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Identification of AP2/ERF Transcription Factor Family Genes and Expression Patterns in Response to Drought Stress in *Pinus massoniana*

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Abstract: *Pinus massoniana* Lamb. is found in 17 Chinese provinces and is an important timber tree species in southern China. The current seasonal drought climate is becoming increasingly severe, threatening *P. massoniana* growth and limiting the development of the *P. massoniana* industry. Plant growth, development, and stress were all regulated by AP2/ERF. We identified 124 AP2/ERF transcription factor family members in this study and discovered that all the genes had their own conserved structural domains and that PmAP2/ERFs were divided into 12 subfamilies with high conservation and similarity in gene structure and evolutionary level. Nine *PmAP2/ERF* genes were constitutively expressed under drought treatment, and it was hypothesized that the *PmAP2/ERF96* gene negatively regulated drought stress, *PmAP2/ERF46* and *PmAP2/ERF49* genes showed a positive or negative response to drought in different tissues, while the remaining six genes were positively regulated. The *PmAP2/ERF* genes responded to drought stress following treatment with the exogenous hormones SA, ABA, and MeJA, but the expression patterns differed, with each gene responding to at least one exogenous hormone to induce up-regulation of expression under drought stress, with *PmAP2/ERF11*, *PmAP2/ERF44*, *PmAP2/ERF77*, and *PmAP2/ERF80* genes significantly induced by three hormones. The genes mentioned above may be involved in hormone signaling pathways in response to drought stress. The results indicate that the *PmAP2/ERF* genes may positively or negatively regulate the corresponding signaling pathways in *P. massoniana* to improve drought resistance.

Keywords: *Pinus massoniana* Lamb.; AP2/ERF transcription factor; bioinformatics; drought stress; exogenous hormone; expression pattern

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1. Introduction

Drought, cold, salt, and other abiotic stressors have a significant impact on plant growth and development [1–3]. When plants are subjected to drought stress, they respond with a comprehensive series of physiological and molecular regulatory mechanisms [4,5]. To reduce plant damage, plants regulated osmoregulatory substances, antioxidant defense systems, and endogenous hormone levels to maintain cell morphology and scavenge excess oxygen radicals [4,6,7]. Drought induced the expression of plant-related genes, and the main regulatory genes that responded to drought were transcription factors. Such genes can respond quickly after the plant becomes stressed and form their own regulatory network by regulating downstream genes or collaborating with one another to resist drought stress [8,9].

AP2/ERF (APETALA2/ethylene -responsive factor) is one of the largest families of transcription factors in plants. AP2/ERF genes were firstly identified in *Arabidopsis thaliana*

and were associated with flower development [10]. AP2/ERF gene families have been discovered in an increasing number of plant species, and AP2/ERF family genes are more numerous and functionally diverse, involved in physiological and biochemical processes such as growth and development, hormone signaling, and the response to biotic and abiotic stresses in plants [11,12]. The AP2 functional structural domain specific to the AP2/ERF gene consists of 60–70 conserved amino acid residues, which include the YRG and RAYD structural domains; the YRG structure is located at the N-terminus of the AP2 structural domain and consists of about 20 amino acid residues; the YRG structure's role is to allow the AP2/ERF gene to contact DNA and recognize cis-acting elements [13]. A structural domain at the C-terminus with about 40 amino acid residues may participate in transcription factor interactions [13]. The AP2/ERF gene family is divided into four subfamilies based on sequence similarity and the number of AP2/ERF functional structural domains [14]. The AP2 subfamily contains two AP2/ERF structural domains that have been linked to plant flower development [15,16]; the RAV subfamily contains one AP2/ERF structural domain and one B3 structural domain; and the ERF and Soloist subfamilies have one AP2/ERF structural domain each.

Genes of the *AP2/ERF* family are considered to be plant-specific transcription factors. In *A. thaliana* [11] and *Oryza sativa* L. [17], a large number of AP2/ERF genes have been discovered; for instance, *AP2/ERF* genes can improve drought tolerance by specifically binding to downstream genes, for example, *SpERF1* activated and regulated downstream genes Meanwhile, the function of *AP2/ERF* genes in model plants such as *Arabidopsis* and rice has been studied more frequently and intensively, and it has been shown that *AP2/ERF* genes play an important role in the molecular regulation mechanism of drought in transgenic plants to improve drought stress tolerance by binding to DRE/CRT elements in the promoters of drought-related genes *HSP101*, *RD29A*, *P5CS*, and others [18]. By regulating hormone signaling pathways such as abscisic acid (ABA) and jasmonic acid (JA), *AP2/ERF* genes play an important role in plant signaling and improve plant drought resistance [19,20]. Plant drought resistance was also improved by *AP2/ERF* genes, which regulate transpiration, photosynthesis, plant development, and endogenous hormone content [21,22]. In contrast, *AP2/ERF* genes have been relatively little studied in forest trees, due to the lack of genomic and related expression data, etc., in many tree species, and functional studies of *AP2/ERF* genes have also been carried out in recent years in forest trees, and the results showed that *AP2/ERF* genes are involved in the process of phellogen activity/phellem differentiation [23], in the early stage of leaf primordium development [24], in signal transduction such as ethylene [23] and gibberellin [25], in phosphorus stress and drought stress [7,26], etc.

Pinus massoniana Lamb. is distributed in 17 Chinese provinces and is an important timber species with significant economic value in southern China [27,28], as well as a pioneer tree species for afforestation [29]. Seasonal drought is common in southern China, which has a negative impact on *P. massoniana* growth and limited the development of the *P. massoniana* industry [6]. The mechanism of *P. massoniana AP2/ERF* genes in response to drought stress is not clear at the moment, and the mechanism of *P. massoniana*-related transcription factors involved in drought resistance from the molecular level study has rarely been investigated [5,30]. Since genome-wide data are not yet available for *P. massoniana*, the identification of the *AP2/ERF* gene family in *P. massoniana* is a feasible approach. In this study, we conducted gene family identification by identifying *PmAP2/ERF* genes in *P. massoniana*, explored related drought resistance genes, and explored the signaling pathways that the genes may be involved in, to provide a reference for revealing the function of *PmAP2/ERF* genes and drought response mechanism studies in *P. massoniana*.

2. Materials and Methods

2.1. Plant Materials and Treatments

Drought-tolerant line 19–220 seedlings and drought-sensitive line 19–214 seedlings were chosen as experimental materials, and seedlings with good development and consis-

tency were chosen and put in pots (18 cm in diameter and 25 cm in height) with substrate (yellow clay soil:coconut coir = 3:1), one plant per pot, and the experiment was carried out after 1 month of normal cultivation in a greenhouse.

Five treatments were established based on the results of the previous pre-experiment: CK1 normal watering; CK2 drought stress; 50 mg/L salicylic acid (SA), SA + drought stress; 0.5 mmol/L methyl jasmonate (MeJA) + drought stress; and 25 mg/L ABA + drought stress, with three replications of each treatment and ten plants in each replicate. Following three days of continuous appropriate watering, the above concentrations of SA, MeJA, ABA, and distilled water (CK1 and CK2) were sprayed for four days, with 10 mL on the above-ground part (stems and needles) and 10 mL on the below-ground part (roots). Following that, all experimental seedlings were rehydrated and recorded as day 0 of drought stress for ongoing natural drought stress, with CK1 serving as the regular watering control group, which was watered once every two days. At soil drought levels [9] of mild drought (55%–70%), moderate drought (45%–55%), severe drought (30%–45%), and 48 h after rehydration, the needles (middle part of the area with needles), stems (3 cm long in the middle), and roots (2 cm in the apical part of the main and lateral roots) of *P. massoniana* were sampled and stored in a refrigerator at −80 °C.

2.2. Identification of PmAP2/ERFs Gene Family

The *AP2/ERF* genes in *P. massoniana* were identified based on the full-length transcriptome, insect resistance transcriptome [31], lateral branch differentiation transcriptome [25], and drought resistance transcriptome (unpublished) of the previous research group. Sequences from the three transcriptome databases were removed from redundant sequences and annotated by NR, SwissProt, and Pfam databases, and genes annotated as AP2/ERF were extracted, and their nucleic acid sequences and protein sequences were extracted using Perl language. Hidden Markov models of the AP2 structural domain were downloaded from the Pfam database [32] (https://pfam.xfam.org/, accessed on 7 June 2021) and the AP2/ERF genes were identified using HMMER software, while the protein sequences of the *A. thaliana AP2/ERF* genes were downloaded from the PlantTFBD database [33] (http://planttfdb.gao-lab.org/index_ext.php, accessed on 7 June 2021) to download the protein sequences of *A. thaliana AP2/ERF* genes, and the AP2/ERF protein sequences of *A. thaliana* and *P. massoniana* were compared using BLAST software, and the protein sequences obtained by the above two methods were taken as a concatenation. After removing the redundant sequences, NCBI CD Search [34] (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 8 June 2021), SMART [35] (http://smart.embl.de/, accessed on 8 June 2021), and Pfam were used to determine whether the candidate protein sequences contained AP2/ERF structural domains, and sequences lacking structural domains or containing incomplete structural domains were removed.

