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Original Paper

Over-expression of MAP3Kδ4, an ABA-inducible Raf-like MAP3K that confers salt tolerance in *Arabidopsis*

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Abstract Mitogen-activated protein kinase (MAPK) cascades play important roles in plant responses to various environmental stimuli, including high salt or drought levels. *Arabidopsis* MAP3Kδ4 with PAS (period circadian protein, arylhydrocarbon receptor nuclear translocator protein and single-minded protein) domain is one of the Raf-type MAPKKKs whose function has not been identified to date. Previous studies have shown that the *MAP3Kδ4* over-expressing transformant exhibits vigorous growth. In this study, RT-PCR analysis showed that *MAP3Kδ4* transcripts are increased through stress treatments, such as high salt, osmosis, drought and cold and the plant hormone, abscisic acid (ABA). The precipitation of MAP3Kδ4 using its specific antibody showed that ABA treatment markedly induces the activity of this enzyme. Furthermore, the ABA-mediated inhibition of seed germination was relieved in transgenic *Arabidopsis* over-expressing *MAP3Kδ4*. These results suggested that MAP3Kδ4 plays an important role in ABA signalling. Transgenic *Arabidopsis* over-expressing *MAP3Kδ4* also exhibited enhanced tolerance to salt stress. The results obtained in this study demonstrated that MAP3Kδ4 was active during ABA-related responses and is involved in both stress tolerance and increased biomass. Therefore, *MAP3Kδ4* and its counterpart genes are important with respect to agricultural developments.

Key words: ABA signalling, biomass, MAPK cascade, MAP3K, salt tolerance.

Plants have developed various signal transduction pathways to modulate cellular responses to environmental changes. Environmental stresses, such as salinity, cold and drought, influence plant growth and limit the yield of crops.

The mitogen-activated protein kinase (MAPK) cascade, a signal transducer, plays an important role in the response against environmental stresses (Bögre et al. 1997; Mizoguchi et al. 1997; Nakagami et al. 2005; Sinha et al. 2011; Taj et al. 2010). The cascade consists of three signal-transducing components: MAP kinase kinase kinases (MAPKKKs, MAP3Ks, or MEKKs), MAPK kinases (MKKs) and MAPKs. External stimuli are converted into cellular responses through this cascade. Eighty different MAP3Ks, which are primarily upstream components of the cascade, have been identified in the *Arabidopsis* genome. (Colcombet and Hirt 2008; MAPK group 2002). MAP3Ks have been classified into two subfamilies: MEKK-like and Raf-like MAP3Ks. Several MEKK-like MAP3Ks in *Arabidopsis* have been characterised. It has been previously reported that the MEKK1-MKK1-MPK4 cascade is stimulated following wounding stress (Hadiarto et al. 2006; Matsuoka et al. 2002). Other studies have also indicated that these cascades are involved in several other stress signalling pathways: the MEKK1-MKK2-MPK4/MPK6 cascade in salt and cold stress signalling (Teige et al. 2004) or the MEKK1-MKK4/MKK5-MPK3/MPK6 cascade following pathogen infection (Asai et al. 2002). However, little is known about Raf-like MAP3Ks. *Arabidopsis* MAP3Kδ4 (At4g23050), which has PAS (period circadian protein, arylhydrocarbon receptor nuclear translocator protein and single-minded protein) and protein kinase domains, is one of the Raf-type MAPKKKs whose function is remains unknown.

It has previously been reported that transgenic plants over-expressing *Arabidopsis MAP3Kδ4* showed more vigorous growth than wild-type plants based on the

Abbreviations: ACC, 1-amino-1-cyclopropane carboxylic acid; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAP3K, MAPKK kinase; MBP, myelin basic protein.

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fresh weight and stem length (Sasayama et al. 2011). Although it was assumed that MAP3Kδ4 functions during plant growth, the effects of MAP3Kδ4 on plant biomass formation and stress tolerance have not been demonstrated. One of the mechanisms used to combat higher salinity is the synthesis of stress hormones, such as abscisic acid (ABA), which triggers secondary responses that lead to the expression of specific sets of genes for plant tolerance to abiotic stresses (Finkelstein et al. 2002; Koornneef et al. 1998; Raghavendra et al. 2010; Xiong et al. 2003; Zhu 2002). Moreover, ABA acts as a negative regulator of seed germination and seedling growth (Koornneef et al. 2002; Rohde et al. 1999; Tucker 1978). The *MAP3Kδ4* expression in *Arabidopsis*, with or without abiotic stress treatments, such as salt, drought, cold and ABA, was monitored in the present study. The affects of ABA on MAP3Kδ4 kinase activity were also analysed. Furthermore, the germination rates of the seeds were monitored to assess the ABA sensitivity in transgenic plants over-expressing *MAP3Kδ4*.

