Innovation and Emerging Roles of Populus trichocarpa TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR Transcription Factors in Abiotic Stresses by Whole-Genome Duplication

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The TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP) family proteins are plant-specific transcription factors that have been well-acknowledged for designing the architectures of plant branch, shoot, and inflorescence. However, evidence for their innovation and emerging role in abiotic stress has been lacking. In this study, we identified a total of 36 TCP genes in Populus trichocarpa, 50% more than that in Arabidopsis (i.e., 24). Comparative intra-genomes showed that such significant innovation was mainly due to the most recent whole genome duplication (rWGD) in Populus lineage around Cretaceous-Paleogene (K-Pg) boundary after the divergence from Arabidopsis. Transcriptome analysis showed that the expressions of PtrTCP genes varied among leaf, stem, and root, and they could also be elaborately regulated by abiotic stresses (e.g., cold and salt). Moreover, co-expression network identified a cold-associated regulatory module including PtrTCP31, PtrTCP10, and PtrTCP36. Of them, PtrTCP10 was rWGD-duplicated from PtrTCP31 and evolved a strong capability of cold induction, which might suggest a neofunctionalization of PtrTCP genes and contribute to the adaptation of Populus lineage during the Cenozoic global cooling. Evidentially, overexpression of PtrTCP10 into Arabidopsis increased freezing tolerance and salt susceptibility. Integrating co-expression network and cis-regulatory element analysis confirmed that PtrTCP10 can regulate the well-known cold- and salt-relevant genes (e.g., ZAT10, GolS2, and SOS1), proving that PtrTCP10 is an evolutionary innovation in P. trichocarpa response to environmental changes. Altogether, our results provide evidence of the rWGD in P. trichocarpa responsible for the innovation of PtrTCP genes and their emerging roles in environmental stresses.

Keywords: TCP, whole-genome duplication, K-Pg extinction event, abiotic stress, adaption, transcriptomics
INTRODUCTION

Plant development and defense to various environmental stresses are determined by the coordinate transcriptional regulation of multiple genes. This is mostly achieved by the action of transcription factors, which show specific DNA binding to regulate downstream genes in response to endogenous and exogenous stimuli (Riechmann et al., 2000). TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP) proteins constitute a plant-specific transcription factor family that plays important roles in multiple developmental processes of plants (Danisman et al., 2013). The TCP family is named after the initially identified members, including TEOSINTE BRANCHED1 (TB1) in maize (Zea mays), CYCLOIDEA (CYC) in snapdragon (Antirrhinum majus), and PCF genes in rice (Oryza sativa) (Luo et al., 1996; Doebley et al., 1997; Kosugi and Ohashi, 1997; Cubas et al., 1999), which share a 59-residue conserved non-canonical basic helix-loop-helix (bHLH) DNA-binding region (i.e., TCP domain) (Cubas et al., 1999). Based on the domain, TCP family proteins can be classified into two subfamilies, including class I (TCP-P) and class II (TCP-C), and the class II subfamily can be further subdivided into two lineages, namely, ubiquitous CINNINATA (CIN) and angiosperm-specific CYC/TB1 (Kosugi and Ohashi, 2002; Navaud et al., 2007; Li, 2015). In addition to the TCP domain, several class II members also have an R-domain, which is predicted to form a hydrophilic α-helix or coiled coil structure involved in protein-protein interactions (Lupas et al., 1991).

The basic region of TCP domain is essential for recognizing GC-rich cis-regulatory elements (Kosugi and Ohashi, 2002; Li et al., 2005; Viola et al., 2011; Danisman, 2016). Random binding site selection experiments suggested that class I proteins can recognize the consensus DNA sequence GTGGGNCC, whereas class II proteins show a preference binding for GTGGNCCC in Arabidopsis and rice (Kosugi and Ohashi, 2002; Viola et al., 2011; Manassero et al., 2013). The different binding preferences between the two classes are dependent on the presence of glycine or aspartic acid at positions 11 or 15, respectively (Viola et al., 2011; Danisman, 2016). TCP transcription factors can form homodimer or heterodimers to bind DNA sequences of target genes, and their dimerization was first described by the interaction of PCF1 and PCF2 in rice (Kosugi and Ohashi, 1997). Later, protein-protein interactions between Arabidopsis TCP transcription factors were provided to demonstrate that TCP proteins from the same class showed a preference to interact with each other (Danisman et al., 2013). Moreover, TCP proteins can also interact with a variety of other proteins, such as immune adaptor SRFR1, components of the circadian clock (TOC1, CCA1, LHY, PRR3, and PRR5), transcriptional repressor TIE1, histidine-containing phosphotransmitters (AHF1, AHF2, and AHF3), and phytoplasma protein effector SAP11 (Pruneda-Paz et al., 2009; Giraud et al., 2010; Sugio et al., 2011; Tao et al., 2013).

