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Running Title: KAT2 functions in ABA signaling

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Arabidopsis 3-Ketoacyl-CoA Thiolase-2 (KAT2), an Enzyme of Fatty Acid β-oxidation, Is Involved in Abscisic Acid Signal Transduction

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Abbreviations: 2, 4-DB, 2, 4-dichlorophenoxybutyric acid; ABA, abscisic acid; ABI5, ABA Insensitive 5; ABRC, Arabidopsis Biological Resource Center; CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; IAA, Indole Acetic Acid; JA, Jasmonic Acid; KAT2, 3-ketoacyl-CoA thiolase-2; ROS, reactive oxygen species

Abstract

The phytohormone abscisic acid (ABA) plays an important role in plant development and adaptation to diverse environmental stresses. Many of the components involved in ABA signaling remain to be discovered and knowledge of these is needed to fully understand the highly complex ABA signaling network. Here, we report that an enzyme catalyzing β-oxidation of fatty acids, 3-ketoacyl-CoA thiolase-2 (KAT2/PED1/PKT3) (EC 2.3.1.16), is involved in ABA signaling. We provided genetic evidence that KAT2 positively regulates ABA signaling in all the major ABA responses, including ABA-induced inhibition of seed germination and postgermination growth arrest, and ABA-induced stomatal closure and stomatal opening inhibition in *Arabidopsis thaliana*. KAT2 was shown to be important for ROS production in response to ABA, suggesting that KAT2 regulates ABA signaling at least partly through modulating ROS homeostasis in plant cells. Additionally, we provided data suggesting that KAT2 may function downstream of an important WRKY transcription repressor WRKY40, which may link KAT2 with the ABA receptor ABAR/CHLH-mediated signaling.

Keywords: ABA signaling, *Arabidopsis thaliana*, KAT2, fatty acid β-oxidation, ROS

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Introduction

The phytohormone abscisic acid (ABA) plays a key role in a wide range of biological processes throughout plant growth and development, including seed germination, postgermination growth and embryo maturation. ABA is also a key hormone in the regulation of plant adaptation to various adverse conditions, such as drought, salt and cold stresses (Leung and Giraudat, 1998; Finkelstein et al., 2002; Himmelbach et al., 2003; Shinozaki et al., 2003). ABA signal transduction has been extensively studied, and numerous signaling components, including three types of ABA receptors, have been identified during the past two decades. These signaling components involve diverse regulators, such as plasma membrane proteins (G-protein coupled receptors, phospholipases C/D and receptor-like kinases) and intracellular proteins (START domain proteins, chloroplast magnesium-chelatase, type 2C/2A protein phosphatases (PP2C/PP2A), SNF1-related protein kinases (SnRK), calcineurin B-like protein kinases (CIPK), calcium-dependent protein kinases (CDPK), mitogen-activated protein kinases (MAPK), E3 ligases, and various classes of transcription factors) (Leung and Giraudat, 1998; Finkelstein et al., 2002; Wang, 2002; Himmelbach et al., 2003; Shinozaki et al., 2003; Fan et al., 2004; Shen et al., 2006; Hirayama and Shinozaki, 2007; Seki et al., 2007; Fujii et al., 2009; Ma et al., 2009; Pandey et al., 2009; Park et al., 2009; Wu et al., 2009; Cutler et al., 2010; Shang et al., 2010). These discoveries deepen significantly our understanding of ABA signaling from primary signal perception events to downstream gene expression. However, it is widely believed that ABA signal transduction involves highly complex signaling pathways. Thus, additional components remain to be identified to understand fully the complex ABA signaling network.

3-ketoacyl-CoA thiolase (KAT) is an important enzyme involved in fatty acid degradation. Fatty acids are stored in the form of triacylglycerides, which can be hydrolyzed by lipases and then activated to fatty acyl-CoA by fatty acyl CoA synthetase before degradation through β-oxidation cycle in peroxisomes. The last step of the β-oxidation cycle requires a KAT enzyme catalyzing fatty ketoacyl-CoA to produce one acetyl-CoA molecule, which further generates succinate through the glyoxylate cycle (Beevers, 1961). In the *Arabidopsis* genome, there are three homogeneous genes in the *KAT*-family: At1g04710, At2g33150 and At5g48880 encoding KAT1, KAT2 and KAT5, respectively (Germain et al., 2001). Despite high identity with each other, KAT2 plays the central role in β-oxidation and its expression is much higher than the other thiolases (Zimmermann et al., 2004; Castillo et al., 2004; Afitlhile et al., 2005).