The results of this study show that MAP3Kδ4 acts during the ABA response and transgenic plants over-expressing *MAP3Kδ4* show increased salt tolerance and biomass production than wild-type plants.

Materials and methods

Plant materials and stress treatments

Arabidopsis thaliana (Columbia ecotype) seeds were surface-sterilised with 70% (v/v) ethanol for 3 min, followed by a solution of NaClO (1% w/v), which included Triton X-100 (0.1% v/v), for 7 min. The seeds were subsequently washed five times with sterile water, plated onto B5 agar (0.8% w/v) medium and incubated for 2 days at 4°C before germination at 22°C.

For the stress treatments, 14-day-old seedlings were carefully removed from the plate and placed in distilled water for 24 h prior to treatment. Subsequently, the seedlings were subjected to 200, 250 and 300 mM NaCl or 15% polyethylene glycol and incubated at room temperature for 1 or 2 h. Similarly, a second set of 14-day-old seedlings were subjected to different stress treatments of 400 mM mannitol, drought, cold (4°C), heat (37°C) and UV for 2 h. A third set of seedlings were treated with plant hormones, i.e., 1 or 10 μM abscisic acid (ABA) and 1-amino-1-cyclopropane carboxylic acid (ACC), for 2 h. The seedlings soaked in the 1 or 10 μM ABA solutions were used for time course analysis through RT-PCR or the immunoprecipitation kinase assay, respectively. After stress treatment, the seedlings were frozen in liquid nitrogen and stored at −80°C until further analysis.

RT-PCR

For the RT-PCR analysis, total RNA was extracted from *Arabidopsis* plants using an RNeasy Plant Mini Kit (Qiagen) and treated with DNase I (Invitrogen) to remove any residual DNA contamination. The cDNA was synthesised from 0.5 μg of *Arabidopsis* total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (TAKARA). *MAP3Kδ4* transcripts were amplified using the forward primer, 5′-GGG AGT CTC TTC AAA ATA CTT CAT -3′, and the reverse primer, 5′-TGC GAT CCG GGG ATT TAA TCC TTC -3′. *Actin8* (At1g49420) transcripts were used as a control and were amplified using the forward primer, 5′-GAA GGA CCT TTA CGG TAA CA-3′, and the reverse primer, 5′-CCA ATC CAG ACA CTG TAC TT-3′. The PCR was performed using TaKaRa *Ex Taq* HS (TAKARA) at 94°C for 2 min, followed by 27 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were separated on 1.6% agarose gels and visualised under UV light.

Antibody production and immunoblot analysis

The anti-MAP3Kδ4 rabbit polyclonal antibody was raised against a synthetic peptide (Cys-Glu-Ser-Arg-Thr-Gly-Lys-Glu-Ser-Ala-Thr-Gly-Leu-Thr), corresponding to amino acid residues 40 to 53 in MAP3Kδ4 (Medical & Biological Laboratories Co., Ltd.). To determine the specificity of the anti-MAP3Kδ4 antibody, the bacterially expressed glutathione *S*-transferase (GST)-fused N-terminal region (amino acid residues 1 to 203) of MAP3Kδ4 was used as an antigen. Immunoblot analyses of the recombinant GST, the GST-fused N-terminal region of MAP3Kδ4 and the crude extracts from the seedlings of wild-type Arabidopsis and transgenic plants over-expressing *MAP3Kδ4* were performed as previously described (Matsuoka et al. 2002). Immunoblot analyses were also performed on MAP3Kδ4 immunoprecipitated from transgenic plants and ABA-treated seedlings. Anti-MAP3Kδ4 serum, diluted 1000-fold, was used as a primary antibody. After extensive washing of the membrane with TBS-T buffer, an alkaline phosphatase-conjugated anti-rabbit second antibody (Promega, Madison, WI) was applied, and the colour reaction was conducted using 5-brome-4-chloro-3-indolyl-phosphate and nitro-blue tetrazolium as substrates.

Immunoprecipitation kinase assay

ABA-treated seedlings or transgenic plants overexpressing *MAP3Kδ4* were ground in liquid nitrogen and thawed in 100 mM Tris-HCl (pH 8.0) extraction buffer containing 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM PMSF, 1 μg/ml leupeptin, 2 mM DTT, 1 mM sodium vanadate, 25 mM sodium fluoride and 50 mM β-glycerophosphate. After centrifugation, the supernatants were used in the immunoprecipitation kinase assay. A total of 2 mg of protein extracts was incubated with the 1 μl of anti-MAP3Kδ4 antiserum in a final volume of 1 ml at 4°C for 1 h, and subsequently 20 μl of Protein A-Sepharose (GE Healthcare) was added. The mixture was further incubated at 4°C for 2 h. The collected resin was washed three times with ice-cold extraction buffer and subjected to immunoblot analysis and the kinase assay. The immunoprecipitates were incubated with 1 μg MBP in a kinase reaction mixture containing 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, 50 μM ATP

and [γ-^{32}P] ATP (37 kBq) at 30°C for 20 min. The samples were separated through SDS-PAGE on a 15% gel, and the phosphorylation of MBP was visualised using a Bioimaging Analyzer BAS2500 (Fuji). For the autophosphorylation assay, the immunoprecipitates were incubated in the kinase reaction mixture at 30°C for 20 min. The samples were subsequently separated through SDS-PAGE on a 7.5% gel and subjected to autoradiography as described above.

