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Activation of ZmMKK10, a maize mitogen-activated protein kinase kinase, induces ethylene-dependent cell death

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ABSTRACT

Mitogen-activated protein kinase (MAPK) cascades play important roles in regulating plant growth, development and stress responses. Here, we report that ZmMKK10, a maize MAP kinase kinase, positively regulates cell death. Sequence comparison to *Arabidopsis* MKKs has led to ZmMKK10 being classified as a group D MKK. Kinase activity analysis of recombinant ZmMKK10 showed that the Mg^{2+} ion was required for its kinase activity. Transient expression of $ZmMKK10^{WT}$ or $ZmMKK10^{DD}$ (the active form of *ZmMKK10*) in maize mesophyll protoplast significantly increased the cell death rate. Inducible expression of $ZmMKK10^{WT}$ or $ZmMKK10^{DD}$ in *Arabidopsis* transgenic plants caused rapid HR-like cell death, whereas induction of $ZmMKK10^{KR}$ (the inactive form of *ZmMKK10*) expression in transgenic plants did not yield the same phenotype. Genetic and pharmacological analysis revealed that ZmMKK10-induced cell death in transgenic plants requires the activation of *Arabidopsis* MPK3 and MPK6 and that it partially depended on ethylene biosynthesis. ZmMPK3 and ZmMPK7, the orthologues of *Arabidopsis* MPK3 and MPK6, interacted with ZmMKK10 in yeast and ZmMKK10 phosphorylated them both *in vitro*. Our results demonstrate that ZmMKK10 induces cell death in an ethylene-dependent manner. Furthermore, ZmMPK3 and ZmMPK7 may be the downstream MAPKs in this process.

1. Introduction

Mitogen-activated protein kinase cascades are highly conserved signal transduction modules in eukaryotic cells [1,2]. In general, extracellular stimuli are sensed by plasma membrane receptors and activate MAPK cascades through the sequential phosphorylation of MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MAPKKs) and MAP kinases (MAPKs). The activated MAPKs then phosphorylate target proteins, such as cytoskeleton-associated proteins, other kinases, enzymes and transcription factors. These phosphorylation signals elicit intracellular responses [3–5].

As convergence points, MAPKKs mediate upstream and downstream signals. Plant MAPKKs contain a conserved N-terminal docking site K/R-K/R-K/R-X_{1-6}-L-X-L/V/I and are phosphorylated by MAPKKKs on two serine/threonine residues in the conserved S/T-X_5-S/T motif [1,6]. The *Arabidopsis* genome contains 10 MAPKK genes that are divided into four groups (A-D) based on their consensus sequence S/T-X_5-S/T and docking sites. Members of MAPKK families in other plant species, e.g. rice, tobacco, *Medicago*, tomato and parsley, were also identified and characterized [3]. They function in plant growth and development, hormonal responses, abiotic stress responses and defense [7–10].

Hypersensitive response (HR) cell death, a form of programmed cell death, occurs rapidly at the site of attempted pathogen invasion and protects plants from infection. HR induction integrates a series of signal events, including the recognition of pathogens and PAMPs by receptors, activation of kinases or other factors, induction of hormones and resistant genes, alteration of morphological and biochemical responses. HR induction then initiates cell death [11,12]. MAPK cascades have been shown to play critical roles in HR cell death regulation. Gain-of-function studies using constitutively active MAPKK mutants revealed that the NtMEK2-SIPK/WIPK/Ntf4 cascade in tobacco, MKK4/MKK5-MPK3/MPK6 in *Arabidopsis* and LeMKK2-LeMPK2/LeMPK3 in tomato induced rapid HR-like cell death [13–16]. Furthermore, MAPKKKα and MAPKKKε are two positive regulators of cell death that might activate the NtMEK2-SIPK/WIPK/Ntf4 module [17,18].

