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Arabidopsis MKK4 mediates osmotic-stress response via its regulation of MPK3 activity

Sun-Ho Kim [a,1], Dong-Hyuk Woo [a,1], Jae-Min Kim [a], Sun-Young Lee [a], Woo Sik Chung [b], Yong-Hwan Moon [a,*]

[a] Department of Molecular Biology, Pusan National University, Busan 609-735, Republic of Korea
[b] Division of Applied Life Science (BK21 Program), Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea

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ABSTRACT

Plants have developed disparate regulatory pathways to adapt to environmental stresses. In this study, we identified MKK4 as an important mediator of plant response to osmotic stress. *mkk4* mutants were more sensitive to high salt concentration than WT plants, exhibiting higher water-loss rates under dehydration conditions and additionally accumulating high levels of ROS. In contrast, *MKK4*-overexpressing transgenic plants showed tolerance to high salt as well as lower water-loss rates under dehydration conditions. In-gel kinase assays revealed that MKK4 regulates the activity of MPK3 upon NaCl exposure. Semi-quantitative RT-PCR analysis showed that expression of *NCED3* and *RD29A* was lower and higher in *mkk4* mutants and *MKK4*-overexpressing transgenic plants, respectively. Taken together, our results suggest that MKK4 is involved in the osmotic-stress response via its regulation of MPK3 activity.

1. Introduction

Plants have sophisticated surveillance systems to sense environmental perturbations, including a variety of biotic and abiotic stress conditions. Upon perceiving a stress, the plant cell converts signals generated at the receptors and sensors to cellular responses through various signal transduction pathways. Mitogen-activated protein kinase (MPK) transduction cascades translate the perception of these external environmental stimuli into physiological cellular responses and are important mechanisms for transcription-dependent stress adaptation. These protein kinase cascades are highly conserved among eukaryotes and consist of three kinases acting in series: MPK kinase kinases (MKKKs), MPK kinases (MKKs), and finally MPKs, each of which phosphorylates its target on specific residues to propagate a signal. MKKKs are serine/threonine kinases that phosphorylate two amino acids in the S/T-X_{3-5}-S/T motif of the MKK activation loop [1]. In turn, MKKs are dual-specificity kinases that can activate MPKs through double phosphorylation of the T-X-Y motif in their activation loop [1]. Lastly, MPKs are serine/threonine kinases able to phosphorylate a wide range of substrates, including other kinases and/or transcription factors.

These evolutionarily conserved signaling pathways are frequently involved in differentiation, stress responses, and the control of cellular growth and division in yeast, humans, and plants [2–6]. The *Arabidopsis* genome contains at least 60 MKKK (AtMKKK), 10 MKK (AtMKK), and 20 MPK genes (AtMPK) [7]. Various environmental stimuli can activate MPKs cascades, and promiscuous interactions among these gene family members can potentially produce thousands of different MPK cascades with multiple permutations of upstream or downstream components. In biotic stress signaling in *Arabidopsis*, flg22-induced defense responses are antagonistically controlled by the MEKK1–MKK1/2–MPK4 and MEKK1–MKK4/5–MPK3/6 MPK cascades [8,9]. Hydrogen peroxide (H_2O_2) stress activates three MPK modules, ANP1–MKK4/5–MPK3/6, MEKK1–MPK4, and MKK3–MPK7, either directly or via the protein kinases NDPK3 and OXI1 [10–12], to mediate the biosynthesis of phytohormones such as salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) by substrate phosphorylation, to evoke responses to biotic stresses.

MPK cascades are additionally involved in plant adaptation to abiotic stresses such as cold, salt, touch/wind, wounding, heat, UV radiation, and osmotic shock. Cold/salt stress induces the MKKK1–MKK2–MPK4 signaling module and drought and wounding MKK1–MPK4 [13,14]. ABA-induced H_2O_2 production and osmotic shock activates MKK1–MPK3 and MPK3/6 via AOS production [15,16]. A number of studies have also revealed the role of MPK cascades in stress-unrelated processes, including developmental and auxin signaling [17,18]. A reverse-genetic approach identified regulation by YODA–MKK4/5–MPK3/6 of stomatal patterning in the epidermis [19,20]. However, despite these studies, only a limited number of MPK modules participating in abiotic stress responses have been analyzed. Thus, further studies are necessary to identify

Abbreviations: ABA, abscisic acid; MKK, MPK kinase; MKKK, MPK kinase kinase; MPK, mitogen-activated protein kinase; NBT, nitroblue tetrazolium; ROS, reactive oxygen species.

