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Phosphorylation by AtMPK6 is required for the biological function of AtMYB41 in *Arabidopsis*

My Hanh Thi Hoang [a], Xuan Canh Nguyen [a], Kyunghee Lee [b,1], Young Sang Kwon [a], Huyen Trang Thi Pham [a], Hyeong Cheol Park [a,b], Dae-Jin Yun [a,b], Chae Oh Lim [a,b], Woo Sik Chung [a,b,*]

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

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ABSTRACT

Mitogen-activated protein kinases (MPKs) are involved in a number of signaling pathways that control plant development and stress tolerance *via* the phosphorylation of target molecules. However, so far only a limited number of target molecules have been identified. Here, we provide evidence that MYB41 represents a new target of MPK6. MYB41 interacts with MPK6 not only *in vitro* but also *in planta*. MYB41 was phosphorylated by recombinant MPK6 as well as by plant MPK6. Ser^{251} in MYB41 was identified as the site phosphorylated by MPK6. The phosphorylation of MYB41 by MPK6 enhanced its DNA binding to the promoter of a *LTP* gene. Interestingly, transgenic plants over-expressing $MYB41^{WT}$ showed enhanced salt tolerance, whereas transgenic plants over-expressing $MYB41^{S251A}$ showed decreased salt tolerance during seed germination and initial root growth. These results indicate that the phosphorylation of MYB41 by MPK6 is required for the biological function of MYB41 in salt tolerance.

1. Introduction

MPK cascades are signal transduction pathways that are highly conserved and widespread in all eukaryotic cells, including yeasts, animals and plants [1–3]. MPKs play a central role for converting extracellular signals, including environmental stresses, into internal signal transduction and activation of intracellular responses [3–6]. It is also well documented that plant MPKs are activated by a variety of environmental stimuli including salt, cold, wounding, heat, osmotic shock, heavy metal, UV, drought and pathogen attack [1,2,7,8]. In *Arabidopsis*, MPK4 and MPK6 are activated by high salt [9]. MPK6-null mutant seedlings were more salt sensitive than wild type [10]. Over-expression of MKK2 generated salt tolerance through the constitutive activation of MPK4 and MPK6 [11]. Furthermore, MKK2 interacted with an upstream MKKK, MEKK1, is activated by high salt [12,13]. These studies demonstrated that the MPK cascade consisting of MEKK1–MKK2–MPK6 plays a role in salt tolerance. However, the direct downstream targets of MPK6 that is involved in salt tolerance signaling pathway are unknown.

The MYB transcription factors represent one of the most complex transcription factor families composed of approximately 125 members in *Arabidopsis* [14]. MYBs are known to be involved in the regulation of plant development and stress responses [15,16]. Among them, AtMYB41 has originally been identified as a transcription factor controlling cell expansion and cuticle deposition in response to abiotic stress [17,18]. However, a post-translational regulation of MYB41 has not been elucidated in response to salt stress. Here, we report that AtMYB41 is phosphorylated and activated by MPK6 in response to salt stress. Interestingly, we showed that the phosphorylation of MYB41 by MPK6 is required for increased salt tolerance in *Arabidopsis*.

2. Materials and methods

2.1. Plant materials and growth conditions

Wild type (*Arabidopsis thaliana*, ecotype Columbia), $MYB41^{WT}$ and $MYB41^{S251A}$ over-expressing transgenic, *mpk3-2* (salk_151594), *mpk6-3* (salk_127507) mutant plants were grown in a controlled culture chamber at 22 °C with a 16 h light/8 h dark cycle at a light intensity of 100 Em^{-2} S^{-1}. To activate MPK6, WT, *mpk3-2* and *mpk6-3* plants were treated with 150 mM NaCl for 30 min.