2.3. Analysis of Physicochemical Properties of PmAP2/ERFs Proteins

ExPASy [36] (https://web.expasy.org/, accessed on 14 December 2021) was used to predict the physicochemical properties of PmAP2/ERF molecular mass, isoelectric point, etc.; Plant-mPLoc [37] (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on 14 December 2021) and WoLF PSORT [38] (https://psort.hgc.jp/, accessed on 14 December 2021) online sites for subcellular localization prediction analysis; TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/, accessed on 14 December 2021) for protein transmembrane structure analysis.

2.4. PmAP2/ERFs Protein Phylogenetic Analysis and Multiple Sequence Alignment

The *A. thaliana* AP2/ERF protein sequences were obtained from the TAIR database [39] and the PlantTFBD database, and the phylogenetic evolutionary trees of *P. massoniana* and *A. thaliana* were generated using MEGA7 software [40] with the following parameters: Using the Neighbor-Joining (NJ) approach, the P-distance model was chosen, and the evolutionary tree was decorated with iTOL [41].

2.5. PmAP2/ERFs Protein Conserved Motif Analysis

The conserved motifs of 124 *P. massoniana* PmAP2/ERFs proteins were evaluated online using the MEME online tool [42] (https://meme-suite.org/meme/, accessed on 26 July 2021), with the following parameters: The expected motif count was ten, and the motif length ranged from 6 to 60 AA.

2.6. Prediction of PmAP2/ERFs Protein Interactions

Using the STRING website [43] (https://string-db.org/cgi, accessed on 27 March 2022) and Cytoscape software [44], potential interaction networks and biological functions between PmAP2/ERFs proteins of *P. massoniana* were predicted based on the AP2/ERF protein analysis of *A. thaliana*.

2.7. RNA-seq Data Analysis of PmAP2/ERF Genes

The expression heat map of *PmAP2/ERF* genes under drought stress was drawn based on the pre-drought transcriptome data. The treatment groups were continuous natural drought stress (D) and the control group (C) was normal watering. The root systems (main and lateral root tips were 2 cm) of seedlings in the treatment and control groups corresponding to the three time points were taken at the 7th d (1), the 8th d before rehydration for 7 h (2), and the 8th d (3) of the drought stress treatment, respectively. Based on the obtained transcriptome data, the expression heat map of *PmAP2/ERFs* gene family under drought stress was drawn using TBtools.

2.8. Expression Analysis of PmAP2/ERFs Gene

RNA extraction was performed using the polyphenol polysaccharide plant RNA extraction kit from Tiangen (Beijing, China), cDNA synthesis was performed using M-MLV reverse transcriptase from Takara (Shanghai, China), and finally, the concentration of all cDNA samples was adjusted to 50 ng/μL. Fluorescent quantitative PCR was performed using the TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) kit (Shanghai, China) from Takara Bio, with the reaction system configured according to the instructions; the internal reference genes were *PmUBI4* (tissue-specific internal reference gene) and *PmCYP* (drought stress-treated internal reference gene) [45], and primer information is provided in (Table S1); real-time fluorescence quantitative PCR was performed using a Bio-Rad CFX96 quantitative PCR instrument (San Diego, CA, USA), and each sample was technically repeated three times, and the relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method [46], and the data were analyzed for significant differences using SPSS software (IBM, New York, NY, USA), and finally plotted using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Identification and Naming of PmAP2/ERFs Gene Family

Non-redundant sequences were obtained based on the previous full-length transcriptome and annotated as *AP2/ERF* genes, and 453 sequences were identified by HMMER software and local Blast. After removing the redundant sequences, the candidate protein sequence structural domains were analyzed using NCBI CD Search, SMART, and Pfam, and 124 PmAP2/ERF genes of *P. massoniana* were finally identified and obtained (Table S2).

3.2. Analysis of Physicochemical Properties of PmAP2/ERFs Proteins

PmAP2/ERFs physicochemical properties and functional structure analysis showed that PmAP2/ERFs protein encoded 101 (PmAP2/ERF58) to 683 (PmAP2/ERF43) amino acids. The molecular weight of PmAP2/ERFs protein ranged from 11.34 (PmAP2/ERF58) to 76.18 (PmAP2/ERF43) kDa. The theoretical isoelectric points of PmAP2/ERFs proteins ranged from 4.48 (PmAP2/ERF27) to 11.65 (PmAP2/ERF89) with an average pI of 7.50; 61 PmAP2/ERFs proteins had isoelectric points < 7, which were acidic, and 63 PmAP2/ERFs proteins had isoelectric points >7, which were alkaline. A total of 9 PmAP2/ERFs proteins

(PmAP2/ERF35\49\71\74\ 101\110\113\121\123) with instability coefficients less than 40 were stable proteins, while the remaining 115 PmAP2/ERFs proteins with instability coefficients greater than 40 were unstable proteins. The average hydrophobic value of 23 PmAP2/ERFs proteins was greater than −0.5, which were hydrophilic proteins, while the average hydrophobic value of the remaining 101 proteins was less than −0.5, which were hydrophobic. The subcellular localization showed that most of the proteins were localized in the nucleus, and some of them were also distributed in the cytoplasm. It was speculated that the *PmAP2/ERF* genes might play different regulatory roles in different organelles, and none of the *PmAP2/ERFs* had transmembrane structures (Table S2).

3.3. PmAP2/ERFs Protein Phylogeny and Multiple Sequence Alignment Analysis

The phylogenetic tree showed that the *AP2/ERF* gene family of *P. massoniana* can be divided into three subfamilies, AP2, RAV, and ERF, and did not contain the Soloist subfamily [11]. Including 8 members of the AP2 subfamily and 9 members of the RAV subfamily, 107 belong to the ERF subfamily. The ERF subfamily was further divided into two subfamilies, DREB and ERF, and in this study the DREB subfamily was divided into subgroups I, II, III, and IV, containing 6, 38, 14, and 4 genes, respectively, for a total of 62 genes; while the ERF subfamily was divided into subgroups V, VI, VII, VIII, IX, and X subfamilies, containing 1, 4, 7, 15, 13, and 5 member genes, respectively, for a total of 45 genes. The AP2 subfamily and the RAV subfamily clustered on one major branch and later on two different minor branches; the DREB and ERF subfamilies clustered on two different major branches, respectively (Figure 1).

The AP2 subfamily genes included two AP2 structural domains (AP2-1 and AP2-2), both containing relatively conserved YRG and RAYD structural domains, with a C-terminal motif deletion in PmAP2/ERF102 in the second AP2 structural domain. The main differences between the ERF and DREB subfamilies were 14 and 19 amino acids of the ERF subfamily were alanine (A) and aspartic acid (D) and 14 and 19 amino acids of the DREB subfamily are valine (V) and glutamic acid (E) [14]. Both the ERF and DREB subfamily genes in the present study also contained the YRG and RAYD structural domains, and most genes were highly conserved in these two structural domains, while a few genes had amino acid residue variants or deletions at positions 14 and 19 (Figure 2).

