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BRIEF COMMUNICATION

Expression Analysis of Segmentally Duplicated *ZmMPK3-1* and *ZmMPK3-2* genes in Maize

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Abstract Gene duplication and alternative splicing (AS) are two evolutionary mechanisms that can increase functional diversification of genes. Here, we found that a previously uncharacterized *ZmMPK4* (*ZmMPK3-1b* in this research) is a splicing variant. *ZmMPK3-1* can undergo AS by retaining the third intron (90 nucleotides) to generate an atypical mitogen-activated protein kinase (MAPK) gene: *ZmMPK3-1b*. Furthermore, we found that *ZmMPK3-1* and *ZmMPK3-2* were segmentally duplicated genes in the maize genome, located on chromosomes 9 and 1, respectively. *ZmMPK3-1* and *ZmMPK3-2* were expressed differentially in maize root, stem, and leaf. *ZmMPK3-1* was expressed predominantly in roots under normal growth conditions, whereas *ZmMPK3-2* accumulated predominantly in stem and leaf. In leaf, both *ZmMPK3-1* and *ZmMPK3-2* were regulated by ABA (100 μM) or NaCl (200 mM). AS of *ZmMPK3-1* occurred mainly in leaves in our tested organs. In leaves, splicing variant *ZmMPK3-1a*, but not *ZmMPK3-1b*, is regulated by ABA (100 μM) or NaCl (200 mM).

Keywords Alternative splicing · Gene expression · Segmental duplication · *ZmMPK3-1* · *ZmMPK3-2*

Abbreviations

ABA	Abscisic acid
CTAB	Chemical cetyl trimethylammonium bromide
MAPK	Mitogen-activated protein kinase
NIP	Nearly identical paralog
UTR	Untranslated region

Introduction

Gene duplication and alternative splicing (AS) are two major evolutionary mechanisms that can increase the functional diversification of genes (Jin et al. 2008; Yuan et al. 2009). Gene duplications, often followed by some sort of divergence, contribute to the establishment of new gene functions. Gene duplications are thought to function in the diversification of gene families (Freeling 2009). AS is another mechanism that contributes to gene diversity by generating different transcripts from a single mRNA by differential inclusion or exclusion of regions of the precursor mRNA (Reddy 2007). Results of recent studies indicate that AS is prevalent in plants, impacting at least 30 % of all expressed protein-coding genes (Chen et al. 2007; Wang and Brendel 2006).

Mitogen-activated protein kinase (MAPK) cascades are universal signaling modules in eukaryotes, including yeasts, animals, and plants (Heinrich et al. 2012; Liu et al. 2011; MAPK Group 2002; Samajova et al. 2011). A typical MAPK cascade consists of three protein kinases, MAPK

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kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. MAPKKK, MAPKK, and MAPK sequentially phosphorylate the corresponding downstream substrates (MAPK Group 2002). MAPK cascades have been involved in plant growth and development, biotic and abiotic stresses (Samajova et al. 2011). The versatile roles of MAPK cascades can be explained, in part, by the fact that there are multiple genes in each of the three tiers of kinases, e.g., in Arabidopsis, there are 20 MAPKs, 10 MAPKs, and about 60 MAPKKKs (MAPK Group 2002). Other plant species have similar numbers. Gene family analysis has revealed that segmental duplication is expected to contribute to the expansion of the MAPK-cascade gene family (Koo et al. 2007; Lin et al. 2010).