2.2. Pull-down assay

Approximately 5 μg of GST–MYB41 was bound to glutathione beads in binding buffer (20 mM Tris–HCl, pH 7.5; 200 mM NaCl; 1% Triton X-100; 0.1 mM EDTA; 0.5 mM DTT) for 2 h at 4 °C. The

* Corresponding author at: Division of Applied Life Science (BK21 Program), Gyeongsang National University, Jinju 660-701, Republic of Korea. Fax: +82 55 759 9363.
E-mail address: chungws@gnu.ac.kr (W.S. Chung).
[1] Present address: College of Pharmacy, Yeungnam University, Daegu, Republic of Korea.

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beads were incubated with 5 μg of His–MPK6 at 4 °C overnight. The proteins bound to the beads were eluted and separated by electrophoresis on 10% SDS–PAGE. Bound protein to GST–MYB41 was detected by Western blotting using an anti-His antibody.

2.3. Firely luciferase complementation imaging assay (LCI)

MPK6 was fused with the N-terminal fragment of firefly luciferase (MPK6–NLuc). MYB41 was fused with the C-terminal fragment of Luc (MYB41–CLuc). STG1a–NLuc and RAR1–CLuc constructs were used as positive interaction control. *Agrobacterium tumefaciens* (strain GV3101) bacteria containing indicated constructs were grown in LB medium at 28 °C overnight. Bacteria were pellet, then washed once with infiltration buffer (10 mM $MgCl_2$; 10 mM MES; 100 μM acetosyringone) and resuspended to a final concentration of OD_{600} = 0.5. Bacterial suspensions were infiltrated into young but fully expanded leaves of *Nicotiana benthamiana* plants using a needleless syringe. After infiltration, plants were immediately covered with plastic caps and placed at 22 °C for 48 h before cap removal. LUC activity was observed with a low-light cooled CCD imaging apparatus (AndoriXon; Andor).

2.4. Kinase and in-gel kinase assays

Recombinant GST–MYB41 and His–MPK6 were expressed in *Escherichia coli* and purified. 1 μg of His–MPK6 was mixed with 2 μg of GST–MYB41 and 1 μg of GST or MBP proteins in 20 μl kinase reaction. The reaction was performed as previously reported [19]. For in-gel kinase assay, gel was embedded with MBP, GST, purified GST–$MYB41^{WT}$ or GST–$MYB41^{S251A}$ proteins. 50 μg crude plant proteins were incubated at 60 °C for 10 min in SDS sample buffer and separated on 10% SDS–PAGE. The in-gel kinase assay was performed as previously reported [20].

2.5. Electrophoretic mobility shift assay (EMSA)

Synthetic DNA oligonucleotide of MYB binding consensus sequence (MCS) from the promoter of a *LTP* gene (At3g22620) was used as a probe. DNA probe labeling and electrophoretic mobility shift assay were performed with modification as previously described [21]. For testing the effect of phosphorylation of MYB41 to the DNA binding, MYB41 was phosphorylated by MPK6 in the kinase buffer. The phosphorylated MYB41 was purified before EMSA assay.

2.6. Transient expression assay

The reporter plasmid was a pUC19-derived plasmid containing the β-glucuronidase (GUS) reporter gene under the control of a CaMV 35S minimal promoter. The 26 bp of MCS motif was cloned and fused to a GUS gene to create the MCS–m35S–GUS reporter construct. For effector plasmids, $MYB41^{WT}$ or $MYB41^{S251A}$ were inserted into a plant expression vector (pHBT95) under the control of CaMV 35S promoter and *nos* terminator. The reporter and effector plasmids were co-transformed into protoplast by polyethylene glycol-mediated transformation. The GUS activity of the cell lysate was divided by the luciferase activity. A construct carrying the CaMV 35S promoter fused to the luciferase gene was used as an internal control. The luciferase assay was performed using the Luciferase Assay System kit (Promega).