Recently, the β-oxidation cycle has been shown to be involved in hormone signaling pathways. *ped1*, a

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single-base substitution mutant, which lacks KAT2 protein and showed abnormal morphology of peroxisomes, could survive in the presence of toxic level of 2, 4-dichlorophenoxybutyric acid (2, 4-DB, precursor of 2, 4-D), but postgermination it required exogenous sucrose (Hayashi et al., 1998; Germain et al., 2001). A T-DNA insertion mutant *kat2-1* showed increased flowering but reduced reproductive success, indicated the balancing role of β-oxidation in the transition of vegetative to reproductive phase (Footitt et al., 2007). Overexpression of *KAT2* increased biosynthesis of JA and accelerated dark-induced senescence of leaves, and the opposite characteristics were observed in antisense transgenic lines for *KAT2* (Castillo and Leon, 2008). Most recently, mutation in a peroxisomal ATP-binding cassette transporter PED3, required for β-oxidation, was shown to impair the seeds germination, and genetic data showed that PED3 functions upstream of ABA-insensitive 5 (ABI5) (Kanai et al., 2010). However, the major ABA-related phenotypes, including ABA-induced inhibition of seed germination and postgermination growth arrest, and ABA-induced promotion of stomatal closure and inhibition of stomatal opening, were not tested in the *ped3* mutants, and only an increase in *ABI5* expression level was demonstrated in the *ped3-3* mutant, which suggests that PED3 may negatively regulate ABA signaling by repressing *ABI5* expression (Kanai et al., 2010). Nevertheless, it remains largely unclear whether KAT2 or KAT2-catalyzed β-oxidation of fatty acids is involved in ABA signaling.

It is well known that reactive oxygen species (ROS) is involved in ABA signaling (Pei et al., 2000; Murata et al., 2001; Mustilli et al., 2002; Kwak et al., 2006; Miao et al., 2006; Zhang et al., 2009). One of the subcellular compartments producing ROS in cells is the peroxisome, in which the photorespiration glycolate oxidase reaction and the fatty acid β-oxidation take place. Fatty acid β-oxidation can produce H_2O_2 in the dark or in non-photosynthetic tissues (Mittler, 2002). On the basis of this information, we presume that KAT2 may be involved in ABA signaling through regulating ROS production in plant cells. Here, we report that KAT2 is important for ROS production in response to ABA, and positively regulates ABA signaling in all the major ABA responses, including ABA-induced inhibition of seed germination and postgermination growth arrest, and ABA-induced stomatal closure and stomatal opening inhibition in *Arabidopsis*. Also, we provided data suggesting that KAT2 may function downstream of an important WRKY transcription repressor, WRKY40, which may link KAT2 with the ABA receptor ABAR/CHLH-mediated signaling.

Results

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Identification of a null mutant of *KAT2* gene and generation of KAT2-overexpression lines

We isolated a mutant line (SALK_021650) (Col ecotype background) by screening the SALK T-DNA insertion mutants of the gene *At2g33150* from the Arabidopsis Biological Resource Center (ABRC). The mutant line is named *kat2-3* and shows an insensitive response to ABA. Genotyping assays showed that the T-DNA segment (or more than one closely linked segments) is inserted in the 14th exon of the *KAT2* gene, which generates a 36-bp deletion from 3109 to 3144 bp downstream of the translation start codon (Fig. 1A). Real-time PCR and immunoblotting analysis showed that the mutant does not produce the corresponding mRNA or protein in contrast to the wild-type plants (Fig. 1B), demonstrating that *kat2-3* is a null mutant allele of the *KAT2* gene due to the T-DNA insertion.

We failed to identify other *kat2* mutant alleles from ABRC lines. To ensure that the *kat2-3* null mutant allele is responsible for the observed ABA-related phenotypes, we crossed the mutant to the wild-type (Col), and found that the inheritance ratio of kanamycin resistance of the seeds from heterozygotes grown on medium containing kanamycin (50 μg/ mL) was about 3:1. This genetic segregation analysis indicated the presence of a single T-DNA insertion site (or more than one closely linked site) in *kat2-3*. Phenotype analysis showed that the heterozygous F1 plants have normal ABA responses, as in the background wild-type, and the F2 progeny segregated in a 3:1 Mendelian ratio (181 normal plants to 55 ABA-insensitive plants, $\chi^2 = 0.36 < \chi^2_{0.95}$), indicating that the mutation was inherited as a single recessive locus. ABA-insensitive phenotypes were identified as homozygote for T-DNA insertion in the *KAT2* gene by genotyping, suggesting that the mutant phenotype is closely linked to the T-DNA insertion locus.

