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Identification of mitogen-activated protein kinase kinase gene family and MKK–MAPK interaction network in maize

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ABSTRACT

Plant mitogen-activated protein kinases (MAPK) are involved in important processes, including stress signaling and development. MAPK kinases (MAPKK, MKK) have been investigated in several plant species including *Arabidopsis thaliana*, *Oryza sativa*, *Populus trichocarpa*, and *Brachypodium distachyon*. In the present study, nine putative maize MKK genes have been identified. Analysis of the conserved protein motifs, exon–intron junctions and intron phase has revealed high levels of conservation within the phylogenetic groups. Next, we defined four new ZmMKK–ZmMPK interactions using yeast two-hybrid. Finally, we examined the biological functions of the *ZmMKK4* gene. Overexpression of *ZmMKK4* in *Arabidopsis* conferred tolerance to oxidative stress by increased germination rate and early seedling growth compared with WT plants. Taken together, we provide a comprehensive bioinformatics analysis of the MKK gene family in maize genome and our data provide an important foundation for further functional study of MAPK and MKK families in maize.

1. Introduction

In the past several years, it has been clearly found that the mitogen-activated protein kinase (MAPK) cascade is evolutionarily conserved from unicellular to complex eukaryotic organisms [1]. MAPK signaling cascade plays a vital role in conferring resistance to the sessile plants besides coordinating the normal growth and developmental cues [2,3]. Usually consisting of protein kinases from three different subfamilies including MAPKKK (MAPK kinase kinase), MKK (MAPK kinase), and MAPK. MAPKKKs are serine/threonine kinases, which phosphorylate two amino acids in the S/T-X_{3-5}-S/T motif of the MKK activation loop. MKKs are dual-specificity kinases that activate MAPK through double phosphorylation of the T-X-Y motif in the activation loop. Eventually, the activated MAPKs phosphorylate various transcription factors and other signaling components that modulate the expression of downstream genes [3].

Available data have shown that MAPK modules are involved in abiotic stress, hormonal responses, the regulation of cell division, differentiation, programmed cell death and growth, as well as in symbiotic and pathogenic biotic interactions [3,4]. Although MAPKs regulate global changes in gene expression, they also affect cytoplasmic infrastructure [5]. Recent study reported that the MEKK1–MKK1/MKK2–MPK4 kinase cascade has dual functions in plant immunity. It positively regulates basal resistance and negatively regulates immunity mediated by SUMM2 [6]. CTR1 was found to inhibit MKK9-MPK3/MPK6 activation during ethylene signaling [7]. YDA–MKK4/MKK5–MPK3/MPK6-SPCH negatively regulates stomatal development [8]. Tobacco NPK1-NQK1-NRK1 and *Arabidopsis* ANP1/2/3-ANQ/MKK6-MPK4 are found in the equatorial region of phragmoplasts and are involved in cytoskeletal regulation [5]. Recently, MKK3/CaM-MPK8 cascades mediate Ca^{2+} and ROS signaling in early wound signaling [9]. OsMEK1/2/6/7b/8a-OsMPK1 has been shown to be involved in growth, development and biotic and abiotic signaling and responses [10].

Maize (*Zea mays*) is an important worldwide crop that is relied upon for human food, animal feed and for starch ethanol production. Recently, MAPKKK and MAPK gene family has been systematic investigated in maize, and the maize genome contains approximately 74 MAPKKK and 19 MAPK genes respectively [11,12]. However, the MKK gene family in maize has not been identified. Sequence and functional analyses of the *Arabidopsis* genome have revealed that there are 20 MAPKs, 10 MKKs and 80 MAPKKKs, with a similar repertoire of genes observed in other plant for which full genome sequences have been determined, such as rice (*Oryza sativa*), poplar (*Populus* sp.), and *Brachypodium distachyon* [3,10,13,14]. The mismatch between the numbers of MKKs and MAPK substrates suggests that individual MKKs have the capacity to address more than one MAPK, an individual MAPK can apparently serve as a substrate for multiple upstream MKKs.

