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Drought-induced activation and rehydration-induced inactivation of MPK6 in Arabidopsis

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ABSTRACT

Mitogen-activated protein kinases (MPKs) have roles in regulating developmental processes and responses to various stimuli in plants. Activations of some MPKs are necessary for proper responses to hyperosmolarity and to a stress-related phytohormone, abscisic acid (ABA). However, there is no direct evidence that MPK activations are regulated by drought and rehydration. Here we show that the activation state of one of the Arabidopsis MPKs, MPK6, is directly regulated by drought and rehydration. An immunoblot analysis using an anti-active MPK antibody detected drought-induced activation and rehydration-induced inactivation of MPK6. MPK6 was activated by drought even in an ABA-deficient mutant, *aba2-4*. In addition, exogenously added ABA failed to suppress the rehydration-dependent inactivation of MPK6. Under drought conditions, elevated levels of reactive oxygen species (ROS), which are known elicitors of MPK6 activation, were detected in both wild type and an MPK6-deficient mutant, *mpk6-4*. These results suggest that ROS, but not ABA, induces MPK6 activation as an upstream signal under drought conditions.

1. Introduction

Mitogen-activated protein kinases (MPKs) are a subset of protein kinases conserved among eukaryotic species. MPKs are phosphorylated and activated via phosphorylation relay from upstream protein kinases, MPK kinase kinases (MKKKs) and MPK kinases (MKKs) upon recognition of a certain elicitor. Activations of MPKs are involved in diverse developmental processes and responses to stimuli in plants [1, for a review].

Some MPK signaling components have roles in responses to dehydration stresses. For example, MPK3, MPK4, and MPK6 are activated by high osmolarity [2–4]. Expressions of constitutively active forms of MKK1-3 enhance promoter activities of osmotic stress-responsive genes, *RD29A*, and *RD29B* [4]. Overexpression of MKK4 or MKKK20 increases the amount of activated MPK3 or MPK6, respectively, and improves plant growth under high salinity [5,6]. Abscisic acid, a phytohormone involved in responses to dehydration stresses, induced activations of MPK1, MPK2, MPK9, and MPK12 [4,7]. Despite all these reports, however, it has been unclear whether drought directly activates MPKs in plants.

Here we provide direct evidence that Arabidopsis MPK6 is activated by drought and inactivated by rehydration in an ABA-independent manner.

2. Material and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as the plant material. An MPK6-deficient mutant, *mpk6-4* (SALK_062471C) [8], and an ABA-deficient mutant, *aba2-4* (CS3835) [9], were obtained from the Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org). Seeds were surface sterilized and sown on 0.8% agar (Wako, Japan) containing 0.5× MS salts (Wako), 1% w/v sucrose and 0.5 g/l MES, pH 5.8, chilled at 4 °C in the dark for 48 h (stratified), and germinated at 22 °C. To compare plant growths under high salinity, plants were grown on MS media above supplied with 110 or 130 mM NaCl. Plants were grown at 22 °C under 16-h light/8-h dark condition (light intensity 120 μmol m^{-2} s^{-1}). After 3 weeks of growth, plants were transferred onto rockwool cubes and grown further with 0.2× MS solution regularly supplied.

Abbreviations: ABA, abscisic acid; MAMP, microbe-associated molecular pattern; MPK, mitogen-activated protein kinase; MKK, MPK kinase; MKKK, MPK kinase kinase; ORF, open reading frame; ROS, reactive oxygen species; RT-PCR, reverse transcription-PCR; SE, standard error.

