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Genome-Wide Analysis of the MAPKKK Gene Family Under Abiotic Stresses in Moso Bamboo (*Phyllostachys edulis*)

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Abstract: Mitogen-activated protein kinase kinase kinases (MAPKKKs) are the upstream components of MAPK cascades and are involved in mediating stress responses and developmental processes. Although *MAPKKK* genes have been investigated in many plants, the identification and characterization of *MAPKKK*s in moso bamboo were still limited. Here, 134 *MAPKKK*s were identified as unevenly distributed on 23 chromosomes (except for chromosome 1) of moso bamboo and divided into three subfamilies by phylogenetic analysis. The gene structure and conserved motif of *PeMAPKKK*s were investigated. The expansions of *PeMAPKKK*s were driven by whole-genome duplication (WGD) or segmental duplication events. The duplicated gene pairs were under purifying selection based on the Ka/Ks ratios, suggesting they underwent functional conservation. Most *PeMAPKKK*s contained *cis*-elements related to development, hormones, and stress responses. Tissue expression patterns showed that *PeMAPKKK*s had multiple expression patterns. The qPCR analysis showed distinct expression patterns of *PeMAPKKK*s under drought, salt, and cold stress conditions. Taken together, this study provides a solid foundation for future functional characterizations of *MAPKKK*s and identifies candidate stress-responsive genes for further study in moso bamboo.

Keywords: evaluation; moso bamboo; MAPKKK; expression profile; abiotic stress

Citation: Huang, F.; Wang, J. Genome-Wide Analysis of the MAPKKK Gene Family Under Abiotic Stresses in Moso Bamboo (*Phyllostachys edulis*). *Forests* **2024**, *15*, 1883. https://doi.org/10.3390/f15111883

Academic Editor: Carol A. Loopstra

Received: 1 October 2024
Revised: 20 October 2024
Accepted: 24 October 2024
Published: 25 October 2024

1. Introduction

With global climatic change, plants face salinity, extreme temperature, and drought stimuli during their life cycle, which greatly impact plant growth and developmental processes [1]. Plants have evolved complex signaling networks over evolutionary time to receive and transmit various environmental stimuli and then adapt to adverse stresses. The MAPK cascade is an important signal transduction module transferring various extracellular signals into intracellular responses [2]. The typical cascade contains MAPKKK (MAP3K), MAPKK (MKK), and MAPK (MPK), which work by phosphorylation [3]. MAPKKK is activated by receptor kinases in response to environmental signals and then phosphorylates and activates a MAPKK. Subsequently, MAPKKs phosphorylate a specific MAPK [2]. Finally, the activated MAPKs lead to the phosphorylation of other signaling components and influence various downstream genes [4].

The cascade is involved in response to multiple biotic and abiotic stresses and plant growth and development [5,6]. *MAPKKK* genes have been identified and characterized in a wide range of plant species, including model organisms and crops [7–12]. *Arabidopsis* MEKK1-MKK4/5-MPK3/6 functions in plant innate immunity [13]. The MKK4/5-MPK3/6 module is activated by MAPKKKs, which act in cold stress response [14]. The MEKK1-MKK1-MPK4 module is identified to act in cold stress response [15]. MAPKKK17/18-MAPKK3-MPK1/2/7/14 is induced by ABA and enhances drought resistance in *Arabidopsis* [16]. In rice, MEK1/2/6/7b/8a-MPK1 is found to participate in various stress responses [17].

As the top of the MAPK pathway, MAPKKKs show great numbers and sequence diversity. MAPKKKs have longer C- or N-terminal regions, which may help to recruit MAPKs and MAPKKs [18]. MAPKKKs consist of three major groups: MEKK-like, ZIK-like, and Raf-like families [6]. Increasingly MAPKKKs have been studied, such as rice (75 members), *Arabidopsis* (80 members), tomato (89 members), maize (71 members), soybean (150 members), and *Fagopyrum tataricum* (56 members) [7–12]. *Arabidopsis MEKK1* participates in stress response during development [19]. *ANP1*, *ANP2*, and *ANP3* belong to the MAPKKK family and positively regulate growth and negatively regulate stress responses [20]. In *Arabidopsis*, *Enhanced Disease Resistance1* (*EDR1*) and *Constitutive Triple Response1* (*CTR1*), a Raf gene, inhibit ethylene signaling transduction and salicylic acid-induced defense responses, respectively [21,22]. The mutant of *raf5* has enhanced resistance to salt [23]. Overexpression of rice *MAPKKK6* has enhanced tolerance to drought [24]. As signaling molecules, *SlMAPKKK*s promote cell death networks related to plant immunity [25]. *GhRaf19* positively regulates cold resistance and negatively regulates salt and drought resistance [26]. Despite these advances in other species, MAPKKK research in moso bamboo (*Phyllostachys edulis*) remains largely unexplored.

Moso bamboo is a vital non-timber forest product in the Bambusoideae subfamily of the Poaceae family, which is widely distributed in China with rapid growth, strong adaptability, easy reproduction, and significant economic value [27]. Environmental stresses like cold, drought, and salinity significantly impact its yield and ecological or economic values. The release of genomic data of moso bamboo helps us to analyze *MAPKKK*s in moso bamboo [28]. While the functions of *MAPKKK* genes have been extensively studied in other plants, no comprehensive genome-wide analysis has been conducted in moso bamboo, leaving a gap in understanding their roles in abiotic stress responses in this species. Herein, the MAPKKK gene family in moso bamboo was analyzed, including its physicochemical property, gene structure, phylogenetic relationship, chromosomal distribution, *cis*-element, and conserved motif. The expression patterns of *PeMAPKKK*s were also detected under abiotic stress treatments and in different organs. This study aims to conduct a genome-wide analysis of the MAPKKK gene family in moso bamboo to elucidate their roles in abiotic stress responses. These results will provide a foundation for understanding the characterization of *PeMAPKKK*s and in-depth studying their functions in moso bamboo.

2. Materials and Methods

2.1. Plant Material and Treatments

The material used here was moso bamboo. The germinated seeds were planted in plastic pots with a medium of soil (loam) and vermiculite (1:1 by volume) in a growth chamber at 23/18 °C (16 h light/8 h dark). Two-month-old seedlings were selected for sampling. Five kinds of tissue materials, including mature and young leaves, stems, roots, and bamboo shoots, were collected from the seedlings. The seedlings were treated with 20% polyethylene glycol (PEG), 250 mM NaCl, and 100 µM ABA, respectively. To induce low temperature stress, the seedlings were changed to a new growth chamber under low temperature (4 °C). The seedlings without treatment were used for control plants. Leaves were collected at 0 h, 1 h, 2 h, 3 h, 4 h, and 8 h with three biological replicates separately and stored immediately at -80 °C.

