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Phosphorylation of the transcriptional regulator MYB44 by mitogen activated protein kinase regulates *Arabidopsis* seed germination

Xuan Canh Nguyen [a,1], My Hanh Thi Hoang [a,1], Ho Soo Kim [a], Kyunghee Lee [a], Xiao-Min Liu [a], Sun Ho Kim [b], Sunghwa Bahk [a], Hyeong Cheol Park [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

The phytohormones abscisic acid (ABA) and gibberellic acid (GA) have antagonistic roles in the control of seed germination and seedling development. We report here that the transcriptional regulator MYB44 has a role in the control of seed germination in *Arabidopsis thaliana*. High levels of the *MYB44* transcript are found in dry seeds but the transcript levels decrease during germination. The decrease in transcript level during germination is inhibited by the GA biosynthesis inhibitor paclobutrazol (PAC). MYB44 is phosphorylated by both recombinant and native forms of MPK3 and MPK6 at Ser^{53} and Ser^{145}. Transgenic overexpression of *MYB44* results in increased sensitivity of seed germination to ABA or PAC treatment. The PAC-insensitive germination phenotype of the *myb44* mutant is complemented by overexpression of wild type MYB44 but not by overexpression of a mutant protein that lacks the MPK-target serines indicating that phosphorylation of MYB44 by MPKs is required for its biological function.

1. Introduction

The mitogen-activated protein kinase (MPK) cascade is a conserved signaling pathway in eukaryotes [1–3]. The MPK cascade consists of three kinases. The most upstream kinase is a MPK kinase kinase (MKKK) which is a serine/threonine kinase that is itself activated by an upstream kinase. MKKK phosphorylates and activates a MPK kinase (MKK) which is a dual specificity kinase that phosphorylates threonine and tyrosine residues at a Thr-X-Tyr motif. MKK phosphorylates and activates MPK, the terminal kinase of the module [4,5]. MPK is a serine/threonine kinase and phosphorylation of its substrates that are often transcription factors, results in a physiological response [6].

Several MPK cascades that are characterized by unique players, and having distinct inputs and outputs, function in eukaryotic cells. There is also crosstalk between the various MPK cascades. In plants, MPK cascades are involved in the regulation of growth and development as well as response to various environmental stimuli [3,5,7–9]. A number of studies have demonstrated that MPKs in *Arabidopsis* are associated with hormone biosynthesis and signaling including ethylene, jasmonic acid, auxin and ABA [10–14]. Accordingly, there are 10 MPK kinases (MKKs) and 20 MPKs in the *Arabidopsis* genome [4]. Every MPK cascade signaling outcome depends on specific MKK–MPK modules and the substrate specificity of the MPK. The characterization of the specific MKK–MPK and MPK–substrate interactions that are involved in the response to an individual stimulus or a particular plant process is a subject of considerable research. Recently, several high throughput investigations of *Arabidopsis* MPK interaction partners and substrates have been reported [15,16]. These investigations have relied on *in vitro* phosphorylation assays, protein microarray-based proteomic methods, and yeast two-hybrid analysis to identify candidates. Several of these candidates have been confirmed as substrates of a specific MPK. However, more studies are required to confirm that the remaining candidates are true MPK substrates, to establish their MPK specificity and to connect them to a specific physiological response.

MYB44 is a transcription factor that belongs to subgroup 22 of *Arabidopsis* R2R3-MYB family. MYB44 has been identified as an interaction partner of MPK3 and MPK6 by yeast two-hybrid screening of library of *Arabidopsis* cDNAs [17]. MYB44 is induced by a variety of abiotic stresses and phytohormones including salt, drought, cold, sugar, abscisic acid (ABA), gibberellic acid (GA), jasmonic acid, ethylene and auxin [18,19]. Plants over-expressing *MYB44* show enhanced tolerance to salt and drought stresses via an ABA signaling pathway [18]. Investigation of transcriptome changes show that the MYB44 homolog in *Euphorbia esula* (leaf spurge) is involved regulator hubs that control in ABA signaling

* Corresponding author at: Division of Applied Life Science, 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] These authors contributed equally to this work.

