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Note

Control of plant growth and development by overexpressing *MAP3K17*, an ABA-inducible MAP3K, in *Arabidopsis*

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Abstract Abscisic acid (ABA) plays an important role in plant growth, development, and stress responses. ABA regulates many aspects of plant growth and development, including seed maturation, dormancy, germination, the transition from vegetative to reproductive growth, leaf senescence and responses to environmental stresses, such as drought, high salinity and cold. It is also known that mitogen-activated protein kinase (MAPK) cascades function in ABA signaling. Recently, we and another group have identified the ABA-inducible MAP3Ks MAP3K17 and MAP3K18 as the upstream MAP3Ks of MKK3, implicating the MAP3K17/18-MKK3-MPK1/2/7/14 cascade in ABA signaling. It has also been reported that overexpression of *MAP3K18* in *Arabidopsis* causes an early leaf senescence phenotype, ABA hypersensitive stomata closing, and drought tolerance. In this study, we generated transgenic plants overexpressing *MAP3K17* (35S:MAP3K17) and its kinase-inactive form (35S:MAP3K17KN). The bolting of 35S:MAP3K17 was earlier than WT, and the fresh weights of the seedlings were smaller, whereas 35S:MAP3K17KN showed the opposite phenotype. These results indicate that the transition from vegetative to reproductive growth can be regulated by overexpression of MAP3K17 and its kinase-inactive form. Moreover, 35S:MAP3K17 showed lower sensitivity to ABA during post-germinated growth, whereas 35S:MAP3K17 KN showed the opposite phenotype, suggesting the negative roles of MAP3K17 in the response to ABA. Our work provides the possibility to regulate plant growth and development by the genetic manipulation of ABA-induced MAPK cascades, leading to improved crop growth and productivity.

Key words: ABA response, Arabidopsis, floral transition, MAP3K, post-germinated growth.

As sessile organisms, plant growth is limited by many aspects of external stress, such as cold, heat, drought, and biotic attacks. To survive them, plants have developed various signal transduction pathways to modulate cellular responses to environmental changes. Abscisic acid (ABA) is one of the major plant hormones that play important roles in plant growth and stress responses. ABA regulates many aspects of plant growth and development, including seed maturation, dormancy, germination, the transition from vegetative to reproductive growth, leaf senescence and responses to environmental stresses, such as drought, high salinity and cold (Finkelstein 2013). ABA signaling comprises the cellular events of ABA perception and subsequent downstream pathways that regulate ABA responses. Various signaling components, for example, second messengers, including Ca^{2+} and reactive oxygen species (ROS), SNF1-related protein kinase 2 (SnRK2), protein phosphatase 2C (PP2C) pathway and G-protein, have been identified in ABA signaling (Mori et al. 2006; Munemasa et al. 2013; Wang et al. 2001). Mitogen-activated protein kinase (MAPK) cascades have also been implicated in this signaling pathway (Liu et al. 2012).

The MAPK cascade is a highly conserved signaling system in eukaryotic cells that converts 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 (MAP3K). In the genome of the model plant *Arabidopsis thaliana*, there are at least 80 MAP3K, 10 MAPKK (MKK1–MKK10) and 20 MAPK (MPK1–MPK20) genes (Colcombet and Hirt 2008). Several MAPK cascades have been identified in stress and developmental signal transduction pathways (Nakagami et al. 2005; Takahashi et al. 2004). We have 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

Abbreviations: ABA, abscisic acid; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAP3K, MAPKK kinase.
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cascades are involved in several other stress signaling pathways: the MEKK1-MKK2-MPK4/MPK6 cascade in salt and cold stress signaling (Teige et al. 2004) or the MEKK1-MKK4/MKK5-MPK3/MPK6 cascade following pathogen infection (Asai et al. 2002).

