Physiology

Mitogen-activated protein kinase 6 controls root growth in *Arabidopsis* by modulating Ca^{2+}-based Na^{+} flux in root cell under salt stress

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A R T I C L E   I N F O

Article history:
Received 16 April 2013
Received in revised form 6 September 2013
Accepted 18 September 2013

Keywords:
mpk6
NaCl
Na^{+} flux
Ca^{2+}
Root growth

A B S T R A C T

Little is known about the role of mitogen-activated protein kinase 6 (MPK6) in Na^{+} toxicity and inhibition of root growth in *Arabidopsis* under NaCl stress. In this study, we found that root elongation in seedlings of the loss-of-function mutants mpk6-2 and mpk6-3 was less sensitive to NaCl or Na-glutamate, but not to KCl or mannitol, as compared with that of wild-type (WT) seedlings. The less sensitive characteristic was eliminated by adding the Ca^{2+} chelator EGTA or the Ca^{2+} channel inhibitor LaCl_{3}, but not the Ca^{2+} ionophore A23187. This suggested that the tolerance of mpk6 to Na^{+} toxicity was Ca^{2+}-dependent. We measured plasma membrane (PM) Na^{+}-conducted currents (NCCs) in root cells. Increased concentrations of NaCl increased the inward NCCs while decreased the outward NCCs in WT root cells, attended by a positive shift in membrane potential. In mpk6 root cells, NaCl significantly increased outward but not inward NCCs, accompanied by a negative shift in membrane potential. That is, mpk6 decreased NaCl-induced the Na^{+} accumulation by modifying PM Na^{+} flux in root cells. Observations of aequorin luminescence revealed a NaCl-induced increase of cytosolic Ca^{2+} in mpk6 root cells, resulting from PM Ca^{2+} influx. An increase of cytosolic Ca^{2+} was required to alleviate the NaCl-induced Na^{+} content and Na^{+}/K^{+} ratio in mpk6 roots. Together, these results show that mpk6 accumulated less Na^{+} in response to NaCl because of the increased cytosolic Ca^{2+} level in root cells; thus, its root elongation was less inhibited than that of WT by NaCl.

Abbreviations: MPK, mitogen-activated protein kinase; NCC, Na^{+}-conducted current; NSCC, non-selective cation channel; PM, plasma membrane; RCP, root cell protoplast.

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Introduction

In environments affected by salts, especially NaCl, some plant species take up Na^{+}. The amount of Na^{+} absorbed exceeds that of Na^{+} extruded through the plasma membrane (PM) cation channels, leading to excessive Na^{+} accumulation in root cells. This can bring about a series of Na^{+}-toxicity events, such as damage to systems that absorb and utilize Ca^{2+} and K^{+} ions. Damage to these systems negatively affects plant growth and development, since Ca^{2+} and K^{+} are essential for various physiological and biochemical processes (Demidchik et al., 2002; Demidchik and Maathuis, 2007; Ebrahimii and Bhatia, 2012; Hilge, 2012).

Na^{+} toxicity results from excess absorption of Na^{+} by root cells, since roots are directly exposed to NaCl in soil. Among the PM cation channels, K^{+} channels synchronously transport K^{+} and Na^{+} ions (Volkov et al., 2009). When *Arabidopsis* seedlings were subjected to excessive NaCl, the outward-rectifying K^{+} channels at the PM of root cells were activated by SOS1 (salt overly sensitive 1, a PM Na^{+}/H^{+} antiporter), an Na^{+} efflux system (Shabala et al., 2005). Unlike K^{+} channels, PM non-selective cation channels (NSCCs) have a relatively high affinity for Na^{+} while allowing unidirectional Na^{+} influx into root cells in *Arabidopsis* (Demidchik et al., 2002; Volkov and Amtmann, 2006). Thus NSCCs are considered to be the major pathway for Na^{+} uptake in root cells (Demidchik and Maathuis, 2007). In previous studies, the instantaneous Na^{+}-conducted currents (NCCs) through the PM NSCCs were revealed in different types of cells, such as guard cells of *Vicia faba* (Zhao et al., 2011) and root cells of *Thalliumia halophila* (Volkov and Amtmann, 2006). These currents shared common characteristics: they are time-independent, voltage-dependent, sensitive to quinine (a non-selective cation channels blocker), and insensitive to tetraethylammonium (TEA; a K^{+} channel blocker) or verapamil (a Ca^{2+} channel blocker). Interestingly, NSCCs activity
is involved in plant root growth under NaCl stress. The halophyte <i>Thellungiella</i> maintains an appropriate Na\(^+\)/K\(^+\) ratio by mediating Na\(^+\) influx through PM NSCCs in root cells, and thus grows normally in high-NaCl environments (Volkov and Amtmann, 2006). Its response differs from that of the glycophyte <i>Arabidopsis</i>, which shows inhibited growth when exposed to NaCl (Demidchik et al., 2002; Yu et al., 2008). These findings showed that the activity of NSCCs is a general mechanism by which plants tolerate NaCl stress.