Ethylene biosynthesis and the accumulation of reactive oxygen species (ROS) have been shown to mediate the MAPK-induced cell death process. The conversion of S-adenosyl-L-Met to 1-aminocyclopropane-1-carboxylic acid (ACC), which is catalyzed by ACC synthase, and the oxidative cleavage of ACC, which is catalyzed by ACC oxidase (ACO) to form ethylene, are two key steps in ethylene biosynthesis [19–21]. Previous studies have shown that phosphorylation of ACS2/ACS6 by MPK3 and MPK6 increased the stability of ACS proteins resulting in ethylene production by *Arabidopsis* [22,23]. Expression of the

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constitutively active *Arabidopsis* MKK5 mutant activates MPK3 and MPK6 and promotes cell death and ethylene biosynthesis. Loss of either *MPK3* or *MPK6* greatly reduces ethylene production and suppresses cell death. Inhibition of ethylene biosynthesis also significantly suppresses cell death [24]. It was noted that H_2O_2 accumulation was correlated with MKK4/MKK5-MPK3/MPK6 activation-induced cell death [15].

Eight OsMKKs in the rice genome and ten MKKs in the *Arabidopsis* genome were found, while only nine MAPKK genes have been identified using sequence comparison and signature motif searching in the maize genome [25]. Based on its genome size, maize may have more than nine MAPKKs in its genome. Of the known ZmMKKs, four of them have associated functional information: ZmMKK4 confers tolerance to cold and salt stress in transgenic *Arabidopsis* plants, and it regulates the osmotic stress response in transgenic tobacco plants [26,27]; ZmMKK3 mediates osmotic stress and ABA signal responses [28]; ZmMKK1 positively regulates chilling tolerance, and it is involved in pathogen defense in transgenic tobacco [29]; ZmMEK1 regulates salicylic acid-dependent leaf senescence [30].

Here, we report the identification and characterization of ZmMKK10, a group D maize MAPKK. Expression of active ZmMKK10 induces cell death in both maize mesophyll protoplasts and transgenic *Arabidopsis* plants. ZmMKK10 led to MPK3 and MPK6 activation, ROS accumulation and ethylene production in *Arabidopsis*. Maize ZmMPK3 and ZmMPK7, the orthologues of *Arabidopsis* MPK3 and MPK6, interact and are phosphorylated by ZmMKK10 *in vitro*. These results suggest that ZmMKK10 and ZmMPK3/ZmMPK7 may function in a MAPK cascade to positively regulate ethylene-dependent cell death processes.

2. Materials and methods

2.1. Plant materials and growth conditions

To prepare protoplasts, maize (*Zea mays*, inbred line B73) seedlings were grown in soil at 28 °C for 13 d in a dark growth chamber.

Arabidopsis wild-type plants, mutants and transgenic lines were all of the ecotype Col-0 background. *Arabidopsis* plants were grown at 22 °C under a 16-h-light/8-h-dark photoperiod at a photon flux density of 100 μmol m^{-2} s^{-1}. Unless otherwise indicated, fully expanded leaves of 4-week-old plants were used in experiments. The generation of *mpk3* (SALK_100651) and *mpk6* (SALK_127507) plants were described previously [31]. *ZmMKK10^{DD}/mpk3* and *ZmMKK10^{DD}/mpk6* plants were generated by crossing *ZmMKK10^{DD}* plants with *mpk3* and *mpk6* plants, respectively.

2.2. Transgenic Arabidopsis plant generation

The complementary DNA (cDNA) of maize *ZmMKK10* (GRMZM2G163217) was obtained using RT-PCR with specific primers and inserted into a modified pBlueScript vector with a Flag epitope tag coding sequence at the 5′-end. The constitutively active and inactive mutants of *ZmMKK10* were generated using the QuickChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). The resulting *Flag-ZmMKK10* mutant fragments were cloned into the steroid-inducible pTA7002 binary vector [32]. All plasmids were electroporated into the *Agrobacterium tumefaciens* strain GV3101, which was used to transform *Arabidopsis* by floral dip [33]. Transgenic plants were screened using 15 mg/liter Hygromycin resistance and immunoblot analysis with an anti-Flag antibody, and T3 homozygote plants were used for further experiments. Gene-specific primers are listed in Table S2 (See Supplementary material Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2017.09.012).