TCP family genes have been identified with different numbers in different plants, such as 24 members in Arabidopsis thaliana (Danisman et al., 2013), 22 in Oryza sativa (Cubas et al., 1999; Xu et al., 2017), 30 in Solanum lycopersicum (Parapunova et al., 2014), 17 in Camellia sinensis (Wu et al., 2017), and 33 in Populus euphratica (Ma et al., 2016). Generally, TCP transcription factors have a conserved role as regulators for branching, floral asymmetry, and cell proliferation (Luo et al., 1996; Doebley et al., 1997; Kosugi and Ohashi, 1997). A classic example is TB1 and its homologs that regulate the architectures of a branch, shoot, and/or inflorescence in maize, rice, wheat, and Arabidopsis (Doebley et al., 1997; Lewis et al., 2008; Dixon et al., 2018). Besides, multiple studies showed that TCP genes are also involved in leaf development, gametophyte development, seed germination, regulation of circadian clock, and influencing of hormone pathways (Takeda et al., 2006; Tatematsu et al., 2008; Pruneda-Paz et al., 2009; Sarvepalli and Nath, 2011; Rueda-Romero et al., 2012; Aguilar-Martinez and Sinha, 2013; Manassero et al., 2013). Moreover, some studies suggested that the TCP-controlled growth responses could also be linked with environmental challenges, such as plant pathogen, high light, salt, and nutritional stresses (Mukhtar et al., 2011; Guan et al., 2014; Mukhopadhyay and Tyagi, 2015; Danisman, 2016; Viola et al., 2016). However, evidence for the innovation and the emerging role of TCP family genes in abiotic stress has been lacking.

With the completion of genome sequencing, Populus trichocarpa has been considered to be an ideal model species for genomic and genetic studies of woody plants (Tuskan et al., 2006). In this study, we visited PtrTCP family genes and traced their evolutionary trajectory, especially their expansion experience around K-Pg extinction event followed by the Cenozoic global cooling. Subsequently, we investigated the family expression profile in different tissues under different abiotic stresses and identified a cold-associated regulatory module including PtrTCP31, PtrTCP10, and PtrTCP36. Accordingly, we selected a rWGD-duplicated and cold-induced PtrTCP gene (PtrTCP10) to study its potential role under abiotic stresses. Our results provide new insights into the studies of TCP genes in woody plants, and they may serve as an impetus to explore the molecular mechanism of TCP genes in response to environmental stresses.

MATERIALS AND METHODS

Identification of TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR Transcription Factors in P. trichocarpa

The genome sequences of Arabidopsis and P. trichocarpa were downloaded from TAIR10 (Huala et al., 2001) and Phytozome v13.1 (Goodstein et al., 2012), respectively. To obtain TCP homologs in P. trichocarpa, we first used the 24 Arabidopsis TCP proteins that have been genome-wide identified (Martin-Trillo and Cubas, 2010) as query sequences to perform BLASTP against P. trichocarpa proteome sequences (P. trichocarpa v3.1), and those genes with E-value < 1e-10 were selected as candidate PtrTCP proteins. In addition, we also downloaded the predicted P. trichocarpa TCP proteins from the Plant Transcription Factor Database (PlantTFDB 5.0) (Tian et al., 2020). Based on the above sequences, we further confirmed TCP domain containing proteins using hmmsearch1 using the Hidden Markov Model

1http://hmmer.org/
Phylogenetic Analysis, Gene Structure, and Conserved Motif Analysis

The TCP proteins of *P. trichocarpa* and *Arabidopsis* were aligned by MUSCLE program (Edgar, 2004). The phylogenetic tree was constructed using the maximum-likelihood (ML) method with the bootstrap of 1,000 implemented in IQ-TREE (Nguyen et al., 2015). Exon/intron information of TCP family genes was obtained from gene annotation files of *P. trichocarpa* and *Arabidopsis*. To investigate the conserved functional domain of PtrTCP proteins, we downloaded the HMMs of all protein domains from the Pfam database (Finn et al., 2016) and used hmmsearch (see text footnote 1) to search TCP proteins against the HMMs with an E-value < 1e-5. The locations of the conserved functional domains across the TCP proteins were further visualized using TBtools version 1.0971 (Chen et al., 2020).

Synteny Analysis and Expansion History of *PtrTCP* Genes

To trace the expansion history of *PtrTCP* genes, MCScanX (Wang et al., 2012) was utilized to identify collinear gene blocks, some of which contained *PtrTCP* genes, and the synteny analysis result was visualized using the Circos software in TBtools version 1.0971 (Chen et al., 2020). In dating the expansion of *PtrTCP* family genes, the synonymous substitution (Ks) value of each pair of the collinear gene blocks was calculated using the YN method by KaKs_Calculator2.0 (Wang et al., 2010). Similarly, the average Ks of all the collinear gene blocks between *P. trichocarpa* and *Arabidopsis* was also calculated.

Gene Ontology Enrichment and *Cis*-Regulatory Element Analysis

Gene ontology (GO) term assignment for *P. trichocarpa* genes was obtained using the eggno-g-mapper version 2 tool, and GO term enrichment analysis of *PtrTCP* genes was performed using GOSeq (Young et al., 2010; Cantalapiedra et al., 2021). The GO terms with a value of p < 0.05 were considered to be significantly enriched. To identify potential cis-regulatory elements within the promoter sequences of *PtrTCP* genes, we searched 1-kb sequence upstream of the translation initiation site (TIS) of *PtrTCP* genes in PlantCARE (Rombauts et al., 1999; Lescot et al., 2002) and visualized the distribution of the enriched elements using TBtools version 1.0971 (Chen et al., 2020).