Growth measurements

Transgenic *Arabidopsis* plants over-expressing *MAP3Kδ4* under the control of the cauliflower mosaic virus 35S promoter were constructed as described in a previous report (Sasayama et al. 2011). In the present study, the FL2-5 line was used for all analysis, and the other lines showed similar phenotypes to FL2-5. The wild type and the *MAP3Kδ4* over-expressing plants were grown on the plate medium for 14 days, subsequently transplanted into vermiculite and grown at 22°C under continuous light. The shoots were harvested at 20, 30 and 40 days after germination and immediately weighed to obtain the fresh weights. The shoots were dried at 80°C for 3 days to obtain the dry weights. Twenty-one-day-old wild type and *MAP3Kδ4* over-expressing plant seedlings were transferred to Gamborg's B5 medium, supplemented with or without 50 mM NaCl, and grown at 22°C under continuous light for 7 days. The fresh weights of the two plant types were measured to compare the differences in the respective plant growth.

Germination assay

Sterilised wild type and *MAP3Kδ4* over-expressing plant seeds were sown in Gamborg's B5 medium, supplemented with the indicated concentrations of ABA, glucose or NaCl. Each plate contained 20 seeds of either the wild type or the *MAP3Kδ4* over-expressing plants. The germination rate was recorded at 7 and 14 days after planting. Every experiment was repeated three times.

Results

Expression of MAP3Kδ4 in response to abiotic stresses

The expression of *MAP3Kδ4* was monitored under stress conditions using RT-PCR. Figure 1 shows that *MAP3Kδ4* transcript levels increased under high-salt conditions and peaked at a salt concentration of 250 mM. The gene expression levels were significantly enhanced with increasing treatment duration from 1 to 2 h. *MAP3Kδ4* was also induced through polyethylene glycol (PEG) treatment, which indicated that the induction was associated with osmotic stress. The mannitol, dehydration and cold treatments also increased gene expression (Figure 2). In general, these stresses affect the ability of plant cells to absorb water. Therefore, *MAP3Kδ4* is likely responsible for combating water stress.

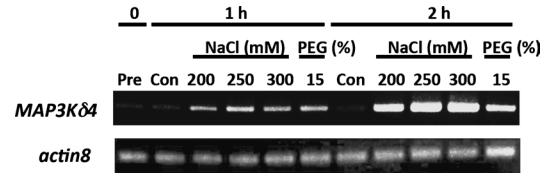

Figure 1. Elevation of *MAP3Kδ4* expression after NaCl or PEG treatment. *Arabidopsis* seedlings at 14 days old were pre-incubated in distilled water for 24 h (Pre) and treated with 200, 250, and 300 mM NaCl or 15% polyethylene glycerol (PEG) for 1 or 2 h. Distilled water was used as the control treatment (Con). Total RNA was extracted from the whole seedling, and the *MAP3Kδ4* transcript levels were examined using RT-PCR. *Actin8* was used as a control template. The RT-PCR analysis involved at least three biological replicates, and the representative data are shown.

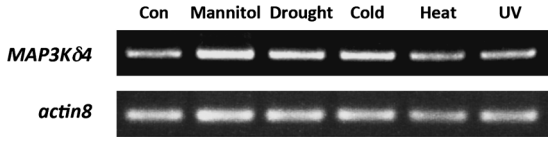

Figure 2. Expression of the *MAP3Kδ4* gene in response to various stress treatments. *Arabidopsis* seedlings at 14 days old were pre-incubated in distilled water for 24 h and treated with 400 mM mannitol or subjected to drought, cold (4°C), heat (37°C), and UV light. Each stress treatment was performed for 2 h. Distilled water was used as a control. Total RNA was extracted from the seedlings, and the *MAP3Kδ4* transcript levels were examined using RT-PCR. *Actin8* was used as a control template. The RT-PCR analysis involved at least three biological replicates, and the representative data are shown.

Induction and activation of MAP3Kδ4 through ABA

RT-PCR was performed to analyse *MAP3Kδ4* transcripts in 14-day-old wild-type *Arabidopsis* seedlings treated with ABA or the ethylene precursor, ACC. The expression of *MAP3Kδ4* was significantly induced through ABA, but not ACC, treatment (Figure 3A). The expression of *MAP3Kδ4* was further analysed along a time course after ABA treatment. The expression of *MAP3Kδ4* gradually increased at 30 min and lasted until 120 min (Figure 3B). These results indicated that *MAP3Kδ4* was an ABA-responsive MAP3K involved in combating water stress in plants cells.