The generation of a high number of different isoforms by AS in MAPK-cascade genes also plays an important role in regulating the function of MAPK cascades. The AS of MAPK-cascade genes may influence kinase activity, protein stability, subcellular localization, and binding properties with substrates and effectors. AS of MAPK-cascade genes is a feature common to both mammals and plants. AS of human MAP kinases *JNK1*, *JNK2*, and *JNK3* yields products containing different carboxy-termini, and thus proteins exhibiting different roles (Gupta et al. 1996). *ERK1* and *p38* also undergo AS to generate different isoforms (Enslen et al. 1998; Yung et al. 2001). In *Arabidopsis*, two isoforms of *cANP1* have been identified. The longer splice variant (*cANP1L*) has an intron-like sequence for the kinase-unrelated region. Protein encoded by *cANP1L* is functional in the mating pheromone-responsive signal pathway of yeast. However, retention of the intron-like sequence in *cANP1L* impairs protein activity when compared with the shorter protein in yeast (Nishihama et al. 1997). Another *Arabidopsis* gene, *AtMPK13*, has also been found to generate at least three splice variants, one with complete splicing of five introns, one with the fourth intron retained, and one with the fifth intron retained. Intron retention of *AtMPK13* generates proteins with no kinase activity (Lin et al. 2010). In rice, *OsMAPK5* has been demonstrated to generate at least two differentially spliced transcripts. *OsMAPK5b* is smaller than *OsMAPK5a* as a result of a 104-amino-acid deletion, leading to *OsMAPK5b* with no kinase activity (Xiong and Yang 2003). *OsBWMK1*, another MAPK of rice, generates three splice variants with different expression and subcellular localization (Koo et al. 2007). The different expression of *OsBWMK1* splice variants is due to the result of alternative promoter usage (Koo et al. 2009). *MIK* is a maize gene coding for a GCK-like MAP4K (Llompart et al. 2003). *MIK* generates at least four different mature mRNAs that accumulate with particular expression during maize development (Castells et al. 2006). Analyses of the maize genome have revealed that, although the average number of introns per gene is about the same in corn and *Arabidopsis*, maize genes have more alternatively spliced isoforms (Alexandrov et al. 2009). Here, we showed that a previously uncharacterized *ZmMPK4* (*ZmMPK3-1b* in this research) is one such splicing variant. *ZmMPK3-1* and *ZmMPK3-2* are segmentally duplicated genes in the maize genome, and the expression profiles of *ZmMPK3-2* and *ZmMPK3-1* splice variants differ.

Materials and Methods

Plant Materials, Growth Conditions, and *Agrobacterium*-Mediated Transient Gene Expression

Maize cultivar Zhengdan958 was used in this research. Maize seedlings (14-days-old) were cultivated and treated as described previously (Gu et al. 2010; Liu et al. 2012). Briefly, maize seeds were washed several times with tap water and soaked in distilled water for germination. Seedlings were grown in Hoagland's solution (pH 6.0) under greenhouse conditions at 22/26 °C (night/day) and a photoperiod of 14/10 h (day/night) for 2 weeks.

Extraction of RNA and DNA

RNA and DNA was extracted as previously described (Liu et al. 2012). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) method. Genomic DNA was isolated by the CTAB method.

Northern and Southern Blot Analyses

Total RNA (30 μg) and genomic DNA (30 μg) were used for northern and Southern blots, respectively. Hybridization analyses were performed as described (Liu et al. 2012). Probes were labeled with [a-^{32}P]dCTP according to the manufacturer's instructions (Primer-a-Gene Labeling System, Promega, Madison, WI). The primers used in the hybridization analyses are listed in Supplementary Table S1.

Dot Blot Assay

To confirm the specificity of probes used for northern blot analysis, we performed dot blot assay as described by Park et al. (2010). Plasmid DNA containing cDNAs of either the *ZmMPK3-1a*, *ZmMPK3-1b* or *ZmMPK3-2* genes was blotted onto nylon membranes (Amersham, Pharmacia, Little Chalfont, UK). The membranes were hybridized with [a-^{32}P]dCTP-labeled probes. Washes and detection were performed as above for northern blots.

Semi-Quantitative RT-PCR Analysis

Total RNA was extracted using Trizol (Invitrogen) from samples collected at various intervals, incubated with RNAse free DNAse I (Invitrogen) overnight, and reverse transcribed employing SuperScript II (Invitrogen) according to the manufacturer's protocols. *Zmactin* was used as control for equal loading. Primers are listed in Supplementary Table S1.

Bioinformatic Analysis

The genomic context, and chromosomal location of *ZmMPK3-1* and *ZmMPK3-2* were performed using sequences from the maize genome sequencing project (http://www.maizegenome.org). Intron and exon organization of *ZmMPK3-1* and *ZmMPK3-2* were determined on http://www.phytozome.net/ (Goodstein et al. 2012). Sequence alignments of introns were displayed as a sequence logo with WebLogo (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al. 2004).

