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ORIGINAL PAPER

Genome-wide identification of *MPK* and *MKK* gene families and their responses to phytohormone treatment and abiotic stress in foxtail millet

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Abstract

The mitogen-activated protein kinase (MAPK) cascade is one of the most important pathways in eukaryotic signaling networks, and it plays a crucial role in plant growth and development, hormonal responses, and responses to various biotic and abiotic stress. Foxtail millet (*Setaria italica*) is a minor cereal with excellent nutritional value and fine adaptability to abiotic stress associated with climate change, and it has also emerged as a C_4 model plant. MAPK cascade genes from several model plant species have been analyzed, but not from foxtail millet. Here, 16 *SiMPKs* and 11 *SiMKKs* were systematically identified and analyzed in foxtail millet. Phylogenetic relationships, conserved protein motifs, and gene structure indicated clearly that both *MPK*s and *MKKs* were divided into four subgroups. RNA-seq data analysis showed that expression profiles of some *SiMPK* and *SiMKK* genes varied in different tissues or developmental stages. Furthermore, the expression levels of *SiMPK* and *SiMKK* genes under abiotic stresses as well as exogenously applied phytohormone were also investigated. The identified abiotic stress and phytohormone responsive genes suggested that the *SiMPKs*, *SiMKKs*, and MKK-MPK interactomes play key roles in abiotic stress and hormone signaling pathways and networks. Our study provides detailed information of *MKK* and *MPK* genes in foxtail millet and lays the foundation to explore their functional characterization for stress-tolerance.

Keywords Genome-wide analysis · *MAPK* · *MAPKK* · Foxtail millet · Phytohormone treatment · Abiotic stress

Introduction

The mitogen-activated protein kinase (MAPK) cascades consist of the three sequential protein kinases MAPKK kinase (MAPKKKs/MEKKs), MAPK kinase (MKKs), and MAPKs, which are highly conserved signal transduction pathways in all eukaryotes (Meng and Zhang 2013; Jonak et al. 2002). MEKKs are located in the upper reaches of the MAPK cascade pathway, which can be phosphorylated and activated by a series of upstream signals or receptor proteins. The activated MEKKs then transmit the signal by phosphorylating the serine/threonine residues in the S/T-xxxxx-S/T motif of the downstream MAPKKs. MAPKKs, which are located at the central position of the MAPK cascade signaling system, are a class of dual-specific protein kinases that activate MAPKs through phosphorylation of the conserved tyrosine and threonine residues in the TEY/TDY motif in the activation loop (T-loop) (Çakır and Kılıçkaya 2015; Rodriguez et al. 2010; Tena et al. 2001). The activated MAPKs phosphorylate the downstream transcription factors and other enzymes or signaling components that are related to numerous cellular processes, which include cell proliferation and differentiation (Jiménez et al. 2007; Xu and Zhang 2015), hormonal responses (Tena et al. 2001), and biotic and abiotic stress (Zhu 2002; Pitzschke et al. 2009b).

To date, several MAPK cascade components have been well characterized and studied in both model plants and crop species. In tobacco, the NPK1-NQK1/NtMEK1-NRK1

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cascade regulated cytokinesis during meiosis and mitosis (Soyano et al. 2003), and the MAP kinase $p45^{ntf4}$ was activated by hydration during pollen germination (Wilson et al. 1997). The *AtMKK5-AtMPK6* cascade plays a vital role in primary root growth and stomatal response by responding to ABA (Li et al. 2017). *AtMPK12* acted as a negative regulator in the auxin signaling pathway (Lee et al. 2009). The OsMKK4-OsMPK1-OsWRKY53 signaling cascade was positively involved in plant wounding (Yoo et al. 2014). In addition, some MAPK cascade genes played essential roles in response to abiotic stress. The AtMEKK1-AtMKK4/5-AtMPK3/6 cascade was involved in regulating biotic stress (Asai et al. 2002; Wang et al. 2007). The AtMEKK1-AtMKK1/AtMKK2-AtMPK4 cascade played a vital role in plant innate immunity, salt stress, and cold stress (Gao et al. 2008; Teige et al. 2004; Furuya et al. 2014; Kong et al. 2012b). Some MAPKs, such as *AtMPK4* and *OsMAPK3*, negatively regulated biotic stress signals (Pitzschke et al. 2009a; Lee et al. 2011). Overexpression of *SlMAPK1* improved the resistance of tomato to drought stress (Wang et al. 2018). *ClMPK7*, *ClMPK20-1*, and *ClMPK9-4* in watermelon were significantly up-regulated after high-temperature treatment (Song et al. 2015).

With the completion of annotated genome databases, it is possible to systematically identify the MAPK cascade genes in plants. Genome-wide analysis revealed that the *Arabidopsis thaliana* genome contained 10 MKKs and 20 MPKs (Colcombet and Hirt 2008; Pitzschke et al. 2009a), whereas 8 MKKs and 17 MPKs were found in rice (Wankhede et al. 2013). MKK and MPK gene families were also analyzed in economic crops. Six MKKs and 14 MPKs were identified in cucumber (Wang et al. 2015), and five MKKs and 16 MPKs were found in tomato (Wu et al. 2014; Li et al. 2014). These studies suggested that the number of MAPKK and MAPK genes varied across species. Foxtail millet (*Setaria italica*), which is cultivated in Asia and Africa's semi-arid regions, is well-known for its high nutritional value and strong adaptability to abiotic stress (Barton et al. 2009). In addition, the high-quality small genome, small stature, prolific seed production, and available genetic transformations have made foxtail millet a potential C_4 model plant (Doust et al. 2009; Li and Brutnell 2011; Muthamilarasan et al. 2020). However, no systematic studies on the MAPK cascade genes in foxtail millet have been reported.

In this study, we characterized 16 *MPK* and 11 *MKK* genes at the genome-wide level in foxtail millet. Subsequently, we analyzed the detailed information of the identified *SiMPK* and *SiMKK* genes, which included the gene structures, conserved motifs, physicochemical properties, chromosomal locations, and phylogenetic relationships. Finally, we evaluated the expression patterns of the *SiMPK* and *SiMKK* genes under phytohormone treatment and abiotic stress using qRT-PCR. Our research lays a foundation for future studies of the roles of *SiMPK* and *SiMKK* genes in response to hormone and abiotic stress. Our data presented here also provide clues to understanding the crosstalk between the *SiMPKs* and *SiMKKs* in foxtail millet and other plant species.

Materials and methods

Identification of family genes of *SiMPKs* and *SiMKKs* in foxtail millet

The reported gene sequences of MPK and MKK genes in *Arabidopsis thaliana* and rice were downloaded from the *Arabidopsis* genome database (https://www.arabidopsis.org/) and Rice Genome Annotation Project (http://rice.uga.edu/). These gene sequences were submitted to the plant genome database Phytozome (https://phytozome-next.jgi.doe.gov/) for BLAST using default parameters (e-value < 1.0) (Goodstein et al. 2012), which resulted in candidate gene sequences for *SiMPK* and *SiMKK*. To further test the presence of intact protein structures of these candidate genes, amino acid sequences of SiMPKs and SiMKKs were submitted to the Pfam database (https://www.sanger.ac.uk/) for identification (Mistry et al. 2021), and genes without complete structural domains were removed. Finally, members of the *SiMPK* and *SiMKK* gene families were identified. Isoelectric point (PI) and the molecular weight (Mw) of *SiMPKs* and *SiMKKs* in foxtail millet were analyzed using the bioinformatics online analysis software ExPASY (https://web.expasy.org/protparam/) (Wilkins et al. 1999).

Phylogenetic tree and chromosomal location of *SiMPK* and *SiMKK* genes in foxtail millet

The amino acid sequences of MPKs and MKKs of *Arabidopsis thaliana*, rice, and foxtail millet were aligned homologously by ClustalX software (Thompson et al. 2002) using default parameters. Phylogenetic trees of the MPK and MKK members of *Arabidopsis*, rice, and foxtail millet were constructed using the neighbor-joining (NJ) method of MEGA 7.0 software (Parameters set as: Poisson model and pairwise deletion, Bootstrap 1000 repetitions) (Kumar et al. 2016). We used Gene Location Visualize in Tbtools to map the chromosome locations of *SiMPK* and *SiMKK* genes based on their gene numbers and GFF files of foxtail millet genome annotations (Chen et al. 2020a).

Analysis of gene structure and conserved motifs of*SiMPKs*and*SiMKKs*in foxtail millet

The amino acid sequences of *SiMPK* and *SiMKK* members were submitted to the Multiple Em for Motif Elicitation (MEME) online program (https://meme-suite.org/meme/index.html) to predict their conserved protein motifs (Parameters: number of repetitions = any, maximum number of motifs = 10) (Bailey et al. 2009). The coding sequences and genome sequences of *SiMPK* and *SiMKK* genes were downloaded from Phytozome, and the gene structures of *SiMPKs* and *SiMKKs* were analyzed using the Gene Structure Display Serve online program (http://gsds.gao-lab.org/index.php) (Hu et al. 2015).

*In silico*transcript profiling analysis of foxtail millet*SiMPKs*and*SiMKKs*in different tissue

To explore the spatial and temporal expression patterns of *SiMPK* and *SiMKK* genes in foxtail millet, transcription data in different tissue parts and growth stages of foxtail millet were downloaded from the Phytozome Datasets GeneAtlas v1 Tissue Sample library, which included seedlings (5 d), germ shoots (6 d), leaves (14 d), panicles (7 d), and roots (10 d). Gene expression was shown by the FPKM (Fragments per kilobase of the exon model per million mapped) value. The downloaded data were imported into the Heatmap program of TBtools to map the expression profiles of *SiMPKs* and *SiMKKs* in different tissues and different developmental stages of foxtail millet (Chen et al. 2020a).