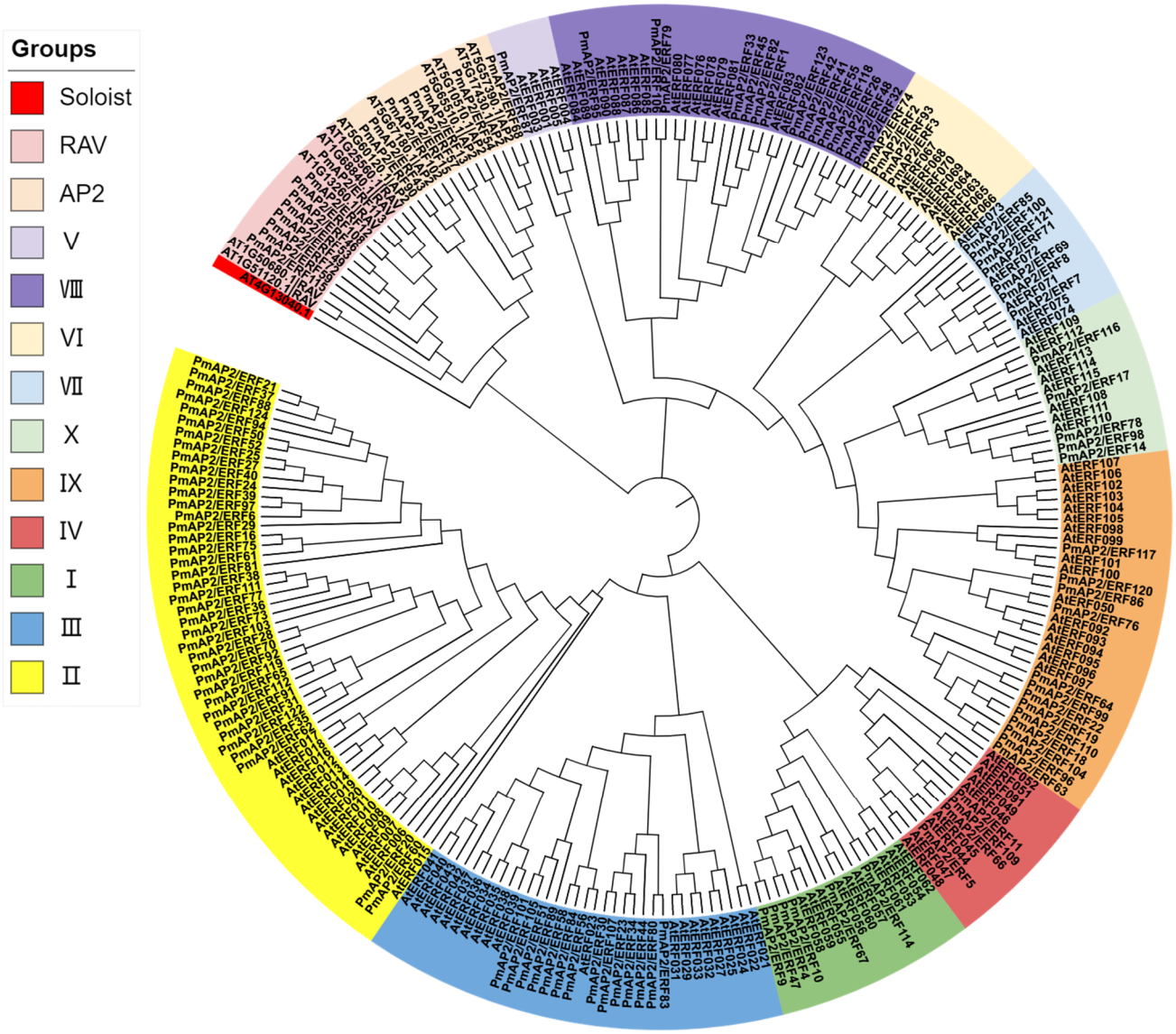


Figure 1. Phylogenetic analysis of PmAP2/ERFs proteins in *P. massoniana*. *A. thaliana* AP2/ERF protein sequences were downloaded from TAIR database and PlantTFBD database, and the phylogenetic evolutionary trees of PmAP2/ERFs of *P. massoniana* and AtERFs of *A. thaliana* were constructed using MEGA7 software and iTOL, and different colors in the figure represent different groupings.

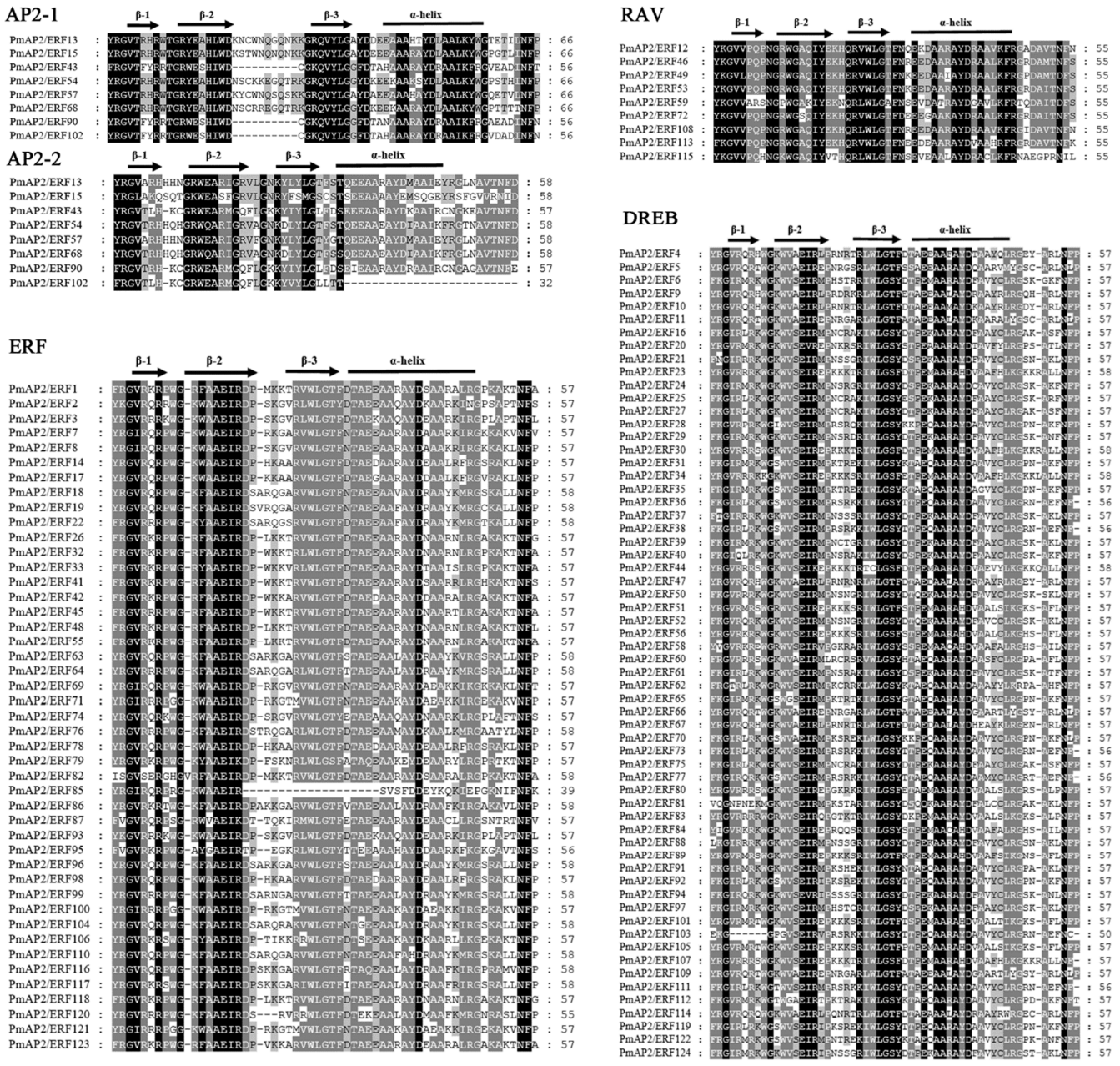


Figure 2. Multiple sequence alignment of AP2 structural domain proteins from each subfamily of PmAP2/ERFs. The sequence comparison results of AP2 structural domain proteins of AP2, RAV, ERF, and DREB subfamilies are shown in the figure, where AP2 subfamily contains two AP2 structural domains, AP2-1 and AP2-2, respectively. Arrows represent β-sheets and horizontal lines represent α-helix.

3.4. Conserved Motif Analysis of PmAP2/ERFs Proteins

The conserved motif analysis of PmAP2/ERFs protein showed that 10 conserved Motifs were obtained, ranging from 29 to 58 amino acids in length. Among them, Motif 8, Motif 1, and Motif 10 formed the first AP2 structural domain of AP2 subfamily, and Motif 6 and Motif 1 formed the second AP2 structural domain. Motifs 6, 1, and 10 formed the AP2 structural domain of RAV subfamily, and Motif 5 and Motif 4 formed the B3 structural domain of RAV subfamily. Motif 2, Motif 1, and Motif 3 formed the AP2 domain of the ERF subfamily. The results showed that the genes of the same subfamily contained basically the same motifs, but there were a few differences, for example, members of the RAV subfamily contained Motif 6, Motif 1, Motif 10, Motif 5, and Motif 4, while PmAP2/ERF115 of the

same RAV subfamily contained one less Motif 10 and one more Motif 4. This phenomenon also exists in other subfamilies, which may be due to mutations during protein evolution (Figure 3A,B).