Results and Discussion

The Previously Identified *ZmMPK4* is One of Two Spliced Transcripts of *ZmMPK3-1*

Maize *ZmMPK4* was a previously isolated cDNA with no characterized function (Berberich et al. 1999). The cDNA of *ZmMPK4* was 1,647 base pairs (bp) long and encoded a protein of 406 amino acids. Remarkably, this gene encoded a protein with an unexpected insertion of 30 amino acids between subdomains IX and X (Berberich et al. 1999). This insertion has not been found in other plant MAPKs identified so far (Supplementary Fig. S1). In our attempt to test the expression of *ZmMPK4*, we detected two DNA bands in leaves and the predominantly amplified DNA products were about 500 bp, although the primers were expected to generate DNA products of 590 bp. This result was confirmed by DNA marker and DNA products from *ZmMEK1* (589 bp) (Fig. 1a).

Upon cloning and sequencing, the DNA products from the larger band were found to be 590 bp and matched most of the *ZmMPK4* sequence (90 bp insertion included). The DNA products from the smaller band were 498 bp, containing two highly similar sequences (93.37 %) and matched the parts of *ZmMPK4* sequence with no 90 bp insertion. From our sequencing results, we presumed that the smaller band (498 bp) contains DNA products from two highly identical genes or nearly identical paralogs (NIPs) (Emrich et al. 2007). Before the availability of the maize genomic sequence (Wilson 2008), we conducted a first round Southern blot to test the copy number of *ZmMPK4*. As shown in Fig. 1b, we detected two bands in *Eco*RI- or *Hin*dIII-digested genomic DNA, indicating that *ZmMPK4* was a two-copy gene. If the insertion was excluded, *ZmMPK4* is the homolog of *Arabidopsis AtMPK3* (Supplementary Fig. S2). To avoid confusion, and according to *Arabidopsis* MAPK nomenclature, we renamed *ZmMPK4* as *ZmMPK3-1*. Without accounting for the 90 bp insertion, we found that the DNA products from the larger band (590 bp) fully match one product of the smaller band (498 bp). Therefore, we concluded that *ZmMPK3-1* has at least two AS variants. We named the splicing variant without the 90 bp insertion *ZmMPK3-1a*, and the splicing variant with the 90 bp insertion *ZmMPK3-1b*.

ZmMPK3-1 and *ZmMPK3-2* are Segmentally Duplicated Genes in the Maize Genome

The availability of the genomic sequence of maize B73 provides a convenient approach to analyze the genomic information of *ZmMPK3-1*. In silico analysis showed that *ZmMPK3-1* is located on the long arm of chromosome 9 (Fig. 1c). In searching for a highly identical gene or NIP of *ZmMPK3-1*, we found another gene located on the short arm of chromosome 1 with 92.48 % identity to *ZmMPK3-1* (Supplementary Fig. S3). Both these genes were homologs of *Arabidopsis AtMPK3* and encoded proteins sharing 90.1 % identity (Supplementary Fig. S2, S4). The gene located on chromosome 1 was named *ZmMPK3-2* (Fig. 1c). One product of the smaller band (498 bp) corresponded to just part of *ZmMPK3-2*. Therefore, the DNA products from the smaller band (498 bp in Fig. 1a) were a mixture of *ZmMPK3-1* and *ZmMPK3-2*.

The genomic organizations of *ZmMPK3-1* and *ZmMPK3-2* were highly similar to each other. Both *ZmMPK3-1* and *ZmMPK3-2* consisted of five exons. The exon organization (sequence and length) was highly conserved. Furthermore, intron length at comparable positions was also highly similar. In silico analyses indicated that *ZmMPK3-1* and *ZmMPK3-2* were extremely closely related to each other and that the two genes have likely evolved as a result of a recent segmental gene duplication event. We conducted a second round of Southern blot analysis using a specific probe generated from the 3′ portion of *ZmMPK3-2*. The result showed that the genomic segment containing the *ZmMPK3-2* gene contributes to the previously identified two-copy gene (Fig. 1b). During our analysis, Wang et al. (2010) published the a paper on *ZmMPK3* (*ZmMPK3-2* in our research). However, the latter authors mistakenly located *ZmMPK3* (*ZmMPK3-2*) on chromosome 2. Furthermore, the primers used by Wang et al. match both *ZmMPK3-1* and *ZmMPK3-2*. Therefore, the expression results from the research of Wang et al. can be recognized as a mixed analysis of *ZmMPK3-1* and *ZmMPK3-2*.

