

An abscisic acid inducible *Arabidopsis* MAPKKK, MAPKKK18 regulates leaf senescence via its kinase activity

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Abstract Abscisic acid (ABA) is a phytohormone that regulates many physiological functions, such as plant growth, development and stress responses. The MAPK cascade plays an important role in ABA signal transduction. Several MAPK and MAPKK molecules are reported to function in ABA signaling; however, there have been few studies related to the identification of MAPKK upstream of MAPKK in ABA signaling. In this study, we show that an *Arabidopsis* MAPKKK, MAPKKK18 functions in ABA signaling. The expression of MAPKKK18 was induced by ABA treatment. Yeast two-hybrid analysis revealed that MAPKKK18 interacted with MKK3, which interacted with C-group MAPK, MPK1/2/7. Immunoprecipitated kinase assay showed that the 3xFlag-tagged MAPKKK18, expressed in *Arabidopsis* plants, was activated when treated with ABA. These results indicate the possibility that the MAPK cascade is composed of MAPKKK18, MKK3 and MPK1/2/7 in ABA signaling. The transgenic plants over-expressing MAPKKK18 (*35S:MAPKKK18*) and its kinase negative mutant (*35S:MAPKKK18 KN*) were generated, and their growth was monitored. Compared with the WT plant, *35S:MAPKKK18* and *35S:MAPKKK18 KN* showed smaller and bigger phenotypes, respectively. Senescence of the rosette leaves was promoted in *35S:MAPKKK18*, but suppressed in *35S:MAPKKK18 KN*. Furthermore, ABA-induced

leaf senescence was accelerated in *35S:MAPKKK18*. These results suggest that MAPKKK18 controls the plant growth by adjusting the timing of senescence via its protein kinase activity in ABA dependent manners.

Keywords ABA · Leaf senescence · MAPK cascade · MAPKKK · Protein kinase assay

Introduction

The phytohormone abscisic acid (ABA) plays important roles in plant growth and stress responses. ABA regulates many aspects of plant growth and development, including seed maturation, dormancy, germination, leaf senescence and responses to environmental stresses, such as drought, high salinity and cold (Finkelstein 2013). These functions are regulated by various signaling components, for example, second messengers, including Ca^{2+} and reactive oxygen species (ROS), SnRK2, PP2C pathway and G-protein (Mori et al. 2006; Munemasa et al. 2013; Wang et al. 2001). In addition, it is also known that MAPK cascades function in ABA signaling (Liu 2012).

Mitogen-activated protein kinase (MAPK) cascades are important signaling modules in eukaryotic cells and convert signals generated from receptors/sensors into cellular responses. In plants, MAPK pathways have been implicated in the responses to various biotic and abiotic stresses, plant hormones, cell division and developmental processes (MAPK Group 2002; Nakagami et al. 2005; Takahashi et al. 2004). A MAPK cascade is composed of three classes of enzymes: MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). In the genome of the model plant *Arabidopsis thaliana*, there are at least 80 MAPKKK, 10 MAPKK (MKK1–MKK10) and 20 MAPK

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(MPK1–MPK20) genes (Colcombet and Hirt 2008), which suggests that the signaling cascades generated by these molecules are complex. Several MAPK cascades have been identified in stress and developmental signal transduction pathways (Nakagami et al. 2005; Takahashi et al. 2004); however, considering the number of MAPKs, MAPKKs and MAPKKKs, only a few MAPK cascades have been characterized to date. Regarding the involvement of the MAPK cascade in ABA signaling, it has been reported that MKK1–MPK6 regulate the ABA-dependent expression of *CAT1* and H₂O₂ production (Xing et al. 2008), MPK9/12 positively regulate stomatal closure via ROS-mediated ABA signaling, MPK4/6 are activated by ABA (Ichimura et al. 2000) and MKK3–MPK1/2 mediate ABA signal and are involved in salt stress tolerance (Hwa and Yang 2008). However, there have been few studies on MAPKKK as the upstream activator of MAPKK in ABA signaling.

In this study, we tried to identify the MAPKKK that mediates ABA signaling and reveal the role of the MAPK cascade in the physiological functions of ABA.

We focused on an *Arabidopsis* MAPKKK, MAPKKK18 and analyzed the expression of *MAPKKK18* on treating the seedlings with ABA. Yeast two-hybrid assay was performed to identify the molecules downstream of MAPKKK18, revealing that MAPKKK18 interacts with MKK3. Moreover, the recombinant MAPKKK18 protein, expressed in *Arabidopsis* plants, was activated by ABA treatments. To investigate the physiological role of MAPKKK18, transgenic plants overexpressing *MAPKKK18* were generated. Interestingly, it was found that MAPKKK18 regulates leaf senescence via its kinase activity in ABA dependent manners.

Materials and methods

Plant materials and ABA treatments

Arabidopsis thaliana (Columbia ecotype) seeds were surface-sterilized with 70 % (v/v) ethanol for 3 min, followed by a solution of NaClO (1 % w/v), containing 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 ABA treatments, 14-day-old seedlings were carefully removed from the plate and placed in distilled water 24 h before the treatment. Time course analysis was performed on the seedlings soaked in the 100 or 10 μM ABA solutions, using either RT-PCR or immunoblot analysis and immunoprecipitated kinase assay, respectively. After the ABA treatment, the seedlings were frozen in liquid nitrogen and stored at –80 °C until further analysis.

RT-PCR

For RT-PCR analysis, total RNA was extracted from *Arabidopsis* plants using an RNeasy Plant Mini Kit (Qiagen) and treated with DNaseI (Invitrogen) to remove residual DNA contamination. The cDNA was synthesized from 0.5 μg of *Arabidopsis* total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (TAKARA). Real-time quantitative PCR amplification (Light Cycler 480 II; Roche Applied Science) was performed in total volume 20 μl with Thunderbird SYBR qPCR Mix (Toyobo) and using gene-specific primers as follows for 1 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 30 s at 60 °C, and 5 s at 95 °C, and then 1 min at 65 °C. Subsequently, samples were heated to 97 °C and then cooled to 40 °C. Samples were run in triplicate, with good reproducibility. Amplification of *Actin8* cDNA in these same samples was used as an internal control for all real-time quantitative PCR amplification reactions. The *MAPKKK18* (At1g05100) specific primers are 5'-TGACG-GATCAAATGTTCTGG-3' and 5'-AGCTTGAACGGTC-CTCTGAC-3'. The *ABI1* (At4g26080) specific primers are 5'-GATGCTCTGCGATGGTGATA-3' and 5'-CAGGAAC-GAGTTGAAAAGAGC-3'. The *ACTIN8* (At1g49420) specific primers are 5'-AAATAACCGAACCGGTTAGA-3' and 5'-GCTTGAACGGTCCTCTGACA-3'. To check the expression level of *MAPKKK18* in each transgenic plant, the *MAPKKK18* (At1g05100) transcripts were amplified using the forward primer 5'-AAATAACCGAACCGGT-TAGA-3' and the reverse primer 5'-GCTTGAACGGTC-CTCTGACA-3'. *ACTIN8* (At1g49420) transcripts served as a control and were amplified using the forward primer 5'-GAAGGACCTTACGGTAACA-3' and the reverse primer 5'-CCAATCCAGACACTGTACTT-3'. PCR was performed with Hot Start *Taq* DNA Polymerase (New England Bio Labs) at 95 °C for 30 s, followed by 25 or 30 cycles (for *MAPKKK18*) and 25 cycles (for *Actin8*) of 95 °C for 30 s, 50 °C for 30 s and 68 °C for 30 s with a final extension at 68 °C for 5 min. The PCR products were separated on 1.6 % agarose gels and visualized under UV light.