An anti-MAP3Kδ4 rabbit polyclonal antibody was raised against a synthetic peptide corresponding to amino acid residues 40 to 53 in MAP3Kδ4. Bacterially expressed GST and the GST-fused N-terminal region of MAP3Kδ4 were separated using SDS-PAGE, and immunoblot analysis was conducted using the polyclonal antibody (Figure 4A). The GST protein and GST-fused N-terminal region of MAP3Kδ4 are indicated with arrows 1 and 2, respectively, on the left side of Panel A in Figure 4. The band showing the GST-fused N-terminal region of MAP3Kδ4 is shown in the lane marked with arrow 3 on the right side of Panel A in Figure 4. Other bands in the lower positions in this lane are likely the degradation products of the GST-

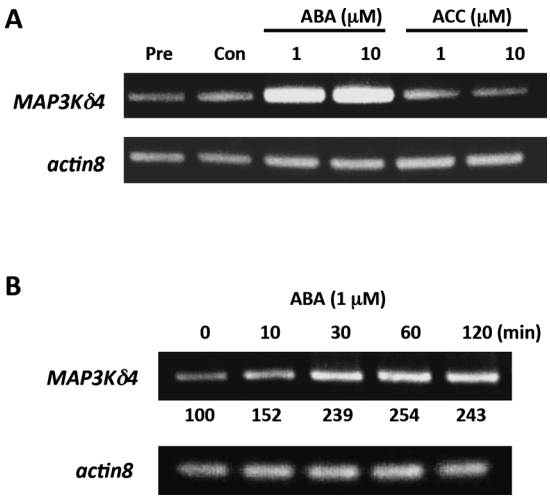

Figure 3. ABA induces *MAP3Kδ4* gene expression. (A) *Arabidopsis* seedlings at 14 days old were pre-incubated in distilled water for 24 h and treated for 2 h with 1 or 10 μM of abscisic acid (ABA) or 1-amino-1-cyclopropane carboxylic acid (ACC), respectively. (B) The time-course for *MAP3Kδ4* expression was examined in response to ABA treatment. *Arabidopsis* seedlings at 14 days old were pre-incubated in distilled water for 24 h and treated with 1 μM ABA for the indicated time. Total RNA was extracted from the seedlings, and the *MAP3Kδ4* transcript levels were examined using RT-PCR. *Actin8* was used as a control template. The expression of *MAP3Kδ4* was quantified using Image Gauge Ver. 4.22 software, and the relative amounts of expression were calculated, with the expression at time 0 considered as 100. The RT-PCR analysis involved at least three biological replicates, and the representative data are shown.

fused N-terminal region of MAP3Kδ4 and contain the antigen peptide sequences, which were strongly visualised on the immunoblots. The immunoblot analysis was also conducted on the crude protein extracts from wild type plants and the transgenic plants over expressing *MAP3Kδ4*. A clear band was detected in the extract from transgenic plants at the position of the predicted molecular mass (83 kDa) for MAP3Kδ4 protein (Figure 4B). This band was also detected in the immnoprecipitates from ABA-treated seedlings and the transgenic plants (Figure 4D). To analyse the activity of endogenous MAP3Kδ4, the immunoprecipitates were incubated in the kinase reaction mixture, separated through SDS-PAGE and subjected to autoradiography. A clear phosphorylation band was detected in the extracts from transgenic plants at the same position as detected through immunoblot analysis (Figure 4C). These results indicated the specificity of the polyclonal antibody.

Immunoprecipitation kinase assay for MAP3Kδ4 in the seedlings after ABA treatment revealed that the protein kinase was active at 30 min after treatment (Figure 4E). The results showed that the enhanced activity was primarily due to the activation of the protein kinase. A slight increase in the detectable MAP3Kδ4 on the western blots was observed after ABA treatment (Figure 4D). The strong phosphorylation of MBP was also detected in the extract from the transgenic plants without ABA treatment (Figure 4D).

Reduced sensitivity to ABA and glucose in the MAP3Kδ4 over-expressing plants during seed germination

The expression and enzymatic activity of *MAP3Kδ4* were induced through ABA, which suggested that MAP3Kδ4 plays a role in ABA signalling. ABA plays an important role in the induction of seed dormancy or seed germination inhibition (Finkelstein et al. 2002; Koornneef et al. 2002). The germination rates for the wild-type seeds and the seeds from transgenic plants over-expressing *MAP3Kδ4* were measured on plant media containing ABA. The germination rate was significantly higher for the transgenic plants than for the wild-type plants (Figures 5A, B). The difference in the sensitivity to ABA was particularly pronounced at a concentration of 1 μM ABA. Sugar affects post-germination growth through the sugar-signalling pathway (Rolland et al. 2002), and a glucose-induced delay in seed germination can be induced through the activation of ABA signalling (Yuan and Wysocka-Diller 2006). The seed germination rates for the transgenic plants over-expressing *MAP3Kδ4* were high, even at high glucose concentrations, whereas the germination rates for the wild-type plants were reduced (Figures 5A, C). These less sensitive phenotypes in the transgenic plants suggest that MAP3Kδ4 is involved in ABA signalling pathways.