Regarding the involvement of the MAPK cascade in ABA signaling, several works have been reported as follows. MKK1-MPK6 regulates the ABA-dependent expression of *CAT1* and H_2O_2 production (Xing et al. 2008). MPK9/12 positively regulates stomatal closure via ROS-mediated ABA signaling. MPK4/6 are activated by ABA (Ichimura et al. 2000), and MKK3-MPK1/2 mediates ABA signaling and is involved in salt stress tolerance (Hwa et al. 2008). Recently, we and another group have identified MAP3K17 and MAP3K18 as the upstream MAP3Ks of MKK3, implicating the MAP3K17/18-MKK3-MPK1/2/7/14 cascade in ABA signaling (Danquah et al. 2015; Matsuoka et al. 2015). Moreover, it has been shown that MAP3K18 directly interacts with ABI1 protein phosphatase, which composes the core signaling module of ABA and is regulated by the ubiquitin-proteasome pathway (Mitula et al. 2015). Overexpression of MAP3K18 in *Arabidopsis* causes an early leaf senescence phenotype (Matsuoka et al. 2015), ABA hypersensitive stomata closing (Mitula et al. 2015), and drought tolerance (Li et al. 2017). The transcripts of *MAP3K17* and *MAP3K18* are induced by ABA (Menges et al. 2008), and these genes seem to have redundant functions in ABA signaling. In this study, we generated transgenic *Arabidopsis* overexpressing *MAP3K17* and kinase-inactive *MAP3K17* (*MAP3K17KN*) to analyze the function of MAP3K17 in ABA signaling. We analyzed their growth characteristics and the response to ABA in post-germinated growth.

Molecular cloning of the full-length cDNA for *MAP3K17* and production of the transgenic plants overexpressing *MAP3K17* and the kinase-inactive form of *MAP3K17 (MAP3K17 KN)* were carried out as follows. *Arabidopsis thaliana* (Col-0) 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 Gamborg's B5 agar (0.8% w/v) medium and incubated for two days at 4°C before germination at 22°C, and the seedlings were grown at 22°C under continuous light conditions. Total RNA was extracted from two-week-old *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). The cDNA for *Arabidopsis MAPKKK17* was isolated using RT-PCR with the forward primer 5′-GAA AGA ATT CAT GGA ATG GAC TAG AGG AAG-3′ and the reverse primer 5′-GTT TCT CGA GTT ACA ATT CCC CCA CCA ATA-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 a cloning vector, pBluescript II SK (−). The plasmid clones were verified using DNA sequencing. The kinase negative mutation of MAP3K17 (K32R), designated MAP3K17 KN, was created using a QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions and verified by DNA sequencing. The cDNA for *MAP3K17* and *MAP3K17 KN* genes without the start codon were translationally fused to the coding sequence of the *3xFLAG-tag* in the N-terminus (Figure 1A). The *3xFLAG-tag* fused *MAP3K17* and the *3xFLAG-tag* fused *MAP3K17 KN*, designated *3xFLAG-MAP3K17* and *3xFLAG-MAP3K17 KN*, respectively, were