In this study, 9 MKK genes were identified from the maize genome. Detailed information on their genomic structures,

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conserved protein motif and phylogenetic trees is provided. In addition, we conducted comprehensive directed yeast two-hybrid (Y2H) using 4 maize MKKs as individual bait proteins and each of 15 MAPKs as prey proteins. Subsequently, we investigated *ZmMKKs* expression profiling in different organs and developmental stages using microarray data. Finally, we examined the biological functions of the *ZmMKK4* gene in responses to oxidative stress. These results will help to guide future analyses of MAPK signaling cascades in maize.

2. Materials and methods

2.1. Identification and characterization of MKK gene family in maize

The completed genome sequence of *Z. mays* was downloaded from the maize sequence database (http://www.maizesequence.org/index.html). For the identification of maize MKK gene family, *Arabidopsis* and rice MKK protein sequences were firstly used as query sequences to search against the maize genome database and NCBI using BLASTP program. The Pfam (http://pfam.sanger.ac.uk/search) and SMART (http://smart.embl-heidelberg.de/) databases were used to confirm each predicted maize MKK protein sequence, and each MKK protein contained the canonical consensus sequences for serine/threonine protein kinases, as well as a plant-specific phosphorylation target site motif (S/TxxxxxS/T) within the activation loop.

2.2. Phylogenetic analysis of maize MKK proteins

Multiple alignments of MKK proteins were carried out using the Clustal X v1.83 program. The protein sequences of *Arabidopsis* and rice MKK were obtained from the TIGR database and phylogenetic analysis was performed with MEGA5.0 program by neighbor-joining method and the bootstrap test was carried out with 1000 replicates.

2.3. Plant materials and growth conditions

Maize seedlings (*Z. mays* L. cv Zhengdan 958) were grown in Hoagland's solution (pH 6.0) under greenhouse conditions at 22/26 °C (night/day) with a photosynthetic active radiation of 200 μmol m^{-2} s^{-1} and a photoperiod of 16/8 h (day/night) for 2 weeks. Samples were collected and were immediately frozen in liquid N_2 for further use.

2.4. cDNA cloning

Total RNAs were extracted according to the instructions of Trizol reagent (Invitrogen, Carlsbad, CA, USA) from leaves of maize seedlings with different treatments. The first strand cDNAs were synthesized using First Strand cDNA Synthesis kit (Fermentas, USA). Fifteen MAPK and four MKK genes were selected to be amplified by PCR using primers designed based on the predicted sequences. PCR amplifications were performed under the condition of pre-denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 50–60 °C for 50 s and 72 °C for 1 min. The expected PCR fragment was cloned into the PMD18-T vector and sequenced. The primers used are described in Table S1.

2.5. Yeast two hybrid assays

The Matchmaker Gold GAL4 Two-Hybrid System (Clontech, TAKARA, Dalian division, China) was used for the analysis of the specific interaction between ZmMPKs and ZmMKKs. Briefly, the ORFs of *ZmMKKs* were independently fused to the GAL4 DNA-binding domain in the bait plasmid pGBKT7, and ORFs of *ZmMPKs* were cloned to the GAL4 activation domain in the prey vector pGADT7. Different combinations of plasmids were transformed into the yeast strain Y2HGold, and transformants were plated on selective SD medium (SD-Leu-Trp and SD-Leu-Trp-His-Ade/AbA/X-α-Gal) and grown for 3–5 d at 30 °C as described in the manufacturer's instructions.

2.6. Expression analyses of MKK genes

Microarray expression data from the Maize eFP database (http://bar.utoronto.ca/efp_maize/cgi-bin/efpWeb.cgi) using identified MKK ID (Table 1). The expression profiles were clustered using Cluster 3.0 with Euclidean distances and the hierarchical cluster method of complete linkage clustering.

2.7. Oxidative stress tolerance assays

Surface-sterilized seeds from *ZmMKK4*-overexpressing lines and WT were germinated on MS plates supplemented with different concentration of MV. Seeds were stratified by incubation in the dark at 4 °C for 3 d prior to placing them in the light. Germination rates were scored after 7 d. Seedlings of transgenic lines and WT grown on MS medium for 7 d were transferred to MS medium supplemented with MV for 10 d before the photographs were taken.

