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RESEARCH ARTICLE

MAP3Kθ1 is Involved in Abscisic Acid Signaling in Drought Tolerance and Seed Germination in *Arabidopsis*

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Abstract

Mitogen-activated protein kinase cascades play pivotal roles in mediating environmental stress responses and plant development. In this study, a loss-of-function mutant of *Arabidopsis*, *map3kθ1*, exhibited wider stomatal openings, reduced root elongation, and increased seed germination rate compared with its wild type under exogenous abscisic acid (ABA) treatment. *MAP3Kθ1* encodes a MAP kinase kinase kinase (MAP3K) with unknown function. Two overexpression lines of *MAP3Kθ1* exhibited inhibited seed germination and narrowed stomata, which were aggravated by ABA treatment. Upregulation of *MAP3Kθ1* also resulted in stronger drought tolerance, whereas *map3kθ1* was more sensitive to water deficiency, partially due to differences in the water-holding capacity of leaves. The *MAP3Kθ1*-overexpressing lines also showed a greater ability to maintain root elongation under exogenous ABA. Expression of *MAP3Kθ1* was inhibited by ABA, H_2O_2, and methyl viologen treatments in roots. The *MAP3Kθ1*-overexpressing lines accumulated more ABA by promoting its biogenesis and inhibiting its catabolism, whereas the *map3kθ1* mutant accumulated less ABA, compared with wild-type plants. These findings indicate that *MAP3Kθ1* promotes ABA accumulation to regulate stomatal movement, root elongation, and seed germination, while the ABA–H_2O_2 signaling module inhibits *MAP3Kθ1* expression through feedback regulation.

Keywords Abscisic acid · *Arabidopsis* · MAP3Kθ1 · MAPK · Seed germination · Stomatal movement

Introduction

Abscisic acid (ABA) is a well-known signaling molecule that mediates many types of abiotic stress, allowing plants to rapidly sense and react to environmental fluctuations (Yoshida et al. 2014). When a plant faces water stress, increased ABA levels promote the closing of stomata and inhibit its opening. The controlled opening of stomata plays an essential role in reducing water loss and maintaining the relative water level of plants for survival (Mishra et al. 2006; Bauer et al. 2013). ABA also participates in many developmental processes, such as root elongation, seed germination, and seedling and fruit development (Liu et al. 2010; Kim et al. 2013). In particular, through antagonistic regulation with gibberellins, ABA coordinates seed dormancy and germination as its classical function (Shu et al. 2016).

In ABA signal transduction, some components have been shown to play important roles as signaling molecules, such as calcium ion, nitric oxide, and reactive oxygen species (ROS) (Zhang et al. 2006, 2014). ROS is the most important molecule in the regulation of antioxidant defense systems induced by abiotic stresses, such as water stress, salinity, and high or low temperature, all of which induce oxidative stress in plant cells. At the beginning of this century, it was found that ABA can trigger the production of H_2O_2 and other ROS in plants (Guan et al. 2000; Jiang and Zhang 2001). Growing evidence suggests that the increased plant drought tolerance under enhanced ABA accumulation results at least partly from the induction of ROS production, which initiates antioxidant defense systems (Zhang et al. 2014).

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Many genes are involved in ABA signaling, in which a mitogen-activated protein kinase (MAPK) cascade plays a pivotal role (Zelicourt et al. 2016). MAPKs are highly conserved in eukaryotes, linking a series of environmental changes to a range of cellular responses. A typical MAPK module includes three types of protein kinase: a MAPK (MPK), a MAP kinase kinase (MAP2K; MAPKK/MEK/MKK), and a MAP kinase kinase kinase (MAP3K; MAPKKK/MAP3K), which form a signaling cascade and are sequentially activated by phosphorylation (MAPK Group, 2002; Fiil et al. 2009). In *Arabidopsis*, there are 20 MAPKs, 10 MAP2Ks, and about 80 genes encoding MAP3K, which are conserved in all eukaryotes (MAPK Group 2002; Su and Krysan 2016). In plants, MAPK cascades have been shown to be activated by many environmental stresses, hormone stimuli, and ROS, as well as developmental processes (Liu 2012; Zelicourt et al. 2016).

ABA has been shown to modulate several MAPK signaling components at the transcriptional and translational levels, as well as kinase activity in various plant species (Zhang et al. 2006; Liu 2012; Danquah et al. 2014). For instance, MKK1 has been indirectly shown to be a positive regulator of ABA signaling (Wu et al. 2015). Furthermore, AtMKK1–AtMPK6 is regarded as an important signaling cascade stimulating H_2O_2 generation and stress responses in an ABA-dependent manner (Xing et al. 2008). Expressing anti-sense MPK3 showed that MPK3 acts downstream of ABA signaling in the ABA-induced H_2O_2 synthesis pathway (Gudesblat et al. 2007). MPK9 and MPK12 have been verified to be expressed preferentially in guard cells, and a *mpk9mpk12* double-mutant exhibited insensitivity to ABA and H_2O_2 regardless of stomatal opening status and increased water loss via transpiration (Jammes et al. 2009). Recently, MPK1 and MPK2 were shown to be activated by ABA in an SRK2D/E/I-dependent manner, thus establishing a direct link between the MAPK modules and ABA signaling (Umezawa et al. 2013).

MAP3K has also been reported to be involved in ABA responses. A study showed that ANP1, a MAPKKK in *Arabidopsis*, activated AtMPK3 and AtMPK6 and was induced by H_2O_2 (Kovtun et al. 2000). Moreover, YODA (MAPKKK4) was verified to regulate stomatal development by activating MKK4/5–MKK7/MKK9 and MPK3/6 as an important MAPK cascade (Wang et al. 2007; Lampard et al. 2009). Recent studies have shown that MAP3K17 and MAP3K18 of *Arabidopsis* function upstream to activate the MKK3 in response to ABA signaling (Danquah et al. 2015; Matsuoka et al. 2015). Through detailed genetic analysis, it has been shown that the MAP3K17/18-MKK3-MPK1/2/7/14 cascade induced by drought stress is mediated by an ABA receptor signaling module at the transcriptional level (Danquah et al. 2015; Zelicourt et al. 2016). Plants overexpressing *MAP3K16* exhibit ABA insensitivity during the seed germination and cotyledon greening stages, but are hypersensitive during root development. Further investigation has shown that MAP3K14–18 are involved in the ABA response, but that the effects might be negative or positive depending on developmental stage (Choi et al. 2017).

There is abundant evidence showing that MAPK cascades are closely related to ABA signaling. Although much progress has been made in recent years, the functions of most MAP3Ks are largely unknown because of the diversity and complexity of the MAP cascade kinases family. Screening and functional analysis of more MAP3Ks is essential for investigating MAPK cascades and understanding the complex mechanisms of ABA signaling.

Results

Screening of *map3kθ1* Mutant and Expression Patterns of *MAP3Kθ1* Gene

To identify additional ABA signaling components, we obtained a T-DNA insertion mutant for the gene encoding MAP3Ks in *Arabidopsis*. According to the nomenclature from the MAPK Group (2002), the gene was named *MAP3Kθ1* (*AT2G31010*). The protein kinase domain of MAP3Kθ1 was predicted and verified using the PROSITE tool (ExPASy) (Fig. 1a). The T-DNA of the screened mutant (SALK_137974) was located in the intron between E8 and E9 in the gene (Fig. 1b). Gene expression of the *MAP3Kθ1* T-DNA insertion mutant at the transcriptional level was determined via quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Although messenger RNA (mRNA) was strongly expressed in wild-type seedlings, transcription was barely detected in *map3kθ1*-homozygous mutant plants (Fig. 1c).