2.7. Seed germination and root growth assays

Arabidopsis seeds used for all experiments were harvested at the same time. For germination assays, seeds from wild type, $MYB41^{WT}$ and $MYB41^{S251A}$ over-expressing plants were sown on 1/2 MS salt medium supplemented with various concentrations of NaCl (100 mM, 125 mM and 150 mM). The plates were maintained for 3 days at 4 °C in the dark then transferred to normal growth condition. The germination yields were calculated after 5 days. For measurement of root length, seeds were germinated on 1/2 MS medium (0.8% agar) supplied with 100 mM NaCl and kept growing vertically for 10 days. Each experiment was repeated at least three times.

3. Results and discussion

3.1. MYB41 interacts with MPK6

Using yeast two-hybrid screening, MYB41 was identified as a MPK6 interacting protein [19]. To test whether MYB41 is a genuine target of MPK6, we analyzed *in vitro* interaction between MYB41 and MPK6 by using pull-down assays. GST–MYB41 was immobilized to glutathione beads and then incubated with His–MPK6. Protein bound to the beads was precipitated and analyzed by Western blotting using anti-His antibody. His–MPK6 input served as a positive control. As shown in Fig. 1A, MYB41 could pull-down MPK6 fusion protein but not GST protein.

To further examine this interaction *in planta*, the luciferase complementation imaging (LCI) assay was performed. MYB41 was fused with the C-terminal fragment of luciferase (CLuc–MYB41), whereas MPK6 was fused with the N-terminal fragment of luciferase (MPK6–NLuc). The result showed that co-infiltration of CLuc–MYB41 and MPK6–NLuc lead to positive luciferase activity in tobacco leaves (Fig. 1B). In contrast, negative controls (NLuc/CLuc–MYB41, MPK6–NLuc/CLuc, NLuc/CLuc) showed only background level of luciferase activity. Quantitation of the average firefly luciferase activity of CLuc–MYB41/MPK6–NLuc was significantly greater than negative controls (Fig. 1C). Taken together, pull-down and LCI assays demonstrated that MYB41 directly interacts with MPK6 in both *in vitro* and *in planta*.

3.2. MPK6 phosphorylates serine-251 of MYB41

To ascertain whether MYB41 is phosphorylated by MPK6, the kinase assay was performed. Purified GST–MYB41 and His–MPK6 proteins were used for *in vitro* kinase assay. MBP and GST proteins were used as positive and negative controls, respectively. The autophosphorylation activity of His–MPK6 (~46 kDa) was observed. GST–MYB41 (~57 kDa) and MBP (~18.5 kDa) were strongly phosphorylated by MPK6, whereas GST protein (~26 kDa) was not (Fig. 2A). This result revealed that MPK6 could specifically phosphorylate MYB41 *in vitro*.

It was documented that the phosphorylation sites of substrates by MPKs are serine or threonine followed by proline (S/T-P motif) [22]. MYB41 contains five potential MPKs phosphorylation sites. To identify the phosphorylation site of MYB41 by MPK6, we performed mass spectrometry after *in vitro* phosphorylation. A phosphopeptide derived from phosphorylated MYB41 was enriched by using TiO_2 chromatography and analyzed by MALDI-TOF mass spectrometry. This phosphopeptide contains two putative phosphorylation sites, Thr^{247} and Ser^{251} (Table 1). Based on the mass spectrometry data, we knew that one of two putative sites is phosphorylated by MPK6. To know the phosphorylation sites of MYB41, we performed site-directed mutagenesis. The substitution of Ser^{251} by Ala completely abolished the phosphorylation of MYB41 by MPK6. However, a strong phosphorylation signal was observed in the $MYB41^{T247A}$ mutant protein as in $MYB41^{WT}$ protein (Fig. 2B). Based on these results, we concluded that Ser^{251} of MYB41 is phosphorylated by MPK6.