3. Results and discussion

3.1. Genome-wide identification of MKK family in maize

A search of published *Z. mays* genome database (http://www.maizesequence.org/index.html) and NCBI database using *Arabidopsis* and rice MKK sequences as query identified 9 MKK genes including 4 already known MKKs in maize. In Table 1, a total

Table 1
Characteristics of maize MKKs and their function.

Name	Other names	ID	cDNA (bp)	Amino acid	MW (kDa)	pI	T-loop	Group	Function
ZmMKK1		GRMZM2G400470_T03	1310	350	38.8	6.3	–	A	Inducible by NaCl, ETH, PEG, ABA, H_2O_2 and 4 °C [26]
ZmMKK3-1	*ZmMKK3*	JN972438[a]	1850	523	58.5	5.7	–	B	Inducible by H_2O_2, PEG and ABA [24]
ZmMKK3-2		GRMZM2G004468_T05	2382	561	62.9	5.9	–	B	
ZmMKK3-3		GRMZM2G367411_T01	900	299	33.3	5.2	–	B	
ZmMKK4		GU942956[b]	1341	357	38.8	9.4	–	C	Inducible by NaCl, PEG, H_2O_2 and 4 °C. Down regulated by ABA [29,30]
ZmMKK5	*ZmMAPKK1*	GRMZM5G834697_T01	1739	347	37.6	9.4	–	C	
ZmMKK6	*ZmMEK1*	GRMZM2G167856_T01	1773	355	40.0	5.6	–	A	Inducible by NaCl, PEG, ABA, SA and 4 °C [31]
ZmMKK10-1		GRMZM2G130213_T01	1838	375	40.1	7.3	–	D	
ZmMKK10-2		GRMZM2G344388_T01	1435	401	43.0	6.5	–	D	

[a] and [b] GenBank accession numbers.

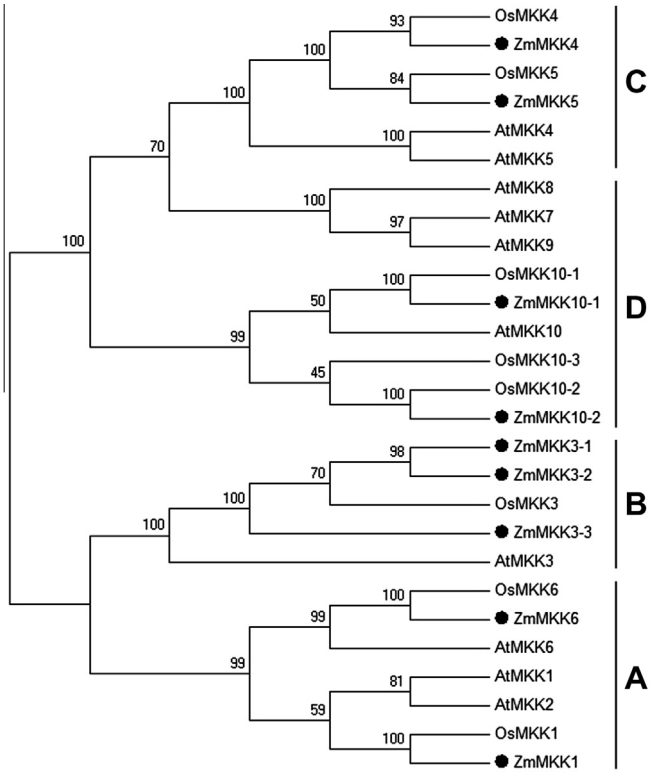

Fig. 1. Phylogenetic tree of MKKs from maize, rice and *Arabidopsis*. Neighbor-joining tree was created using MEGA5.0 program with 1000 bootstrap using full length sequences of 9 maize, 8 rice, and 10 *Arabidopsis* MKK proteins.

of 9 MKKs were listed, including accession number, amino acid number, predicted protein size and isoelectric point (pI) for each MKK gene.