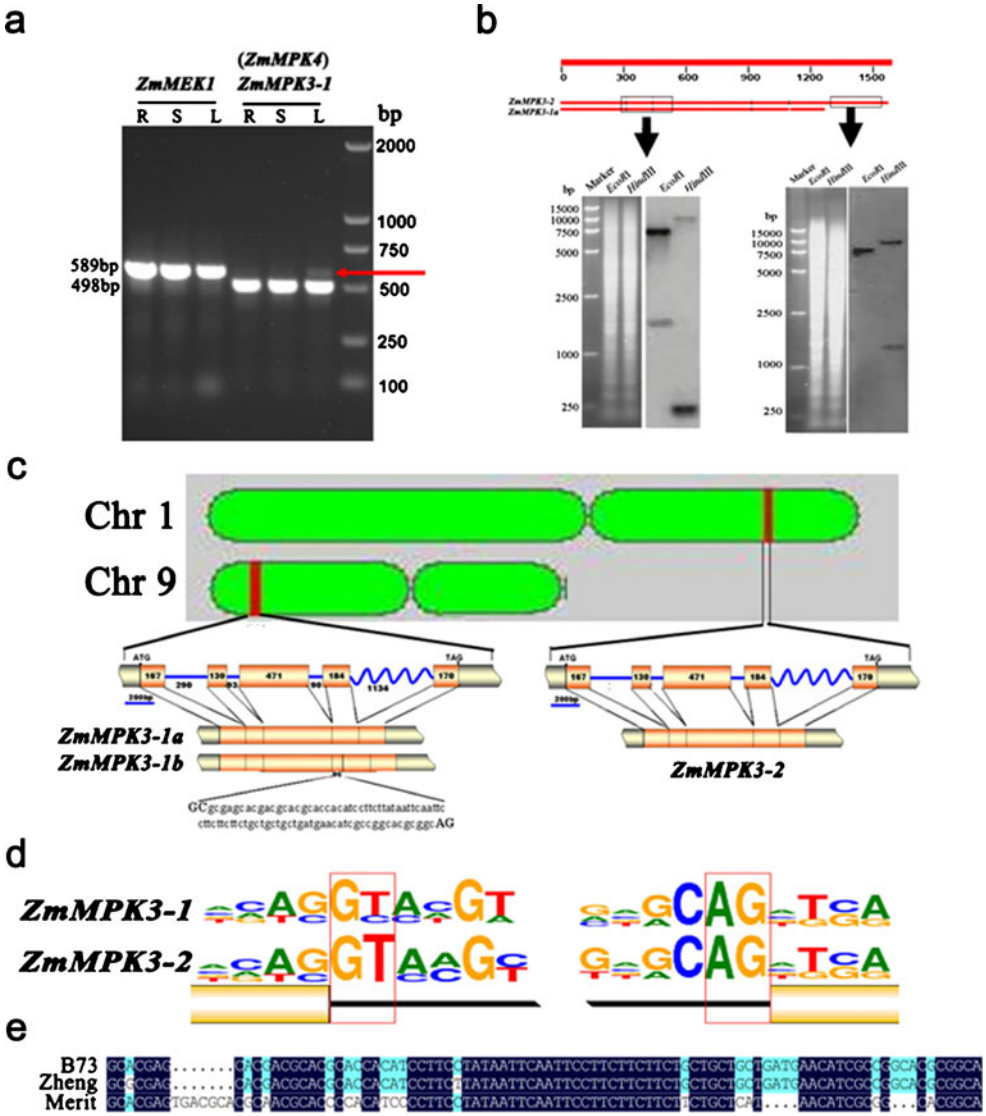

Fig. 1a–e Genomic analysis of *ZmMPK3-1* and *ZmMPK3-2* genes. **a** Expression analysis of *ZmMEK1* and *ZmMPK3-1*. *ZmMEK1* and *ZmMPK3-1* were amplified from root (*R*), stem (*S*), or leaf (*L*) by 38 cycles of PCR. *ZmMEK1* was used to mark the position of the 589 bp band. *Arrow* indicates the splicing variant of *ZmMPK3-1b*. **b** Southern blot analysis of *ZmMPK3-1* and *ZmMPK3-2*. Genomic DNA (30 μg) from leaves was digested with *Eco*RI or *Hin*dIII, separated by electrophoresis on an agarose gel, and blotted onto nylon membranes. The common region or divergent region of *ZmMPK3-1* and *ZmMPK3-2* was used as a probe. **c** Exon–intron organization and chromosome localization of *ZmMPK3-1* and *ZmMPK3-2*. The size of genomic DNA was reduced by the ratio indicated. Genomic DNA is presented as *lines* (introns) and *boxes* (exons). Start (ATG) codon, stop (TAG) codon and numbers of base pairs are marked. The retention of the third intron in *ZmMPK3-1b* is *outlined*. **d** Sequence logo and comparative analysis of the exon–intron junction of *ZmMPK3-1* and *ZmMPK3-2*. Sequence alignments of the exon–intron junction (ten nucleotides) were displayed as a sequence logo with WebLogo (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al. 2004). The height of each nucleotide represents the relative frequency of the nucleotide at that position. *Boxes* represent the position of the exon sequence, and the line indicates the intron sequence. **e** Sequence alignment of the third intron of *ZmMPK3-1* from maize cultivar B73, Zhengdan 958, and Merit

Since *ZmMPK3-1* can undergo AS, and since *ZmMPK3-1* and *ZmMPK3-2* are highly similar to each other, it is reasonable to ask whether *ZmMPK3-2* can undergo AS. Analysis of the sequencing results revealed only the 590 bp product matching part of *ZmMPK3-1* among the 100 clones sequenced. We further analyzed the introns of *ZmMPK3-1* and *ZmMPK3-2* and found that most of the introns (7 of 8) were the GU–AG type—the type found most typically in plants (Sheth et al. 2006). However, the third intron of *ZmMPK3-1* was the GC–AG type (Fig. 1c, d), indicating that AS of *ZmMPK3-1* may correlate with the type of third intron. We next examined the introns of *ZmMPK3-1* and *ZmMPK3-2* in three different inbred lines (B73, Zhengdan 958, and Merit). Most of the introns in the three inbred lines were conserved. Although all of the third introns of *ZmMPK3-1* in the three inbred lines were of the