To examine whether MYB41 is phosphorylated by plant MPK6, we performed in-gel kinase assay. Total proteins were extracted

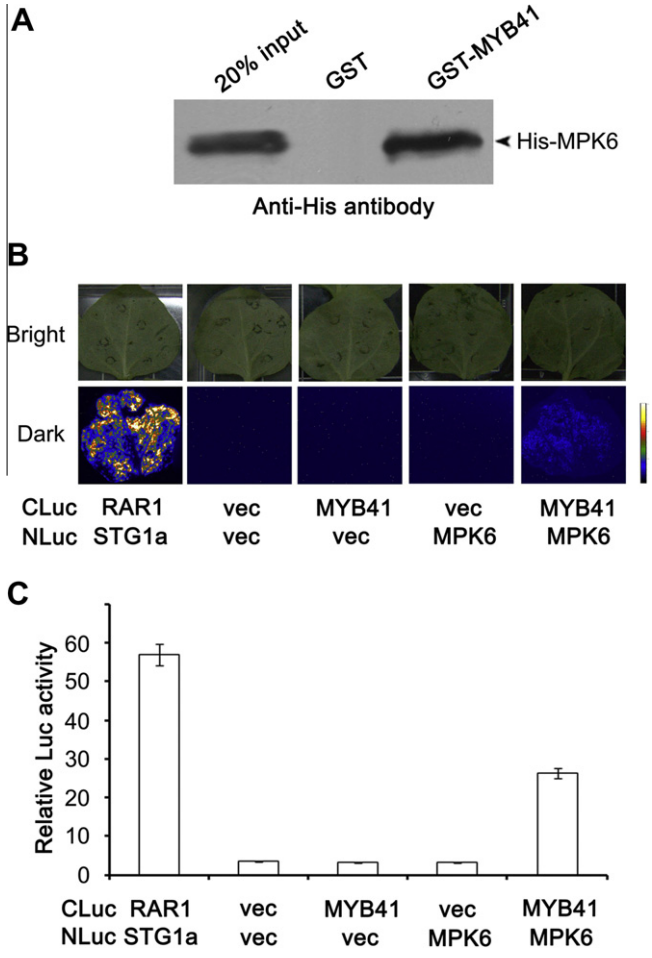

Fig. 1. MYB41 physically interacts with MPK6 *in vitro* and *in vivo*. (A) The interaction of MYB41 with MPK6 in pull-down assay. The equal amount of GST and GST–MYB41 proteins were incubated with glutathione beads, then incubated with His–MPK6 in binding buffer. The protein complex was eluted and the association of MYB41 and MPK6 was determined by Western blot with the anti-His antibody. Twenty percent input of His–MPK6 (20% input) and purified GST were used as positive and negative control, respectively. (B) The interaction of MYB41 with MPK6 *in planta*. Leaves of *N. benthamiana* were co-infiltration with the *Agrobacterium tumefaciens* culture harboring the indicated construct. CLuc- RAR1/STG1a–NLuc was used as positive control and CLuc/MYB41–NLuc, MPK6–CLuc/NLuc, NLuc/CLuc were used as negative controls. Pseudocolor bar, right, shows the range of luminescence intensity from weak to strong. Data was collected 2 days after infiltration. (C) Quantitation of the average firely luciferase activities in (B).

