

Research Article

Gene Structures, Classification, and Expression Models of the *DREB* Transcription Factor Subfamily in *Populus trichocarpa*

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Received 6 August 2013; Accepted 17 September 2013

Academic Editors: C. Baumgartner and B. X. Ling

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We identified 75 dehydration-responsive element-binding (*DREB*) protein genes in *Populus trichocarpa*. We analyzed gene structures, phylogenies, domain duplications, genome localizations, and expression profiles. The phylogenetic construction suggests that the *PtrDREB* gene subfamily can be classified broadly into six subtypes (*DREB* A-1 to A-6) in *Populus*. The chromosomal localizations of the *PtrDREB* genes indicated 18 segmental duplication events involving 36 genes and six redundant *PtrDREB* genes were involved in tandem duplication events. There were fewer introns in the *PtrDREB* subfamily. The motif composition of *PtrDREB* was highly conserved in the same subtype. We investigated expression profiles of this gene subfamily from different tissues and/or developmental stages. Sixteen genes present in the digital expression analysis had high levels of transcript accumulation. The microarray results suggest that 18 genes were upregulated. We further examined the stress responsiveness of 15 genes by qRT-PCR. A digital northern analysis showed that the *PtrDREB17*, 18, and 32 genes were highly induced in leaves under cold stress, and the same expression trends were shown by qRT-PCR. Taken together, these observations may lay the foundation for future functional analyses to unravel the biological roles of *Populus*' *DREB* genes.

1. Introduction

Environmental stresses, such as drought, high salt, and low temperature, have adverse effects on plant growth and development. During evolution, plants established physiological and metabolic defense system responses to adverse conditions. In essence, stress induced the expression of specific genes and their products play a role in the plant's stress defense mechanism. Transcription factors (TFs) can upregulate a set of genes under their control by interacting with cis-elements present in the promoter region of targeted genes. The products act as regulatory proteins, consequently enhancing the stress tolerance of the plant. The dehydration-responsive element-binding (*DREBs*) protein TFs play an important role in regulating plant growth and the response to external environmental stresses.

In the plant kingdom, *DREB* is a large subfamily belonging to the AP2/ERF/ethylene-responsive element-binding protein (AP2/EREBP) family. *DREB* genes contain the highly conserved AP2/ERF DNA-binding domain [1]. The AP2/ERF

domains, which consist of 50 to 60 amino acids, are found in proteins involved in a variety of regulatory mechanisms throughout the plant life cycle. The *DREB* subfamily play an important role in the resistance of plants to abiotic stresses by recognizing the dehydration-responsive element (DRE), which has a core motif of A/GCCGAC [2], and some members of this gene subfamily recognize the cis-acting element AGCCGCC, known as the GCC box [3]. The *DREB* subfamily TFs have been identified in various plant species, including *mangrove* [4], *soybean* [5], and *potato* [6]. The roles of *DREB* proteins in the plant's response to abiotic stress have also been extensively documented [7]. In the genomes of *Arabidopsis* [3], *Vitis vinifera* [8], and *rice* [9], 56, 36, and 57 AP2/ERF-related proteins, respectively, are encoded. Genetic and molecular approaches have been used in combination to characterize a series of *DREB* family regulatory genes involved in many different pathways, including genes related to cold, drought, high salinity, heavy metals, and abscisic acid (ABA) [4].

TABLE 1: The primers of *DREB* genes were generated in Primer 5 for qRT-PCR.

| Name | Sequence (5'-3') |
|------------|---------------------------|
| PtrDREB4F | GTATTGAGGGGAGAAATGGATGG |
| PtrDREB4R | CATATCATGGTCGGAAGACAAGC |
| PtrDREB16F | AATCTTGCTACCACCACATCACAGT |
| PtrDREB16R | ATGCCTCCGCCTGACTCCTCTAT |
| PtrDREB17F | ACTCTGGCTTGGCACATTGAC |
| PtrDREB17R | GGCTTGATATCGCCGATGTAGGA |
| PtrDREB18F | GGCTCCAAAACCTGTCCCTATGA |
| PtrDREB18R | CCCAATGTCTCTGCCTCACTCCT |
| PtrDREB19F | GAGGAGGCGGCTTTGGCTTAT |
| PtrDREB19R | AACCGAGGAATGGAGAGGCTTG |
| PtrDREB28F | CAGTCAAAAAGTTTCAGAGGGGT |
| PtrDREB28R | CTCTTCTGCTGTTTCAAATGTGC |
| PtrDREB30F | GCATGTAACGGTAGAAAGGAGGGGG |
| PtrDREB30R | AGATTGGCGGTAGATCAAGAGTG |
| PtrDREB32F | AGAAGGAAGTCATCAACAAGGGG |
| PtrDREB32R | ATTTGGTGCAGGCTGAGGCAA |
| PtrDREB38F | GTGAGAGGCAATACAAGGGGA |
| PtrDREB38R | CGCTACTGGTGTGAGTAGGAA |
| PtrDREB51F | TGACCCGACCTCAAACCTCTCCAG |
| PtrDREB51R | TCAGACACCCATTTCACCCACCT |
| PtrDREB55F | GATTCTCAACCAACCAAAACCTC |
| PtrDREB55R | GGCTCTCTAATTTCAGACACCCA |
| PtrDREB60F | GAAGAAGAACAAGCGGGAAGGA |
| PtrDREB60R | CATTTCTGGGCTCTTGAAGTCC |
| PtrDREB61F | GCAGGAAGGAAGAAGTTCAAGGA |
| PtrDREB61R | GGCTAGTGAAGTCCCTAACCAAAT |
| PtrDREB62F | TCTTCTTCTCCGATAGCAGCAC |
| PtrDREB62R | CACCCTATTGTTACCATTCTCT |
| PtrDREB68F | TCTAAGCGAAACCAAGACCCGAA |
| PtrDREB68R | TTTGCCCCATTGACGCATTCT |
| ActinF | CATCAAAGCATCGGTGAGGTC |
| ActinR | GTTGCCATCCAGGCTGTCC |

The characterization of the *DREB* subfamily of genes in *Populus* can aid the understanding of the molecular mechanisms of stress resistance and thus aid in the development of *Populus* varieties, using transgenic technology, with a greater tolerance to many adverse environments. Some *DREB* subfamily genes have been isolated from rice, *Arabidopsis*, and other plants [10], but they have not been isolated from *Populus*. Thus, their functions remain to be determined in *Populus*. The completion of the high-quality sequencing of the *Populus* genome [11] has provided an excellent opportunity for genome-wide analysis of genes belonging to specific gene families. Therefore, we present a comprehensive and specific analysis of gene structure, chromosome localization, and expression of the *Populus*' *DREB* subfamily for the first time. Here, we identified 75 *PtrDREB* genes in *Populus* using database searches and classified these genes according to their homology with known genes. We describe *DREB* subtypes

