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ZmMKK3, a novel maize group B mitogen-activated protein kinase kinase gene, mediates osmotic stress and ABA signal responses

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ABSTRACT

Mitogen-activated protein kinase (MAPK) cascades are important intracellular signaling modules and function as a convergent point for crosstalk during abiotic stress signaling. In this article, we isolated a novel group B MAPKK gene, *ZmMKK3*, from *Zea mays*. ZmMKK3 protein might be localized in both the cytoplasm and the nucleus. RNA blot analysis indicated that the *ZmMKK3* transcription was up-regulated by abscisic acid (ABA), hydrogen peroxide (H_2O_2) and PEG, and that H_2O_2 mediated PEG-induced expression of *ZmMKK3*. Constitutive expression of *ZmMKK3* in *Nicotiana tabacum* reduced H_2O_2 accumulation under osmotic stress by affecting antioxidant defense systems and alleviated reactive oxygen species-mediated injury under oxidative stress. Transgenic tobacco exhibited attenuated ABA sensitivity by means of an increased germination rate and main root growth. Taken together, these results indicate that ZmMKK3 is a positive regulator of osmotic tolerance and ABA signaling in plants.

Introduction

Plants are continuously exposed to biotic and abiotic stresses during their whole life and they have developed sophisticated signaling machineries to adapt their cellular metabolism to cope with adverse environment. One of the universal signaling modules function in response to such external stimuli is the mitogen-activated protein kinase (MAPK) cascades (Tena et al., 2001; MAPK group, 2002). A typical MAPK cascade consists of three sequentially activated kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAPK. MAPKKK phosphorylates a serine or threonine residue on a MAPKK, which in turn activates a MAPK, the last protein in the cascade. MAPKs are activated by the dual phosphorylation of threonine and tyrosine residues by MAPKKs (MAPK group, 2002). Different MAPK pathways can be activated in response to a wide variety of extracellular stimuli, including pathogens, drought, salt, cold, wounding, reactive oxygen species (ROS) and hormone (Rodriguez et al., 2010).

In recent years, several putative MAPKKs as well as downstream factors have been isolated from different plant species. In general, all plants use a more limited number of MKKs compared to other MAPK components (Rodriguez et al., 2010). The *Arabidopsis* genome harbours: 80 MAPKKKs, 10 MAPKKs, and 20 MAPKs (Colcombet and Hirt, 2008). The small number of MAPKKs suggests that MAPKKs may have multiple MAPK targets and the same MAPKK may function in several different MAPK modules (Rodriguez et al., 2010). It has previously been shown that the MEKK1–MKK1/MKK2–MPK4 kinase cascade negatively regulates cell death and defense responses (Gao et al., 2008; Qiu et al., 2008). More recently, Zhang et al. (2012) 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 the NB-LRR protein SUMM2. *Arabidopsis* group C MAPKs, including MPK1, MPK2, MPK7 and MPK14, were reported to be activated by upstream MKK3, MKK3–MPK7 participates in pathogen signaling (Doczi et al., 2007), whereas MKK3–MPK6 plays an important role in JA signaling (Takahashi et al., 2007). Recently, studies showed that MKK3/CaMMPK8 cascades mediate Ca^{2+} and ROS signaling in early wound signaling (Takahashi et al., 2011).

Plants possess a sophisticated ROS network, comprising ROS producing enzymes, antioxidative enzymes and antioxidants which allow them to keep ROS levels under tight control. Several studies have shown that MAPK signaling pathways are not only induced by ROS but can also regulate ROS production (Pitzschke and Hirt, 2009). In *Arabidopsis*, for example, hydrogen peroxide (H_2O_2) was able to activate AtMPK6 and AtMPK3 (Colcombet and

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; CaMV, cauliflower mosaic virus; DMTU, dimethylthiourea; DAB, 3,3′-diaminobenzidine; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; MV, methyl-viologen; NBT, nitroblue tetrazolium; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Hirt, 2008), and in tobacco H_2O_2 activated the ortholog of AtMPK6, SIPK (Samuel et al., 2000). The AtMKK1–AtMPK6 signal module regulated H_2O_2 metabolism through CAT1 (Xing et al., 2008), and MEKK1–MPK4 cascade was also an essential component in ROS metabolism (Nakagami et al., 2006). H_2O_2 induced the transcription of *ZmMPK7*, *ZmMPK5*, *ZmMPK3* and *ZmMKK4* in maize, and over expressing of *ZmMPK7* in *Nicotiana tabacum* provided protection against ROS-mediated injury by enhancing peroxidase (POD) activity under osmotic stress (Zong et al., 2009; Zhang et al., 2010; Wang et al., 2010a,b; Kong et al., 2011a). In rice, the MAPKKK DSM1 functions as an early signaling component in response to drought stress by regulating scavenging of ROS (Ning et al., 2010).

Maize (*Zea mays*) is an important economically grain crop, and its growth and yield are severely inhibited under various biotic and abiotic stress conditions. To date, few MAPKKs have been identified in maize, and their function has not been well documented. In this study, we identified and characterized a homologue of AtMKK3, ZmMKK3, in maize. The expression of *ZmMKK3* was induced by PEG, H_2O_2 and abscisic acid (ABA). Over expression of *ZmMKK3* conferred tolerance to osmotic and oxidative stresses and alleviated ABA sensitivity in transgenic tobacco plants.

