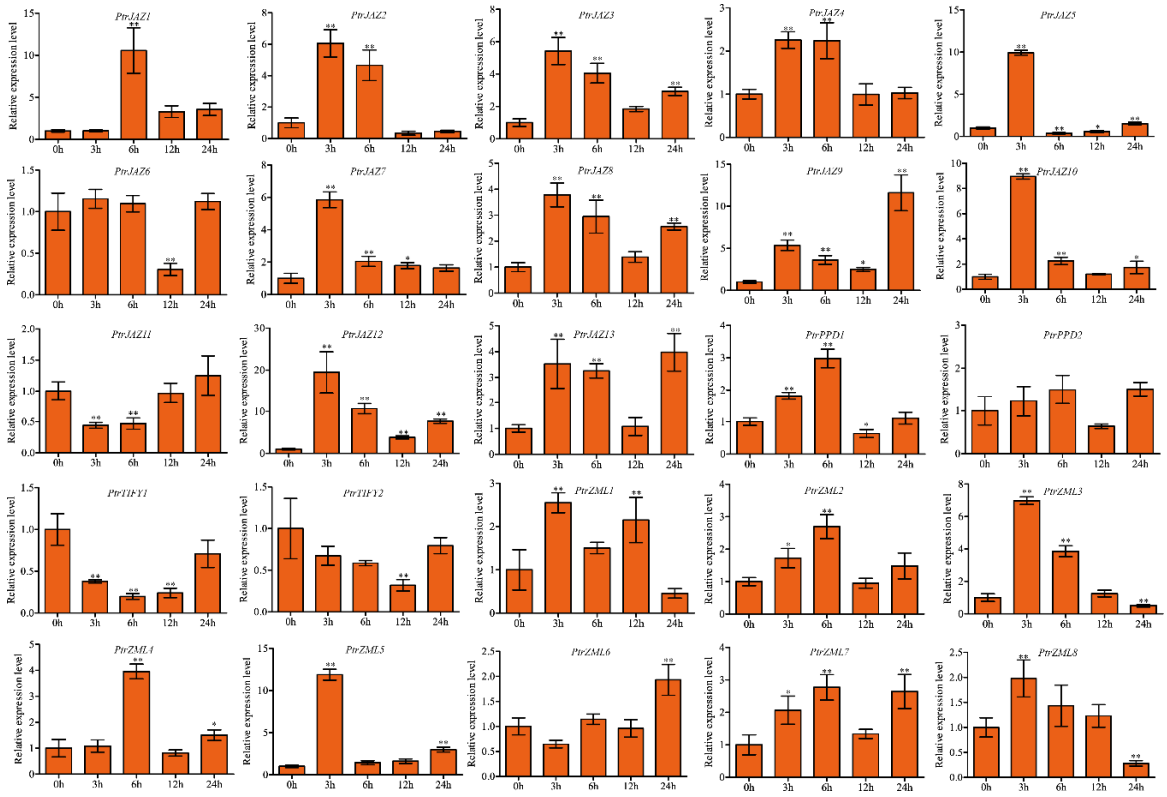

Figure 6. Expression analysis of *PtrTIFY* genes in root under low-temperature stress by qRT–PCR. Error bars represent the standard deviations from three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* $p < 0.05$, ** $p < 0.01$).