Yeast two-hybrid assays

Yeast two-hybrid assay was performed using a Hybri-ZAP-2.1 two-hybrid vector system (Stratagene). The cDNA for *Arabidopsis* *MAPKKK18* was isolated using RT-PCR using the forward primer 5'-GAAAGAATTCA-TGAATTGGACTAGAGGAAA-3' and the reverse primer 5'-GTTTGTGACCTAATTCCGTCGAACCGTGA-3'. PCR was performed with KOD-Plus-Neo DNA polymerase (TOYOBO) at 98 °C for 2 min, followed by 30 cycles of 94 °C for 10 s, 50 °C for 30 s and 68 °C for 1 min. The PCR product was cloned into the pAD-GAL4-2.1

vector for the expression of GAL4 transcriptional activation domain fusion proteins. The plasmid clones were verified using DNA sequencing. The kinase negative mutation of MAPKKK18 (K32R), designated as MAPKKK18 KN, was created using a Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions and verified by DNA sequencing. 10 MKK genes (MKK1–MKK10) were inserted into the pBD-GAL4 Cam vector for the expression of GAL4 DNA-binding domain fusion proteins. Each pair of bait and prey vectors was co-transformed into yeast (strain YRG-2). Each transformant was spotted onto synthetic media plate lacking leucine and tryptophan (SD Trp⁻ Leu⁻). The interaction was monitored using the filter assay method of β-galactosidase activity, by following the manufacturer's instructions (Stratagene).

Expression and purification of recombinant proteins

The cDNA for *MAPKKK18* and *MAPKKK18 KN* genes were inserted into a pGEX4T1 vector (GE Healthcare) for translational fusion to glutathione S-transferase (GST). Each vector was transformed into *Escherichia coli* (strain JM109). The recombinant GST-fusion proteins were purified as described previously (Matsuoka et al. 2002) and were designated as GST-MAPKKK18 and GST-MAPKKK18 KN, respectively. After purification, the recombinant proteins were used for kinase assays.

In vitro kinase assay

GST-MAPKKK18 and GST-MAPKKK18 KN were incubated with MBP in the kinase reaction mixture containing 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM MgCl₂, 50 μM ATP and [γ-³²P] ATP (37 kBq) at 30 °C for 30 min. The samples were resolved using SDS-PAGE on a 15 % gel and subjected to autoradiography. The phosphorylation of MBP and GST-MAPKKK18 was visualized with a Bioimaging Analyzer BAS2500 (Fuji).

Generation of *Arabidopsis* transgenic plants

The cDNA for MAPKKK18 and MAPKKK18 KN genes without the start codon were translationally fused to the coding sequence of 3xFLAG-tag in the N-terminus. The 3xFLAG-tag fused *MAPKKK18* and the 3xFLAG-tag fused *MAPKKK18 KN*, designated as 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN, respectively, were inserted between the CaMV 35S promoter and the NOS terminator of the plant expression vector pBI121 (Clontech). The resulting constructs were introduced into *Agrobacterium tumefaciens* (strain C58) by triparental mating with *E. coli* (strain DH5α) containing pRK2013 (Wise et al. 2006) and transferred into wild-type (WT) *Arabidopsis* (Columbia ecotype) by the

vacuum infiltration method (Bechtold and Pelletier 1998). The transgenic plants were germinated on 0.5 × Murashige and Skoog medium with 20 μg ml⁻¹ kanamycin. All transgenic lines used in this study were T₄ homozygous plants.

Total protein extraction

The seedlings of the WT and transgenic plants or the seedlings of the ABA-treated transgenic plant overexpressing 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN were ground in liquid nitrogen and then thawed in an extraction buffer (100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 % Triton X-100, 150 mM NaCl, 1 mM PMSF, 1 μg ml⁻¹ leupeptin, 2 mM DTT, 1 mM sodium vanadate, 25 mM sodium fluoride and 50 mM β-glycerophosphate). After centrifugation, the supernatants were used for immunoblot analysis and immunoprecipitated kinase assay.

Immunoblot analysis

The aliquots of the crude extracts from the seedlings of the WT and transgenic plants or the ABA-treated transgenic plant overexpressing 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN were resolved using SDS-PAGE, and immunoblot analysis was performed as previously described (Matsuoka et al. 2002). An anti-FLAG-tag antibody was used as the primary antibody. After extensive washing of the membrane with TBS-T buffer, an alkaline phosphatase-conjugated anti-mouse secondary antibody (Promega, Madison, WI) was employed, and the color reaction was conducted using 5-bromo-4-chloro-3-indolyl-phosphate and nitro-blue tetrazolium as substrates. Each immunoblot analysis was repeated at least three times, and the results from one representative experiment are shown.

Immunoprecipitated kinase assay of 3xFLAG-MAPKKK18

Crude extracts from the seedlings of ABA-treated transgenic plant overexpressing 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN were incubated with the anti-FLAG tag antibody in a final volume of 1 ml at 4 °C for 1 h, followed by the addition of 10 μl of Protein G Sepharose (GE Healthcare). The mixture was further incubated at 4 °C for 2 h. The collected resin was washed three times with the ice-cold extraction buffer and subjected to kinase assays. The 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN immunoprecipitates were incubated with MBP, in the kinase reaction mixture containing 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM MgCl₂, 50 μM ATP and [γ-³²P] ATP (37 kBq) at 30 °C for 30 min. The samples were analyzed by SDS-PAGE on a 15 % gel, and the phosphorylation of the substrates was visualized and quantified with a Bioimaging Analyzer BAS2500 (Fuji).

Growth measurements

WT and transgenic plants were germinated as described above. After 1 week of growth on the plates at 22 °C under continuous light, the seedlings were transplanted to soil. Rosette parts of the WT and transgenic plants were photographed every 10 days from 20 days after the transfer to 22 °C. After photography, each harvested plant was immediately weighed to obtain the fresh weights. The plants were dried at 70 °C for 3 days to obtain the dry weights. To analyze seed productivity, the mature plants were harvested and dried, the seeds were counted, and 100 seeds were weighed. Chlorophyll contents of WT and transgenic plants were quantified according to Porra et al. (1989). Rosette parts of WT and transgenic plants were harvested every 10 days from 20 days after the transfer to 22 °C. At least six rosette parts from each sample were mixed and *N, N'*-dimethylformamide was used for the extraction of chlorophyll.

