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The mitogen-activated protein kinase cascade MKK1–MPK4 mediates salt signaling in rice

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ABSTRACT

Mitogen-activated protein kinase (MAPK) pathways have been implicated in signal transduction of both biotic and abiotic stresses in plants. In this study, we found that the transcript of a rice (*Oryza sativa*) *MAPKK* (*OsMKK1*) was markedly increased by salt stress. By examining the survival rate and Na^+ content in shoot, we found that *OsMKK1*-knockout (*osmkk1*) mutant was more sensitive to salt stress than the wild type. OsMKK1 activity in the wild-type seedlings and protoplasts was increased by salt stress. Yeast two-hybrid and *in vitro* and *in vivo* kinase assays revealed that OsMKK1 targeted OsMPK4. OsMPK4 activity was increased by salt, which was impaired in *osmkk1* plants. In contrast, overexpression of *OsMKK1* increased OsMPK4 activity in protoplasts. By comparing the transcription factors levels between WT and *osmkk1* mutant, *OsMKK1* was necessary for salt-induced increase in *OsDREB2B* and *OsMYBS3*. Taken together, the data suggest that OsMKK1 and OsMPK4 constitute a signaling pathway that regulates salt stress tolerance in rice.

1. Introduction

Soil salinity has been regarded as a serious agricultural problem. One-fifth of irrigated agriculture is affected by high soil salinity [1]. The injury by salinity to plants includes ionic toxicity (such as Na^+), osmotic stress, and ionic imbalance [2]. To survive on saline soil, plants have evolved a complex adaptive mechanism at the molecular, cellular, physiological, and biochemical level to perceive and respond to salt stress [3], but the sensing and signal transduction mechanisms for salt stress is largely unknown.

Mitogen-activated protein kinase (MAPK) cascades are conserved signaling pathways in transducing extracellular stimuli into cellular responses in eukaryotes [4]. MAPK cascades consist of three sequentially phosphorylating and activating components, a MAP kinase kinase kinase (MEKK/MAPKKK), a MAP kinase kinase (MKK/MAPKK), and a MAP kinase (MPK/MAPK) [5]. MAPKs phosphorylate a variety of substrates including transcription factors, protein kinases, cytoskeleton-associated proteins, and transporters [6,7]. Signaling through MAP kinase cascades can lead to cellular responses, including cell division, differentiation as well as response to various stresses [8]. In recent years, it is learnt that stress-activated MAPK pathways play a pivotal role in osmostress signal transduction, in both yeast and mammals [8,9]. When yeast cells are exposed to high osmotic conditions, they respond by producing high intracellular concentrations of glycerol and reducing membrane permeability to this solute in an effort to reestablish osmotic equilibrium with the environment [10]. The signaling pathways mediating these processes include MAPK homolog *Hog1* and the MAPKK homolog *Pbs2*, which are required for cell growth in high-osmolarity medium [11]. The HOG pathway is activated predominantly by two independent mechanisms that lead to the activation of Ssk2 and Ssk22 or the Ste11 MAPKKKs, respectively [12]. In mammalians, three different MAPKs are activated in response to osmostress: p38MAPK, c-Jun N-terminal kinases (JNK), and ERK5 (extracellular signal-regulated kinase5). MKK3 and MKK6 activate p38 MAPKs, whereas MKK4 and MKK7 are mainly responsible for the activation of JNK; all four MAPKKs can be activated by osmostress [9].

MAPK has also been related with biotic and abiotic stresses in plants. An increasing body of evidence has shown that MAPKs play important roles in signal transduction in response to salt, drought, reactive oxygen species, wounding, and low temperature in plants [5]. On the basis of the fully sequenced *Arabidopsis* genome, 20 *MAPKs*, 10 *MAPKKs*, and 60 *MAPKKKs* were identified, and a unified nomenclature was made [4].