Salt tolerance and growth stimulation phenotype of transgenic plants over-expressing MAP3Kδ4

Salt stress is a severe stress that reduces plant productivity. Therefore, salt tolerance is an important phenotype for agriculture. This study evaluated the effects of salt on seed germination. Wild-type seeds produced cotyledons on NaCl-free plates. However, the rates were gradually reduced as the concentration of NaCl increased and showed almost complete inhibition at 100 mM NaCl. In contrast, the germination inhibition of the seeds from plants over-expressing *MAP3Kδ4* was relieved and some seeds were able to germinate on the NaCl plate. Although the germination rates of both seed types were reduced with increasing salt concentration, those of the transgenic plants were more gradually reduced compared with the seeds from wild-type plants (Figures 6A, B). The green seedlings grown on the normal B5 plate for 3 weeks were transferred to the plate containing 50 mM NaCl. Salt tolerance in the plants was evaluated by measuring the fresh weight at 0, 2 and 7 days after transfer. The fresh weights of the wild-type plants were hardly increased on the plate containing salt, whereas the transgenic plants over-expressing *MAP3Kδ4* showed almost the same growth with or without salt (Figure 6C), indicating that the constitutive expression of

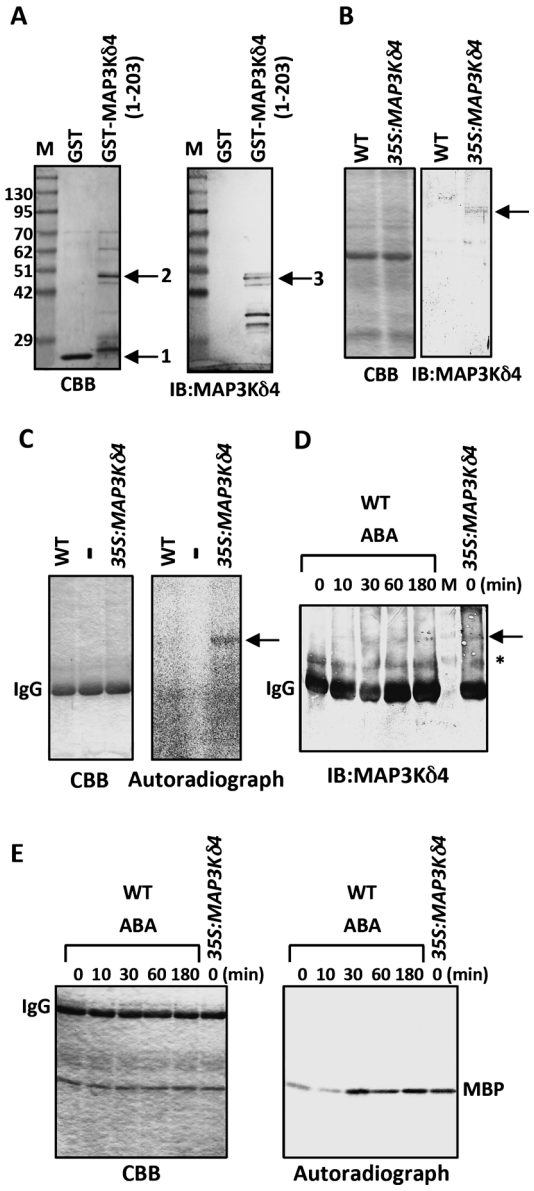

Figure 4. ABA induces the activation of MAP3Kδ4, as determined using the immunoprecipitation kinase assay. (A) The specificity of anti-MAP3Kδ4 antibody; the bacterially expressed GST and GST-fused N-terminal region (1–203 amino acid) of MAP3Kδ4 were separated using SDS-PAGE, subjected to protein staining with Coomassie brilliant blue (CBB) (left side of Panel A) and immunoblot analysis (IB) using the anti-MAP3Kδ4 antibody (right side of Panel A). Arrow 1 shows the CBB-stained GST band. Arrows 2 and 3 show the band for the GST-fused N-terminal region (1–203 amino acids) of MAP3Kδ4 visualised through CBB staining and immunoblot analysis, respectively. (B) Immunoblot analysis of endogenous MAP3Kδ4; 20 μg of the total protein extracts from the 14-day-old seedlings of WT and transgenic plants over-expressing *MAP3Kδ4* (*35S: MAP3Kδ4*) were separated through SDS-PAGE on 10% gel and visualised with CBB staining (left) and immunoblot analysis (IB, right) using the anti-MAP3Kδ4 antibody. The arrow indicates the position of MAP3Kδ4. (C) Autophosphorylation of MAP3Kδ4; 2 mg of the total protein extracts from the 14-day-old seedlings of WT and *35S: MAP3Kδ4* plants were