Plant material and experimental treatment

In this experiment, "Jingu 21" was used as the experimental material, which were provided by Prof. Xingchun Wang's lab at Shanxi Agricultural University. The seeds of "Jingu 21" were cultivated in a 1:1 vermiculite and soil mixture of nutrient soil at 26/23°C, 50,000 lx light (16 h/8 h period), and 30–50% relative humidity in greenhouse. After seed germination, seedlings with uniform growth were selected and transferred to nursery pots with three plants in each pot. Seedlings at the double-leaf stage were ready for the subsequent treatment. For hormonal stress and for drought and salt stress, seedlings were washed and transferred to a liquid 1/2 MS medium for culture. To acclimatize the plants to the hydroponic environment, hormonal stress treatments were performed after 3 d of hydroponics (the nutrient solution was changed once a day). Specific stress types included 100 µM ABA, 100 µM BR, 1 Mm GA3, 100 µM melatonin, 100 µM MeJA, 10 mM SA, 10 µM IAA, 75 µM 6-BA, 10 nM NAA (Wu et al. 2014), 10% PEG6000, and (150 mM/200 mM) NaCl. For extreme temperature stress, double-leaf stage seedlings were grown in soil for another 3 d, and then they were subjected to low (4 °C) and high temperature (40 °C day/32°C night) in constant temperature plant incubators. Three biological replicates were used in each set of experimental treatments. Samples (approximately 100 ng) that were harvested after 24 h of treatment were frozen instantaneously with liquid nitrogen and stored in a -80°C refrigerator.

RNA extraction and reverse transcription, qRT-PCR expression analysis

Total RNA of foxtail millet leaves was extracted using TransZolTM UP Plus RNA Kit (Transgen Biotech, China, Beijing), according to the instructions for use. The synthesized cDNAs were stored at -20 °C for subsequent experiments. The primers used in the experiments (Supplementary Table 1) were synthesized commercially, with the *SiActin* (*Seita.8G043100*) gene as an internal reference. cDNA was diluted to 300ng/µL as the template for qRT-PCR analysis. The reaction system with 10 µL, which included 5 µl of TransStart Tip Green qPCR SuperMix, 1 µL of template, 0.4 µL of upstream and downstream primers, and 3.2µL of each ddH_2O was set. The reaction procedure of qRT-PCR contained two steps: 94 °C for 30 s, 94 °C for 15 s, and 60 °C for 30 s (40 cycles). The $2^{-\Delta\Delta CT}$ method was used to analyze the qRT-PCR results, and a t-test was used to analyze any significant differences (Livak and Schmittgen 2001).

Results

Identification of*SiMPK*and*SiMKK*gene families in foxtail millet

Following a BLASTP search in the foxtail millet Phytozome database using the protein sequences of the *MPK* and *MKK* genes from *Arabidopsis* and rice, the candidate loci of *SiMPKs* and *SiMKKs* were obtained. After a Pfam search with the protein kinase domains (PF00069.28) to exclude the loci without any kinase domain, 16 *SiMPK* and 11 *SiMKK* genes were identified in foxtail millet (Table 1). The open reading frame (ORF) of the 16 *SiMPKs* ranged from 1044 to 1836 bp, and the resulting protein molecular weights ranged from 39.13 kDa to 69.60 kDa. SiMPK3 had the minimum Mv with 347 amino acids, and SiMPK10 had the maximum with 611 amino acids, which was approximately a two-fold difference. The range of protein isoelectric points for SiMPKs varied from 5.46 to 9.34. The *SiMKKs* family consisted of 11 genes with ORF lengths that ranged from 996 to 1572 bp. The protein length of the SiMKK members was from 331 to 523 amino acids, which resulted in molecular

Table 1 Characteristics of predicted *MPKs* and *MKKs* in foxtail millet

Family	Gene name	Gene ID(Phytozome)	Orthologous Os gene ID	Orthologous At gene ID	ORF (bp)	Length (aa)	PI	Mw (KDa)
MAPK	*SiMPK1*	*Seita.9G444100*	*OsMPK3*	*AtMPK3*	1128	375	5.46	43.43
	SiMPK2	*Seita.9G344000*	*OsMPK4*	*AtMPK11*	1119	372	5.96	42.29
	SiMPK3	*Seita.4G069900*	*OsMPK6*	*AtMPK6*	1044	347	5.71	39.13
	SiMPK4	*Seita.4G243400*	*OsMPK7*	*AtMPK1*	1110	369	6.63	42.34
	SiMPK5	*Seita.6G056700*	*OsMPK11*	*AtMPK12*	1173	390	6.44	44.10
	SiMPK6	*Seita.1G089400*	*OsMPK14*	*AtMPK2*	1173	390	7.21	44.67
	SiMPK7	*Seita.8G104500*	*OsMPK16-1*	*AtMPK18*	1608	535	8.79	61.17
	SiMPK8	*Seita.4G273900*	*OsMPK17-1*	*AtMPK9*	1725	574	6.64	65.25
	SiMPK9	*Seita.1G095300*	*OsMPK17-2*	*AtMPK8*	1521	506	7.67	57.85
	SiMPK10	*Seita.5G235700*	*OsMPK20-1*	*AtMPK20*	1836	611	9.14	69.60
	SiMPK11	*Seita.3G058000*	*OsMPK20-2*	*AtMPK16*	1674	557	8.83	63.64
	SiMPK12	*Seita.J012000*	*OsMPK20-3*	*AtMPK19*	1626	541	8.76	61.91
	SiMPK13	*Seita.5G261300*	*OsMPK20-4*	*AtMPK20*	1770	589	9.34	67.38
	SiMPK14	*Seita.3G145200*	*OsMPK20-5*	*AtMPK20*	1776	591	9.20	67.26
	SiMPK15	*Seita.3G136100*	*OsMPK21-1*	*AtMPK15*	1773	590	6.74	66.86
	SiMPK16	*Seita.5G241700*	*OsMPK21-2*	*AtMPK8*	1527	508	7.64	57.78
MAPKK	*SiMKK1*	*Seita.4G036300*	*OsMKK1*	*AtMKK2*	1053	350	6.36	39.06
	SiMKK2	*Seita.1G307500*	*OsMKK3*	*AtMKK3*	1572	523	5.80	58.52
	SiMKK3	*Seita.1G345400*	*OsMKK4*		1101	366	9.45	39.56
	SiMKK4	*Seita.4G043500*	*OsMKK5*		1362	453	10.02	49.47
	SiMKK5	*Seita.5G301500*	*OsMKK6*	*AtMKK6*	1068	355	5.47	39.87
	SiMKK6	*Seita.1G281300*	*OsMKK10-1*		1002	333	8.63	34.69
	SiMKK7	*Seita.9G484700*	*OsMKK10-2*		996	331	6.08	35.18
	SiMKK8	*Seita.9G117500*	*OsMKK10-3*	*AtMKK7*	1005	334	8.43	35.07
	SiMKK9	*Seita.3G062300*		*AtMKK1*	1068	355	5.57	39.95
	SiMKK10	*Seita.1G307400*	*OsMKK3*	*AtMKK3*	1572	523	5.72	58.41
	SiMKK11	*Seita.5G231200*		*AtMKK4*	1026	341	8.68	37.24

weights from 34.69 kDa to 58.52 kDa. The corresponding isoelectric point of the 11 SiMKKs ranged from 5.47 to 10.02.

Phylogenetic tree and chromosomal location analysis of*SiMPK*s and*SiMKK*s in foxtail millet

To reveal and to classify the evolutionary relationships of the *SiMPK* and *SiMKK* genes in foxtail millet, unrooted phylogenetic trees were created by sequence alignment of the 16 *SiMPKs* and 11 *SiMKKs* with homologous genes in *Arabidopsis* and rice using the MEGA7.0 neighbor-joining (NJ) method. The phylogenetic tree showed that the *SiMPK* genes were divided into four groups that labelled A, B, C, and D (Fig. 1a). Two members were assigned to group A (*SiMAPK2* and *SiMAPK5*), two members belonged to group B (*SiMPK1* and *SiMPK3*), and two members were placed in group C (*SiMPK4* and *SiMPK6*). The remaining eight members were classified as group D. Similarly, the 11 *SiMKK* genes were also classified into four groups that labelled A, B, C, and D (Fig. 1b). Group A contained *SiMKK1*, *SiMKK5*, and *SiMKK9*, group B had *SiMKK2* and *SiMKK10*, group C contained *SiMKK3*, *SiMKK4*, and *SiMKK11*, and the remainder were members of group D. The evolutionary tree revealed that *MPKs/MKKs* in foxtail millet and rice were near to each other evolutionarily, which resulted in more direct homologous gene pairs (e.g., *OsMPK11*/*SiMPK5, OsMKK1*/*SiMKK1*, etc.), but they were somewhat far from *MPKs/MKKs* in *Arabidopsis*, which is a characteristic that could be related to plant evolution (Fig. 1b).

Next, the chromosome distributions of 16 *SiMPKs* and 11 *SiMKKs* were analyzed by using the Gene Location Visualize of Tbtools (Fig. 2). Our results revealed that these genes existed individually or in sub-groups on chromosomes, rather than being distributed randomly. Based on Holub's definition of the gene cluster as ≥4 genes within 200 kb on a chromosome, no gene clusters were found in either the SiMAPK and SiMAPKK gene families (Holub 2001). *SiMPKs* and *SiMKKs* were distributed on 7 of the 9 chromosomes, and none of these genes were found on chromosome 2 and 7. More interestingly, *SiMKK2* and *SiMKK10* were linked tightly on chromosome 1, and they showed > 98% similarity in amino acid sequence, which suggested that gene duplication may have occurred.