NaCl-induced toxic Na\(^+\) uptake through PM NSCCs can be antagonized by increased Ca\(^{2+}\) influx and cytosolic Ca\(^{2+}\) levels (Volkov and Amtmann, 2006; Demidchik and Maathuis, 2007), and this antagonism contributed to the growth of plant roots (Demidchik and Tester, 2002; Demidchik et al., 2002). New research on biochemical profiles indicated that sunflower roots depend on Ca\(^{2+}\) to block NaCl-imposed toxic Na\(^+\) uptake (Ebrahimie and Bhatla, 2012). However, little is known about how plants increase cytosolic Ca\(^{2+}\) to tolerate Na\(^+\) toxicity under NaCl stress.

Mitogen-activated protein kinase (MPK) cascades consist of three sequential components, MPK kinase kinase (MKKK), MPK kinase (MKK), and MPK. Various combinations of MPK cascades mediate plant tolerance to NaCl, and play roles in cell wall biosynthesis and cell growth and differentiation (Antonsson et al., 2006; Colcombet and Hirt, 2008). Activation of the MKKK-MPK6 cascade mediates sensing of NaCl in <i>Arabidopsis</i>, as revealed by the following experimental findings: (1) MKK9 directly phosphorylated and activated MPK6 in <i>Arabidopsis</i> seedlings (Xu et al., 2008); (2) NaCl increased the kinase activity of MPK6 in <i>Arabidopsis</i> seedlings (Ichimura et al., 2000); and (3) seedlings of the loss-of-function mutant mkk9 were insensitive to NaCl inhibition of root elongation (Alzwey and Morris, 2007; Xu et al., 2008). These results showed that the activity of MPK6 negatively regulates tolerance to NaCl stress in <i>Arabidopsis</i>. Nevertheless, MPK6-activated SOS1 (Yu et al., 2010) likely control PM K\(^+\) and H\(^+\) flux, but not Na\(^+\) flux (Shabala et al., 2005). Thus, there is still a missing link between the activation of MPK6 and the flux of Na\(^+\) through the PM of plant cells in the signaling pathway for NaCl stress. Activation of SOS1 bypassed the Ca\(^{2+}\)-sensing SOS3, indicating that the signaling pathways of the Na\(^+\) flux system SOS1–SOS2–SOS3 are highly branched (Shabala et al., 2005). Besides its involvement in activating SOS5, MPK6 was shown to mediate cytosolic Ca\(^{2+}\) homeostasis by modifying the activity of PM Ca\(^{2+}\) transporters, such as the glutamate receptors (GLRs), the cyclic nucleotide gated channels (CNGCs), in <i>Arabidopsis</i> seedling roots in response to fungus stimuli (Vadassery et al., 2009). However, it remains to be determined whether NaCl-modified cytosolic Ca\(^{2+}\) homeostasis is involved in NaCl-activated MPK6 signaling (Ichimura et al., 2000) or MPK6-activated SOS1 activity (Yu et al., 2010).

In this study, we investigated root growth in the loss-of-function mutants mpk6-2 and mpk6-3 seedlings in response to NaCl stress. We analyzed NaCl-initiated events such as PM Na\(^+\) flux, cytosolic Ca\(^{2+}\) homeostasis, and variations in the Na\(^+\)/K\(^+\) ratio, and compared the responses between the mutants and WT. Our findings provided the evidence that the inactivation of MPK6 allowed <i>Arabidopsis</i> seedlings to increase the cytosolic Ca\(^{2+}\) level and to buffer toxic Na\(^+\) accumulation in root cells exposed to NaCl.

**Materials and methods**

**Plant materials and growth conditions**

<i>Arabidopsis thaliana</i> L. (Columbia-0) was used as WT. The T-DNA insertion lines were mpk6-2 (Salk_073907) and mpk6-3 (Salk_127507). Their homozygotes were indented by our colleagues, and the expression of MPK6 in these mutants is null (Wang et al., 2010). Sequence data from this article have been deposited in <i>Arabidopsis</i> Genome Initiative databases under the accession number At2g43790 for mitogen-activated protein kinase 6 (MPK6).

All seeds were surface-sterilized with 0.1% HgCl\(_2\) and washed with distilled water, and sown on Murashige-Skoog (MS) medium (0.6% agar, 1% sucrose). The seeds were kept for 3 d at 4 °C in the dark to break dormancy, and then transferred to a growth chamber for a further 4 days. The growth conditions in the chamber were as follows: day/night temperatures of 22°C/18°C, respectively, approximately 70% relative humidity, 16/8-h light/dark photoperiod with a light intensity of approximately 100 μmol m\(^{-2}\) s\(^{-1}\). The seedlings were then transferred to fresh MS (1.2% agar, 1% sucrose) or containing NaCl and/or other treatments. The plates were placed vertically.