3.6. Expression Pattern of PtrTIFY Genes Under ABA, MeJA, and SA Treatments

We employed qRT–PCR to determine the relative expression levels of all *PtrTIFY* members under 200 μM ABA treatment in *P. trichocarpa* roots at 0, 3, 6, 12, and 24 h. Up- or downregulation of the relative expression level >2.0-fold was regarded as significantly differentially expressed. In roots, most *PtrJAZ* subfamily members were induced by 200 μM ABA treatment. The relative expression levels of *PtrJAZ1* and *PtrJAZ9* were upregulated about 17-fold at 12 h compared with control. *PtrJAZ3* and *PtrJAZ6* had similar expression patterns, with the expression level being gradually upregulated at 3 and 6 h and then gradually downregulated at 12 and 24 h. *PtrJAZ13* was downregulated at all time points in the root. *PtrPPD1* and *PtrPPD2* were upregulated by 200 μM ABA treatment at 12 h. *PtrTIFY1* was slightly up-regulated at 3 and 12 h, *PtrTIFY2* was suppressed by ABA at all time points. *PtrZML1*, -5, -6, -7 were significantly upregulated by ABA. The remaining five *PtrZML* subfamily members exhibited opposing expression patterns. The relative expression level of *PtrZML2* showed gradual upregulation at 3 and 6 h, then peaked at 12 h before declining (Figure 7).

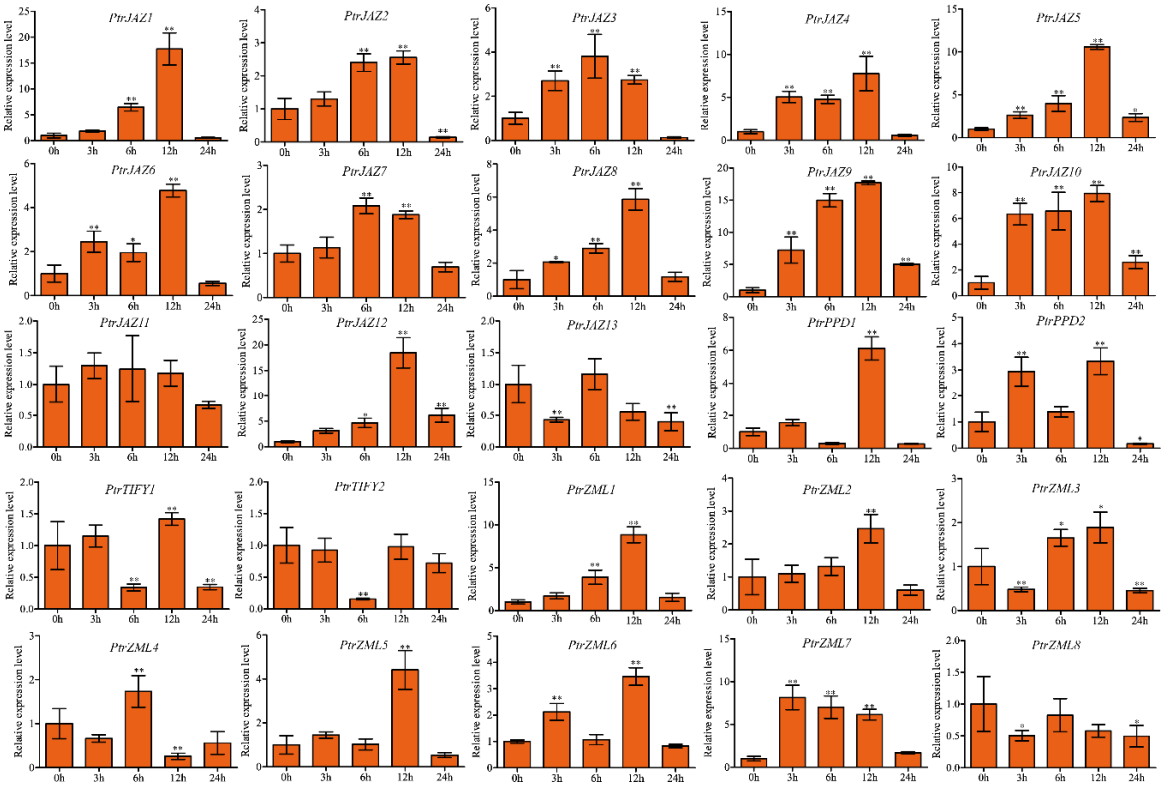

Figure 7. Expression analysis of *PtrTIFY* genes in root under 200 μM ABA treatment by qRT–PCR. Error bars represent the standard deviations from three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* $p < 0.05$, ** $p < 0.01$).