The qRT-PCR method was used to analyze the expression pattern of *MAP3Kθ1* at the transcriptional level in various tissues. Our determination showed that it was expressed at the highest levels in roots, followed by flowers, siliques, and buds, with a lower expression in leaves and shoot (Fig. 2a). We also constructed a chimeric gene by fusing a *GUS* reporter gene with the promoter of *MAP3Kθ1* (*MAP3Kθ1pro-GUS*), and transferred it to wild-type *Arabidopsis* plants. The GUS activity of transgenic plants driven by the *MAP3Kθ1* promoter was further used for expression pattern analysis through histochemical staining. The results showed that GUS activity in *MAP3Kθ1pro-GUS* lines was most abundant in the radicle of germinating seeds (Fig. 2b), in the root tip and shoot tip (shoot apical meristem and young leaves) at the seedling stage (Fig. 2c), and in the axillary bud meristem, young cauline leaves, young silique, and pollen of adult plants (Fig. 2d).

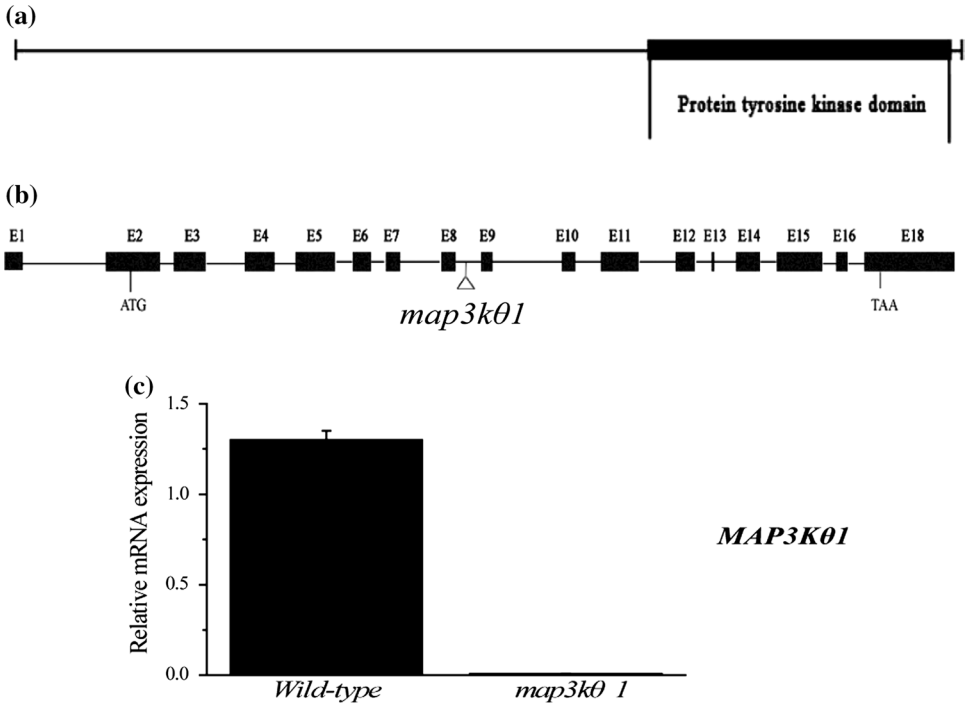

Fig. 1 Prediction of the kinase domain of MAP3Kθ1 protein and verification of the *map3kθ1* mutant by qRT-PCR. **a** Predicted tyrosine kinase domain of MAP3Kθ1 protein. **b** Schematic presentation of T-DNA insertion site in the *map3kθ1* mutant. Black boxes represent exons, and 'ATG' and 'TAA' represent the start codon and stop codon of *MAP3Kθ1*, respectively. **c** qRT-PCR analysis of *MAP3Kθ1* expression at the transcriptional level. The *ACTIN* gene was amplified in the wild-type and *map3kθ1* mutant as a control

Response of *MAP3Kθ1* Expression to Exogenous ABA Signaling

The transcript levels of *MAP3Kθ1* in *Arabidopsis* roots were assayed by qRT-PCR from 1 to 24 h under ABA and H_2O_2 treatments. Expression was initially induced (within 1 h), and then dramatically downregulated over time. The transcript levels exhibited similar tendencies under ABA and H_2O_2 treatments (Fig. 3a, b). Methyl viologen (MV) is commonly used as an H_2O_2 inducer for its capacity to disturb the electron transport chain (Jia et al. 2012). When treated with 0.5 μM MV, the transcript level of *MAP3Kθ1* decreased sharply over time. Unlike under the ABA and H_2O_2 treatments, we did not detect any increase in expression within the first hour (Fig. 3c).

GUS activity in the roots of *MAP3Kθ1pro-GUS* transgenic lines was also assayed under ABA, H_2O_2, and MV treatments. In accordance with the qRT-PCR results, MAP3Kθ1 expression was downregulated in wild-type *Arabidopsis* roots after 6 h, and almost no staining was observed under the H_2O_2 and MV treatments (Fig. 3d, e). Overall, these results indicate that *MAP3Kθ1* is inhibited by ABA–H_2O_2 cascade signaling.

Endogenous ABA Content in *map3kθ1* Mutant and Overexpressing *Arabidopsis* Plants

To further confirm the function of MAP3Kθ1, the construct of the full-length cDNA sequence of *MAP3Kθ1* guided by a CaMV 35S promoter was transformed into wild-type *Arabidopsis*. A total of 21 independent transgenic lines (T_2) were obtained, among which 17 lines showed significantly elevated *MAP3Kθ1* mRNA levels. Two lines (denoted as *OE2* and *OE7*) were chosen as overexpressing lines for further experiments. *MAP3Kθ1* was expressed at higher levels in *OE2* and *OE7* lines than in the wild type based on reverse transcription (RT-PCR) analysis (Fig. 4a).

The ABA contents in the *map3kθ1* mutant, wild-type, and *OE2*/*OE7* plants were measured using a radioimmunoassay method. More ABA accumulated in *MAP3Kθ1*-overexpressing plants, and less accumulated in the *map3kθ1* mutant, compared with the wild type (Fig. 4b). To investigate the cause of these effects, both ABA biogenesis and catabolism genes were investigated. The results showed that an ABA biogenesis gene, *NCED3*, exhibited higher expression in *MAP3Kθ1*-overexpressing lines and lower expression in the *map3kθ1* mutant (Fig. 4c). Conversely, two ABA catabolism genes (*CYP707A1* and *CYP707A3*) were downregulated in *OE2* and *OE7*, but significantly upregulated in the *map3kθ1* mutant compared with the wild-type plant (Fig. 4d). The ABA-responsive gene ABI1 was upregulated in the *map3kθ1* mutant but downregulated in *MAP3Kθ1*-overexpressing lines (Fig. 4e), and ABI3 and ABI5 exhibited the opposite tendency as ABI1 (Fig. 4f, g).

Increased ABA Sensitivity of *map3kθ1* Mutant in Response to Water Stress

Shoot fresh weight of the *map3kθ1* mutant decreased compared with the wild type in 4-week-old plants, while the