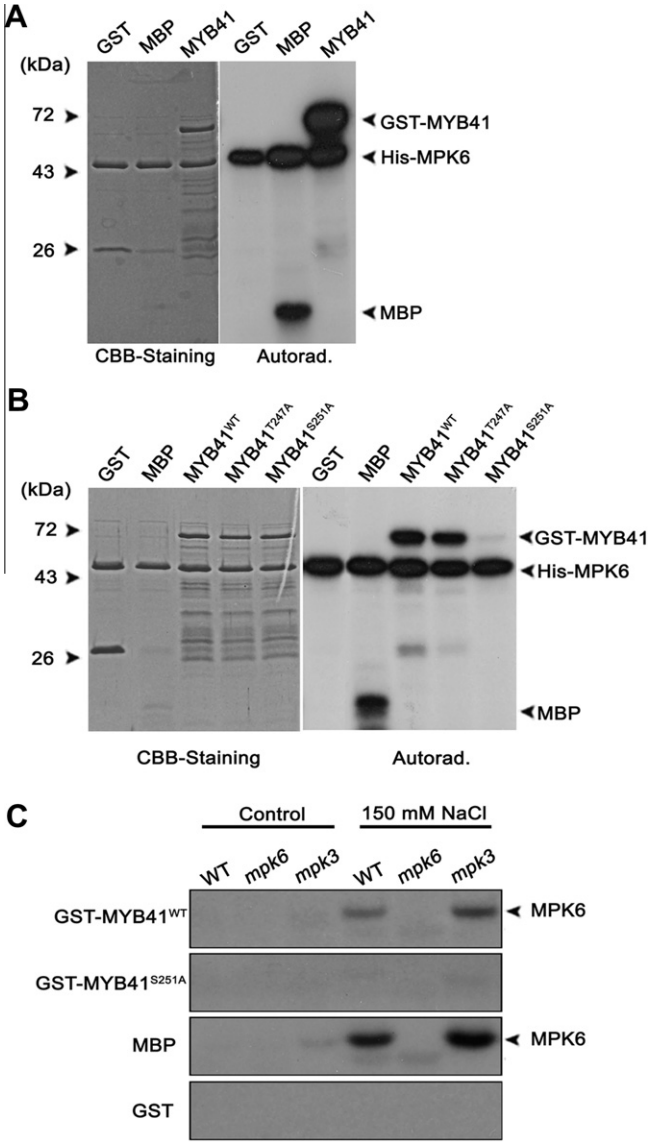

Fig. 2. MPK6 phosphorylates Serine-251 of MYB41. (A) *In vitro* phosphorylation of MYB41 by MPK6. Purified recombinant His–MPK6 and GST–MYB41 were mixed in kinase reaction buffer and reacted for 30 min at 30 °C. The position of molecular weight marker is indicated on the left. The arrowheads on the right indicate position of His–MPK6, GST–MYB41 and MBP protein. (B) The 251st Ser residue of MYB41 is phosphorylated by MPK6. The MYB41^{WT}, MYB41^{T247A} and MYB41^{S251A} proteins were used as substrates. (C) In-gel kinase assay of MYB41. Three week old plants of WT, *mpk3-2* and *mpk6-3* were treatment with water (control) or 150 mM NaCl for 30 min. Total (50 μg) extracted protein from whole plants were denatured and separated in SDS gel. In-gel kinase assay was performed by using recombinant MYB41^{WT} and MYB41^{S251A} proteins as embed substrates. MBP and GST proteins are a positive and negative control, respectively.

from wild type (WT) plant, the null mutant plants of MPK3 (*mpk3-2*) and MPK6 (*mpk6-3*) as well [20]. To activate endogenous MPK6, WT, *mpk3-2* and *mpk6-3* plants were treatment with 150 mM NaCl. Recombinant GST–MYB41^{WT} and GST–MYB41^{S251A} proteins were embedded in the SDS–PAGE gel. MBP and GST protein were used as positive and negative controls, respectively. MBP and MYB41^{WT} were strongly phosphorylated by ~46 kDa protein kinase band in wild type and *mpk3-2* plants but not in *mpk6-3* plant (Fig. 2C). Therefore, we knew that ~46 kDa protein kinase corresponded to MPK6. However, GST and MYB41^{S251A} were not phosphorylated by MPK6. These results indicated that the 251st Ser residue of MYB41 protein is phosphorylated by both recombinant and native plant MPK6.

3.3. The DNA binding of MYB41 to the promoter of a LTP is increased by phosphorylation

Previously the transcript of a lipid transfer protein (*LTP*) gene (At3g22620) was found to be highly up-regulated in MYB41^{WT} over-expressing plants [17]. *LTP* proteins are known to function in cuticle deposition which is important to confer resistance to abi-

Table 1
Phosphopeptides isolated by TiO_2 chromatography from chymotryptic digestion of MYB41 phosphorylated by MPK6.