For MeJA treatment, all but *PtrJAZ10* among *PtrJAZ* subfamily members were strongly induced by 100 μM MeJA in roots. *PtrJAZ10* showed no change compared to control. *PtrJAZ4*, *-5*, *-7*, *-11*, *-12*, and *-13* were significantly upregulated at both 3 and 6 h, indicating these *PtrJAZ* subfamily genes are early-responding genes for MeJA treatment. *PtrPPD1* was suppressed by MeJA at 3, 6, and 24 h, while its homologous gene *PtrPPD2* showed no significant change in expression compared to control. *PtrTIFY1* and *PtrTIFY2* were markedly downregulated at 12 h. Only *PtrZML4*, *-7*, and *-8* were induced by MeJA treatment. *PtrZML5* and *PtrZML6* did not respond to MeJA treatment at any time point (Figure 8).

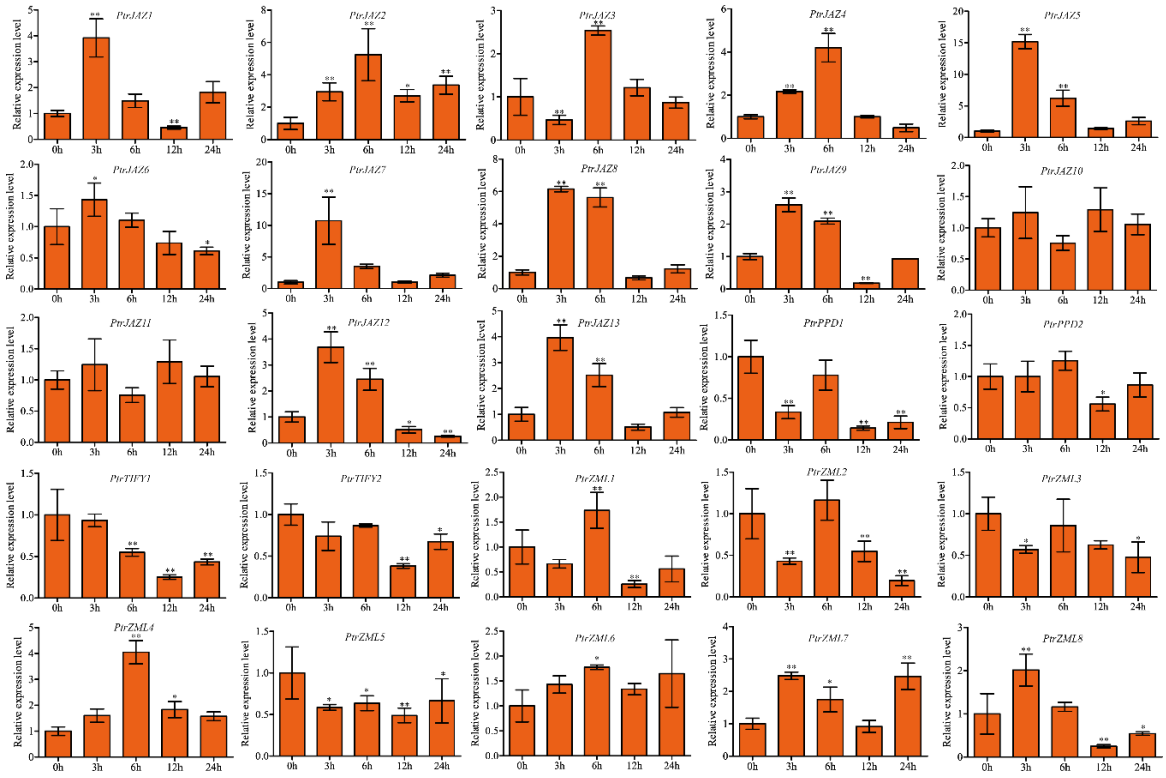

Figure 8. Expression analysis of *PtrTIFY* genes in root under 100 μM MeJA treatment by qRT–PCR. Error bars represent the standard deviations from three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* $p < 0.05$, ** $p < 0.01$).