* Corresponding author. Fax: +82 51 513 9258.
E-mail address: moonyh@pusan.ac.kr (Y.-H. Moon).

[1] These authors contributed equally to this work.

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and characterize the roles of additional MPK cascades in abiotic stress signal transduction.

In this study, we characterized the role of MKK4 in the *Arabidopsis* osmotic-stress response. We found that *mkk4* loss-of-function mutants and *MKK4*-overexpressing transgenic plants (*MKK4* OXs) produce aberrant responses to osmotic stress. In-gel kinase assays for MPK3 phosphorylation in *mkk4* mutants and *MKK4* OXs demonstrated that MKK4 plays an important role in osmotic-stress responses via regulation of MPK3 activity.

2. Materials and methods

2.1. Plant materials and growth conditions

All *Arabidopsis* (*Arabidopsis thaliana*) plants used in this study were ecotype Columbia. T-DNA insertion-mutant seeds of *MKKK*, *MKK*, and *MPK* genes were obtained from the Salk T-DNA mutant collection (Supplementary Table S1) [21]. *Arabidopsis* seeds were surface-sterilized, placed in the dark for 2 days at 4 °C, and germinated on agar plates containing one-half strength MS medium [22], supplemented with B5 vitamins, 1.5% (w/v) sucrose, and 0.7% (w/v) agar. The plates were then placed under short-day (SD) conditions (cycles of 8-h light/16-h dark) at 22 °C. Ten- to twelve-day-old seedlings were transplanted to soil and grown under long-day (LD) conditions (cycles of 16-h light/8-h dark) at 22 °C.

2.2. Transgenic plant generation

To generate *MKK4* OXs, we amplified the coding sequence of *MKK4* (TAIR ID: AT1G51660) from *Arabidopsis* genomic DNA by PCR, using forward primer 5′-ACTGTCGACATGAGACCGATTCAATCGCC-3′ (*Sal*I sequence is underlined) and reverse primer 5′-AGAGGTACCCTATGTGGTTGGAGAAGAAG-3′ (*Kpn*I sequence is underlined), and inserted it into the pFGL571 vector with a modified *cauliflower mosaic virus* (*CaMV*) *35S* promoter [23]. The resulting plasmid, pFGL1001, was introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by a freeze–thaw method [24], then transformed into *Arabidopsis* wild-type (WT) plants by a floral-dipping method [25]. Transgenic plants were selected on MS medium containing 25 mg/L kanamycin.

2.3. Stress treatments

To study response to NaCl treatment, 7-day-old seedlings grown under SD conditions were transferred to MS-agar medium supplemented with 0, 120, 130, 150, or 160 mM NaCl. NaCl response was estimated by measuring the fresh weight (FW) of seedlings after 7 or 14 days of treatment. For LiCl treatment, 7-day-old seedlings of WT and *MKK4* OX grown under SD conditions were transferred to MS-agar medium supplemented with 0 or 25 mM LiCl. FW was measured after 8 days of treatment. For water-loss measurements, 4-week-old plant shoots grown under LD conditions were detached from their roots and immediately weighed. Each shoot was then placed on a plate and weighed at the times designated.

2.4. Detection of superoxide production in Arabidopsis seedlings

For nitroblue tetrazolium (NBT) staining, 10-day-old WT and mutant seedlings grown under SD conditions were placed on MS media supplemented with 0, 50, or 100 mM NaCl for 2 h. Whole seedlings were then incubated in HEPES buffer (pH 7.5) containing 6 mM NBT for 2 h; pale yellow NBT reacts with superoxide to form dark blue insoluble formazan within 20 min [26]. Chlorophyll was then extracted by treatment with 95% (v/v) ethanol for 2 h at 50 °C. Seedlings were observed by microscopy.