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[20]. However, the mechanism by which MYB44 activity is regulated remains unknown.

We report here that, in *Arabidopsis*, MYB44 is a substrate of MPK3 and MPK6. Phosphorylation of MYB44 by MPKs was found to be necessary for its function as a negative regulator of GA- and ABA-mediated control of seed germination.

2. Materials and methods

2.1. Plant materials

Arabidopsis thaliana plants were grown at 22 °C in a growth chamber under 100 μE m^{-2} s^{-1} light intensity a 16-h–light/8-h–dark cycle. T-DNA insertion mutants of *MPK3* (*mpk3-2*, SALK_151594), *MPK6* (*mpk6-3*, SALK_127507), and *MYB44* (*myb44*, SALK_039074) were obtained from the *Arabidopsis* Biological Resource Center. All *Arabidopsis* lines used in this study were of the Columbia ecotype (Col-0). Three-week-old plants of WT, *mpk3-2*, *mpk6-3* were sprayed with 100 μM ABA to activate MPKs.

2.2. Seed germination assay

Mature seeds of all genotypes were harvested at the same time and stored at 4 °C for at least 3 months before testing. Seeds were sown on Murashige and Skoog medium containing 2% sucrose and solidified with 0.8% agar. Supplements such as ABA (A1049, Sigma–Aldrich), paclobutrazol (PAC, 46046, Sigma–Aldrich), or GA3 (G7646, Sigma–Aldrich) were added to this medium before solidifying at the concentrations indicated in the figures. Plates were kept at 4 °C for 4 d in the dark to promote germination and then transferred to the growth chamber. A seed was regarded as germinated when the radical protruded through the seed coat. The germination results are presented as average values from three independent experiments. More than 300 seeds for each genotype were used in each experiment.

2.3. Plasmid construction

The MYB44 ORF was cloned into *BamHI*/*EcoRI* sites of the vector to generate the fusion construct GST-MYB44. The MYB44 mutant constructs GST-MYB44^{S53A}, GST-MYB44^{S145A}, GST-MYB44^{AA} were then generated by using the QuickChange site-directed mutagenesis kit (Stratagene). The GST-MYB44^{AA} construct has two point mutations, namely Ser-to-Ala substitutions at Ser^{53} and Ser^{145}. ORFs of the MAP kinases MPK3 and MPK6 were cloned between the *BamHI*/*SalI* sites of pQE-30 vector to generate His-tagged fusion constructs. All constructs were sequenced to confirm mutant sites before using in kinase assays.

2.4. Kinase assay and in-gel kinase assay

The substrate proteins (GST-MYB44 and its derivatives) and the kinases (His-tagged MPK3 or MPK6) used for *in vitro* kinase assays were expressed in *Escherichia coli* and purified by affinity chromatography on glutathione agarose and Ni-NTA affinity resin. *In vitro* kinase assays were performed as previously described [17] by incubating 2 μg of purified substrate protein and 1 μg of purified kinase in kinase assay buffer at 30 °C for 30 min. Glutathione S-transferase (GST) and myelin basic protein (MBP) proteins were used as negative and positive controls, respectively.

For the in-gel kinase assay, 30 μg crude plant proteins were incubated at 60 °C for 10 min in SDS sample buffer and then fractionated by electrophoresis on 10% SDS–polyacrylamide gels embedded with purified GST-MYB44, GST-MYB41^{S53A}, GST-MYB41^{S145A} or GST-MYB41^{AA} proteins. Gels embedded with GST and MBP were used as negative and positive controls, respectively. After electrophoresis, the in-gel kinase assay was performed as previously reported [21].

2.5. Mass spectrometry

GST-MYB44 was phosphorylated by His-MPKs in a kinase assay before performing mass spectrometry. The reaction mixture was resolved by SDS–PAGE as described above. Then MYB44 protein bands were excised, alkylated and digested with trypsin. Phosphorylated peptides were enriched by passage through a TiO_2 microcolumn and then analyzed by MALDI-TOF as previously described [22].