**Measurement of primary root length**

As described by Bai et al. (2009), the lengths of the primary roots of seedlings were measured under a FV1000 microscope (Olympus, Tokyo, Japan) each day.

**Isolation of root cell protoplasts (RCPs) and whole-cell configuration patch-clamp recordings**

RCPs were isolated from 10-d-old seedlings as described by Demidchik and Tester (2002). The whole-cell voltage-clamp current in root protoplasts was recorded with an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Whole-cell currents were obtained in response to 3-s voltage pulses (ranging from −190 to +110 mV in 20-mV steps). After establishing the whole-cell configuration, the membrane potential was clamped to −52 mV (holding voltage), and the Na\(^+\) and Ca\(^{2+}\) currents were recorded 15 min before and after treatments with NaCl and/or other compounds.

To record whole-cell Na\(^+\)-conducted currents (NCNs), the patch-clamp pipette solution contained 50 mM Na-glucosinate, 2 mM NaCl, 5 mM HEPES, and 2 mM MgCl\(_2\) (pH 7.2), with osmolality at 510 mOsmol kg\(^{-1}\) adjusted with d-sorbitol. The bath solution contained 25 mM Na-glucosinate, 0.05 mM CaCl\(_2\), and 2 mM MES (pH 5.5). NaCl were added to bath solution to create salt-stress conditions. In both cases (salt stress or not), the final osmolality was adjusted to 490 mOsmol kg\(^{-1}\) with d-sorbitol. To evaluate antagonism between cytosolic Ca\(^{2+}\) and Na\(^+\) uptake in root cells, various concentrations of CaCl\(_2\) was added to the pipette solution. The final osmolality was 380 mOsmol kg\(^{-1}\) adjusted with d-sorbitol.

For measurement of whole-cell Ca\(^{2+}\)-permeable channel currents, the pipette solution contained 10 mM BaCl\(_2\), 0.1 mM dithiothreitol (DTT), 2 mM EGTA, and 10 mM Hepes-Tris (pH 7.2), with osmolality adjusted to 510 mOsmol kg\(^{-1}\) with d-sorbitol. The bath solution contained 100 mM CaCl\(_2\), 0.1 mM DTT, and 10 mM MES-Tris (pH 5.5). NaCl were added to the bath solution to create salt-stress conditions, and the final osmolality was adjusted to 490 mOsmol kg\(^{-1}\) with d-sorbitol.

Whole-cell data were low-pass filtered with a cut-off frequency of 2.9 kHz and analyzed with the software PULSE and PULSEFIT (version 8.3). The final whole-cell currents are expressed as currents per unit capacitance (pA pF\(^{-1}\)) to account for variations in cell surface area.

**Creation of aequorin-expressing plants**

WT and mpk6-3 plants constitutively expressing aequorin were generated by transformation with pMAQ2, a gift from Dr. M. Knight according to Bai et al. (2009). The F2 generation was screened and used to measure aequorin luminescence.
Measurements of cytosolic Ca\(^{2+}\) content in transgenic seedling roots

To evaluate aequorin luminescence, roots were dissected from 10-d-old transgenic seedlings grown on MS. The roots were incubated in 2.5 mM native coelenterazine (Promega, Madison, WI, USA) overnight in the dark at approximately 25 °C. The next day, 10 roots were placed in a transparent plastic cuvette without liquid, and then transferred into a T20/20n digital luminometer (Turner Biosystems, Sunnyvale, CA, USA). Luminescence was recorded every 0.2 s. After 20 s, the buffer with or without NaCl was added to the cuvette and the luminescence was measured. At the end of each experiment, the remaining aequorin was discharged by adding an equal volume of 2 M CaCl\(_2\) and 20% ethanol. Luminescence values were converted to Ca\(^{2+}\) concentrations according to Knight et al. (1991).