3.2. Phylogenetic analysis, conserved domain and gene structural organization of MKK genes in maize

To study the phylogenetic relationship among maize, *Arabidopsis*, and rice, we constructed an unrooted phylogenetic tree of MKK proteins via MEGA5.0. As shown in Fig. 1, phylogenetic analysis indicated that MKKs could be divided into four groups (A, B, C, D). Among them, the orthologs of *AtMKK7-9* were not found in maize (Fig. 1), which was as the case with rice and *B. distachyon* MKKs [1,10,14]. Compare with *MKK3* from *Arabidopsis* and rice, maize *MKK3* expanded with three genes. All 9 MKK proteins also contained 11 subdomains of protein kinases with serine/threonine specificity that was conserved in eukaryotic organisms (Fig. 2). The S/TxxxxxS/T motif, which includes the serine/threonine residues whose phosphorylation is necessary for MKK activation and is a characteristic feature of MKK, was also conserved in the all protein sequences between subdomains VII and VIII except ZmMKK10-1 and ZmMKK10-2. Furthermore, ZmMKK3-1 and ZmMKK3-2 had a unique C-terminal NTF2-like domain, which may be essential for its nuclear localization [15]. However, ZmMKK3-3 did not possess the NTF2 domain in its C- terminus.

The patterns of exon–intron structure can provide important insights into the evolution of gene families. Therefore, we determined the data regarding exon and intron distribution for the coding regions of *ZmMKKs*. The *MKK* genes displayed two strikingly different exon–intron structural patterns in maize (Fig. S1). Members of group C and D *MKKs* only had one exon, whereas the

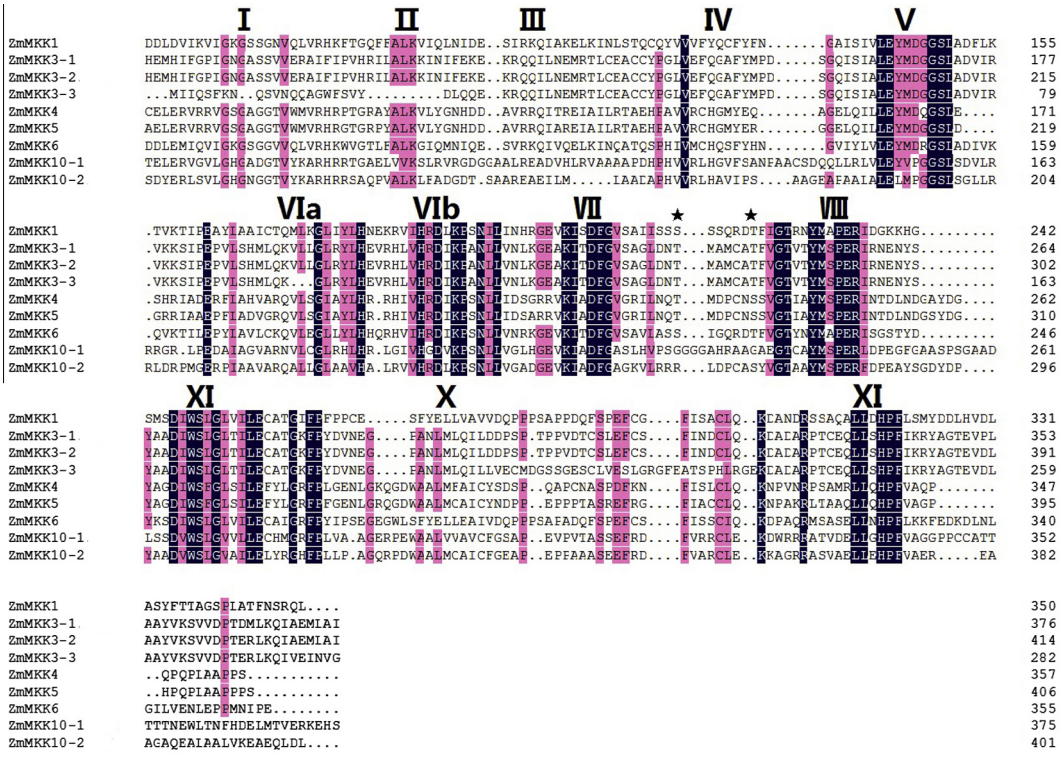

Fig. 2. Alignment of ZmMKKs. Protein kinase subdomains are shown with numerals (I–XI) on top and the conserved sequence motif S/TxxxxxS/T for phosphorylation is indicated by asterisks.