2.2. Identification of PeMAPKKKs

The moso bamboo protein sequences from the Bamboo Genome Database (http://gigadb.org/dataset/view/id/100498, accessed on 29 August 2018) were downloaded to establish a local protein database. The protein sequences of AtMAPKKKs and OsMAPKKKs from the *Arabidopsis* information resource database and the rice genome annotation project were used as query sequences [8,12]. The local BLASTP search was used to identify *PeMAPKKK* genes with a threshold E value < 1×10^{-10}. These sequences were aligned to build an HMM profile using the HMMER 3.0 program to match the local database. The BLASTP results were compared with HMMER hits with the kinase domain (PF00069) to find putative moso bamboo MAPKKK sequences. The InterProScan (v36.0) (https://www.ebi.ac.uk/interpro/, accessed on 20 April 2024) and SMART (v9.0) (http://smart.

embl-heidelberg.de/, accessed on 20 April 2024) were used to further analyze the protein sequences.

2.3. Sequence Analysis

The lengths, molecular weights, and isoelectric points of *PeMAPKKK*s were employed using the ExPASy website (v3.0) (https://web.expasy.org, accessed on 15 June 2024) (Table S1). The Plant-mPLoc (v2.0) (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on 20 June 2024) was used for predicting the locations of PeMAPKKK proteins. The genomic and CDS sequences of *PeMAPKKK*s were used to analyze the structures and visualized by TBtools [29]. The protein sequences of PeMAPKKKs were studied using the MEME program (http:/meme.nbcr.net/meme/intro.html) with the default parameters. The conserved motif analysis was visualized by TBtools [29].

2.4. Phylogenetic Tree Construction

The protein sequences of AtMAPKKKs and OsMAPKKKs were used to contrast PeMAPKKK proteins. Multiple alignments of these proteins were performed by Clustal X following our previous report [30]. For the phylogenetic analysis, a neighbor-joining (NJ) tree was built by MEGA 7.0 software with 1000 bootstrap replicates.

2.5. Cis-Regulatory Element Analysis

The sequences (2000 bp sequence upstream of the translational start codon) of promoters were submitted to PlantCARE (v1.0) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 25 June 2024) to analyze *cis*-regulatory elements in *PeMAPKKK* promoter regions. The *cis*-regulatory elements on *PeMAPKKK* promoters were summarized and counted, and then visualized using the TBtools software (v2.127) (Table S2) [29].

2.6. Chromosome Localization and Gene Duplication

The location information of *PeMAPKKK*s was collected from the genome database [28]. The distribution of *PeMAPKKK*s was drawn by TBtools software [29]. Gene duplication events of *PeMAPKKK*s were analyzed by MCScanX and Advanced Circos in TBtools with the default parameters.

2.7. Calculation of Ka/Ks Ratios

The synonymous (Ks), non-synonymous (Ka) substitution rate, and Ka/Ks ratio of the duplicated genes were determined by utilizing a simple Ka/Ks calculator in Tbtools [29]. A Ka/Ks ratio <1 indicates the purifying selection. The time of evolutionary divergence of the MAPKKK gene family in moso bamboo was calculated by the formulae $T = Ks/2\lambda$, and $\lambda = 6.5 \times 10^{-9}$ bases/generation in bamboo. The selection pressure of the gene pairs was detected using the Ka/Ks ratio [31].

2.8. qPCR Analysis

RNA isolation, cDNA synthesis, and qPCR reaction were carried out following our protocol [30]. The reaction was performed with the following conditions: 95 °C, 60 s; 40 cycles for 95 °C, 10 s; and 60 °C, 30 s. The melting curve was implemented with the default setting. The internal reference gene was *PeNTB* [32]. Primers used in qPCR are listed in Table S3. The expression levels were calculated by $2^{-\Delta\Delta CT}$ [33]. The data were analyzed using three independent biological replicates.

2.9. Statistical Analysis

The data were represented as means ± standard deviation. The statistical analysis was tested using analysis of variance (ANOVA). The least significant difference (LSD) test was set as $p < 0.01$ to compare the differences between treated and control samples.

3. Results

3.1. Identification of MAPKKKs in Moso Bamboo

One hundred thirty-four dependable *MAPKKK*s were identified as *PeMAPKKK*s based on homology comparison and domain analysis. *PeMAPKKK*s were divided into three subgroups and then named *PeMEKK1*–*PeMEKK40*, *PeZIK1*–*PeZIK15*, and *PeRAF1*–*PeRAF79*, depending on their grouping situation and blast scores. The information of *PeMAPKKK*s are shown in Table S1, including their CDS length, protein size, chromosome position, isoelectric point, molecular weight, and subcellular localization. The CDS lengths of *PeMAPKKK*s ranged from 780 (*PeMEKK2*) to 5286 bp (*PeRAF3*), with protein sizes and molecular weights within the range of 259 to 1761 amino acids and 27.06–193.28 kDa, respectively. The isoelectric points of all identified P*eMAPKKK*s had a range of 4.43 to 9.67. The instability index was from 25.93 to 69.16. The aliphatic index spanned 62.47 to 104.62. The grand average of hydropathicity varied from -0.68 to -0.23. The predicted localization results suggested that all PeMAPKKKs are located in the nucleus.

3.2. Phylogenetic Analysis of MAPKKKs

To examine the evolutionary relationships among the MAPKKKs in moso bamboo, rice, and *Arabidopsis*, a phylogenetic tree was built by the neighbor-joining (NJ) method (Figure 1). The analysis showed that PeMAPKKKs were classified into three clades: MEKK, ZIK, and Raf, which was consistent with the AtMAPKKKs and OsMAPKKKs [8,12]. The quantitative distribution of each clade of MAPKKKs is shown in Table S4. Among these subfamilies, the Raf subfamily has the most genes, containing 79 PeMAPKKKs, 48 AtMAPKKKs, and 43 OsMAPKKKs. There were 40 PeMEKKs, 21 AtMEKKs, and 22 OsMAPKKKs grouped in the MEKK subfamily, while 15 PeZIKs, 11 AtZIKs, and 10 OsMAPKKKs grouped in the ZIK subfamily. The gene number of moso bamboo was much greater than that of rice and Arabidopsis, suggesting that the MAPKKK gene family may undergo significant tandem duplication in evolution, leading to tetraploid moso bamboo possessing more MAPKKK genes. Some orthologs of *MAPKKK*s between moso bamboo and rice have obtained, implying that some ancestral *MAPKKK*s existed before the divergence of moso bamboo and rice.

3.3. Conserved Motifs and Gene Structure Analysis

To elucidate the structural diversity of PeMAPKKKs, the phylogenetic tree of PeMAPKKKs was constructed. PeMAPKKKs were categorized into three subfamilies (Figure 2). The differences in the PeMAPKKK proteins were determined by MEME. Ten distinct motifs were found and named motif 1 to 10. The motif numbers of PeMAPKKKs were similar, mainly ranging from 8 to 10, except PeMEKK39, PeRAF62, and PeMEKK20 with 6, 7, and 7 motifs. Motifs 1, 2, 4, and 7 were the most conserved motifs in all PeMAPKKKs, which formed the kinase domain (PF00069). The PeMAPKKKs contained similar motif distribution in the same subfamilies, indicating functional similarity. However, the motif distributions in MEKKs differed with those in the other groups, half of which was distributed at the N-end and the remaining was distributed at the C-end. The motif distribution differences may lead to the functional diversification of PeMAPKKKs.