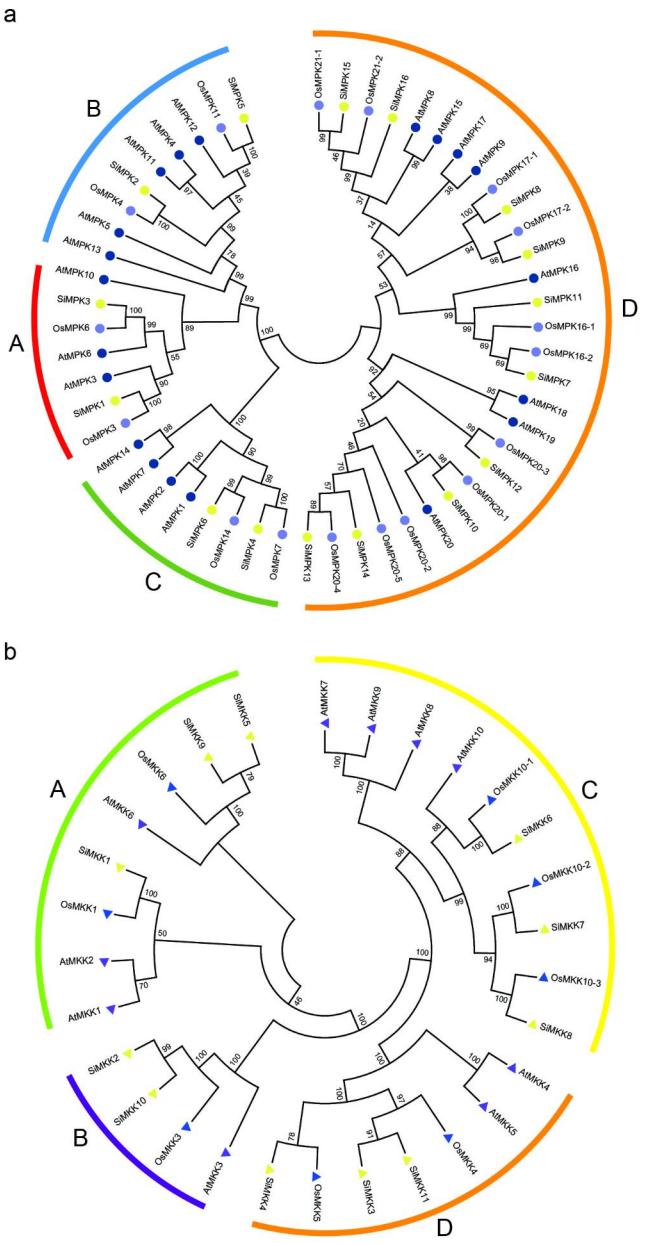

Fig. 1 Phylogenetic relationships of MPK and MKK proteins in *Arabidopsis*, rice, and foxtail millet. Using MEGA7.0 software and neighbor-joining (NJ) method, *Arabidopsis thaliana*, rice, and foxtail millet protein sequences were constructed as unrooted phylogenetic trees. Bootstrap values of 1000 repeats were calculated on each branch. (a) phylogenetic tree for MPK and (b) phylogenetic tree MKK

Analysis of the conserved motifs and gene structure in SiMPK and SiMKK families of foxtail millet

Using amino acid sequence alignment, we found that all the SiMPKs and SiMKKs have conserved activation loops of protein kinase (Supplementary Fig. 1). SiMPKs had an evolutionarily conserved T-X-Y motif, which is a typical phosphorylation site required for MAPK activation. The SiMPKs in groups A, B, and C contained the conserved TEY motifs, whereas group D contained the conserved TDY motif. SiMPK5 was an exception that had the MEY motif instead of the TEY motif. The MEY motif was identified in tomato (Kong et al. 2012a) and *Brachypodium* (Chen et al. 2012a), but not in rice and *Arabidopsis*. In addition, we discovered that the SiMPKs in A and B groups all had a CD domain conserved as (LH)DXXDE(P), which is the anchoring site of MKKs, while no such domain was found in groups C and D (Supplementary Fig. 1a). Alignment of amino acid sequences revealed that all 11 SiMKKs had the conserved D(L/I/V)K active site and the VGTxxxYMSPER motif. The activation-loop motif (S/T-X5-S/T) was present in the SiMKKs in groups A and B, but not in the other groups (Supplementary Fig. 1b).

Next, MEME analyses were performed to get further insight into the functional motifs of both gene families. A total of 10 different conserved motifs were detected in SiMPKs. Among them, motifs 6, 9, and 10 were found only in group D, but the other seven motifs were found in all four groups (Fig. 3a). Motif analysis revealed that motifs 1, 2, 4, and 5 were conserved in all SiMKKs (Fig. 3b). Meanwhile, specific motifs were found in different groups. For example, motif 6 was identified only in groups A and B, and motif 7 was detected in group D specifically. These unique motifs may indicate their specific gene functions (Zhang et al. 2020).

Analysis of exon/intron organization was then performed to understand the gene structures of the *SiMPKs* and *SiMKKs*. All the *SiMPK* genes contained introns that varied in number, location, and length. The *SiMPK* members in Group D contained the most, usually 9–11, but Group C had the least number of introns, 2–3. (Fig. 4a). The gene structures of *SiMKKs* showed that the exon-intron organizations were the same in the classified groups. The *SiMKK* members in group A all had seven introns, and the members in group B all had eight introns. The SiMKKs in groups C and D had no introns, except for *SiMKK6*, which had one intron (Fig. 4b). Above all, both the conserved motif analysis and the gene structures validated the phylogenetic tree-based evolutionary relationship.

In silico temporal and spatial transcript profiling of *SiMPK* and *SiMKK* genes

Numerous studies showed that the MAPK cascade genes were involved in various life processes in different organisms. To further study the roles of *SiMPK* and *SiMKK* genes in the growth and development of foxtail millet, the expression levels of *SiMPK* and *SiMKK* genes in diverse tissues were collected from the RNA-seq database, which included etiolated seedlings (5 d), germ shoots (6 d), leaves (14 d),

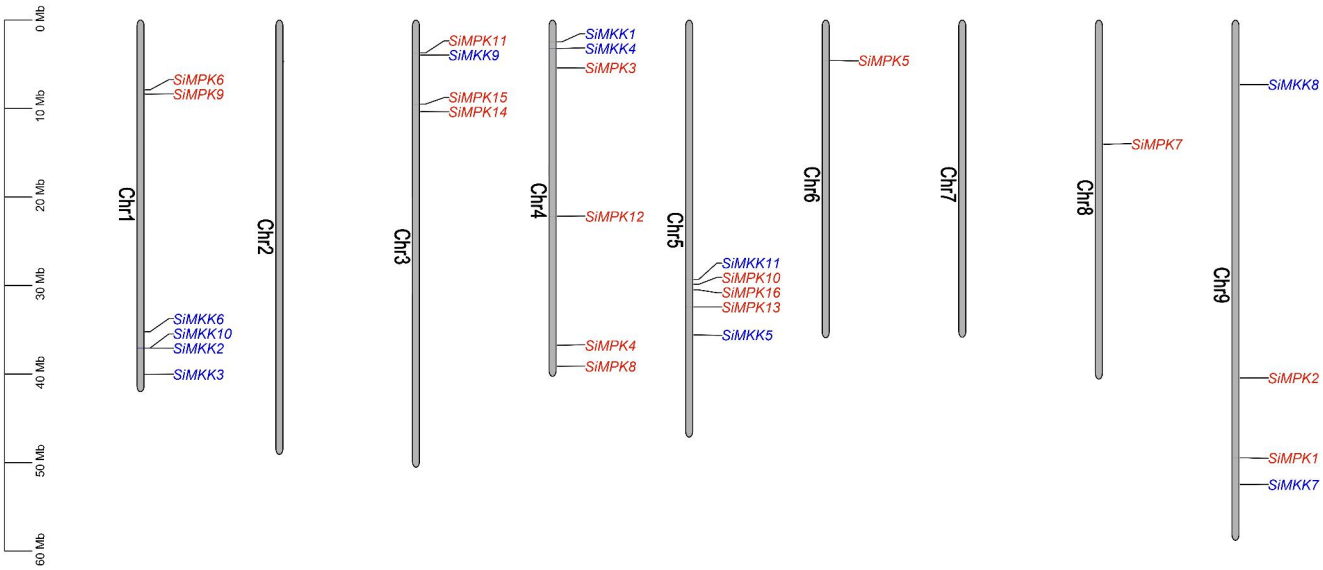

Fig. 2 Chromosome distribution of*SiMPK*and*SiMKK*genes in foxtail millet.*SiMPK* and *SiMKK* genes were mapped on nine chromosomes of foxtail millet using TBtools software. The scale on the left represents chromosome length

panicles (7 d), and roots (10 d). The heatmap results revealed that the expression levels of *SiMPK* and *SiMKK* genes varied in different tissues and developmental stages (Fig. 5). In the *SiMPK* family, *SiMPK5*, *SiMPK6*, *SiMPK7*, *SiMPK9*, *SiMPK15*, and *SiMPK16* genes were expressed constitutively, and the *SiMPK1*, *SiMPK4*, *SiMPK8*, *SiMPK13*, and *SiMPK14* genes had expression patterns that were highly tissue-specific in all the tested tissues. *SiMPK2* and

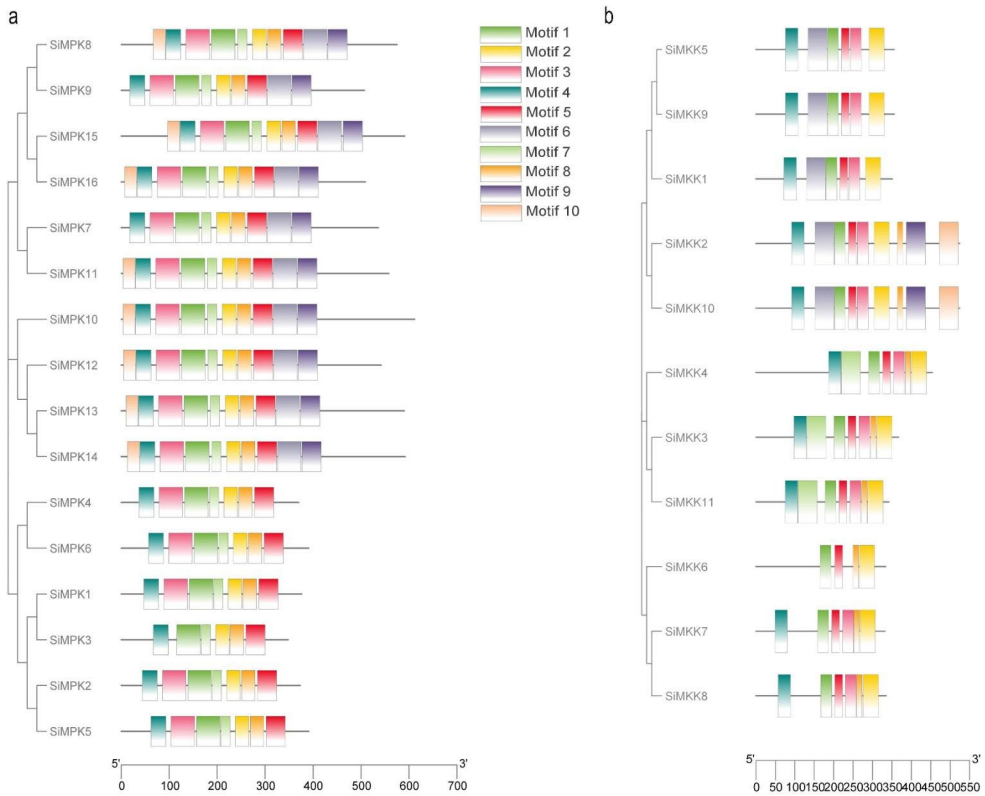

Fig. 3 Schematic diagram of the conserved motifs of amino acids of (a) SiMPKs and (b) SiMKKs. A total of 10 motifs of SiMPKs and SiMKKs were identified by the Meme program. The Grey solid lines represent SiMPK and SiMKK genes and their lengths.The boxes with different colors represent 10 different motifs and their positions in each gene sequence. The results of SiMPK and SiMKK motifs correspond to the evolutionary tree by TBtools software, and the two family motifs correspond to the same legend