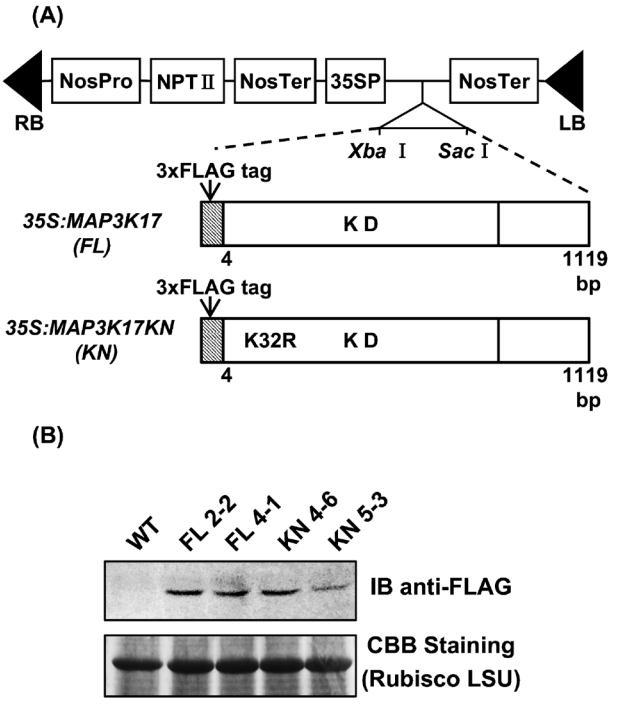

Figure 1. Overexpression of *MAP3K17* and its kinase-inactive mutant in *Arabidopsis*. (A) Schematic representation of the constructs for the overexpression of *3xFLAG-MAP3K17* (*FL*) and *3x-FLAG MAP3K17KN* (K32R, *KN*). The 3xFLAG-tag was translationally fused to the N-terminus of the coding sequence, without an initiation codon, of *MAP3K17* and *MAP3K17 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 sites. RB: right border, LB: left border, NOS Pro: NOS promoter, NPTII: neomycin phosphotransferase II gene, NOS Ter: NOS terminate, KD: kinase domain (B) Detection of the 3xFLAG-MAP3K17 and 3xFLAG-MAP3K17KN proteins. Total protein extracts from WT and each transgenic plant were resolved by SDS-PAGE. Immunoblot analysis was conducted using the anti-Flag tag antibody. Equal amounts of the samples were resolved by SDS-PAGE and stained with CBB, and the large subunit (LSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is shown as the loading control for equal protein amounts in the WT and transgenic plants.

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 *Escherichia coli* (strain DH5α) containing pRK2013 (Wise et al. 2006) and transferred into wild-type *Arabidopsis* (Columbia ecotype) by the vacuum infiltration method (Bechtold et al. 1998). The transgenic plants were germinated on 0.5× Murashige and Skoog medium with 20 μg ml^{-1} kanamycin. Eight and seven independent lines of the transgenic plant overexpressing *3xFLAG-MAP3K17* and *3xFLAG-MAP3K17 KN*, respectively, were generated. All transgenic lines used in this study were T_4 homozygous plants. Two-week-old seedlings of WT and transgenic plants overexpressing *3xFLAG-MAP3K17* and *3xFLAG-MAP3K17 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. The aliquots of the crude extracts from the seedlings of the WT and transgenic plants overexpressing *3xFLAG-MAP3K17* and *3xFLAG-MAP3K17 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) 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. Equal amounts of the sample were resolved on SDS-PAGE and stained with CBB for a loading control. The immunoblot results showed that both 3xFLAG-MAP3K17 and 3xFLAG-MAP3K17 KN proteins were expressed in each transgenic plant (Figure 1B). We used both lines of transgenic plants overexpressing *3xFLAG-MAP3K17* (FL 2-2 and FL 4-1) and *3xFLAG-MAP3K17 KN* (KN4-6 and KN 5-3) for further analysis.

To analyze the effects of overexpressing *3xFLAG-MAP3K17 and 3xFLAG-MAP3K17 KN* on plant growth, WT and both transgenic plants were germinated on soil and grown at 22°C under continuous light. Bolting time was monitored daily and calculated as the number of days from sowing to the first elongation of the floral stem at 1 mm height. Rosette leaf numbers were counted at bolting day. Representative plants (28 days after germination) are shown in Figure 2A. The bolting of both FL lines preceded that of WT by approximately 3 days