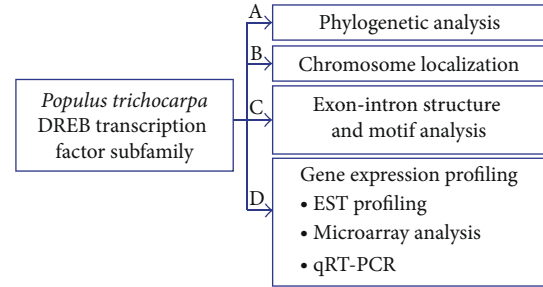


FIGURE 1: Integrated systems analysis workflow for elucidation of the role of *DREB* subfamily in the bioinformatics and data analysis in *Populus*. A: A phylogenetic analysis was performed using all the *DREB* subfamily amino acid sequences from *Arabidopsis* and *Populus* by MEGA 4 software. B: Each of the *DREB* genes' chromosomal position in *Populus* was using the Phytozome and Joint Genome Institute websites. C: The exon/intron organization for individual *DREB* genes was using the gene structure display server (GSDS) program and motif analysis was performed using the program MEME (v4.3.0). D: Gene expression profiling of *Populus DREB* subfamily was used to characterize differentially expressed genes.

more specifically and present novel information from different tissues and/or developmental stages. Some subtypes of this gene subfamily were differentially expressed under abiotic stress conditions. *PtrDREB* genes play an important role in the cross-talk of signaling pathways responding to different kinds of stress. We analyzed the phylogenetic relationships of the *DREB* genes in *Populus* and attempted the complete alignment of the subtypes. We examined gene structure and conserved motifs of *DREB* genes. Taken together, our results will be helpful in determining the functions of each *DREB* gene.

2. Materials and Methods

2.1. Database Search and Sequence Retrieval. The *P. trichocarpa* genome DNA database was downloaded from Phytozome (<http://www.phytozome.net/>). The database of the *A. thaliana DREB* subfamily was downloaded from the *Arabidopsis* Information Resource (TAIR, <http://www.arabidopsis.org/>, release 10.0). A local BLAST search was performed using the amino acid sequences of the AP2/ERF domains from *Arabidopsis* as the queries for the identification of the *DREB* genes from *Populus*. All of the located sequences were further manually analyzed to confirm the presence of an AP2 domain using the InterProScan program (<http://www.ebi.ac.uk/Tools/InterProScan/>). The *Arabidopsis At4g13040* was used as an outlier.

2.2. Phylogenetic Analysis. A phylogenetic analysis was initially performed using all the *DREB* genes from *Arabidopsis* and *Populus*. To construct the phylogenetic trees, full-length *Arabidopsis* and *Populus* amino acid sequences were aligned using ClustalX 1.83 software [12] and manually edited using Jalview to reduce gaps [13]. The phylogenetic analysis was performed by the maximum parsimony method with 1,000 bootstrap replicates using MEGA 4 software [14].

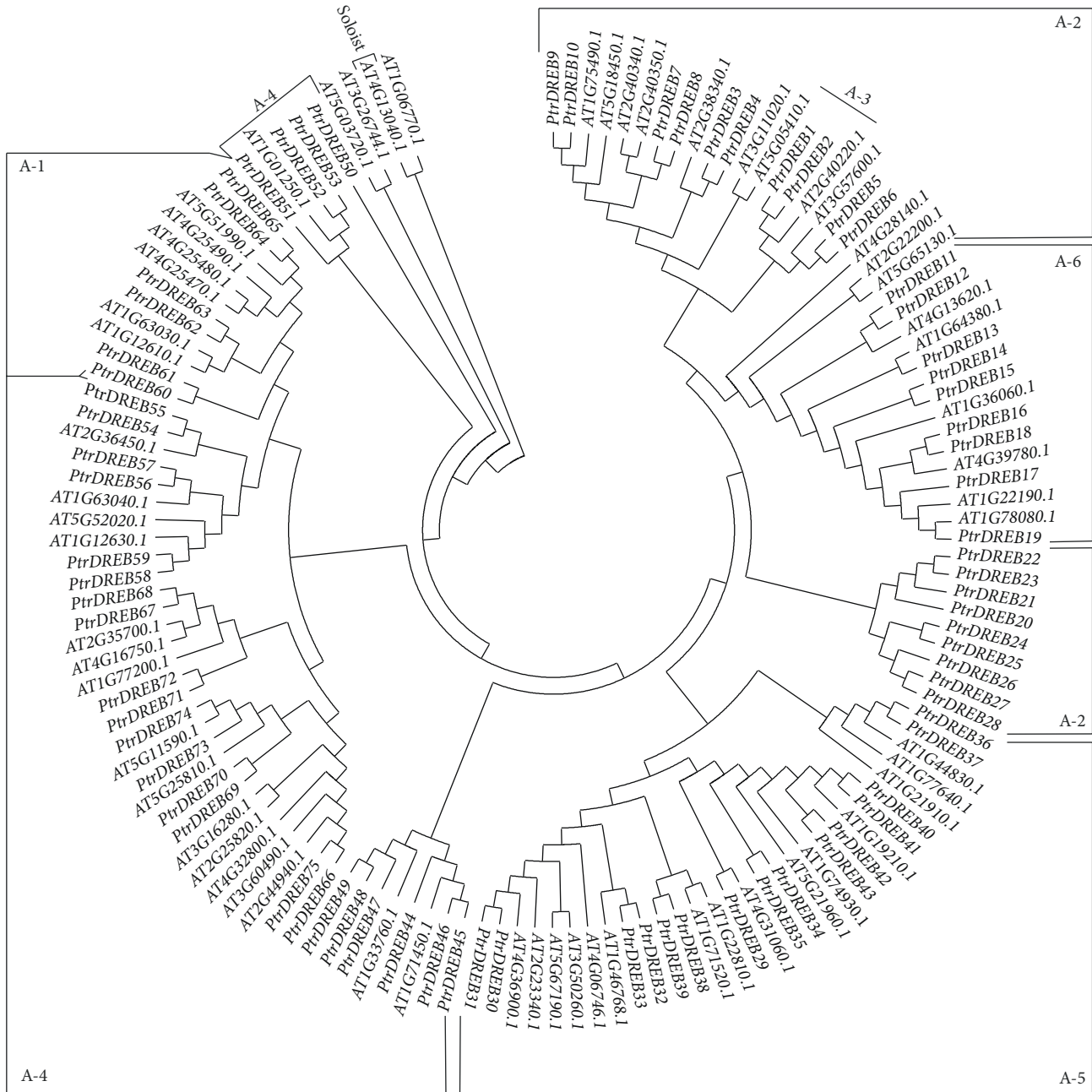


FIGURE 2: Relationships among *Populus* DREBs proteins after alignment with ClustalW. Proteins were allocated to six distinct subgroups of DREB, A-1 to A-6.

2.3. *Chromosome Localization.* Each of the DREB genes' chromosomal position in *Populus* was identified and plotted using the Phytozome (<http://www.phytozome.net/>) and Joint Genome Institute (<http://genome.jgi-psf.org/pages/blast.jsf?db=Poptr1.1>) websites. This information is provided in Table 2. A schematic view of the chromosomes was reorganized by the most recent whole-genome duplication in *Populus*.

2.4. *Exon/Intron Structure and Motif Analysis.* The exon/intron organization for individual DREB genes was illustrated using the Gene structure display server (GSDS)

program (<http://gsds.cbi.pku.edu.cn/>). The CDS and genome sequences of the *P. trichocarpa* genes were obtained from NCBI (<http://www.ncbi.nlm.nih.gov/>). The program MEME (v4.3.0) (<http://meme.sdsc.edu/>) was used to deduce 75 *Populus* DREB protein sequences.