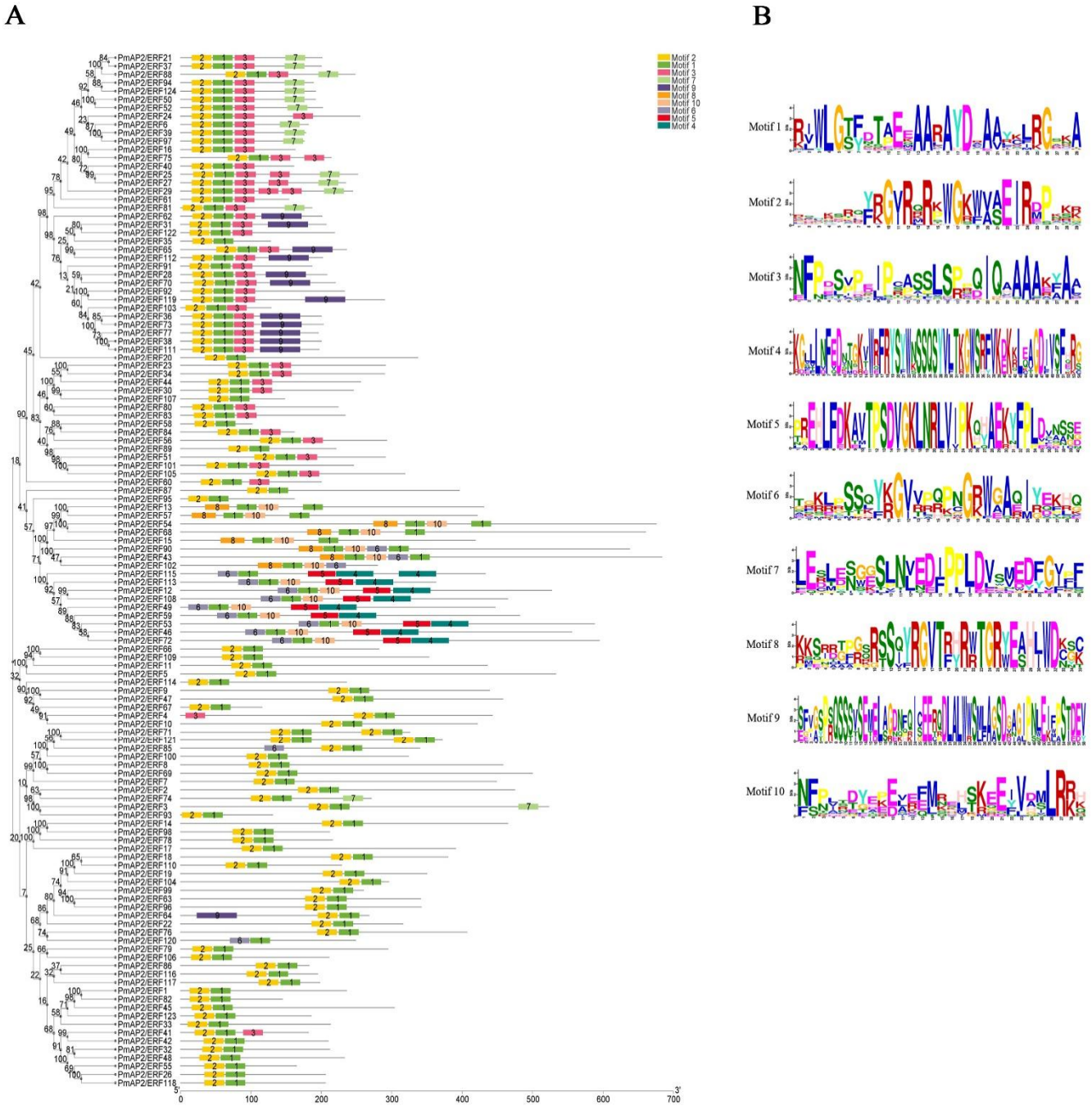


Figure 3. Conserved motif analysis of PmAP2/ERFs proteins of *P. massoniana* and multiple sequence alignment of AP2 structural domain proteins: (**A**): Distribution of conserved motifs of PmAP2/ERFs proteins of *P. massoniana*. A total of 10 conserved Motifs were obtained, indicated by different numbers and colors, and arranged in order. (**B**): Conserved motifs of PmAP2/ERFs proteins of *P. massoniana*. Corresponds to Motif in A.

3.5. Protein Interaction Analysis of PmAP2/ERFs

The results of the protein interaction network map (Figure 4) showed that most of the PmAP2/ERFs could interact with more than one protein, among which there were

interactions between PmAP2/ERF102 and PmAP2/ERF109. PmAP2/ERF102 (AT4G36920) and PmAP2/ERF109 (AT5G05410) played a vital role in the overall reciprocal network.

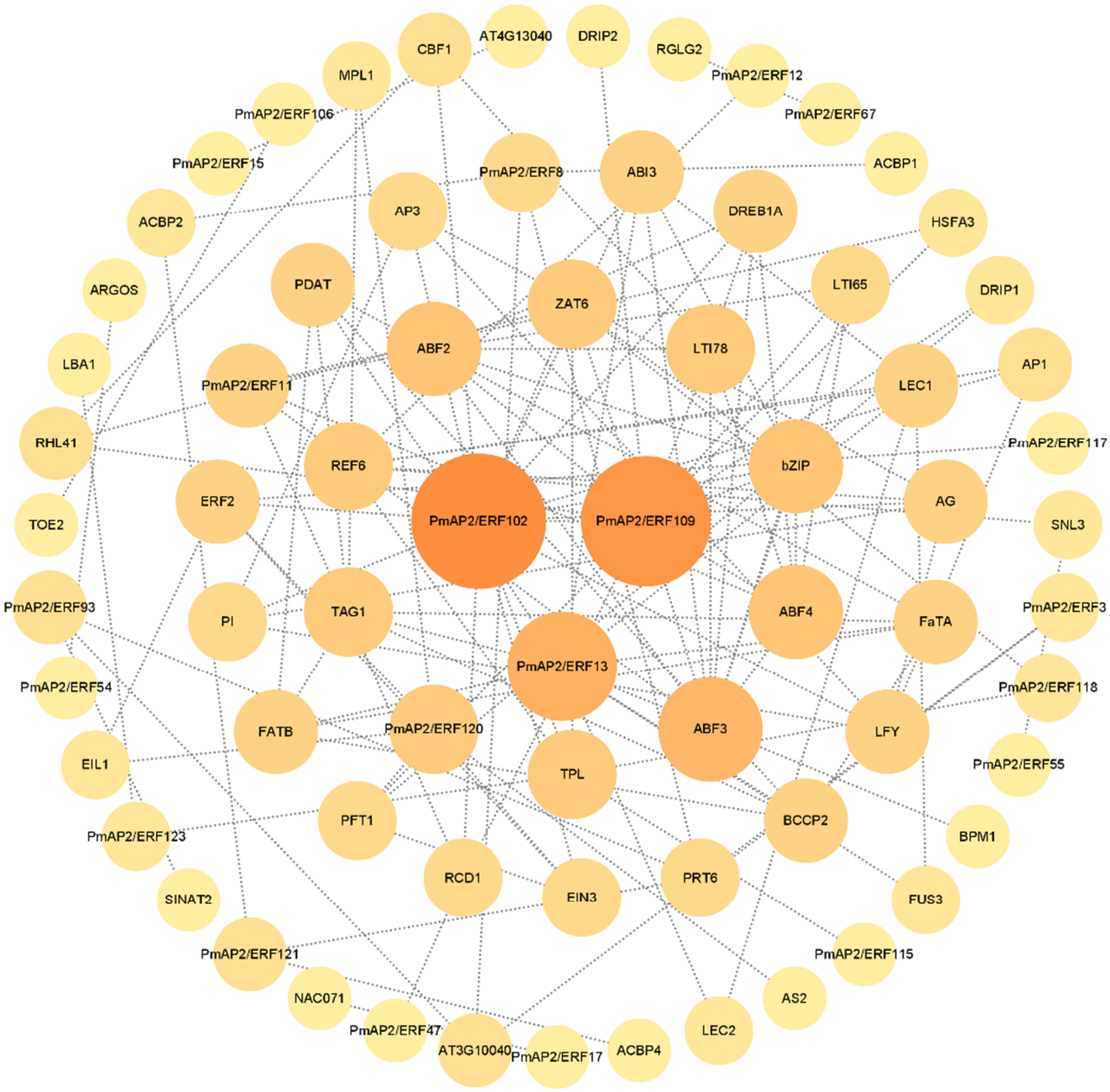


Figure 4. Prediction of the protein interaction network between PmAP2/ERFs of *P. massoniana* and AP2/ERFs of *A. thaliana*. The protein interaction network map of PmAP2/ERFs was constructed by STRING and Cytoscape software, which was based on the AP2/ERFs proteins of *A. thaliana* for analysis and prediction. The circles of different colors and sizes represent the importance of different proteins in the whole interaction network, and the dashed lines represent the possible interactions between the proteins.