For SA treatment, all but *PtrJAZ5* were induced by SA treatment in roots. *PtrJAZ1* showed gradual upregulation with time, peaked at 12 h (26-fold) then declined. *PtrJAZ13* was significantly upregulated at 3 and 6 h, then declined to normal expression level at 12 and 24 h. *PtrJAZ1* and *PtrJAZ9* were dramatically upregulated at all time points. *PtrPPD1* was induced by SA treatment in roots and *PtrPPD2* was slightly up-regulated at 3 and 6 h, and repressed at 12 and 24 h. *PtrTIFY2* was suppressed by SA. For *PtrZML*s, only *PtrZML5* was upregulated at the 6-h time point; *PtrZML1*, *-3*, *-4*, *-6*, *-7*, and *-8* were downregulated compared to the untreated control, while *PtrZML2* was slightly increased by the 100-μM SA treatment. *PtrZML1* and *PtrZML6* were suppressed by MeJA at all the time points (Figure 9).

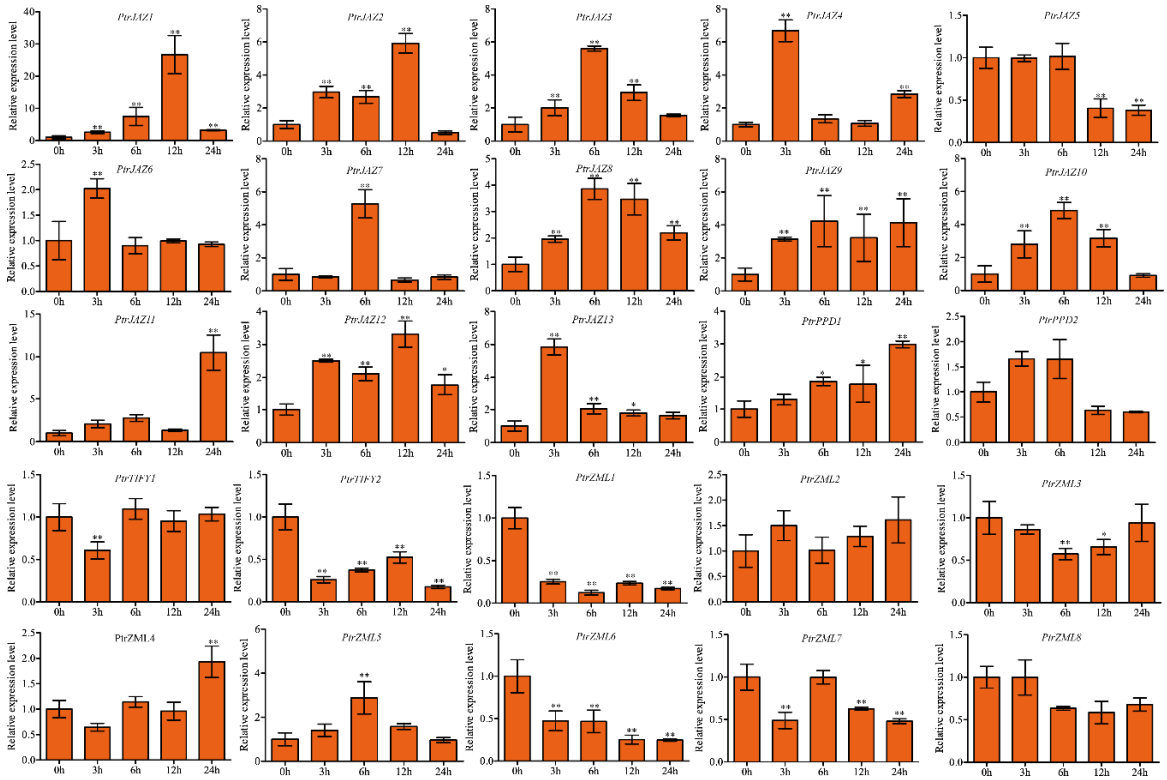

Figure 9. Expression analysis of *PtrTIFY* genes in root under 100 μM SA treatment by qRT–PCR. Error bars represent the standard deviations from three biological replicates. Asterisks indicate stress treatment groups that showed a significant difference in transcript abundance compared with the control group (* $p < 0.05$, ** $p < 0.01$).