2.6. Western blot analysis

Total proteins were extracted from leaves of two-week old plants and separated by 10% SDS–PAGE. The anti-FLAG antibody used for Western blot was commercially obtained (Sigma, USA). Western blot analysis was performed as described [23].

2.7. RT-PCR

Total RNAs were extracted from dry seeds, imbibed seeds, or leaves and converted to cDNAs using MMLV reverse transcriptase (Fermentas) according the manufacturer's protocol. Equal amounts of cDNA were used as template for PCR. Primers were designed for *MYB44* as follows: forward 5′-ATGGCTGATAGGATCAAAGG-3′ and reverse 5′-CTCGATTCTCCCAACTC-3′. The RT-PCR reaction was performed under the following conditions: pre-denaturation at 94 °C for 5 min, 28 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. Equivalence of cDNA in different samples was verified by an RT-PCR reaction for *Tubulin2*.

3. Results and discussion

3.1. Identification of MPK3 and MPK6 phosphorylation sites in MYB44

MYB44 has been identified as an interaction partner of MPK3 and MPK6 by yeast two-hybrid screening [17]. The interaction of MYB44 with MPK3 and MPK6 was verified here by a luciferase (LUC) complementation imaging assay (Fig. 1). Co-expression of MYB44 with MPK3 or MPK6 led to LUC activity in tobacco leaves, similar to the positive control STG1a-RAR1 interaction [24]. Expression of MYB44, MPK3 and MPK6 in the absence of the interaction partner showed only the background level of LUC activity. This result indicated that MYB44 directly interacts with MPK3 and MPK6 *in planta*. Therefore, we hypothesized that MYB44 is a target substrate of MPK3 and MPK6. We performed an *in vitro* kinase assay to ascertain whether MYB44 could be phosphorylated by MPK3 and MPK6 using purified recombinant GST-MYB44 as substrate and His-MPK3 or His-MPK6 as enzyme. GST and MBP were used as negative and positive control substrates, respectively. We observed strong autophosphorylation bands at approximately 43 kDa and 46 kDa with all substrates as His-MPK3 and His-MPK6, respectively (Fig. 2A). With both His-MPK3 and His-MPK6, strong phosphorylated bands were observed on the autoradiograph that corresponded to the GST-MYB44 (~60 kDa) and MBP (~17.5 kDa) bands on the Coomassie-stained gel, but not the GST (~25 kDa) band (Fig. 2A). This result indicated that MPK3 and MPK6 could specifically phosphorylate MYB44 *in vitro*.

Mass spectrometry was then performed to identify the MPK3/MPK6-target phosphorylation sites in MYB44. For this, the phosphopeptides derived from phosphorylated MYB44 were enriched

