Annual Review & Research in Biology
3(4): 540-550, 2013

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Different Expression of Segmentally Duplicated *ZmMPK6-1* and *ZmMPK6-2* Paralogues in Maize

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Authors' contributions

This work was carried out in collaboration between all authors and all authors read and approved the final manuscript.

Research Article

Received 21st March 2013
Accepted 8th May 2013
Published 18th June 2013

ABSTRACT

Aims: *ZmMPK6-1* and *ZmMPK6-2* are two genes resulting from segmental duplication in the maize genome. To investigate whether *ZmMPK6-1* and *ZmMPK6-2* had different expression or functions, we designed and conducted this experiment.
Methodology: Adult plants and 10-day-old seedlings of maize were used to study the expression of *ZmMPK6-1* and *ZmMPK6-2* in different tissues. The responses of *ZmMPK6-1* and *ZmMPK6-2* to polyethylene glycol (PEG6000, 25%, w/v), NaCl (200 mM), abscisic acid (ABA, 100 ìM), salicylic acid (SA, 1 mM), or hydrogen peroxide (H_2O_2, 2 mM) were performed in 10-day-old seedlings.
Results: Although *ZmMPK6-1* and *ZmMPK6-2* are highly similar to each other throughout the entire coding sequence (95.32% identity), gene-specific primers can be generated based on the portions of 5'-untranslated region (UTR) of the two genes which share only 37.97% identity. The organ-specific expression of *ZmMPK6-1* and *ZmMPK6-2* in adult plants and 10-day-old showed that, although the two genes share high sequence identity, they have differently expressional patterns in different tissues. They have also dissimilarly expressional patterns in response to PEG6000 (25%, w/v), NaCl (200 mM), ABA (100 ìM), SA (1 mM), or H_2O_2 (2 mM).
Conclusion: *ZmMPK6-1* and *ZmMPK6-2* paralogoues displayed markedly different patterns of expression in different tissues or in response to different stimuli, suggesting that

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differently functional roles may have been distributed to the two genes after evolutionary duplication.

Keywords: ZmMPK6-1; ZmMPK6-2; maize; gene expression.

ABBREVIATIONS

ABA: Abscisic acid; CTAB: Cetyltrimethylammonium bromide; EDTA: Ethylenediaminetetraacetic acid; H_2O_2: Hydrogen peroxide; LOX: Lipoxygenase; MAPK: Mitogen-activated protein kinase; MAPKK: MAPK kinase; MAPKKK: MAPK kinase kinase; MPK: MAPK; NIPs: Nearly identical paralogues; PEG: Polyethylene glycol; SA: Salicylic acid; UTR: Untranslated region.

1. INTRODUCTION

Gene duplication is a prominent feature in the evolution of plant genomes and plays an important role in the evolution of new functions of proteins [1,2]. As one mechanism of the gene duplication, segmental duplication is resulted from the duplication of large segments of genomic DNA [3]. The duplicated genes are often referred to as paralogoue genes. Most of the duplicated genes are members of multigene families [1,4]. In the evolutionary processes, many duplicated genes have been lost. For those that do become fixed, the long-term evolutionary fate will still be determined by functions [1]. The duplicated genes may have three kinds of fate: (1) Duplicated genes may persist in the genome with perfect sequence identity; (2) One copy is suppressed and becomes a pseudogene; (3) One of the duplicated genes may diverge functionally, either by finding a novel functional role, or by specializing some aspect of its previous role [5]. The fate of duplicated genes can produce gene families containing similar genes carrying out similar or divergent functions. Nearly identical paralogs (NIPs) are used to define the paralogous genes that exhibit at least 98% identity. Sequence analyses of the maize (*Zea mays* L., an ancient segmental tetraploid) genome have revealed that at least 1% of maize genes have a NIP, a rate substantially higher than that in Arabidopsis [6].