Table 2
Comparative analyses of four ZmMKKs with fifteen ZmMPKs using yeast two-hybrid. The yeast strain Y2HGold containing the indicated plasmid combinations was grown on the selective medium minus His, Leu, Trp and plus Aureobasidin A and X-α-Gal (SD/-Ade-His-Leu-Trp/AbA/X-α-Gal, QDO/X/A).

	pGBKT7	ZmMKK1	ZmMKK3-1	ZmMKK6	ZmMKK10–2
pGADT7	–	–	–	–	–
ZmMPK6-2	–	–	++	–	++
ZmMPK3-1	–	–	–	–	–
ZmMPK3-2	–	–	–	–	–
ZmMPK4	–	++++	–	–	–
ZmMPK7	–	–	++++++	–	–
ZmMPK2	–	–	–	–	–
ZmMPK12	–	–	–	–	–
ZmMPK20-1	–	–	–	–	–
ZmMPK20-2	–	–	–	–	–
ZmMPK18-2	–	–	–	–	–
ZmMPK19	–	–	–	–	–
ZmMPK17-1	–	–	–	–	–
ZmMPK17-3	–	–	–	–	–
ZmMPK16	–	–	–	–	–
ZmMPK15		–	–	–	–

– Represents no interaction; ++++++ represents strong interaction.

group A and group B *MKKs* possessed 8–9 exons in maize, poplar and *Arabidopsis*. In addition, *ZmMKK3-2* and *ZmMKK3-3* had a completely unique exon–intron structure. Intron phase was also assessed for all *ZmMKK* gene models. For *ZmMKKs* (82.8%), the majority of introns were within phase 0, while 17.2% of phase 2 was found in *ZmMKKs*. This conserved exon numbers and intron phase in each subgroup among species supported their close evolutionary relationship and the introduced classification of subgroups.

3.3. Analyses of the interactions between MAPK and MKK family members

At present, only a few MAPK cascade have been studied in detail in various plant species. AtMKK2-AtMPK4/MPK6 cascade was involved in cold and salt stress [16]. AtMKK1/2-AtMPK4 cascade and AtMKK4/5-AtMPK3/6 cascade were shown to play an important role in plant innate immunity [17,18]. OsMKK4-OsMPK3/6 was reported to mediate the MAMP signal [19], whereas OsMKK6-OsMPK3 can be activated by cold stress [20]. In *Malus domestica*, MdMKK1–MdMPK1 signaling cascade was involved in ABA signaling [21]. Since the nature of protein kinase activities depends on their direct physical encounters, we conducted a comprehensive directed yeast two-hybrid to define the new ZmMKK-ZmMPK interactions. Four MKK genes and 15 MAPK genes were cloned into DNA binding domain and activation domain plasmids respectively. As shown in Table 2, ZmMKK3-1 exhibited a significant interaction with ZmMPK7. The interactive partner between ZmMKK3-1 and ZmMPK7 was also found in their homologs of *Arabidopsis*. In *Arabidopsis*, MKK3–MPK7 cascade participated in pathogen signaling [22]. More recently, Chen et al. also detected that BdMPKK3-1 exhibited a significant interaction with BdMPK7-1 [14]. Furthermore, in previous studies, both *ZmMKK3* and *ZmMPK7* transcriptions were up-regulated by ABA, H_2O_2 and PEG, and *ZmMKK3* and *ZmMPK7* conferred osmotic stress in transgenic tobacco through the reduced H_2O_2 accumulation [23,24]. In addition, ZmMKK3 also interacted with ZmMPK6-2 which was involved in response to diverse environment cues. In *Arabidopsis*, AtMKK1/2-AtMPK4 cascade negatively regulates plant immune response [17,25]. We also found that ZmMKK1 interacted only with ZmMPK4 among the 15 ZmMPKs, and ZmMKK1 and ZmMPK4 showed the highest similarity with AtMKK1 and AtMPK4 respectively. More recently, Cai et al. reported that *ZmMKK1* conferred