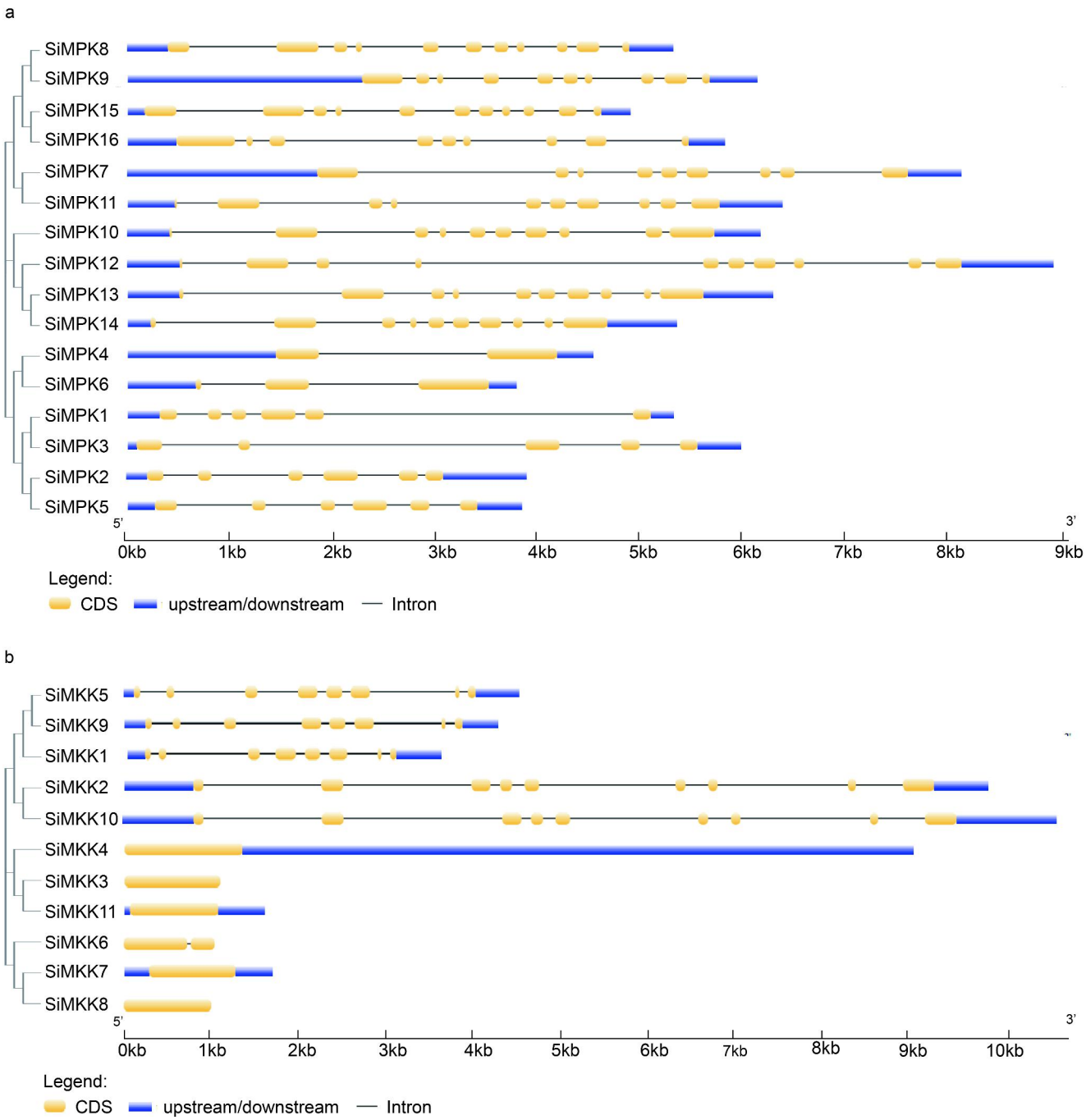

Fig. 4 Gene structures of (a)*SiMPK*and (b)*SiMKK*in foxtail millet. The exon-intron structure of *SiMPK* and *SiMKK* genes was predicted by GSDS 2.0. The yellow boxes represent the gene coding region (CDS), the black lines represent introns, and the blue boxes represented untranslated Regions (UTR)

SiMPK10 showed relatively higher expression levels than others in shoot and root tissues. For the 11 *SiMKK* genes, the expression levels of *SiMKK1*, *SiMKK3*, and *SiMKK5* varied in all the tissues, whereas *SiMKK4*, *SiMKK6*, *SiMKK8*, and *SiMKK10* were constitutive with lower expression. The *SiMKK* genes also showed developmental, stage-specific, expression patterns. For example, *SiMKK7* was highly expressed in all the leaves at different stages, but *SiMKK2* and *SiMKK11* were only found in particular leaves at specific stages. These results suggested that the expression profiles of the MAPK cascade genes depended on their particular functions rather than their sequence similarities and evolutionary relationships and further more efforts are needed to determine their biological functions.

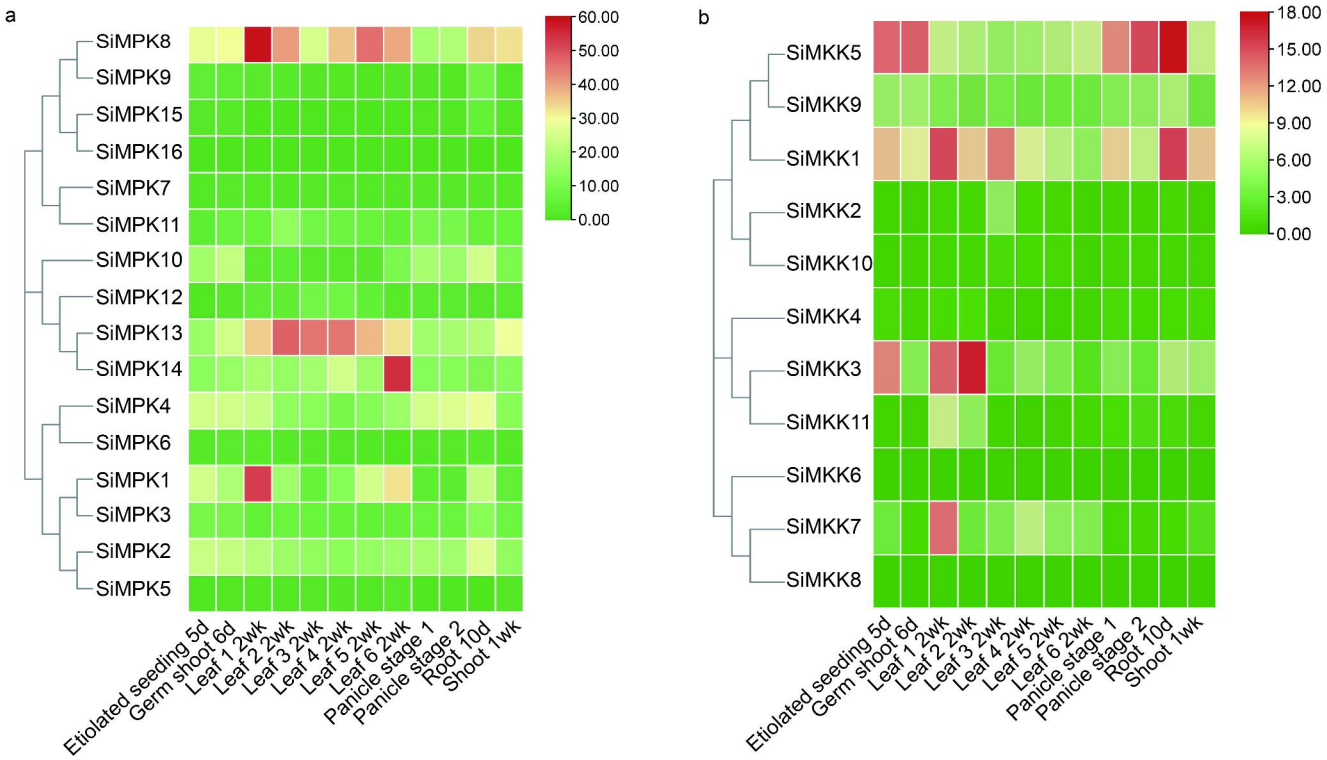

Fig. 5 Expression profiles of (a)*SiMPK*and (b)*SiMKK*genes in different tissues and developmental stages of foxtail millet. Genes expression data were downloaded from the Phytozome Datasets GeneAtlas v1 Tissue Sample database, which included etiolated seedlings (5 d), germ shoots (6 d), leaves (14 d), panicles (7d), roots (10 d), and shoots (7 d). The gene expression maps of *SiMPKs* and *SiMKKs* were drawn using the Heatmap program in TBtools

Expression pattern of*SiMPK*and*SiMKK*genes in response to phytohormones

Hormones affect plant physiological and biochemical responses through a variety of signaling pathways, which include MAPK cascades (Chen et al. 2020b). Gene expression patterns are frequently used to predict gene function. To explore the gene expression patterns of *SiMPKs* and *SiMKKs* in foxtail millet after exogenous hormone treatment, we harvested the samples after 24 h of treatment with nine different phytohormones (ABA, BR, GA3, MT, MeJa, SA, IAA, 6-BA, and NAA). We determined their expression patterns using qRT-PCR technology (Supplementary Fig. 2).