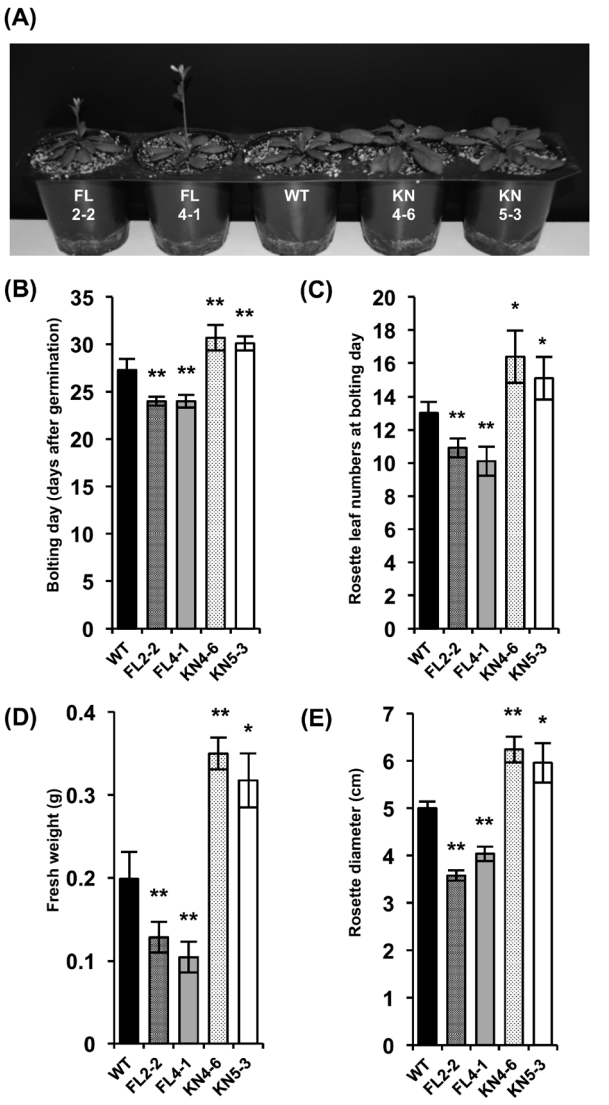

Figure 2. Growth characteristics of transgenic *Arabidopsis* plants overexpressing *MAP3K17* and *MAP3K17KN*. WT and the transgenic plants were germinated on soil and grown at 22°C under continuous light conditions. (A) Representative (28-day-old) image of WT and transgenic plants. Bolting days (B), rosette leaf numbers at bolting day (C), fresh weights at 4 weeks (D), and rosette diameters at 4 weeks (E) of WT and transgenic plants. Bolting time was monitored daily and determined as the number of days from sowing to the first elongation of the floral stem at 1 mm height. All results are presented as the means. The bars indicate the standard errors from ten (B, C) and five (D, E) replicates. Asterisks indicate significant differences (Student's *t*-test, * $p<0.05$, ** $p<0.01$) between WT and each transgenic line.

(Figure 2B). On the other hand, the bolting of both KN lines was approximately 3 days later than that of WT. Similarly, the numbers of rosette leaf at bolting day were smaller in both FL lines and bigger in both KN lines (Figure 2C). To evaluate plant growth, the shoots were harvested at 4 weeks after germination and immediately measured the fresh weights and the diameter of rosette leaves. The fresh weights and the size (rosette diameter) of both FL plants were significantly lower than those of WT plants at 4 weeks after germination, on the other

hand, those of both KN plants were clearly greater to WT (Figure 2D, E).

Next, we analyzed the ABA-related response of transgenic *Arabidopsis* plants overexpressing *MAP3K17* and *MAP3K17KN*. The sterilized seeds of WT and transgenic *Arabidopsis* plants overexpressing *MAP3K17* and *MAP3K17KN* were sown in Gamborg's B5 medium supplemented with or without 1 μm ABA. The cotyledon greening was recorded at 7 days after transfer to 22°C. Every experiment was repeated three times (50 seeds for each repeat). Representative pictures are shown in Figure 3A (Gamborg's B5 medium) and Figure 3B (Gamborg's B5 medium plus 1 μm ABA). On Gamborg's B5 medium plates, all plants germinated and grew without significant differences among them (Figure 3C). In the presence of ABA, the cotyledon greening rates of FL 2-2 and FL 4-1 were 84.4±0.7% and 73.9±2.5%, respectively, which was significantly higher than that of WT (45.8±7.2%). The cotyledon greening rates of KN 4-6 and KN 5-3 were 22.1±4.2% and 5.4±1.4%, respectively, indicating a sensitive phenotype of both KN plants to ABA (Figure 3C).

In this study, we generated transgenic plants overexpressing ABA-inducible MAP3K, *MAP3K17* (35S:MAP3K17) and its kinase-inactive form (35S:MAP3K17KN). The bolting of 35S:MAP3K17 was earlier than WT, and the fresh weights of the seedlings were smaller, whereas 35S:MAP3K17KN showed the opposite phenotype (Figure 2). These results indicate that the transition from vegetative to reproductive growth can be regulated by overexpression of *MAP3K17* and its kinase-inactive form. Floral transition is one of the most important decisions in the plant life cycles and four regulatory pathways, the photoperiod, vernalization, autonomous pathways, and gibberellic acid, have been identified in *Arabidopsis* (Mouradov et al. 2012). The inhibitory role of ABA in regulating the floral transition was initially proposed based on the early-flowering phenotype of ABA-deficient mutant (Martinez-Zapater et al. 1994). It has been also reported that the inhibitory effect of ABA on floral transition is mediated by ABI5 in *Arabidopsis* (Wang et al. 2013). 35S:MAP3K17 showed an early bolting phenotype and a lower sensitivity to ABA in cotyledon greening (Figure 3), suggesting the negative roles of MAP3K17 in the response to ABA. Transcriptome and proteome analysis of 35S:MAP3K17 and 35S:MAP3K17KN may lead to the identification of the downstream signaling pathways of the MAP3K17/18-MKK3-MPK1/2/7/14 cascade and the regulatory components of floral transition. As described above, ABA regulates many aspects of plant growth, development, and responses to environmental stresses. (Finkelstein 2013). It has been reported that several MAP3K genes were upregulated by ABA treatment (Menges et al. 2008). Previously, we reported that one of the *Arabidopsis*