The intron–exon organizations of *PeMAPKKK*s were analyzed (Figure S1). The intron numbers were highly variable in *PeMAPKKK*s, which varied from 0 to 23. A majority of the *PeMAPKKK*s (83.33%) had more than two introns. Only *PeRAF33* had no intron in the Raf subfamily, whereas *PeRAF79* had 23 introns. In the MEKK group, *PeMEKK24*, *PeMEKK25*, and *PeMEKK26* contained no intron, while *PeMEKK34* and *PeMEKK36* had 23 introns. Members of the ZIK group contained two to nine introns. The majority of ZIK genes contained 7 introns, MEKK genes mostly ranged from 2 to 12 introns, and the Raf genes mostly had 2 to 13 introns with the highest level of variation, which was consistent with previous studies in *OsMAPKKK*s and *AtMAPKKK*s [8,12]. The ZIK subfamily had less sophisticated structure than the other two subfamilies, suggesting that ZIK genes had preserved a relatively constant structure during the evolution of the moso bamboo genome. Genes showed similar gene structures within the same phylogenetic cluster but structurally differed between different subgroups and even in the same groups, suggesting that duplication events of *MAPKKK*s may have occurred in ancient times and offspring genes may have different roles.

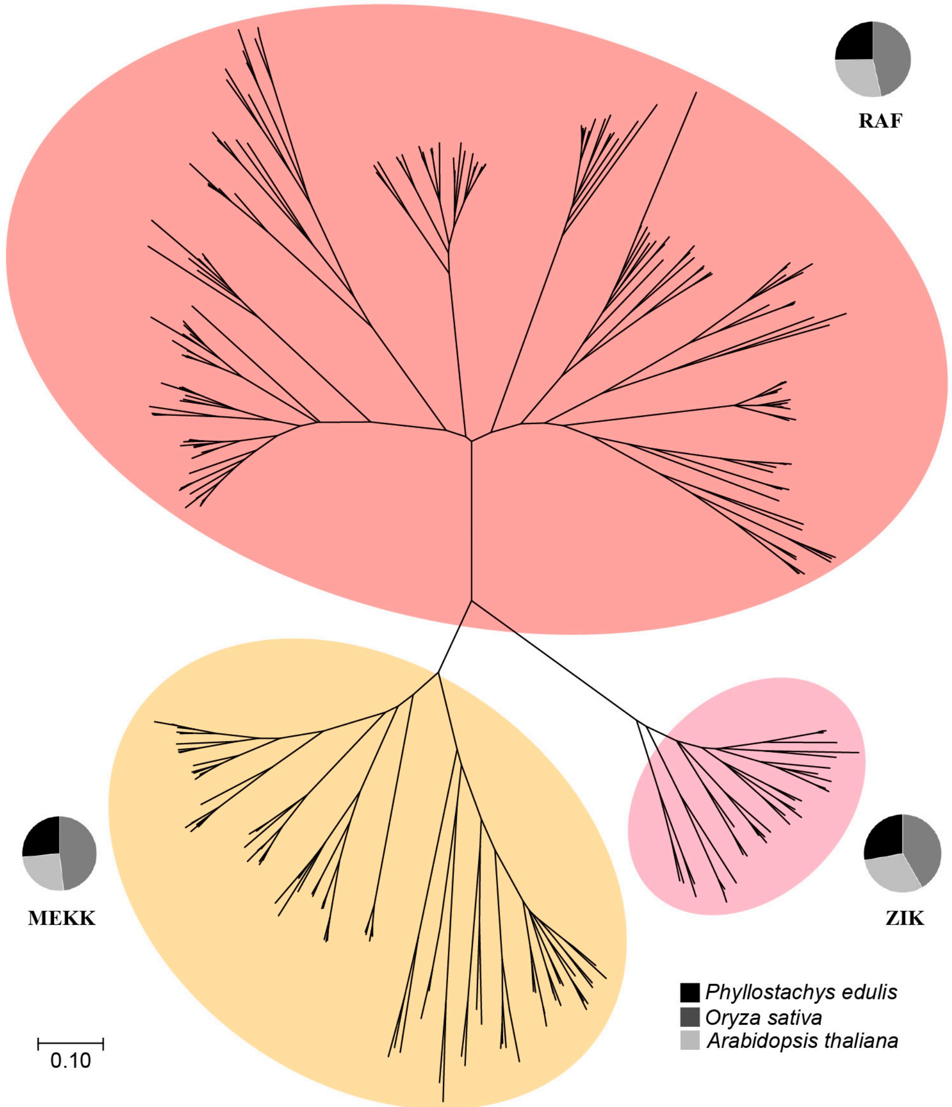

Figure 1. Phylogenetic analysis of OsMAPKKKs, AtMAPKKKs, and PeMAPKKKs. The tree was built by the neighbor-joining (NJ) method. The branched lines of the subtrees were colored to indicate different subgroups. The details of this tree are available in Supplementary Table S4. Member size for each clade is illustrated in the pie chart.

3.4. Chromosome Location and Gene Duplication of PeMAPKKKs

One hundred thirty-four *PeMAPKKK*s were mapped on 23 chromosomes of moso bamboo with different densities, except for chromosome 1 (Figure 3). Chromosome 17 had the largest number of *PeMAPKKK*s. Chromosome 19 only had one gene. In addition, the distribution of ZIK, MEKK, and Raf genes was not random in moso bamboo. 15 ZIK genes were found on 10 chromosomes, whereas 40 MEKK genes were distributed across the 17 chromosomes. Raf genes were present on 21 chromosomes. Interestingly, members of the Raf, ZIK, and MEKK subfamilies all map to chromosomes 3, 10, 13, and 17. The information on chromosome distributions is given in Table S1. The expansion mechanism of *PeMAPKKK* genes was used to investigate gene duplication events with the MCScanX program (Table S5). Among the *PeMAPKKK*s, a total of 126 genes underwent WGD or segmental duplication; 5 underwent dispersed duplications (*PeZIK5*, *PeRAF1*, *PeRAF23*, *PeRAF66*, and *PeRAF78*); 2 underwent proximal duplication (*PeMEKK24* and *PeMEKK30*); and one underwent tandem duplication (*PeRAF52*). Thus, WGD, or segmental duplication, was the main driving force for *PeMAPKKK* genes.