3.7. Analysis of the TIFY Protein Interaction Network in P. trichocarpa

The STRING database was used to investigate the relationship of interaction and association in PtrTIFY proteins with a medium confidence score of 0.40 and the interaction sources "experiments" and "co-expression". Thirteen putative protein networks were constructed within three kinds of subfamilies (*PtrJAZ*, *PtrZML*, and *PtrTIFY*). This result showed that PtrTIFY proteins could interact with other proteins to respond to phytohormone treatment and environmental stress in *P. trichocarpa*. Interestingly, the pairs PtrJAZ10 and PtrJAZ11, PtrTIFY1 and PtrTIFY2, and PtrZML5 and PtrZML7 shared similar protein interaction networks, indicating a conserved protein interaction domain may exist in each protein pair (Figure 10).

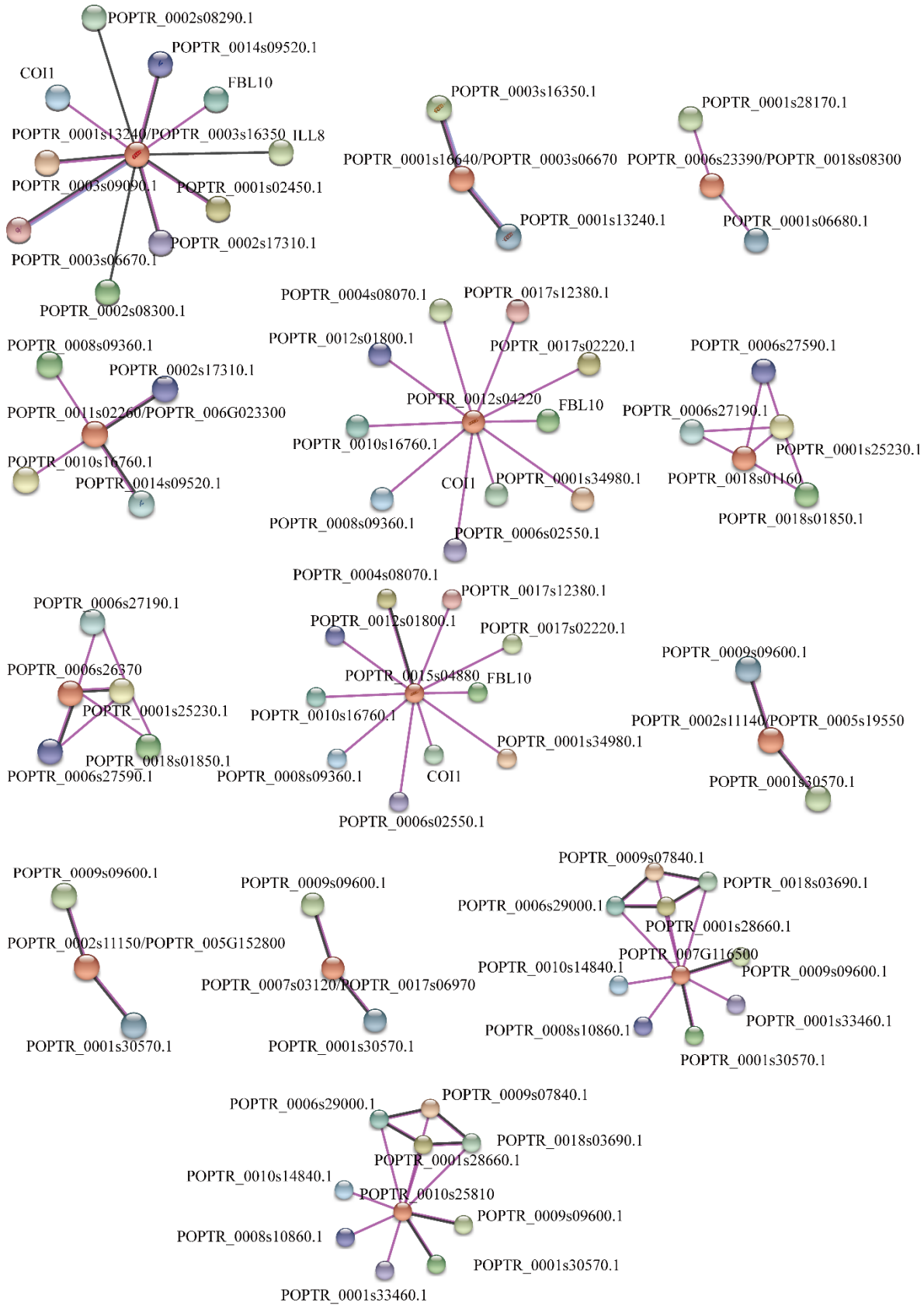

Figure 10. The predicted protein interaction network of PtrTIFY proteins by STRING database. Different colored lines represent different evidence of interaction.

4. Discussion

TIFY is a plant-specific transcription factor that has been identified in many model plants, such as *Arabidopsis*, *Rice*, and *Maize* [1,3,5]. Previous studies have given a comprehensive analysis of 24 *PtrTIFY* genes in leaves of *P. trichocarpa* under various phytohormone treatments and abiotic stresses [19,20]. In this study, we found a new *PtrTIFY* gene, *PtrJAZ13*, that belongs to the important *JAZ* subfamily. Additionally, we examined in roots, for the first time, the expression pattern of the entire *PtrTIFY* family under ABA, MeJA, and SA treatments plus drought, heat, and cold stresses.