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To generate transgenic plants expressing GFP-fused MPK6 (MPK6-GFP), pBS-35SMCS-GFP [10] was digested by *Xba*I and *Sac*I to obtain DNA fragments of the open reading frame (ORF) of GFP with a multi cloning site. The DNA fragments were inserted into the *Xba*I–*Sac*I site of pBI121, generating pBI121-35SMCS-GFP. The ORF of *MPK6* was amplified by reverse transcription-PCR (RT-PCR) using cDNA synthesized from total RNA from 2 week-old Arabidopsis seedlings as template and the following primer pair: 5′-CCATCTAGACCATGGACGGTGGTTCAGGTC-3′ and 5′-CTCCGTCGACGTTGCTGATATTCTGGATTGAAAG-3′ (*Xba*I and *Sal*I sites are underlined). The PCR products were digested by *Xba*I and *Sal*I, and inserted into the *Xba*I–*Sal*I site of pBI121-35SMCS-GFP. This plasmid was used for Arabidopsis transformation, which was performed as previously described [11]. T2 transgenic (heterozygous) plants showing GFP fluorescence were used for RT-PCR and growth tests. The expression of MPK6-GFP was confirmed by RT-PCR and immunoblotting using an anti-GFP antibody (MBL, Japan) or an anti-MPK6 antibody (Sigma–Aldrich) (Supplementary Fig. S1).

2.2. Immunoblot analysis of MPK activations

For drought treatment, two-week old plants were dried on a filter paper for 30 min at room temperature. For rehydration treatment, plants were submerged into 20 mM Tris–HCl, pH 6.8, after the drought treatment, and incubated for 30 min at room temperature. For flg22 treatment, plants were submerged in 20 mM Tris–HCl, pH 6.8, with 1 μM flg22 (QRLSTGSRINSAKDDAAGLQIA) and incubated for 10 min at room temperature. For ABA, mannitol or NaCl treatment, plants were submerged into 20 mM Tris–HCl, pH 6.8, with 100 μM ABA, 0.8 M mannitol or 0.4 M NaCl, respectively, before or after the drought treatment, and incubated for 30 min at room temperature. For phosphatase inhibitor treatment, plants were submerged into 20 mM Tris–HCl, pH 6.8, with 1× PhosSTOP (Roche, Germany) after the drought treatment, and incubated for 30 min at room temperature. Plants were lysed immediately after each treatment and used for immunoblot analyses of MPK activations as previously described [12].

2.3. ROS detection

To detect drought-induced production of reactive oxygen species (ROS), 2 week-old plants were submerged into 20 mM Tris–HCl, pH 6.8, with 5 μM APF, which is a fluorescent probe for ROS [13], incubated for 10 min at room temperature, washed with distilled water, transferred to wet (with distilled water) or dry filter paper, incubated for another 30 min at room temperature, and used for fluorescence microscopy. APF fluorescence (excitation: 490 nm; emission: 515 nm) was observed using an epifluorescence microscope (BX51, Olympus, Japan).

2.4. RT-PCR

Two-week old plants were sampled for RNA extraction before and after the drought treatment described above. RNA was extracted as previously described [14], and cDNA was synthesized from 2 μg of total RNA with PrimeScript Reverse Transcriptase (Takara Bio, Japan) using oligo (dT) primer. Reaction mixtures were diluted 25 times with distilled water and used as templates for RT-PCR. GoTaq Green Master Mix (Promega) was used for semi-quantitative RT-PCR, and GoTaq qPCR Master Mix (Promega) for quantitative RT-PCR. Primers used for RT-PCR are given in Supplementary Table S1. In quantitative RT-PCR, relative expression levels were calculated by the comparative C_T method using *UBQ5* as an internal control gene.

3. Results and discussion

3.1. Drought-induced activation and rehydration-induced inactivation of MPK6

To examine whether any MPK is activated by drought, an immunoblot analysis of activated MPKs was performed using drought-stressed Arabidopsis plants. This analysis revealed that a ~45 kDa MAPK was activated by drought treatment. The size of the drought-activated MPK was the same as the size of an MPK activated by flg22 (Fig. 1A). Because flg22 was known to elicit the activations of MPK3 (~43 kDa) and MPK6 (~45 kDa) [15], the drought-activated MPK was thought to be MPK6. To confirm this, an MPK6-deficient mutant, *mpk6-4* [8], was subjected to the immunoblot analysis of drought-induced MPK activations. As expected, the activation of the ~45 kDa MPK was not detected in *mpk6-4*. Interestingly, a ~43 kDa MPK was more strongly activated in *mpk6-4* than in the wild type (Fig. 1B). MPK3 and MPK6 are activated by common upstream MKKs and have redundant physiological functions [1,16, for reviews]. Therefore, the drought-activated ~43 kDa MPK in *mpk6-4* is thought to be MPK3.

