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Comparative phospho-proteomics analysis of salt-responsive phosphoproteins regulated by the MKK9-MPK6 cascade in *Arabidopsis*

Zhenbin Liu, Yuan Li, Hanwei Cao, Dongtao Ren*

State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China

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ABSTRACT

Mitogen-activated protein kinase (MAPK) cascades are involved in the salt stress response in plants. However, the identities of specific proteins operating downstream of MAPKs in the salt stress response remain unclear. Our studies showed that *mkk9* and *mpk6* null mutant seedlings are hyposensitive to salt stress. Moreover, we showed that MPK6 was activated by salt stress, indicating that the MKK9-MPK6 cascade mediated the salt stress response in *Arabidopsis*. To identify phosphoproteins downstream of the MKK9-MPK6 cascade in the salt stress response pathway, we performed two-dimensional electrophoresis (2-DE) with Pro-Q phosphoprotein staining and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) to identify phosphoproteins induced by salt treatment in *mkk9*, *mpk6*, and wild-type seedlings. Phosphorylation of 4 proteins, including Rubisco activase (RCA), plastid ribosomal protein S 1 (PRPS1), plastid division protein (FtsZ2-2), and tortifolia2 (TOR2), was found to be regulated by activation of MKK9-MPK6 cascade. Further Phospho-proteomics analysis of *MKK9^{DD}* mutant seedlings revealed that RCA phosphorylation was up-regulated as a result of MKK9 activation. The finding that the MKK9-MPK6 cascade functions in the salt stress response by regulating phosphorylation of RCA, PRPS1, FtsZ2-2, and TOR2, provides a novel insight into the MAPK-related mechanisms underlying the salt stress response in plants.

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1. Introduction

High soil salinity is a major obstacle to agricultural development [1,2]. High soil salinity induces osmotic stress, ionic stress, and secondary oxidative stress in plant cells, eventually affecting plant growth and development [3]. Photosynthesis is a major factor in the determination of plant growth. Salt stress results in the decreased stomatal opening, and thereby restricts the diffusion of CO_2 into chloroplast and reduces the leaf photosynthetic rate [4,5]. Salt stress can also inhibit photosynthesis by affecting the activity of photosynthesis-related proteins. For example, salt treatments inhibit the activity of and reduce the content of Rubisco, a crucial enzyme in CO_2 fixation process [6,7]. Plants are generally immobile and unable to select their environments. Therefore, in order to adapt to the environment, plants have evolved molecular mechanisms to perceive and respond to various biotic and abiotic stresses. Protein phosphorylation by kinases is an important post-translational modification that regulates cellular signal transduction in response to changes in the environment. In eukaryotes, activation of mitogen-activated protein kinase (MAPK) signaling cascades is a general mechanism through which external stimuli, including biotic and abiotic stresses, are translated into cellular responses.

MAPK cascades are highly conserved signaling processes in eukaryotes that function downstream of sensors and receptors by converting signals generated at the sensors/receptors into cellular responses [8–10]. MAPK cascades are minimally composed of 3 types of kinase, MAP kinase kinase kinases (MAPKKK), MAP kinase kinases (MAPKK), and MAPK (denoted MKKK, MKK, and MPK in Arabidopsis, according to accepted systematic nomenclature), which are linked in various ways to upstream receptors and downstream targets [8,10]. Numerous MAPK pathways that respond to a variety

Abbreviations: 2-DE, two-dimensional electrophoresis; AAA^+, ATPase associated with various cellular activities; BAM1, beta-amylase 1; DEX, dexamethasone; FtsZ2-2, plastid division protein; IPGAM, 2, 3-biphosphoglycerate-independent phosphoglycerate mutase; LC, liquid culture; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MAPK, mitogen activated protein kinase; MBP, myelin basic protein; PRPS1, plastid ribosomal protein S1; RCA, Rubisco activase; ROC4, cyclophilin 20-3; TCA, trichloroacetic acid; TOR2, tortifolia2.