Each of the 25 *TIFY* genes in the *P. trichocarpa* genome contains at least one conserved TIFY domain. The length of these sequences varied significantly, implying a high degree of complexity among the *TIFY* genes. WoLF PSORT analysis helped to predict the location of PtrTIFY proteins. Most PtrTIFY proteins (20/25) were predicted to localize to the nucleus. In addition, *PtrZML1*, *-5*, and *PtrJAZ6* on one hand, and *PtrZML8* and *PtrJAZ10* on the other, were localized in cytoplasm and chloroplast, respectively. Interestingly, *PtrJAZ6* and *PtrJAZ12* were in cytoplasm and nucleus, respectively, although they were placed in the JAZIII clade as homologous genes; it is the same with *PtrZML1* and *PtrZML3*, *PtrZML6* and *PtrZML8*. These results indicate that the same phylogenetic group identified based on sequence similarity does not necessarily correspond to the same subcellular localization [28]. Therefore, homologous genes may show differences in gene function and signal transduction.

In this study, we compare the members of the *TIFY* genes in *Populus*, *Rice*, and *Arabidopsis*. Our results show that the phylogenetic tree is divided into two groups; Group I contains the GATA zinc-finger, and Group II lacks it. All *PtrZML* members, together with *OsTIFY1a*, *-1b*, *-2a*, and *-2b*, *AtTIFY1*, *-2a*, and *-2b* belong to the Group I, which contain not only the TIFY domain but also the GATA zinc-finger and CCT motif. The remainder are in Group II. PtrTIFY and AtTIFY proteins are more closely related than those of rice, indicating that they may have a common ancestor. Interestingly, *OsTIFY1a*, *-1b*, *-2a*, *-2b*, *AtTIFY1*, *-2a*, *-2b* not only have a TIFY domain but also a GATA zinc-finger and a CCT motif; the remaining members in *Rice* and *Arabidopsis* do not have the CCT motif. However, almost all the PtrZML proteins contain the CCT motif, suggesting that woody plants might have undergone many different changes during the evolutionary process [37].