Peptide sequence	$(M + H)^+$ (Expected)	$(M + H)^+$ (Measured)	Number of phosphate group	Number of Met oxidation
MS**T**PMS**S**PRQNSIEAETNSSTF	2498.021	2498.048	1	1
MS**T**PMS**S**PRQNSIEAETNSSTF	2514.016	2514.051	1	2

Potential phosphorylation sites by MPK6 are bold and underlined.

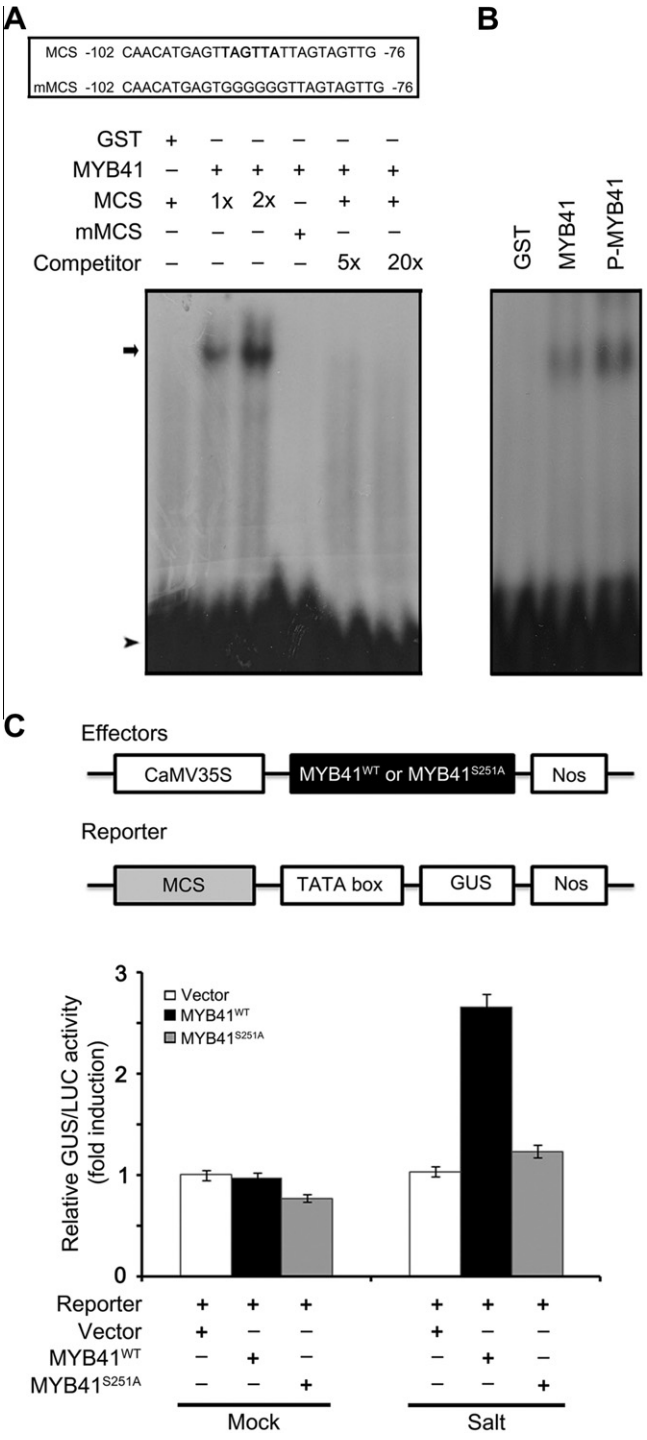

Fig. 3. The phosphorylation of MYB41 is required for the transcriptional regulation of the *LTP* gene. (A) MYB41 directly binds to MCS of a *LTP* (At3g22620) promoter *in vitro*. EMSA was performed by using recombinant MYB41^{WT} protein and MCS probe. Excess amount of unlabeled MCS competition or mutant MCS (mMCS) were added. DNA–protein complex and free probes were indicated with arrow and arrowhead, respectively. (B) The DNA binding of MYB41 was enhanced by its MPK6 phosphorylation. The phosphorylated and non-phosphorylated MYB41 were applied in EMSA. (C) The transactivation of the *LTP* promoter–GUS gene by MYB41^{WT} and MYB41^{S251A} proteins in a transient assay. Schematic representation of reporter and effector constructs used in transient expression assay (upper). The expression constructs were expressed transiently in *Arabidopsis* protoplasts. The transformed protoplasts were treated with water (Mock) or 50 mM NaCl (Salt). Reporter activities were normalized as GUS/LUC activity in each transformed sample, and the activities were calculated relative to that of the reporter construct alone. The bars indicate the mean ± S.D. for each set of three independent experiments.

otic stresses such as osmotic stress and drought stress [23,24]. We hypothesized that MYB41 might regulate the expression of the *LTP* gene through direct binding to the promoter. By promoter analysis, we identified a conserved MYB binding consensus sequence (MCS) in the promoter of the *LTP*. The ^{32}P-labeled MCS motif was used as a probe for electrophoresis mobility shift assays (EMSA). The results indicated binding of MYB41 to the MCS motif, whereas GST protein could not (Fig. 3A). To investigate the specificity of binding by MYB41 to the MCS motif, the unlabeled probe was used as a competitor, which was effective. The unlabeled probe competed with the labeled probe proportional to its concentration. To confirm the specific binding of MYB41 to the MCS motif, we examined a mutated MCS (mMCS). As expected, interactions of MYB41 and the mMCS were not detected (Fig. 3A). Taken together, these results indicated that MYB41 specifically bind to the MCS motif on the promoter of a *LTP* (At3g22620).