We also created *KAT2*-overexpression lines under the control of the cauliflower mosaic virus (CaMV) 35S promoter and obtained eight transgenic lines. We took *35S-KAT2* line4 (OE4) and *35S-KAT2* line6 (OE6) as representatives, because all of the overexpression transgenic lines exhibited upregulated *KAT2* expression profiles and similar ABA-related phenotypes. Real-time PCR and immunoblotting assays showed that both mRNA and protein levels of KAT2 are significantly increased in OE4 and OE6 plants in comparison with those in wild-type plants (Fig. 1B).

Disruption of *KAT2* reduces, but overexpression of *KAT2* enhances, ABA sensitivity of the major ABA responses

The seeds of the loss-of-function mutant *kat2-3* and the *KAT2*-overexpression lines OE4 and OE6

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germinated normally, as did the wild-type Col seeds, in the ABA-free MS media (Fig. 1C). However, in the media supplemented with different concentrations of (±)-ABA (0.5, 1 or 3 μM), the *kat2-3* seeds germinated rapidly, even in the medium containing high levels of ABA (1 and 3 μM), showing obvious ABA insensitive phenotype compared with the wild-type seeds (Fig. 1C). On the contrary, the OE4 and OE6 seeds germinated slowly, showing ABA hypersensitive phenotype compared with the wild-type seeds (Fig. 1C).

To investigate the ABA response of early seedling growth after germination, seeds were directly planted in (±) ABA-containing medium (0.3, 0.5 or 0.8 μM). When grown for 10 days (after stratification) in the medium supplemented with 0.8-μM (±) ABA, seedlings of *kat2-3* grew slightly, but significantly, better than wild-type Col seedlings (Fig. 1D and 1E). In contrast, the growth of OE4 and OE6 seedlings was significantly more reduced than that of Col seedlings in the ABA-containing media (Fig. 1D and 1E). To ensure that the effect of ABA on postgermination growth (Fig. 1D and 1E) is not due to the differences in seed germination speed when directly planted on ABA-containing MS medium (Fig. 1C), we used another approach to assess the response of seedling growth to ABA. Germinating seeds were transferred 48 h after stratification from the ABA-free MS medium to (±) ABA-containing MS (0.5, 1, 2 μM) medium, and similar phenotypes were observed 14 days later (Fig. 1F and 1G).

Stomatal apertures were investigated in 30-μM-(±) ABA treated leaves of the *kat2-3* mutant, OE4 and OE6 lines, and wild-type plants (Fig. 1H-I). Compared with wild-type plants, the *kat2-3* mutant plants showed ABA-insensitive phenotypes, but the KAT2-overexpression transgenic lines showed strong ABA-hypersensitive phenotypes, in both ABA-induced promotion of stomatal closure (Fig. 1H) and ABA-induced inhibition of stomatal opening (Fig. 1I).

ABA-induced stimulation of *KAT2* expression is abolished in *wrky40-1* mutant

The *KAT2*-promoter-GUS transgenic lines showed that the *KAT2* gene is expressed ubiquitously in different tissues (Fig. 2A), which is in agreement with the previous reported immunoblot analysis (Footitt et al., 2007). Previous northern blot analysis showed that mRNA levels of *KAT2* increased in response to exogenous ABA application (Castillo et al., 2004). We confirmed this observation with both the *KAT2*-promoter-GUS transgenic manipulation and real-time PCR analysis (Fig. 2B and 2C), and further showed, with immunoblotting assays, that the levels of KAT2 protein were also stimulated by exogenous ABA application (Fig. 2C). Interestingly, we observed that the KAT2 mRNA levels increased in a loss-of-function *wrky40-1* mutant of the *WRKY40* gene, but the ABA-induced stimulation of *KAT2*

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expression was abolished by the loss-of-function *wrky40-1* mutation (Fig. 2C), suggesting that WRKY40 is important for KAT2 expression in response to ABA.