Several *SiMPK* and *SiMKK* genes responded rapidly to specific hormones, with more *SiMPKs* involved than *SiMKKs* (Figs. 6 and 7). For example, 15 out of 16 *SiMPKs* (except for *SiMPK3*) were induced by different hormones, but only 6 out of 11 *SiMKK* genes were induced. The transcriptions of *SiMKK6* and *SiMKK8* were too low to be detected by qRT-PCR in the samples. Both up-regulated and down-regulated genes were detected in some treatments. When treated with ABA, the expression levels of nine *SiMPK* genes (*SiMPK1*, *SiMKP2*, *SiMPK5*, *SiMPK6*, *SiMPK7*, *SiMPK10*, *SiMPK11*, *SiMPK12*, and *SiMPK13*) and four *SiMKK* genes (*SiMKK2*, *SiMKK4*, *SiMKK10*, and *SiMKK11*) changed significantly, and all of them were up-regulated. With the BR treatment, only four genes, which included *SiMPK7*, *SiMKP15*, *SiMKK2*, and *SiMKK4*, were up-regulated. After being treated with GA3, five *SiMPKs* (*SiMPK3*, *SiMPK5*, *SiMPK8*, *SiMPK13*, and *SiMPK14*) showed no significant response, and the other *SiMPK* genes (except for *SiMPK16*) showed increased expression, whereas *SiMKK2* and *SiMKK4* in the *SiMKK* family showed significant differences in transcription levels. Additionally, three SiMPKs (*SiMPK5*, *SiMPK6*, and *SiMPK16*) and two SiMKKs (*SiMKK3* and *SiMKK11*) were up-regulated after MT treatment. After 24 h of MeJA treatment, eleven *SiMPK* genes and two *SiMKKs* were up-regulated significantly, while *SiMPK16* showed decreased expression. Seven *SiMPKs* and five *SiMKKs* were induced by 6-BA treatment, and none of them were down-regulated. Unlike other phytohormones, all the *SiMPK* and *SiMKK* genes induced by SA were down-regulated, except for *SiMPK5* and *SiMKK4*. Similar expression patterns were observed after exogenous IAA and NAA treatment, where *SiMPK5* and *SiMPK15* showed relatively higher increases than others. Taken together, the *SiMPK* and *SiMKK* genes exhibited specific expression patterns in response to various hormone stress. *SiMPK5* and *SiMKK4* responded to the most

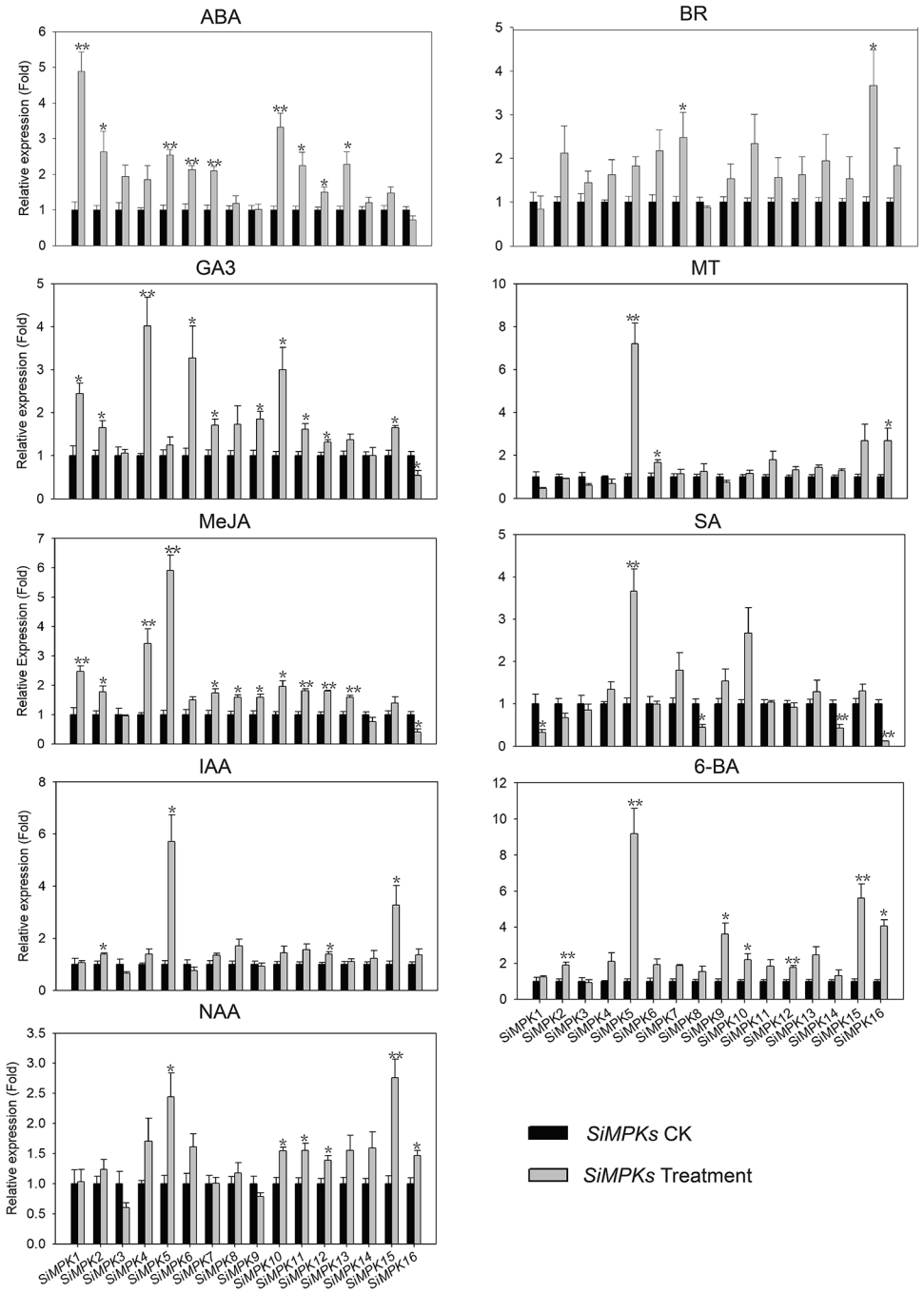

Fig. 6 Analysis of expression patterns of*SiMPK*s under hormonal stress. Transcript levels of the *SiMPK* gene familiy under hormonal stress were analyzed using qRT-PCR. Hormones included 100 µM ABA, 100 µM BR, 1 mM GA3, 100 µM MT, 100 µM MeJA, 10 mM SA, 10 µM IAA, 75 µM 6-BA, and 10 nM NAA. Three biological replicates and three technical replicates were set up for all samples. Each bar represents the mean ± SE normalized to *SiActin* (Seita.8G043100). Asterisks denote significant differences using a t-test (* for $p < 0.05$ and ** for $p < 0.01$)

hormones, which suggested they played significant roles in the phytohormone signaling pathways.

Expression pattern of*SiMPK*and*SiMKK*genes under abiotic stress

To investigate the roles of *SiMPK* and *SiMKK* genes under abiotic stress, we conducted qRT-PCR analysis to examine their expression levels in response to four abiotic treatments