Water loss assay

Aerial parts of 4-week-old WT and each transgenic plant were detached and used to measure the water loss. At least eight plants were used for WT and each transgenic plant. The weights of the sample were monitored immediately to obtain the fresh weights and every 30 min to analyze the water loss rate.

ABA-induced leaf senescence

The rosette leaves of 2-week-old WT and each transgenic plant were detached and incubated under continuous light in 50 µM ABA or distilled water (Mock) for 4 days. Chlorophyll contents of WT and each transgenic plant were monitored as described above.

Results

The transcript of *MAPKKK18* is induced by ABA, and *MAPKKK18* possesses a protein kinase activity

We performed real-time quantitative RT-PCR analysis to investigate *MAPKKK18* transcripts in 14-day-old WT *Arabidopsis* seedlings treated with ABA. The expression of *MAPKKK18* was gradually increased at 30 min and lasted until 180 min (Fig. 1). *ABI1* is a type-A Protein Phosphatase 2C (PP2C) and a negative regulator of ABA responses. It has been reported that the expression of *ABI1* is induced in early ABA responses (Hubbard et al. 2010). The expression of *ABI1* was compared with the expression of *MAPKKK18*. The expression of *ABI1* also increased at

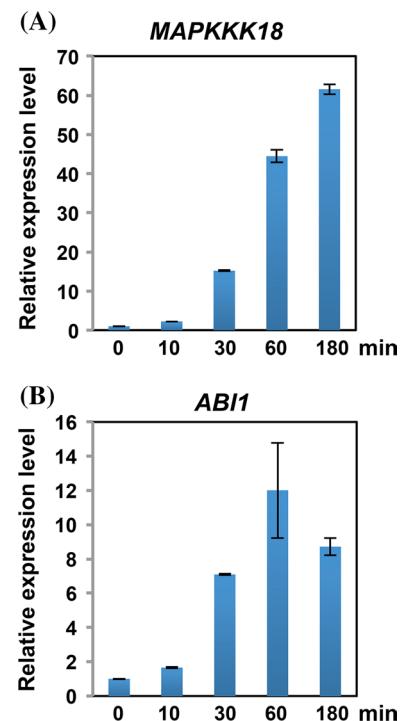


Fig. 1 ABA induces MAPKKK expression. 14-day old *Arabidopsis* seedlings were pre-incubated in distilled water for 24 h and treated with 100 µM ABA for the times indicated. Total RNA was extracted from the seedlings, and the *MAPKKK18* transcript levels **a** were analyzed by quantitative RT-PCR. **b** The expression of *ABI1*, an ABA-inducible protein phosphatase gene, was monitored for the validation of ABA treatments. The data are expressed as relative fold change to non-treated control (0 min). All results are presented as the means. The bars indicate the standard errors of three replicates

30 min and the same transcript level was maintained until 180 min. The ABA induced expression of *MAPKKK18* showed a similar pattern to that of *ABI1*.

To analyze the kinase activity of *MAPKKK18*, *MAPKKK18* and its kinase-negative mutant proteins were expressed as GST-fusion proteins in *E. coli* and affinity purified by glutathione-Sepharose. The proteins showed an approximate molecular mass of 64 kDa by CBB staining after SDS-PAGE (Fig. 2). The kinase activities of these GST-fusion proteins were measured by using MBP as a substrate. GST-*MAPKKK18* phosphorylated MBP, and a phosphorylated band was detected at the same position as the GST-*MAPKKK18* band. These results indicated that *MAPKKK18* has a kinase activity and has the ability to autophosphorylate. No phosphorylation band was detected in the GST-*MAPKKK18 KN* lane as expected (Fig. 2).

MAPKKK18 interacts with MKK3 and activated by ABA

To identify the downstream target of *MAPKKK18*, the protein interaction between *MAPKKK18* and 10 MAPKK

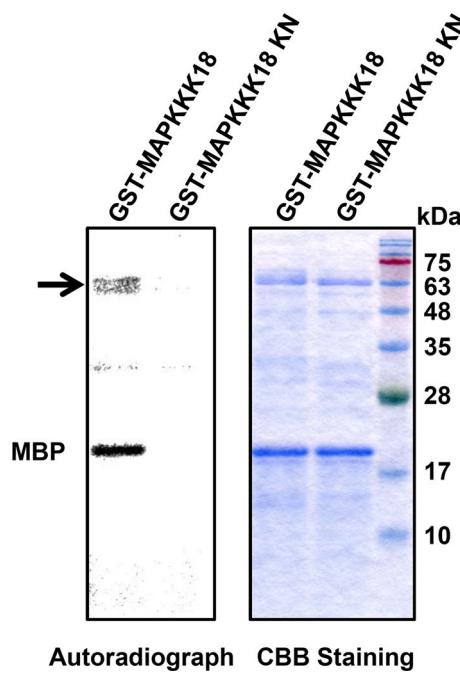


Fig. 2 The protein kinase activity of the recombinant MAPKKK18. MAPKKK18 and the kinase-negative mutant of MAPKKK18 (designated as MAPKKK18 KN) were expressed as GST-fusion proteins in *E. coli* and purified by affinity chromatography. Recombinant MAPKKK18 (GST-MAPKKK18) and MAPKKK18 KN (GST-MAPKKK18 KN) were allowed to react in the kinase reaction buffer with MBP. After the phosphorylation reaction, the samples were resolved using SDS-PAGE, stained with CBB (right) and visualized by autoradiography (left). The arrow indicates the position of the phosphorylation band of GST-MAPKKK18

(MKK1–MKK10) was analyzed by using yeast two-hybrid assay. The blue color from the β -galactosidase activity was detected at the MKK3 position (Fig. 3a). To analyze the effects of the MAPKKK18 kinase activity on the interaction with MKKs, the interaction between MAPKKK18 KN and MKKs was monitored using the yeast two-hybrid assay. Interaction with the other MKKs was not detected for MAPKKK18 KN (Fig. 3a). These results indicated that MAPKKK18 interacted with MKK3. Additionally, MKK3 interacted with MPK1/2/7 (C-group MPK), indicating the possibility that the MAPK cascade is composed of MAPKKK18, MKK3 and MPK1/2/7 (Fig. 3b).