RNA-Seq Analysis of *P. trichocarpa* Leaf, Stem, and Root Samples Under Abiotic Stresses

Transcriptome data of *P. trichocarpa* under abiotic stresses were downloaded from the NCBI BioProject (accession ID: PRJEB19784) (Filichkin et al., 2018). Trimmomatic software was utilized to remove the Illumina adapter contamination and filter the low-quality bases (Bolger et al., 2014), and then, the obtained clean reads were mapped to *P. trichocarpa* genome (version 3.1) by HISAT2 (Pertea et al., 2016). StringTie was further utilized to calculate the Transcripts Per Kilobase Million (TPM) value for each gene in the genome (Pertea et al., 2016). According to the gene expression, we calculated Pearson correlation coefficients of the leaf, stem, and root samples under cold, heat, salt, and drought stresses using R programming and visualized the expression patterns of *PtrTCP* genes using TBTools v1.0971 (Chen et al., 2020). The differentially expressed genes (DEGs) between two groups were obtained using edgeR, DESeq2, and Ballgown (Robinson et al., 2010; Trapnell et al., 2013; Love et al., 2014; Frazee et al., 2015). Those genes that had an adjusted p < 0.05 and an absolute value of fold change ≥ 2 from at least two of the methods were considered to be DEGs. Finally, we obtained the DEGs of *PtrTCP* genes in *P. trichocarpa* leaf, stem, and root samples under cold, heat, salt, and drought stresses.

Validation of *PtrTCP* Genes in Response to Abiotic Stresses by Quantitative Real-Time PCR

*Populus trichocarpa* seedlings were cultured in an artificial climate chamber with 25°C at a photoperiod of 16/8 h light/dark cycle, and the 2-month-old *P. trichocarpa* seedlings with a similar growth status were utilized for abiotic stress treatments, including cold (4°C), heat (39°C), salt (200 mM NaCl), and drought (20% PEG6000). The fourth expanded leaves of *P. trichocarpa* seedlings were separately collected after short period (24 h for cold, salt, and drought, while 12 h for heat) and long period (7 days) of the corresponding abiotic stress treatments. Total RNA was extracted using the TRizol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer’s procedure, and cDNA was synthesized by the PrimeScript RT™ Reagent Kit with gDNA Eraser (TaKaRa, Japan). Quantitative Real-time PCR (qRT-PCR) was performed using a CFX96 real-time PCR system with ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China), and PCR reaction was performed under the following conditions: 95°C for 10 min, 45 cycles of 95°C for 30 s, and 60°C for 10 s. Relative expression level of each gene was normalized to *PtrACTIN2* (Potri.019G010400), *Ptr60S* (Potri.005G072300), *PtrACTIN* (Potri.019G010400), and *PtrACTIN2* (Potri.001G309500), and the achieved data were analyzed using the 2^−ΔΔCT method (Livak and Schmittgen, 2001).

Gene Co-expression Network Analysis

Based on the above RNA-seq analysis of *P. trichocarpa* under abiotic stresses, R package WGCNA was utilized to identify modules of highly correlated genes that were associated with each abiotic stress treatment (Langfelder and Horvath, 2008). First, Pearson’s correlation coefficients were calculated for all pairwise genes, and soft threshold was then obtained to construct an adjacency matrix. Subsequently, the topological overlap matrix (TOM) was calculated from the resulting adjacency matrix, and the genes were hierarchically clustered based on TOMSimilarity. Finally, the dynamic tree cut algorithm was utilized to cut the hierarchical clustering tree into gene modules...
Generation of 35S::PtrTCP10 Transgenic Lines in Arabidopsis

The full length of PtrTCP10 coding sequence (CDS) without the stop codon was amplified from the wild-type P. trichocarpa cDNA using the PtrTCP10 specific primers designed with KpnI and SalI sites, and then, the purified PCR product was inserted into the pCAMBIA1300-sGFP vector under the control of CaMV35S promoter. After transferring pCAMBIA1300-PtrTCP10-sGFP plasmid into Agrobacterium tumefaciens EHA105 competent cells, transformation of PtrTCP10 into Arabidopsis was further performed using the floral dip method. Finally, the positive seedlings screened on the hygromycin-resistant plates were selected as candidate 35S::PtrTCP10 overexpression (OE) lines, and each of the candidate 35S::PtrTCP10 OE lines was verified by PCR and qRT-PCR.

Freezing Tolerance Assay

For freezing tolerance assay, 3-week-old plotted cultivation 35S::PtrTCP10 OE lines and wild-type plant (Col-0) that were cultured under a 12/12 h light/dark photoperiod were treated with or without cold acclimation (4°C for 3 days), and then, freezing tolerance assays were performed as described by Jia et al. (2016). In brief, the program was set at 4°C for 10 min and 0°C for 20 min and dropped 1°C/h to the desired temperatures. After freezing treatment, the plants were grown at 4°C in dark for 12 h and then were transferred to 22°C for additional 3 days. Freezing tolerance phenotype of each line was observed, and the survival rate of each line was also calculated.