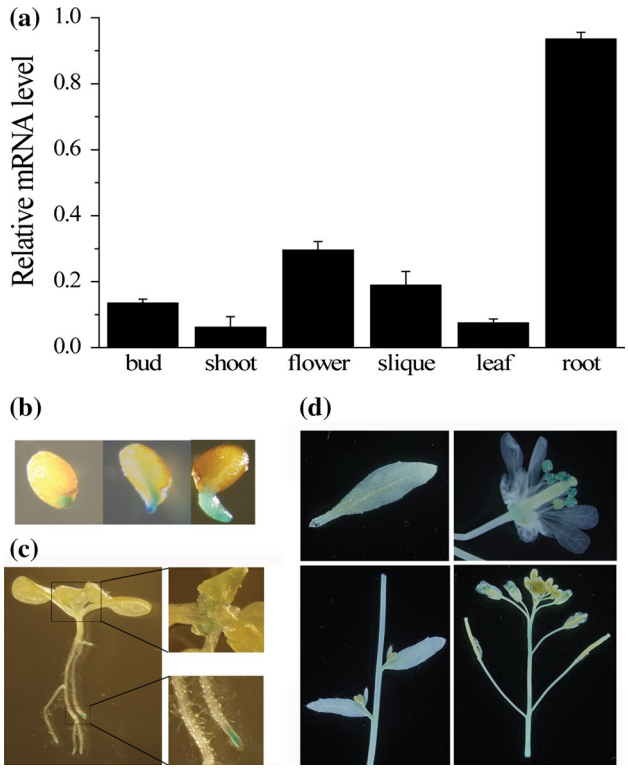

Fig. 2 Organ-specific expression of *MAP3Kθ1* in *Arabidopsis*. **a** Relative mRNA levels of *MAP3Kθ1* in bud (shoot apical meristem), shoot (primary inflorescence stem), flower, silique, leaf, and root of 6-week-old seedlings. Transcript levels were determined by qRT-PCR and normalized to *ACTIN* expression. Values presented are the means of three replications and standard errors. Tissue-specific expression patterns of MAP3Kθ1 in **b** germinating seed, **c** bud and root of 2-week-old seedlings, and **c** leaf, shoot, and flower of 6-week-old seedlings determined by histochemical GUS staining of *MAP3Kθ1pro-GUS* transgenic plants

expression of *MAP3Kθ1* increased. Root elongation exhibited a similar tendency, and was longer in the mutant but shorter in the overexpression lines compared to the wild type (Table 1). All seedlings exhibited symptoms in response to 20 days of water deficiency: *OE2* and *OE7* maintained expanded leaves, some leaves on wild-type plants curled, and all leaves curled and dehydrated on *map3kθ1* mutant plants (Fig. 5a). Because ABA is a major hormone that responds to water stress, we speculated that it plays an important role in the function of *MAP3Kθ1* in the drought response. To investigate this possibility, we analyzed the root elongation of the wild type, *map3kθ1* mutant, and *OE2* and *OE7* under 10 μM or 50 μM ABA in Murashige and Skoog (MS) medium. There was no significant difference in relative root length under 10 μM ABA treatment, although it decreased in all groups compared to the control. However, compared with the wild type, relative root length was significantly lower in the *map3kθ1* mutant and higher in the *MAP3Kθ1*-overexpressing lines when treated with 50 μM ABA (Fig. 5b). This may partially explain why the *map3kθ1* mutant was more sensitive to water stress. We also compared the water-holding capacity of leaves among the wild type, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing lines. The results showed that the *map3kθ1* mutant easily lost water from its leaves and exhibited the lowest water-holding ability, while the *MAP3Kθ1*-overexpressing lines had the highest leaf water retention based on measurement of detached rosette leaves (Fig. 5c).

Stomata openings were analyzed to further assess differences in the water-retention capacity of leaves. Under normal growth conditions, stomata openings decreased with increased MAP3Kθ1 expression, although there was no significant difference in the width/length ratio of stomata among the wild type, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing line *OE2* (Fig. 6a, b). The stomata openings were significantly smaller in *map3kθ1*, wild-type, and *MAP3Kθ1*-overexpressing plants when treated by ABA. Stomata were also significantly less narrow in *map3kθ1* plants under ABA treatment, although no significant difference was found between the wild type and *MAP3Kθ1*-overexpressing line (Fig. 6).

MAP3Kθ1 is Involved in ABA-regulated Seed Germination

To determine the sensitivity of *MAP3Kθ1* to ABA during seed germination, dormancy-broken *Arabidopsis* seeds were chosen for investigation under ABA concentration gradients. The germination behavior of the *map3kθ1* mutant, wild-type plants, and *OE2* line was observed under 0 μM, 1 μM, 2 μM, and 5 μM ABA supplemented into MS medium (Fig. 7a). No difference in germination rate was found between *map3kθ1* and the wild type, but germination rate was lower for *OE2* without ABA supplementation after 7 days. The germination rates for all seed lots decreased with increasing ABA concentration in MS medium, with particular differences at 2 μM ABA: 95.1%, 62.8%, and 42.2% for the *map3kθ1* mutant, wild type, and *OE2*, respectively. At 5 μM ABA, the mutant maintained a higher germination rate, while those of the wild type and *OE2* were very low (Fig. 7b).

Discussion

MAPK cascades are involved in almost all biological processes in plants, including organ development, responses to environmental variations, and plant immunity (Zhao et al. 2017; Bi et al. 2018). In *Arabidopsis*, there are about 80 genes encoding MAP3Ks, which are conserved among all eukaryotes (Su and Krysan 2016). ABA is a signaling molecule involved in the response to many environmental stresses and accumulates in seedlings during the stress response

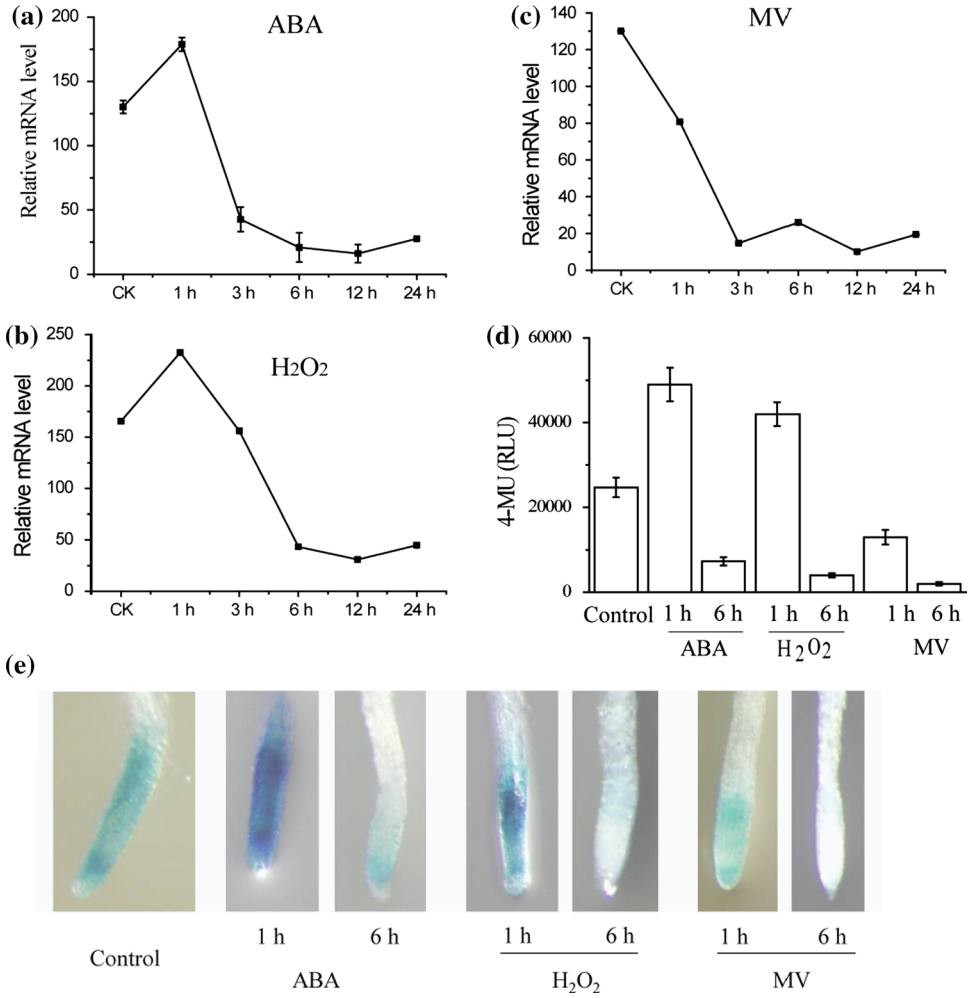

Fig. 3 Induced expression patterns of *MAP3Kθ1* in roots of 2-week-old seedlings under chemical treatments. Relative mRNA levels under **a** 0.1 mM ABA, **b** 20 mM H_2O_2, and **c** 0.5 μM MV treatment after 1 h, 3 h, 6 h, 12 h, and 24 h. Seedlings grown under normal conditions were used as a control (CK). Relative expression levels of *MAP3Kθ1* normalized to *ACTIN* levels determined by qRT-PCR. Values presented are the means of three replications and standard errors of the mean. **d** GUS activity and **e** GUS staining in situ of *MAP3Kθ1* promoter-GUS after treatment with ABA, H_2O_2, or MV for 1 h and 6 h. The seedlings were incubated with the substrate 4-MUG (1 mM) for the indicated time periods before the reaction was terminated by the addition of stop solution (Na_2CO_3). The released reaction product, 4-MU, was directly quantified by its fluorescence using a microplate reader

(Nakashima and Yamaguchishinozaki 2013). Many genes take part in the ABA signaling pathway, in which a MAPK cascade is an important module (Zelicourt et al. 2016).