ABA responsiveness of ROS homeostasis is altered by disruption mutation or overexpression of *KAT2*

ROS levels were assayed in the *kat2-3* mutant and OE4 plants to gain insight into the mechanism of KAT2-mediated ABA signaling. The intensity of ROS staining in leaves was shown to increase after treatment with 5-µM (±) ABA in the wild-type Col plants (Fig. 3A and 3B), which is consistent with previous studies (Pei et al., 2000; Murata et al., 2001). However, the ROS level decreased in the *kat2-3* mutant, and the responsiveness of the ROS level to ABA, as observed in wild-type leaves, was substantially abolished in this mutant (Fig. 3A and 3B). In contrast to the *kat2-3* mutant, overexpression of *KAT2* (OE4 line) resulted in a significant increase in ROS level (Fig. 3A and 3B). Surprisingly, however, the ROS level in OE4 leaves decreased by 5-µM (±) ABA treatment (Fig. 3A and 3B). The function of ABA in ROS homeostasis may involve both ROS production and degradation (Pei et al., 2000; Murata et al., 2001; Mustilli et al., 2002; Kwak et al., 2006; Miao et al., 2006; Zhang et al., 2009). Downregulation or upregulation of KAT2 expression may affect the balancing mechanism of ROS production and degradation in plant cells, suggesting that KAT2 is involved in the ABA signaling pathway through regulation of ROS homeostasis in response to ABA.

Disruption of *KAT2* alters the expression of a set of ABA-responsive genes

To investigate the expression of ABA-responsive genes in the knockout mutant *kat2-3*, we treated plants of wild type Col and the *kat2-3* mutant with 100 µM (±) ABA. These assayed ABA-responsive genes were: *ABAR* (Shen et al., 2006, Wu et al., 2009), *ABF*s (*ABF1*, *ABF2*/*AREB1*, *ABF3* and *ABF4*/*AREB2*) (Choi et al., 2000; Uno et al., 2000), *ABI1* (Leung et al., 1994; Meyer et al., 1994; Gosti et al., 1999), *ABI2* (Leung et al., 1997), *ABI4* (Finkelstein et al., 1998), *ABI5* (Finkelstein and Lynch, 2000), *DREB1A* and *DREB2A* (Liu et al., 1998), *EM1* and *EM6* (Gaubier et al,. 1993; Devic et al., 1996), *ERD10* (Kiyosue et al. 1994), *KIN1* and *KIN2* (Kurkela and Borg-Franck, 1992), *MYB2* and *MYC2* (Abe et al., 2003), *RAB18* (Lang and Palva, 1992), *RD29A* (Yamaguchi-Shinozaki and Shinozaki, 1994), three *SnRK*s (*SnRK2.2*, *SnRK2.3*, *SNRK2.6*/*OST1*) (Fujii and Zhu, 2009), and *WRKY40* (Shang et al., 2010). Consistent with previous reports, expression of the most ABA-responsive genes, except for *DREB2A* and *WRKY40,* was stimulated by the ABA treatment in wild-type plants (Fig. 4). WRKY40 is a negative ABA signaling regulator and its

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expression is repressed by ABA (Shang et al., 2010), which was confirmed in this assay with wild-type plants (Fig. 4).

Disruption of KAT2 in the *kat2-3* mutant reduced expression levels of the most ABA-positive-regulator-encoding genes *ABF1*, *ABI4*, *ABI5*, *DREB1A*, *DREB2A*, *ERD10*, *KIN1*, *KIN2*, *RD29A*, *SnRK2.2*, *SnRK2.3* and *SnRK2.6*, and enhanced expression levels of the ABA-negative-regulator-encoding genes *ABI2* and *WRKY40,* and also those of the ABA-positive-regulator-encoding genes *ABAR*, *MYB2* and *MYC2* (Fig. 4). This is essentially consistent with the positive role of KAT2 in the ABA signaling pathway, and also suggests the complexity of KAT2-mediated ABA signaling.