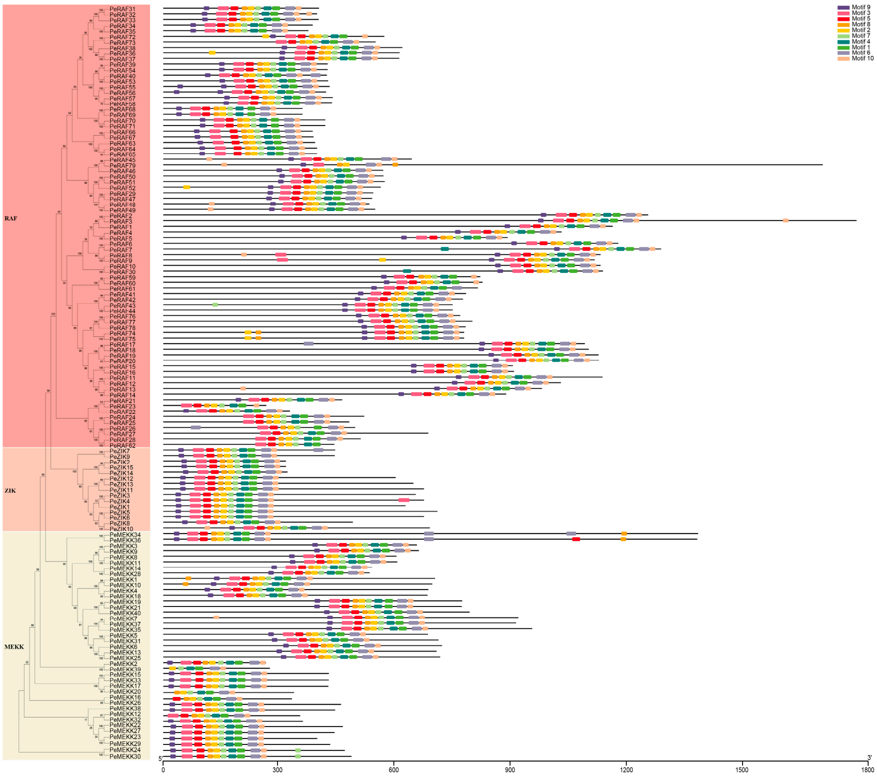

Figure 2. Phylogenetic relationships (the left) and motif distributions (the right) of PeMAPKKKs. Scale bar: 300 amino acids.

To further estimate the selection constraints among the *PeMAPKKK*s, the Ka, Ks, and Ka/Ks ratios were calculated for 100 pairs of duplicated genes and are listed in Table S6. The Ka/Ks values were substantially lower than 1, ranging from 0.071 to 0.663, suggesting that these genes have experienced strong purifying selection. The Ks values of *PeMAPKKK* gene pairs varied from 0.089 to 1.522, showing that a large-scale gene duplication event occurred 6.85–117.04 million years ago (MYA). The divergence time for most *PeMAPKKK* gene pairs (63 of 100) was about 12.09 to 117.04 MYA, differed with the whole-genome duplication 7–12 MYA, suggesting that *PeMAPKKK* gene expansions had less relative to the WGD event. Moreover, Ks values of five gene pairs (like *PeMEKK7* and *PeMEKK37*) were less than 0.1, suggesting that the generation of these homologous gene pairs came from a recent segmental duplication/WGD event.

3.5. Analysis of PeMAPKKK Promoters

To further understand the potential functions of *PeMAPKKK*s, the cis-acting elements at its promoters were analyzed. In addition to basic elements, cis-elements related to environmental stress, hormones, light, and development were identified in *PeMAPKKK* promoters (Figure 4 and Table S2). The varieties and locations of *cis*-regulatory elements on the *PeMAPKKK* promoters were manifold, implying that *PeMAPKKK*s functioned in multiple metabolic processes. The most abundant were development-related elements, represented by as many as 24 types, including meristem expression (CAT-box), meristem-specific activation (CCGTCC box), seed-specific regulation (RY elements), cell cycle regulation (MSA-like), and endosperm expression (GCN4 motif) elements. Numerous environmental stress-related elements were also identified, including salicylic acid stress response elements (TCA-element), drought stress response elements (MBS and MYB), low temperature stress response elements (LTR), and anaerobic responsive elements (ARE). MYB was the most abundant stress-related element, indicating that *PeMAPKKK*s may play roles in drought stress response. *PeRAF9*, *PeMEKK8*, and *PeRAF5* contained the largest number of stress-related elements, suggesting that they may act in stress response. Furthermore, of all hormone-related element types, ABA-responsive element (ABRE) was the most frequent, which was found in 88.8% of *PeMAPKKK* promoters, and the total number reached 503, suggesting that *PeMAPKKK*s may exert their biological functions mainly through the ABA pathways. Interestingly, except for basic and light responsive elements, *PeRAF24* contained no elements. Each *PeMAPKKK* promoter contained different types and numbers of cis-acting elements, indicating that they may have different functions.