To analyze the kinase activity of MAPKKK18 in ABA signaling and to clarify the physiological role of MAPKKK18, transgenic plants overexpressing *MAPKKK18* (*35S:MAPKKK18*) were generated. We also created the transgenic plants overexpressing *MAPKKK18 KN* (*35S:MAPKKK18 KN*) to examine the role of kinase activity for its physiological function. The 3xFLAG tag was fused to the N-terminus of MAPKKK18 and its kinase-negative mutant to detect and purify the expressed proteins easily (Fig. 4a). Both gene forms were expressed under the

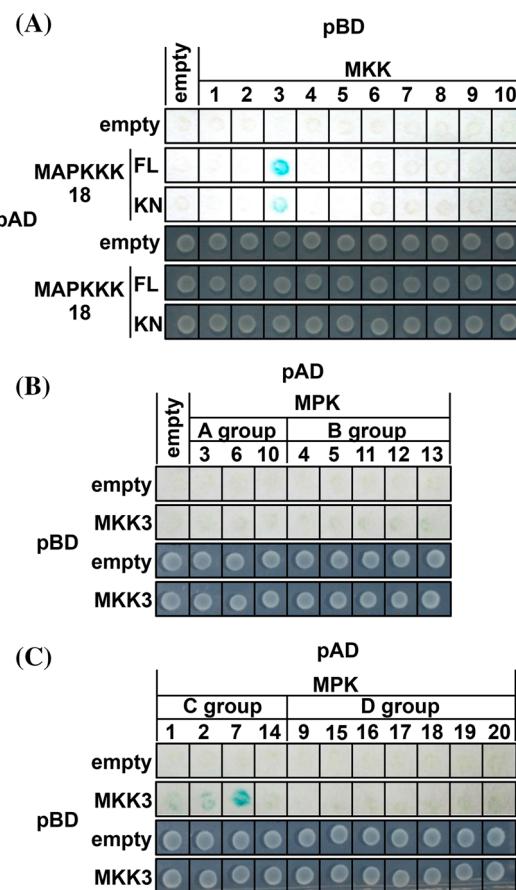
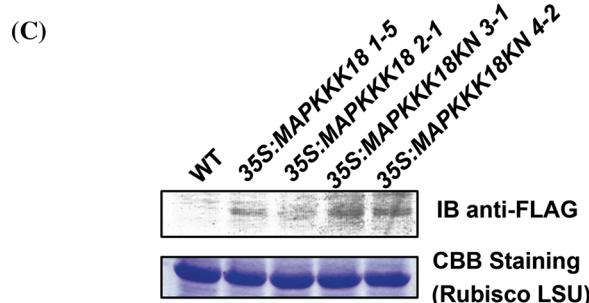
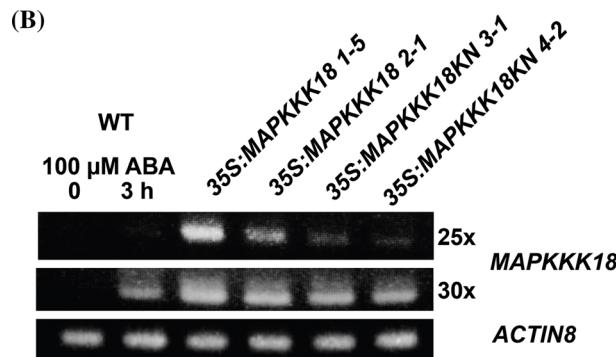
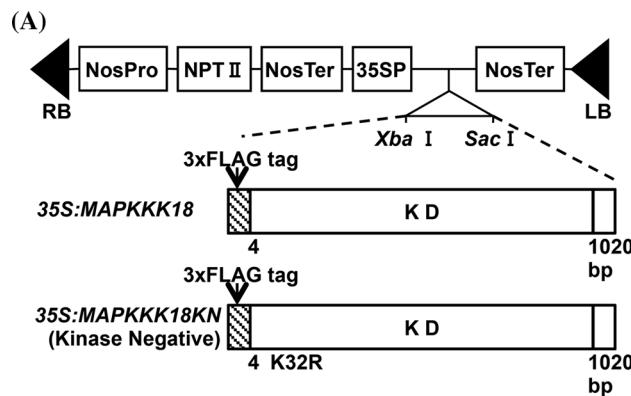


Fig. 3 Identification of the possible MAPKKK18 cascade by yeast two-hybrid analysis. **a** Analysis of interaction between MAPKKK18 and MKKs. GAL4 DNA-binding domain (BD) fused MKKs (MKK1–MKK10) and GAL4 transcriptional activating domain (AD) fused MAPKKK18 or MAPKKK18 KN were co-transformed in yeast cells. Each transformant was spotted onto synthetic medium plates lacking tryptophan and leucine (SD Trp[–] Leu[–], lower panel). The interaction was monitored using the filter assay method of β -galactosidase activity. **b** Analysis of the interaction between MKK3 and MPKs. BD-fused MKK3 was co-transformed in yeast cells with AD-fused MPKs. The interactions were monitored as described in **a**

constitutive cauliflower mosaic virus 35S promoter. Both homozygous lines, *35S:MAPKKK18* and *35S:MAPKKK18 KN*, were used in subsequent experiments. Overexpression was confirmed using RT-PCR and immunoblot analysis with the anti-FLAG tag antibody. The RT-PCR result indicated that the expression of *MAPKKK18* in the transgenic plants was higher than that in the WT plants treated with ABA for 3 h (Fig. 4b). The immunoblot result showed both 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN proteins were expressed in each transgenic plant (Fig. 4c).

As described above in Fig. 1a, the transcript of *MAPKKK18* was induced by ABA treatments. To analyze the effects of the ABA treatments on the MAPKKK18 activity, 14-day-old *35S:MAPKKK18 1-5* and *35S:MAPKKK18 KN 4-2* seedlings were treated with ABA. The expression level



of 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN proteins were monitored by immunoblot analysis, using the anti-FLAG tag antibody. Immunoblot analysis was carried out at three times and the intensity of each band was quantified (data not shown). The ABA treatment did not change the expression level of both 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN (Fig. 5a). Next, 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN were immunoprecipitated from the same extracts using the anti-FLAG tag antibody and tested for their ability to phosphorylate MBP. Phosphorylation of MBP by 3xFLAG-MAPKKK18 was detected from 10 min after the treatment and peaked at 30 min and then decreased until 120 min after the treatment (Fig. 5). No ABA induced phosphorylation of MBP was detected in 35S:MAPKKK18 KN 4-2 seedlings. These results indicate that kinase activity of MAPKKK18 is regulated by ABA, suggesting that MAPKKK18 mediates ABA signal via MKK3-MPK1/2/7 cascade.