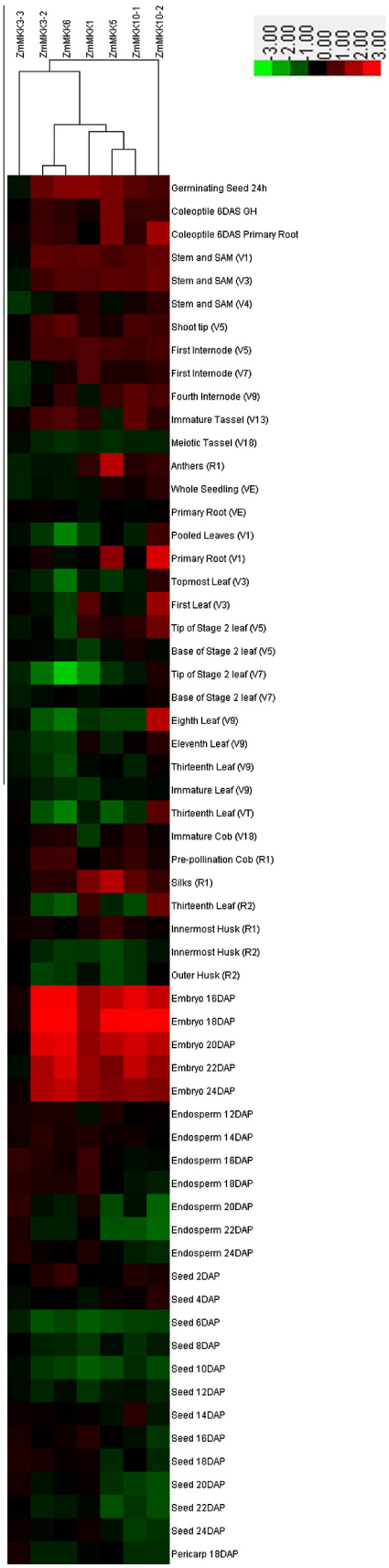

Fig. 3. The expression profile of 7 *MKK* genes in maize different tissues and developmental stages. The scale representing the relative signal intensity values is shown above.

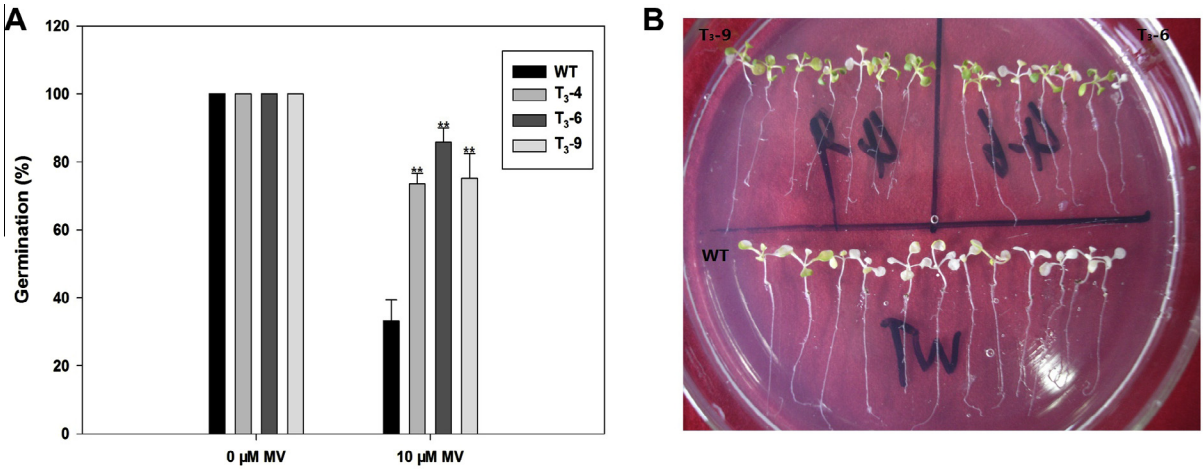

Fig. 4. Germination and early seedling development of *ZmMKK4*-overexpressing lines and WT plants under oxidative stress. A, Germination rate under oxidative stress. WT and over-expression seeds on the medium containing 10 μM MV. Germination rate was counted 7 d and after sowing. B, Phenotypic analysis of 7 d old WT and over-expression early seedlings in the presence of 10 μM MV for 10 d. Each column represents an average of three replicates, and bars indicate SDs. **Indicates significant differences in comparison with the WT at $P < 0.01$.