Salt Stress Assay

For salt stress assay, seeds of the 35S::PtrTCP10 OE lines and wild-type plant (Col-0) were cultured on the 1/2 MS media containing different concentrations of NaCl (0, 100, 125, and 150 mM). After being treated at 4°C for 2 days, seeds were cultured in a light incubator with 23°C for a photoperiod of 12/12 h light/dark cycle. The number of the germinated seeds of each line under different salt stress treatments was counted every day, and the germination rate of each line was finally calculated. In addition, we also measured root length and fresh weight of the salt-treated seedlings.

Construction of PtrTCP10-Mediated Regulatory Network

The Pearson correlation coefficient (PCC) between PtrTCP10 and other genes was calculated by using the above transcriptome data of P. trichocarpa under cold and salt stresses, and the genes with |PCC| > 0.7 and p < 0.0001 were identified as PtrTCP10 co-expressed genes. GO enrichment analysis of the co-expressed genes was performed and visualized by ClueGO (Bindea et al., 2009). To further predict the putative target genes of PtrTCP10, 1-kb sequence upstream of the TIS ofPtrTCP10 co-expressed genes was utilized to search for PtrTCP10 binding site with at most one-mismatch across the consensus sequence GTGGNCCC. Accordingly, a regulatory network centrally mediated by PtrTCP10 was constructed based on PtrTCP10 and its putative co-expressed target genes that contained at least two TCP binding sites. In addition, the well-known cold- and salt-relevant genes (e.g., ZAT10, GolS2, HY5, CBL1, and SOS1) that contained one TCP binding site were also included in this regulatory network (Wu et al., 1996; Cheong et al., 2003; Sakamoto et al., 2004; Nishizawa et al., 2008; Zhang et al., 2020).

RESULTS

Exon/Intron Structures and Protein Domains of PtrTCP Family Show Significant Conservation but Also Evolutionary Divergence

Based on a homolog search in combination with previous reportedPtrTCP genes (Ma et al., 2016), we gave an updated list of the family, designating PtrTCP1 toPtrTCP12 and PtrTCP14 toPtrTCP37. As a total, we obtained 36PtrTCP genes (Supplementary Table 1), which showed 50% more than that in Arabidopsis (Dahman et al., 2013). Among them, PtrTCP37 (Potri.006G207700) was a newly identified member that had not been reported previously, and a 41-amino acid length protein identified asPtrTCP13 (Potri.005G140600) by Ma et al. (2016) was excluded. To elucidate phylogenetic relations ofPtrTCP family, we constructed a phylogenetic tree by including Arabidopsis TCP genes as references (Figure 1A). The tree showed thatPtrTCP genes can be classified into two major groups (i.e., class I and class II), and the members within class II were further divided into two lineages (i.e., CIN and CYC), supporting the previous results from other plants (Kosugi and Ohashi, 2002; Navaud et al., 2007; Li, 2015).

As the gene structure is considered to provide important clues for the conservation and evolution of homologous genes (Xu et al., 2017), we analyzed the gene structures ofPtrTCP genes (Figure 1B).PtrTCP genes were likely to have relatively simple exon/intron structures, with the exon number from one to five, and most of thePtrTCP genes within the same subfamily shared similar gene structures. Approximately, 86% ofPtrTCP genes contained one or two exons, while only two genes (i.e.,PtrTCP29 andPtrTCP37) contained five exons. In addition, we also detected the conserved domains or motifs ofPtrTCP proteins, and a total of seven conserved motifs were identified (Figure 1C and Supplementary Figure 1). Of the motifs, motifs 1 and 2 constituted the TCP domain. Interestingly,PtrTCP29 andPtrTCP37 have an order of motif 1 followed by motif 2 in contrast to all of the otherPtrTCP proteins sharing an order of motifs 2 and 1, demonstrating thatPtrTCP29 andPtrTCP37 underwent an evolution of reverse positions of motifs 1 and 2. Except for the two motifs, it is worthy to note that motif 4 with an enrichment of Gln (Q) is the most widely distributed in thePtrTCP proteins (Figure 1C and Supplementary Figure 1). Together,PtrTCP genes have a similar composition of exon/intron structures and...
protein domains but also some divergences especially between the subfamilies.

### Three Whole Genome Duplication Events Are Responsible for the Innovation of PtTCP Family Genes

As described above, *P. trichocarpa* has 50% more TCP genes than *Arabidopsis*. To investigate the innovation of PtTCP family genes, we detected their chromosomal distribution and gene duplication events. PtTCP genes were distributed unequally on *P. trichocarpa* chromosomes, with 34 genes on 17 chromosomes and two genes on the unmapped scaffolds (Figure 2A). Intra-specie genomic comparison generated hundreds of collinear gene blocks; Of which, 30 blocks were identified to contain 32 PtTCP genes (Figure 2A, gene block pairs connected by color lines). Accordingly, WGD is the major duplication mechanism to generate PtTCP genes in *P. trichocarpa* (Figure 2B).