Fig. 4 Overexpression of MAPKKK18 and its kinase negative mutant in *Arabidopsis*. **a** Schematic representation of the constructs for the overexpression of 3xFLAG-MAPKKK18 and 3x-FLAG MAPKKK18 KN (K32R). The 3xFLAG-tag was translationally fused to the N-terminal of the coding sequence, without initiation codon, of MAPKKK18 (3xFLAG-MAPKKK18) and MAPKKK18 KN (3xFLAG-MAPKKK18 KN) and then inserted between the CaMV 35S promoter (35SP) and the NOS terminator (NOS Ter) of the plant expression vector pBI121 (Clontech) using *Xba*I and *Sac*I restriction enzyme site. Transformation of *Arabidopsis* plant (Col-0) was performed using the vacuum infiltration method (Bechtold and Pelletier 1998). RB right border, LB left border, NOS Pro NOS promoter, NPTII neomycin phosphotransferase II gene, NOS Ter NOS terminat, KD kinase domain. **b** Overexpression of 3x-FLAGMAPKKK18 and 3x-FLAG MAPKKK18 KN in transgenic plants was confirmed by RT-PCR. Total RNA was extracted from 14-day-old seedlings of each transgenic plant. To compare the MAPKKK18 expression with the ABA-induced expression of the endogenous MAPKKK18, RT-PCR reaction was performed at 25 and 30 cycles. ACTIN8 expression was used as a control. **c** Detection of the 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN proteins. Total protein extracts from WT and each transgenic plant were resolved on SDS-PAGE. Immunoblot analysis was conducted using the anti-Flag tag antibody. Equal amounts of the samples were resolved on SDS-PAGE and stained with CBB, and the large subunit (LSU) of ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) is shown as the loading control for equal protein amounts in the WT and transgenic plants

MAPKKK18 regulates plant aging via its kinase activity

Transgenic and WT *Arabidopsis* plants were grown at 22 °C under continuous light, and both fresh and dry weights were measured every 10 days, from 20 to 60 days after the transfer to 22 °C. Both the fresh and dry weights were similar in the WT and transgenic plants for 30 days (Fig. 6a). Fifty days later, the fresh weight of 35S:MAPKKK18 was lesser than that of the WT plant. Between 40 to 50 days, the fresh weight of 35S:MAPKKK18 KN was greater than that of the WT plant (Fig. 6a). Similarly, after 60 days of growth, the dry weight of 35S:MAPKKK18 was lesser than that of the WT plant, and after 50 days, the dry weight of 35S:MAPKKK18 KN was greater than that of the WT plant (Fig. 6b). Next, we measured the seed numbers and weights of the WT and transgenic plants. 35S:MAPKKK18 had similar seed numbers per plant as that in the WT plant. However, the seed numbers per plant for 35S:MAPKKK18 KN was greater than that in the WT plant and 35S:MAPKKK18 (Fig. 7a). There was no significant difference in seed weights between the WT and transgenic plants (Fig. 7b). To compare the growth differences between the WT and transgenic plants in detail, we observed the phenotypes of the rosette leaves of the WT and transgenic plants from 20 to 60 days after the transfer to 22 °C (Fig. 8a). After 20 days, there was no difference in the phenotypes of the rosette leaves. From 30 to 40 days after the transfer to 22 °C, the rosette

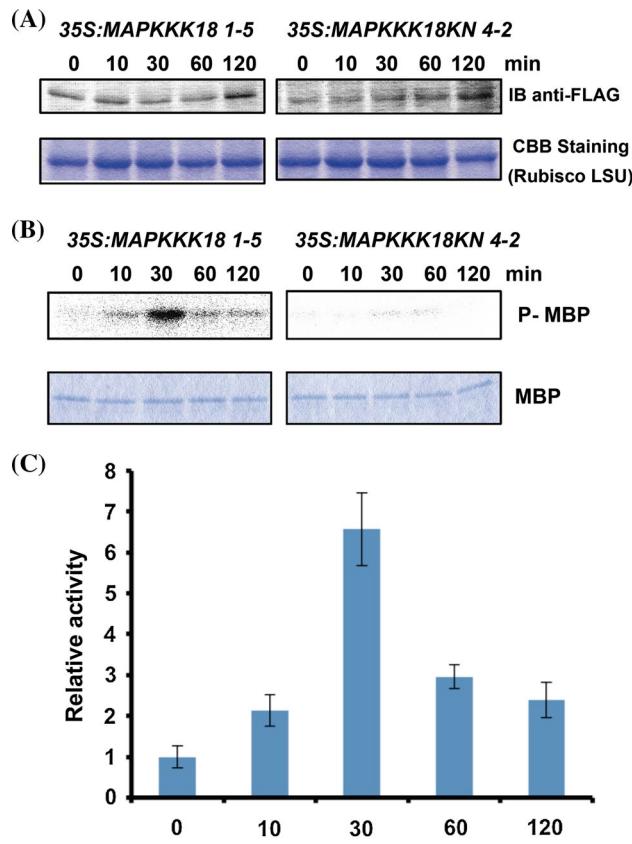


Fig. 5 ABA induces the activation of MAPKKK18. **a** Immunoblot analysis of 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN protein levels of the transgenic plant overexpressing MAPKKK18 with the ABA treatments. Fourteen-day-old seedlings of the transgenic plant overexpressing 3xFLAG-MAPKKK18 (*35S: 3xFLAG-MAPKKK18 1-5*) and 3xFLAG-MAPKKK18 KN (*35S: 3xFLAG-MAPKKK18 KN 4-2*) were treated with 10 µM ABA for the times indicated. Total protein extracts were separated on SDS-PAGE. Immunoblot analysis (*upper panel*) and CBB staining (*lower panel*) of the samples were performed as in Fig. 4. **b** The ABA-induced activation of MAPKKK18. 3xFLAG-MAPKKK18 and 3xFLAG-MAPKKK18 KN were immunoprecipitated from the same protein extracts in a. Aliquots of the immunoprecipitates were incubated in the kinase reaction mixture containing MBP as a substrate. After the phosphorylation reaction, the samples were resolved using SDS-PAGE and subjected to autoradiography. The phosphorylation (P-MBP) and CBB staining of MBP are shown in the upper and lower panels, respectively. **c** The phosphorylation of MBP was quantified and relative amounts of each phosphorylation were calculated when the MBP phosphorylation by 3xFLAG-MAPKKK18 from seedlings treated with ABA for 0 min was 1. Vertical lines on each bar indicate ±SE ($n = 3$)

leaves of *35S:MAPKKK18* were smaller and those of *35S:MAPKKK18 KN* were larger, compared with the WT plants. 40 days after the transfer to 22 °C, the rosette leaves of WT and *35S:MAPKKK18 KN* remained green; however, the rosette leaves of *35S:MAPKKK18 1-5* with a high protein expression lost leaf color severely. After