2.5. Semi-quantitative reverse-transcription (RT)-PCR and real-time RT-PCR

Ten-day-old WT seedlings grown under SD conditions were subjected to a range of stress treatments, including 300 mM NaCl (0, 1, 2, 4, or 8 h), 10 μM methyl viologen (MV; 0, 1, 2, 4, or 8 h), 300 mM mannitol (0, 1, 2, 4, or 8 h), and 100 μM abscisic acid (ABA; 0, 1, 2, 4, or 8 h). Total RNA was prepared using TRIzol (Molecular Research Center). RNA was treated with RNase-free DNase I (Promega) according to the manufacturer's instruction. cDNA was synthesized from 5 μg of total RNA using M-MLV Reverse Transcriptase (Promega) and a poly-T primer.

MKK4 mRNA levels were determined by semi-quantitative RT-PCR analysis with 26–30 PCR cycles, and *GAPc* with 23–26 cycles. The number of PCR cycles used was demonstrated to be in the linear range for amplification (data not shown). The PCR procedure included an initial 5-min denaturation at 94 °C, followed by cycles of 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s, with a final 10-min extension at 72 °C. Primers used for the PCR reaction were as follows: for *MKK4*, forward, 5′-GGTGATATACGGTAACCACG-3′ and reverse, 5′-TTACACGGATCCATAGTCTG-3′; for *GAPc*, forward, 5′-CACTTGAAGGGTGGTGCCAAG-3′ and reverse, 5′-CCTGTTGTCGCCAACGAAGTC-3′.

Real-time RT-PCRs were performed in a 15-μL volume containing 1 μL RT product as template, 10 pmol gene-specific primers, and 7.5 μL SsoFast EvaGreen Supermix (Bio-Rad), using the CFX96™ Real-Time System (Bio-Rad). The reaction included an initial 5-min pre-incubation at 95 °C, 45 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s followed by melting curves with stepwise increases of 0.5 °C/cycle, 90 cycles at 55 °C, and final extension for 10 min at 72 °C. PCR primers were as follows: for *NCED3*, forward, 5′-TGATACGACGACCACGAATC-3′ and reverse, 5′-AGTAACTCCAAAGCGACACG-3′; for *RD29A*, forward, 5′-TATGACGACGGATGCTCATG-3′ and reverse, 5′-CTCCATAAGGACACGTCATC-3′; for *Rab18*, forward, 5′-GATGACGACGTATCGTTATGG-3′ and reverse, 5′-TACACTCGTTTCTCAGTTTTACAAAC-3′.

2.6. In-gel kinase assay

In-gel kinase assays were performed essentially as previously described [27], with the following modifications: 18-day-old seedlings of WT, *mkk4*, and *MKK4* OX grown under LD conditions were treated with 150 mM NaCl. Total proteins were extracted from NaCl-treated seedlings, and 30 μg of proteins were separated on SDS–PAGE using 10% gels added with 0.5 mg/mL myelin basic protein (MBP) as a substrate. After electrophoresis, gels were washed three times with wash buffer (25 mM Tris–HCl, pH 7.5; 0.5 mM DTT; 0.1 mM Na_3VO_4; 5 mM NaF; 0.5 mg/mL bovine serum albumin; 0.1% Triton X-100) to remove SDS. Proteins were then renatured overnight at 4 °C in renaturing buffer (25 mM Tris–HCl, pH 7.5; 1 mM DTT; 0.1 mM Na_3VO_4; 5 mM NaF). Gels were incubated in reaction buffer (25 mM Tris–HCl, pH 7.5; 2 mM EGTA; 12 mM $MgCl_2$; 1 mM DTT; 0.1 mM Na_3VO_4) at room temperature for 30 min, and then phosphorylated in 20 mL reaction buffer containing 0.5 μM ATP and 50 μCi [γ-^{32}P] ATP at room temperature for 1 h. Reactions were stopped by transferring gels into stop solution (5% trichloroacetic acid and 1% sodium pyrophosphate). Gels were dried on 3 M paper and then subjected to autoradiography.