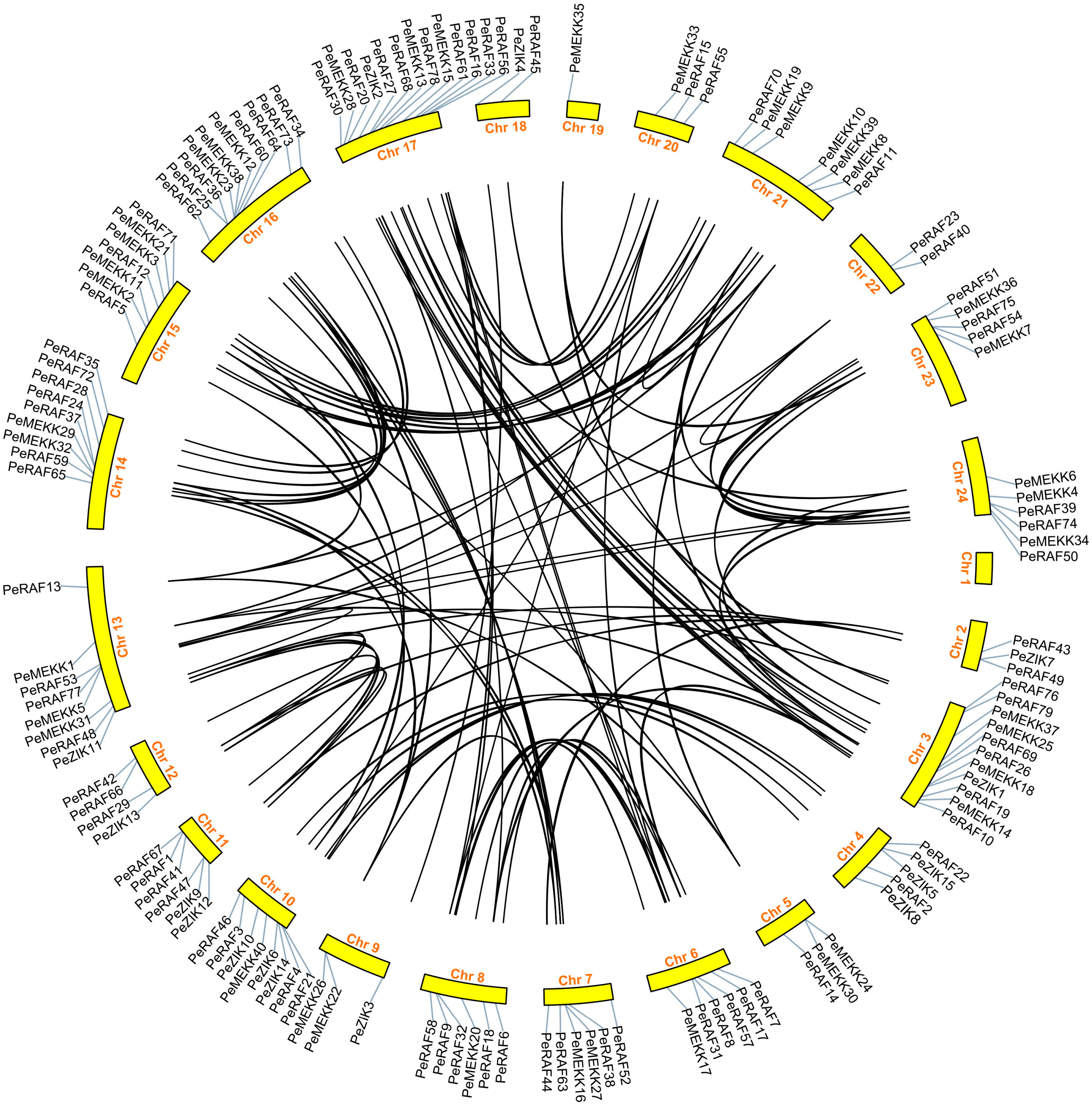

Figure 3. Chromosome distribution and syntenic relationships of *PeMAPKKK*s. The *PeMAPKKK* gene pairs are connected by black lines.

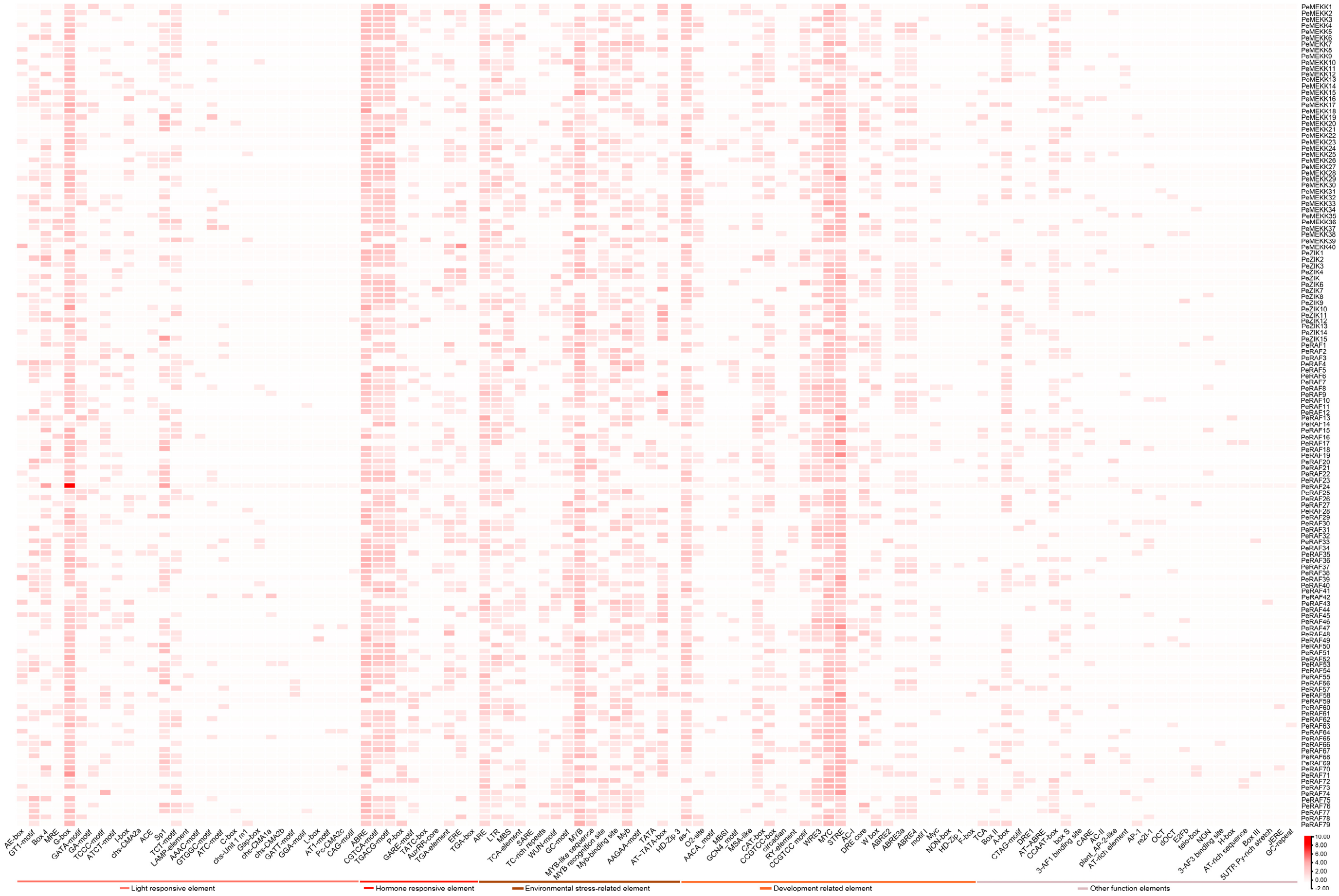

Figure 4. The cis-acting elements of *PeMAPKKK* promoters.

3.6. Tissue-Specific Expression Patterns of PeMAPKKKs

The *cis*-acting element analysis showed that *PeMAPKKK*s may act in plant development and respond to multiple stresses. To investigate the roles of *PeMAPKKK*s in moso bamboo development, the levels of *PeMAPKKK*s in MEKK, ZIK, and Raf groups in five tissues (roots, stems, mature leaves, young leaves, and bamboo shoots) were assessed by qPCR (Figure 5). The levels of *PeMAPKKK*s varied significantly in different tissues. Most *PeMAPKKK*s were expressed in at least one organ except *PeMEKK24*, *PeMEKK30*, and *PeRAF28*, which were not detected in any tissues, suggesting that they may be pseudogenes or only expressed at specific developmental stages or under special conditions. The majority of MEKK and Raf genes were highly expressed in leaves and roots, while most ZIK genes were highly expressed in bamboo shoots. It was noteworthy that *PeRAF50* and *PeRAF52* were predominantly expressed in roots, while *PeZIK13* showed preferential expression in stems. Similarly, *PeMEKK16*, *PeMEKK23*, *PeMEKK26*, *PeMEKK27*, and *PeMEKK29* had relatively high expression levels in stems and roots, and *PeMEKK1* showed preferential expression in bamboo shoots. In addition, *PeMEKK5*, *PeMEKK15*, *PeMEKK33*, *PeZIK5*, *PeRAF21*, *PeRAF22*, and *PeRAF44* had higher expression in both young and mature leaves. Thus, these *PeMAPKKK*s may play various roles during moso bamboo growth and development. Furthermore, most *PeMAPKKK* duplicated gene pairs showed similar expression profiles, and other paralogs (like *PeRAF34* and *PeRAF35*, *PeMEKK14*, and *PeMEKK28*) were not the case. These results suggested that although the protein sequences of the duplicated genes had highly similarity, they may have different functions or be involved in different developmental processes.