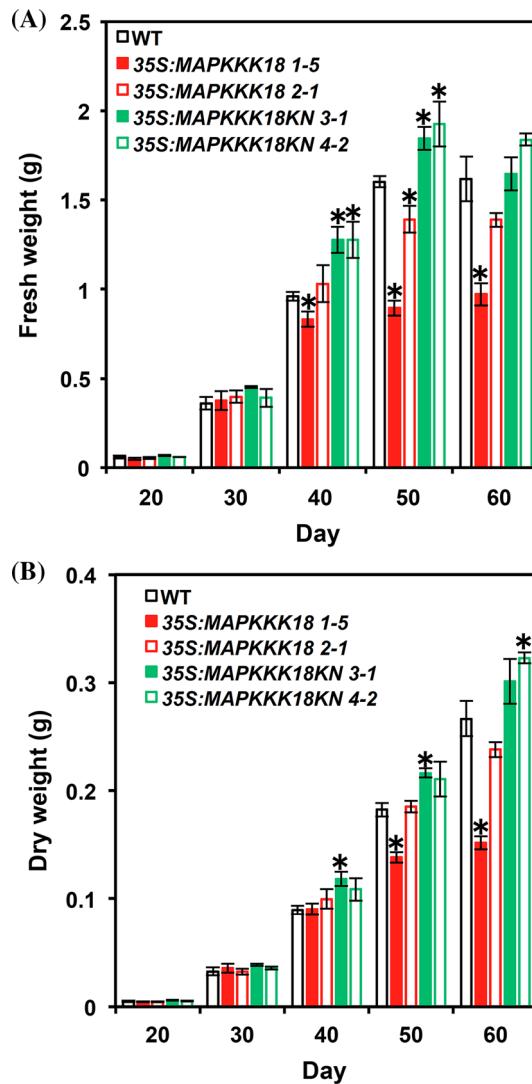


Fig. 6 Evaluation of plant growth. **a** WT and transgenic plants were grown at 22 °C under continuous light for the indicated number of days. The plants were harvested, and the fresh weights were measured. **b** After measurement of fresh weights, the plant samples were dried at 70 °C for 3 days, and the dry weights were measured. All results are presented as the means. The bars indicate the standard errors of four replicates. Asterisks indicate significant differences (Student's *t* test, $P < 0.05$) between WT and each transgenic line

50 days, the rosette leaves of all plants turned yellow, the rosette leaves of *35S:MAPKKK18 2-1* with a relatively low expression did not differ from those of the WT plants, and compared with the WT plants, the rosette leaves of *35S:MAPKKK18 1-5* turned significantly more yellow. In contrast, the rosette leaves of *35S:MAPKKK18 KN* were greener than the WT plants (Fig. 8a).

We calculated the yellowing rate for the leaves of these plants for 40 to 60 days after the growth (Fig. 8b). Compared with the WT plants, the rate for *35S:MAPKKK18*

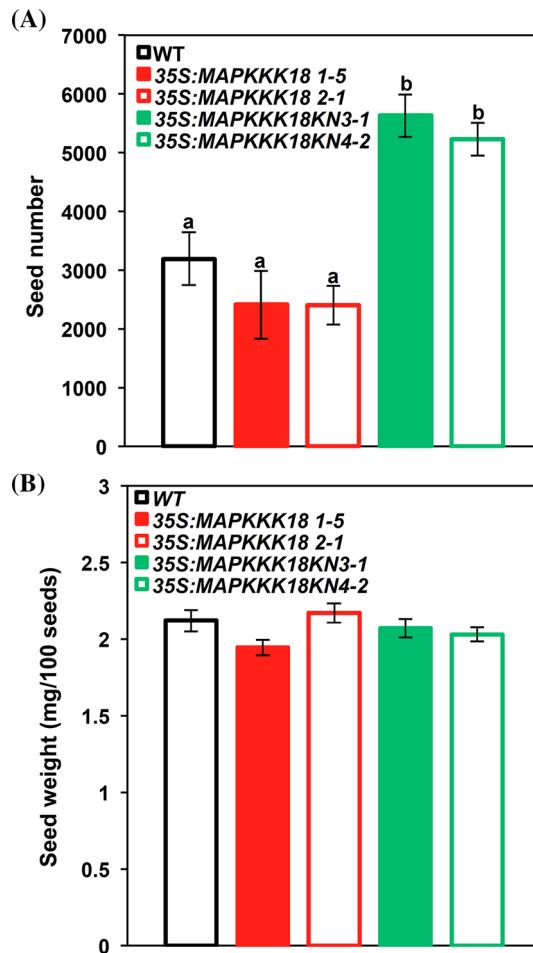


Fig. 7 Evaluation of seed productivity. The WT and transgenic plants were grown at 22 °C under continuous light until the growth of all plants stopped. These plants were harvested and dried. Seed weights and seed numbers per plant were measured. **a** The seed weights are presented as the means for 100 seeds. The bars indicate the standard errors of nine or ten replicates. **b** The seed numbers are presented as the means. The bars indicate the standard errors of ten replicates. Different letters indicate significant differences ($P < 0.05$) calculated with data from all replicates using one-way analysis of variance and Tukey's honestly significant difference test for multiple comparisons between the means

was higher, and the rate for 35S:MAPKKK18 KN was lower. We also monitored the chlorophyll contents of WT and the transgenic plants every 10 days from 20 day after the transfer to 22 °C (Fig. 8c). After 40 days, chlorophyll contents of 35S:MAPKKK18 1-5 and 35S:MAPKKK18 2-1 were lower than that of WT plants, on the other hand, those of 35S:MAPKKK18 KN 3-1 and 35S:MAPKKK18 KN 4-2 were higher than WT plants. Similar results were shown in 50 and 60 days old plants. Senescence was promoted in 35S:MAPKKK18 and suppressed in 35S:MAPKKK18 KN. These results indicate that MAPKKK18 positively regulates senescence with its kinase activity.

ABA related response of 35S:MAPKKK18 and 35S:MAPKKK18 KN plants

To analyze the role of MAPKKK18 in ABA signaling, the ABA related response of WT and each transgenic plant was monitored. Since ABA signaling controls water loss in plants, we measured the water loss rate in the detached aerial parts of WT and each transgenic plant. Water loss rate of 35S:MAPKKK18 was lower than that of WT, whereas 35S:MAPKKK18KN lost significantly much water than WT (Fig. 9). We also monitored the ABA-induced leaf senescence of WT and each transgenic plant to see the effect of the overexpression of MAPKKK18 on ABA signaling. The rosette leaves of 2-week-old WT and each transgenic plant were detached and incubated under continuous light in 50 μM ABA or distilled water (Mock) for 4 days. After the treatment, the color of the leaves in 35S:MAPKKK18 changed to yellow, whereas that in 35S:MAPKKK18KN still green (Fig. 10a). The chlorophyll contents of 35S:MAPKKK18 1-5 and 2-1 decreased at 52 and 63 % to mock treatment, respectively (Fig. 10b). On the other hand, the 87 % of chlorophyll were remained in both 35S:MAPKKK18KN 3-1 and 4-2, which was significantly higher than that of WT. These results indicate that MAPKKK18 positively regulates ABA signaling and overexpression of the kinase-negative mutant of MAPKKK18 caused the interference of ABA signaling.