3.6. Expression Analysis of PmAP2/ERF Genes in RNA-seq

The expression heat map of PmAP2/ERF genes under drought based on transcriptome data (Figure 5) revealed that 118 genes expressed during drought stress, while 6 genes did not express. A total of 13 genes peaked at D1, 14 genes peaked at D2, 29 genes peaked at D3, and the rest of the genes peaked at CK1. Further, FDR $\leq$ 0.001 and $|\log2FC| \geq 2$ were used

as the screening criteria for significantly different genes, and *PmAP2/ERF11*, *PmAP2/ERF14*, *PmAP2/ERF44*, *PmAP2/ERF46*, *PmAP2/ERF49*, *PmAP2/ERF77*, *PmAP2/ERF80*, *PmAP2/ERF96*, and *PmAP2/ERF109* genes were studied for their expression patterns under hormonal and drought stresses.

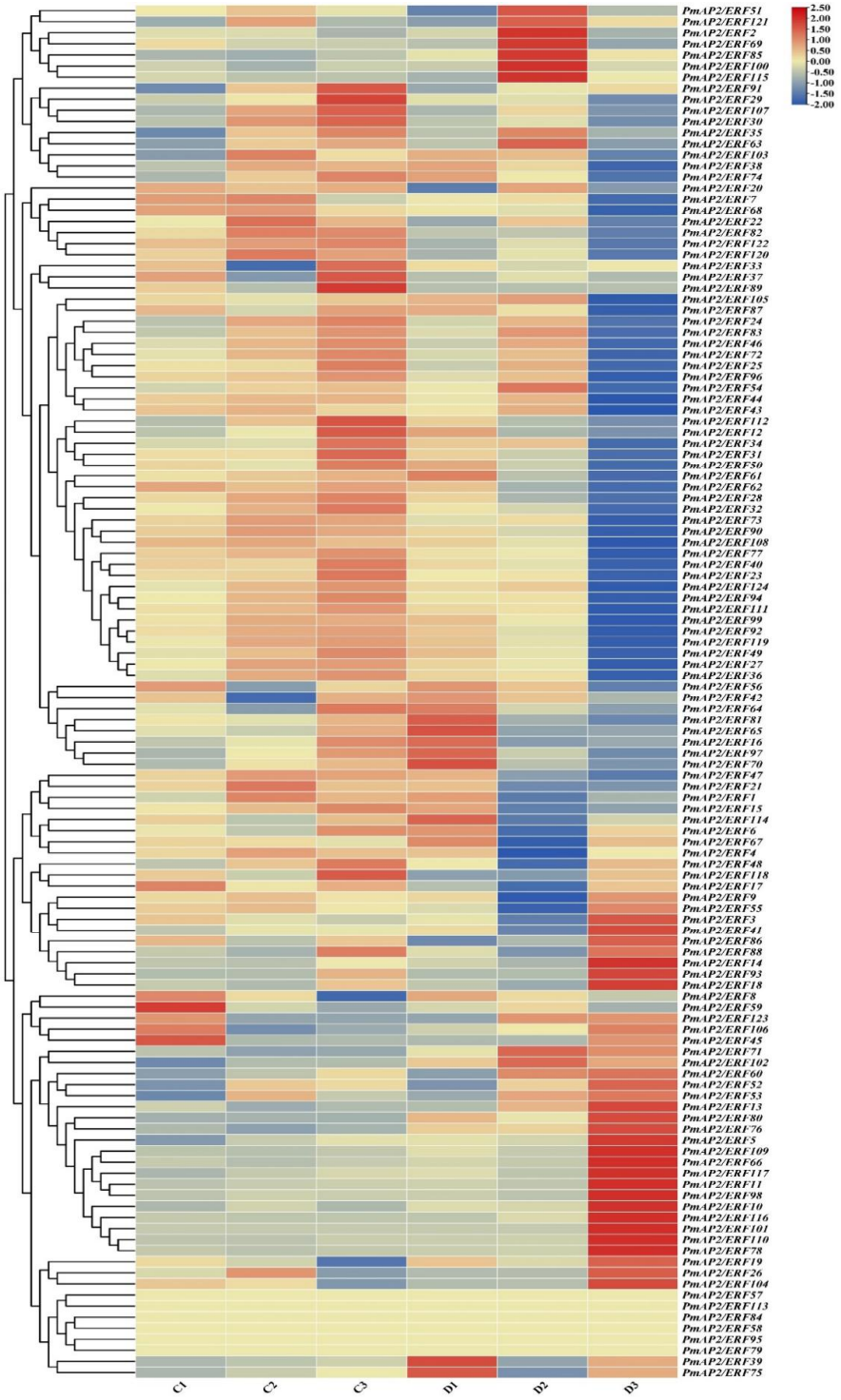


Figure 5. Expression heat map of *PmAP2/ERFs* gene expression in RNA-seq data. Blue represents low expression levels and red represents high expression levels. C and D indicate control (normal growth) and treatment (drought stress) groups, respectively; 1, 2, and 3 indicate control and drought treatment groups on the 7th day, 8th day rehydration at 7 h, and 8th day, respectively. Gene expression heat map was plotted using TBtools.

3.7. Tissue-Specific Analysis of PmAP2/ERF Genes

The tissue-specific results showed that *PmAP2/ERF* genes were expressed in needles, stems, and roots, but the expression levels differed (Figure 6). The genes *PmAP2/ERF14*, *PmAP2/ERF44*, *PmAP2/ERF49*, and *PmAP2/ERF109* were expressed in the roots of different drought-resistant materials. The highest expression was found in needle leaves. These genes may play an important role in regulating the growth and development of roots or needles (Figure 6A,B).