Total RNA extraction and RT-PCR

Seedlings grown on MS or containing 120 mM NaCl for 7 days. RNA was extracted from seedling roots using TRIzol reagent (Invitrogen). Reverse transcription was performed using 5 μg RNA and SuperScript II Reverse Transcriptase (Takara). The cDNA was diluted for RT-PCR. The RT-PCR was performed with the PCR system (Biorad S1000). The transcript level of ACTIN2 was used as the control, which was amplified using the primers 5′-ATTACCGAGGGCGAATCTACA-3′ (forward, F) and 5′-CACAAGCAG GCTG GAAAACA-3′ (reverse, R). The other gene-specific primers used for PCR amplifications were as follows: CNGC2 (F: 5′-GAAGCC GCAGGCTTCTGTG, R: 5′-GTGTATAGCCACCAACAG); CNGC10 (F: 5′-GGGTATGGTCA AAGTGGAAGCA, R: 5′-ACGCGGGGCACACCTACA); CNGC15 (F: 5′-ACCCCTGTTGTAACCCGAG, R: 5′-ACCTGACTT GCTCAGGCC); CNGC20 (F: 5′-CTCCAGAACTCGTTCCTGTA, R: 5′-ATGTCA TTAG CTTTCTGTTGA); GLR1.3 (F: 5′-GGGCGGAACTCCTGTTGA, R: 5′-GGACGTACGAAACCCAT); GLR2.5 (F: 5′-AGGAGGCCCCATCGAGA GCCT, R: 5′-AAGCCAAAGCCATCAGCCTA); GLR3.1 (F: 5′-AACGTAATGTCGTTCTCCAGC, R: 5′-CACCACATTCTCGACCGCA).

Measurements of Na\(^+\) and K\(^+\) contents

Roots were collected from 10-d-old seedlings grown on MS or containing indicated compounds, their Na\(^+\) and K\(^+\) contents were determined by inductively coupled plasma–atomic emission spectrometry (ICP–OES; Perkin-Elmer Optima 2100DV, Shelton, CT, USA). To kill root tissues, they were subjected to 110 °C for 10 min, and then dried at 70 °C for 48 h. The dried tissues were incinerated at 550 °C for 6 h. An aliquot of sample ash was dissolved in 0.5 M HCl solution, and then concentrations of Na\(^+\) and K\(^+\) were determined.

Statistical analysis

Differences in various parameters were compared using Student’s t-test (**P<0.01, *P<0.05).

Results

mpk6 seedling root elongation was insensitive to NaCl

To investigate the role of mpk6 in NaCl-inhibited root elongation, the elongation of the mpk6-2 or mpk6-3 mutants was examined in response to various concentrations of NaCl. The root length was not significantly different between WT and mpk6 seedlings grown on MS for 10 d (Fig. 1A). However, on MS containing ≤120 mM NaCl, roots of mpk6-2 or mpk6-3 seedlings were longer than those of WT seedlings from 5 d after transfer (Fig. 1C and D). At 10 days on MS containing 120 mM NaCl, the lengths of mpk6-2 roots (2.85 ± 0.29 cm) or mpk6-3 roots (2.89 ± 0.28 cm) were approximately twice that of WT (1.43 ± 0.13 cm) (Fig. 1B–D). On MS containing ≥150 mM NaCl, mpk6 mutants and WT showed similarly inhibited root elongation (Fig. 1C). These observations showed that the mpk6 mutants lost their sensitivity to moderate concentrations of NaCl, as determined by inhibition of seedling root elongation.

Alleviation of Na\(^+\)-inhibited root elongation in mpk6 required cytosolic Ca\(^{2+}\)

The mechanisms of plant adaptations to salinity include tolerance to osmotic stress and to Na\(^+\) toxicity. Thus we tested whether mpk6 tolerated Na\(^+\) toxicity. On MS containing 120 mM Na-Glutamate, the mean primary root length of mpk6-2 or mpk6-3 was approximately 1.9-fold that of WT (Fig. 2A), similar to the root lengths on MS containing 120 mM NaCl (Fig. 1A). However, root lengths were similar in WT and mpk6 seedlings grown on MS containing 120 mM KCl or 300 mM mannitol (Fig. 2A). These results suggested that mpk6 was able to tolerate toxic Na\(^+\) accumulation.

We investigated whether the lower sensitivity of root elongation to NaCl in mpk6 was dependent on Ca\(^{2+}\). Various concentrations of CaCl\(_2\) (5–20 mM) did not result in differences in root elongation between mpk6-3 and WT during a 10 d period (Fig. 2B). In contrast, when 0.5 mM EGTA (a Ca\(^{2+}\) chelator) was added to MS containing 120 mM NaCl, root elongation was similarly inhibited in mpk6-2 (0.22 ± 0.05 cm), mpk6-3 (0.21 ± 0.04 cm), and WT (0.21 ± 0.05 cm) seedlings (Fig. 2B). A similar trend was observed when 1 mM LaCl\(_3\) was added to MS containing 120 mM NaCl (Fig. 2B). Conversely, when the Ca\(^{2+}\) ionophore A23187 (10 μM) was added to MS containing 120 mM NaCl, the mean root length of mpk6 seedlings was significantly longer than that of WT seedlings (Fig. 2B). These observations suggested that Ca\(^{2+}\) was required for NaCl-insensitive root elongation in mpk6.

mpk6 reduced Na\(^+\) uptake under NaCl stress by mediating plasma membrane (PM) NCCs in root cells

To illuminate the less-sensitivity of mpk6 root elongation to toxic Na\(^+\) uptake, root cell protoplasts (RPCs) were isolated from 10-d-old seedlings grown on MS, and the patch-clamp technique with the whole-cell configuration was used to evaluate NCCs in root cells in response to NaCl.