Mitogen-activated protein kinase (MAPK) cascades are universal signaling modules in eukaryotes, including yeasts, animals, and plants [7,8]. A MAPK cascade consists of three consecutively acting protein kinases, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK [9]. MAPK cascades have been involved in plant growth and development, biotic and abiotic stresses [10]. There are multiple genes in each of the three tiers of kinases. For example, in *Arabidopsis*, there are 20 MAPKs, 10 MAPKKs, and about 60 MAPKKKs [9]. Rice genome contains at least 17 MAPKs and 8 MAPKKs [11]. Poplar genome contains 21 MAPKs and 11 MAPKKs [12]. Comparatively genomic analysis showed that, in some cases, two MAPK paralogues could be identified in one species for a single MAPK gene in another species, e.g., *PtMPK16-1* and *PtMPK16-2* versus *AtMPK16* [11], indicating the segmental duplication may have occured after the divergence of species. Although theoretical and experimental studies have advanced our understanding of the generation and evolutionary dynamics of duplicated genes, we still do not know much about the functions, especially the specific functions, of duplicated gene paralogues. Previously, we have showed that *ZmMPK3-1* and *ZmMPK3-2* are segmentally duplicated genes in maize and are the paralogues of *AtMPK3* of *Arabidopsis*. *ZmMPK3-1* and *ZmMPK3-2* are

differently regulated by abscisic acid (ABA) or NaCl [13]. Here, we report another segmentally duplicated paralogues, *ZmMPK6-1* and *ZmMPK6-2*.

2. MATERIALS AND METHODS

2.1 Plant Materials and Growth Conditions

In this study, maize cultivar Zhengdan958 was used. Maize seedlings were cultivated and treated as previously described [14]. Briefly, maize seeds were washed several times with tap water and soaked in distilled water for germination. Seedlings were grown in sterile water for 10 days under greenhouse conditions at 22/26ºC (night/day) prior to sample collection. Adult maize plants were raised in soil under greenhouse conditions.

For treatments, 10-day-old seedlings were used. The seedlings were dipped in sterile water containing 25% polyethylene glycol (PEG6000, w/v), NaCl (200 mM), ABA (100 μM), salicylic acid (SA, 1 mM), or hydrogen peroxide (H_2O_2, 2 mM). Seedlings treated with sterile water for the entire study period served as controls. Samples were collected at different time intervals after treatment and were immediately frozen in liquid nitrogen for RNA extraction.

2.2 Extraction of RNA

For RNA extraction, samples of various plant tissues were collected, frozen in liquid nitrogen, and processed immediately for total RNA isolation. Total RNA was isolated using a cetyltrimethylammonium bromide (CTAB) solution (2% CTAB, 0.1 M TRIS, 0.2 M ethylenediaminetetraacetic acid (EDTA), 1.4 M NaCl, 1% mercaptoethanol, 0.1% spermidine, pH 9.5). DNase I (Tiangen, Shanghai) was added to remove genomic DNA and RNase-free columns (Tiangen, Shanghai) were used for purifying total RNA. The concentration of the purified RNA was quantified by a nucleic acid analyzer (GeneQuant Pro, Amersham). The quality of purified RNA was further checked on a denaturing formaldehyde gel.

2.3 Semi-quantitative RT-PCR Analysis

Semi-quantitative RT-PCR analysis was performed as previously described [13,14]. Total RNA was reverse-transcribed by employing SuperScriptII (Invitrogen) according to the manufacturer's protocols. The PCR temperature scheme was adjusted according to the oligonucleotide primers employed for the experiments. The *ZmACTIN* was used as control for equal loading. PCR products were separated on 1.0% agarose gels with ethidium bromide in 1×TAE buffer (Tris base, acetic acid, and EDTA) and visualized under UV light. Primers were listed in Table 1. All PCR experiments were repeated three times to confirm reproducibility of results.