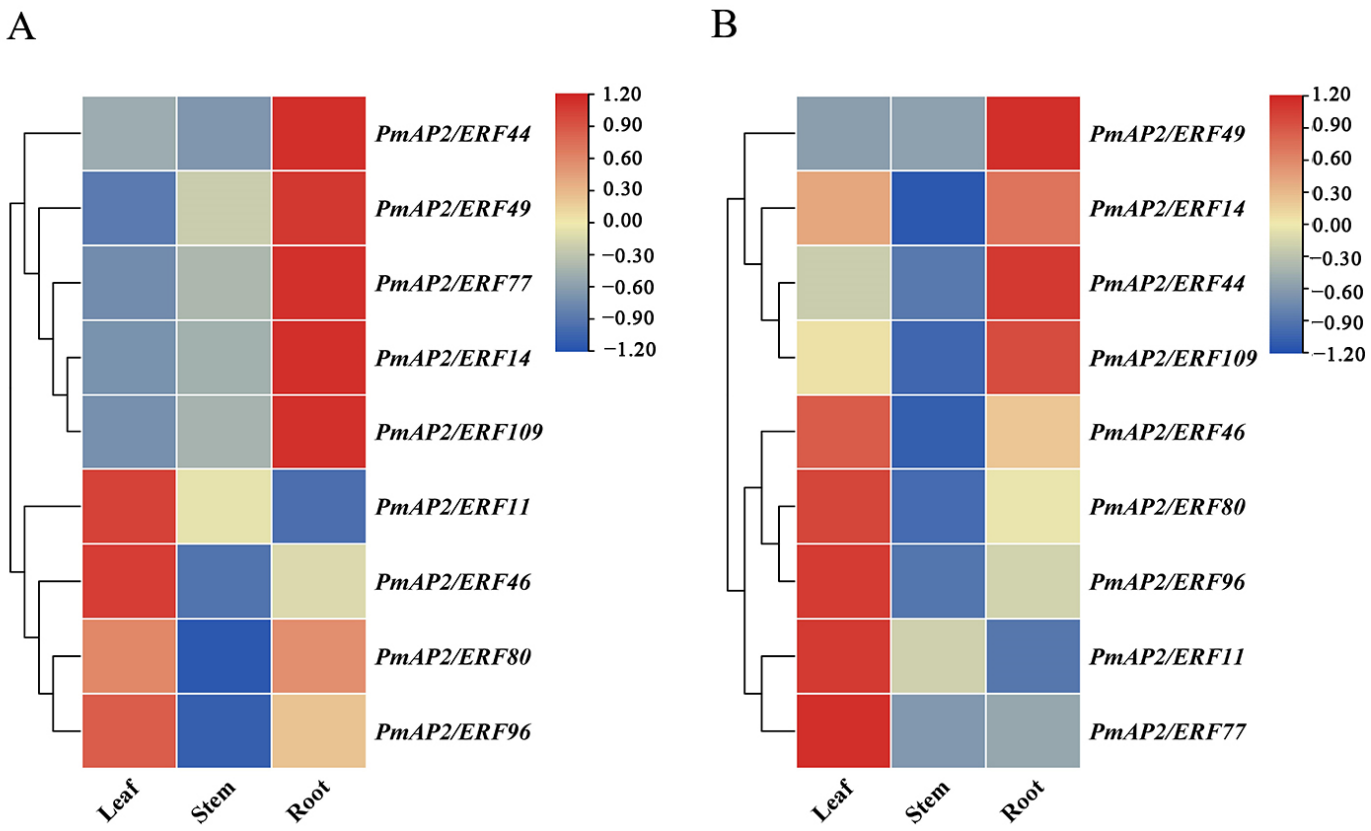


Figure 6. Tissue-specific analysis of *PmAP2/ERF* genes in *P. massoniana*: (**A**): Heat map of *PmAP2/ERFs* gene expression in needle leaves, stems, and roots of drought-sensitive lines. (**B**): Heat map of *PmAP2/ERFs* gene expression in needles, stems, and roots of drought-resistant lines. The expression levels of *PmAP2/ERF* genes in needles, stems, and roots of *P. massoniana* were analyzed by real-time fluorescence quantitative PCR, and the expression of each gene in leaves was used as a control for quantification in stems and roots, respectively. Blue color represents low expression levels and red color represents high expression levels. Plotting was performed using TBtools software.

3.8. Expression Pattern Analysis of PmAP2/ERF Genes under Hormone Treatment and Drought Stress

Expression pattern studies showed that *PmAP2/ERF* genes expressed in all tissues as constitutive expression, but there were differences in expression patterns. In different drought-tolerant lines, drought stress (CK2) induced up-regulated expression of *PmAP2/ERF11*, *PmAP2/ERF14*, *PmAP2/ERF44*, *PmAP2/ERF77*, *PmAP2/ERF80*, and *PmAP2/ERF109* genes, and down-regulated expression of *PmAP2/ERF46*, *PmAP2/ERF49*, and *PmAP2/ERF96*. Expression patterns of PmAP2/ERF genes induced by hormones differed in different tissues of different families (Figures 7 and 8).

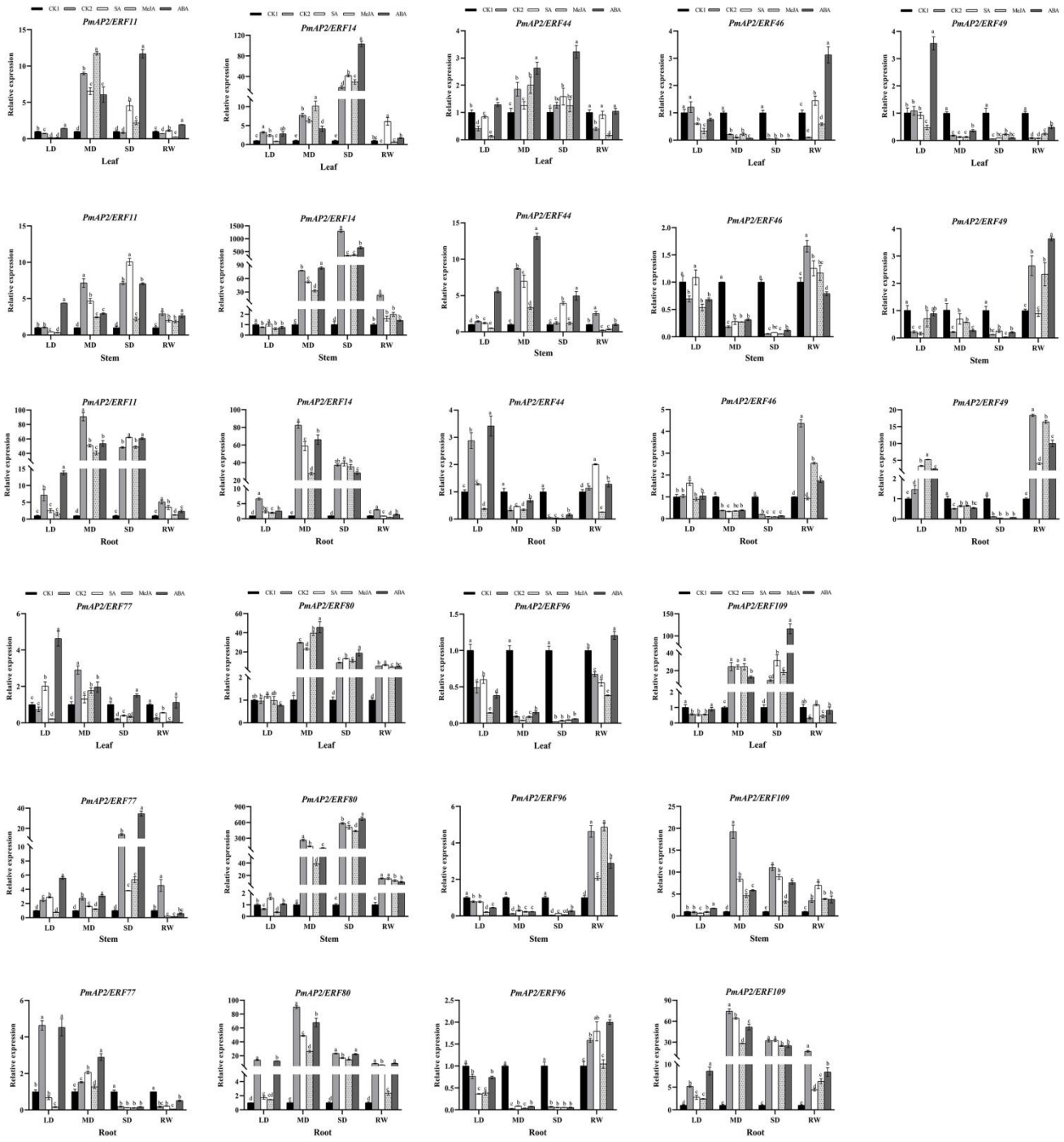


Figure 7. Expression patterns of *PmAP2/ERF* genes in drought-sensitive lines during drought stress. LD indicates light drought, MD indicates moderate drought, SD indicates severe drought, and RW indicates rehydration. Different lowercase letters indicate differences in gene expression at the $p < 0.05$ level between treatments at each sampling site. The standard error of the mean for three biological replicates is represented by the error bars.