ABA-responsiveness of expression of these genes was also altered by disruption of *KAT2*. The ABA-stimulation responsiveness of some positive ABA-response/signaling genes was significantly reduced in the *kat2-3* mutant compared with that in wild-type plants, which included *ABF1*, *ABF2*, *ABF3*, *ABF4*, *RAB18*, and *ABAR* (Fig. 4), suggesting that ABAR and the ABFs/AREB1/2 transcription factors may play important roles in the KAT2-mediated ABA signaling, and that ABAR may function upstream of KAT2, but KAT2 may act on ABAR expression, possibly by a feed-back effect. ABA-stimulation responsiveness of other ABA-response/signaling genes (*ABI2*, *ABI4*, *ABI5*, *DREB1A*, *DREB2A*, *EM1*, *EM6*, *ERD10*, *KIN1*, *KIN2*, *MYB2*, *MYC2*, *RD29A*, *SnRK2.2*, *SnRK2.3*, and *SnRK2.6*) was also altered in the *kat2-3* mutant, though to a similar extent to that in wild-type plants (Fig. 4). This suggests that these ABA signaling regulators may be involved in the KAT2-mediated ABA signaling, but either with a limited role or functioning upstream of KAT2. The ABA responsiveness of *ABI1* and *WRKY40* remained substantially unchanged (Fig. 4), most likely because these two regulators may function upstream of KAT2. Taken together, these data essentially support the positive role of KAT2 in the ABA signaling pathway, which may, however, involve complex feed-forward and feed-back mechanisms.

Discussion

KAT2 positively regulates ABA signaling

In the present study, we provided genetic evidence to reveal that KAT2 positively regulates ABA signaling in all the major ABA responses in *Arabidopsis,* including ABA-induced inhibition of seed germination and postgermination growth arrest, and ABA-induced promotion of stomatal closure and inhibition of stomatal opening (Fig. 1). Also, we showed that disruption or upregulation of *KAT2* expression altered ABA

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responsiveness of ROS homeostasis in plant cells (Fig. 3), suggesting that KAT2 is involved in the ABA signaling pathway, at least partly, through regulating ROS homeostasis in response to ABA. However, since the KAT-mediated β-oxidation pathway produces ROS as a by-product and also produces other metabolites, such as phytohormones IAA and JA, we do not exclude other possible mechanisms by which KAT is involved in ABA signaling. It is also noteworthy that the *KAT2*-overexpression lines displayed ABA-hypersensitive phenotypes, which suggest that these transgenic lines should have a more sensitively positive response of ROS accumulation to ABA, but the opposite phenomenon was observed (Fig. 3). Indeed, this aspect is difficult to explain by ROS accumulation in the leaves, which may also imply that mechanisms other than ROS regulation are involved in this KAT2-mediated ABA signaling.

Two other KAT genes, *KAT1* and *KAT5*, have been identified from the *Arabidopsis* genome (Germain et al., 2001), but expression analysis data from a microarray database showed that *KAT2* expression is several-fold higher than that of its two paralogues in most tissues (Zimmermann et al., 2004), suggesting that KAT2 function is more important than that of other KAT members. In the present experiments, the ABA-insensitive phenotype of *kat2-3* in seedling growth was not obvious, though it was statistically significant, in comparison with the ABA-insensitive phenotypes in seed germination and stomatal movement (Fig. 1). However, the overexpression lines of *KAT2* exhibited obvious ABA-hypersensitive phenotypes in all the ABA responses (Fig. 1). These data suggest that all three KAT proteins may redundantly regulate ABA signaling, though KAT2 may play the most important role.

It is noteworthy that the mutation in an ATP-binding cassette transporter PED3, which also impaired β-oxidation of fatty acids, appeared to function as a negative regulator of ABA signaling in seed germination (Kanai et al., 2010), which is in contrast to a positive role of KAT2 in ABA signaling. The different roles of KAT2 and PED3 in ABA signaling may be attributed to the possible different mechanisms by which these two proteins are involved in β-oxidation of fatty acids. PED3 is required for the import of long-chain fatty acids into the peroxisomes, and thus is indirectly involved in β-oxidation of fatty acids, while KAT2 is involved directly in β-oxidation of fatty acids. So, the responses of ROS homeostasis or other related signaling molecules to ABA may be different between the loss-of-function mutants of *kat2* and *ped3*, which may result in different physiological responses. This aspect needs further study. Whatever the mechanisms, the different function of KAT2 and PED3 may establish a feed-forward and feed-back regulation loop to coordinate ABA signaling.

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How does KAT2 work in ABA signaling?