The promoter is a specific DNA region located about 2000 bp upstream of the initiation codon that contains a variety of *cis*-elements, including AREB, MBS, LTR, HSE, CGTCA, and TGACG. These help plants respond to exogenous phytohormone treatment and environmental stress [38,39]. In our study, *PtrTIFY* genes possessed at least one stress- or phytohormone-related promoter *cis*-element, indicating *PtrTIFY* genes respond to exogenous phytohormone treatments and environmental stresses. It is worth noting that *PtrJAZ3*, -5 and -8 did not possess the MBS *cis*-element in their promoter regions, although our qRT–PCR data showed these three genes were strongly induced in the root by drought stress; we can thus speculate that they may interact with other genes to respond to drought stress.

Abiotic stresses, such as drought, cold, and heat, along with important phytohormones, always influence plant growth and development in the life cycle, and may even cause fatal damage [40]. To adapt and survive under adverse environments, woody plants have evolved a number of responses, including reprogramming of gene expression [40]. In this study, *PtrJAZ1, -3, -4, -5, -7, -8, -9 -10, -12* showed high expression levels during drought, heat, and cold stresses, indicating that the *PtrTIFY* family has important functions in responding to these abiotic stresses. *PtrJAZ2* and *PtrJAZ13* were induced by drought and cold stresses but suppressed by heat stress, whereas *PtrJAZ11* was upregulated in drought and heat stresses but downregulated in cold stress. These results suggest that *PtrTIFY* sets up different physiological and biochemical functions to respond to various environmental challenges. Notably, *OsTIFY9*, a *PtrJAZ1* and *PtrJAZ4* orthologous gene, was strongly induced by drought and cold stresses [3], suggesting the homologous genes have similar functions in different species. A previous study has shown the relative expression level of *VvJAZ11* was downregulated by cold stress [2], but according to our qRT–PCR data, its homologous genes in *P. trichocarpa* (*PtrJAZ7, -8, -10*) are strongly induced by cold stress. This result suggests that their functions might vary in different plant species. Many previous studies had shown that ABA takes part in response to drought stress [41],

with multiple drought stress-responsive genes being induced by ABA [42]. In this study, qRT–PCR analysis showed almost all *PtrJAZ* subfamily members were induced by ABA treatment and by drought stress. In *Rice*, *OsJAZ1* was induced by ABA treatment and drought stress, over-expression of *OsJAZ1* lead to a drought-sensitive phonotype under ABA treatment [43]. *PtrJAZ6* and its homologous gene *PtrJAZ12* were simultaneously induced under ABA treatment and drought stress. *PtrJAZ6/12* was the homologous gene of *OsJAZ1*; this result suggests that *PtrJAZ6/12* may negatively function in drought stress under ABA treatment in *P. trichocarpa*. Salicylic acid (SA) is a key molecule in the signal transduction pathway of abiotic responses [44,45]. In this work, qRT–PCR shows that most *PtrJAZ* subfamily members are induced by SA and by heat stress. *PtrJAZ1*, as well as *PtrJAZ4*, were remarkably upregulated at all time points both by SA treatment and heat stress. A previous study showed that SA functions as a plant growth regulator and alleviates the effects on photosynthesis under heat stress [46]. When plants suffer from heat stress, SA could help to increase proline production through the increase in γ-glutamyl kinase and decrease in proline oxidase activity to help protect photosynthesis from heat stress [47]. *PtrJAZ* subfamily genes may play important roles in the cross-talk between SA and heat stress.

JA is also involved in a wide range of plant growth and developmental processes, including root development [48]. Previous research had shown that JA could regulate lateral root formation in *Arabidopsis* and petunia cuttings [49,50]. Furthermore, *PtrJAZ5* was dramatically upregulated at early response time points (3 and 6 h) under MeJA treatment. *GaJAZ5* was the homologous gene of *PtrJAZ5* in *Gossypium arboretum*; previous research had shown that when *GaJAZ5*-overexpression lines were treated with exogenous MeJA, the lateral root and root hair were higher in number compared to untreated transgenic lines in cotton [51]. OsJAZ1 could regulate root development by interacting with OsMYC2 through JA signaling in *Rice* [52]. In this research, besides *PtrJAZ5, PtrJAZ7, -8, -9, -11, -12, -13* were also dramatically upregulated at early response time points (3 and 6 h); moreover, *PtrJAZ2* was significantly upregulated at all time points under MeJA treatment. These results suggest the *PtrJAZ* subfamily members may take part in root growth and development through the JA signaling pathway.