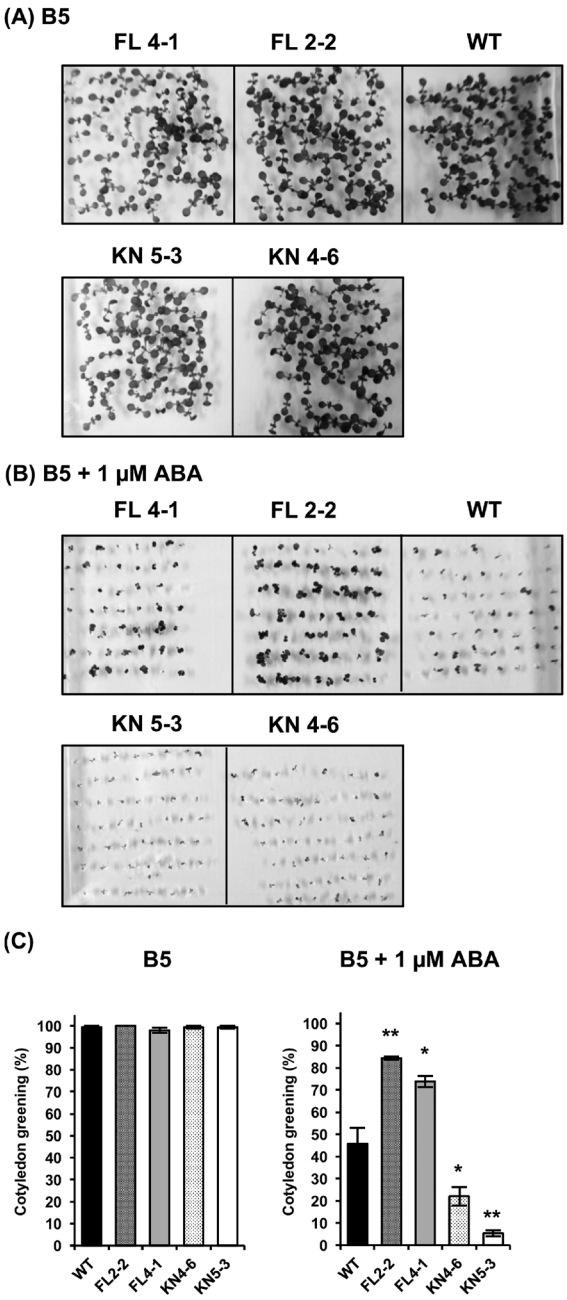

Figure 3. Effect of ABA on the post-germinated growth of WT and transgenic *Arabidopsis* plants overexpressing *MAP3K17* and *MAP3K17KN*. The seeds of WT and transgenic *Arabidopsis* plants overexpressing *MAP3K17* (FL 2-2, FL 4-1) and *MAP3K17KN* (KN 4-6, KN 5-3) were sown onto Gamborg's B5 medium plates with or without 1 μM ABA. Images of 7-day-old germination on B5 medium plates (A) and B5 medium plates containing 1 μM ABA (B) are shown. (C) The cotyledon greening rates were scored at 7 days after cultivation. All results are presented as the means. The bars indicate the standard errors from three independent experiments (50 seeds for each repeat). Asterisks indicate significant differences (Student's *t*-test, * $p<0.05$, ** $p<0.01$) between WT and each transgenic line.

MAPKKK genes, *MAP3Kδ4* (At4g23050), was induced and activated by ABA treatment (Shitamichi et al. 2013). Overexpression of *MAP3Kδ4* in *Arabidopsis* enhanced tolerance to high salinity and showed vigorous growth

(Sasayama et al. 2011). Recently, it has been reported that transgenic tobacco overexpressing cotton *GhMKK3* enhanced drought tolerance (Wang et al. 2016), and two recessive mutations in MKK3 orthologs have been identified in both wheat and barley using QTL mapping for seed dormancy (Nakamura et al. 2016; Torada et al. 2016). These results suggest that ABA-induced MAP3Ks, including MAP3K17, and the components of their downstream pathways are targets of genetic modification for regulating plant growth and enhancing stress tolerance.

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