To further estimate the time of WGD-expanded PtTCP genes, we calculated the synonymous substitutions per synonymous site (Ks) of collinear gene blocks (Supplementary Table 2). We found that the gene blocks could be mainly classified into three categories (Figure 2C). The first category included...
nine pairs of *PtrTCP* genes (shown in blue) with *Ks* value ranging from 3.43 to 4.66, which were derived from the ancient angiosperm-wide or seed plant-wide WGD (Jiao et al., 2011). The second category included seven pairs of *PtrTCP* genes (shown in green) that were derived from the γ whole-genome triplication (γ-WGT) shared by core eudicots (Tang et al., 2008). The third category included 14 pairs of *PtrTCP* genes (shown in red) with *Ks* value ranging from 0.17 to 0.31, suggesting that these genes blocks were originated from the most recent WGD (rWGD) in *Populus* lineage after its divergence from *Arabidopsis*, which explained well 50% more *PtrTCP* genes in *P. trichocarpa* than that in *Arabidopsis*. The findings demonstrated that the three WGD events are the dominant underlying mechanism for expanding *PtrTCP* family genes, and rWGD is the main
event to lead the innovation of \textit{PtrTCP} family genes in contrast to \textit{Arabidopsis}.

Interestingly, the rWGD event occurred around K-Pg extinction event \sim66 million years ago, which was followed by the Cenozoic global cooling (Wu et al., 2020). Those rWGD-duplicated \textit{PtTCP} genes might have undergone subfunctionalization or neofunctionalization, which contributed to their retention and plant adaptation during the cooling stress. Thus, we investigated the functional enrichments of \textit{PtTCP} genes and their regulation under abiotic stresses, especially cold stress.

**Enrichments of Gene Ontology Terms and Cis-Regulatory Elements Reveal That \textit{PtrTCP} Genes Might Be Involved in Abiotic Stresses**

To investigate the biological pathways that \textit{PtrTCP} genes might participate in, GO term enrichment analysis of \textit{PtrTCP} genes was performed (Figure 3A). We found that the GO terms related to the regulation of various developmental processes were significantly enriched, such as shoot system development, morphogenesis of a branching structure, timing of transition from vegetative to reproductive phase, inflorescence development, seed germination, and embryonic morphogenesis, which were consistent with previous studies (Takeda et al., 2006; Tatematsu et al., 2008; Sarvepalli and Nath, 2011; Rueda-Romero et al., 2012; Aguilar-Martinez and Sinha, 2013; Manassero et al., 2013). In addition, GO terms related to the regulation of response to hormone and environmental stresses were also enriched, such as response to gibberellin, response to cytokinin, regulation of response to stress, and regulation of response to stimulus. These results indicated that \textit{PtrTCP} genes may not only be involved in plant growth and development but also participate in response to hormones and environmental stresses.

Cis-regulatory elements in promoters are essential for regulating gene expression (Lescot et al., 2002). We predicted potential cis-regulatory elements in the 1-kb sequence upstream of TIS of \textit{PtrTCP} genes using PlantCARE (Rombaud et al., 1999). In addition to the common cis-regulatory elements (e.g., TATA-box and CAAT-box), we found cis-regulatory elements in relation to plant growth and development (e.g., endosperm expression, meristem expression, and circadian control), and in response to environmental stresses (e.g., low-temperature, drought, and light) and hormone responses (e.g., abscisic acid, salicylic acid, MeJA, gibberellin, and auxin) (Figure 3B). This suggested that the expression of \textit{PtrTCP} genes might be dynamically regulated in plant growth and development under normal and/or environmental stresses. Then, we investigated the expression profiles of \textit{PtrTCP} genes in leaf, stem, and root tissues under normal and stress conditions (e.g., cold, heat, salt, and drought).

**Expression Profiles of \textit{PtrTCP} Subfamilies Are Diversified in Leaf, Stem, and Root Tissues**

Correlation analysis on transcriptome-scale gene expression from \textit{P. trichocarpa} samples showed three clusters, which were classified by leaf, stem, and root tissues rather than by abiotic stresses (Figure 4A). This demonstrated that the tissue type was the main factor to classify the samples, and meanwhile, the genes in these tissues were likely to be elaborately controlled in response to abiotic stresses. Inspecting the expression profiles of \textit{PtrTCP} genes in the tissues under abiotic stresses (Figure 4B), we found that most of the class I genes were highly expressed, while the genes in CYC lineage were lowly expressed in the three tissues despite all of the abiotic stresses. However, \textit{PtrTCP} genes in CYC lineage showed diversified expression profiles. In detail, 50% of CYC lineage genes including \textit{PtrTCP24}, \textit{PtrTCP34}, \textit{PtrTCP4}, \textit{PtrTCP9}, and \textit{PtrTCP19} were highly, moderately, and lowly expressed, respectively, in leaf, root, and stem, and some CYC lineage genes (e.g., \textit{PtrTCP10}, \textit{PtrTCP36}, \textit{PtrTCP31}, and \textit{PtrTCP27}) were especially expressed in leaf tissue, suggesting an important role of CYC lineage in leaves. Moreover, \textit{PtrTCP} genes were likely to be elaborately regulated in response to abiotic stresses (Figure 4B). For example, \textit{PtrTCP10}, \textit{PtrTCP36}, \textit{PtrTCP35}, and \textit{PtrTCP5} were obviously upregulated in leaves after cold stress. Then, we investigated the abiotic stress-affected \textit{PtrTCP} genes in detail.