Interestingly, we observed that the expression of *KAT2* was upregulated by the *WRKY40* loss-of-function mutation, but the ABA-stimulating effect on expression of *KAT2* was abolished in the *wrky40-1* knockout mutant (Fig. 2). WRKY40 was previously identified as an ABA-responsive WRKY transcription repressor involved in ABA signaling directly downstream of the ABA receptor ABAR/CHLH, which inhibits expression of a set of positive ABA signaling regulators (Shang et al., 2010). A search of the Arabidopsis genomic sequence showed that eight W-boxes [(T)(T)TGAC(C/T)] are present in the promoter region of the *KAT2* gene, which can recruit WRKY transcription factors. These data suggest that ABA may stimulate *KAT2* expression through the ABAR-WRKY40 coupled signaling pathway: WRKY40 represses *KAT2* expression; ABA inhibits WRKY40 expression via promotion of ABAR-WRKY interaction (Shang et al., 2010), and in this way ABA derepresses *KAT2* expression. KAT2 functions in ABA signaling at least partly by regulating ROS homeostasis in plant cells. However, we did not observe a significant difference in the *KAT2* expression levels between the *cch* mutant (a mutant allele of the *ABAR* gene) and wild-type plants. This may be explained by complex feed-forward and feed-back mechanisms in the ABAR-mediated signaling: ABAR may regulate, besides the WRKY40 transcription repressor, other downstream signaling components that may function to maintain KAT2 homeostasis*,* and the *cch* mutation may not disrupt the ability of plant cells to balance *KAT2* expression. Further studies will be needed to elucidate this interesting working model of the ABA signaling pathway.

Materials and Methods

Plant material and growth conditions

Arabidopsis KAT2 gene T-DNA insertion lines in the Col ecotype were obtained from the Arabidopsis Biological Resource Center (ABRC). The mutant lines were genotyped by amplifying the genomic DNA with the left genomic primer (LP) and right genomic primer (RP). These two genomic primers were used together with a T-DNA left border primer (LBa1) and a right border primer (Rba1) to constitute specific primer pairs for genotyping the T-DNA insertion line. The sequences for these primers are presented in Supplemental Table 1. The T-DNA insertion in the mutant was identified by PCR and the exact position was determined by sequencing. We identified a homozygous T-DNA insertion allele, SALK_024922, in the 14th exon of the KAT2 gene, designated *kat2-3*. For the *kat2-3* mutant, the PCR products could be generated with the primer pairs LP-RBa1 and RP-LBa1, but not with the primer pair LP-RP, indicating that a single

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copy (or more than one copy closely linked) of T-DNA was inserted into the genome.

To create transgenic plant lines overexpressing the *KAT2* gene, the open reading frame (ORF) for the *KAT2* gene was isolated by PCR using primers presented in Supplemental Table 1. The ORF of KAT2 was inserted into the pCAMBIA-1300-221 vector (http://www.Cambia.Org/daisy/cambia/materials/vectors/585.html) at the *Xba*I and *Sac*I sites under the control of a constitutive CaMV 35S promoter. The construct was verified by sequencing and introduced into the GV3101 strain of *Agrobacterium tumefaciens*. The construction was transformed by floral infiltration into plants of wild-type Col. Transgenic plants were selected by hygromycin resistance and confirmed by PCR. The homozygous T3 seeds of the transgenic plants were used for further analysis.

Plants were grown in a growth chamber at 21°C on MS medium (Sigma) at ~80 μmol photons m^{-2} s^{-1} or in compost soil at ~120 μmol photons m^{-2} s^{-1} cool-white fluorescent lamps with a photoperiod of 16h light/8h dark and 60% relative humidity.

Segregation analysis

To test co-segregation of the mutation in the *KAT2* gene and the mutant phenotype, *kat2-3* was crossed to the wild-type (Col), and 236 F2 plants were analyzed by an ABA-induced stomatal closure assay. As a result, 181 plants showed normal ABA response while 55 plants showed ABA-insensitive phenotype. The ratio was about 3:1, $\chi^2 = 0.36 < \chi^2_{0.95}$, which means that the data fall well within the range expected for 95% of sample sets occurring from segregation of single recessive genes. Furthermore, each plant was genotyped and the genotype was in accordance with the mutant phenotype and vice versa.