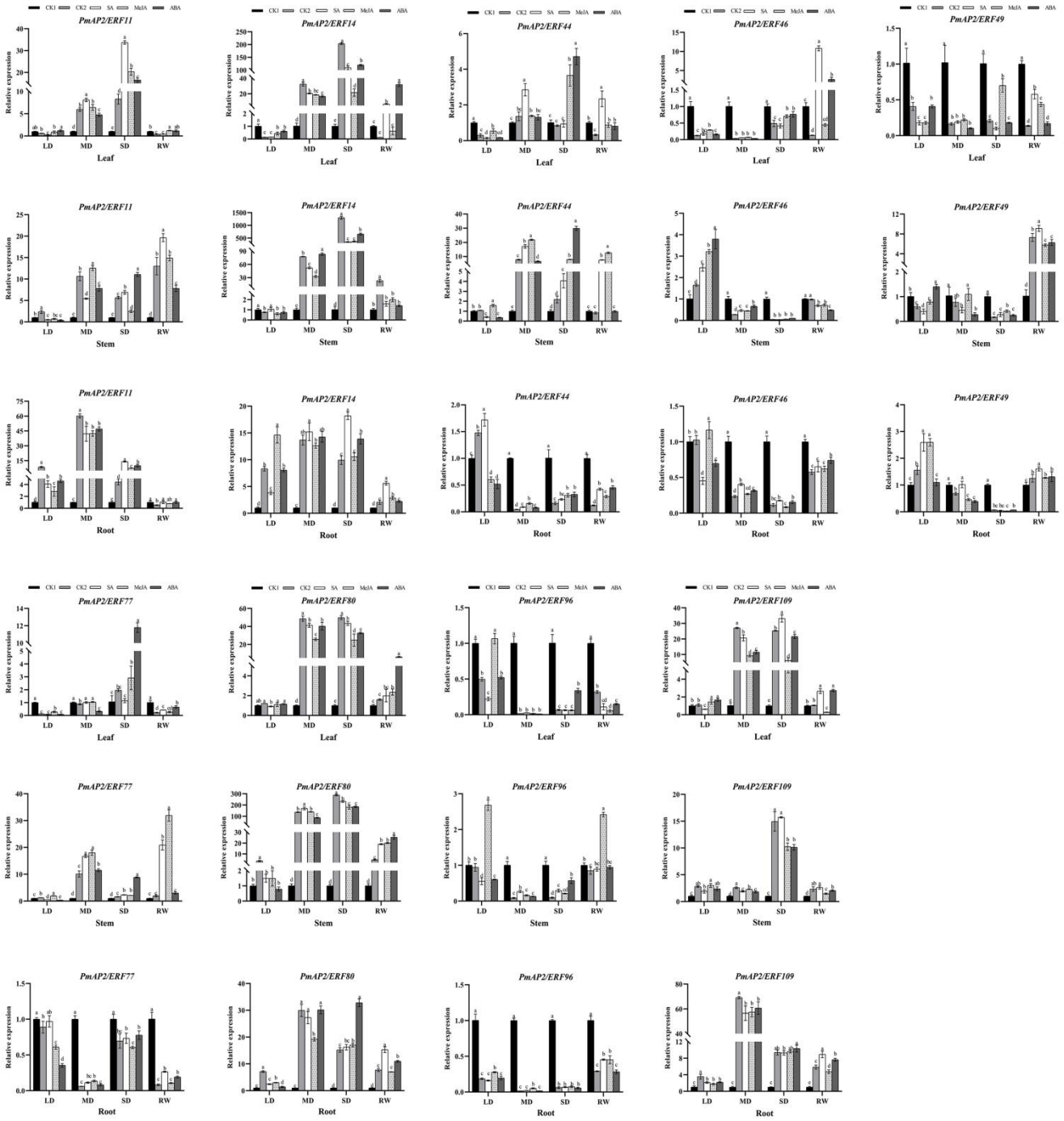


Figure 8. Expression patterns of *PmAP2/ERF* genes in drought-resistant lines during drought stress. LD indicates mild drought, MD indicates moderate drought, SD indicates severe drought, and RW indicates rehydration. Different lowercase letters indicate differences in gene expression at the $p < 0.05$ level between treatments at each sampling site. The standard error of the mean for three biological replicates is represented by the error bars.

In needle leaves, 6 *PmAP2/ERF* genes were up-regulated in leaves of two lines, with *PmAP2/ERF11* and *PmAP2/ERF14* genes expressing more in drought-resistant lines than in drought-sensitive lines. The expression patterns of *PmAP2/ERF* genes induced by hormones under drought stress were similar and different in drought-sensitive and drought-resistant lines. Compared with CK2, *PmAP2/ERF11* and *PmAP2/ERF109* genes were significantly up-regulated when induced by SA, MeJA, and ABA in both lines, while *PmAP2/ERF11* gene expression was higher in drought-resistant lines than in drought-sensitive lines.

PmAP2/ERF14 and *PmAP2/ERF80* genes were up-regulated by three hormones in drought-sensitive lines, while they were not significantly up-regulated by hormones in drought-resistant lines and both were smaller than CK2. *PmAP2/ERF44* and *PmAP2/ERF77* gene expression was induced by three hormones in drought-resistant lines, whereas *PmAP2/ERF44* was induced by ABA at a higher expression level than CK2 in drought-sensitive lines; the *PmAP2/ERF77* gene was significantly expressed when induced by SA and ABA during mild drought. The remaining three genes did not significantly express when induced by hormones. After rehydration, *PmAP2/ERF* genes were expressed at higher levels in drought-resistant lines (Figures 7 and 8).

In the stems, there were similarities and also differences in the expression patterns of *PmAP2/ERF* genes induced by hormones in different lines. Compared with CK2, *PmAP2/ERF11*, *PmAP2/ERF77*, and *PmAP2/ERF80* genes were significantly up-regulated when induced by SA, MeJA, and ABA in drought-sensitive lines, and *PmAP2/ERF11*, *PmAP2/ERF44*, *PmAP2/ERF46*, and *PmAP2/ERF77* genes were significantly expressed when induced by three exogenous hormones in drought-resistant lines. The *PmAP2/ERF14* gene was expressed when induced by SA and ABA in drought-sensitive lines, but only by ABA in drought-tolerant lines. The *PmAP2/ERF49* gene was expressed when induced by ABA, and *PmAP2/ERF96* gene was expressed when induced by MeJA. The expression of the *PmAP2/ERF109* gene in drought-sensitive lines significantly increased by ABA treatment at mild drought and then decreased, which was always lower than CK2. In drought-resistant lines, the expression of the *PmAP2/ERF109* gene induced by hormones was less than CK2 or not significantly different from CK2. After rehydration, the expression of *PmAP2/ERF* genes induced by different hormones was significantly higher than CK1 and CK2 after rehydration (Figures 7 and 8).

In the roots, there were significant differences in expression levels of *PmAP2/ERF* genes in the two lines under hormone treatments. Compared with CK2, the *PmAP2/ERF49* gene was significantly up-regulated by three hormones in drought-sensitive lines, whereas *PmAP2/ERF49* genes were expressed when induced by SA and MeJA in the drought-resistant line. The *PmAP2/ERF11*, *PmAP2/ERF14,* and *PmAP2/ERF80* genes were significantly expressed when induced by the three hormones in the drought-resistant lines, while the *PmAP2/ERF11* gene was significantly induced by MeJA and ABA in the drought-sensitive lines, and the *PmAP2/ERF14* and *PmAP2/ERF80* genes were not significantly induced by the hormones. Both were basically smaller than CK2. In drought-sensitive lines, the *PmAP2/ERF44* and *PmAP2/ERF109* genes were expressed when induced by ABA, and the *PmAP2/ERF46* gene was expressed when induced by SA; while in the drought-resistant lines, the *PmAP2/ERF44* and *PmAP2/ERF46* genes were induced by SA and MeJA, respectively, with the highest expression at mild drought, and *PmAP2/ERF109* did not express. The *PmAP2/ERF77* and *PmAP2/ERF96* genes were less or not significantly different from CK2 in both lines, while the *PmAP2/ERF44* and *PmAP2/ERF46* genes were significantly expressed when induced by SA and MeJA, respectively, and the *PmAP2/ERF109* gene was not significantly expressed in the drought-sensitive lines after rehydration (Figures 7 and 8).

4. Discussion

AP2/ERF is a major transcription factor in plants that is involved in plant growth, development, biotic, and abiotic stresses [47–49]. Based on transcriptome data identification, 124 *PmAP2/ERF* genes were identified in this study, which was comparable to 122 in *A. thaliana* [11], less than *Oryza sativa* L. (163) [17], *Zea mays* L. (292) [3], and more than *Taxus wallichiana* var. *chinensis* (49) [20], implying that the number of *AP2/ERF* gene family may not be directly related to species and genome size. The current analysis was more detailed than previous investigations, which revealed 88 *AP2/ERF* genes in *P. massoniana* [50]. There were eight AP2 subfamily genes, nine RAV subfamily genes, and 107 ERF subfamily genes in *PmAP2/ERFs*. The ERF subfamily was divided into two subfamilies, DREB and ERF [11], which included 62 and 45 genes, respectively, and it has been shown that DREB genes bound to DRE elements in the promoter regions of downstream genes to regulate the

expression of related genes, and ERF genes activated downstream gene expression by binding to GCC-box elements in the promoter regions of downstream target genes [51]. ERF subfamily genes were implicated in both plant hormone response and abiotic stress response [48,52]. The result showed that around half of the proteins were acidic, the majority were hydrophobic, and the protein structures were unstable. PmAP2/ERFs may perform various regulatory roles in different organelles based on their subcellular location in the nucleus or cytoplasm. Conservative motifs revealed that each cluster had its own distinct distribution pattern, and the motifs contained in each cluster branch and the same subfamily of genes were basically the same, implying that their roles may be similar, which was consistent with earlier research [51,53]. The current findings indicated that *AP2/ERF* genes were highly conserved and structurally and evolutionary comparable.