To further explore the functional components, we screened a MAP3K, defined as *MAP3Kθ1* according to the nomenclature from the MAPK Group (2002). Expression analyses at the transcriptional level and by GUS staining showed that *MAP3Kθ1* was expressed at the highest levels in the radicle of germinating seeds and roots, and was also expressed in the flowers, siliques, buds, leaves, and shoots of seedlings (Fig. 2). Its transcription was downregulated under exogenous ABA treatment (Fig. 3). Conversely, MAPKKK18 is activated by an ABA core signaling module through protein synthesis (Danquah et al. 2015; Tajdel et al. 2016), which is upregulated by ABA at the transcriptional level along with MAP3K16 (Choi et al. 2017).

Recent evidence has shown that ROS production is induced by the ABA signaling pathway (Jiang and Zhang 2001; Zhang et al. 2006; Jia et al. 2012; Arve et al. 2014). Our results also showed that the expression of *MAP3Kθ1* was downregulated by H_2O_2 and MV (a ROS production inducer) (Fig. 3). It is possible that ROS mediates the regulation of *MAP3Kθ1* via the phytohormone ABA. However, in a previous study, it appeared that another MAP3K, NPK1 (tobacco ANP1 ortholog), was specifically activated by H_2O_2 without activating the drought, cold, or ABA signaling pathways (Kovtun et al. 2000). Although MAP3K has not been reported to be involved in the ABA–ROS signaling module, many MAPKs and MAP2Ks have been shown to be downstream of the ABA-induced H_2O_2 synthesis pathway (Gudesblat et al. 2007; Xing et al. 2008; Jammes et al. 2009; Wu et al. 2015).

Many studies have observed that the MAPK cascade is involved in the ABA signaling module, but there are fewer reports on the possible regulation of ABA metabolism by MAPKs. Although the expression of MAP3Kθ1 is also regulated by ABA, endogenous ABA content changes with ectopic expression of *MAP3Kθ1* in *Arabidopsis*. More ABA accumulated in the *MAP3Kθ1*-overexpressing lines, but less in the *map3kθ1* mutant, compared with the wild type. Further investigation showed that this was due to the upregulation of ABA biosynthesis genes and downregulated catabolic

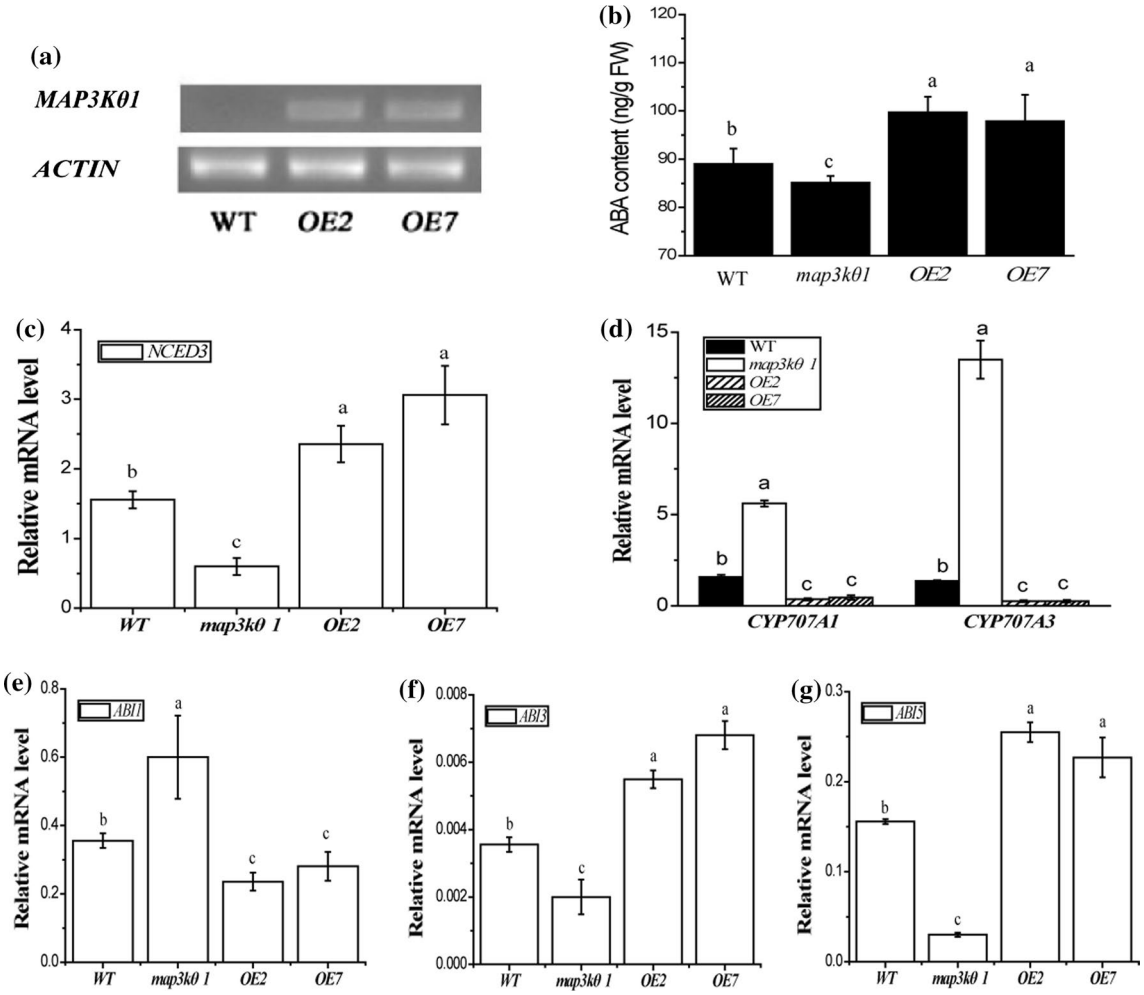

Fig. 4 Endogenous ABA levels regulated by *MAP3Kθ1*. **a** Transcript levels of *MAP3Kθ1* in two overexpression lines analyzed by RT-PCR. The expression of *MAP3Kθ1* in wild-type (WT) plants was used as a control and *ACTIN* expression was used as an internal standard. **b** Determination of endogenous ABA level in wild-type, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing seedlings. The transcript levels of **c** an ABA biogenesis gene (*NCED3*), **d** ABA catabolism genes (*CYP707A1* and *CYP707A3*), and the ABA-responsive genes **e** *AB11*, **f** *ABI3*, and **g** *ABI5* were determined by qRT-PCR in WT, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing seedlings. Values presented are the mean ± standard error of the mean of three replicates for **b**–**g**

Table 1 Phenotype of *map3kθ1 and* MAP3Kθ1-overexpressing lines under normal growth conditions

	Fresh weight of shoot (g seedling^{-1})	Root length (cm seedling^{-1})
Wild type	0.112 ± 0.007 b	1.20 ± 0.11 b
map3kθ1	0.129 ± 0.013 a	1.51 ± 0.16 a
OE2	0.082 ± 0.002 c	1.04 ± 0.04 c
OE7	0.089 ± 0.003 c	1.06 ± 0.03 c

Note: Four-week-old plants were used for shoot fresh weight analyses under normal growth conditions. Seven-day-old seedlings in MS medium were used for root length determination. Values within a column with the same letter denote no statistically significant difference at $P = 0.05$

genes by *MAP3Kθ1* expression (Fig. 4c, d). Meanwhile, the expression of genes in the ABA-response pathway demonstrated that MAP3Kθ1 was involved in ABA signaling. A negative regulator of ABA signaling, *ABI1*, was upregulated in the *map3kθ1* mutant, and positive regulators of ABA signaling (*ABI3* and *ABI5*) were downregulated in the mutant (Fig. 4e–g).