GC–AG type, the sequence of the introns did not fully match with one another (Fig. 1e), indicating that further evolution had occurred in the third intron of *ZmMPK3-1* after the divergence of the sweet corn (Merit) and the corn lines B73 and Zhengdan 958.

Our analyses support the conclusion that *ZmMPK3-1*, but not *ZmMPK3-2*, can retain the third intron for AS. Although there is no evidence, it is possible that AS of *ZmMPK3-1* is correlated with the type of third intron. It seems that GC–AG type intron here could influence splice site recognition or other mechanisms, leading to the retention of the intron.

Organ-Specific Expression of *ZmMPK3-1a*, *ZmMPK3-1b*, and *ZmMPK3-2*

We next tested the expression profile of *ZmMPK3-1a*, *ZmMPK3-1b*, and *ZmMPK3-2* in maize seedlings. Although the coding sequences of *ZmMPK3-1* and *ZmMPK3-2* shared high identity, the 3′-untranslated region (UTR) of the two genes shared only 46.3 % identity (Supplementary Fig. S5). Therefore, gene-specific probes were generated based on a portion of the 3′-UTR. A dot-blot assay was used to test the specificity of the probes and showed that the *ZmMPK3-1* probe did not cross-hybridize with plasmid DNA containing cDNA of *ZmMPK3-2*. The *ZmMPK3-2* probe did not cross-hybridize with plasmid DNA containing cDNA of *ZmMPK3-1a* or *ZmMPK3-1b* (Fig. 2a).

Northern blot analysis showed that transcripts of *ZmMPK3-2* accumulated predominantly in stems and leaves, whereas transcripts of *ZmMPK3-1* accumulated mainly in roots (Fig. 2b). Although the probe of *ZmMPK3-1* could detect both *ZmMPK3-1a* and *ZmMPK3-1b*, we detected only one band in our Northern blot analysis. The RNA band represented a mixture of *ZmMPK3-1a* and *ZmMPK3-1b* (Fig. 2b). Therefore, we used RT-PCR to test the specific expression of *ZmMPK3-1a* and *ZmMPK3-1b*. As shown in Fig. 2c. *ZmMPK3-1b* was expressed in stem and leaf and was almost undetectable in root. *ZmMPK3-1a*, however, was expressed in root, stem, and leaf, with higher amounts detectable in root. These results showed that