We constructed the protein interaction network to investigate their interactions and associations in *P. trichocarpa* using the STRING database. The results showed that ten PtrJAZ (PtrJAZ1/4, PtrJAZ2/3, PtrJAZ6/12, PtrJAZ9/13, PtrJAZ10, and PtrJAZ11), two PtrTIFY (PtrTIFY1 and PtrTIFY2), and eight PtrZML (PtrZML1/3, PtrZML2/4, PtrZML5, PtrZML6/8, and PtrZML7) subfamily members were predicted to interact with other proteins. Two members of the PtrPPD subfamily could not interact with any other proteins; nevertheless, *PtrPPD1* was induced by ABA, SA, and cold stress, and *PtrPPD2* was upregulated by ABA at 12 h and heat stress at 1 h, indicating that the two *PtrPPD* subfamily members may function by regulating their target genes to respond to abiotic stress and phytohormone treatment. Increasing evidence supports the idea that Arabidopsis COI 1 (CORONATINE IN SENSITIVE 1), NINJA CNOVEL IN TEACTIOR OF JAZ and two bHLH protein family members (MYC2/MYC3) play important roles in JA signaling and function in the JA pathway, such as root growth inhibition, wound response, and abiotic stress response [10,53,54]. *POPTR_0003s09090* is a homologous gene of *AtMYC2*; moreover, *PtrJAZ1/4* shared a high degree of homology with *AtTIFY9*, which had been reported to interact with AtMYC2 to restrain the activity of transcription factor and then promote the expression of JA-relative genes [55]. Meanwhile, according to our expression pattern, *PtrJAZ1/4* was induced by ABA treatment and drought, heat stresses, so we speculate that *PtrJAZ1/4* may interact with *PtrMYC2* in response to phytohormone treatment and abiotic stress by taking part in JA signaling. PtrJAZ1/4, PtrJAZ10, and PtrJAZ11 shared similar protein interaction networks with PtrZML5 and PtrZML7, PtrJAZ2/3 and PtrZJAZ6/12, PtrTIFY1 and PtrTIFY2, PtrZML2/4 and PtrZML6/8, as well as PtrZML5 and PtrZML7. This phenomenon indicates that a conserved protein interaction domain may exist in these proteins that share the same network.

5. Conclusions

In summary, we identified 25 *PtrTIFY* genes in the *P. trichocarpa* genome and performed comprehensive bioinformatics analyses, including phylogenetic analysis, conserved motif analysis, and promoter *cis*-elements analysis. Importantly, a new TIFY member (*PtrJAZ13*) was found. Then, for the first time, we analyzed the expression pattern of *PtrTIFY* genes in roots under ABA, MeJA, and SA treatments plus drought, heat, and cold stresses. Almost all the *PtrTIFY* genes responded to at least one abiotic stress and phytohormone treatment in the root of *P. trichocarpa*. These results indicate that the *PtrTIFY* genes may play important roles in response to phytohormone treatment and abiotic stress. Our study will help to determine which genes deserve further functional characterization.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/3/315/s1. Table S1: Primer pairs for real-time quantitative PCR. Table S2: Motif sequences of *PtrTIFY* genes. Table S3: Phytohormone- and abiotic stress-related *cis*-elements.

Author Contributions: Conceptualization, H.W., and C.L.; experiment design, C.L.; data curation, W.H., L.X., X.X., and C.L.; contribution of materials/analysis tools, H.W., X.L., X.X., and C.L.; writing—original draft, H.W., and X.L.; writing—review and editing, C.L. All authors have read and agreed to the published version of the manuscript.

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