Discussion

The MAPK cascade plays an important role in ABA signal transduction. It has been reported that the MKK1–MPK6 pathway (Xing et al. 2008), the MKK3–MPK1/2 pathway (Hwa et al. Hwa and Yang 2008) and MPK9/12 pathway positively regulate stomatal closure via Ca²⁺ (Jammes et al. 2009), and MPK4/6 is activated by ABA (Ichimura et al. 2000). However, there are few reports regarding MAPKK that mediates ABA signal. To identify the MAPKK that functions in ABA signaling, we focused on the MAPKKs whose gene expression is induced by ABA treatments, using databases that describe the expression patterns of *Arabidopsis* genes. Several MAPKK genes were upregulated by the ABA treatment (Menges et al. 2008). Recently, we reported that one of the *Arabidopsis* MAPKK, MAP3K84 (At4g23050) was induced and activated by ABA treatment (Shitamichi et al. 2013). Overexpression of this MAPKK in *Arabidopsis* enhanced the tolerance to high salinity. Additionally, the transgenic plants overexpressing MAP3K84 showed vigorous growth (Sasayama et al. 2011), indicating the crucial roles of MAP3K84 in both stress response and growth regulation. In this study, we revealed that the

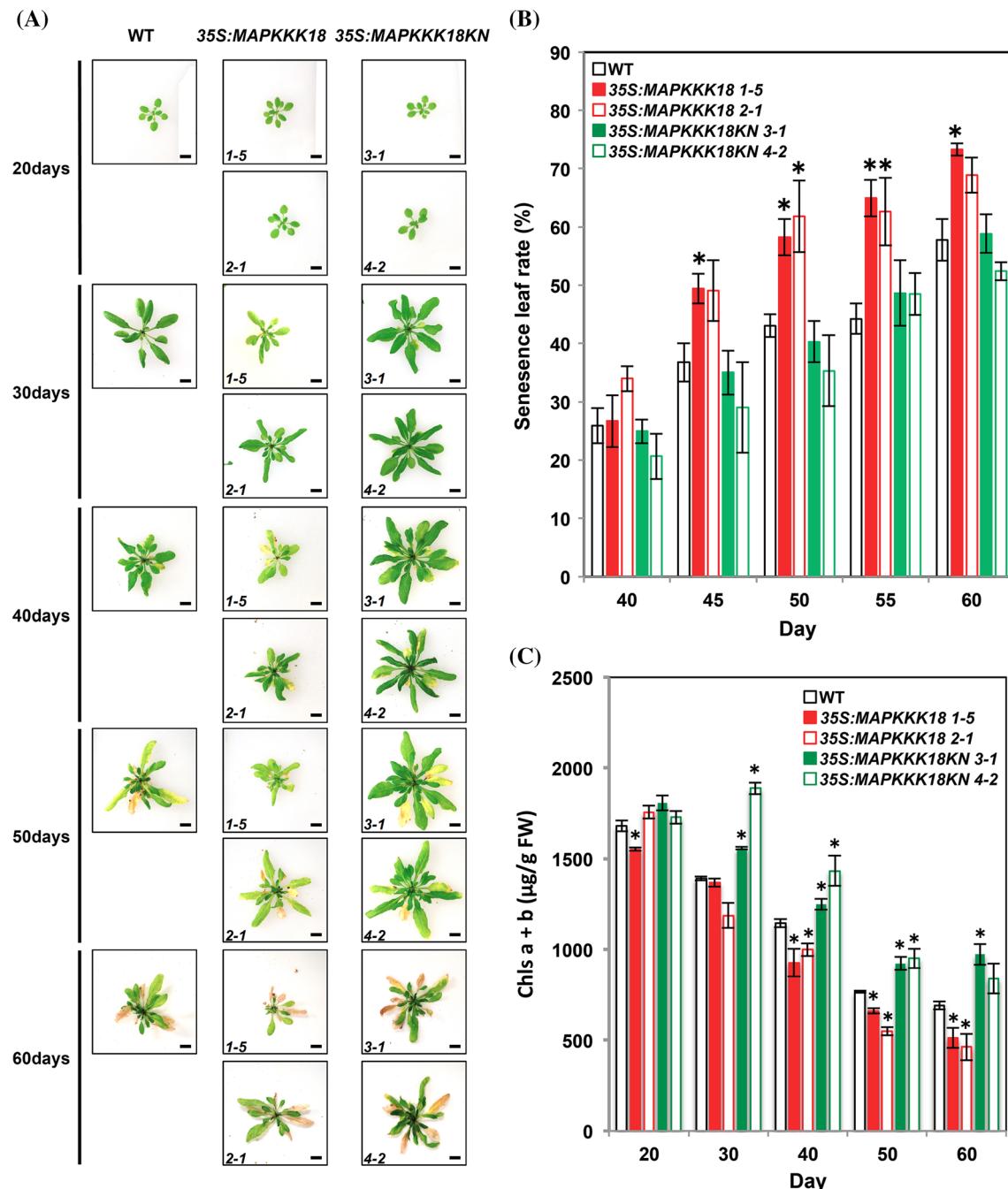


Fig. 8 Leaf senescence of WT and the transgenic plants. **a** Picture of the rosette leaves of WT and transgenic plants grown at 22 °C under continuous light for the indicated number of days. Bar 1 cm. **b** Rate of leaf senescence for the WT and transgenic plants. Each plant was harvested and its flowering stem was removed. The senescent and the total leaves were counted every 5 days, from 40 days after the transfer to 22 °C, and the rates of leaf senescence were calculated. All results

are presented as the means. The bars indicate the standard errors of six replicates. **c** Chlorophyll contents of WT and the transgenic plants. Chlorophyll a + b contents were quantified every 10 days from 20 days after the transfer to 22 °C. All results are presented as the means. The bars indicate the standard errors of three replicates. Asterisks indicate significant differences (Student's *t* test, $P < 0.05$) between WT and each transgenic line

expression of another MAPKKK, *MAPKKK18*, was also induced by ABA (Fig. 1). The kinase activity of this MAPKKK, overexpressed in *Arabidopsis* cells, was transiently increased by ABA treatments (Fig. 5b). The timing of ABA

induced expression of the *MAPKKK18* transcript was similar to the activation of *MAPKKK18* protein expressed in *Arabidopsis*. We also showed that the immunoprecipitated 3xFLAG-MAPKKK18 proteins did not show a kinase