used for the immunoprecipitation of endogenous MAP3Kδ4. The autophosphorylation reaction was conducted as described in the Materials and methods section. The same reaction was performed without the protein extract (−). The samples were separated through SDS-PAGE on a 7.5% gel and subjected to autoradiography (right). The CBB staining of the samples is shown on the left. The arrow indicates the position of the phosphorylation band of MAP3Kδ4. The position of heavy chain of IgG is indicated. (D) ABA response of MAP3Kδ4 protein; Arabidopsis seedlings at 14 days old were treated with 10 μM ABA for the indicated time. A total of 2 mg of protein extracts from the 14-day-old seedlings of WT and *35S: MAP3Kδ4* plants were used for the immunoprecipitation of endogenous MAP3Kδ4. The samples were separated through SDS-PAGE on a 7.5% gel and subjected to immunoblot analysis as described above. M indicates the pre-stained molecular marker lane. The asterisk shows the non-specific band, and the arrow indicates the position of MAP3Kδ4. The position of heavy chain of IgG is indicated. (E) ABA-induced activity of MAP3Kδ4. ABA treatments and the immunoprecipitation were performed as in (D). The kinase activity of the immunoprecipitates was analysed using MBP as a substrate. After the kinase reaction, the samples were separated through SDS-PAGE on a 15% gel and subjected to autoradiography. The CBB staining of the samples (left) and phosphorylation of MBP (right) are shown. The position of the heavy chain of IgG is indicated. All analyses in this figure involved at least three biological replicates, and the representative data are shown.

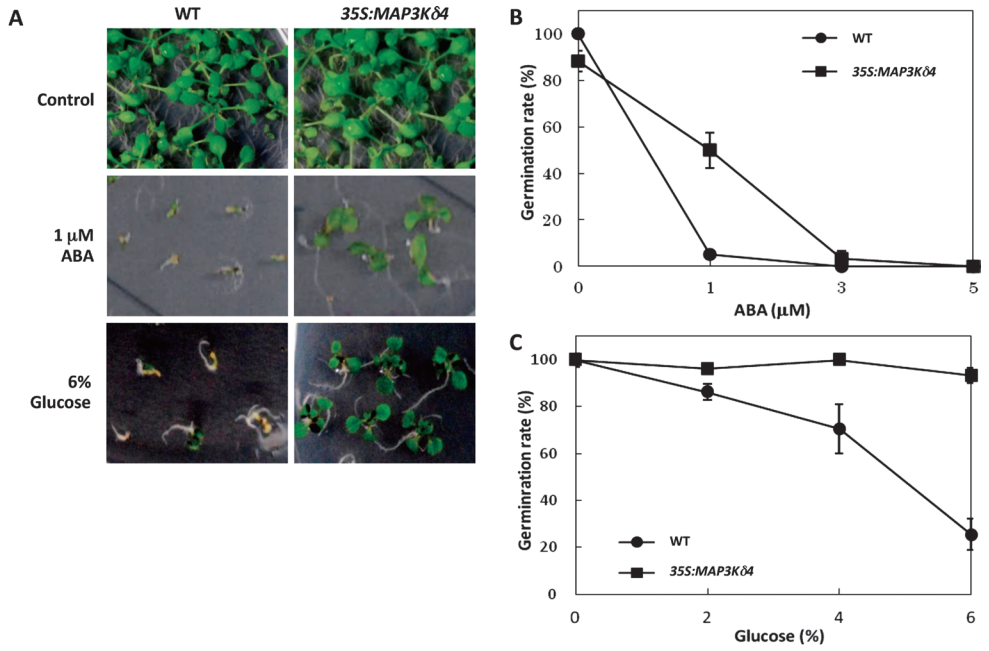

Figure 5. Effect of ABA or glucose on the seed germination of the wild type or *MAP3Kδ4* over-expressing *Arabidopsis* seeds. (A) Pictures of the seed germination of wild-type (WT, left side) plants and transgenic *Arabidopsis* plants over-expressing *MAP3Kδ4* (*35S: MAP3Kδ4*, right side) treated with 1 μM ABA or 6% glucose; dose-dependent effects of ABA and glucose on seed germination of the WT (circle) and the *35S: MAP3Kδ4* (square) plants are depicted in Panels B and C, respectively. The seeds of WT and *35S: MAP3Kδ4* plants were sown onto Gamborg's B5 medium plates containing the indicated concentrations of ABA and glucose. The germination rates (emergence of leaves) were scored at 14 (B) or 7 (C) days after cultivation. All results are presented as the means. The bars indicate the standard errors from three independent experiments (20 seeds for each repeat).