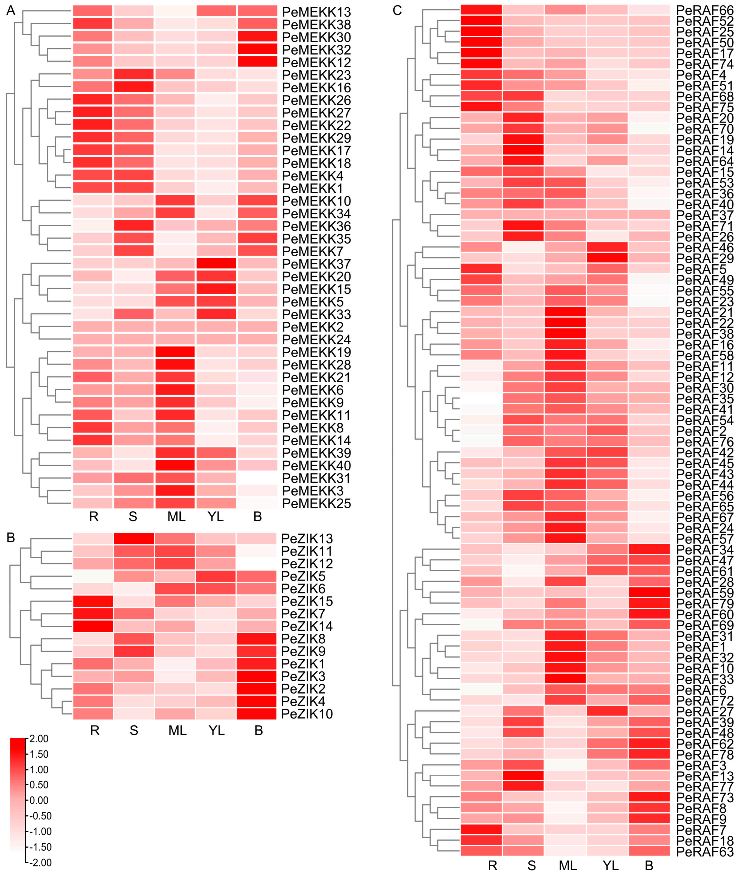

Figure 5. Heatmaps of *PeMAPKKK* expression in roots, stems, mature leaves, young leaves, and bamboo shoots. The levels of *PeMAPKKK*s in MEKK, ZIK, and Raf groups (**A**–**C**) are shown with different colors.

3.7. Expression Profiles of PeMAPKKK Genes Under Abiotic Stresses

To explore the response of *PeMAPKKK*s to abiotic stress, their expression patterns were verified by qPCR analysis (Figure 6). *PeMAPKKK*s responded differently to these abiotic stresses, and some *PeMAPKKK* expression were significantly changed. After salt stress, 28 *PeMAPKKK*s were induced (15 *PeMEKK*s, 3 *PeZIK*s, and 10 *PeRAF*s), whereas 15 *PeMAPKKK*s were repressed (6 *PeMEKK*s, 3 *PeZIK*s, and 6 *PeRAF*s). In the drought stress, the levels of 18 *PeMAPKKK*s were significantly up-regulated (11 *PeMEKK*s, 1 *PeZIK*, and 6 *PeRAF*s) and 16 *PeMAPKKK*s were down-regulated (4 *PeMEKK*s, 4 *PeZIK*s, and 6 *PeRAF*s). Under cold stress, only 14 *PeMAPKKK*s (9 *PeMEKK*s, 3 *PeZIK*s, and 2 *PeRAF*s) were upregulated. The expression peaked at 8 h for *PeMEKK*s and *PeRAF*s except *PeRAF27*, with a trend of up-regulation and then down-regulation followed by continuously increasing, with *PeRAF27* peaking at 4 h. *PeZIK* expression peaked earlier than the other genes, at 1 h. Interestingly, most *PeMAPKKK* expression peaked before 8 h under salt and drought treatment, indicating that the response to cold was not as rapid as to drought and salt stresses for these *PeMAPKKK*s. Some *PeMAPKKK*s exhibited opposite expression patterns in response to salt, cold, and drought treatments. *PeZIK11*, *PeZIK12*, and *PeZIK13* were induced under cold treatment but reduced under salt and drought treatment. ABA acts in plant growth and stress signal response. Therefore, the expression profiles of *PeMAPKKK*s under ABA treatment were also detected. 19 *PeMAPKKK*s (6 *PeMEKK*s, 4 *PeZIK*s, and 9 *PeRAF*s) were up-regulated, and 14 *PeMAPKKK*s (2 *PeMEKK*s, 5 *PeZIK*s, and 7 *PeRAF*s) were down-regulated by this treatment. Moreover, *PeMEKK14*, *PeMEKK23*, *PeMEKK29*, *PeMEKK31*, *PeMEKK32*, and *PeRAF27* were up-regulated under salt, drought, and cold treatment, of which *PeMEKK14*, *PeMEKK23*, *PeMEKK29*, and *PeMEKK32* also responded to ABA treatment. The above results showed that *PeMAPKKK*s could respond to multiple abiotic stresses, of which *PeMEKK14*, *PeMEKK23*, *PeMEKK29*, and *PeMEKK32* may play more important roles against different abiotic stresses.