3. Results and discussion

3.1. Response of mkkk, mkk, and mpk loss-of-function mutants to high salt stress

To identify MKKKs, MKKs, and MPKs involved in osmotic-stress response, T-DNA insertion mutants of nine MKKKs, four MKKs, and

eleven MPKs were analyzed for their response to high concentration of NaCl. For this, the seeds of T-DNA insertion mutants of MKKKs, MKKs, and MPKs were obtained from ABRC, and homozygous mutant lines of nine MKKKs, four MKKs, and eleven MPKs were selected for analysis (Supplementary Table S1). For NaCl treatment, 7-day-old mutant seedlings were treated with 120 or 130 mM NaCl for selection of sensitive mutants. Among the mutants analyzed, *mkkk20*, *mkk4*, and *mpk6* mutants were more sensitive to NaCl than wild-type (WT) plants (Supplementary Table S2 and Supplementary Fig. S1). We selected the *mkk4* mutant for further analysis as significantly sensitive to high salt stress (Supplementary Fig. S1A); additionally the role of MKK4 in abiotic stress signal transduction is not well characterized. Previously the involvement of MKK4 in biotic stress response was reported [28], but not in abiotic stress response. The MKKK1–MKK4/5–MPK3/6 modules are shown to be activated by flg22 peptide through the flagellin receptor FLS2, which in turn activates the plant immune responsive genes, WRKY29 and FRK1 [28].

3.2. Loss-of-function mutants of MKK4 are sensitive to salt and drought stresses

RT-PCR analysis showed that the homozygous *mkk4* mutant (Salk_058307) had lost expression of *MKK4* (Supplementary Fig. S2A). To confirm the response of the *mkk4* mutant to high salt stress, we treated 120 or 130 mM NaCl to *mkk4* mutants. *mkk4* mutants displayed a salt-sensitive phenotype and a reduction in fresh weight compared with WT plants (Fig. 1A and B). We then compared water-loss rates in *mkk4* mutants and WT plants under dehydration conditions. As a result, we found that this rate was augmented in *mkk4* mutants compared with WT plants (Fig. 1C). Taken together, *mkk4* mutants appeared to be sensitive to osmotic stresses such as high salt and drought, suggesting that *MKK4* is involved in the osmotic-stress response.

Most abiotic stresses evoke an increased production of reactive oxygen species (ROS) in plants [29]. We therefore determined the generation of superoxide radicals in *mkk4* mutants under NaCl treatment. Before NaCl treatment, the superoxide produced in *mkk4* mutants was higher than that produced in WT plants. This difference was augmented by NaCl treatment (Fig. 1D). These results are consistent with the previous conclusion that *mkk4* mutants are sensitive to high salt stress.

3.3. MKK4-overexpressing plants are tolerant to high salt and drought stresses

To confirm the role of *MKK4* in the response to osmotic stress, we generated *MKK4* OXs in which *MKK4* was overexpressed under the control of *CaMV 35S* promoter. Using RT-PCR analysis, we selected two *MKK4* OX T_1 lines for gain-of-function studies of *MKK4* (Supplementary Fig. S2B). *MKK4* OXs were resistant to treatment with 150 or 160 mM NaCl (Fig. 2A), and their fresh weight was also heavier compared with WT plants (Fig. 2B). Additionally, *MKK4* OXs were resistant to 25 mM LiCl (Supplementary Fig. S3), indicating that their salt-resistance is not ion-specific. We compared water-loss rates in *MKK4* OX and WT plants under dehydration conditions. Water-loss in *MKK4* OXs was slower than that in WT plants (Fig. 2C). These results and those for *mkk4* loss-of-function mutants indicate that *MKK4* plays an important role in osmotic-stress signal transduction.