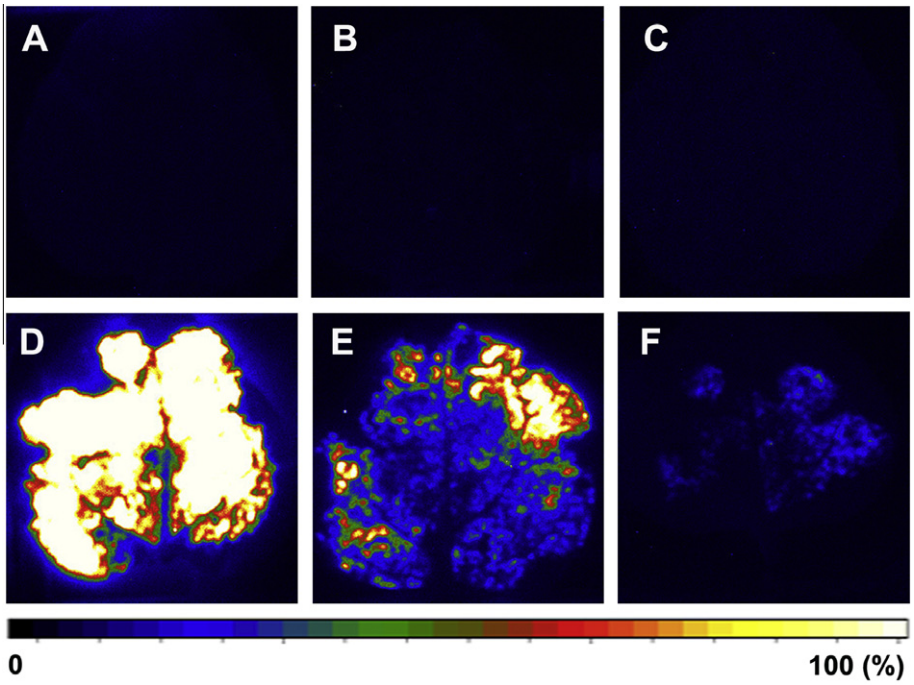

Fig. 1. Luciferase complement imaging assay for interaction of MYB44 with MPK3 and MPK6 *in planta*. Shown are luminescence images of tobacco leaves that were transiently transformed via *Agrobacterium* infiltration with the construct pairs of CLuc-MPK3 plus NLuc vector (A), CLuc-MPK6 plus NLuc vector (B), MYB44-NLuc plus CLuc vector (C), STG1a-NLuc plus RAR1-CLuc (D) (positive control), MYB44-NLuc plus CLuc-MPK3 (E) and MYB44-NLuc plus CLuc-MPK6 (F). Pseudocolor bar shows the range of luminescence intensity in the images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by using TiO_2 chromatography and analyzed by MALDI-TOF mass spectrometry. Based on the mass spectrometry analysis, Ser^{53} and Ser^{145} of MYB44 were identified as putative phosphorylation sites by MPKs (Table 1). These serines lie within Ser/Thr-Pro protein sequence motifs that are likely to be phosphorylation sites of MPKs substrates [25]. To confirm the mass spectrometry result, we generated three plasmid constructs, GST-$MYB44^{S53A}$, GST-$MYB44^{S145A}$ and GST-$MYB44^{S53A/S145A}$ (GST-$MYB44^{AA}$) by site-directed mutagenesis. In these constructs, one or both of the putative MPK substrate phosphorylation sites were erased by substitution with alanine. An *in vitro* kinase assay was then performed with His-MPK3 and proteins expressed from these constructs. As shown in Fig. 2B, strong bands corresponding to phosphorylated GST-MYB44 and GST-$MYB44^{S53A}$, were observed on the autoradiograph (Fig. 2B, Right). The intensity of the band corresponding to phosphorylated GST-$MYB44^{S145A}$ was significantly lower and the band corresponding to phosphorylated GST-$MYB44^{AA}$ was barely visible even though approximately equal amounts of substrate proteins were used in every case (Fig. 2B, Left). The same data was observed in an *in vitro* kinase assay using His-MPK6 instead of His-MPK3 (data not shown). Based on these results, we concluded that MPK3 and MPK6 phosphorylate MYB44 at Ser^{53} and Ser^{145}.

To test whether MYB44 is phosphorylated by native MPKs derived from plant tissues, an in-gel kinase assay was performed using the same substrates as above (Fig. 2C). For this, total proteins were extracted from rosette leaves of wild type, *mpk3-2* and *mpk6-3* plants 30 min after they had been sprayed with 100 μM ABA. The purpose of the ABA treatment was to induce MPK activities. The results of the in-gel kinase assay are depicted in Fig. 2C. Radioactive phosphorylated bands of approximately 43 kDa and 46 kDa corresponding to MPK3 and MPK6, respectively, were detected in extracts of the wild type leaves with GST-MYB44 as embedded substrate. The 46 kDa band was absent in *mpk6-3* extracts and the 43 kDa band was absent in *mpk3-2* extracts, as expected. Similar results were seen in in-gel kinase assays with GST-MYB^{S53A} or GST-MYB^{S145A} as embedded substrate, except that the intensity of the signal in every lane was greatly reduced. The in-gel kinase assay with MBP as embedded substrate confirmed that the 43 kDa and 46 kDa bands corresponded to MPK3 and MPK6, respectively and that both activities were present in extracts of wild type leaves. No phosphorylated bands were observed with embedded GST protein (negative control) or GST-$MYB44^{AA}$ protein. Thus Ser^{53} and Ser^{145} of MYB44 protein were confirmed as phosphorylation sites of both recombinant and plant-derived MPK3 and MPK6.