In WT RPCs, the amplitude of NCCs ranged from –18.4 (±1.5) pA/pF\(^{-1}\) (at –190 mV) to +40.1 (±4.6) pA/pF\(^{-1}\) (at +110 mV) during 15 min in NaCl-free solution (Fig. 3A and D). The addition of NaCl to the bath solution resulted in significant increases in inward NCCs, but not outward NCCs (Fig. 3A, B, D and E). For example, 50 mM NaCl increased the maximum inward Na\(^+\) currents to –59.9 (±5.5) pA/pF\(^{-1}\) at –190 mV, while the maximum outward NCCs remained at +44.9 (±4.9) pA/pF\(^{-1}\) at +110 mV (Fig. 3A and D). Meanwhile, the membrane potential showed a positive shift from –52 mV to –0.5 mV (Fig. 3D).

In mpk6-3 RPCs, the amplitude of NCCs ranged from –6.3 (±1.5) pA/pF\(^{-1}\) (at –190 mV) to 27.9 (±2.6) pA/pF\(^{-1}\) (at +110 mV) in NaCl-free bath solution (Fig. 3B and E). The addition of ≤20 mM NaCl did not increase inward or outward NCCs of mpk6-3 RPCs (Fig. 3B and E). In the presence of ≥50 mM NaCl, the increase of the outward NCCs was greater than that in inward NCCs. For example, 50 mM NaCl increased the maximum inward NCCs to –23.8 (±2.5) pA/pF\(^{-1}\) at –190 mV (Fig. 3B and E), while the maximum outward NCCs increased to –62.5 (±3.9) pA/pF\(^{-1}\), 2.6-times that of the maximum inward NCCs (Fig. 3B and E). Moreover, the
reversal potential showed a negative shift from −52 mV to −86 mV in *mpk6* root cells (Fig. 3E), reflecting that root cells of *mpk6* seedlings had a reduced driving force for Na⁺ excretion through non-selective cation channels (NSCCs).

These measured NCCs shared the traits of NSCCs-catalyzed currents: the magnitude was dependent on NaCl concentrations and voltage but independent of time, and the current-voltage curves were linear (Fig. 3A, B, D and E). To exclude the involvement of K⁺- or Ca²⁺-selective channels, we added various pharmacological agents and obtained the following results: these NCCs were inhibited by 0.5 mM quinine, but not by 10 mM TEA⁺ or 0.1 mM verapamil, as measured 15 min after addition of these compounds to the bath solution (Fig. 4A and B).

**Internal Ca²⁺ reduced NaCl-increased PM NCCs in root cells**

We checked whether there was an antagonistic relationship between intracellular Ca²⁺- and Na⁺ uptake in response to NaCl. The amplitude of NCCs in WT RCPs with 50 mM NaCl in the bath solution served as the control. When 5 μM CaCl₂ was added to the

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**Fig. 1.** Sensitivity of root elongation to external NaCl. Root elongation was compared among *mpk6-3*, *mpk6-2*, and WT seedlings. All Arabidopsis seeds were germinated on MS for 3 d, and then seedlings were transferred to fresh MS or MS containing indicated additives. Root length was measured daily. Representative samples are shown. (A)–(B) Root phenotypes of WT, *mpk6-3*, and *mpk6-2* grown on MS (A) or MS containing 120 mM NaCl (B) for 10 d. (C) Elongated roots of 10-d-old WT, *mpk6-2*, or *mpk6-3* seedlings grown on MS containing 0, 60, 90, 120, 150, or 180 mM NaCl. (D) Comparison of root lengths among WT, *mpk6-2*, and *mpk6-3* seedlings grown on MS or MS containing 120 mM NaCl for 2, 4, 8, and 10 d. Values in (C) and (D) are means ± SD of 70–90 seedlings from at least five independent experiments.

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**Fig. 2.** Effects of Ca²⁺ on the less sensitivity of root elongation to NaCl in *mpk6*. (A) Elongated roots of 10-d-old *mpk6-2*, *mpk6-3*, or WT seedlings grown on MS containing 120 mM NaCl, 120 mM Na-Glutamate, 120 mM KCl, or 300 mM mannitol. (B) Comparison of increases in root length of 10-d-old *mpk6-2*, *mpk6-3*, or WT seedlings grown on MS containing 10 mM CaCl₂, 120 mM NaCl + 0.5 mM EGTA, 120 mM NaCl + 10 μM A23187, or 120 mM NaCl + 1 mM LaCl₃. Values are means ± SD of 50–70 seedlings from five independent experiments.
Fig. 3. NaCl-induced Na⁺ flux in root cells of mpk6 and WT. Whole-cell Na⁺-conducted currents (NCCs) in root cell protoplasts (RCPs) from 10-d-old seedlings measured using patch-clamp technique. (A) and (B) Amplitudes of inward and outward NCCs through plasma membrane (PM) of WT (A) or mpk6-3 (B) RCPs with 0, 20, or 50 mM NaCl in the bath solution. Representative current curves from at least five independent experiments are shown. (C) Voltage protocols in 20-mV-increments between −190 mV and +110 mV (from holding voltage of −52 mV). (D)–(E) Relationship between current density of NCCs (pA pF⁻¹) and reversal potential (mV) in WT (D) and mpk6-3 (E) root cells. Values are mean currents from at least five independent recordings ± SE.