* Corresponding author at: 2 West Yuanming yuan Rd., Haidian District, Beijing 100193, China. Fax: +86 010 62733794.
E-mail address: ren@cau.edu.cn (D. Ren).

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of external stimuli have been characterized in yeast, animals, and plants [11].

Plant MAPK cascades have previously showed to be important for the regulation of salt stress responses. SIMK (salt stress-induced MAPK) and SIMKK (SIMK kinase) in alfalfa cells are activated by salt stress [12,13]. Salt and osmotic stress can enhance the activity of SIPK (salicylic acid-induced protein kinase) in tobacco protoplasts [14]. Three salt stress-induced MAPKs have been identified in *Zea mays*: ZmMPK3, ZmMPK5, and ZmSIMK1 [12,15,16]. In Arabidopsis, the most completely characterized MAPK cascade functioning in response to abiotic stress is the MEKK1-MKK2-MPK4/MPK6 cascade, which is activated by salt or cold stress [17]. Previous studies demonstrated that activation of MKK9 enhanced the sensitivity of transgenic seedlings to salt stress [18,19]. However, it is unclear how MKK9, MPK6, and other kinases regulate the response to salt stress. To understand how kinases mediate the salt stress response, it is vital to identify their downstream components.

Phospho-proteomics is a powerful tool that can be used to identify MAPK substrates and downstream proteins, because it allows unbiased localization and site-specific quantification of many phosphorylated proteins in a single *in vivo* experiment. We used Pro-Q Diamond phosphoprotein gel stain [20–23] to study protein phosphorylation in Arabidopsis under salt stress [24]. The intensity of the Pro-Q Diamond stain was proportional to the degree of phosphorylation of each phosphoprotein, but not to the protein concentration [25].

In this study, we found that *mkk9* and *mpk6* mutants were hyposensitive to salt stress in comparison with wild-type plants. The fresh shoot weight of *mkk9* and *mpk6* seedlings was significantly heavier than that of the wild-type seedlings. Comparative analysis of the two-dimensional electrophoresis (2-DE) patterns in wild-type seedlings, *mkk9* and *mpk6* mutants under salt treatment was used to identify proteins with different phosphorylation statuses among the groups. Twenty salt- responsive phosphoproteins were identified with a high level of confidence in Arabidopsis wild-type seedlings. Four of these proteins was found to be regulated by activation of MKK9-MPK6 cascade.

2. Material and methods

2.1. Plant materials and treatments

Wild-type (Col-0) and mutant *Arabidopsis thaliana* (ecotype Columbia) seeds were surface-sterilized. After cold treatment in the dark for 2 d at 4 °C, the seeds were germinated and grown on Murashige and Skoog medium (containing 2.5% sucrose) with 0.5% phytagel agar (Sigma–Aldrich, St. Louis, MO, USA) plates. For the investigation of the mutant phenotype, wild-type and mutant seedlings were transferred to new plates containing 100 mM NaCl and grown at 22 °C in a growth room with a 12-h photoperiod (photon flux density, 100 $\mu E/m^{-2}/s^{-1}$). Three biological replicates were done for each treatment.

For kinase assays and phospho-proteomics studies, seedlings were transferred to liquid culture medium (0.5 × Murashige and Skoog medium containing 0.025% 2-(*N*-morpholino) ethanesulfonic acid (MES) and 0.25% sucrose, pH 5.7) [18] and grown at 22 °C under continuous light (photon flux density, 70 $\mu E/m^{-2}/s^{-1}$). Two-week-old seedlings were treated with liquid culture medium containing NaCl to obtain final concentration of 100 mM. The control group was treated with liquid culture medium (LCM) only. The MKK9 active mutant ($MKK9^{DD}$), MKK9 inactive mutant ($MKK9^{KR}$), and $MKK9^{DD}$/*mpk6* crossed seedlings were treated with 2 μM dexamethasone (DEX) for 4 and 8 h [26,27]. Plant samples were collected at different time points, flash-cooled in liquid nitrogen, and stored at −80 °C.