When treated with exogenous ABA, the *map3kθ1* mutant became more sensitive in terms of root elongation. Especially under high ABA concentrations, the mutant exhibited less root elongation than the wild-type and *MAP3Kθ1*-overexpressing plants (Fig. 5b). Moreover, the water-holding capacity of leaves decreased when *MAP3Kθ1* gene expression was downregulated, which was validated by the observation of detached rosette leaves (Fig. 5c). The guard cells of

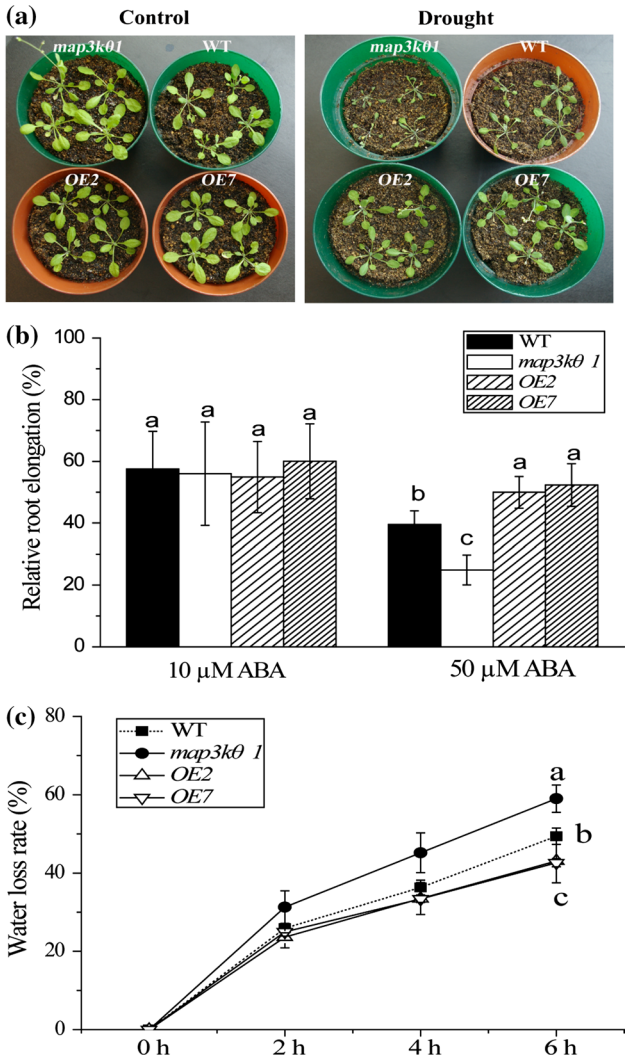

Fig. 5 Phenotypes of wild-type, *map3kθ1* mutant, and MAP3Kθ1-overexpressing *Arabidopsis* plants in response to water stress and ABA treatment. **a** Wild-type, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing seedlings (2 weeks old) were subjected to dehydration for 20 days. **b** Relative root length of seedlings on day 7 after transfer to MS medium containing 3% (w/v) sucrose with ABA (10 or 50 μM) compared to the control (without ABA). **c** Water loss as a percentage of fresh weight of detached rosettes from 4-week-old plants after 2 h, 4 h, and 6 h in a flow laminar hood. Values presented are the mean ± standard error of the mean for four replicates for **b**, **c**

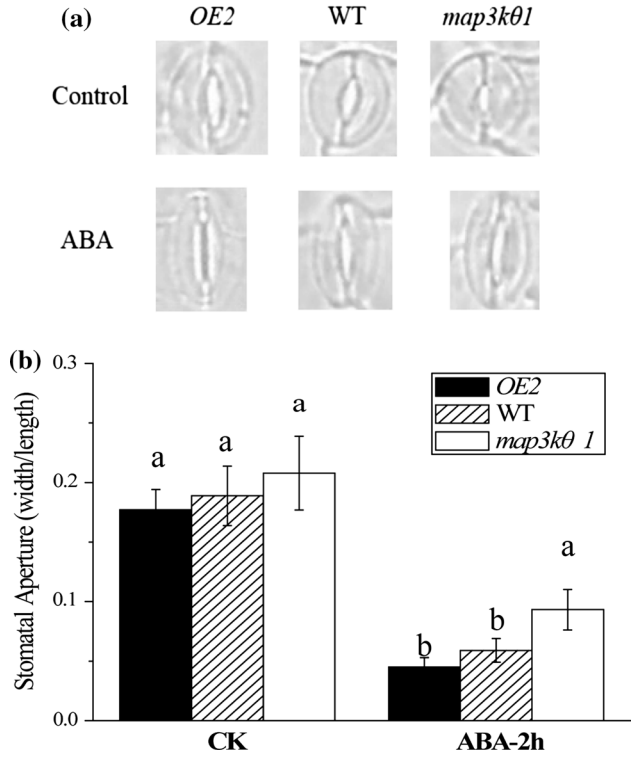

Fig. 6 Effects of ABA on stomatal closure in wild-type, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing *Arabidopsis* plants. **a** Representative photos of stomatal openings for the wild type, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing line (*OE2*) with or without 20 μM ABA treatment for 2 h. **b** Stomatal apertures (width/length) were measured with a project microscope. Fifty stomata were chosen for each determination and three determinations were performed for each group

stomata also possess the entire ABA biosynthesis pathway, which is regulated autonomously when plants are exposed to low air humidity, and is necessary and sufficient for stomatal closure (Bauer et al. 2013; Li et al. 2018). Thus, the low water-holding capacity of leaves in the *map3kθ1* mutant was likely related to ABA signaling. Indeed, the stomata openings were relatively narrow in the *MAP3Kθ1*-overexpressing lines compared with the wild type under normal growth conditions. The absence of a significant difference in stomata openings among the *map3kθ1* mutant, wild type, and *MAP3Kθ1*-overexpressing lines might be attributed to insufficient changes in ABA content. Under treatment with ABA at some concentrations, there was a degree of opening in the wild type and *map3kθ1* mutant, but stomata were mostly closed in the *MAP3Kθ1*-overexpressing line *OE2* (Fig. 6). The shortened roots and low water-holding capacity of leaves due to the loss-of-function of *MAP3Kθ1* made the *map3kθ1* mutant more sensitive to water stress (Fig. 5a). Another MAP3K, YODA, also plays a role in drought tolerance and water use efficiency by regulating stomatal density and root length (Meng and Yao 2015). Although *MAP3Kθ1* appeared to have a similar function to that of *YODA*, we did not identify a role in regulating stomatal density.