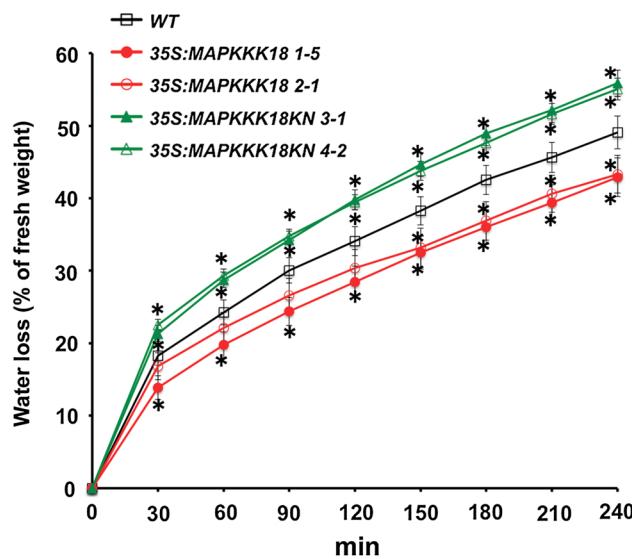


Fig. 9 Analysis of water-loss rate in WT and the transgenic plants. Aerial parts of 4-week-old WT and each transgenic plant were detached and used to measure water losses. All results are presented as the means. The bars indicate the standard errors of eight replicates. Asterisks indicate significant differences (Student's *t* test, $P < 0.05$) between WT and each transgenic line

activity to MBP without ABA treatments. Considering these results, we hypothesize that *MAPKKK18* is expressed by ABA treatment and then the resulting MAPKKK18 protein is further regulated by ABA signaling. To identify the MKK partners of MAPKKK18, the yeast two-hybrid analysis was performed, revealing that MAPKKK18 selectively interacts with MKK3 (Fig. 3a). To identify downstream MAPK targets of MKK3, we studied its direct interaction with 19 different MPKs using yeast two-hybrid analysis. MKK3 interacted with MPK1/2/7 (Fig. 3b), in agreement with the previous report (Dóczki et al. 2007). Our results and the report that the MKK3–MPK1/2 pathway is involved in ABA signaling (Hwa and Yang 2008) indicated that MAPKKK18 mediates ABA signaling via MKK3–MPK1/2. It has been reported that the germination of the transgenic plants overexpressing the constitutively active MKK3 increased sensitivity to ABA and overexpression of its downstream MPK1 or MPK2 also showed a hypersensitivity to ABA. These results indicate that MKK3 and its downstream MPK1 and MPK2 positively regulate ABA signaling. In this study, we revealed that overexpression of *MAPKKK18* strengthened the effects of ABA in water loss (Fig. 9) and senescence of the detached leaves (Fig. 10), indicating the positive role of MAPKKK18 in ABA signaling. It is necessary to analyze the activity of MKK3, MPK1 or MPK2 in the transgenic plant overexpressing *MAPKKK18* and also *mapkkk18* mutant plants.

To investigate the physiological roles of MAPKKK18 in *Arabidopsis*, the transgenic plants overexpressing *MAPKKK18* and its kinase negative mutant were generated,

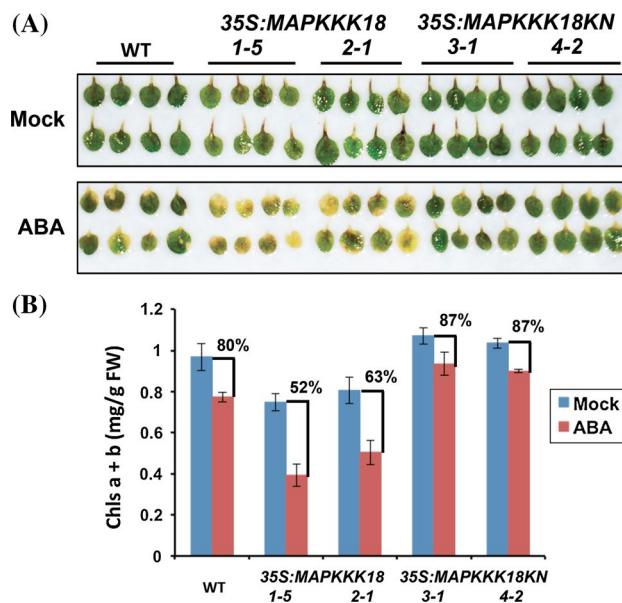


Fig. 10 ABA-induced leaf senescence of WT and the transgenic plants. The rosette leaves of 2-week-old WT and each transgenic plant were detached and incubated under continuous light in 50 μ M ABA or distilled water (Mock) for 4 days. **a** The picture of the leaves of WT and each transgenic plant. **b** Chlorophyll contents of WT and the transgenic plants. All results are presented as the means. The bars indicate the standard errors of eight replicates. ABA-induced decrease (%) of chlorophyll contents is shown

and the phenotypes of these plants were analyzed. Compared with the WT plants, senescence of the rosette leaves was promoted in 35S:MAPKKK18 but suppressed in 35S:MAPKKK18KN (Fig. 8b, c). Furthermore, ABA-induced leaf senescence was accelerated in 35S:MAPKKK18 (Fig. 10). These results indicate that MAPKKK18 positively regulates leaf senescence in ABA dependent manners. As the results of positive effects of MAPKKK18 on the ABA signaling, the timing of leaf senescence in 35S:MAPKKK18 became faster and the plant size was smaller than those of WT. The opposite effects were observed in the case of 35S:MAPKKK18KN and showed larger phenotype (Figs. 6, 8a). It is known that leaf senescence is regulated by many phytohormones, including ethylene, cytokinins and ABA (Khan et al. 2014). Further research is required to clarify how MAPKKK18 positively regulates the leaf senescence. MKK9 has been shown to positively regulate leaf senescence, and the transgenic plants overexpressing MKK9 showed earlier leaf senescence and a reduced stature (Zhou et al. 2009). It has been reported that the transcript of MKK9 was induced by ABA (Menges et al. 2008), and MKK9 is known to function in ethylene signaling (Yoo et al. 2008). In *Arabidopsis* publicly available microarray data (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>; Winter et al. 2007), the transcript of MAPKKK18 is increased in senescing leaf, indicating the roles of MAPKKK18 in leaf senescence.

It needs to analyze the senescing phenotype of *mapkkk18* mutants. In this study, we revealed that MAPKKK18 is activated by ABA treatments without any change in the protein amounts (Fig. 5), indicating that post-translational modifications of MAPKKK18 are associated with the ABA signal. It has been reported that an *Arabidopsis* receptor kinase, RPK1 mediates senescence in old leaves (Lee et al. 2011) and also reported that, *SAG113/HAI1* gene, which encodes a Golgi-localized PP2C, promotes ABA-resistant stomatal opening and water loss, leading to dehydration during senescence (Zhang et al. 2012). It will be important to analyze the relation between MAPKKK18 and RPK1 or SAG113. Further investigation on the regulatory mechanisms controlling MAPKKK18 activity in response to ABA signal may improve the understanding of the roles of the MAPK cascade in ABA signaling.

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