T-DNA insert mutants (*mkk9*, Salk_060_H06; *mpk3*, Salk_100651; *mpk6*, Salk_062471) were obtained from the Arabidopsis Biological Resource Center. $MKK9^{KR}$, $MKK9^{DD}$, and $MKK9^{DD}$/*mpk6* mutant plants were generated as previously described [18].

2.2. Protein extraction

Samples were ground to a fine powder in liquid nitrogen and transferred to a tube with precooled 10% TCA/acetone and 1% β-mercaptoethanol, vortexed briefly, and incubated overnight at −20 °C. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the supernatants were discarded and the pellets were washed twice with precooled acetone containing 0.1% β-mercaptoethanol. After incubation for 30 min at −20 °C, the samples were centrifuged at 10,000 × *g* for 20 min at 4 °C. The final pellets were freeze–dried under vacuum and solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS detergent, 60 mM dithiothreitol (DTT), and 0.8% IPG buffer) to extract proteins. The solution was centrifuged (13,000 × *g* for 10 min at 25 °C) and the supernatants were collected for 2-DE. Total protein concentrations were estimated using 2D Quant kits (GE Healthcare Life Sciences).

2.3. 2-DE and Pro-Q staining

Total protein samples (1 mg) in rehydration buffer (8 M urea, 2% CHAPS detergent, 60 mM DTT, 0.8% IPG buffer, and a trace amount of bromophenol blue) were loaded onto 18-cm IEF linear strips (pH 4–7, GE Healthcare Life Sciences). Rehydration was performed at room temperature for 18 h. The IEF voltage was 100 V for 3 h, 300 V for 3 h, 1000 V for 1.5 h, 3000 V for 1.5 h, and finally 8000 V for a total of 75,000 Vh. When IEF was completed, the strips were equilibrated as previously described [28]. Proteins in the strips were separated on 11% polyacrylamide gels, fixed overnight in 500 mL of a solution of 50% methanol and 10% acetic acid, washed in 500 mL deionized water for 30 min, and stained with 3-fold-diluted Pro-Q stain for 2 h. After staining, the gels were destained with 250 mL of a solution of 50 mM sodium acetate (pH 4.0) in 20% acetonitrile (ACN) 4 times for 30 min. All steps from staining to washing were performed in a dark room. Gels were scanned with a Typhoon 9410 fluorescence scanner, stained with a blue-silver staining method as previously described [29], scanned with a UMAX 2000 scanner, and analyzed with PD-Quest 8.0 software. Three biological replicates were done for each condition. Protein spots were considered credible when they were detected in at least three biological replicates.

2.4. Protein identification by MALDI-TOF MS

In-gel protein digestion was performed using trypsin (Roche) as previously described with minor modifications [28]. First, excess trypsin solution was removed, after which the gel pieces were submerged in working solution (1 mM $CaCl_2$ and 25 mM NH_4HCO_3). Supernatants were collected after incubation in the working solution for 12 h at 37 °C. The gel pieces were extracted twice with 70% acetonitrile and 0.1% trifluoroacetic acid for 15 min with sonication. Supernatants were pooled and concentrated by freeze–drying. Samples were loaded onto an Anchor Chip target plate (Bruker Daltonics). Matrix solution (1 mg/mL of a-cyano-4-hydroxycinnamic acid dissolved in 70% acetonitrile and 0.1% trifluoroacetic acid) was added to the dried peptide samples. After fast evaporation, the peptides were washed with 0.1% trifluoroacetic acid and analyzed using an AutoFlexII TOF/TOF mass spectrometer. Spectrum masses of 700 to 4000 D were acquired. All mass spectra were externally calibrated with a peptide calibration standard. Monoisotopic peaks were collected and used for peptide fingerprinting identification with Flex Analysis 3.0 software (Bruker Daltonics). Proteins were identified by searching the National Center for Biotechnology Infor-

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