Many reports have shown that ABA plays an important regulatory role in maintaining seed dormancy and inhibiting germination (Liu et al. 2010; Kim et al. 2013; Shu et al. 2016). Our results showed that the wild type and *map3kθ1* mutant germinated well under normal conditions, whereas the *MAP3Kθ1*-overexpressing line had a reduced germination rate. Under exogenously applied ABA, we observed a more rapid decrease in germination

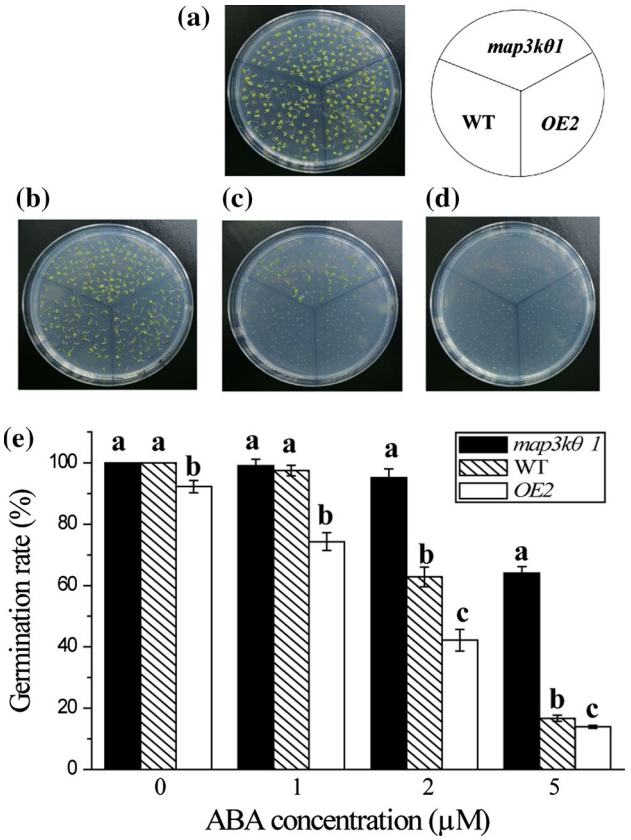

Fig. 7 Effects of ABA on seed germination for wild-type, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing *Arabidopsis* plants. **a**–**d** Images of wild-type, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing (*OE2*) 7-day-old seedlings on MS medium with and without 1 μM, 2 μM, and 5 μM ABA supplementation. **e** Germination rates of the wild-type, *map3kθ1* mutant, and *OE2* strains treated by ABA on day 7. Values are expressed as the means and standard errors of the mean ($n=3$) for **e**. Means denoted by different letters significantly differ at $P<0.05$ based on Duncan's multiple range test

rate for the *MAP3Kθ1*-overexpressing line than in the wild type. Moreover, the germination rate of *map3kθ1* was significantly lower than that of the wild type when treated with exogenous ABA (Fig. 6). This further demonstrated that MAPK cascades are involved in ABA signaling in seed germination (Xing et al. 2007; Zhang et al. 2007).

Based on our findings, we propose that *MAP3Kθ1* promotes ABA accumulation by inducing its biosynthesis and inhibiting its catabolism, and that the ABA signaling module is located downstream of *MAP3Kθ1* and regulates stomatal movement, root elongation, and seed germination. Accumulated ABA modulates the expression of *MAP3Kθ1* in a feedback inhibition manner, while H_2O_2 mediates the regulation of *MAP3Kθ1* via ABA (Fig. 8).

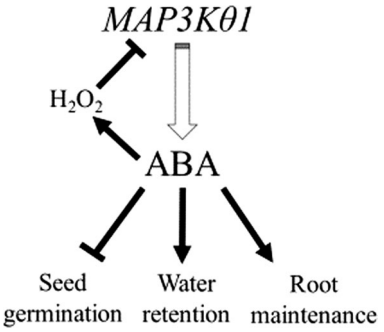

Fig. 8 Proposed working models for ABA signaling involving MAP3Kθ1 and its regulation mediated by the ABA–H_2O_2 signaling module in *Arabidopsis*

Materials and Methods

Plant Materials

We used *Arabidopsis thaliana* (L.) Col-0 ecotype and mutant with the Col-0 background in our experiments. Plants were cultured in a controlled room with a 16/8-h light/dark cycle at 22 ± 2 °C and a relative humidity of 80%. The photon flux density was 200–500 μmol m^{-2} s^{-1} at daytime for cultivation. All seed lots used for experimental material were harvested concurrently. All seed samples were put in a dehumidifier container to break dormancy for at least 2 months until further experiments.

Identification and Isolation of the *map3kθ1* Mutant

The T-DNA insertion mutant (SALK_137974) of the *MAP3Kθ1* (*AT2G31010*) gene was acquired from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA). Kanamycin-resistant plants were screened first on kanamycin-containing medium, and surviving plants were transferred to soil. The plants were cultured normally until harvest, and seeds were stored separately for individual plants.

The chloroform extraction method was used to extract genomic DNA from main leaves to confirm homozygosity by PCR. Gene-specific primers (LP, 5′-TGAGCTGTTTTC ATCCTCTCG-3′; RP, 5′-ATACATTGGCACGACCTCTTG-3′) and a left-border-specific primer (LBb1.3, 5′-ATTTTG CCGATTTCGGAAC-3′) were used for identification.

Generation of *MAP3Kθ1*-overexpressing Lines

Full-length cDNA of the *Arabidopsis MAP3Kθ1* gene was cloned by RT-PCR, fused with the pENTR-TOPO cloning vector (Invitrogen), and validated by sequencing. The primers used to confirm *MAP3Kθ1* overexpression by PCR were as follows: forward primer, 5′-CACCATGGAAGAGAG

ACGAGATGATG-3′; and reverse primer, 5′- TTAGCACAG TGTGTACTCACAATCGA-3′. After LR reaction using the Gateway system, *MAP3Kθ1* cDNA was linked with the 35S promoter in the pGWB5 vector. The constructs in *Agrobacterium tumefaciens* (GV3101) were then transferred into Col0 wild type using the floral dip method (Clough and Bent, 1998). Transformed plants were screened by growth on hygromycin-containing medium, and homozygous T3 transgenic lines were used for further analyses.

GUS Activity Analysis

An 1868 bp DNA fragment from the upstream region of the *MAP3Kθ1* gene in *Arabidopsis* was amplified via PCR. Two restriction sites (XbaI and BamHI) were introduced at the ends of each of two primers for the construction of the plant expression vector. The forward and reverse primers were as follows: forward primer, 5′-AAtctagaGTATTGGGA ACCACCGAAAA-3′ (the underlined portion is the XbaI site); and reverse primer, 5′-AAggatccCTCACCATCTCT TTACCCCAAA-3′ (the underlined portion is the BamHI site). The amplification fragments and GUS expression vector (pBI101.3; Clontech) were separately digested with XbaI and BamHI and then linked. The resulting construct, *MAP3Kθ1pro-GUS*, was transferred into *Agrobacterium tumefaciens* GV3101. Genetic transformation of wild-type *Arabidopsis* plants was performed using the floral dip method as previously described (Clough and Bent, 1998). Independent transgenic lines were used for histochemical localization by GUS staining as described by Bechtold (1993). A 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) buffer (50 mM sodium phosphate buffer, 2% dimethyl sulfoxide, 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100, 2 mg mL^{-1} X-gluc, pH ~ 7.0) was prepared, and plant tissues were immersed in the buffer for 6–12 h at 37 °C. The stained plants were observed after washing with 50–100% ethanol for about 30 min. In situ GUS activity was monitored as described by Halder and Kombrink (2015).

For histochemical analysis of GUS activity of *MAP3Kθ1*, 0.1 mM ABA, 0.5 μM MV, or 20 mM H_2O_2 was sprayed onto the seedlings growing on MS medium. Seedlings were then cultivated for 5 h under normal growth conditions as previously described (Xing et al. 2007; Jia et al. 2012).

Drought Tolerance Analysis

For the drought assays, watering was withheld from 2-week-old soil-grown plants for a specified period. We used an equal number of plants per pot to minimize experimental variation. Photographs were taken after 20 days of water deprivation when phenotypes visibly differed among the wild-type, *map3kθ1* mutant, and *MAP3Kθ1*-overexpressing plants. Short-term assays were performed to detect the water-holding capacity of leaves. The detached rosettes of well-watered 4-week-old plants were allowed to dehydrate in a laminar flow hood for 2 h, 4 h, and 6 h. Then, fresh weight was measured at the beginning of treatment and various timepoints thereafter (Catala et al. 2007). The percentage loss of fresh weight was calculated based on the initial weight of the leaves.