A cascade consisting of AtMEKK1–AtMKK4/AtMKK5–AtMPK3/AtMPK6 has been found to participate in flagellin-mediated innate immune signaling [13]. AtMKK3–AtMPK6 is activated by jasmonic acid (JA) in *Arabidopsis*, and plays a key role in JA-dependent negative regulation of *AtMYC2/JIN1* expression [14]. The

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AtMKK9–AtMPK3/AtMPK6 cascade participates in the regulation of the biosynthesis of ethylene and camalexin and may be an important axis in the stress responses of *Arabidopsis* [15].

MAPK in the salt stress signal transduction has been reported in a variety of plants [16]. Salt stress activates the salt stress-induced MAPK (SIMK) in *alfalfa* [17], which is mediated by a MAPKK homolog SIMKK [18]. In tobacco protoplasts, salt stress enhances the activation of a 48-kDa MAPK, the salicylic acid-induced protein kinase (SIPK) [19]. Three MAPKs, *ZmMPK3*, *ZmMAPK5*, and *ZmSIMK1* can be induced by salt stress in *Zea mays* [20]. The MAPK activation by salt stress was also reported in *Chorispora bungeana* [21] and cotton [22]. In *Arabidopsis thaliana*, AtMPK4 and AtMPK6 are activated by salt treatment [16]. Recently, AtMEKK1 (MAPKKK) has been defined to activate AtMPK4 and AtMPK6 through AtMKK2 when *Arabidopsis thaliana* was exposed to cold and salt stress [23]. In our previous study, we reported that PLDα1-derived PA binds to AtMPK6 and leads to its activation. The activated AtMPK6 phosphorylates the Na^+/H^+ anti-porter SOS1, which may contribute to reduced Na^+ accumulation in *Arabidopsis* leaves under salt stress [7].

In the rice (*Oryza sativa L.*) genome, 17 *MAPKs*, 8 *MAPKKs*, and 75 *MAPKKKs* genes have been annotated [24,25]. Increasing evidence suggests that rice MAPK cascade is an essential system to regulate both biotic and abiotic stress responses. Shi et al. (2014) demonstrated that OsMPK1 regulated the activities of antioxidant enzymes in abscisic acid signaling [26]. Both OsMPK3 and OsMPK6 have been implicated in salt stress response [27,28]. OsMPK6 is activated during defense response, and is up-regulated by OsMKK4, which is also a gain development regulator [29–31]. Kumar et al. (2008) showed that rice MKK1, 4, 6, 10-2 were induced by salt stress [32]. Furthermore, overexpression of *OsMKK6* enhances salt tolerance in rice [33]. For a MAPKKK, overexpression of *DSM1* (a putative MAPKKK gene in rice) increases the tolerance to dehydration stress [34].

In this study, we present several lines of evidence that OsMKK1 and OsMPK4 constitute a MAPK cascade for salt stress signaling by regulating the expression of transcription factors and regulating salt tolerance in rice.

2. Materials and methods

2.1. Plant materials and salt treatments

Wild-type rice (*Oryza sativa, japonica* cv. Dongjin) and *OsMKK1* T-DNA insertion mutant *osmkk1* were used in this work. T-DNA insertion mutant line was identified from the rice T-DNA Insertion Sequence Database (http://cbi.khu.ac.kr/RISD_DB.html). Germinated seeds were grown in hydroponics with Hoagland medium in a growth chamber at 28°C/25°C, 16 h of light/8 h of dark, and 50% humidity. After 2 weeks, the rice seedlings were transferred to a solution containing 130–200 mM NaCl for treatment.

2.2. Gene cloning and vector construction

All primer sequences used in this paper are listed in Supplementary Table 1.

OsMKK1 and *OsMPK4* were cloned from rice cDNA by PCR. The primers for cloning were *HA-OsMPK4-L/R* and *flag-OsMPKK1-L/R*, respectively. The PCR products were first cloned into a Promega pGEM-T Easy Vector according to the kit's instructions and were then verified by sequencing. *OsMKK1* was cloned into pRT105 vector with flag-tagged between the *Spe*I and *Bam*HI sites. *OsMPK4* was cloned into pRT105 vector with HA-tagged between the *Spe*I and *Bam*HI sites.