3.2. Phosphorylation of MYB44 by MPKs is required for the effect of ABA on seed germination

ABA inhibits seed germination and transgenic overexpression of *MYB44* increases sensitivity to ABA during seed germination [14]. We found that the level of *MYB44* transcript was high in dry seeds and during seed imbibition at 4 °C (Fig. 3A). After transfer to 22 °C to induce seed germination, steady state levels of the *MYB44* transcript decreased rapidly over time (Fig. 3A). To investigate the physiological function of MYB44 in seed germination, we obtained a MYB44 knock-out mutant (*myb44*). RT-PCR analysis confirmed that the *MYB44* transcript was undetectable in *myb44* plants (Supplementary Fig. 1A). We also generated transgenic plants in *myb44* mutant background over-expressing MYB44-Flag or $MYB44^{AA}$-Flag fusion proteins from the CaMV35S promoter (MYB44/*myb44*, $MYB44^{AA}$/*myb44*) in order to test the relevance of the phosphorylation sites of MPK3 and MPK6 on MYB44 for ABA-inhibition of seed germination. Homozygous plants from three independent lines of each transgene that showed constitutively elevated levels of MYB44 protein were selected for further analyses (Fig. 3B). No difference was observed between these seeds when germinated on MS plates. The germination rates of wild type and *myb44* seeds were about 48%, showing that inactivation of *MYB44* had no effect on ABA-inhibition of seed germination (Fig. 3C). The germination rate of MYB44/*myb44* lines ranged between 15% and 20%, as expected from the earlier report that overexpression of MYB44 increases ABA-sensitivity in the germination assay [18]. However,