Epidermal Strip Bioassay

A stomatal bioassay experiment was performed to assess the effects of ABA on stomatal movement as described by Xing et al. (2007) with slight modifications. Fresh abaxial epidermis was prepared and immersed in 10 mM MES and 50 mM KCl solution (pH 6.15), then incubated for 2 h under light (photon flux density of 0.2–0.3 mmol m^{-2} s^{-1}, 22–25 °C) to open the stomata. Epidermal samples were then transferred to ABA-containing (20 μM) MES–KCl buffer as described above. After incubation for 2 h, photos of stomatal apertures from ten separate plants were immediately taken under the microscope. Fifty randomly selected stomata per treatment were measured using ImageJ® software (http://rsbweb.nih.gov/ij/), and three replicates were used for each treatment.

Seed Germination Assays

Seed samples of the wild type, *map3kθ1* mutant, and *MAP3Kθ1* overexpression lines (*OE2/OE7*) were stored for 2 months after harvest, and then sterilized by washing with 70% (v/v) ethanol for 30 s and 2% hypochlorite for 10 min. Germination behavior was analyzed after rinsing the seeds four times with sterile water. About 100 seeds were chosen and spread in MS medium containing 0.8% (w/v) Bacto Agar (Difco/BD) and 1% sucrose, with or without 1 μM, 2 μM, or 7 μM ABA. Plates containing spread seeds were kept at 4 °C for stratification and, after 3 days in darkness, the plates were transferred to a growth chamber for cultivation under normal conditions. Germination was recorded daily for seven sequential days, and the germination rates were calculated according to three independent experiments (Xi et al. 2010).

Root Elongation Analysis

Seedlings germinated on MS medium were transferred to half-strength MS medium (1.0% agar) without or with 10 μM or 50 μM ABA. Six seedlings of the wild type, *map3kθ1* mutant, and *MAP3Kθ1* overexpression line were transferred to one half-strength MS medium plate and grown vertically for 7 days before measurement of primary root length. Four replicate plates were used. The percentage elongation of primary root was calculated based on the control (no ABA supplication in medium).

Total RNA Extraction, Semi-quantitative RT-PCR, and qRT-PCR

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. A 2-μg aliquot of RNA was reverse-transcribed into cDNA using a SuperScript III RTS first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Semi-quantitative RT-PCR for *MAP3Kθ1* transcript analysis was performed using the following primers: forward primer, 5′-CCACCTGAGAAGGTTGTCTTTG -3′; and reverse primer, 5′-CCTGTGAGAACATGAATCACACC-3′. *ACTIN* was amplified as an internal control using gene-specific oligonucleotides: forward primer, 5′- GTGAAGGCTGGATTTGCAGGA-3′; and reverse primer, 5′-CGAGCAAAACCAGTCCCA A-3′.

Transcript levels of *MAP3Kθ1* were measured for mutant screening, organ-specific expression analysis, and ABA treatment by qRT-PCR using an iCycler (Bio-Rad, Hercules, CA, USA). The *MAP3Kθ1* primers for qRT-PCR included the forward primer 5′-GGTGATTAAGAAAATAGCAGGAACGG-3′ and reverse primer 5′-TCGACAGCAACCACGCTTAATCT-3′. The primers for gene expression of ABA biogenesis and catabolism were as follows: NCED3-F, 5′-CGGTGGTTTACGACAAGAACAA-3′; NCED3-R, 5′-CAGAAGCAATCTGGAGCATCAA-3′; CYP707A1-F, 5′-TTGGAAAGAGGAGACTAGAG-3′; CYP707A1-R, 5′-GTGAACCACAAAAGAGGAAC-3′; CYP707A3-F, 5′-ATTCTTGTCCAGGCAATGAG-3′; and CYP707A3-R, 5′-ATAGGCAATCCATTCTGAGG-3′. The primers for gene expression of ABA signaling were as follows: ABI1-F, 5′-GGTTCGATGTTAGATGGTCGG-3′; ABI1-R, 5′-GAGCATCGGTTTCTCCTTAGCT-3′; ABI3-F, 5′-ACATCTCCAGCTCCTGTCA-3′; ABI3-R, 5′-TGCACCAGAAGAGTCGTCACAG-3′; ABI5-F, 5′-ATCAAGAACCGCGAGTCTGC-3′; and ABI5-R, 5′-TCCAACTCCGCCAATGCA-3′. The data were normalized based on the transcriptional levels of *ACTIN* in *Arabidopsis* using specific primers: forward primer, 5′- GGTGGTTCCATTCTTGCTTC-3′; and reverse primer, 5′-GGAGATCCACATCTGTAACCAAC-3′. The mean value of three replicates for each qRT-PCR reaction was used for the analyses.

Determination of Endogenous ABA Content

The radioimmunoassay method was used for ABA determination (Quarrie et al. 1988). Seedlings growing on MS agar plates were ground in liquid nitrogen and shaken overnight at 4 °C after adding 1 mL of water per 200 mg ground sample. The homogenates were centrifuged at 4 °C for 10 min at 12,000×*g*, and the supernatant was collected and used for ABA determination. The 450-μL reaction mixtures included 200 μL of phosphate buffer (pH 6.0), 100 μL of diluted antibody (Mac 252) solution [5 mg mL^{-1} bovine serum albumin and 4 mg mL^{-1} soluble polyvinylpyrrolidone in phosphate buffer (pH 6.0)], 100 μL of [^{3}H] ABA (~8000 cpm) solution, and 100 μL of crude extract. The solution was mixed thoroughly and held for 45 min at 4 °C, then precipitated with 50% saturated $(NH_4)_2SO_4$. The samples were centrifuged for 5 min at 12,000 g and the supernatant was poured off, after which 200 μL of double-distilled H_2O and 1.2 mL of scintillation fluid were added to the pellets. The bound radioactivity in pellets was measured using a liquid scintillation counter (Ye et al. 2011).

Statistical Analysis

All data were analyzed by analysis of variance using SigmaPlot 8.0 (Systat Software, San Jose, CA, USA) and the SPSS statistical analysis package (ver. 19.0; IBM Corp., Armonk, NY, USA). Means were compared through Duncan's multiple range tests at a 5% probability level.

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References

Arve LE, Carvalho DR, Olsen JE, Torre S (2014) ABA induces H_2O_2 production in guard cells, but does not close the stomata on *Vicia faba* leaves developed at high air humidity. Plant Signal Behav 9(7):1–9

Bauer H, Ache P, Lautner S, Fromm J, Hartung W, Al-Rasheid KA, Sonnewald S, Sonnewald U, Kneitz S, Lachmann N, Mendel RR, Bittner F, Hetherington AM, Hedrich R (2013) The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. Curr Biol 23(1):53–57

Bechtold N (1993) In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. Methods Mol Biol 82(10):259

Bi G, Zhou Z, Wang W, Li L, Rao S, Wu Y, Zhang X, Menke FLH, Chen S, Zhou JM (2018) Receptor-like cytoplasmic kinases directly link diverse pattern recognition receptors to the activation of mitogen-activated protein kinase cascades in *Arabidopsis*. Plant Cell 30:1543–1561

Catala R, Ouyang J, Abreu IA, Xu Y, Seo H, Zhang X, Chua NH (2007) The *Arabidopsis* E3 SUMO ligase SIZ1 regulates plant growth and drought responses. Plant Cell 19(9):2952–2966

Choi SW, Lee SB, Na YJ, Jeung SG, Kim SY (2017) *Arabidopsis* MAP3K16 and other salt-inducible MAP3Ks regulate ABA response redundantly. Mol Cells 40(3):230–242