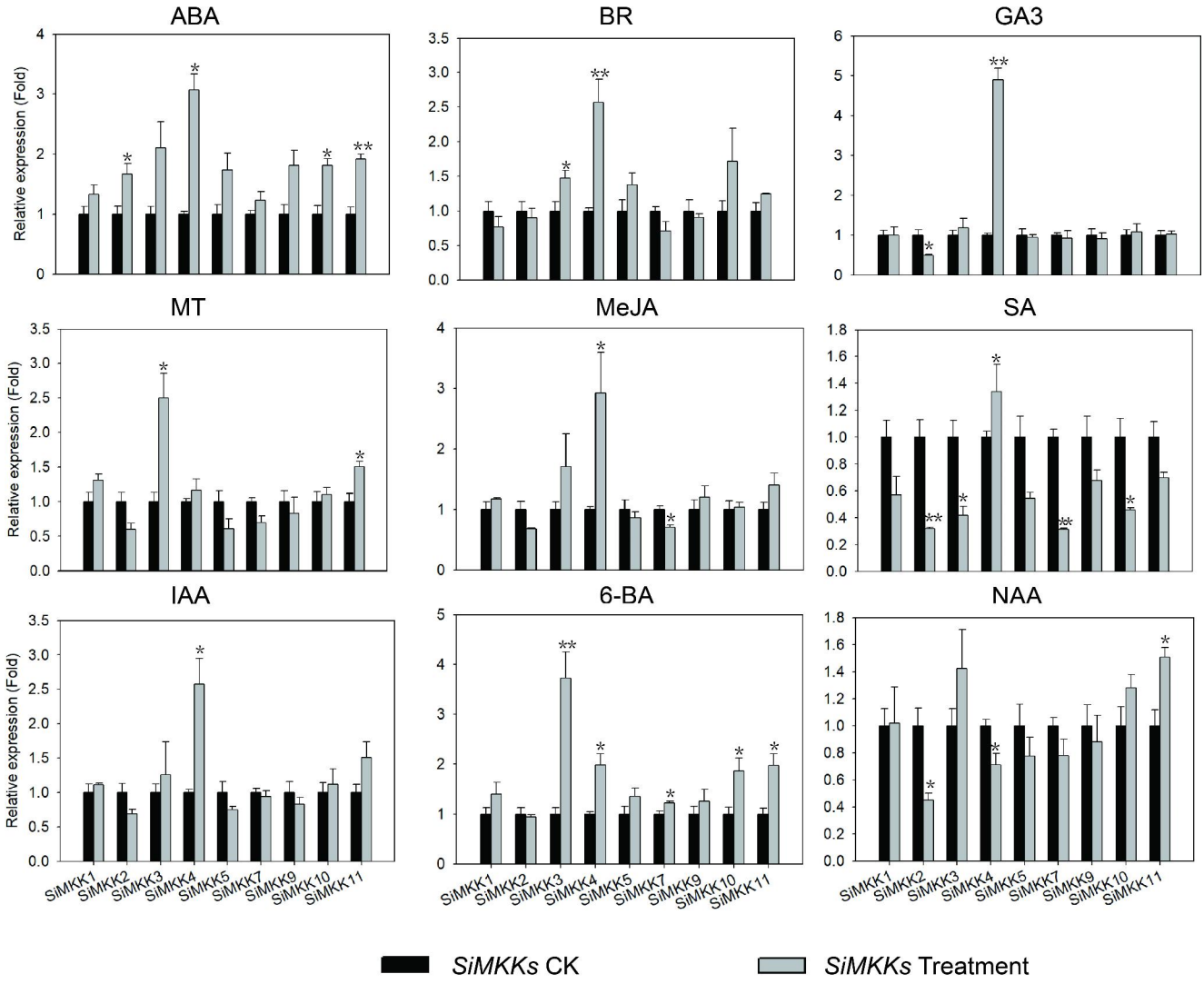

Fig. 7 Analysis of expression patterns of*SiMKKs*under hormonal stress. Transcript levels of *SiMKK* gene families under hormonal stress were analyzed using qRT-PCR. Hormones included 100 µM ABA, 100 µM BR, 1 mM GA3, 100 µM MT, 100 µM MeJA, 10 mM SA, 10 µM IAA, 75 µM 6-BA, and 10 nM NAA. Each bar represents the mean ± SE normalized to *SiActin* (*Seita.8G043100*). Asterisks denote significant differences using a t-test (* for $p < 0.05$ and ** for $p < 0.01$)

(cold, heat, salt, and PEG) (Supplementary Fig. 2). Almost all *SiMPK* and *SiMKK* genes exhibited differential expression levels after the four abiotic challenges (Figs. 8 and 9). When we subjected the young seedlings to cold, all the responsive eleven *SiMPKs* and five *SiMKKs* were up-regulated. For the heat treatment, all six *SiMPK* genes (except for *SiMPK11*) were significantly down-regulated, and no significant changes were obtained in the *SiMKK* families under heat stress. The expression levels of *SiMPK* and *SiMKK* genes changed irregularly following PEG treatment, with eight *SiMPKs* (*SiMPK1*, *SiMPK3*, *SiMPK4*, *SiMPK5*, *SiMPK6*, *SiMPK9*, *SiMPK10*, and *SiMPK13*) and *SiMKK1* up-regulated, and two *SiMPKs* (*SiMPK11* and *SiMPK14*) and *SiMKK4* were down-regulated. We used two concentrations of NaCl (150 mM and 200 mM) to simulate salt stress, and similar expression patterns of the two gene families were obtained. Nine *SiMPKs* and four *SiMKKs* with significantly changed transcription levels were detected under both concentrations. Overall, our findings revealed that *SiMPKs* and *SiMKKs* responded to a variety of abiotic stress, which provides a basis for further studies on the mechanism of *SiMPK* and *SiMKK* genes under abiotic stress.

Discussion

Abiotic stress can cause a reduction in crop yield or even extinction; therefore, understanding how plants respond to adversity is critical for global food security (Chen et al. 2012b). The external stress received by upstream receptors

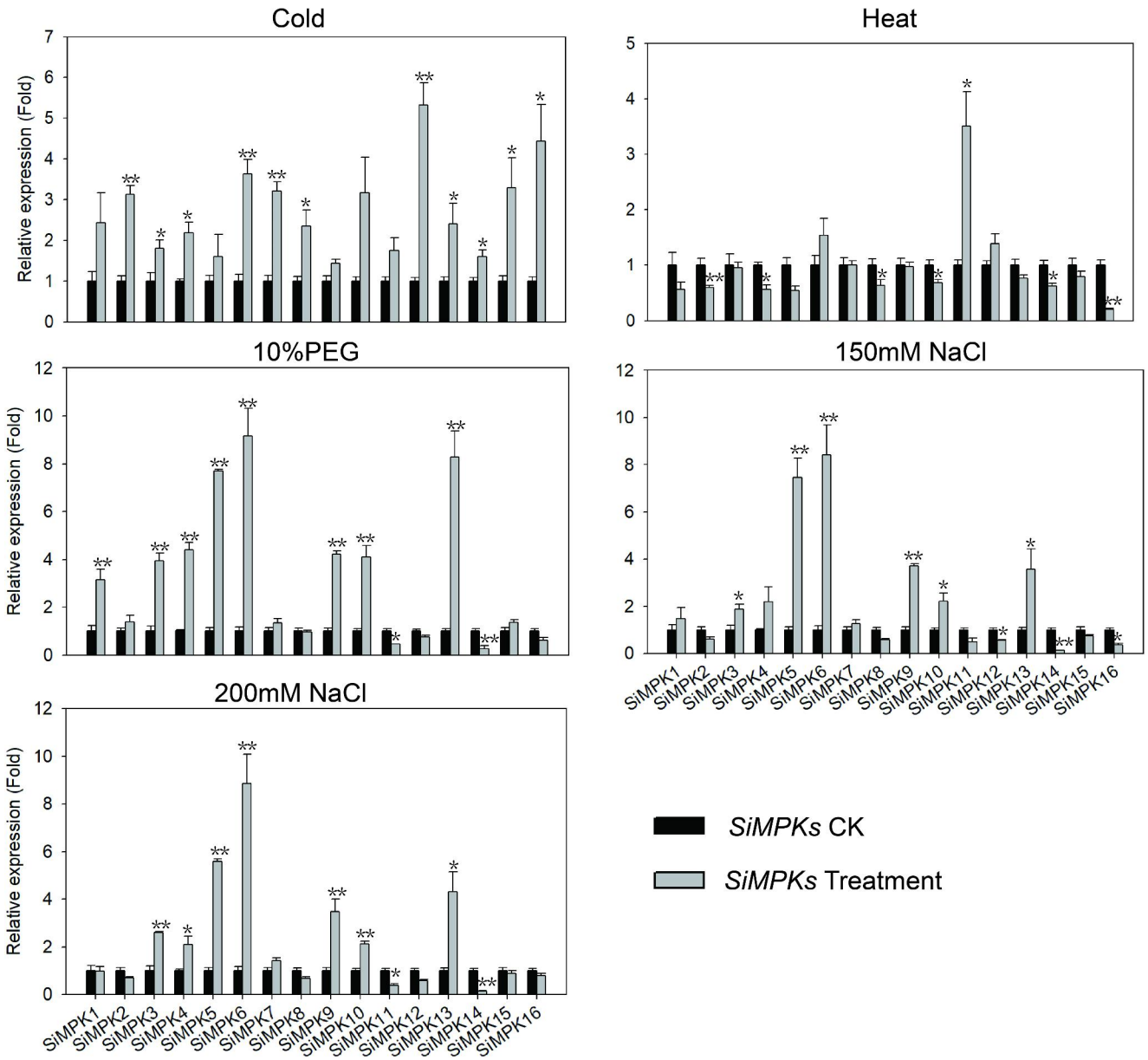

Fig. 8 Analysis of expression patterns of*SiMPKs*under abiotic stresses. Transcript levels of *SiMKKs* gene families under abiotic stresses were analyzed using qRT-PCR. Abiotic stresses included 10% PEG6000, (150 mM/200 mM) NaCl, cold (4 °C) and heat (40 °C day/32°C night) stress. Three biological replicates and three technical replicates were set up for all samples. Each bar represents the mean ± SE normalized to *SiActin* (*Seita.8G043100*). Asterisks denote significant differences using a t-test (* for $p < 0.05$ and ** for $p < 0.01$)

is transmitted to the downstream stress resistance genes through several signal transduction pathways (Genot et al. 2017; Bari and Jones 2009). The MAPK cascade, which comprises many MAPKKK-MAPKK-MAPK modules, is an essential signaling pathway that transmits and amplifies the external signal through sequential phosphorylation (Rodriguez et al. 2010; Kong et al. 2013; Hamel et al. 2006). Genome-wide analyses of the MAPK cascade genes have been done in several plant species, which provides a crucial basis for subsequent functional characterization. However, little information on the MAPK cascade genes was available for foxtail millet. In this study, we systematically identified the *MPK* and *MKK* genes in foxtail millet, defined their fundamental characteristics, and analyzed the specific expression patterns in different tissues as well as under hormonal and abiotic stress.