2.5. *EST Profiling and Microarray Analysis.* The expression profile for each gene was obtained by a Digital Northern tool at PopGenIE (<http://www.popgenie.org/>) that generated a digital northern expression profile heat map based on the EST representations of 19 cDNA libraries derived from different tissues and/or developmental stages [15]. The heat

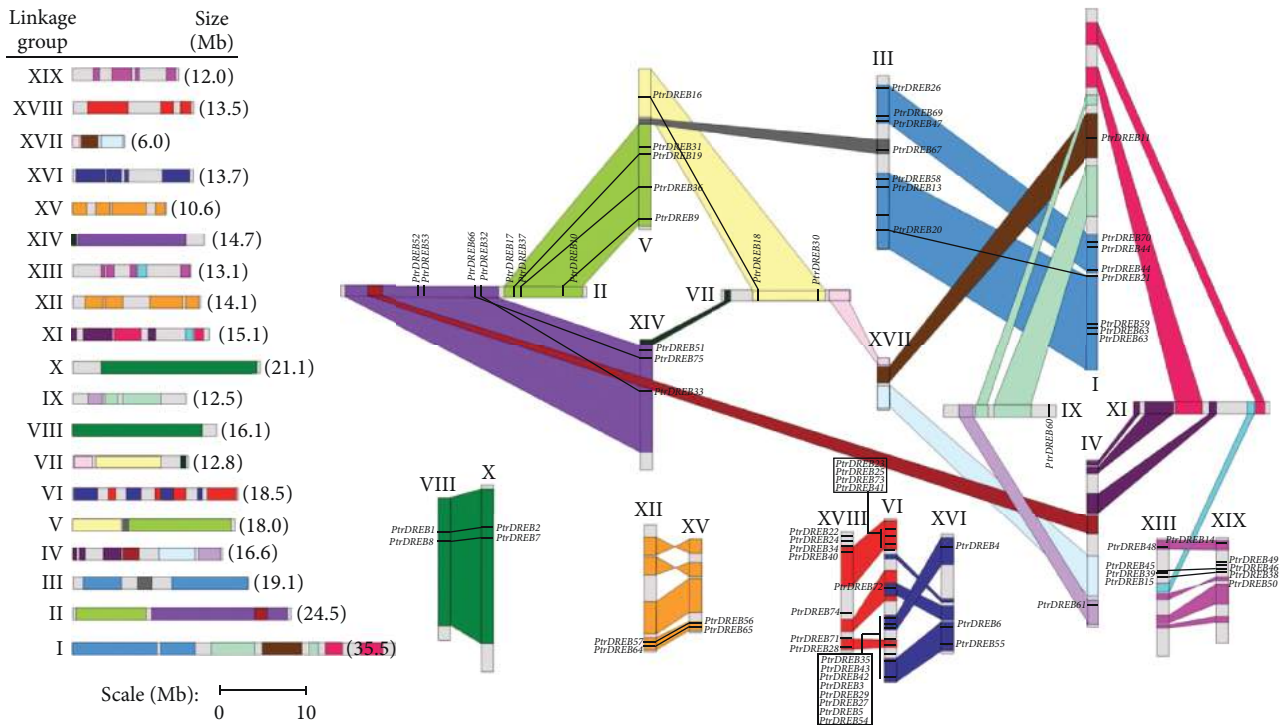


FIGURE 3: Locations of *P. trichocarpa* DREB genes on the chromosomes LGI-XIX. A schematic view of chromosome reorganization by recent whole-genome duplication in *Populus* is shown (adapted from [21]). Regions that are assumed to correspond to homologous genome blocks are shaded in the same color and connected with lines.

map was visualized using the Heatmapper Plus tool at the Bio-Array Resource for Plant Functional Genomics (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi/) [16]. The microarray data for various tissues/organs available at NCBI Gene Expression Omnibus (GEO) database under the series accession number GSE6422 were used for the tissue-specific expression analysis.

2.6. Plant Treatment and qRT-PCR Analysis. For expression pattern analysis of the *Populus* DREB gene subfamily under abiotic stresses, plants were exposed to 42°C for 0, 0.5, and 1 h; 4°C for 0, 12, and 24 h; 200 mM NaCl for 0, 4, and 8 h; 100 μM ABA for 0, 2, 4, and 6 h; and 4% PEG6000 for 0, 4, and 8 h. Young leaves were harvested at various time points. All samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Total RNA from leaf was extracted using the CTAB method. Synthesized cDNAs were used for qRT-PCR, which was performed using the TaKaRa ExTaq RT PCR Kit and SYBR green dye (TaKaRa, Dalian, China) in 96-well optical reaction plates (Applied Biosystems, USA). The results obtained for the different stages were standardized to the levels of the *actin* gene using the $2^{-\Delta\Delta CT}$ method. We selected 15 DREB genes to observe tolerance under stress conditions in *Populus* via EST profiling and microarray analysis. We designed the primers for gene expression analysis using Primer Premier 5 to produce amplified lengths of 180 to 200 bp (Table 1).

3. Results and Discussion

3.1. Identification of DREB Subfamily TFs in *Populus*. To identify putative DREB genes in *Populus*, we performed a BLASTP search against *Populus* genome release v2.1 using DREB protein sequences from *Arabidopsis*. By removing the redundant sequences, 75 DREB genes were identified in the *Populus* genome. All DREB candidates were analyzed using the smart database (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) to verify the presence of AP2/ERF domains. Seventy-five DREB genes were used for the analysis of bioinformatics and gene expression profiling. The overall strategy used in this study was depicted in Figure 1 and presented in detail below. We designated *Populus* DREB genes as *PtrDREB* following the nomenclature proposed in a previous study [17]. *A. thaliana* *At4g13040* was used as a query sequence and it includes an AP2/ERF-like domain sequence; however, its homology appears quite low in comparison with the other AP2/ERF genes. Therefore, this gene was also designated as an outlier. Detailed information on the DREB subfamily of genes in *Populus* and *Arabidopsis* are listed in Table 1.

3.2. Phylogenetic Relationships and Alignments of the DREB Subfamily in *Populus*. Based on the alignment of the AP2/ERF coding region protein sequences of all *Populus* and *Arabidopsis* DREB subfamily genes, 75 DREB subfamily genes of *Populus* were classified into six groups, A1, A2, A3, A4, A5,

TABLE 2: The *DREB* genes identified from the *P. trichocarpa* genome.