Table 1. List of primers used in this study

Name	Sequence 5' to 3'	Purpose
ZmACTIN-1	TGCAGGGATTCTTGGTGTTC	RT-PCR of *ZmACTIN*
ZmACTIN-2	TCTCTTGGTCTTGATGGTTGC	RT-PCR of *ZmACTIN*
ZmMPK6-1-1	GCTTCAGCCTCGAAAGCAAC	RT-PCR of *ZmMPK6-1*
ZmMPK6-1-2	CTCGTCGCGATCTAAATCCG	RT-PCR of *ZmMPK6-1*
ZmMPK6-2-1	GAGCCTCTCTTCTGCTAAAATAG	RT-PCR of *ZmMPK6-2*
ZmMPK6-2-2	CTCGTCGCGATCTAAATCCG	RT-PCR of *ZmMPK6-2*

2.4 Bioinformatic Analysis

Sequence alignments of nucleotides and proteins were constructed using DNAMAN 6.0 software (Lynnon Biosoft, Quebec, Canada).

3. RESULTS AND DISCUSSION

3.1 Sequence Analysis of *ZmMPK6-1* and *ZmMPK6-2*

Previously, we have showed that *ZmMPK3-1* and *ZmMPK3-2* are segmentally duplicated genes in maize [13]. In order to find more duplicated paralogues in MAPK gene family of maize, we performed multiple database searches and found *ZmMPK6-1* and *ZmMPK6-2* share more sequence similarity in both nucleotides and proteins than that of *ZmMPK3-1* and *ZmMPK3-2*. Both *ZmMPK6-1* and *ZmMPK6-2* contain a open reading frame (ORF) of 1,197 bp encoding predicted 398 amino acids. *ZmMPK6-1* and *ZmMPK6-2* share 95.32% identity throughout the entire coding sequence (Fig. 1).

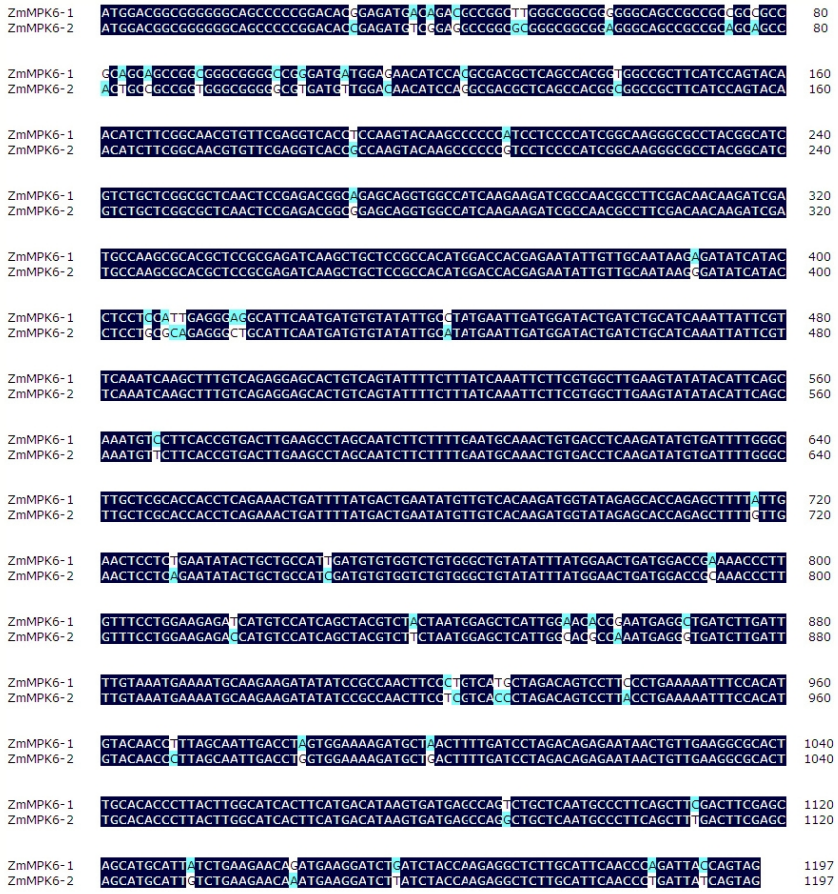

Fig. 1. Nucleotide alignment of open reading frames of *ZmMPK6-1* and *ZmMPK6-2*

For the alignment analysis, the DNAMAN 6.0 software is used. Identical nucleotides are shown in black.