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Danquah A, De ZA, Colcombet J, Hirt H (2014) The role of ABA and MAPK signaling pathways in plant abiotic stress responses. Biotechnol Adv 32(1):40–52

Danquah A, Zélicourt A, Boudsocq M, Neubauer J, Frei Dit Frey N, Leonhardt N, Pateyron S, Gwinner F, Tamby JP, Ortiz-Masia D, Marcote MJ, Hirt H, Colcombet J (2015) Identification and characterization of an ABA-activated MAP kinase cascade in *Arabidopsis thaliana*. Plant J 82(2):232–244

Fiil BK, Petersen K, Petersen M, Mundy J (2009) Gene regulation by MAP kinase cascades. Curr Opin Plant Bio 12(5):615–621

Guan LM, Zhao J, Scandalios JG (2000) Cis-elements and trans-factors that regulate expression of the maize Cat1 antioxidant gene in response to ABA and osmotic stress: H_2O_2 is the likely intermediary signaling molecule for the response. Plant J 22(2):87–95

Gudesblat GE, Iusem ND, Morris PC (2007) Guard cell-specific inhibition of *Arabidopsis* MPK3 expression causes abnormal stomatal responses to abscisic acid and hydrogen peroxide. New Phytol 173(4):713–721

Halder V, Kombrink E (2015) Facile high-throughput forward chemical genetic screening by in situ monitoring of glucuronidase-based reporter gene expression in *Arabidopsis thaliana*. Front Plant Sci 6(13):1–12

Jammes F, Song C, Shin D, Munemasa S, Takeda K, Gu D, Cho D, Lee S, Giordo R, Sritubtim S, Leonhardt N, Ellis BE, Murata Y, Kwak JM (2009) MAP kinases MPK9 and MPK12 are preferentially expressed in guard cells and positively regulate ROS-mediated ABA signaling. Proc Natl Acad Sci USA 106(48):20520–20525

Jia L, Sheng Z, Xu W, Li Y, Liu Y, Xia Y, Zhang J (2012) Modulation of anti-oxidation ability by proanthocyanidins during germination of *Arabidopsis thaliana* seeds. Mol Plant 5:472–481

Jiang M, Zhang J (2001) Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. Plant Cell Physiol 42(11):1265–1273

Kim D, Cho YH, Ryu H, Kim Y, Kim TH, Hwang I (2013) BLH1 and KNAT3 modulate ABA responses during germination and early seedling development in *Arabidopsis*. Plant J 75(5):755–766

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci USA 97:2940–2945

Lampard GR, Lukowitz W, Ellis BE, Bergmann DC (2009) Novel and expanded roles for MAPK signaling in *Arabidopsis* stomatal cell fate revealed by cell type-specific manipulations. Plant Cell 21:3506–3517

Li W, De CO, Dodd IC (2018) Long-distance ABA transport can mediate distal tissue responses by affecting local ABA concentrations. J Integr Plant Biol 60(1):16–33

Liu Y (2012) Roles of mitogen-activated protein kinase cascades in ABA signaling. Plant Cell Rep 31:1–12

Liu Y, Ye N, Liu R, Chen M, Zhang J (2010) H_2O_2 mediates the regulation of ABA catabolism and GA biosynthesis in *Arabidopsis* seed dormancy and germination. J Exp Bot 61(11):2979–2990

MAPK Group (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci 7(7):301–308

Matsuoka D, Yasufuku T, Furuya T, Nanmori T (2015) An abscisic acid inducible *Arabidopsis* MAPKKK, MAPKKK18 regulates leaf senescence via its kinase activity. Plant Mol Biol 87(6):565–575

Meng L, Yao S (2015) Transcription co-activator *Arabidopsis* ANGUSTIFOLIA3 (AN3) regulates water-use efficiency and drought tolerance by modulating stomatal density and improving root architecture by the transrepression of YODA (YDA). Plant Biotechnol J 13(7):893–902

Mishra G, Zhang W, Deng F, Zhao J, Wang X (2006) A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in *Arabidopsis*. Science 312(5771):264–266

Nakashima K, Yamaguchishinozaki K (2013) ABA signaling in stress-response and seed development. Plant Cell Rep 32(7):959–970

Quarrie SA, Whitford PN, Appleford NEJ, Wang TL, Cook SK, Henson IE, Loveys BR (1988) A monoclonal antibody to (s)-abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. Planta 173(3):330–339

Shu K, Liu XD, Xie Q, He ZH (2016) Two faces of one seed: hormonal regulation of dormancy and germination. Mol Plant 9(1):34–45

Su SH, Krysan PJ (2016) A double-mutant collection targeting map kinase related genes in *Arabidopsis* for studying genetic interactions. Plant J 88(5):867–878

Tajdel M, Mituła F, Ludwików A (2016) Regulation of *Arabidopsis* MAPKKK18 by ABI1 and SnRK2, components of the ABA signaling pathway. Plant Signal Behav 11(4):e1139277

Umezawa T, Sugiyama N, Takahashi F, Anderson JC, Ishihama Y, Peck SC, Shinozaki K (2013) Genetics and Phosphoproteomics Reveal a Protein Phosphorylation Network in the Abscisic Acid Signaling Pathway in *Arabidopsis thaliana*. Sci Signal 6(270):rs8

Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S (2007) Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in *Arabidopsis*. Plant Cell 19:63–73

Wu Y, Li Y, Liu Y, Xie Q (2015) Cautionary notes on the usage of abi1-2 and abi1-3 mutants of *Arabidopsis* ABI1 for functional studies. Mol Plant 8(2):335–338

Xi D, Liu W, Yang G, Wu C, Zheng C (2010) Seed-specific overexpression of antioxidant genes in arabidopsis enhances oxidative stress tolerance during germination and early seedling growth. Plant Biotechnol J 8(7):796–806

Xing Y, Jia W, Zhang J (2007) AtMEK1 mediates stress-induced gene expression of CAT1 catalase by triggering H_2O_2 production in *Arabidopsis*. J Exp Bot 58(11):2969–2981

Xing Y, Jia W, Zhang J (2008) AtMKK1 mediates ABA-induced CAT1 expression and H_2O_2 production via AtMPK6-coupled signaling in *Arabidopsis*. Plant J 54(3):440–451

Ye N, Zhu G, Liu Y, Li Y, Zhang J (2011) ABA controls H_2O_2 accumulation through the induction of OsCATB in rice leaves under water stress. Plant Cell Physiol 52(4):689–698

Yoshida T, Mogami J, Yamaguchishinozaki K (2014) ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. Curr Opin Plant Bio 21(21C):133–139

Zelicourt AD, Colcombet J, Hirt H (2016) The role of MAPK modules and ABA during abiotic stress signaling. Trends Plant Sci 21(8):677–685

Zhang A, Jiang M, Zhang J, Tan M, Hu X (2006) Mitogen-activated protein kinase is involved in abscisic acid-induced antioxidant defense and acts downstream of reactive oxygen species production in leaves of maize plants. Plant Physiol 141:475–487

Zhang A, Jiang JH, Ding HD, Xu SC, Hu XL, Tan MP (2007) Nitric oxide induced by hydrogen peroxide mediates abscisic acid-induced activation of the mitogen-activated protein kinase cascade involved in antioxidant defense in maize leaves. N Phytol 175(1):36–50

Zhang H, Liu Y, Feng W, Yao D, Wang L, Guo J, Ni L, Zhang A, Tan M, Jiang M (2014) A novel rice C2H2-type zinc finger protein, ZFP36, is a key player involved in abscisic acid-induced antioxidant defence and oxidative stress tolerance in rice. J Exp Bot 65(20):5795–5809

Zhao C, Wang P, Si T, Hsu CC, Wang L, Zayed O, Yu Z, Zhu Y, Dong J, Tao WA, Zhu JK (2017) Map kinase cascades regulate the cold response by modulating ICE1 protein stability. Dev Cell 43(5):618–829