MAP3Kδ4 improves resistance to salt stress.

To further analyse the growth stimulation in the transgenic plants, both the fresh and dry weights were monitored. The wild type and *MAP3Kδ4* over-expressing plants were grown for 20, 30 and 40 days, harvested and immediately weighed to obtain the fresh weights. After the weights were obtained, the samples were dried at 80°C and the dry weights were measured. As shown in Figure 6D, the average fresh weight for transgenic *MAP3Kδ4* over-expressing plants after 40 days of growth was 133.0 mg, whereas the fresh weight was only 113.3 mg for the wild-type plants. The average dry weight of the same samples was 35.8 and 24.0 mg, respectively. The biomass contents (dry weight/fresh weight×100) were calculated as 29.8 and 21.1%, respectively. However, after 20 days, the biomass contents were 21.2 and 20.5%, respectively. The biomass content of wild-type plants was not significantly different between 20 and 40 days, whereas that of the transgenic plants increased dramatically as growth progressed. These data indicated that the increase in the fresh weight, rather than water intake, of the transgenic plants was primarily responsible for the increase in the biomass content.

Discussion

The analysis of the gene expression in response to various stresses is important when clarifying the function of the gene. An analysis of the *MAP3Kδ4* expression under various stress conditions was performed in this study, and the results showed that the expression of the *MAP3Kδ4* gene was increased when treated with NaCl, PEG and mannitol or during dehydration and cold (Figures 1, 2). These results indicated that the *MAP3Kδ4* gene was up-regulated at the transcriptional level in response to water stress. The RT-PCR analysis and immunoprecipitation kinase assay revealed that *MAP3Kδ4* transcription increased and the enzymatic activity was induced after ABA treatment (Figures 3, 4). These results indicate that MAP3Kδ4 functions in an ABA-dependent manner during the water stress response. Recent studies have shown the relationship between the MAPK cascade and ABA signalling (Liu 2012). It has been reported that the expression of the *AtMKK3*, *AtMPK1* and *AtMPK2* genes is induced through cold, salt, mannitol and ABA treatments. The protein kinase activities of AtMPK1 and AtMPK2 were elevated through cold, salt and ABA treatments (Hwa and Yang 2008), and AtMKK1, the other MAPKK, is activated through ABA treatment (Xing et al. 2007). ABA also activated AtMPK4 and AtMPK6 (Ichimura et al. 2000). Furthermore, the AtMPK12 kinase activity was increased through ABA and H_2O_2 treatments (Jammes et al. 2009). To date, there have been no studies showing that MAP3K acts in response to ABA in *Arabidopsis*. However, in the present study, we observed that MAP3Kδ4 is one of the candidate MAP3Ks that functions in ABA signalling. Further investigations, such