Real-time PCR analysis

Total RNA was isolated from leaves of 3 to 4-week-old *Arabidopsis* seedlings using Total RNA Rapid Extraction Kit (BioTeke, China) and treated with RNase-free DNaseI (Takara) at 37°C for 30 min to degrade genomic DNA and purified by using RNA Purification Kit (BioTeke, China). Two micrograms of RNA was subjected to first-strand cDNA synthesis using M-MLV reverse transcriptase (Promega), and an oligo (dT21) primer. The primers of various ABA-responsive genes used for real-time PCR are listed in the Supplemental Table 2. Analysis was performed using the BioRad Real-Time System CFX96TM C1000 Thermal Cycler (Singapore). All experiments were repeated at least three times along with three

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independent repetitions of the biological experiments.

Extraction of proteins and immunoblotting

Total protein extracts were obtained from *Arabidopsis* plants by grinding whole seedlings or leaf tissue first in liquid nitrogen and then on ice for 3 h in 1 volume of the extraction buffer. The extraction buffer consisted of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride, and 10 µL/mL protease inhibitor cocktail (FOCUS-Protease Arrest™ Cocktail, Merck). Lysates were cleared of debris by centrifugation at 12,000 g for 30 min at 4°C. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as a standard. The SDS-PAGE and immunoblotting assays were done essentially according to our previously described procedures (Wu et al., 2009; Shang et al., 2010). The antibody against KAT2 (see below for antibody production) was used for immunoblotting.

Production of anti-KAT2 serum

The full-length cDNA of *KAT2* was isolated using primers listed in the Supplemental Table 1. In brief, the PCR product was cloned into the EoRI (5′end) and XhoI (3′end) sites of pGEX-4T-1 (Amersham Pharmacia Biotec) for isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible tac promoter. To ensure that no errors were introduced by PCR, the construct was checked by sequencing. To produce glutathione *S*-transferase (GST) tag fusion protein, *Escherichia coli* DH5α cells transformed with pGEX-4T-1/KAT2 construct was induced with 0.2 mM IPTG for 4h at 30°C. The fusion protein was purified from IPTG-induced cell pellets by a Glutathione Sepharose 4B column (Amersham Pharmacia Biotech) and analyzed by SDS-PAGE. The purified fusion protein was used for standard immunization protocols in rabbits. Polyclonal antiserum obtained was affinity-purified by HiTrap Protein-A HP (Amersham Pharmacia Biotech). The affinity-purified antiserum was evaluated by immunoblotting and shown to be highly specific to KAT2.

***KAT2*-promoter-GUS transgenic assay**

The promoter fragments of the *KAT2* gene was amplified by PCR using primers listed in Supplemental Table 1, and linked to GUS. The construct was introduced into *Agrobacterium tumefaciens* strain GV3101 for transforming *Arabidopsis* (Col) plants by floral infiltration. Hygromycin-resistant transgenic seedlings of T3 plants were used for the analysis of GUS activity. For GUS staining, whole plants or tissues were

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immersed for 8h at 37°C in the medium containing 1 mM 5-bromo-4-chloro-3-indolyl-b-GlcUA (X-gluc) solution in 100 mM sodium phosphate (pH 7.0), 2 mM EDTA, 0.05 mM ferricyanide, 0.05 mM ferrocyanide, and 0.1% (v/v) Triton X-100. Chlorophyll was cleared from the tissues with a mixture of 30% acetic acid and 70% ethanol.

To investigate the expression of the *KAT2* gene after exogenous ABA treatment, 3 to 4-week-old T3 transgenic plants were sprayed with different concentrations of (±) ABA, and sampled 6h after spraying. Leaves were stained for 4h as described above.

Phenotypic analysis

For the germination assay, about 100 seeds each from wild type plants and mutants or transgenic lines were sterilized and planted in triplicate on MS medium. The medium contained 3% sucrose and 0.8% agar (pH 5.9) and supplemented with or without different concentrations of (±) ABA. The seeds were incubated at 4°C for 3 days and then placed at 21°C under light conditions. Germination (emergence of radicals) was scored at the indicated times.

We used two techniques to test the postgermination growth. One was that the seedling growth was assessed after directly planting the seeds in ABA-containing MS medium, and another was that germinating seeds were transferred 48 h after stratification from the common MS medium to ABA-containing MS medium, and seedling growth was examined 14 d after the transfer. The length of primary roots was measured using a ruler.