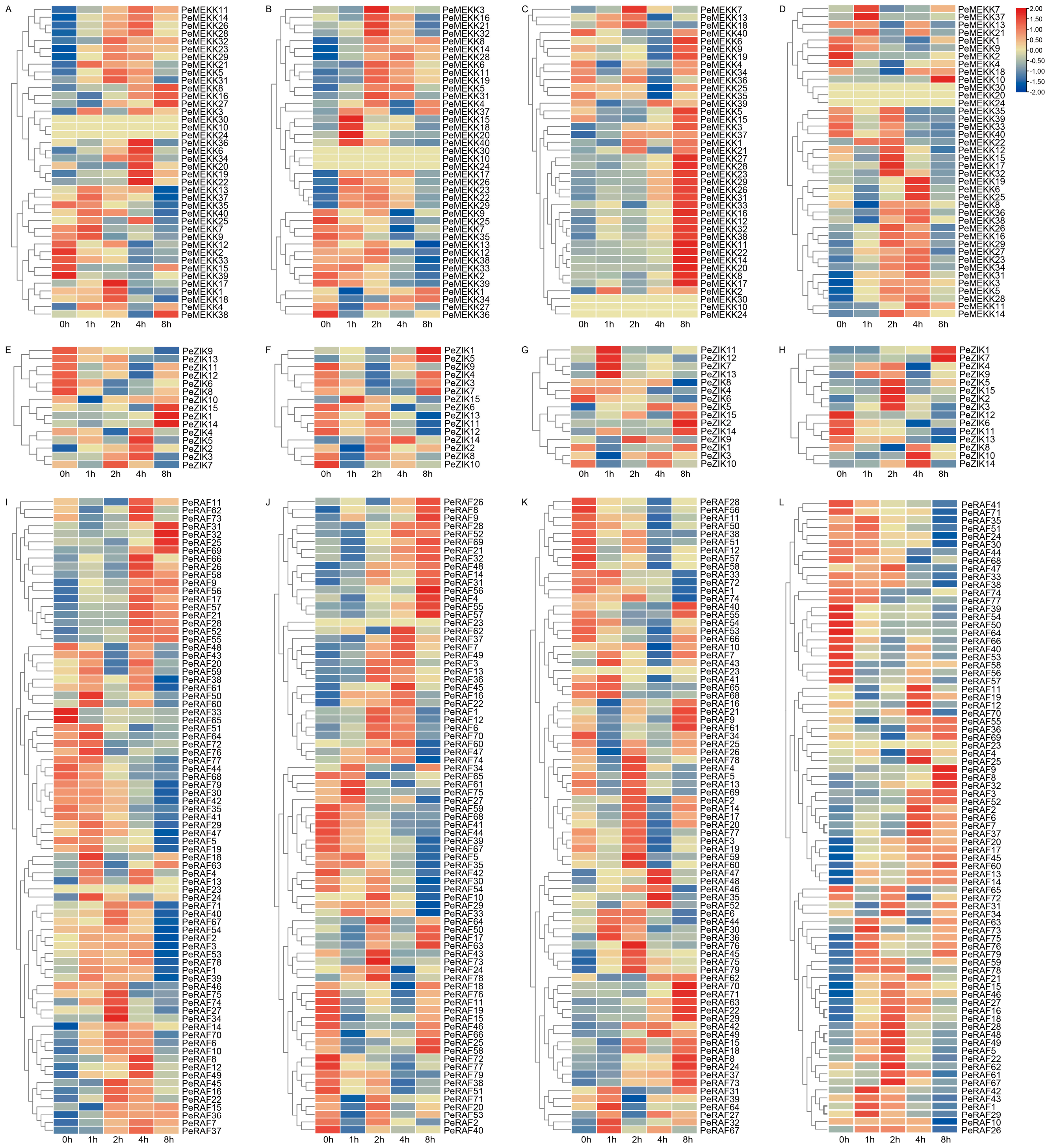

Figure 6. Heatmaps of *PeMAPKKK* expression in MEKK, ZIK, and Raf groups under NaCl (**A**,**E**,**I**), PEG (**B**,**F**,**J**), cold (**C**,**G**,**K**) and ABA (**D**,**H**,**L**) treatment. The levels of *PeMAPKKK*s are shown with different colors.

4. Discussion

Adverse environmental conditions can significantly inhibit plant growth and development, prompting the evolution of complex stress resistance mechanisms mediated by specific genes. MAPK signal transduction modules regulate various biological functions, such as development and abiotic stress response [6]. MAPK cascade is the oldest conserved signal transduction pathway, and MAPKKKs are the first component [18]. The MEKK1-MKK1-MPK4 module is identified to act in cold stress response [15]. MAPKKK17/18-MAPKK3-MPK1/2/7/14 is induced by ABA and enhances drought resistance in *Arabidopsis* [16]. *MAPKKK* genes of many plant species have been excavated, like rice, *Arabidopsis*, maize, soybean, tomato, and *Fagopyrum tataricum* [7–12]. However, no data set of *MAPKKK*s is available for moso bamboo. Therefore, a bioinformatic analysis of moso bamboo *MAPKKK*s and their expression profiles will help us to further study *PeMAPKKK*s.

In this study, the evolution, conserved motifs, and expression of 134 *PeMAPKKK*s were analyzed. The lengths of PeMAPKKKs ranged from 259 to 1761 amino acids, which may be due to their sequence variability and complexity. The instability index of most *PeMAPKKK*s was more than 40, indicating that the structures of *PeMAPKKK*s were unstable. The number of *PeMAPKKK*s was more than that of *Arabidopsis* (80 members), rice (75 members), tomato (89 members), maize (71 members), and *Fagopyrum tataricum* (56 members), and similar to that of soybean (150 members) [7–12]. The genome size of the above plants is 2021 Mb for moso bamboo, 164 Mb for Arabidopsis, 441 Mb for rice, 950 Mb for tomato, 2300 Mb for maize, 490 Mb for *Fagopyrum tataricum*, and 1013 Mb for soybean [27,34–39]. This phenomenon suggested that the number of *MAPKKK* genes had no direct correlation with the plant genome size, and *PeMAPKKK*s may have undergone large-scale duplication events. The presence of more *MAPKKK*s in the moso bamboo genome may imply the great need for *MAPKKK*s acting in the complicated signal transduction in this perennial herbaceous plant.

Chromosome distribution analysis showed that *PeMAPKKK*s were unevenly distributed on the twenty-three chromosomes (except for chromosome 1) (Figure 3 and Table S1). PeMAPKKKs were classified into three subgroups, and the Raf subfamily was the largest (Figure 1), consistent with *Arabidopsis* and rice MAPKKKs [8,12], demonstrating that MAPKKKs were highly conserved among species and genes in the Raf group underwent a more rapid expansion in evolution. Phylogenetic analysis showed that PeMAPKKKs had close affinity to rice MAPKKKs and a relatively low distant affinity to Arabidopsis MAPKKKs, suggesting that MAPKKKs may be conserved in monocotyledonous plants. Compared with *Arabidopsis* and rice, the number of the three subgroups expanded nearly twice in moso bamboo (Table S4). *MAPKKK*s in different groups with different gene numbers may be under different evolutionary constraints, and these expansions could relate to environmental adaptation.