**The Emerging Roles of \textit{PtrTCP} Genes in Response to Abiotic Stresses**

We compared the expression changes of \textit{PtrTCP} genes in the tissues between abiotic stresses and normal condition (Figure 5 and Supplementary Table 3). In the aspect of \textit{PtrTCP} genes under each of the four abiotic stresses, there were 19, 21, 18, and 13 differentially expressed (DE) ones, respectively, in response to cold, heat, salt, and drought stresses. Under the same stress, different tissues showed an overlap but also a great divergence of DE \textit{PtrTCP} genes (Figure 5A). For cold, heat, and salt stresses, most of the DE \textit{PtrTCP} genes were determined in leaf, while for drought, the counterpart was determined in the root. In addition, some of those \textit{PtrTCP} genes could be induced by multiple abiotic stresses. For example, 22 genes could respond to at least two of the stresses, and in particular, five genes were differentially regulated by all the four abiotic stresses (Figure 5B). In the aspect of \textit{PtrTCP} genes in each of the tissues, 23, 17, and 20 genes were differentially regulated by at least one of the four abiotic stresses, respectively, in leaf, root, and stem (Figure 5C). Of them, 11 DE \textit{PtrTCP} genes could respond to abiotic stresses in the three tissues (Figure 5D). In leaf, 13, 14, 15, and 5 \textit{PtrTCP} genes were DE under the treatment of cold, heat, drought, and salt, respectively (Figure 5E). In addition to the overlaps of DE \textit{PtrTCP} genes between the abiotic stresses, some \textit{PtrTCP} genes could uniquely respond to one of cold, heat, and salt in leaf (Figure 5F). Moreover, of the DE \textit{PtrTCP} genes, some showed different expression patterns in response to short periods and/or long periods of abiotic stresses (Figure 5G and Supplementary Table 3). Likewise, in stem and root, we also observed similar patterns of DE \textit{PtrTCP} genes in response to abiotic stresses of cold, heat, salt, and drought at different time points (Figures 5H–M).

To further validate the expression of \textit{PtrTCP} genes in response to abiotic stresses, 16 DE \textit{PtrTCP} genes that could be induced or repressed by at least one of the abiotic stresses
**FIGURE 3** | GO enrichment and cis-regulatory element analysis of PtrTCP family genes. **(A)** Gene ontology (GO) enrichment analysis of PtrTCP family genes. The dot size represents the number of enriched genes, and the colored bar represents the significant enrichment level of GO term. **(B)** Distribution of the cis-regulatory elements in the 1-kb promoter sequence upstream of the translation initiation site (TIS) of PtrTCP genes.
were selected to be for experimentally qRT-PCR confirmation in leaf (Supplementary Table 4). Except for *PtrTCP33*, the selected *PtrTCP* genes could respond to at least one of the abiotic stresses, showing similar expression trends under abiotic stresses with those detected by RNA-seq (Figure 6 and Supplementary Table 3). For example, *PtrTCP5*, *PtrTCP10*, and *PtrTCP35* were significantly upregulated by cold treatments at 24 h and 7 days, while *PtrTCP34* was downregulated. *PtrTCP1*, *PtrTCP4*, *PtrTCP2*, *PtrTCP7*, *PtrTCP8*, *PtrTCP11*, and *PtrTCP35* responded to both the short period and the long period of...
heat treatments, PtrTCP1, PtrTCP7, PtrTCP10, PtrTCP26, and PtrTCP35 were significantly induced by salt stress, and in contrast, PtrTCP4, PtrTCP11, PtrTCP18, PtrTCP20, PtrTCP24, and PtrTCP34 were repressed. These findings suggested that at least half of the PtrTCP genes could respond to abiotic stresses, and they might be involved in regulating abiotic stress tolerance.
Then, we sought to explore the potential hub *PtrTCP* genes in an abiotic process using the gene co-expression network.

**Co-expression Network Identifies a Cold-Associated Module Including Three *PtrTCP* Genes (***PtrTCP10*, **PtrTCP31**, and **PtrTCP36**)**