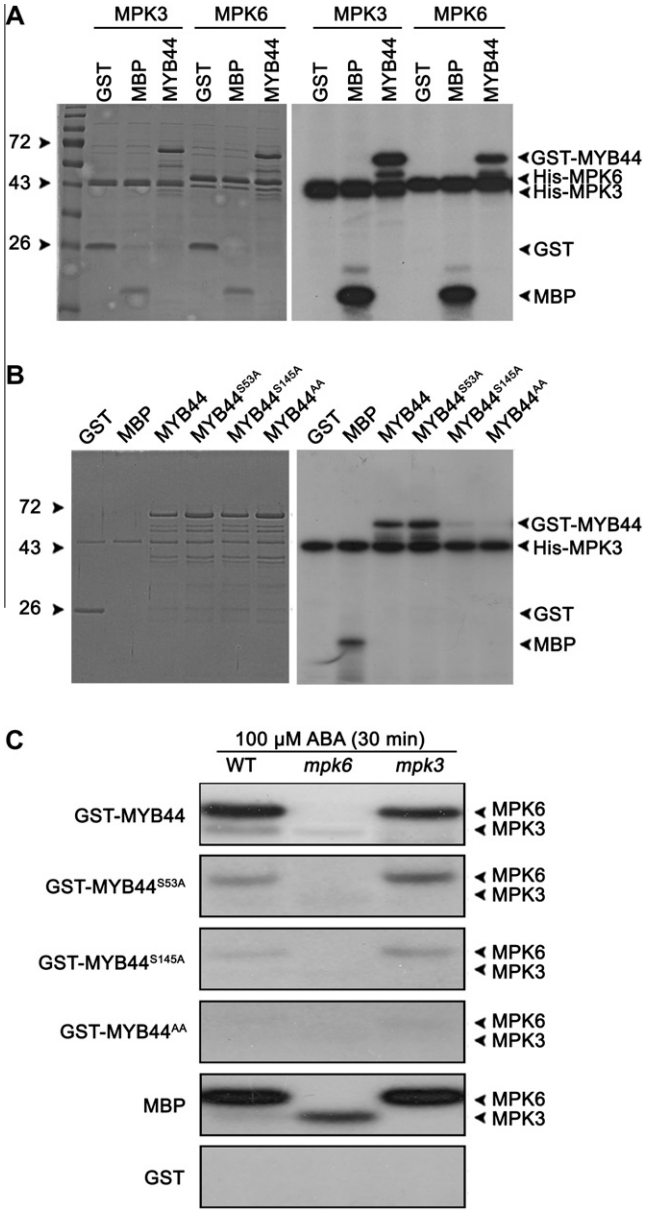

Fig. 2. MYB44 is phosphorylated by MPK3 and MPK6 *in vitro*. (A and B) MYB44 is phosphorylated at Ser^{53} and Ser^{145} by recombinant MPK3 and MPK6. Kinase reactions were carried out using purified His-tagged MPK3 (MPK3) or His-tagged MPK6 (MPK6) as enzyme and purified GST, MBP, GST-MYB44 (MYB44), GST-$MYB44^{S53A}$ ($MYB44^{S53A}$), GST-$MYB44^{S145A}$ ($MYB44^{S145A}$), or GST-$MYB44^{AA}$ ($MYB44^{AA}$) as substrate. At the end of the reaction, proteins were resolved on 12% SDS–PAGE. Shown is a gel stained with Coomasie Brilliant Blue (Left) and its autoradiograph (Right). Protein molecular sizes are shown on the left by arrowheads. The arrowheads on the right indicate position of GST-MYB44, His-MPK3, His-MPK6, MBP and GST proteins. (C) In-gel kinase assay showing phosphorylation of MYB44 at Ser^{53} and Ser^{145} by native MPK3 and MPK6. Proteins (30 μg) that were extracted from leaves of three-week-old plants of WT, *mpk3-2* and *mpk6-3* null mutants 30 min after spraying with 100 μM ABA, were resolved by 10% SDS–PAGE on gels embedded with the purified substrates indicated on the left. Arrowheads on the right of the autoradiographs indicate the positions of native MPK6 and MPK3. GST and MBP were used as negative and positive controls, respectively.