3.4. MKK4 mediates phosphorylation of MPK3 under high salt stress

It has been demonstrated that MPK3, MPK4, and MPK6 are involved in abiotic stress responses [30–32]. MKK4 was previously reported to regulate the activity of MPK3 and MPK6 in response to flg22 challenge [28]. To investigate whether MPK3, MPK4, and MPK6 are involved in MPK cascades including MKK4 in osmotic-

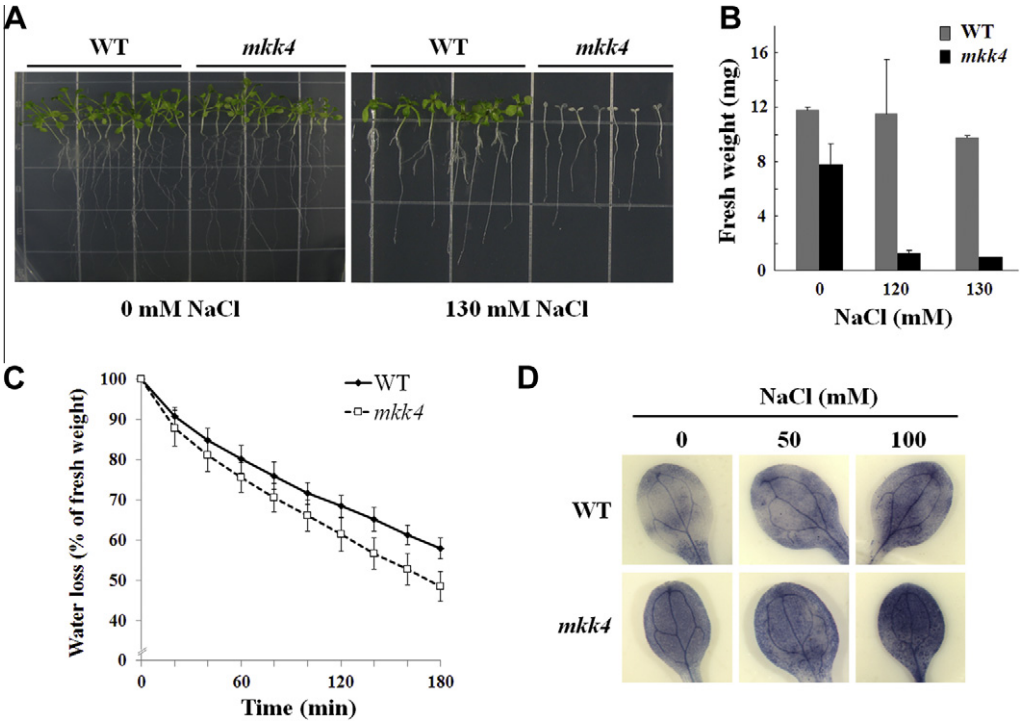

Fig. 1. Response of *mkk4* mutants to osmotic stress. (A) Response of WT and *mkk4* seedlings to 130 mM NaCl. (B) Fresh weights of WT and *mkk4* seedlings measured after 0, 120, or 130 mM NaCl treatment. (C) Analysis of water-loss rate in WT and *mkk4* plants. Fresh weights of aerial parts were measured after detachment. (D) Superoxide accumulation in cotyledons of 10-day-old WT and *mkk4* seedlings after 2-h treatment with 0, 50, or 100 mM NaCl. In (A) and (B), 7-day-old WT and *mkk4* seedlings were treated with 120 or 130 mM NaCl for 14 days. In (B) and (C), error bars represent standard deviation ($n = 24$ plants).