The proteins of *ZmMPK6-1* and *ZmMPK6-2* share 94.22% identity different at 23 residues. The non-identity amino acids locate discretely in protein sequence of ZmMPK6-1 and ZmMPK6-2, except that a cluster of non-identity amino acids located between 25th and 42nd amino acids from the N-terminal (Fig. 2). These data indicated that *ZmMPK6-1* and *ZmMPK6-2* are extremely closely related to each other. They may be evolved as a result of recent segmental gene duplication event, as *ZmMPK6-1* and *ZmMPK6-2* are discretely located on chromosomes 6 and 9 in the maize genome, respectively.

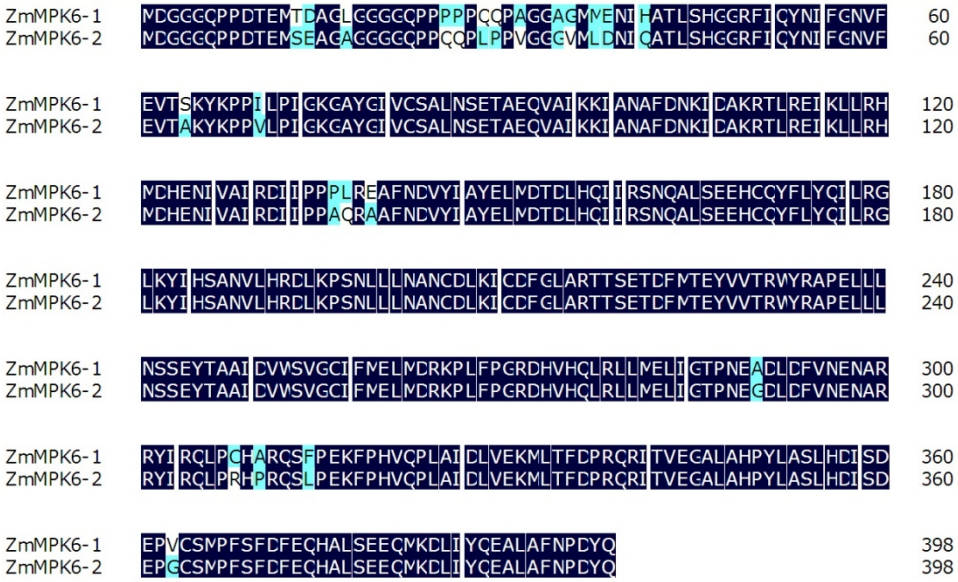

Fig. 2. Alignment of amino acid sequences of *ZmMPK6-1* and *ZmMPK6-2*

For the alignment analysis, the DNAMAN 6.0 software is used. Amino acids are shown in single letter code. Identical amino acids are shown in black.

When characterizing the closely related genes, it is important to take into account that maize genome of B73 inbred line contains a substantial number of nearly identical paralogues (NIPs). In most instances, both members of maize NIP pairs are expressed and are therefore at least potentially functional [6]. Although *ZmMPK6-1* and *ZmMPK6-2* share 95.32% identity, these highly similar genes are prevalent, as shown by *ZmLOX4* and *ZmLOX5* genes [15]. To determine whether there are any NIPs that are highly identical to *ZmMPK6-1* and *ZmMPK6-2* genes, we performed whole-genome searches of the B73 genome. The highly similar MAPK genes share no more than 78% similarity with *ZmMPK6-1* or *ZmMPK6-2*, indicating that no NIP exists in the maize genome that is closely related to *ZmMPK6-1* or *ZmMPK6-2*.