| Gene symbol | Gene Locus | PF00847 AP2 domain | <i>Arabidopsis</i> ortholog locus |
|-------------|------------------|--------------------|-----------------------------------|
| PtrDREB1 | POPTR_0008s07120 | 102–151 | AT1G12610.1 |
| PtrDREB2 | POPTR_0010s19370 | 147–196 | AT1G63030.1 |
| PtrDREB3 | POPTR_0006s10510 | 67–125 | AT4G25470.1 |
| PtrDREB4 | POPTR_0016s13380 | 74–132 | AT4G25480.1 |
| PtrDREB5 | POPTR_0006s05320 | 28–78 | AT4G25490.1 |
| PtrDREB6 | POPTR_0016s05360 | 29–78 | AT5G51990.1 |
| PtrDREB7 | POPTR_0010s19100 | 78–128 | AT1G75490.1 |
| PtrDREB8 | POPTR_0008s07360 | 78–128 | AT2G38340.1 |
| PtrDREB9 | POPTR_0005s25470 | 43–92 | AT2G40340.1 |
| PtrDREB10 | POPTR_0002s03090 | 40–89 | AT2G40350.1 |
| PtrDREB11 | POPTR_0001s32250 | 285–333 | AT3G11020.1 |
| PtrDREB12 | POPTR_0017s08250 | 276–324 | AT3G57600.1 |
| PtrDREB13 | POPTR_0003s13910 | 107–155 | AT5G05410.1 |
| PtrDREB14 | POPTR_0019s13330 | 232–281 | AT5G18450.1 |
| PtrDREB15 | POPTR_0013s13920 | 232–281 | AT2G40220.1 |
| PtrDREB16 | POPTR_0005s07900 | 173–222 | AT1G01250.1 |
| PtrDREB17 | POPTR_0002s09480 | 163–211 | AT1G12630.1 |
| PtrDREB18 | POPTR_0007s05690 | 173–222 | AT1G33760.1 |
| PtrDREB19 | POPTR_0005s16690 | 120–168 | AT1G63040.1 |
| PtrDREB20 | POPTR_0003s17830 | 7–56 | AT1G71450.1 |
| PtrDREB21 | POPTR_0001s14720 | 46–95 | AT1G77200.1 |
| PtrDREB22 | POPTR_0018s02280 | 7–56 | AT2G25820.1 |
| PtrDREB23 | POPTR_0006s27710 | 7–56 | AT2G35700.1 |
| PtrDREB24 | POPTR_0018s01680 | 5–55 | AT2G36450.1 |
| PtrDREB25 | POPTR_0006s26990 | 21–71 | AT2G44940.1 |
| PtrDREB26 | POPTR_0003s02750 | 5–55 | AT3G16280.1 |
| PtrDREB27 | POPTR_0006s06870 | 5–55 | AT3G60490.1 |
| PtrDREB28 | POPTR_0018s13130 | 5–55 | AT4G16750.1 |
| PtrDREB29 | POPTR_0006s08000 | 22–71 | AT4G32800.1 |
| PtrDREB30 | POPTR_0007s10750 | 22–71 | AT5G11590.1 |
| PtrDREB31 | POPTR_0005s18430 | 22–71 | AT5G25810.1 |
| PtrDREB32 | POPTR_0002s12550 | 35–84 | AT5G52020.1 |
| PtrDREB33 | POPTR_0014s02530 | 35–84 | AT1G19210.1 |
| PtrDREB34 | POPTR_0018s00700 | 14–63 | AT1G21910.1 |
| PtrDREB35 | POPTR_0006s14110 | 14–63 | AT1G22810.1 |
| PtrDREB36 | POPTR_0005s15830 | 31–80 | AT1G44830.1 |
| PtrDREB37 | POPTR_0002s08610 | 26–76 | AT1G46768.1 |
| PtrDREB38 | POPTR_0019s10220 | 29–78 | AT1G71520.1 |
| PtrDREB39 | POPTR_0013s10420 | 20–70 | AT1G74930.1 |
| PtrDREB40 | POPTR_0018s08320 | 18–68 | AT1G77640.1 |
| PtrDREB41 | POPTR_0006s23480 | 23–73 | AT2G23340.1 |
| PtrDREB42 | POPTR_0006s14090 | 16–66 | AT3G50260.1 |
| PtrDREB43 | POPTR_0006s14100 | 16–66 | AT4G06746.1 |
| PtrDREB44 | POPTR_0001s18180 | 15–65 | AT4G31060.1 |
| PtrDREB45 | POPTR_0013s10340 | 14–64 | AT4G36900.1 |
| PtrDREB46 | POPTR_0019s10420 | 11–62 | AT5G21960.1 |

TABLE 2: Continued.

| Gene symbol | Gene Locus | PF00847 AP2 domain | <i>Arabidopsis</i> ortholog locus |
|-------------|------------------|--------------------|-----------------------------------|
| PtrDREB47 | POPTR_0003s05300 | 16–66 | AT5G67190.1 |
| PtrDREB48 | POPTR_0013s10330 | 49–99 | AT1G36060.1 |
| PtrDREB49 | POPTR_0019s10430 | 15–65 | AT1G64380.1 |
| PtrDREB50 | POPTR_0019s09530 | 36–84 | AT1G78080.1 |
| PtrDREB51 | POPTR_0014s09540 | 38–88 | AT2G22200.1 |
| PtrDREB52 | POPTR_0855s00200 | 20–68 | AT4G13620.1 |
| PtrDREB53 | POPTR_0002s17330 | 20–68 | AT4G28140.1 |
| PtrDREB54 | POPTR_0006s02180 | 44–93 | AT4G39780.1 |
| PtrDREB55 | POPTR_0016s02010 | 50–98 | AT5G65130.1 |
| PtrDREB56 | POPTR_0015s13840 | 84–132 | AT1G22190.1 |
| PtrDREB57 | POPTR_0012s13880 | 115–163 | AT4G13040.1 |
| PtrDREB58 | POPTR_0003s12120 | 14–61 | |
| PtrDREB59 | POPTR_0001s08740 | 14–61 | |
| PtrDREB60 | POPTR_0009s14990 | 57–108 | |
| PtrDREB61 | POPTR_0004s19820 | 59–110 | |
| PtrDREB62 | POPTR_0001s08710 | 66–116 | |
| PtrDREB63 | POPTR_0001s08720 | 62–111 | |
| PtrDREB64 | POPTR_0012s13870 | 85–135 | |
| PtrDREB65 | POPTR_0015s13830 | 79–129 | |
| PtrDREB66 | POPTR_0002s14210 | 94–143 | |
| PtrDREB67 | POPTR_0003s07700 | 96–146 | |
| PtrDREB68 | POPTR_0001s15550 | 71–122 | |
| PtrDREB69 | POPTR_0003s04920 | 87–137 | |
| PtrDREB70 | POPTR_0001s18800 | 54–103 | |
| PtrDREB71 | POPTR_0018s09270 | 69–119 | |
| PtrDREB72 | POPTR_0006s17670 | 67–117 | |
| PtrDREB73 | POPTR_0006s25500 | 59–109 | |
| PtrDREB74 | POPTR_0018s00270 | 59–109 | |
| PtrDREB75 | POPTR_0014s05500 | 90–139 | |

and A6, containing six, 17, two, 26, 15, and nine members, respectively. The phylogenetic trees of the *DREB* subfamilies of *Populus* and *Arabidopsis* are shown in Figure 2. The alignment analysis indicates that *DREBs* share high homology in the AP2/ERF domain (see Supplementary Figure 1; available online at <http://dx.doi.org/10.1155/2013/954640>) and contain a conserved WLG motif in the AP2/ERF domain of *Populus*. In the proteins encoded by *DREBs*, position 14 is normally valine and position 19 is glutamic acid. This region may play an important role in the recognition of different DNA-binding sites by the DRE and GCC box cis-elements of the *DREB* subfamily [3]. Also, the *DREBs* contain alanine at position 319 in the β -sheet. Group A-1 possesses a conserved C/SEV/LR amino acid sequence between V319 and E324, and this region is converted into an AEIR amino acid sequence in groups A-2, A-3, and A-6. In Group A-4, Ser-324 is crucial for the specific binding of the ERE element, and Ser-324/Ala-324 is crucial in Group A-5.