Fig. 4. Characteristics of NCCs in WT Arabidopsis seedling root cells. (A) Whole-cell instantaneous currents in RCPs from 10-d-old WT seedlings grown on MS. Data were obtained after 10-min exposures to indicated treatments, including 0.5 mM quinine (PM Ca²⁺ channel inhibitor), 10 mM tetraethylammonium (TEA⁺; K⁺ channel blocker), or 0.1 mM verapamil (Ca²⁺ channel blocker). Representative current curves from at least five independent experiments are shown. (B) Relationship between whole-cell current density of NCCs (pA pF⁻¹) and membrane potential (mV). NCCs values are means from at least five independent recordings ± SE. (C) Voltage protocols as described in caption of Fig. 3C.
pipette solution, the amplitude of NCCs in WT RCPs was lower than that of the control (Fig. 5A and B). When 50 μM CaCl₂ was added to the pipette solution, the inward and outward NCCs decreased 4.9-fold and 1.2-fold, respectively, compared with those in the control (Fig. 5A and B). This result suggested that Ca²⁺ inside root cells antagonized NaCl-imposed Na⁺ uptake from the intracellular space.

*Increased NaCl-induced cytosolic Ca²⁺ accumulation in root cells of mpk6*

To examine whether *mpk6* increased cytosolic Ca²⁺ levels in response to NaCl stress, aequorin (Aq)-expressing *mpk6* and WT plants were created. Roots of 10-d-old seedlings were cut from transgenic plants, and the luminescence of cytosolic Ca²⁺ was monitored. In the absence of NaCl, the cytosolic Ca²⁺ content was ~0.15 (±0.12) μM in WT roots or 0.25 (±0.13) μM in *mpk6-3* roots (Fig. 6A and B). Addition of 120 mM NaCl caused a steep increase in Ca²⁺ content, with peak values of 3.17 (±0.23) μM in roots of transgenic *mpk6-3* and 1.45 (±0.22) μM in roots of WT (Fig. 6A and B). During the whole observation period, the NaCl-induced cytosolic Ca²⁺ increase in root cells was grater in *mpk6* than in WT (Fig. 6).

We also observed Ca²⁺ accumulation in the root cell using the Ca²⁺-sensitive fluorescence probe Fluo3-AM staining. Confocal microscope images indicated that the NaCl-elevated Ca²⁺ level was higher in *mpk6* roots than in WT roots, and this increase was mainly concentrated in the cytosol of root cells (Supplemental data). These suggested that the increase of the cytosolic Ca²⁺ level was positively correlated with the external NaCl concentration.

*Increased NaCl-induced PM Ca²⁺ influx in root cells of mpk6*

We further investigated PM Ca²⁺ influx through Ca²⁺-permeable channels by measuring voltage-independent whole-cell currents. In NaCl-free conditions, the magnitude of Ca²⁺ currents in root cells did not differ significantly between 10-d-old *mpk6-3* (−25.7 ± 7.1 pA pF⁻¹) and WT (−30.7 ± 6.3 pA pF⁻¹) at −190 mV (Fig. 7A–D). The NaCl-increased Ca²⁺ currents in root cells were higher in *mpk6-3* than in WT. When 50 mM NaCl was added to the bath solution, the maximum Ca²⁺ current in root cells (at −190 mV) in *mpk6-3* (−88.6 ± 7.2 pA pF⁻¹) was 1.65-fold that in WT (−53.7 ± 4.6 pA pF⁻¹) (Fig. 7A–D). These results indicated that NaCl promoted greater apoplastic Ca²⁺ influx into the cytosol of *mpk6* root cells than into the cytosol of WT root cells.

We also evaluated the activity of PM-located Ca²⁺-transporters. RT-PCR analyses showed that the transcript levels of CNGC3/10 or GLR2.5/1.3 were markedly higher in *mpk6* seedling roots than in WT seedling roots under NaCl stress (Fig. 7E). These provided further evidence that activation of PM-located Ca²⁺ channels was greater in *mpk6* roots than in WT roots under NaCl stress.