3.2 Organ-Specific Expression of *ZmMPK6-1* and *ZmMPK6-2*

Since *ZmMPK6-1* and *ZmMPK6-2* are highly similar to each other throughout the entire coding sequence, it is difficult to find specific primers for each gene. We compared the 5'-UTR and 3'-UTR of *ZmMPK6-1* and *ZmMPK6-2* and found the portion of 3'-UTR of the two genes share only 37.97% identity (Fig. 3). Thus, gene-specific primers were generated based on the portion of 5'- UTR for further expression analysis (Table 1). To test whether the primers were gene-specific, we cloned and sequenced the DNA fragments generated by the

primers. The sequencing results showed the DNA fragments were unique as predicted, suggesting the primers were gene-specific (data not shown).

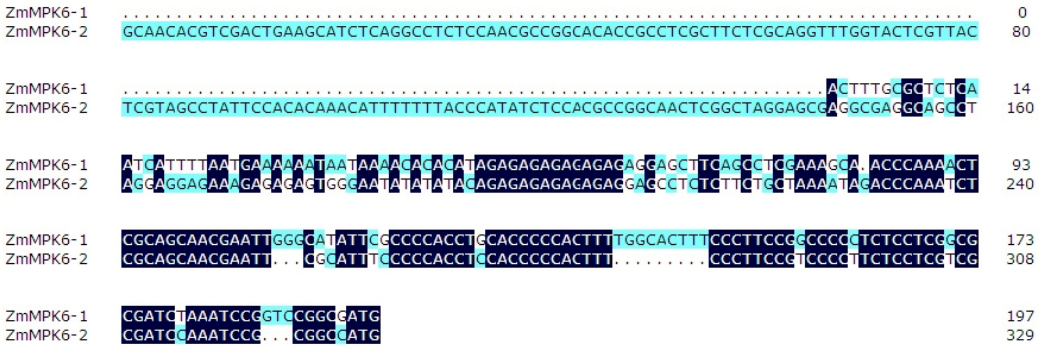

Fig. 3. Nucleotide alignment of 5'- UTRs of *ZmMPK6-1* and *ZmMPK6-2*

For the alignment analysis, the DNAMAN 6.0 software is used. Identical nucleotides are shown in black.

As shown in Fig. 4, *ZmMPK6-1* and *ZmMPK6-2* have different expression profiles. In adult plants, the tissue in which most *ZmMPK6-1* gene expressed is style (silk), followed by pericarp, leaf, ear, and caryopsis. Expression of *ZmMPK6-1* in culm, adventitious root, or anther is less than that in style (silk), pericarp, leaf, ear, or caryopsis. No expression of *ZmMPK6-1* was detectable in internode, primary root, or tassel. *ZmMPK6-2* was undetectable in leaf and anther. Specifically, transcripts of *ZmMPK6-2* were highly accumulated in anther, whereas no transcript of *ZmMPK6-1* was detectable. *ZmMPK6-2* was expressed in pericarp, culm, internode, primary root, adventitious root, tassel, ear, style, and caryopsis, with higher amount of transcripts could be detectable in adventitious root and caryopsis. Both *ZmMPK6-1* and *ZmMPK6-2* were expressed in pericarp, adventitious root, ear, style and caryopsis. In 10-day-old seedlings, transcripts of *ZmMPK6-1* were highly accumulated in root, whereas *ZmMPK6-2* was mainly expressed in leaf. Both *ZmMPK6-1* and *ZmMPK6-2* were expressed in stem. *ZmMPK6-2* was undetectable in root (Fig. 4). The organ-specific expression of *ZmMPK6-1* and *ZmMPK6-2* in adult plants and 10-day-old seedlings suggested that, although the two genes share high sequence identity, they might play different roles in different tissues. The paralogoue of *ZmMPK6-1* and *ZmMPK6-2* in *Arabidopsis*, *AtMPK6*, has been shown to function in anther and ovule development [16,17], stomatal development and patterning [18], leaf senescence [19], floral organ abscission [20]. A gene expressed abundantly in a tissue may indicate its function related to forming, growth, or development of this tissue.