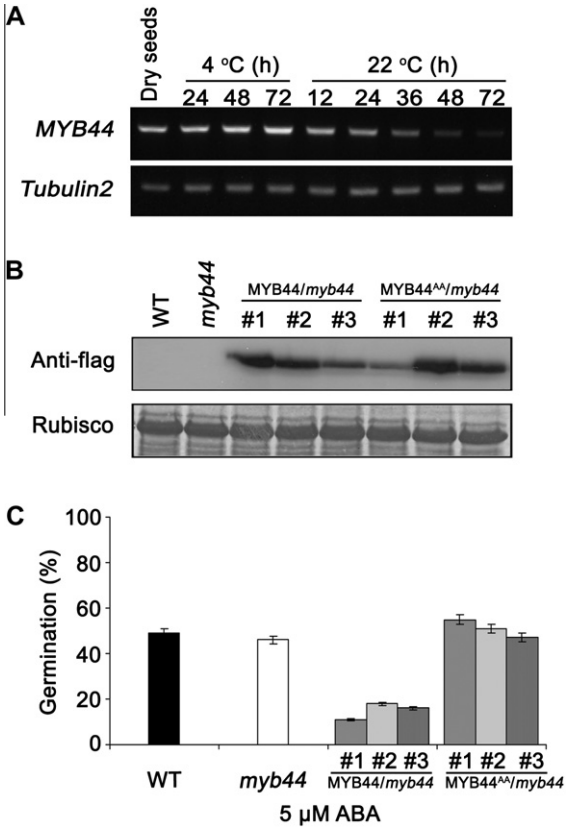

Fig. 3. Phosphorylation of MYB44 by MPKs is required for its role in ABA-mediated inhibition of germination. (A) *MYB44* transcript is reduced during seed germination. Total RNA was isolated from seeds of the wild type before imbibition (Dry seeds), after incubation in sterile water at 4 °C in the dark for imbibition (4 °C) for the indicated periods and after transfer to the growth chamber at 22 °C to induce germination (22 °C) for the indicated periods. Shown are the results of an RT-PCR that was performed using the *MYB44* gene-specific primers. The *Tubulin2* transcript level is shown as a control for quantifying input cDNA. (B) Characterization of MYB44/*myb44* and $MYB44^{AA}$/*myb44* transgenic lines. Protein expression levels in leaf extracts of T3 generation transgenic plants of three independent lines expressing Flag-tagged MYB44 or Flag-tagged $MYB44^{AA}$ in the *myb44* background (MYB44/*myb44* and $MYB44^{AA}$/*myb44*, respectively) was examined after SDS–PAGE by Western blot using anti-Flag antibody (anti-Flag). The intensity of the Rubiso band after Coomassie Brilliant Blue staining of the same gel was used as loading control (Rubisco). Wild type (WT) and *myb44* extracts were used as negative controls. (C) MPK phosphorylation sites are required for the function of MYB44. Shown are germination rates of WT, *myb44*, MYB44/*myb44* and $MYB44^{AA}$/*myb44* seeds in growth medium supplemented with 5 μM ABA. Three independent transgenic lines of each transformant were used for the experiment. Germination yields were scored after 4 days. For each experiment, approximately 300 seeds were scored. Values represent the average of three experiments ± SE.

the germination rate of $MYB44^{AA}$/*myb44* lines was close to that of the wild type showing that the phosphorylation of MYB44 by MPKs is essential for its biological function.

3.3. Phosphorylation of MYB44 by MPK3 and MPK6 is required for the effect of PAC on seed germination

It known that endogenous GA biosynthesis is required for breaking seed dormancy and inducing germination [26]. Thus the

Table 1
Phosphopeptides isolated by TiO_2 chromatography from trypsin digestion of MYB44 phosphorylated by MPKs.

Peptide sequence	$(M+H)^+$ (Expected)	$(M+H)^+$ (Measured)	Number of phosphate group	Number of Met oxidation
CNQL<u>S</u>PQVEHRPF	1691.73	1692.79	1	0
PG<u>S</u>PTGSDVSDSSTIPILPSVELFK	2569.16	2570.73	1	1

Potential phosphorylation sites by MPKs are underlined.