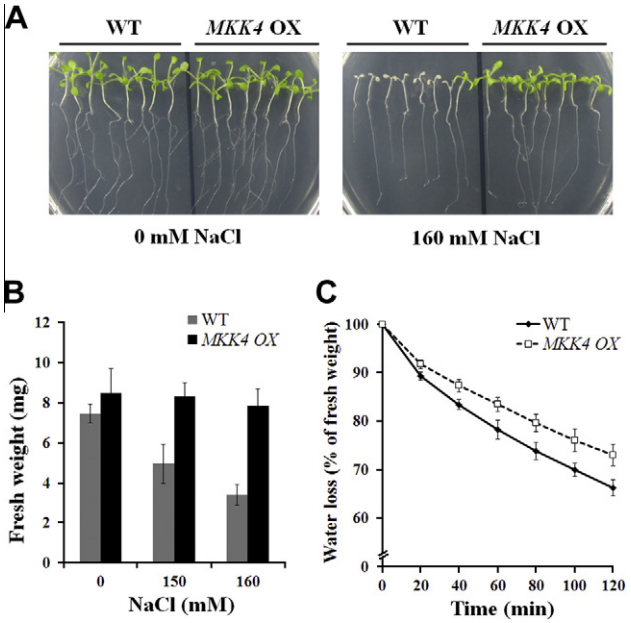

Fig. 2. Response of *MKK4* OXs to osmotic stress. (A) Response of WT and *MKK4* OX seedlings to 160 mM NaCl. (B) Fresh weights of WT and *MKK4* OX seedlings measured after 0, 150, or 160 mM NaCl treatment. (C) Analysis of water-loss rate in 4-week-old WT and *MKK4* OX plants. Fresh weights of aerial parts were measured after detachment. In (A) and (B), 7-day-old WT and *MKK4* OX seedlings were treated with 150 or 160 mM NaCl for 7 days. In (B) and (C), error bars represent standard deviation ($n = 24$ plants).

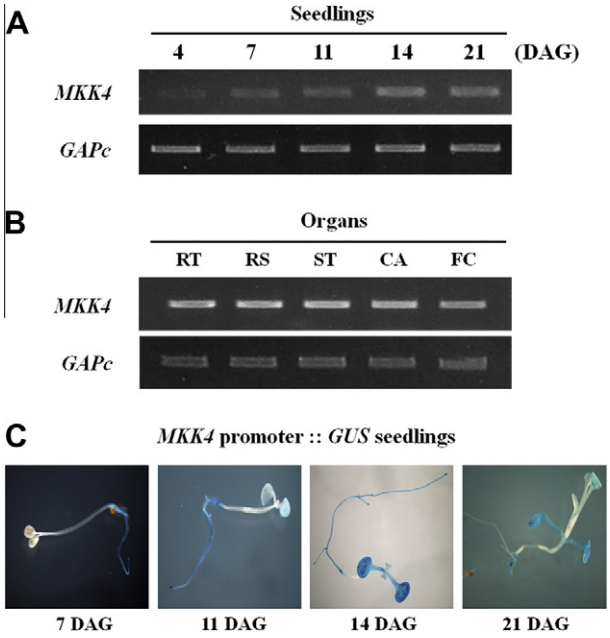

Fig. 4. Analysis of *MKK4* expression patterns during development and in organs of mature plants. (A) Semi-quantitative RT-PCR analysis of *MKK4* expression in 4-, 7-, 11-, 14-, or 21-day-old WT seedlings grown under SD conditions. DAG: days after germination. (B) Semi-quantitative RT-PCR analysis of *MKK4* expression in organs of 8-week-old WT plants grown under LD conditions. RT: roots; RS: rosette leaves; ST: stems; CA: cauline leaves; FC: floral clusters. In (A) and (B), similar results were obtained from at least two independent biological replicates; one experiment is depicted here. *GAPc* was used as an internal control. (C) Histochemical GUS-staining of transgenic seedlings harboring *MKK4* promoter::*GUS* fusion at 7, 11, 14, or 21 DAG under SD conditions.

stress signal transduction, we characterized the activities of MPK3, MPK4, and MPK6 in *mkk4* mutants and *MKK4* OXs under NaCl treatment using in-gel kinase assays. Under NaCl treatment, the activity of MPK4 and MPK6 in the *mkk4* mutants was nearly the same as that in WT, while MPK3 activity was lower in *mkk4* than WT after 15 min (Fig. 3A). In *MKK4* OX, only MPK3 activity was higher than WT after 5 min (Fig. 3A). These results suggest that MKK4 is involved in osmotic-stress signal transduction via the regulation of MPK3 activity.

3.5. MKK4 regulates the transcription of NCED3 and RD29A

Osmotic stress can increase the cellular level of the phytohormone ABA, with concomitant induction of many osmotic stress-responsive genes [33,34]. MPK3 is known to be involved in ABA signaling [15]. Because our results implied MKK4's involvement in osmotic-stress signal transduction through its regulation of MPK3 activity, we investigated whether MKK4 also participates in the regulation of ABA-dependent stress-responsive gene induction. We analyzed the expression patterns of ABA-dependent stress-responsive genes such as *NCED3* and *RD29A* in *mkk4* mutant and *MKK4* OX seedlings under NaCl treatment by real-time RT-PCR. As a result, the transcripts of both *NCED3* and *RD29A* genes were reduced in *mkk4* mutants and increased in *MKK4* OXs compared with WT (Fig. 3B). These results suggest that MKK4 might mediate osmotic-stress response and promote *NCED3* and *RD29A* expression.