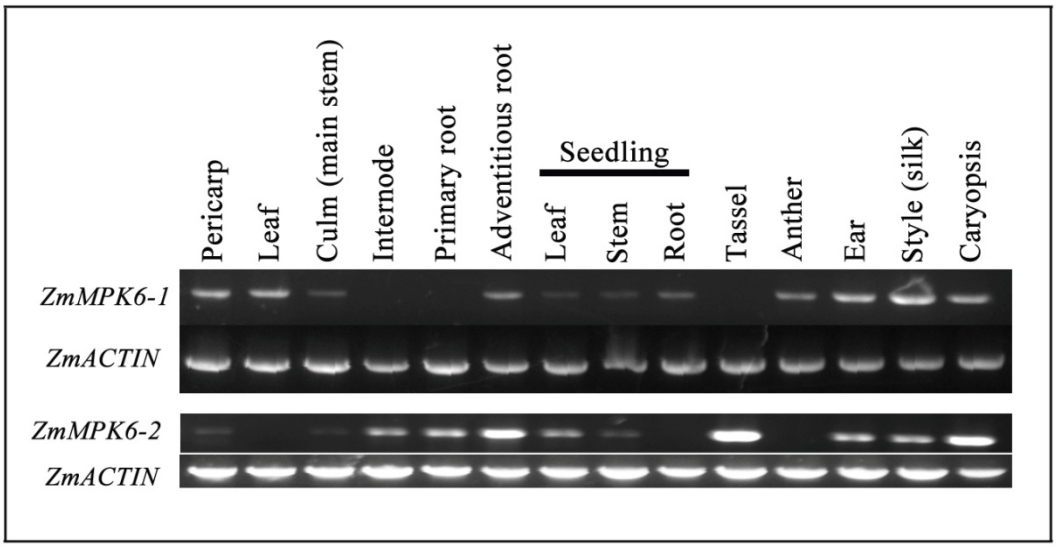

Fig. 4. RT-PCR analyses of organ-specific expression of *ZmMPK6-1* and *ZmMPK6-2* in maize seedlings and adult plants

For organ-specific expression analysis, total RNA was isolated from roots, stems, and leaves of 10-day-old maize seedlings and from pericarp, leaf, culm, internode, primary root, adventitious root, tassel, anther, ear, style and caryopsis of adult plants. The ZmACTIN was used as control for equal loading. The reactions were amplified for 28 cycles. The experiments were repeated at least three times with similar results and the representative results were shown.

3.3 Expression of *ZmMPK6-1* and *ZmMPK6-2* in Response to PEG6000 and NaCl

In previous studies, it was reported that *AtMPK6*, the paralogoue of *ZmMPK6-1* and *ZmMPK6-2*, was involved in osmotic [21] and salt stresses [22,23]. To test the functions of *ZmMPK6-1* and *ZmMPK6-2* in maize osmotic or salt stresses, we conducted the expression analysis using 10-day-old seedlings treated with PEG6000 (25%, w/v) or NaCl (200 mM). As shown in Fig. 5, PEG6000 could up-regulate (induce) the expression of *ZmMPK6-1*. The induction was occurred at 2 h and 3 h, followed by returning to background levels at 4 h. At 6 h, 12 h, and 24 h, the expression of *ZmMPK6-1* was again highly induced. The induction of *ZmMPK6-2* was observed only at 1 h and 2 h. At 3 h, 4 h, 6 h, and 12 h, it seemed that PEG6000 could down-regulate (block) the expression of *ZmMPK6-2*, whereas the expression of *ZmMPK6-2* returned to background levels at 24 h. For NaCl treatments, it was observed that both *ZmMPK6-1* and *ZmMPK6-2* could be up-regulated by NaCl. Two peaks were detectable in the time course (24 h) for *ZmMPK6-1*. Transcription levels of *ZmMPK6-1* increased and reached a peak at 2 h and then declined. Another peak emerged at 12 h. Long-time treatment (24 h) by NaCl could likely down-regulate the expression of *ZmMPK6-1*. The induction of *ZmMPK6-2* was more delayed than that of *ZmMPK6-1* and was observed at 12 h (Fig. 5). At 1 h, 2 h, 3 h, and 4 h, *ZmMPK6-2* was slightly down-regulated by NaCl. These results indicated that the expression patterns of *ZmMPK6-1* and *ZmMPK6-2* in response to PEG6000 or NaCl were different, suggesting that different functional roles may have been distributed to *ZmMPK6-1* and *ZmMPK6-2* after evolutionary duplication.