Based on the expressed genes including *PtrT* in leaf, root, and stem under abiotic stresses, we constructed a co-expression network (Figure 7A) using weighted correlation network analysis (WGCNA; Langfelder and Horvath, 2008). In the network, 28 modules were obtained, and some modules were significantly correlated with the tissue traits (Figure 7B). For example, the module MElavenderblush3 was significantly positively correlated with the trait (Leaf_cold_24h), which indicated that the genes in MElavenderblush3 were especially upregulated in the same orientation in leaf under cold stress at 24 h. In the case of *PtrTCP* family genes, 30 members were clustered into six modules, e.g., ten in MEBlue, six in MEBrown, six in MEdarkolivegreen, four in MEGrey, three in MELavenderblush3, and one in MEgrey60 (Figure 7B and Supplementary Table 5).
FIGURE 7  Distribution of PtrTCP genes in the regulatory modules of *P. trichocarpa* tissues under abiotic stresses. (A) Gene regulatory modules identified by weighted gene co-expression network analysis (WGCNA). (B) Relationships of module eigengenes (ME, rows) and traits of the tissues under abiotic stresses (columns). Red/blue represents positive/negative correlations between the modules and the traits. The six modules containing PtrTCP genes were marked in red, and the number of PtrTCP genes was shown nearby. Of the six modules, the ones significantly correlated with one of the traits were especially shown in the box, and the corresponding traits were colored in red. (C) Expression conservation and divergence of *PtrTCP*31/10/36 orthologs in dicots under cold stress at different time points (0 h, 24 h, and 7 days). The cold-treated samples were from *Arabidopsis thaliana* (*A. thaliana*), *Cucumis sativus* (*C. sativus*), *Carya illinoinensis* (*C. illinoinensis*), *Betula pendula* (*B. pendula*), *Glycine max* (*G. max*), and *Populus trichocarpa* (*P. trichocarpa*). (D) The generation process of *PtrTCP*10 gene. *PtrTCP*10 was rWGD-duplicated from *PtrTCP*31 and underwent an inversion, where the *P. trichocarpa* region containing *PtrTCP*31 (Chr17:10.55–10.94) showed collinear orthologous gene pairs with *Arabidopsis* homologous region (Chr5:23.93–24.03 Mb) containing AtTCP5.
However, of the six modules, only two modules (i.e., MEgrey60 and MElavenderblush3) including four PtrTCP genes were significantly correlated with abiotic stress treatments. Although PtrTCP12 in MEgrey60 was correlated with Root_salt_24h, the gene was not significantly upregulated (<2 fold change) by salt treatment at 24 h (Supplementary Table 3). In contrast, PtrTCP10, PtrTCP31, and PtrTCP36 in MElavenderblush3 were correlated and significantly upregulated in leaf under cold stress at 24 h (Leaf_cold_24h), demonstrating a potential role of the genes in MElavenderblush3 under cold stress.

Notably,_PTRTCP10,_PTRTCP31, and_PTRTCP36 genes were _P. trichocarpa_ inparalogs related to other dicot orthologs including _Arabidopsis_ singular ortholog _AtTCP5_ and _Glycine_ five co-orthologs (the left panel in Figure 7C). This demonstrated the three genes evolved from gene duplications within _P. trichocarpa_ lineage after the divergence from other dicot lineages, which was consistent with the above WGD analysis (Figure 2). Further expression analysis of those genes showed that_PTRTCP10 and_PTRTCP36 were significantly upregulated (>5-fold change) by cold stress, whereas the corresponding co-orthologs in other close dicots (e.g., _Arabidopsis, Cucumis sativus, Carya illinoinensis, Betula pendula, and Glycine max_) were not or less affected (the right panel in Figure 7C and Supplementary Table 6). Based on the expansion history of_PTRTCP genes (Figure 2),_PTRTCP36 was mapped on a scaffold and predicted to be produced by dispersed duplication from_PTRTCP10, whereas_PTRTCP10 was mapped on Chr4 and duplicated from_PTRTCP31 by rWGD ~66 million years ago (Figure 7D). After rWGD, the Cenozoic global cooling and other environmental factors might have driven subfunctionalization and/or neofunctionalization of these_TCP genes in regulation and function, and of those_TCP orthologs in the six plants, only_PTRTCP10 and_PTRTCP36 acquired a strong capability of cold induction. We questioned whether the acquisition of cold-induced capability in_PTRTCP10 and_PTRTCP36 contributed to the cold adaptation of the plants. Then, we selected the WGD-duplicated_PTRTCP10 for OE in _Arabidopsis_ to investigate its potential role in cold stress.

**Overexpression of_PTRTCP10 Increases Freezing Tolerance and Salt Sensitivity**

To further investigate the role of_PTRTCP10 under cold stress, we generated 35S::_PTRTCP10 OE lines in _Arabidopsis_. The candidate positive seedlings screened on the hygromycin-resistant plates were verified by PCR and qRT-PCR (Supplementary Figure 2), and three independent 35S::_PTRTCP10 OE lines (i.e., OE1, OE3, and OE6) with high expression levels of_PTRTCP10 were selected for further study. Under normal condition, we found that the OE lines showed a similar growth as the wild-type (Col-0), and in contrast, under freezing condition, they exhibited freezing tolerance phenotypes with or without cold acclimation (Figure 8A). Statistically, their survival rates were significantly higher than the wild type (Figure 8B). Given that_PTRTCP10...
could also be significantly induced by salt treatment at 7 days (Figures 4, 6), we examined the phenotypes of 35S::PtrTCP10 OE lines under salt treatments. Compared with the wild type, the OE lines were more sensitive to salt stress (Figure 9). Their germination rate, root length, and fresh weight were significantly lower than the wild type. The findings suggested that PtrTCP10 was positively involved in plant resistance to cold stress but also might be a negative regulator involved in salt stress. Then, we elucidated the regulatory molecular mechanism of PtrTCP10 in response to cold and salt stresses.
**FIGURE 10** | PtrTCP10-mediated regulatory network under cold and salt stresses. (A) Functionally grouped networks on the enriched GO terms from the co-expressed genes of PtrTCP10 under cold and salt stresses. The enriched terms were linked according to their K score (≥0.3). (B) An enrichment of PtrTCP10 binding cis-regulatory elements in promoters (1-kb) of the 813 co-expressed genes. The sequence in dotted box indicates the core region potentially bind byPtrTCP10. Approximately, 44% (359 out of 813) of the PtrTCP10 co-expressed genes’ promoters contained this cis-regulatory element, and 118PtrTCP10 co-expressed genes contained at least two elements. (C)PtrTCP10-mediated regulatory network based on the high-confidence target genes of PtrTCP10. The red and green circles represent the well-known cold and salt responsive gene, and the purple circles represent the genes that could respond to both the cold and salt stresses.