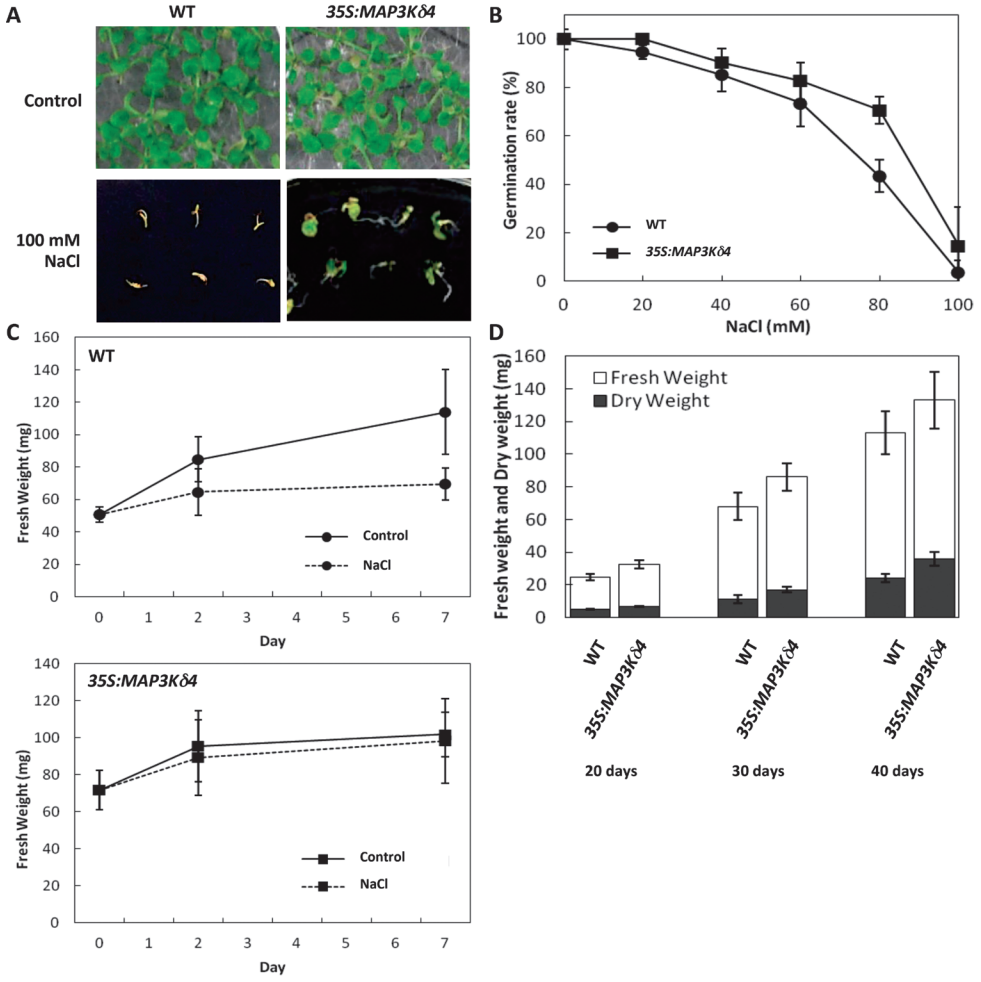

Figure 6. Over-expression of *MAP3Kδ4* gene in *Arabidopsis* increases tolerance to salt stress and biomass production. (A) Pictures of the seed germination of wild type (WT, left side) and transgenic *Arabidopsis* plants over-expressing *MAP3Kδ4* (*35S: MAP3Kδ4*, right side) treated without (Control, upper) or with 100 mM NaCl (lower). (B) Dose-dependent effects of NaCl on seed germination. The seeds of WT and *35S: MAP3Kδ4* plants were sown onto Gamborg's B5 medium plates containing the indicated concentrations of NaCl. The germination rates (emergence of leaves) were scored at 14 days after cultivation. All results are presented as the means. The bars indicate the standard errors from three independent experiments (20 seeds for each repeat). (C) Growth of WT and *35S: MAP3Kδ4* seedlings under high-salt conditions; WT and *35S: MAP3Kδ4* seedlings at 21 days old were transferred to Gamborg's B5 medium plates without (control, solid line) or with 50 mM NaCl (dotted line) and cultured for the indicated number of days. All results are presented as the means. The bars indicate the standard errors from three independent experiments. (D) Evaluation of plant growth. WT and *35S: MAP3Kδ4* plants were grown at 22°C under continuous light for the indicated number of days. The plants were harvested, and the fresh weights were measured. After measurement, the plant samples were dried at 80°C for 3 days, and the dry weights were recorded. All results are presented as the means. The bars indicate the standard errors from three independent experiments.

as the identification of downstream MAPKK and MAPK molecules, are needed to determine whether there is a crosstalk between MAP3Kδ4 and ABA signalling.

ABA has been recognised as a negative regulator of seed germination and seedling growth (Koornneef et al. 2002; Rohde et al. 1999; Tucker 1978). Therefore, the ABA sensitivity in the transgenic plants during the germination process was examined by scoring the germination rate on ABA-containing mediums. The results showed that the *MAP3Kδ4* over-expressing plants were less sensitive to ABA than the wild-type plants (Figures 5A, B). The over-expression of *MAP3Kδ4* also reduced the sensitivity to glucose (Figures 5A, C). Glucose inhibits seed germination in an ABA-related manner (Yuan and Wysocka-Diller 2006). As mentioned above, the kinase activity of MAP3Kδ4 was induced through ABA treatment (Figure 4). These results strongly support the idea that MAP3Kδ4 plays an important role in ABA signalling. In transgenic plants overexpressing MAP3Kδ4, the kinase activity was maintained at high levels (Figure 4E). Further analysis is needed to clarify the relationship between the reduced sensitivity of the transgenic plants and the increased kinase activity of MAP3Kδ4.

The over-expression of *MAP3Kδ4* increased the tolerance to salt stress compared with wild-type plants, as demonstrated through the increased germination rate and the stress resistance during seedling growth

(Figures 6A–C). The vigorous growth of *MAP3Kδ4* over-expressing *Arabidopsis*, which has been previously shown (Sasayama et al. 2011), reflected an increase in the dry weight of the transgenic *Arabidopsis* plants. Further research into the mechanisms underlying the high biomass production and the salt stress tolerance of this transgenic plant would be beneficial to agriculture.

Thus, this study showed that *Arabidopsis* MAP3K*δ*4, a novel ABA-inducible MAP3K, plays an important role in plant adaptation to stresses. Further studies are needed to elucidate how MAP3K*δ*4 functions during stress signalling.

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