2.3. Plant treatment

To induce transgenes expression, plants were sprayed with 15 μM dexamethasone (DEX) or detached leaves were incubated in 15 μM DEX solutions. Evans blue staining and 3,3′-diaminobenzidine (DAB) staining were performed as described previously [24].

As an inhibitor of ethylene biosynthesis and reception pretreatment, detached leaves were incubated in 2 μM AVG, 100 μM $CoCl_2$ or 10 μM STS solutions for 1 h before 15 μM DEX was added. For dimethylthiourea (DMTU) pretreatment, detached leaves were incubated in 15 mM DMTU solutions for 6 h before DEX was added.

2.4. Measurement of membrane ion leakage and ethylene production

The membrane ion leakage was detected as previously described [30]. Ethylene production was measured as described previously [31].

2.5. Recombinant protein expression and purification

The coding regions of the *ZmMKK10* mutants were inserted into the pGEX4T-1 vector to obtain Glutathione S-transferase (GST)-tagged ZmMKK10 mutant proteins. The coding regions of *ZmMPKs* were cloned into the pET28a vector to obtain His-tagged ZmMPK proteins. The resultant plasmids were transformed into the *Escherichia coli* BL21 strain. GST-ZmMKK10 mutant proteins were induced at 16 °C for 30 min using 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified using glutathione-agarose beads (GE Healthcare). His-ZmMPK proteins were induced at 16 °C for 10 h using 0.1 mM IPTG and purified using the Ni^+-Chelating Sepharose Fast Flow (GE Healthcare). Primers used are listed in Table S2 (see Supplementary Material Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2017.09.012).

2.6. In vitro autophosphorylation and kinase activity assay

The *in vitro* autophosphorylation assay was performed as previously described [31]. Equal amounts (5 μg) of purified recombinant ZmMKK10 were incubated at 25 °C for 30 min in a total volume of 30 μL of reaction mixture containing 20 mM HEPES (pH 7.5), 1 mM dithiothreitol (DTT), 50 μM ATP, 1 μCi [γ-^{32}P] ATP and various concentrations of $MgCl_2$ or $MnCl_2$. Then, SDS loading buffer was added to stop the reaction, and the samples were subjected to SDS-PAGE (polyacrylamide gel electrophoresis) and autoradiography.

The *in vitro* kinase activity assay was performed by incubating 1 μg of recombinant ZmMKK10 mutant proteins with 5 μg of individual ZmMPK proteins in 30 μL of reaction buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 μM ATP, 1 mM DTT and 1 μCi [γ-^{32}P] ATP at 25 °C for 30 min. The reactions were stopped by adding SDS loading buffer. Proteins were separated using SDS-PAGE and kinase activity was determined by autoradiography.

2.7. Protein extraction, in-gel kinase activity assay and immunoblot analysis

Protein was extracted and subjected to an in-gel kinase activity assay and immunoblot analysis as described previously [30]. Anti-Flag antibody (Sigma-Aldrich) was used to detect ZmMKK10 mutant protein expression in transgenic plants. Anti-GST (GE Healthcare) and anti-His (Sigma-Aldrich) antibodies were used to detect recombinant GST-tagged ZmMKK10 and His-tagged ZmMPKs, respectively. Anti-MPK3 and anti-MPK6 antibodies (Sigma-Aldrich) were used to detect *Arabidopsis* MPK3 and MPK6, respectively.

2.8. Yeast two-hybrid assay

For the yeast two-hybrid assay, the *ZmMKK10^{KR}* fragment was inserted into the pGADT7 vector to produce the prey plasmid and the coding regions of *ZmMPKs* were cloned into the pGBKT7 vector to obtain bait plasmids. The bait and prey plasmids were cotransformed into the yeast strain AH109. Transformants were screened by measuring the