**PtrTCP10-Mediated Regulatory Network Provides Molecular Mechanisms for Emerging Roles of PtrTCP Genes in Response to Cold/Salt Stresses**

First, we identified a total of 813 significantly co-expressed genes ofPtrTCP10 from the above transcriptome data under cold and salt stresses. The co-expressed genes were significantly enriched in the GO terms related to environmental stresses, such as response to cold, response to salt stress, response to osmotic stress, regulation of cell death, potassium ion transmembrane transport, and leaf senescence (Figure 10A). TCP transcription factors have been shown to recognize GC-rich cis-acting regulatory elements, and the class II TCP proteins show a preference binding for the motif GTGGNCCC (Kosugi and Ohashi, 2002; Viola et al., 2011; Manassero et al., 2013). To predict the putative target genes ofPtrTCP10, we searched for the motif allowing a degenerate mutation against the promoters (1-kb upstream of TIS) of the 813 co-expressed genes. Approximately, 44% (359 out of 813) of the genes contained the motif, and of which, 118 genes contained at least two binding sites (Figure 10B). Based on the putativePtrTCP10 target genes (Supplementary Table 7), we constructed a regulatory network mediated byPtrTCP10. The network presented some well-acknowledged cold- and salt-responsive genes (e.g., ZAT10, GolS2, HY5, CBL1, SOS1, RCI2A, and SnRK3.9) directly regulated byPtrTCP10, which suggested thatPtrTCP10 might be involved in response to cold and salt stresses by regulating those stress-related genes (Figure 10C; Wu et al., 1996; Capel et al., 1997; Guo et al., 2001; Cheong et al., 2003; Sakamoto et al., 2004; Nishizawa et al., 2008; Zhang et al., 2020).

**DISCUSSION**

Since the discovery ofTCP genes, TCP transcription factors have been widely studied to be involved in multiple processes of plant growth and development, such as branching, floral organ morphogenesis, cell proliferation, leaf development, seed germination, regulation of circadian clock, and influencing of hormone pathways (Danisman et al., 2013). Recent studies suggested that they are also involved in environmental challenges, such as plant pathogen, high light stress, salt, and nutritional stress (Danisman et al., 2013; Guan et al., 2014; Mukhopadhyay and Tyagi, 2015; Danisman, 2016; Viola et al., 2016), but evidence for their innovation and evolutionary role in abiotic
stress has been lacking. In this study, we identified PtrTCP family genes and traced their evolutionary trajectory. WGD events were the main underlying mechanism for expanding and innovating PtrTCP family genes. Comparative genomes showed 50% more TCP genes in *P. trichocarpa* than in *Arabidopsis*, and such significant innovation was produced by rWGD in *Populus* lineage after its divergence from *Arabidopsis*. Interestingly, the rWGD occurred around K-Pg extinction event ~66 million years ago, which was followed by global cooling (Wu et al., 2020). Those rWGD-duplicated PtrTCP genes might have undergone subfunctionalization and/or neofunctionalization during the severe cooling stress. Further expression profiles of PtrTCP genes from RNA-seq and qRT-PCR analysis showed at least one half can be differentially regulated by cold, heat, salt, and/or drought stresses. Moreover, the co-expression network identified a cold-associated regulatory module including PtTCP31, PtTCP10, and PtTCP36. Of the three genes, PtTCP10 was rWGD-duplicated from PtTCP31, evolved a strong capability of cold induction, and produced PtTCP36 with the capability of cold induction as well. In contrast, their co-orthologs in the other five dicots showed monotonous expression profiles after cold treatment. We questioned that the innovation of PtTCP10 and PtTCP36 with a strong capability of cold induction might contribute to the cold adaptation of the plants during global cooling.

Evidently, OE of PtTCP10 in *Arabidopsis* increased freezing tolerance and salt sensitivity, indicating that PtTCP10 may be a regulator that plays a vital role in plants’ response to cold and salt stresses. Reconstruction of a PtTCP10-mediated regulatory network indicated that PtTCP10 might be involved in the regulation of plants response to cold and salt stresses by directly regulating the cold- and salt-relevant genes (e.g., ZAT10, GoS2, HYS, CB11, SOS1, RC12A, and SnRK3.9) (Wu et al., 1996; Capel et al., 1997; Guo et al., 2001; Cheong et al., 2003; Sakamoto et al., 2004; Nishizawa et al., 2008; Zhang et al., 2020). Overall, our work provides new insights into the innovation of PtrTCP genes and their emerging roles under abiotic stresses in woody plants, and meanwhile, it may provide valuable information for exploring the molecular mechanisms of TCP genes in improving plants resistance to abiotic stresses.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

**AUTHOR CONTRIBUTIONS**

SW, SL, and WW designed the research. SW, YS, and XZ performed the experiments. LG, LT, YN, and DD performed the bioinformatic analysis. YY and XY analyzed RNA-seq data. SW and WW wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

**FUNDING**

This study was supported by the National Natural Science Foundation of China (grant number 31871233).

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.850064/full#supplementary-material

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