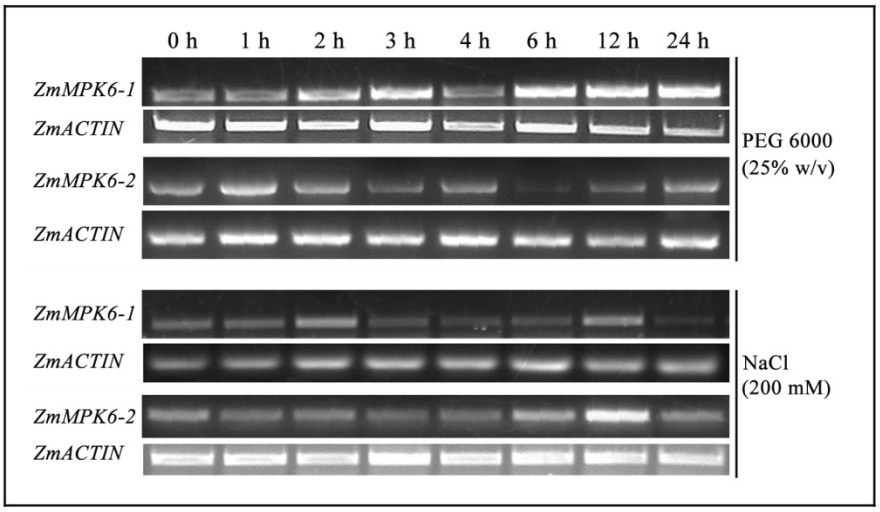

Fig. 5. RT-PCR analyses of *ZmMPK6-1* and *ZmMPK6-2* in response to PEG6000 (25%) and NaCl (200 mM) in maize seedlings

Total RNA was isolated from leaves of 10-day-old maize seedlings treated with PEG6000 (25%, w/v) or NaCl (200 mM). The ZmACTIN was used as control for equal loading. The reactions were amplified for 28 cycles. The experiments were repeated at least three times with similar results and the representative results are shown.

3.4 Expression of *ZmMPK6-1* and *ZmMPK6-2* in Response to Signal Stimuli

It was also reported that *AtMPK6* participated in ABA signaling [24,25] and immunity of *Arabidopsis* [26]. We next tested the expression of *ZmMPK6-1* and *ZmMPK6-2* in response to ABA (100 μM), SA (1 mM), or H_2O_2 (2 mM). As shown in Fig. 6, the transcriptions *ZmMPK6-1* could be down-regulated by ABA at 1 h, 3 h, and 6 h, despite the increased transcriptions could be detectable at 12 h and 24 h. By contrast, the transcriptions *ZmMPK6-2* could be up-regulated rapidly (at 1 h) and down-regulated at 6 h. Both *ZmMPK6-1* and *ZmMPK6-2* could be up-regulated by SA. High amount of *ZmMPK6-1* transcriptions accumulated at 1 h, 3 h, and 12 h when 10-day-old seedlings were treated with SA (1 mM). SA induced the expression of *ZmMPK6-2* at 24 h, although a slight down-regulation was detectable at 3 h. For H_2O_2 treatments, the transcriptions of *ZmMPK6-1* seemed unchanged at the first 3 hours and were up-regulated at 12 h. At 6 h or 24 h, however, it was likely that H_2O_2 could completely block the expression of *ZmMPK6-1* (Fig. 6). When compared to transcriptions of *ZmMPK6-1*, the transcriptions of *ZmMPK6-2* seemed also unchanged at the first 3 hours, but slightly up-regulated at followed time course (6 h, 12 h, and 24 h). These results indicated that the expression patterns of *ZmMPK6-1* and *ZmMPK6-2* in response to ABA, SA, or H_2O_2 were different, suggesting it is likely that the two genes have different functional roles in signaling of ABA, SA, or H_2O_2.