3.3. Chromosomal Locations of DREB Subgroups. To examine the genomic distribution of *DREB* genes on *Populus* chromosomes, we identified their positions by a Phytozome database search. *In silico* mapping of the gene loci showed that 75 *Populus DREB* genes were mapped to linkage groups (LG) (Figure 3). Previous studies revealed that the *Populus* genome has undergone genome-wide duplications followed by multiple segmental duplications, tandem duplications, and transposition events [18]. It was very clear that three pairs of genes were arranged in tandem repeats, LG I (*PtrDREB63* and 64), LG II (*PtrDREB52* and 53), and LG VI (*PtrDREB43* and 42), and 18 pairs of genes were duplicated. The dN/dS ratios from the 18 segmental duplication pairs were less than 0.5. About 56% (42 of 75) of *Populus DREBs* were preferentially retained tandem duplications and multiple segmental duplication events. Tandem duplications and segmental duplications were relatively underrepresented in groups A-1 to A-6 with rates of 67% (4 of 6), 47% (8 of 17), 100% (2 of 2), 53% (14 of 26), 40% (5 of 15), and 89% (8 of 9), respectively. This finding corroborates previous findings that genes involved in transcriptional regulation and signal transduction are preferentially retained following duplications [19].

3.4. Gene Structure and Conserved Motifs of Populus DREB Genes. To gain further insights into the structural diversity of *Populus DREB* genes, we constructed a phylogenetic tree using the full-length *DREB* protein sequences of *Populus* (Figure 4(a)). We compared the exon/intron organization in the coding sequences of each *Populus DREB* gene (Figure 4(b)). All but nine *Populus DREB* members had no introns in their coding regions and the nine *Populus DREB* genes had one intron (*PtrDREB60*, 61, 62, 24, 25, 26, 27, 28, and 3). We predicted conserved motifs using MEME motif detection software that revealed the diversification of the *P. trichocarpa DREB* genes (Figure 4(c)), and 15 distinct motifs were identified (Table 3). The AP2/ERF domain consists of three β -sheet and one α -helix at the N termini [20, 21]. In this study, motif 3, specifying β -sheet strand 1; motif 1, specifying β -sheet strand 2 and 3; and motif 2, corresponding to the α -helix, were present in all of the *Populus DREB* subfamily members. The CBF signature sequences (motif 14) were found in *DREB* Group A-1 [20, 21]. Alignment of the deduced amino acid sequences of *Arabidopsis DREB* Group A-1 TFs demonstrated significant similarity in the AP2/ERF binding domain and the CBF signature sequences [22]. These results suggest that the *P. trichocarpa DREB* Group A-1 share remarkable similarities at the amino acid sequence level with known CBF/DREB proteins of *Arabidopsis* and carry critical amino acids that are needed for binding to the CRT elements in the target genes. The CMIV domain (motif 7) was found in the *DREB* Group A-2. Alignment of the deduced amino acid sequences of *Arabidopsis DREB* Group A-2 TFs in the N-terminal region included the conserved motif CMIV-1 and the DNA-binding domain [23]. These results suggest that most of the closely related members in the phylogenetic tree shared a common motif composition with each other, suggesting functional similarities among the *DREB* proteins within the same subfamily.

TABLE 3: Motif sequences of *DREB* genes identified in *P. trichocarpa* by MEME tools.

| Motif | Width | Best possible match |
|-------|-------|------------------------|
| 1 | 21 | WGKVVCEIREPRKKSRIWLG |
| 2 | 24 | FPTPEMAARAHDVAALCIKGD |
| 3 | 11 | KHPVYRGVVRM |
| 4 | 21 | LPVPASTSPRDIQAAAAASAA |
| 5 | 8 | LNFPDLVH |
| 6 | 25 | EEALFDMPNLLVDMAGGMLLSP |
| 7 | 29 | GDGGNKPVRKVPKAGSKKGC |
| 8 | 15 | EDHHIEQMIEELLDR |
| 9 | 18 | YKPLHSSVDAKLQAICQS |
| 10 | 25 | HIGVWQKKAGSRSSSNWVMK |
| 11 | 15 | GPITVRLSPSQIAI |
| 12 | 30 | DMSAASIRKRATEVGAHVDAI |
| 13 | 29 | STSLTSLVSLMDLSSQEEELCE |
| 14 | 21 | EVMLASRNPKKRAGRKKFRET |
| 15 | 21 | FESGNFMLQKYPSEIDWASI |

3.5. EST Profiling and Microarray Analysis. The expression profile for each gene was obtained by the Digital Northern tool, which generates a digital northern expression profile heat map based on the ESTs, and is a useful additional means of inferring gene function by examining expression patterns based on the frequency of ESTs in libraries prepared from various tissues [15] (Figure 5). Such analysis yielded 32 *Populus DREB* genes in the available cDNA libraries. Of the 32 *DREBs* examined, 16 genes in the digital expression analysis had high transcript accumulation. A comparison of the digital northern expression analysis revealed that *PtrDREB16* and 63 had high transcript accumulation in flower buds, *PtrDREB51* in apical shoots, *PtrDREB24*, 25, and 51 in petioles, *PtrDREB39* and 65 in dormant buds, *PtrDREB33* and 69 in senescing leaves, *PtrDREB11* and 30 in roots, *PtrDREB37* in active cambium, *PtrDREB71* in shoot meristem, *PtrDREB13*, 16, and 49 in female catkins, *PtrDREB36* in bark, and *PtrDREB13* in imbibed seeds. On the whole, the remaining genes had low transcript accumulation in the different libraries examined. The low-abundance TFs had relatively low EST frequencies [24] and most of the *DREBs* were represented by only one single EST in the cDNA libraries. Nevertheless, this expression analysis demonstrated that most of the *DREBs* have a broad expression pattern across different tissues.

To gain more insights into the expression profiles of *DREB* genes, we then reanalyzed the previously published microarray data in *Populus*. We first investigated the global expression profiles of *DREB* genes by examining Affymetrix (GSE6422) [25] microarray data from Gene Expression Omnibus. Seventy-five *DREB* genes were included in GSE6422, and the *DREB* genes showed a distinct tissue-specific expression pattern (Figure 6). A comparison of the different tissues revealed that *PtrDREB13*, 15, 33, 51, and 53 were overrepresented, however *PtrDREB23* and 28 were under-represented in mature leaves. *PtrDREB13*, 27, 28, 67, and 69 were over-represented, however *PtrDREB30* was under-represented in young leaves. *PtrDREB20*, 30, 49, 70, and 74 were over-represented in