*Requirement for Ca²⁺ to alleviate NaCl-induced increase of Na⁺/K⁺ ratio in mpk6 root cells*

To verify that the increase in cytosolic Ca²⁺ played a role in alleviating NaCl-imposed Na⁺ uptake in *mpk6*, roots were collected from 10-d-old seedlings grown on MS or contained various additions, and the Na⁺ content, K⁺ content, and Na⁺/K⁺ ratio were analyzed. In NaCl-free conditions, the Na⁺ contents in root tissues of *mpk6-2* or *mpk6-3* were similar to that in root tissues of WT (Fig. 8A).
Fig. 6. Cytosolic Ca\(^{2+}\) level in aequorin-expressing mpk6 and WT seedling roots in response to NaCl. (A) and (B) Seedling roots were cut from aequorin-expressing WT (A) or mpk6-3 (B) seedlings grown on MS for 10 days (after transfer). Cytosolic Ca\(^{2+}\) levels in roots were quantified by measuring Aq luminescence intensity in response to NaCl treatment. Recording started at 20 s after addition of NaCl. Values shown are means from at least five independent experiments (n=50–70 roots). Values are means ± SD.

Fig. 7. NaCl-induced PM Ca\(^{2+}\) influx and the expression of genes encoding PM Ca\(^{2+}\) transporters in mpk6 and WT root cells. Whole-cell Ca\(^{2+}\) currents through PM Ca\(^{2+}\)-permeable channels of RCPs from 10-d-old WT or mpk6-3 seedlings were detected using patch-clamp technique with whole-cell configuration. (A) and (C) Whole-cell Ca\(^{2+}\) currents in WT (A) or mpk6-3 (C) RCPs with 0, 20, or 50 mM NaCl in the bath solution as indicated in the figures. Representative traces of Ca\(^{2+}\) currents are from at least five independent experiments. (B) and (D) Comparison of Ca\(^{2+}\) current density (pA/pF) between WT RCPs (B) and mpk6-3 RCPs (D). Values are mean ± SD. Voltage ramps were from −190 mV to +32 mV. (E) Reverse transcription (RT)-PCR analysis of transcript levels of CNGCs and GLRs encoding Ca\(^{2+}\) transporters in 7-d-old mpk6-3 or WT seedling roots in the absence or presence of NaCl. Samples shown were randomly selected from three independent experiments.

Fig. 8. Requirement of mpk6-2 or mpk6-3 mutants for Ca\(^{2+}\) to reduce NaCl-induced increase in Na\(^{+}\) content or Na\(^{+}\)/K\(^{+}\) ratio in root tissue. Roots were collected from 10-d-old mpk6-2, mpk6-3, and WT seedlings grown on MS or MS containing NaCl and subjected to treatments as indicated. Na\(^{+}\) content (A), K\(^{+}\) content (B) and Na\(^{+}\)/K\(^{+}\) ratio (C) in seedling roots. Mean values from seven independent experiments ± SD are shown.
In the presence of 120 mM NaCl, the Na⁺ content in roots of WT (118.9 ± 7.2 mg gDW⁻¹) was approximately double that in roots of mpk6-2 (58.4 ± 6.8 mg gDW⁻¹) or mpk6-3 (60.1 ± 6.6 mg gDW⁻¹) (Fig. 8A). Unexpectedly, the K⁺ content in root tissue was not significantly different between mpk6 and WT seedlings, with or without NaCl (Fig. 8B). As a result, the Na⁺/K⁺ ratio in roots of mpk6-2 (0.50 ± 0.1) or mpk6-3/0.53 ± 0.1 was higher than that in WT roots (1.1 ± 0.1) (Fig. 8C).

Next, we analyzed how Ca²⁺ affected on Na⁺ accumulation in mpk6 under NaCl stress. When 0.5 mM EGTA was added to MS contained 120 mM NaCl, the Na⁺ content in seedling roots of mpk6-2 (120.4 ± 7.3 mg gDW⁻¹) and mpk6-3 (122.6 ± 7.4 mg gDW⁻¹) were similar to that of WT (124.1 ± 7.2 mg gDW⁻¹) (Fig. 8A). Similar results were obtained when 1 mM LaCl₃ was added to the bath solution (Fig. 8A). As a result, the Na⁺/K⁺ ratio was similar in mpk6 mutants and WT (Fig. 8C). Conversely, addition of 10 μM A23187 caused greater decrease in Na⁺ content (Fig. 8A) and the Na⁺/K⁺ ratio (Fig. 8C) in root tissue of mpk6 than in root tissue of WT. These results indicated that increment of the cytosolic Ca²⁺ level was required to decrease NaCl-induced Na⁺ uptake in root cells of mpk6.