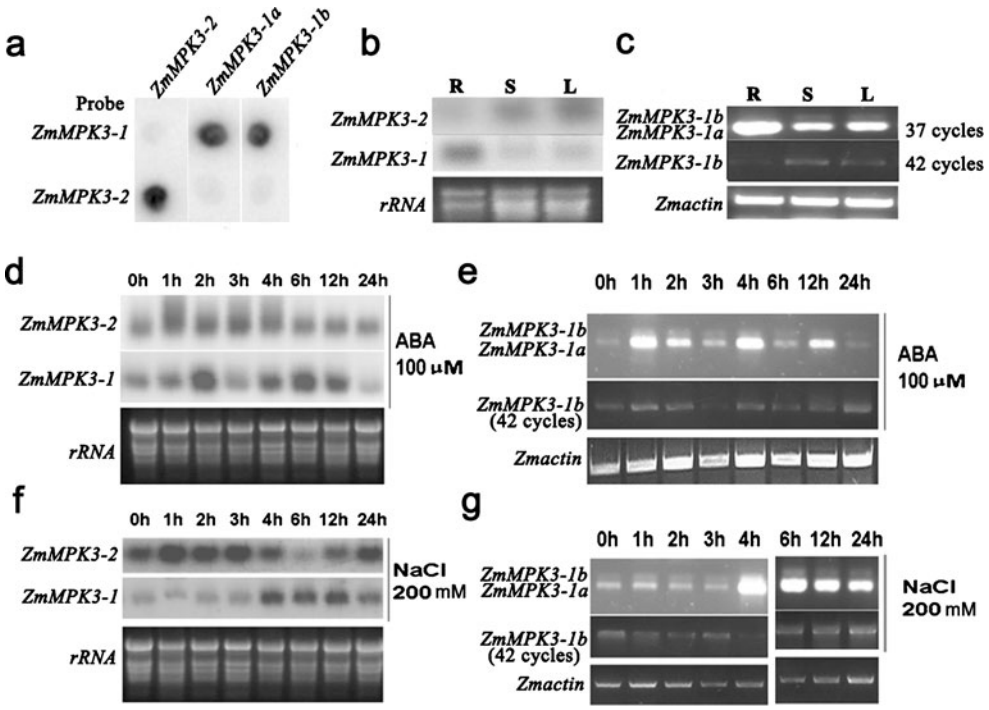

Fig. 2a–g Expression analysis of *ZmMPK3-2*, *ZmMPK3-1a*, and *ZmMPK3-1b*. **a** Dot blot assay of probes for *ZmMPK3-1* and *ZmMPK3-2*. Specific probes were generated from the 3′ UTR portions of *ZmMPK3-1* and *ZmMPK3-2*. The probe for *ZmMPK3-1* recognized plasmid DNA of both *ZmMPK3-1a* and *ZmMPK3-1b*. **b** RNA gel blot analysis of *ZmMPK3-1* and *ZmMPK3-2* expression in maize root (*R*), stem (*S*), and leaf (*L*). **c** RT-PCR analysis of *ZmMPK3-1a* and *ZmMPK3-1b* expression in maize root (*R*), stem (*S*), and leaf (*L*). **d** Time course analysis of *ZmMPK3-1* and *ZmMPK3-2* expression in leaf under abscisic acid (ABA) (100 μM) treatment. **e** RT-PCR analysis of *ZmMPK3-1a* and *ZmMPK3-1b* expression in leaf under ABA (100 μM) treatment. **f** Time course analysis of *ZmMPK3-1* and *ZmMPK3-2* expression in leaf under NaCl (200 mM) treatment. **g** RT-PCR analysis of *ZmMPK3-1a* and *ZmMPK3-1b* expression in leaf under NaCl (200 mM) treatment. For RNA gel blot analysis, root (*R*), stem (*S*), and leaf (*L*, untreated or treated with ABA/NaCl) were collected from 14-day-old maize seedlings. Total RNA (30 μg) was loaded into each lane of a 1.5 % formaldehyde RNA gel and separated by electrophoresis. Ethidium bromide staining of gels confirmed equal loading of RNA samples. For RT-PCR analysis, total RNA was isolated from roots of 14-day-old maize seedlings. *Zmactin* was used as control for equal loading. The reactions were amplified for cycles as indicated. The experiments were repeated at least three times with similar results and representative results are shown

ZmMPK3-1a, *ZmMPK3-1b*, and *ZmMPK3-2* were expressed differentially in root, stem, and leaf of maize seedlings. The expression of *ZmMPK3-1* in root was due to the transcript of *ZmMPK3-1a*. AS of *ZmMPK3-1* occurred mainly in stem and leaf. The organ-specific expression of *ZmMPK3-1* and *ZmMPK3-2* suggested that, although the two genes share high sequence identity, they may play different roles in different tissues.