We identified 16 *SiMPK* and 11 *SiMKK* genes in foxtail millet based on the MAPK cascade genes in *Arabidopsis* and rice. Both SiMPKs and SiMKKs were classified into four groups by phylogenetic analysis, which was supported by the conserved motif analysis and exon/intron organization. The same classification with other plant species suggested

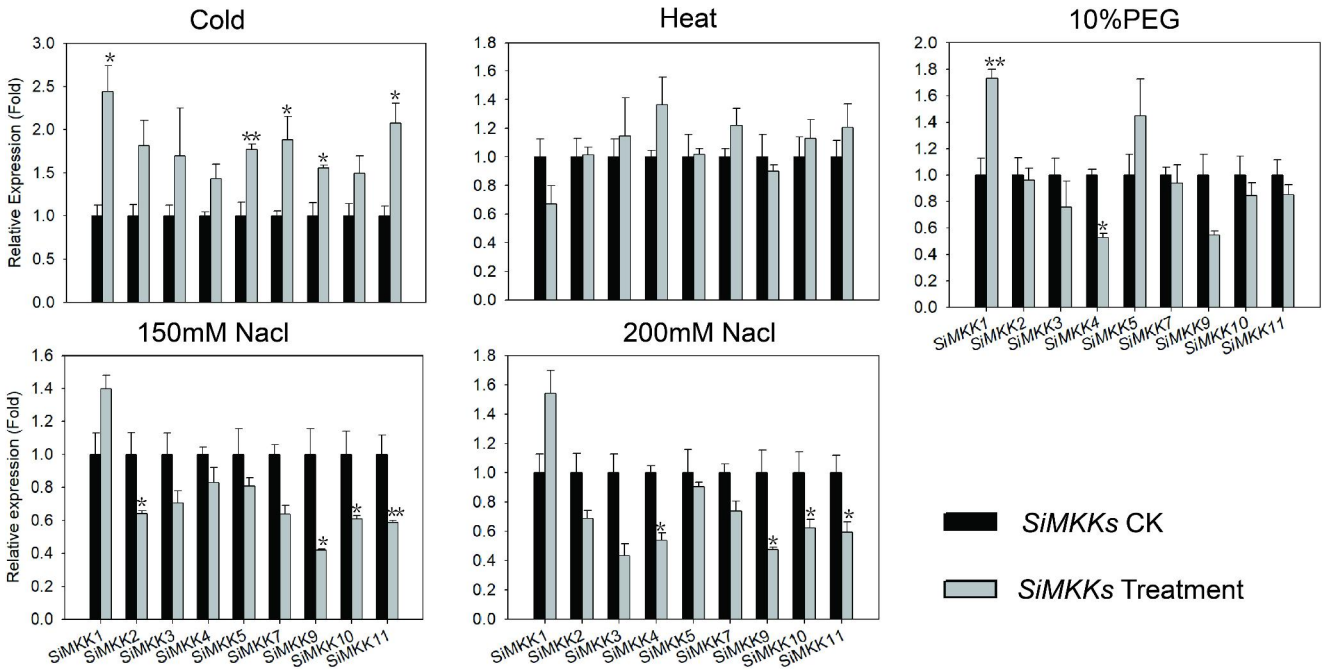

Fig. 9 Analysis of expression patterns of*SiMKKs*under abiotic stresses. Transcript levels of *SiMKKs* gene families under abiotic stresses were analyzed using qRT-PCR. Abiotic stresses included 10% PEG6000, (150 mM/200 mM) NaCl, cold (4 °C) and heat (40 °C day/32°C night) stress. Three biological replicates and three technical replicates were set up for all samples. Each bar represents the mean ± SE normalized to *SiActin* (Seita.8G043100). Asterisks denote significant differences using a t-test (* for $p < 0.05$ and ** for $p < 0.01$)

their evolutionary conservation in different organisms.We also discovered that the MAPK cascade genes changed during the evolution of foxtail millet. Compared with other species, there were certain differences in quantity (Supplementary Table 2). For example, in Arabidopsis and rice, there was only one gene in the B group of MKKs, while two genes in foxtail millet, *SiMKK2* (*Seita.1G307500*) and *SiMKK10* (*Seita.1G307400*). We also checked the copy number of SiMKK2 and SiMKK10 in other species (Supplementary Fig. 3). There was one copy maize and sorghum, while two copies in *Setaria viridis* and Brachypodium. In *Setaria italica and Setaria viridis*, the two copy genes were closely connected on chromosome 1, and the amino acid sequence homology approaches 98%. The double-copy genes in Brachypodium genomes were located on different chromosomes, and have an amino acid sequence similarity of more than 90%. As a result, we speculated that gene duplication may have occurred between SiMKK2 and SiMKK10. Gene duplication is an essential mechanism for providing genetic material for functional evolution of genes. This may provide evidence for a gene duplication event during the evolution of the MAPK cascade in *Setaria* grasses. Moreover, we found that the *SiMPK5* contained an M-E-Y motif in the protein kinases activation loop, which is different from the conserved T-X-Y domain in other SiMAPKs (Supplementary Fig. 1). SiMPK5 reacted to the majority of hormones in nine hormone treatments, indicating that it plays an essential role in phytohormone signal transduction pathways. In rice MAPKs, SA stimulated the expression of *OsMPK17-1* and *OsMPK17-2* (Singh and Jwa 2013), but in foxtail millet, SA only induced the expression of *SiMPK8* (the homolog of *OsMPK17-1*) to regulate the defensive response. *In silico*, RNA-seq data showed that most *SiMPKs* and *SiMKKs* were expressed constitutively in all the tested tissues, which suggested their essential roles in maintaining basic life processes. Some genes showed significant spatio-temporal expression characteristics. For example, *SiMPK8* showed variable transcription levels in almost all tissues, and *SiMPK13* was highly expressed in leaves, which exhibited their tissue-specific function.

Plant hormones are involved in complex signaling pathways that regulate plant growth and development as well as play a vital role in responses to biotic and abiotic stress (Bari and Jones 2009; Zhang et al. 2012; Bennetzen et al. 2012; Liu et al. 2015). The MAPK cascade pathway responds to hormonal signals and coordinates with the hormonal signal pathway to regulate responses to abiotic stress (Jagodzik et al. 2018). For example, the MEKK1-MKK2-MPK4/MPK6 pathway in *Arabidopsis* participated in the tolerance of salt stress and cold stress (Teige et al. 2004). The MEKK6-MAPK5 cascade pathway in rice was involved in root development in response to salt stress (Schmidt et al. 2013). In this study, we analyzed the expression patterns of *SiMPK* and *SiMKK* genes under nine different phytohormones

and four abiotic stresses. Our results indicated that all the *SiMPK* and *SiMKK* genes were involved in the hormone and abiotic treatment. (Singh and Jwa 2013)Previous studies have shown that *AtMAPK1/2/7/14* was induced by ABA and mediated drought signaling in *Arabidopsis* (Danquah et al. 2015; Matsuoka et al. 2018). In foxtail millet, *SiMPK4/6*, which belongs to the same subfamily as *AtMAPK1/2/7/14*, responded positively to ABA and 10% PEG stress and was up-regulated. Therefore, we hypothesize that *SiMPK* and *SiMKK* genes may be in a crosstalk between hormones and abiotic stress. The complicated interactions between different MAPK cascade components maintain their high-fidelity and specific response to certain signals. For example, AtMKK1/AtMKK2 interacted with AtMPK4 to form the AtMKK1/AtMKK2-AtMPK4 cascade, which was involved in regulating low temperature and salt stress in *Arabidopsis* (Teige et al. 2004; Lee et al. 2008; Popescu et al. 2009; Kong et al. 2012b). In our study, *SiMKK1* and *SiMPK5*, which are homologs of *AtMKK1*/*AtMKK2* and *AtMPK4*, respectively, were up-regulated in expression under both salt and cold stress. As a result, we speculated that SiMKK1-SiMPK5 may have protein-protein interactions. In conclusion, our findings provide a foundation for further investigation of the regulatory network of the MAPK cascade in response to hormonal stress or abiotic stress for various biological functions.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415(6875):977–983

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS (2009) MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37:202–208

Bari R, Jones JD (2009) Role of plant hormones in plant defence responses. Plant Mol Biol 69(4):473–488

Barton L, Newsome SD, Chen FH, Wang H, Guilderson TP, Bettinger RL (2009) Agricultural origins and the isotopic identity of domestication in northern China. Proc Natl Acad Sci U S A 106(14):5523–5528

Bennetzen JL, Schmutz J, Wang H, Percifield R, Hawkins J, Pontaroli AC, Estep M, Feng L, Vaughn JN, Grimwood J, Jenkins J, Barry K, Lindquist E, Hellsten U, Deshpande S, Wang X, Wu X, Mitros T, Triplett J, Yang X, Ye CY, Mauro-Herrera M, Wang L, Li P, Sharma M, Sharma R, Ronald PC, Panaud O, Kellogg EA, Brutnell TP, Doust AN, Tuskan GA, Rokhsar D, Devos KM (2012) Reference genome sequence of the model plant Setaria. Nat Biotechnol 30(6):555–561

Çakır B, Kılıçkaya O (2015) Mitogen-activated protein kinase cascades in *Vitis vinifera*. Front Plant Sci 6:556

Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, Xia R (2020a) TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. Mol Plant 13(8):1194–1202

Chen L, Hu W, Tan S, Wang M, Ma Z, Zhou S, Deng X, Zhang Y, Huang C, Yang G, He G (2012a) Genome-wide identification and analysis of *MAPK* and *MAPKK* gene families in Brachypodium distachyon. PLoS ONE 7(10):e46744

Chen T, Zhu H, Ke D, Cai K, Wang C, Gou H, Hong Z, Zhang Z (2012b) A MAP kinase kinase interacts with SymRK and regulates nodule organogenesis in *Lotus japonicus*. Plant Cell 24(2):823–838

Chen YH, Wang NN, Zhang JB, Zheng Y, Li XB (2020b) Genome-wide identification of the mitogen-activated protein kinase (MAPK) family in cotton (*Gossypium hirsutum*) reveals GhMPK6 involved in fiber elongation. Plant Mol Biol 103(4–5):391–407

Colcombet J, Hirt H (2008) Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. Biochem J 413(2):217–226

Danquah A, de Zélicourt A, Boudsocq M, Neubauer J, Frei Dit Frey N, Leonhardt N, Pateyron S, Gwinner F, Tamby JP, Ortiz-Masia D, Marcote MJ, Hirt H, Colcombet J (2015) Identification and characterization of an ABA-activated MAP kinase cascade in *Arabidopsis thaliana*. Plant J 82(2):232–244

Doust AN, Kellogg EA, Devos KM, Bennetzen JL (2009) Foxtail millet: a sequence-driven grass model system. Plant Physiol 149(1):137–141

Furuya T, Matsuoka D, Nanmori T (2014) Membrane rigidification functions upstream of the MEKK1-MKK2-MPK4 cascade during cold acclimation in *Arabidopsis thaliana*. FEBS Lett 588(11):2025–2030

Gao M, Liu J, Bi D, Zhang Z, Cheng F, Chen S, Zhang Y (2008) MEKK1, MKK1/MKK2 and MPK4 function together in a mitogen-activated protein kinase cascade to regulate innate immunity in plants. Cell Res 18(12):1190–1198

Genot B, Lang J, Berriri S, Garmier M, Gilard F, Pateyron S, Haustraete K, Van Der Straeten D, Hirt H, Colcombet J (2017) Constitutively Active Arabidopsis MAP Kinase 3 Triggers Defense Responses Involving Salicylic Acid and SUMM2 Resistance Protein. Plant Physiol 174(2):1238–1249

Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N, Rokhsar DS (2012) Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res 40:1178–1186

Hamel LP, Nicole MC, Sritubtim S, Morency MJ, Ellis M, Ehlting J, Beaudoin N, Barbazuk B, Klessig D, Lee J, Martin G, Mundy J, Ohashi Y, Scheel D, Sheen J, Xing T, Zhang S, Seguin A, Ellis BE (2006) Ancient signals: comparative genomics of plant *MAPK* and *MAPKK* gene families. Trends Plant Sci 11(4):192–198

Holub EB (2001) The arms race is ancient history in Arabidopsis, the wildflower. Nat Rev Genet 2(7):516–527

Hu B, Jin J, Guo AY, Zhang H, Luo J, Gao G (2015) GSDS 2.0: an upgraded gene feature visualization server. Bioinformatics 31(8):1296–1297

Jagodzik P, Tajdel-Zielinska M, Ciesla A, Marczak M, Ludwikow A (2018) Mitogen-Activated Protein Kinase Cascades in Plant Hormone Signaling. Front Plant Sci 9:1387

Jiménez C, Cossío BR, Rivard CJ, Berl T, Capasso JM (2007) Cell division in the unicellular microalga Dunaliella viridis depends on phosphorylation of extracellular signal-regulated kinases (ERKs). J Exp Bot 58(5):1001–1011

Jonak C, Okrész L, Bögre L, Hirt H (2002) Complexity, cross talk and integration of plant MAP kinase signalling. Curr Opin Plant Biol 5(5):415–424

Kong F, Wang J, Cheng L, Liu S, Wu J, Peng Z, Lu G (2012a) Genome-wide analysis of the mitogen-activated protein kinase gene family in *Solanum lycopersicum*. Gene 499(1):108–120

Kong Q, Qu N, Gao M, Zhang Z, Ding X, Yang F, Li Y, Dong OX, Chen S, Li X, Zhang Y (2012b) The MEKK1-MKK1/MKK2-MPK4 kinase cascade negatively regulates immunity mediated by a mitogen-activated protein kinase kinase kinase in Arabidopsis. Plant Cell 24(5):2225–2236

Kong X, Pan J, Zhang D, Jiang S, Cai G, Wang L, Li D (2013) Identification of mitogen-activated protein kinase kinase gene family and MKK-MAPK interaction network in maize. Biochem Biophys Res Commun 441(4):964–969

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33(7):1870–1874

Lee JS, Huh KW, Bhargava A, Ellis BE (2008) Comprehensive analysis of protein-protein interactions between Arabidopsis MAPKs and MAPK kinases helps define potential MAPK signalling modules. Plant Signal Behav 3(12):1037–1041

Lee JS, Wang S, Sritubtim S, Chen JG, Ellis BE (2009) Arabidopsis mitogen-activated protein kinase MPK12 interacts with the MAPK phosphatase IBR5 and regulates auxin signaling. Plant J 57(6):975–985

Lee SK, Kim BG, Kwon TR, Jeong MJ, Park SR, Lee JW, Byun MO, Kwon HB, Matthews BF, Hong CB, Park SC (2011) Overexpression of the mitogen-activated protein kinase gene OsMAPK33 enhances sensitivity to salt stress in rice (*Oryza sativa* L.). J Biosci 36(1):139–151

Li K, Yang F, Zhang G, Song S, Li Y, Ren D, Miao Y, Song CP (2017) AIK1, A Mitogen-Activated Protein Kinase, Modulates Abscisic Acid Responses through the MKK5-MPK6 Kinase Cascade. Plant Physiol 173(2):1391–1408

Li P, Brutnell TP (2011) *Setaria viridis* and *Setaria italica*, model genetic systems for the Panicoid grasses. J Exp Bot 62(9):3031–3037

Li X, Zhang Y, Huang L, Ouyang Z, Hong Y, Zhang H, Li D, Song F (2014) Tomato *SlMKK2* and *SlMKK4* contribute to disease resistance against Botrytis cinerea. BMC Plant Biol 14:166

Liu Z, Shi L, Liu Y, Tang Q, Shen L, Yang S, Cai J, Yu H, Wang R, Wen J, Lin Y, Hu J, Liu C, Zhang Y, Mou S, He S (2015) Genome-wide identification and transcriptional expression analysis of mitogen-activated protein kinase and mitogen-activated protein kinase kinase genes in *Capsicum annuum*. Front Plant Sci 6:780

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402–408

Matsuoka D, Soga K, Yasufuku T, Nanmori T (2018) Control of plant growth and development by overexpressing MAP3K17, an ABA-inducible MAP3K, in Arabidopsis. Plant Biotechnol (Tokyo) 35(2):171–176

Meng X, Zhang S (2013) MAPK cascades in plant disease resistance signaling. Annu Rev Phytopathol 51:245–266

Mistry J, Chuguransky S, Williams L, Qureshi M, Salazar GA, Sonnhammer ELL, Tosatto SCE, Paladin L, Raj S, Richardson LJ, Finn RD, Bateman A (2021) Pfam: The protein families database in 2021. Nucleic Acids Res 49:412–d419

Muthamilarasan M, Singh RK, Suresh BV, Rana S, Dulani P, Prasad M (2020) Genomic dissection and expression analysis of stress-responsive genes in C_4 panicoid models, *Setaria italica* and *Setaria viridis*. J Biotechnol 318:57–67

Pitzschke A, Schikora A, Hirt H (2009a) MAPK cascade signalling networks in plant defence. Curr Opin Plant Biol 12(4):421–426

Pitzschke A, Schikora A, Hirt H (2009b) MAPK cascade signalling networks in plant defence. Curr Opin Plant Biol 12(4):421–426

Popescu SC, Popescu GV, Bachan S, Zhang Z, Gerstein M, Snyder M, Dinesh-Kumar SP (2009) MAPK target networks in *Arabidopsis thaliana* revealed using functional protein microarrays. Genes Dev 23(1):80–92

Rodriguez MC, Petersen M, Mundy J (2010) Mitogen-activated protein kinase signaling in plants. Annu Rev Plant Biol 61:621–649

Schmidt R, Mieulet D, Hubberten HM, Obata T, Hoefgen R, Fernie AR, Fisahn J, San Segundo B, Guiderdoni E, Schippers JH, Mueller-Roeber B (2013) Salt-responsive ERF1 regulates reactive oxygen species-dependent signaling during the initial response to salt stress in rice. Plant Cell 25(6):2115–2131

Singh R, Jwa NS (2013) The rice MAPKK-MAPK interactome: the biological significance of MAPK components in hormone signal transduction. Plant Cell Rep 32(6):923–931

Song Q, Li D, Dai Y, Liu S, Huang L, Hong Y, Zhang H, Song F (2015) Characterization, expression patterns and functional analysis of the *MAPK* and *MAPKK* genes in watermelon (*Citrullus lanatus*). BMC Plant Biol 15:298

Soyano T, Nishihama R, Morikiyo K, Ishikawa M, Machida Y (2003) NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. Genes Dev 17(8):1055–1067

Teige M, Scheikl E, Eulgem T, Dóczi R, Ichimura K, Shinozaki K, Dangl JL, Hirt H (2004) The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. Mol Cell 15(1):141–152

Tena G, Asai T, Chiu W-L, Sheen J (2001) Plant mitogen-activated protein kinase signaling cascades. Curr Opin Plant Biol 4(5):392–400

Thompson JD, Gibson TJ, Higgins DG (2002) Multiple sequence alignment using ClustalW and ClustalX.Curr Protoc Bioinformatics Chap.2:Unit 2.3.

Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S (2007) Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. Plant Cell 19(1):63–73

Wang J, Pan C, Wang Y, Ye L, Wu J, Chen L, Zou T, Lu G (2015) Genome-wide identification of *MAPK*, *MAPKK*, and *MAPKKK* gene families and transcriptional profiling analysis during development and stress response in cucumber. BMC Genomics 16(1):386

Wang L, Zhao R, Li R, Yu W, Yang M, Sheng J, Shen L (2018) Enhanced drought tolerance in tomato plants by overexpression of *SlMAPK1*. Plant Cell, Tissue and Organ Culture (PCTOC) 133 (1):27–38

Wankhede DP, Misra M, Singh P, Sinha AK (2013) Rice mitogen activated protein kinase kinase and mitogen activated protein kinase interaction network revealed by in-silico docking and yeast two-hybrid approaches. PLoS ONE 8(5):e65011

Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF (1999) Protein identification and analysis tools in the ExPASy server. Methods Mol Biol 112:531–552

Wilson C, Voronin V, Touraev A, Vicente O, Heberle-Bors E (1997) A developmentally regulated MAP kinase activated by hydration in tobacco pollen. Plant Cell 9(11):2093–2100

Wu J, Wang J, Pan C, Guan X, Wang Y, Liu S, He Y, Chen J, Chen L, Lu G (2014) Genome-wide identification of *MAPKK* and *MAPKKK* gene families in tomato and transcriptional profiling analysis during development and stress response. PLoS ONE 9(7):e103032

Xu J, Zhang S (2015) Mitogen-activated protein kinase cascades in signaling plant growth and development. Trends Plant Sci 20(1):56–64

Yoo SJ, Kim SH, Kim MJ, Ryu CM, Kim YC, Cho BH, Yang KY (2014) Involvement of the OsMKK4-OsMPK1 Cascade and its

Downstream Transcription Factor OsWRKY53 in the Wounding Response in Rice. Plant Pathol J 30(2):168–177

Zhang M, Pan J, Kong X, Zhou Y, Liu Y, Sun L, Li D (2012) *ZmMKK3*, a novel maize group B mitogen-activated protein kinase kinase gene, mediates osmotic stress and ABA signal responses. J Plant Physiol 169(15):1501–1510

Zhang X, Li Y, Xing Q, Yue L, Qi H (2020) Genome-wide identification of mitogen-activated protein kinase (MAPK) cascade and expression profiling of *CmMAPKs* in melon (*Cucumis melo* L.). PLoS ONE 15(5):e0232756

Zhu J-K (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273