Phosphorylation of a transcription factor by a kinase can change the DNA binding activity of the transcription factor [25]. To determine the effect of phosphorylation on the DNA binding activity of MYB41, we performed EMSA by using phosphorylated MYB41 by MPK6. The unphosphorylated MYB41 was used as a control. The result revealed that the DNA binding activity of MYB41 to the promoter of a *LTP* gene was enhanced after phosphorylation by MPK6 (Fig. 3B).

To test whether this phosphorylation might be of functional importance for the transcriptional activity of MYB41, we generated a GUS reporter plasmid by fusion of the MCS motif from the *LTP* promoter and performed transient assays in protoplasts using MYB41^{WT} and MYB41^{S251A}, respectively. In the absence of salt, MYB41^{WT} and MYB41^{S251A} showed no significant differences in expression of the GUS reporter gene. However, at high salt concentrations in the medium, MYB41^{WT} increased transcription of GUS reporter gene by about 2.5-fold, whereas MYB41^{S251A} showed no significant change over the no stress condition (Fig. 3C). These results strongly indicated that phosphorylation by MPK6, which is activated by salt, is important for the transcriptional activity of MYB41.

3.4. MPK6 phosphorylation is required for the biological function of MYB41

MYB41 was reported to be involved in a complex network of transcription factors controlling cell expansion and cuticle deposition. Over-expression of MYB41 resulted in a pleiotropic phenotype resembling that of numerous cuticle mutants [17]. In order to examine the biological consequence of the phosphorylation of MYB41 by MPK6, we generated transgenic plants over-expressing MYB41^{WT} and MYB41^{S251A}. Three independent homozygous transgenic plants were used for phenotypic assessment. MYB41^{WT} and MYB41^{S251A} over-expressing transgenic seeds were examined for their ability to germinate under high salinity condition (Fig. 4A). After 5 days, the germination rate of all seeds reached 100% in the absent of salt. However, on the medium including 150 mM NaCl, the germination rate of wild type seeds was approximately 48% while that of MYB41^{WT} over-expressing seeds reached ~80%. Surprisingly, the germination rate of MYB41^{S251A} over-expressing seeds at 150 mM NaCl medium was reduced to about 20% (Fig. 4B). In addition, we examined root growth of transgenic plants under high salt conditions. On normal medium, root growth of all plants was similar. However, on the medium including 100 mM NaCl, MYB41^{WT} over-expressing plants showed more enhanced salt tolerance than wild type plants. In contrast, MYB41-^{S251A} over-expressing plant exhibited a hypersensitive phenotype to salt (Fig. 4C and D). We consider that the phenotype caused by the highly expressed MYB41^{S251A} is based on a dominant negative effect of the mutant protein overriding effects by the moder-