All PeMAPKKKs contained motifs 1, 2, 4, and 7 (Figure 2). Most PeMAPKKKs displayed high similarity motif patterns in the same group, indicating a potential functional similarity between these PeMAPKKKs. The motifs of MEKK genes were highly divergent. The intron numbers in *PeMAPKKK*s ranged from 0 to 23 (Figure S1). The variable intron-exon structures of *PeMAPKKK*s indicated that the moso bamboo genome was significantly variable during the long process of evolution, which was also observed in cassava (from 0 to 16), *Fagopyrum tataricum* (from 0 to 16), and maize (from 1 to 17) [7,11,40]. The intron numbers in MEKK genes and Raf genes ranged from 0 to 23, and that in the ZIK group was 1 to 8 introns, suggesting that structure divergence and functional differentiation of MEKK and Raf group genes were more complicated than that of ZIK group genes. Compared with intron loss, segmental duplication may lead to a slower rate of intron gain [41]. The Raf subfamily had significantly more exons than the other two subfamilies, implying that the Raf subfamily was the most conserved and may contain the original genes, the same as *MAPKKK*s in cassava [40]. The conserved motifs and gene structures of *PeMAPKKK*s in the same clade supported the classification.

Gene duplication acts in gene expansion [42]. *PeMAPKKK*s primarily originated from WGD or segmental duplication and strong purifying selection (Table S5), implying that WGD or segmental duplication may be the principal reason for the expansion of *PeMAPKKK*s, similar to *MAPKKK*s in pear [43]. The Ka/Ks ratio for *PeMAPKKK* gene pairs was less than 1 (Table S6), implicating mainly purifying selection, consistent with

*MAPKKK*s in pear. These results indicated that tetraploidization was the force for the evolution of *PeMAPKKK*s.

Numerous reports showed that MAPK signal transduction modules act in development and abiotic stress response [6]. To predict *PeMAPKKK* functions, *cis*-elements in *PeMAPKKK* promoters were analyzed. Many development-related and stress-related *cis*-elements were identified in *PeMAPKKK* promoters (Figure 4 and Table S2), indicating that *PeMAPKKK*s may act in development and abiotic stresses. ABA is a major hormone response to stress [44]. ABRE was the most abundant in *PeMAPKKK* promoters, suggesting a direct regulatory mechanism by ABA. Interestingly, *PeRAF24* only contained basic and light responsive elements, suggesting that it may not respond to any abiotic stress, which was consistent with its expression results (Figure 6). The variety and quantity of *cis*-elements in *PeMAPKKK* promoters were different, responsible for functional differentiation in *PeMAPKKK*s.

Thus, the expression profiles of *PeMAPKKK*s were further analyzed. The spatio-temporal expression patterns of *PeMAPKKK*s demonstrated a distinct tissue specificity (Figure 5). The expression of most *PeMAPKKK*s were highest in leaves and roots, which may be related to stress resistance. *PeRAF50* and *PeRAF52* were only expressed in roots. Likewise, *PeZIK13* was only expressed in stems. Moreover, *PeMEKK16*, *PeMEKK23*, *PeMEKK26*, *PeMEKK27*, and *PeMEKK29* were highly expressed in stems and roots. These differential expression patterns indicated that *PeMAPKKK*s acted in growth and development in moso bamboo.

Biochemical and genetic studies showed that some *MAPKKK*s are positively resistant to abiotic stresses in *Arabidopsis*, rice, soybean, and tobacco. The MEKK1-MAPKK2-MPK4 module enhances cold tolerance in *Arabidopsis* [14]. *CRT1*, a MAPKKK gene, participates in regulating freezing tolerance [45]. *NtNPK1* enhances tolerance to high salinity and freezing through mediating the oxidative stress response [46]. *OsDSM1* enhances dehydration tolerance [24]. In this study, 28, 18, and 14 *PeMAPKKK*s were strongly induced under salt, drought, and cold treatments, respectively (Figure 6), implying their functional divergence, consistent with the difference in *cis*-acting elements of their promoters. Most upregulated *PeMAPKKK*s were Raf and MEKK genes, suggesting that Raf and MEKK genes in moso bamboo acted in response to abiotic stresses. Most differentially expressed *PeMAPKKK*s were also changed under ABA treatment. MAPK cascade is a crucial component of the ABA signaling transduction network. The MAPKKK17/18-MAPKK3-MPK1/2/7/14 is controlled by the ABA core signaling pathway at the transcriptional and post-translational levels [47]. 19 *PeMAPKKK*s were up-regulated under ABA treatments, suggesting that these genes may act in the ABA signaling pathway. The transcripts of *PeMEKK14*, *PeMEKK23*, *PeMEKK29*, *PeMEKK31*, *PeMEKK32*, and *PeRAF27* showed an increasing pattern under salt, drought, and cold treatments. The most obvious was *PeMEKK14*, *PeMEKK23*, *PeMEKK29*, and *PeMEKK32*, which were also significantly and continuously increase after ABA treatment, indicating that they may act in various stresses and mediated by ABA signaling. *OsMAPKKK63*, the orthologous gene of *PeMEKK32*, is induced by drought, cold, and high salinity. *OsMAPKKK63* overexpressing plants showed that it can enhance salt tolerance [48]. *PeMEKK32* was highly expressed in roots and stems and significantly induced by all treatments (Figure 6A–D), suggesting that *PeMEKK32* may be the key gene in response to abiotic stresses. These results will help us to select valuable candidate genes and further investigate stress tolerance in moso bamboo. Future studies may analyze the function of the identified candidate genes by overexpression assays.

5. Conclusions

This study represents a pioneering exploration of the PeMAPKKK gene family in moso bamboo, revealing critical insights into its role in abiotic stress responses. One hundred thirty-four *PeMAPKKK*s were identified and systematically investigated for the first time. The *PeMAPKKK*s were classified into three groups, with highly conserved motif compositions and exon/intron structures. The PeMAPKKK gene family may be expanded by chromosome segment duplication. The promoter and expression analysis revealed the involvement of *PeMAPKKK*s in development and responses to abiotic stress. Four *PeMAPKKK*s were identified as important candidate resistance genes. This work will help us to understand *PeMAPKKK*-mediated abiotic stress responses. Future studies should focus on functional validations of the identified candidate genes and their roles in enhancing moso bamboo's resilience to environmental stressors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15111883/s1, Figure S1: Phylogenetic relationships (the left) and exon–intron distribution (the right) of PeMAPKKKs. Scale bar: 10,000 bp, Table S1: Characteristics of *PeMAPKKK*s, Table S2: The information of cis-elements in *PeMAPKKK* promoters, Table S3: Primers used in the paper, Table S4: Quantitative distribution of *PeMAPKKK*s, *OsMAPKKK*s, and *AtMAPKKK*s, Table S5: The mode of gene duplication in *PeMAPKKK*s, Table S6: The KaKs ratios and estimated divergence times for duplicate gene pairs of *PeMAPKKK*s.

Author Contributions: Writing—original draft, F.H.; visualization, F.H.; methodology, F.H.; formal analysis, F.H.; funding acquisition, F.H.; conceptualization, F.H.; writing—review and editing, F.H.; supervision, F.H.; software, J.W.; data curation, J.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grants from the Key research projects of Yibin, Research and Integrated Demonstration of Key Technologies for Smart Bamboo Industry (YBZD2024-1) and the National Natural Science Foundation of China (32102410).

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Conflicts of Interest: No conflicts of interest exist in the submission of this manuscript.

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