Protein interactions revealed that PmAP2/ERF102 (AT4G36920) and PmAP2/ERF109 (AT5G05410) played important roles in the overall interplay network. PmAP2/ERF10 may have a similar function to the AT4G36920 (*APETALA 2*) gene, which was discovered to be important in the development of the floral meristem, embryo, endosperm, and seed coat [54,55]. AT5G05410 (*DREB2A*) was associated with drought and high temperature [56,57], and *A. thaliana* plants with overexpressing *DREB2A* had significant drought tolerance [56], implying that *PmAP2/ERF109* may have similar function with AT5G05410 (*DREB2A*), and the *PmAP2/ERF109* gene was up-regulated under drought stress in our study, which indicated the result was accuracy.

Tissue-specific analysis of nine *PmAP2/ERF* genes revealed that *PmAP2/ERF* genes were expressed in all tissues, and there were differences in the expression levels of genes in different tissues of different lines, while the expression levels of some genes in the same tissues of different lines also differed significantly. For example, in drought-sensitive lines, *PmAP2/ERF77* gene expression was highest in roots and lowest in needle leaves, whereas in drought-tolerant lines, *PmAP2/ERF77* gene expression was the highest in needle leaves and the lowest in stems, and the tissue-specific expression of *PmAP2/ERF77* gene was completely different in the two lines, which could be related to the difference in drought resistance.

The results of this study showed that nine *PmAP2/ERF* genes constitutively expressed under drought stress, *PmAP2/ERF46*, *PmAP2/ERF49,* and *PmAP2/ERF96* genes were negatively regulated by drought stress, and the remaining six *PmAP2/ERF* genes were positively regulated to respond to drought stress. It was shown that the DREB subfamily of *TtAP2/ERF* genes, *TtAP2/ERF-176*, *TtAP2/ERF-206,* and *TtAP2/ERF-227*, were significantly up-regulated under drought stress [47]. Five DREB subfamily genes in this study (*PmAP2/ERF11*, *PmAP2/ERF44*, *PmAP2/ERF77*, *PmAP2/ERF80*, and *PmAP2/ERF109*) were up-regulated under drought stress and positively regulated drought stress. *GmDREB1* regulated the expression of downstream stress-related genes by interacting with *GmERF008* and *GmERF106* to form a heterodimer, which significantly improved drought tolerance and increases yield in transgenic soybean [58]. The *NtERF172* gene is directly bound to the promoter region of the downstream *NtCAT* gene and positively regulates *NtCAT* gene expression, resulting in higher catalase activity and less H_2O_2 accumulation in transgenic plants, indicating that the *NtERF172* gene significantly improved the drought tolerance of the plants [59]. The *PalERF2* gene directly regulates drought response genes *PalRD20* and *PalSAG113* to improve drought resistance in poplar. The above studies suggest that *AP2/ERF* genes can activate and regulate downstream gene expression in response to drought stress through intergenic interactions. Whether there are interactions between the nine PmAP2/ERF genes in this study and the regulatory mechanisms in response to drought needs to be further investigated.

Hormones played a crucial role in plant response to abiotic stresses such as drought. SA, ABA, and JA act as hormone signaling molecules in plant drought resistance [60–62]. *AP2/ERF* genes can improve plant resistance by participating in hormone signaling networks, e.g., the *ZmEREB160* gene increased survival and proline accumulation in transgenic *A. thaliana* under drought stress. The expression levels of the ABA signaling pathway and drought-related

genes such as *ABI2*, *ABI5*, and *DREB2A* were also found to be significantly up-regulated, indicating that the *ZmEREB160* gene was involved in the ABA signaling pathway to improve drought resistance [63]. In this study, *P. massoniana* was pretreated with exogenous hormones SA, ABA, and MeJA, and the expression pattern of the *PmAP2/ERF* genes in *P. massoniana* was found to be different in different tissues of different lines induced by hormones under drought stress, in which the *PmAP2/ERF96* gene significantly expressed when induced by MeJA only in the stem of the drought-resistant line at mild stress, and was not significantly affected by hormones in other tissues. *PmAP2/ERF* gene expression was induced by at least one exogenous hormone in response to drought stress in both lines and its expression was higher than that of CK2. It was hypothesized that *PmAP2/ERF* genes may be involved in the corresponding hormone signaling pathways.

The *MdDREB2* gene directly bonded to the DRE motif in the promoters of *MdNCED6* and *MdNCED9* genes, activating the transcription of ABA biosynthesis genes to promote ABA synthesis, and the *MdDREB2* gene interacted with the *MdCoL* gene to more effectively promote the expression of *MdNCED6/9* for ABA synthesis [64]. *A. thaliana* overexpressing the sweet potato *IbRAP2-12* gene showed up-regulated expression of genes related to ABA and JA signaling pathways under drought stress, while the *IbRAP2-12* gene improved *A. thaliana* tolerance during abiotic stress [23]. *PmAP2/ERF11*, *PmAP2/ERF44*, *PmAP2/ERF77,* and *PmAP2/ERF80* genes were significantly up-regulated when induced by three hormones, and it was hypothesized that these four *PmAP2/ERF* genes enhance drought tolerance in hormone signaling in *P. massoniana*, but the specific regulatory mechanisms need to be further investigated. The *PmAP2/ERF46* and *PmAP2/ERF49* genes were members of the RAV subfamily of the *AP2/ERF* gene family, and *PmAP2/ERF11* and *PmAP2/ERF109* were members of subfamily IV. In this study, we found similarities in expression patterns between the above two groups of genes under drought stress and hormone induction, further demonstrating that genes of the same subfamily may have similarities. The difference in expression of *PmAP2/ERF* genes induced by hormones in two different families was similar to the expression of *CsPRX* genes in two different *Camellia sinensis* varieties [65], which may be caused by the genetic background of two families with different drought tolerance levels. We also suggest that the high expression of some *PmAP2/ERF* genes in drought-resistant lineages increased the drought resistance of *P. massoniana*, and caused differences in drought resistance phenotypes.

5. Conclusions

In this study, we successfully identified 124 *PmAP2/ERF* genes and analyzed the physicochemical properties, phylogeny, and conserved motifs of the gene family members. The expression patterns of nine *PmAP2/ERF* genes were also analyzed under drought treatment, and it was found that all nine genes underwent expression changes in response to drought stress, but the expression patterns were different. The expression patterns showed that *PmAP2/ERF11*, *PmAP2/ERF14*, *PmAP2/ERF44*, *PmAP2/ERF77*, *PmAP2/ERF80,* and *PmAP2/ERF109* genes were up-regulated in response to drought stress and were positively regulated; *PmAP2/ERF96* gene was negatively regulated; *PmAP2/ERF46* and *PmAP2/ERF49* genes are up-regulated and down-regulated. The expression pattern of *PmAP2/ERF* genes induced by hormones differs in different tissues of different families, and the *PmAP2/ERF* genes responded to at least one hormone signal, suggesting that the *PmAP2/ERF* genes may positively or negatively regulate the response to hormones to improve drought tolerance in *P. massoniana*. Our study will provide a theoretical basis for the functional study of the *AP2/ERF* gene family and help to further investigate the molecular mechanism of *PmAP2/ERF* gene regulation in response to drought in *P. massoniana*. At the same time, this study also provides a reference for exploring the molecular mechanism of *AP2/ERF* genes in response to drought in other gymnosperms because of the close kinship between gymnosperms and the similarity in phylogeny and gene functions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13091430/s1, Table S1: qRT-PCR Primer sequences of the genes; Table S2: Physical and chemical analysis of AP2/ERF in Pinus massoniana.

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