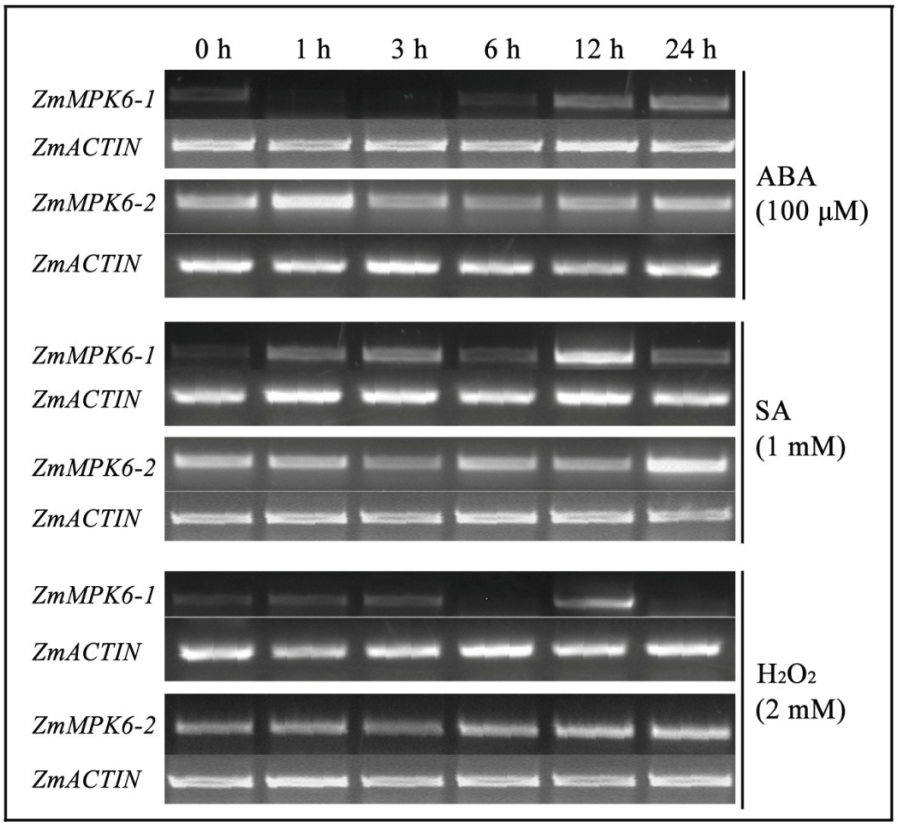

Fig. 6. RT-PCR analyses of *ZmMPK6-1* and *ZmMPK6-2* in response to signal stimuli in maize seedlings

Total RNA was isolated from leaves of 10-day-old maize seedlings s treated with ABA (100 μM), SA (1 mM), or H_2O_2 (2 mM).The ZmACTIN was used as control for equal loading. The reactions were amplified for 28 cycles. The experiments were repeated at least three times with similar results and the representative results are shown

4. CONCLUSION

In this study, we report that *ZmMPK6-1* and *ZmMPK6-2* are segmentally duplicated genes in the maize genome. RT-PCR analyses show that the two genes have dissimilarly expressional patterns in different tissues of adult plants or 10-day-old seedlings (Fig. 4). *ZmMPK6-1* and *ZmMPK6-2* have also dissimilarly expressional patterns in response to PEG6000 (25%, w/v), NaCl (200 mM), ABA (100 μM), SA (1 mM), or H_2O_2 (2 mM) (Fig. 5 and 6). These results indicate that unique functional roles may have been distributed to the two genes after evolutionary duplication. This study provides a useful reference for further functional analysis of *ZmMPK6-1* and *ZmMPK6-2* genes in maize.

ACKNOWLEDGEMENTS

This work was supported by the Scientific Research Foundation of Southwest Forestry University (No. 111169).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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