chilling stress tolerance, and enhanced the resistance to biotrophic pathogens while improved the sensitivity to the necrotrophic pathogens [26]. Furthermore, *ZmMPK4* also was involved in systematic acquired resistance (our unpublished data). Surprisingly, ZmMKK10-2, a family member that lacked the MKK conservative sequence (S/TxxxxxS/T), was found to interact with ZmMPK6-2, which was not found in their homologs of *Arabidopsis* and other plants. However, *in vitro* phosphorylation assays are needed to confirm their connections. Intriguingly, we found that there were no interactions with ZmMKK6, since MKK6 has been proposed to use MPK4, MPK11 and MPK13 as substrates in a signal transduction pathway involved in regulation of cytokinesis in *Arabidopsis* [27,28].

3.4. Expression pattern of MKK genes in maize different tissues and developmental stages

MKKs are known to be involved in various physiological processes and at different stage of plant development. Expression profiling can provide useful clues to gene functions. To investigate the expression profiles of ZmMKK genes in maize different tissues, we performed a comprehensive expression analysis using the publicly available microarray data (Fig. 3). *ZmMKK3-2* and *ZmMKK6* had lower expression in leaf, but higher expression in embryo, suggesting that those two genes may play an important role in embryo development. *ZmMKK10-1* and *ZmMKK10-2* had lower expression in seed than in other organs, which indicated that *ZmMKK10* may negatively control seed development. *ZmMKK5* was expressed with high abundance in anthers and silks, but had lower expression in endosperm and seed, which suggested that *ZmMKK5* may play different role in different development stage. Genes expressed in specific tissues may indicate that the specific function of these genes in related to forming specific tissue or corresponding stage development. From these results, most of MKK genes may play important roles in maize development.

3.5. Overexpression of ZmMKK4 enhanced oxidative stress tolerance

In previous report, *ZmMKK4* has been found to enhance osmotic stress through reactive oxygen species (ROS) scavenging in transgenic plants [29]. To determine whether *ZmMKK4* could confer increased oxidative stress tolerance in plants, transgenic *Arabidopsis* plants constitutively over expressing *ZmMKK4* were generated.

Homozygous T_3 *ZmMKK4*-overexpressing lines and wild-type (WT) seeds were germinated on MS plates supplemented with 10 μM methyl-viologen (MV), an agent that produces ROS and causes oxidative stress. On MS plates without MV, the *ZmMKK4*-overexpressing lines did not show any significant difference from WT during germination (Fig. 4A). Compared with the WT, *ZmMKK4*-overexpressing lines showed a significantly higher germination rate on the medium containing 10 μM MV. T_3-4, T_3-6 and T_3-9 showed 78%, 90% and 80% germination rates, respectively, while the WT had a germination rate of 33% on the medium containing 10 μM MV (Fig. 4A). The sensitivity of early seedling growth to oxidative stress was also assayed. Seedlings grown on MS medium for 7 d were transferred to MS medium supplemented with 10 μM MV for 10 d. As shown in Fig. 4B, on medium containing10 μM MV, the WT seedlings showed much more severe cotyledon bleaching or chlorosis than the transgenic plants. These results suggest that overexpression of *ZmMKK4* appears to confer oxidative stress tolerance in seed germination and early seedling growth of transgenic *Arabidopsis*.

4. Conclusion

Overall, our work identified 9 MKK genes in maize. Phylogenetic analysis of MKKs from maize, rice and *Arabidopsis* has classified them into four subgroups. We also characterized the conserved protein motif, gene duplications, exon–intron organization of MKK family, and our results suggest that the genome duplications contributed to the expansion of *ZmMKKs*. Using yeast two-hybrid, we defined four ZmMKK–ZmMPK interactions. Finally, in the present study, we focus on the expression patterns of *ZmMKK* genes under different tissues and developmental stages, although plant MAPK cascade also play key roles in responses to environmental cues. It will be interesting to explore their functions in abiotic and biotic stresses in future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.008.

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