Expression of *ZmMPK3-1a*, *ZmMPK3-1b*, and *ZmMPK3-2* in Response to ABA and NaCl in Leaves

We next tested whether expression of *ZmMPK3-1a*, *ZmMPK3-1b*, and *ZmMPK3-2* could be regulated by external stimuli. In our preliminary experiments, we found that expression of *ZmMPK3-1* and *ZmMPK3-2* was clearly induced by ABA (100 μM) (Liu 2012) or NaCl (200 mM), but was not affected significantly by salicylic acid (SA; 1 mM), H_2O_2 (10 mM), 30 % PEG6000 (w/v), or methyl jasmonate (MeJA; 100 μM) (data not shown). *ZmMPK3-1* was regulated slightly by high temperature (40 °C) (data not shown). Figure 2d–g shows the effects of ABA (100 μM) or NaCl (200 mM) on expression of *ZmMPK3-1a*, *ZmMPK3-1b*, and *ZmMPK3-2* in maize seedlings within 24 h. ABA (100 μM) could induce expression of both *ZmMPK3-2* and *ZmMPK3-1* (Fig. 2d). The induction of *ZmMPK3-2* by ABA (100 μM) was more transient (within 1 h) than that of *ZmMPK3-1*. As shown in Fig. 2e, the increased expression of *ZmMPK3-1* in response to ABA (100 μM) was contributed mainly by *ZmMPK3-1a*, as ABA (100 μM) did not significantly alter the expression of *ZmMPK3-1b* within 24 h. The increased expression of *ZmMPK3-2* and *ZmMPK3-1* in response to NaCl (200 mM) was likely complementary. NaCl (200 mM) induced strong expression of *ZmMPK3-2* at 1–3 h and down-regulated *ZmMPK3-2* at 6 h. The increased expression of *ZmMPK3-1* was detectable from 4 h to 24 h (Fig. 2f). No clear alteration of *ZmMPK3-1b* was observed within 24 h, indicating that the significant increase of *ZmMPK3-1* was contributed by *ZmMPK3-1a* but not *ZmMPK3-1b* (Fig. 2g).

Expression analysis showed that the segmentally duplicated *ZmMPK3-1* and *ZmMPK3-2* had a dissimilar expression pattern in root, stem, leaf, and in response to ABA (100 μM) or NaCl (200 mM), indicating that unique functional roles may have been distributed to the two genes after evolutionary duplication.

Conclusion

In this research, we reported that previously uncharacterized *ZmMPK4* (*ZmMPK3-1b* in this research) was one of the two splicing variants of *ZmMPK3-1*. Generation of *ZmMPK3-1b* was due to retention of the third intron (90 bp) of *ZmMPK3-1* (Fig. 1c). *ZmMPK3-1b* was expressed in the stem and leaf of maize seedlings (Fig. 2c). In leaf, we did not detect significant alteration in expression of *ZmMPK3-1b* in response to ABA (100 μM), NaCl (200 mM), SA (1 mM), H_2O_2 (10 mM), 30 % PEG6000 (w/v), or MeJA (100 μM) (Fig. 2d–g and data not shown). *ZmMPK3-1* and *ZmMPK3-2* were segmentally duplicated genes in the maize genome. ZmMPK3-1 and ZmMPK3-2 proteins shared 90.1 % identity (Supplementary Fig. S4). *ZmMPK3-1* and *ZmMPK3-2* had a dissimilar expression pattern in root, stem, leaf, and in reponse to ABA (100 μM) or NaCl (200 mM), indicating that unique functional roles may have been distributed to the two genes after evolutionary duplication (Fig. 2). Further study is needed to elucidate the mechanism of AS of *ZmMPK3-1* and to explore the biological function of ZmMPK3-1a, ZmMPK3-1b, and ZmMPK3-2.

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