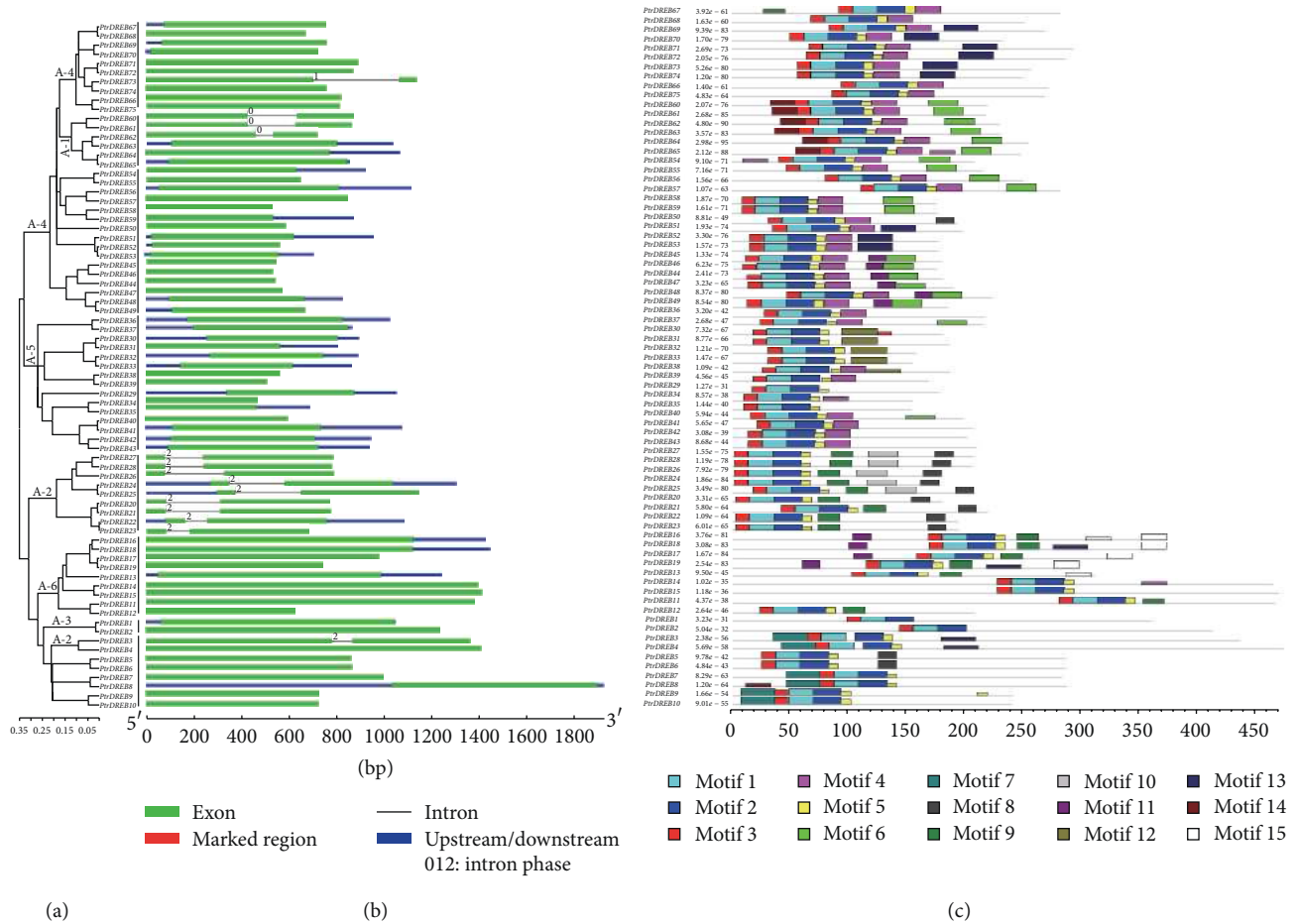


FIGURE 4: Phylogenomic analysis of 75 DREB genes in *P. trichocarpa* (a) with the integration of exon/intron structures (b) and MEME motifs (c). Exon/intron structure was obtained from the Gene Structure Display Server. Motifs were identified with the MEME software using the complete amino acid sequences of the DREB genes.

internodes. *PtrDREB28*, 73, and 74 were over-represented in nodes. *PtrDREB7*, 11, and 18 were over-represented, however *PtrDREB28* was under-represented in roots. On the whole, the remaining genes showed low-abundance transcription levels in the different tissues. EST profiling and microarray analysis showed that *PtrDREB28*, 73, 74, 37, and 71 had high-abundance transcripts in the cambium. Previous studies using genome-wide transcriptional profiling in *Arabidopsis* revealed that stress-related and touch-inducible genes are upregulated in stem regions where secondary growth takes place [26].

3.6. Expression of DREB Genes under Abiotic Stress Conditions in Populus. Studies have previously been conducted to evaluate the expression of the DREB *Populus* genes in response to stresses such as low temperature, high temperature, salt, and dehydration [7]. However, the genes that were evaluated in our study were predicted to be candidate genes in *Populus* via EST profiling and microarray analysis. We have quantified the expression levels of the genes in leaf tissue after exposure to different abiotic stress conditions. The results provide an abundant set of information regarding the expression of

these genes in *Populus* in response to low temperature, high temperature, ABA, salt, and dehydration (Figure 7). Among 15 selected DREB genes, there was evidence of induced expression under different abiotic stresses conditions, with the exception of *PtrDREB30*.

PtrDREB60, 61, and 62, which belong to the A-1 subgroup, were stress-inducible by low temperature, salt, and dehydration (Figure 7). In addition, *PtrDREB62* was stress-inducible by high temperature and *PtrDREB60* was stress-inducible by ABA. These findings are consistent with those of Dubouzet et al. (2003), who reported the increased expression of an A-1 subgroup gene (*OsDREB1*) of rice [10]. It was a major regulator of cold-stress responses in the DREB1/CBF (A-1) subgroup [7]. In a recent study, they indicated that ethylene signaling plays a negative role in the adaptation of *Arabidopsis* to freezing stress [26]. Additionally, some studies indicated that the *AmCBF2* was inducible by heavy metals (Pb²⁺ or Zn²⁺) [4].

PtrDREB4 and *PtrDREB28* belong to the A-2 subgroup (Figure 7), and they were stress-inducible by ABA, salt, and dehydration. In addition, *PtrDREB28* was stress-inducible by high temperature and low temperature. This finding is consistent with those of Dubouzet et al. (2003) [10], who