Materials and methods

Plant cultivation and treatment

Maize Seedlings (*Zea mays* L. cv Zhengdan958) were grown in Hoagland's solution (0.51 g/l KNO_3, 0.82 g/l $Ca(NO_3)_2$, 0.49 g/l $MgSO_4{\cdot}7H_2O$, 0.136 g/l KH_2PO_4, 0.6 ml/l $FeSO_4$, 2.86 mg/l H_3BO_3, 1.81 mg/l $MnCl_2{\cdot}4H_2O$, 0.08 mg/l $CuSO_4{\cdot}5H_2O$, 0.22 mg/l $ZnSO_4{\cdot}7H_2O$, and 0.09 mg/l $H_2MoO_4{\cdot}4H_2O$) (pH 6.0) under hydroponic greenhouse conditions at 22/26 °C (night/day), a photoperiod of 14/10 h (day/night), and photosynthetic active radiation of 200 μmol m^{-2} s^{-1} for 2 weeks.

Tobacco plants (*Nicotiana tabacum* cv NC 89) were also used in this study. The transgenic and wild-type (WT) tobacco plants were grown for six weeks in quartz sand under controlled environmental condition with a photoperiod of 16/8 h (day/night), a temperature of 25/20 °C (day/night) and photosynthetic active radiation of 200 μmol m^{-2} s^{-1}.

Two-week-old maize seedlings were incubated with Hoagland's solution containing 20% PEG6000 (w/v), 10 μM abscisic acid (ABA), 10 mM hydrogen peroxide (H_2O_2) or Hoagland's solution alone at 26 °C with a continuous light intensity of 200 μmol m^{-2} s^{-1}. To investigate the roles of reactive oxygen species (ROS) manipulators, the seedlings were pretreated with 20 mM imidazole, 10 mM Tiron, or 5 mM dimethylthiourea (DMTU) for 12 h and then exposed to 20% PEG6000 (w/v) under the same conditions. Samples were collected at the indicated times and all samples were frozen in liquid nitrogen immediately after collection and stored at −80 °C. Six-week-old transgenic and the wild-type tobacco lines incubated in quartz sand were treated with 20% PEG6000 or 10 μM ABA under the conditions described above, after which second and third leaf samples were taken.

Isolation and characterization of ZmMKK3

Total RNA from untreated maize leaves was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First strand cDNAs were synthesized using first strand cDNA Synthesis kit (Fermentas, USA). Polymerase chain reaction (PCR) was performed with gene-specific primers (forward GAGTTCGGACCTCGCTCTGA and reverse CAAAACCAGGTCCGAAAGGA), the products were cloned into pMD18-T vectors (TaKaRa, Beijing, PR China) and sequenced. The amino acid sequences of the mitogen-activated protein kinase (MAPKK) proteins were retrieved from GenBank. Sequence alignments and phylogenetic trees were constructed using DNAMAN 6.0 software.

Sub cellular localization of ZmMKK3

The whole coding sequence of *ZmMKK3* was amplified with primers (forward CTCTAGAATGGCAGGGCTGGAGGAGTTGA *Xba* site underlined and reverse CGGTACCGGATTGGATAATGTACAGATC *Kpn* site underlined). The coding sequences were inserted into the reconstructed binary vector pBI121-GFP, which generated a C-terminal fusion with the green fluorescent protein (GFP) gene controlled by the cauliflower mosaic virus (CaMV) 35S promoter. The recombinant plasmid and GFP alone were introduced into the *Agrobacterium tumefaciens* strain LB4404, which was used for transient transformation of onion (*Allium cepa*) epidermal cells. After transformation, tissues were incubated on MS agar medium under dark condition at 23 °C for 16 h. Transformed onion cells were observed under a confocal microscope (Olympus, Tokyo, Japan).

RNA gel blot analysis

Total RNA was extracted using the RNeasy plant mini kit (TIANGEN, Beijing, PR China) according to the manufacturer's instruction and stored at −80 °C. 20 μg of total RNA was separated by denaturing 1.0% (w/v) agarose gel, stained with ethidium bromide to ensure equal loading and then transferred to nylon membranes (Hybond-N+, Amersham, USA). Specific fragment of 500 bp at the 5′ end of *ZmMKK3* cDNA was used as probe. RNA blotting was carried out as described earlier (Zong et al., 2009).

Over expression of ZmMKK3 in transgenic tobacco

The full correct coding regions of *ZmMKK3* ligased into the binary pBI121 expression vector under the control of the CaMV 35S promoter. The recombinant plasmid, pBI121-*ZmMKK3*, was introduced into the *A. tumefaciens* strain LBA4404. Transformation of tobacco was performed using an *Agrobacterium*-mediated leaf disc transformation (Zong et al., 2009).

Germination experiments

About 50 surface-sterilized seeds each from transgenic tobacco lines and wild-type line were sowed on MS plates supplemented with or without different concentration of mannitol or ABA, and placed at a photoperiod of 16/8 h (day/night) at a temperature of 25/20 h (day/night). The rates of seed germination were evaluated (root emergence) every day and each experiment was repeated three times at least with identical results.

Root length analysis

Seedlings of transgenic tobacco and WT line grown on MS medium for 5 days were transferred to MS medium supplemented with or without different concentration of ABA for 15 days, and the length of roots were measured and calculated.

In situ detection of H_2O_2 and O_2^-

H_2O_2 and O_2^- accumulation were detected by the 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining methods. The seedlings were infiltrated with 5 mg mL^{-1} DAB at pH 3.8 or 0.5 mg mL^{-1} NBT for 20 h in the dark to detect H_2O_2 and O_2^-, respectively. Then the seedlings were decolorized by boiling in ethanol (96%) for 10 min. After cooling, the leaves were extracted