Discussion

This study focused on how inactivation of MPK6 affected NaCl-induced Na⁺ uptake and cytosolic Ca²⁺ homeostasis in root cells of Arabidopsis seedlings. Based on our results, we propose a working model to explain the role of Arabidopsis MPK6 in mediating NaCl-imposed Na⁺ accumulation in root cell. This process was dependent on PM Ca²⁺ flux and the cytosolic Ca²⁺ level (Fig. 9).

MPK6 activity plays a key role in tolerance to NaCl stress in Arabidopsis. Here we found that root elongation of the mpk6 mutant was insensitive to NaCl, unlike that of WT (Fig. 1). This was consistent with the previous findings that NaCl increased the kinase activity of Arabidopsis MPK6 in a time- and dose-dependent manner (Ichimura et al., 2000), and that the NaCl-induced kinase activity of MPKs was positively correlated with salt resistance in Arabidopsis (Nakagami et al., 2005). Moreover, in seedlings of various mkk9 mutants, root elongation was not inhibited by NaCl (Alzwyi and Morris, 2007; Xu et al., 2008), implying that loss of MPK6 activity conferred NaCl tolerance on mkk9. Therefore, it is reasonable that root elongation of mpk6 seedlings was insensitive to NaCl.

The less-sensitive characteristic of the mpk6 mutants to NaCl could result from decreased Na⁺ uptake in root cells. Changes in NCCs through PM NSCCs are essential for Na⁺ absorption by plants (Demidchik et al., 2002; Shabala et al., 2005; Volkov and Amtmann, 2006; Demidchik and Maathuis, 2007). Here, the traits of these NCCs measured in the mutants and WT (Figs. 3 and 4) were identical to those described in previous studies (Demidchik et al., 2002; Zhao et al., 2011). That is, the Na⁺ currents recorded here reflected the activity of NSCCs, but not by K⁺- or Ca²⁺- selective channels. Importantly, in the presence of 50 mM NaCl, the magnitude of inward NCCs decreased while that of outward NCCs dramatically increased in mpk6 root cells, compared with those in WT (Fig. 3). This finding was consistent with the lower Na⁺ content in root tissues of mpk6 than in those of WT (Fig. 8). These data reflected that mpk6 alleviated Na⁺ accumulation in root cells under NaCl stress, supporting the idea that the restriction of unidirectional Na⁺ influx through NSCCs in roots is a major strategy for tolerance to NaCl stress in plant (Knight et al., 1997; Chen et al., 2007). Moreover, NaCl shifted the reverse potential to a more negative value in mpk6 root cells than in WT root cells (Fig. 3 D and E). This negative shift could reduce the electrochemical force driving Na⁺ excretion, since one of the early responses of plants to excessive NaCl is membrane depolarization (Chen et al., 2007).

Besides alleviating Na⁺ accumulation (Figs. 3 and 7), mpk6 significantly increased PM Ca²⁺ influx (Fig. 7) and the cytosolic Ca²⁺ level in root cells under NaCl stress (Fig. 6 and Supplemental data). Since the less-sensitivity of mpk6 root elongation to NaCl stress was diminished in the absence of Ca²⁺ (Fig. 2), both PM Ca²⁺ influx (Fig. 7) and an increase in cytosolic Ca²⁺ (Fig. 6) were assumed to be critical mechanisms underlying the alleviation of NaCl-imposed Na⁺ uptake in root cells of mpk6 (Fig. 3). This is because increased cytosolic Ca²⁺ not only blocks Na⁺ accumulation in root cells (Demidchik and Maathuis, 2007; Ebrahim and Bhatia, 2012), but is also essential for cell elongation (Knight et al., 1997; Demidchik and Maathuis, 2007).

In response to NaCl, mpk6 showed a lower level of Na⁺ accumulation and a smaller increase of Na⁺/K⁺ ratio in root tissue compared with those in the WT (Fig. 8), which was consistent with the less-sensitive characteristic of mpk6 root elongation to NaCl stress (Fig. 1). Both processes were similarly influenced by cytosolic Ca²⁺ homeostasis (Figs. 2 and 8). The mpk6 seedlings mimicked some salt-tolerant plant species, such as Thellungiella (Volkov and Amtmann, 2006) or barley (Chen et al., 2007), to limit Na⁺ uptake by root cells in a saline environment. Likewise, an increase in cytosolic Ca²⁺ allows these plants to retain a reasonable Na⁺/K⁺ ratio and cell growth under NaCl stress (Shabala et al., 2006; Demidchik and Maathuis, 2007).

In short, our results provided details about the mechanism by which MPK6 negatively regulates salt tolerance in Arabidopsis. These findings contribute to our understanding of salt-tolerance mechanism in plant.

Acknowledgments

We thank Dr Xiang Zhao for helpful discussions and Dr M. Knight (Oxford University) for the kind gift of PMAQ2. This work was supported by the National Natural Science Foundation of China (No. 30971509 and No. 31271510 to J.J.).
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2013.09.023.

References