2.3. Rice protoplast isolation and transient expression assays

For protoplast isolation, rice seeds (Nipponbare) were grown (28°C, 85% humidity) in the dark for 12–14 d. Stems and leaves were cut into 0.5 mm pieces and digested with 25 mL of enzyme solution containing 1.5% w/v cellulose (Sigma) and 0.3% (w/v) macerozyme (Sigma). Further preparation of protoplasts was performed as described [35].

The collected protoplasts were resuspended in an appropriate volume of suspension medium (0.4 M mannitol, 20 mM $CaCl_2$, and 5 mM MES, adjusted to pH 5.7 with KOH). A total of 10 μL of plasmid DNAs (about 10 μg DNA of each construct) were mixed with 100 μL of suspended protoplasts (usually 1.5–2.5 × 10^5 cells/mL). The DNA and protoplasts mixture was added to 110 μL of 40% PEG solution [40% PEG4000, 0.4 M mannitol, and 100 mM $Ca(NO_3)_2$, adjusted to pH 7.0 with KOH], then incubated for 20 min at room temperature. After incubation, 440 μL W5 medium (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES, adjusted to pH 5.8 with KOH) was added to the tube to dilute PEG. After centrifugation at 150 × *g* for 2 min to remove PEG, the protoplasts were resuspended, and incubated in 2 mL of WI solution (500 mM mannitol, 4 mM MES, 20 mM KCl, pH5.7) for 16 h.

2.4. Measurement of Na^+ and K^+ content

Fifteen-day-old rice plants were treated with 130 mM NaCl 7 d. Shoots were collected and dried at 80 °C for 72 h. The samples were digested with the mixture of HNO_3 and $HClO_4$ (87:13). The contents of Na^+ and K^+ were determined with an atomic absorption spectrometer.

2.5. RNA isolation from rice leaves

Leaves from 15-day-old rice plants were detached and immediately frozen in liquid nitrogen. One hundred milligrams of leaf material was processed in one sample. RNA was isolated according to manufacturer's instruction using TRIzol reagent (Invitrogen). Concentration and purity of RNA was determined by measuring OD at 260 nm and 280 nm.

2.6. Molecular cloning and construction of expression vectors

The full-length of *OsMKK1*, *OsMPK3*, *OsMPK4*, and *OsMPK7* were cloned from the wild-type rice cDNA by PCR. The primers for cloning were *His-OsMKK1-L/R*, *GST-OsMPK3-L/R*, *GST-OsMPK4-L/R*, and *GST-OsMPK7-L/R*, respectively. The cDNAs of *OsMKK1* and *OsMPK3* (*OsMPK4* and *OsMPK7*) were introduced in to the pET28 (a) and pGEX-4T-1 vector, respectively.

Fusion proteins with His tags (for pET28a vector) and GST tags (for pGEX-4T-1 vector were expressed in *Escherichia coli* strain BL21 (DE3; Promega) according to the manufacturer's instructions. The bacterial cells grown at 37 °C to an OD_{600} at 0.5 were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for an additional 6 h at 25 °C. The His- and GST-recombinant proteins were purified with Ni-affinity agarose (Qiagen) and glutathione-sepharose beads (GenScript, Piscataway, New Jersey, USA), respectively, according to the manufacturer's instructions.

2.7. Protein extracts from rice protoplasts and leaves

Fifteen-day-old rice plants were used for salt stress treatments by 200 mM NaCl solution and treated for 15, 30, 60, and 120 min. Total protein extracts were prepared from the protoplasts or leaves with 2 volumes of immunoprecipitation buffer (100 mmol/L HEPES, 5 mmol/L EDTA, 5 mmol/L EGTA, 10 mmol/L DTT, 10 mmol/L