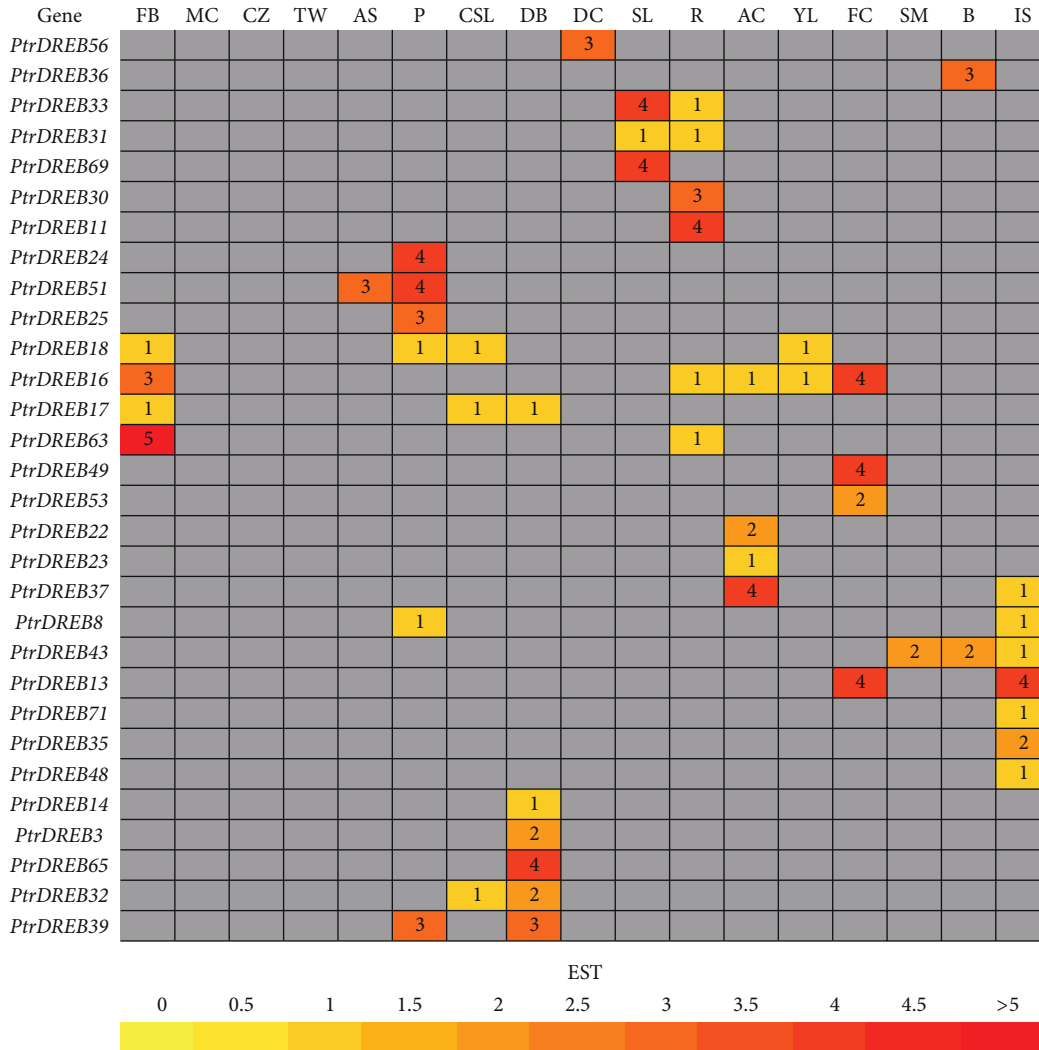


FIGURE 5: In silico EST analysis of *Populus DREB* genes. Color bar at bottom represents the frequencies of EST counts. FB: flower buds, MC: male catkins, CZ: cambial zone, TW: tension wood, AS: apical shoot, R: roots, CSL: cold stressed leaves, DB: dormant buds, DC: dormant cambium, SL: senescing leaves, P: petioles, AC: active cambium, YL: young leaves, FC: female catkins, SM: shoot meristem, B: bark, IS: imbibed seeds. Gene names are shown on the left.

reported increased expression of an A-2 subgroup gene (*MsDREB2C*) of *Malus sieversii* Roem [27]. It was a major regulator of dehydration and heat shock responses in the DREB2 subgroup [7]. This indicated that ethylene signaling plays a negative role in the adaptation of *Arabidopsis* to freezing stress [26]. The oxidative stress tolerance of DREB2C-overexpressing transgenic *Arabidopsis* plants was regulated by heat shock factor A3 (*HsfA3*) and *HsfA3* is regulated at the transcriptional level by *DREB2* [28].

PtrDREB51, 55, and 68 belong to the A-4 subgroup, and they were stress-inducible by salt and dehydration. However, *PtrDREB51*, 55, and 68 were down represented by high temperature *PtrDREB68* was down represented by low temperature. Genes *PtrDREB30*, 32, and 38 belonged to subgroup A-5. *PtrDREB32* was stress-inducible by cold and ABA, whereas *PtrDREB38* was induced by hot and cold temperatures. *PtrDREB30* was down represented by high

temperature, whereas *PtrDREB38* was down represented by salt. *PtrDREB16*, 17, 18, and 19 belonged to the A-6 subgroup. *PtrDREB16* was induced by drought and high salt, and *PtrDREB17* was induced by drought, high temperature, low temperature, and ABA treatment. *PtrDREB18* was induced by high temperature, low temperature, and ABA. In addition, *PtrDREB19* was only induced by low temperature. *PtrDREB18* was down represented by by salt, whereas *PtrDREB16* was down represented by high temperature and cold temperature (Figure 7). In our study, a digital northern analysis showed that the genes *PtrDREB17*, 18, and 32 were expressed in cold-stressed leaves, and showed the same expression trends based on qRT-PCR. The expression patterns of *Populus DREB* genes detected by qRT-PCR are generally consistent with microarray analyses and digital northern analyses.

Some studies indicate that the *HARDY* (*At2g36450*) gene belongs to the A-4 subgroup. The overexpression of the

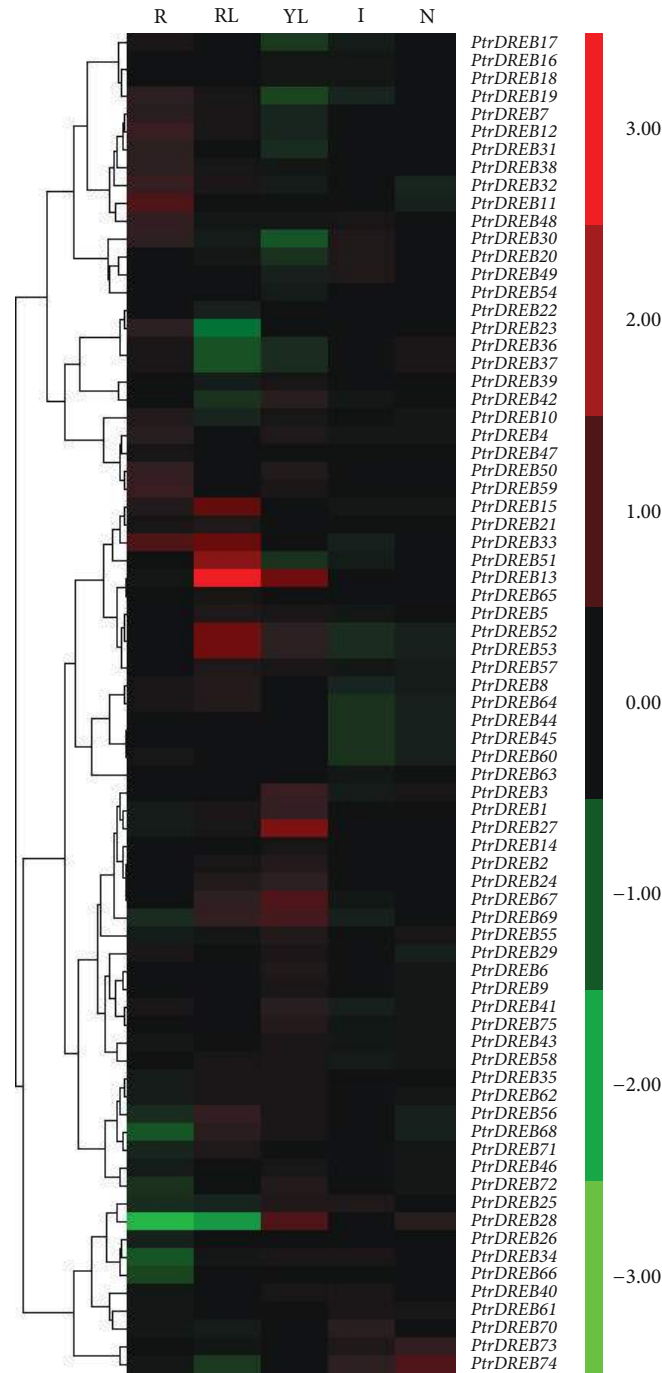


FIGURE 6: Expression profiles of *Populus DREB* genes across different tissues. Heat map showing hierarchical clustering of 75 *PtrDREB* genes across various tissues analyzed. The Affymetrix microarray data were obtained from NCBI Gene Expression Omnibus (GEO) database under the series accession number GSE16422. ML: mature leaf; YL: young leaf; R: root; I: internodes; N: nodes. Color scale represents log₂ expression values, green represents low level, and red indicates high level of transcript abundances.