Next, the amount of activated MPK6 in drought-treated plants was compared with the amounts of activated MPK6 in hyperosmolarity-treated plants. Previously, both 0.5 M mannitol and 0.3 M NaCl were shown to activate MPK3, MPK4, and MPK6 in Arabidopsis suspension cells [4]. In this study, higher concentrations of mannitol (0.8 M) and NaCl (0.4 M) were used. The amount of activated MPK6 in mannitol- or NaCl-treated plants was lower than that in drought-treated plants (Fig. 2A), raising the possibility that drought activates MPK6 by a mechanism that does not depend only on dehydration.

Next, the effects of rehydration on drought-induced MPK6 activation were characterized. The amount of activated MPK6 clearly

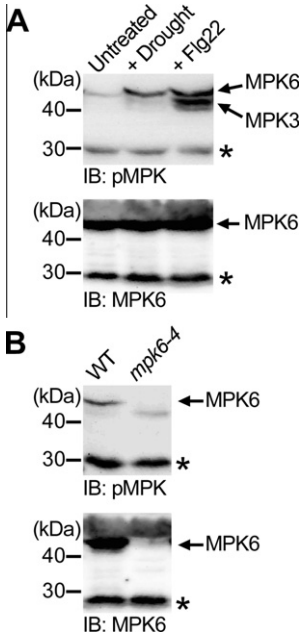

Fig. 1. Drought induces MPK6 activation. (A) Activation of a ~45 kDa MPK by drought. Wild-type Arabidopsis plants were subjected to an immunoblot analyses of activated MPKs (IB: pMPK) and an immunoblot analysis of MPK6 (IB: MPK6) before (untreated) or after drought treatment (+Drought). For a control, flg22 treated plants were used for the analysis (+Flg22). Non-specific signals (*) are shown as loading controls. (B) Confirmation of the drought-induced activation of MPK6. Wild type (WT) and *mpk6-4* were subjected to the immunoblot analysis after the drought treatment. Experiments were performed in triplicate and representative results are shown.

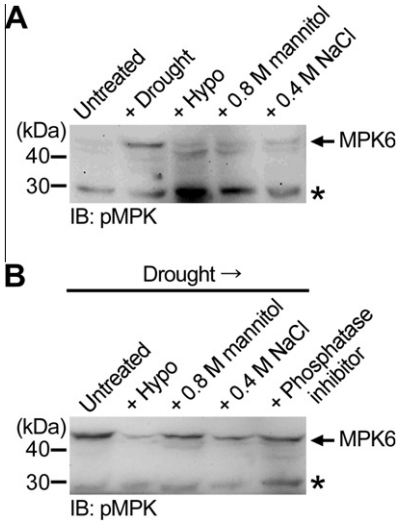

Fig. 2. Effects of hypotonic and hypertonic solutions on the MPK6 activation state. (A) Mere dehydration does not activate MPK6. Wild-type plants were subjected to an immunoblot analysis of activated MPKs (IB: pMPK) before (untreated) or after being treated with drought (+Drought), 20 mM Tris–HCl (+Hypo), 0.8 M mannitol (+0.8 M mannitol) or 0.4 M NaCl (+0.4 M NaCl). Non-specific signals (∗) are shown as loading controls. (B) MPK6 is inactivated by rehydration. Drought-treated plants were subjected to the immunoblot analysis before (untreated) and after being submerged into 20 mM Tris–HCl (+Hypo), or 20 mM Tris–HCl containing 0.8 M mannitol (+0.8 M mannitol), 0.4 M NaCl (+0.4 M NaCl) or 1× PhosSTOP (a mixture of protein phosphatase inhibitors) (+Phosphatase inhibitor). Experiments were performed in triplicate and representative results are shown.

decreased when plants were submerged into hypotonic solution after the drought treatment, suggesting that rehydration causes inactivation of MPK6. This rehydration-induced MPK6 inactivation was inhibited when 0.8 M mannitol, 0.4 M NaCl or a mixture of protein phosphatase inhibitors was present in the solution given to drought-treated plants (Fig. 2B). These results suggest that a rehydration-responsive protein phosphatase is responsible for the MPK6 inactivation.