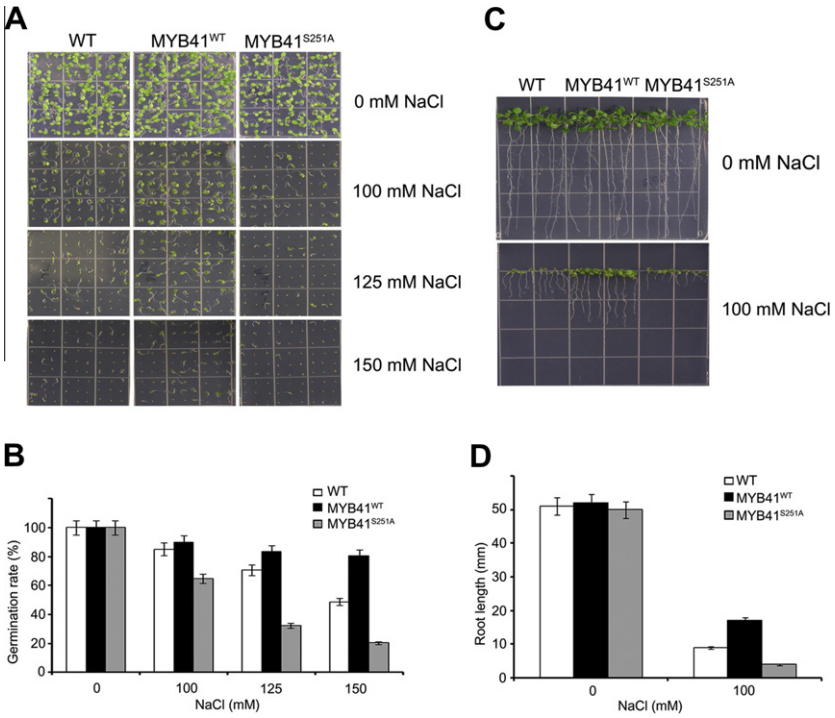

Fig. 4. Effect of salt stress on germination and root growth of wild type, MYB41^{WT} ox and MYB41^{S251A} ox plants. (A) Effect of salt stress on the germination of wild type, MYB41^{WT} ox and MYB41^{S251A} ox seeds. Seeds were germinated on 1/2 MS with different concentration of NaCl for 5 days. (B) The average germination yields in (A). (C) Root growth of wild type, MYB41^{WT} ox and MYB41^{S251A} ox plants at high salt condition. Seeds were germinated and grown on 1/2 MS medium supplied with or without 100 mM NaCl for 10 days. (D) The average root lengths in (C). Representative data was obtained from three independent experiments with similar results.

ately expressed endogenous MYB41. The results strongly imply phosphorylation by MPK6 as a requirement for the biological function of the MYB41 transcription factor as a positive regulator in a pathway leading to salt stress tolerance. Arguably, phosphorylation of MYB41 will increase its DNA binding affinity to the promoters of target genes, or alter its capacity for interactions with other positive regulatory proteins.

Our study provides conclusive evidence for the transcription factor MYB41 as a new target for the MAP kinase, MPK6. Phosphorylation by MPK6 plays a decisive role in determining the biological function of MYB41 through regulation of the DNA binding ability of the transcription factor.

Acknowledgments

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