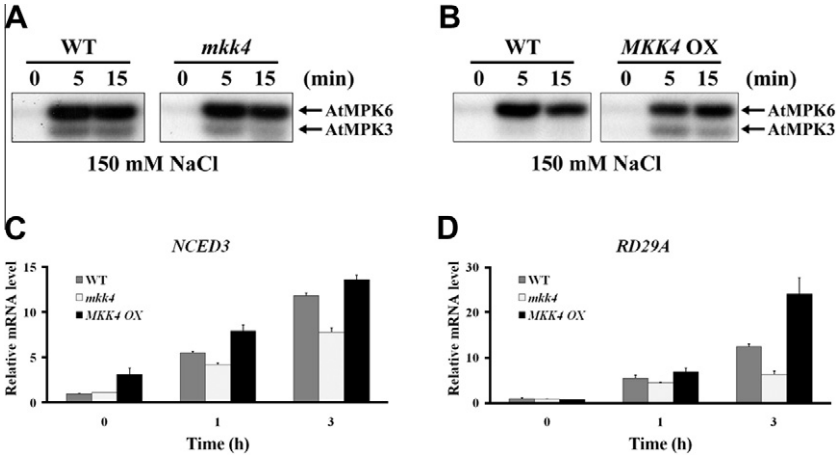

Fig. 3. Analysis of MPK activity and *NCED3* and *RD29A* transcription in *mkk4* mutants and *MKK4* OXs under high salt stress. (A) In-gel kinase assays in WT, *mkk4*, and *MKK4* OX seedlings under 150 mM NaCl treatment. Eighteen-day-old WT, *mkk4*, and *MKK4* OX seedlings were treated with 150 mM NaCl for 0, 5, or 15 min. Real-time RT-PCR analysis of *NCED3* (B) and *RD29A* (C) in WT, *mkk4*, and *MKK4* OX seedlings under NaCl treatment. Ten-day-old seedlings were treated with 150 mM NaCl for 0, 1, or 3 h. Data shown are mean ± SD ($n = 3$). Similar results were obtained from at least two independent biological replicates; one experiment is depicted here.

3.6. Determination of MKK4 transcript levels during development, in adult plant organs, and in response to abiotic stress

We investigated the expression patterns of *MKK4* during *Arabidopsis* development and in different organs of mature plants using semi-quantitative RT-PCR and *MKK4* promoter::*GUS* transgenic plants. Total RNA were extracted from 4, 7, 11, 14, and 21-day-old seedlings grown under SD conditions, and *MKK4* transcript levels were analyzed by semi-quantitative RT-PCR. The results showed that *MKK4* transcripts continue to be expressed over the 21-day time period shown (Fig. 4A). The expression patterns of *MKK4* during vegetative development were also supported by *MKK4* promoter::*GUS* transgenic plants (Fig. 4C). Moreover, in 8-week-old mature plants grown under LD conditions, *MKK4* transcripts could be detected in all organs investigated, including roots, rosette leaves, stems, cauline leaves, and floral clusters (Fig. 4B).

To explore how *MKK4* responds to abiotic stresses at the transcriptional level, semi-quantitative RT-PCR was performed using 10-day-old WT seedlings under treatment with 300 mM NaCl, 10 μM MV, 300 mM mannitol, or 100 μM ABA. Transcript levels of *MKK4* did not change significantly under any treatment (Supplementary Fig. S4). These results suggest that MKK4's participation in the osmotic-stress response might be contingent on its post-transcriptional modification, possibly by phosphorylation, as it is common among MPK signaling cascades [30].

Cumulatively, our results suggest that MKK4 is a mediator of osmotic-stress signal transduction via regulation of MPK3 activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.064.

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