3.2. Drought-induced MPK6 activation and rehydration-induced MPK6 inactivation are independent of ABA

Effects of ABA on the activation state of MPK6 were examined. Drought caused MPK6 activation even in an ABA biosynthesis mutant, *aba2-4* (Fig. 3, left panel). In addition, MPK6 was inactivated when plants were submerged into a hypotonic solution containing 100 μM ABA (Fig. 3, right panel). Drought increases the ABA level [17] and ABA is a known elicitor of MPKs in Arabidopsis [4,7], but our results suggest that drought-induced MPK6 activation and rehydration-induced MPK6 inactivation are independent of ABA.

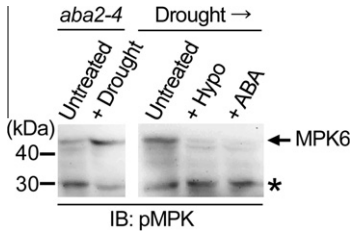

Fig. 3. Drought-induced activation and rehydration-induced inactivation of MPK6 are independent of ABA. Drought-induced MPK6 activation in a ABA biosynthesis mutant, *aba2–4* is shown in the left panel. The mutant plants were subjected to an immunoblot analyses of activated MPKs (IB: pMPK) before (untreated) or after drought treatment (+Drought). Non-specific signals (∗) are shown as loading controls. The right panel shows that ABA does not inhibit rehydration-induced MPK6 inactivation. Wild-type plants were subjected to the immunoblot analysis before (untreated) and after being submerged into 20 mM Tris–HCl containing 0 (+Hypo) or 100 μM ABA (+ABA). Experiments were performed in triplicate and representative results are shown.

3.3. ROS is a possible elicitor of MPK6 activation under drought conditions

H_2O_2 is a ROS that activates MPK6 [12,18]. Endogenous ROS production is enhanced by various stimuli such as flg22 [15] and high salinity [5,6]. To examine whether ROS production is involved in the drought-induced MPK6 activation, ROS in drought-treated