HRD gene for the improvement of water-use efficiency is coincident with drought resistance in rice. The analogous genes *At2g36450* and *PtrDREB68* were stress-inducible in drought [29]. The homologous genes, *RAP2.4* (*At1g78080*) and *PtrDREB19* and *RAP2.4B* (*AT1G22190*) and *PtrDREB17*, were expressed in response to dehydration, high salinity, and

heat [30, 31]. Overexpression or mutation of *RAP2.4* and *RAP2.4B* in *Arabidopsis* acts at or downstream of a point of convergence for light and ethylene signaling pathways that coordinately regulates multiple developmental processes and stress responses [30]. It is noteworthy that the expression of several other genes associated with lipid transport was altered

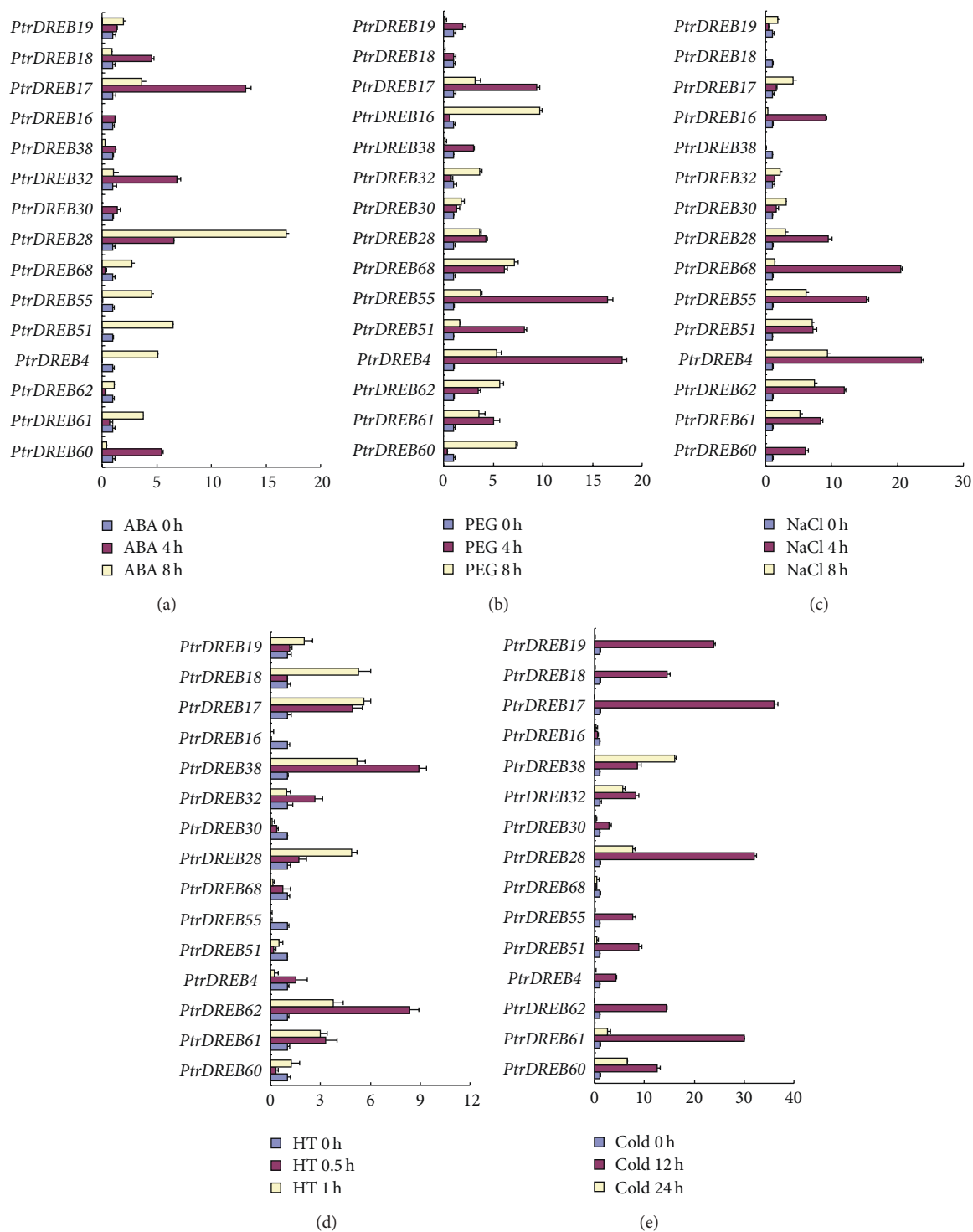


FIGURE 7: Expression analysis of fifteen selected *P. trichocarpa* DREB genes in mature leaves under ABA (a), drought stresses (b), salinity (c), high temperature (d), and low temperature (e) by qRT-PCR. The data were normalized using the *P. trichocarpa* actin gene. Standard deviations were derived from three replicates of each experiment.

in the *RAP2.4* and *RAP2.4B* overexpression lines, further supporting the link between *DREB* TFs and adaptive alterations in lipid metabolism [31]. Hence, we think that the *PtrDREB16* and *17* TFs are probably associated with enhanced drought tolerance by modulating the wax biosynthetic pathway.

4. Conclusions

Understanding the plant *DREB* subfamily is important for elucidating the mechanisms of a variety of stress responses. Therefore, we present a comprehensive and specific analysis

of gene structure, chromosome localization, and expression of the *Populus DREB* subfamily for the first time. We predicted *P. trichocarpa DREB* gene expression and function through comparisons with similar genes that have been well studied in model or other plants. The chromosomal localizations of the *PtrDREB* genes involved in transcription regulation and signal transduction are preferentially retained following duplications. The conserved motif composition of *PtrDREB* genes were highly conserved in the same subtype. EST profiling and microarray analysis of this gene subfamily from different tissues and/or developmental stages showed the same expression trends based on qRT-PCR. The results in the present study indicate that *DREBs* function as important transcriptional activators and may be useful in improving plant tolerance to abiotic stresses. Taken together, these observations may lay the foundation for future functional analyses of *Populus DREB* genes to unravel their biological roles.

Conflict of Interests

The authors declare no conflict of interests.

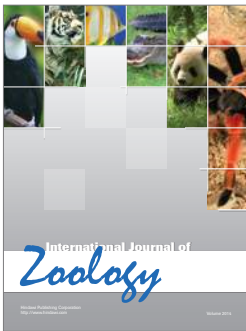
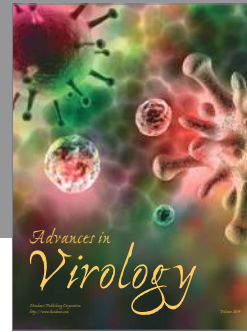
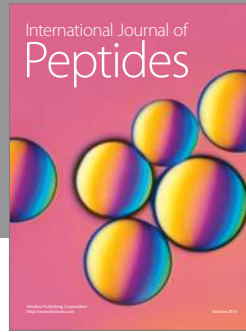
Acknowledgments

This research was supported by the Key Project of Chinese National Programs for Fundamental Research and Development (973 Program: 2009CB119100), the National High Technology Research and Development Program of China (2011AA100203), and the Fundamental Research Funds for the Central Universities (DL11EA02).

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