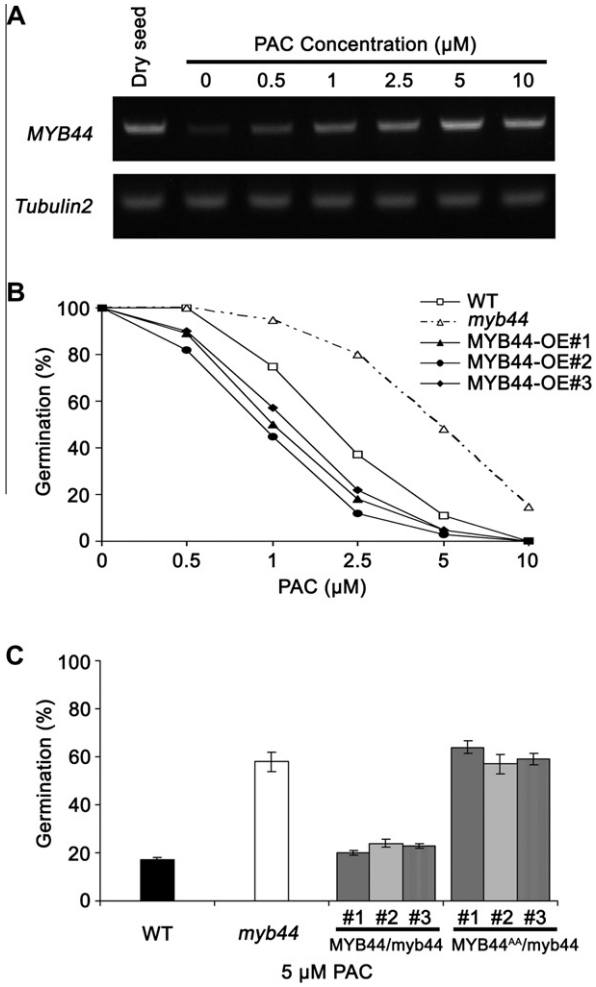

Fig. 4. Phosphorylation of MYB44 by MPKs is required for its role in PAC-mediated inhibition of germination. (A) Effect of PAC on the expression of *MYB44*. Wild-type seeds were imbibed as described in Fig. 3 and then germinated for 72 h on plates containing indicated concentrations of PAC before extraction of total RNA for RT-PCR analysis of *MYB44* and *Tubulin2* transcript abundance. (B) Effect of PAC on germination. Seeds of wild type (WT), *myb44* null mutant (*myb44*) and transgenic *MYB44* overexpression lines (MYB44-OE) were sown on MS medium with indicated concentrations of PAC. Percent germination was scored after 4 d incubation at 22 °C. (C) Phosphorylation by MPKs is required for the function of MYB44. Shown are germination rates of WT, *myb44*, MYB44/*myb44* and MYB44^{AA}/*myb44* seeds in growth medium supplemented with 5 μM PAC. Three independent transgenic lines of each transformant were used for the experiment. Germination yields were scored after 4 days. For each experiment, approximately 300 seeds were scored. Values represent the average of three experiments ± SE.

GA biosynthesis inhibitor PAC inhibits germination. Since germination is accompanied by a decline in *MYB44* expression and since inactivation of *MYB44* had no effect on ABA-inhibition of seed germination (Fig. 3), we explored the possibility that it has a role in GA-mediated signaling during germination. An examination of the transcript level of *MYB44* in seeds germinated in the presence of PAC showed the transcript level of *MYB44* was very low in seeds germinating in the absence of PAC but was increased upon germination in the presence of different levels of PAC, in concentration dependent manner (Fig. 4A). These results suggest that GA biosynthesis is required for the decrease in transcript level of *MYB44* during germination. We therefore hypothesized that MYB44 may be involved in the GA response during seed germination.

To test this hypothesis, we generated transgenic lines over-expressing a Flag-tagged MYB44 protein in the wild type background (MYB44-OE). Over-expression of the MYB44-Flag fusion protein in MYB44-OE lines was verified by Western blot analysis by using anti-Flag antibody (Supplementary Fig. 1B). We performed germination assays in presence of PAC dependent manner. Seeds of the *myb44* mutant were more resistant to PAC than the wild type in the germination assay showing that *MYB44* is required for the inhibitory effect of PAC on seed germination. On the other hand, seeds of the transgenic MYB44-OE lines were more sensitive than the wild type, showing that the PAC response depends on dosage of *MYB44* although PAC also affects the level of the MYB44 transcript (Fig. 4A).

Next, we tested the importance of the MPK3/MPK6 phosphorylation sites on MYB44 for this effect (Fig. 4C). In this experiment, the percent germination of wild type and *myb44* plants were 20% and 60%, respectively, which was similar to the earlier results (Fig. 4B). The percent germination of wild type and *MYB44*/*myb44* plants was comparable, as expected if *MYB44* complements the null *myb44* allele. The percent germination *myb44* and *MYB44*^{AA}/*myb44* plants was comparable, as expected if *MYB44*^{AA} fails to complement the null *myb44* allele. These results indicate that the phosphorylation sites of MPK3 and MPK6 on MYB44 are required for its function in PAC-inhibition of seed germination.

In conclusion, we provide the evidence for the transcription factor MYB41 as a novel target for the MPK3 and MPK6. Our results show that MYB44 and the phosphorylation of MYB44 by MPK3/MPK6 are required for inhibition of germination in response to ABA reduced GA biosynthesis.

Acknowledgments

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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.2012.06.019.

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