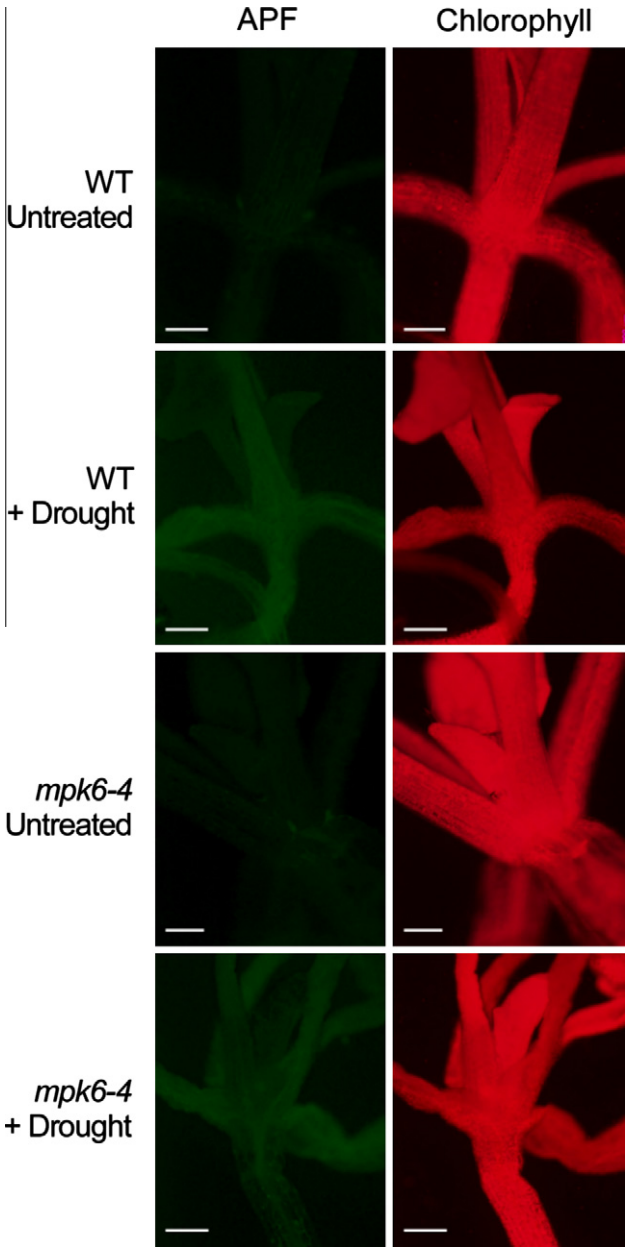

Fig. 4. Drought-induced ROS production. Wild type (WT) and *mpk6-4* were incubated in a solution containing 5 μM APF, a fluorescent ROS probe, and incubated on a wet (untreated) or a dry filter paper (+Drought) for 30 min. APF fluorescence (APF) was observed by fluorescence microscopy. Chlorophyll autofluorescence (Chlorophyll) is shown as control. Experiments were performed in triplicate and representative results are shown. Scale bars = 200 μm.

plants was visualized by a fluorescent ROS probe, APF. APF reacts only with ROS of especially high reactivity converted from other kinds of ROS such as H_2O_2 [13]. In both the wild type and *mpk6-4*, the intensities of APF fluorescence were higher in drought-treated plants than in untreated plants. No clear difference was observed in the intensities of APF fluorescence between the wild type and *mpk6-4* (Fig. 4). These results indicate that drought increases ROS production independently of MPK6 activation. An increase of ROS is thought to activate MPK6 under drought conditions.

3.4. Knockout or overexpression of MPK6 does not affect gene expressions or growth under dehydration conditions

Transgenic plants expressing GFP-fused MPK6 (MPK6-GFPox) were generated to examine physiological roles of MPK6 activation (Supplementary Fig. S1). Gene expressions under the drought conditions where MPK6 was activated were examined by RT-PCR using wild type, *mpk6-4* and MPK6-GFPox. The expression levels of *RD29A*, which is a putative target of MPK6 [4] and which is up-regulated by drought [19], were increased by drought treatment, but no significant difference was observed in *RD29A* induction levels among the three genotypes (Supplementary Fig. S2A). The expression levels of *RAB18*, which reflect the endogenous ABA level [19], were increased after the drought treatment, but no large difference was observed in *RAB18* induction levels among the three genotypes (Supplementary Fig. S2B). These results suggest that drought can induce *RD29A RAB18* expressions in an MPK6-independent manner. Expressions of two putative MPK6 target genes, *FRK1* [15] and *CAT1* [20] were not increased by drought and were comparable among the three genotypes (Supplementary Fig. S2C and D). In previous studies, flg22-activated MPK3 and MPK6 were suggested to induce *FRK1* expression [15], while ABA-activated MPK6 was suggested to induce *CAT1* expression [20]. Taken together, these results suggest that MPK6 activation by itself is insufficient to induce gene expressions downstream of MPK6, and that other elicitor-specific reactions may be required.

Because high salinity appears to inhibit MPK6 inactivation (Fig. 2B), the growths of wild type, *mpk6-4* and MPK6-GFPox under high salinity were compared. However, no clear difference was observed in phenotypes among the three genotypes (Supplementary Fig. S3). In previous studies, overexpressions of MKK4 and MKKK20 increased the amounts of activated MPK3 and MPK6 and improved plant growth under high salinity, while knockouts of MKK4 and MKKK20 caused growth defects under high salinity [5,6]. Our finding that neither knockout nor overexpression of MPK6 affects plant growth under high salinity may be due to upstream protein kinases such as MKK4 and MKKK20 that limit the activations of MPK3 and MPK6.

Previous studies showed that activated MPK3 and MPK6 suppress stomatal differentiation [1, for a review). Thus the drought-induced activation of MPK6 may contribute to decreasing transpirational water loss by limiting the number of stomata.

In conclusion, our results show that MPK6 and possibly MPK3 are activated under drought conditions and inactivated upon rehydration, probably due to increased ROS production but not to increased ABA level. Neither overexpression nor knockout of MPK6 affected gene expressions under drought conditions or plant growth under high salinity. Because MPK3 and MPK6 have diverse roles in plant development, further studies are required to elucidate the physiological importance of drought-induced MPK6 activation.

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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.bbrc.2012.08.141.

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