For the stomatal aperture assays, 3 to 4-week-old leaves were used. To observe ABA-induced stomatal closure, leaves were floated in buffer containing 50 mM KCl and 10 mM MES-KOH (pH 6.15) under a halogen cold-light source for 3h followed by addition of different concentrations of (±) ABA. Apertures were recorded on epidermal strips to estimate ABA-induced closure. To study ABA-inhibited stomatal opening, leaves were floated on the same buffer in the dark for 3h before they were transferred to the cold-light for 2.5h in the presence of ABA and the apertures were determined.

Measurements of ROS production

ROS production in leaves was detected by the nitroblue tetrazolium (NBT) staining method essentially as previously described (Lee et al., 2002). Leaves from 3 to 4-week-old plants were incubated in buffer composed of 50 mM KCl, 10 mM MES-KOH (pH 6.15) supplemented with 0 or 5 μM concentration of (±)

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ABA under light at 200 μmol m^{-2} sec^{-1} for 1 h, and then were vacuum-infiltrated with 0.1 mg/mL NBT (Amresco, Solon, OH, USA) in 100 mM potassium phosphate buffer (pH 7.6). Samples were subsequently transferred to 30°C in the dark for 2 h and boiled in 80% ethanol for 10min to remove chlorophylls. The stained samples were placed in 50% (v/v) glycerin and photographed.

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Figure Legends

Fig. 1 KAT2 positively regulates ABA signaling in seed germination, postgermination growth and stomatal movement. (A) T-DNA insertion site in *kat2-3* mutant (Col ecotype background). Boxes and lines represent exons and introns respectively. Arrows indicate the locations of the left genomic primer (LP) and right genomic primer (RP) for identification of the mutant. LB, left border of the T-DNA; RB, right border of the T-DNA; LBa1,left border primer for T-DNA; RBa1, right border primer for T-DNA; nt, nucleotides. One single copy of T-DNA was inserted into the promoter region at nt -3109 to -3144 in the last exon relative to the translation start codon (ATG) of *KAT2* gene with a 36-bp fragment deleted in the *kat2-3* mutant. (B) Real-time PCR and immunoblotting analysis of wild-type Col, homozygous *kat2-3* and two KAT2-overexpressing lines OE4 and OE6. For the real-time PCR analysis (top panel). The value obtained from the 3 to 4-week-old Col seedlings after stratification was taken as 100%, and all the other values were normalized relative to this value. Each value for real-time PCR is the mean ± SE of three independent biological determinations. Immunoblotting was performed with anti-KAT2 serum and the total proteins extracted from the leaves of the seedlings grown for 3-4 weeks after stratification (bottom panel); actin was used as a control. The immunoblotting assay was repeated three times with independent biological experiments and similar results were obtained. (C) Seed germination. Germination rates were recorded on MS medium supplemented with 0, 0.5, 1 or 3 μM (±) ABA, from 24h to 72h after stratification, for wild-type Col, *kat2-3* and two KAT2-overexpressing lines (OE4 and OE6). Each value is the mean ± SE of at least three biological determinations. (D) - (G) Postgermination growth. (D) and (E) Seeds were directly planted in MS medium supplemented with different concentrations of (±) ABA (0, 0.3, 0.5, or 0.8 μM), and the growth was investigated 10d after stratification. Photographs (E) were taken after the seedlings were removed from the media and rearranged on agar plates. Primary root lengths (D) were measured and each value is the mean ± SE of at least 30 seedlings. (F) and (G) Seedlings were transferred from ABA-free medium to medium supplemented with different concentrations of (±) ABA (0, 1.0, or 2.0 μM) 48 h after stratification, and the growth was investigated 14d after transfer. Photographs (G) were taken after the seedlings were removed from the media and rearranged on agar plates. Primary root lengths (F) were measured and each value is the mean ± SE of at least 30 seedlings. (H) and (I) Phenotypic analysis of ABA-induced stomatal closure (H) and ABA-inhibited stomatal opening (I) for wild-type Col, *kat2-3* and two KAT2-overexpressing lines (OE4 and OE6). Values are the means ± SE from three independent experiments; n = 60 apertures per experiment.

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Fig. 3 ABA-responsiveness of ROS production is altered by disruption or upregulation of *KAT2* expression. ROS was assayed in leaves of wild type Col, *kat2-3* mutant and a KAT2-overexpressor OE4 plants. Detached leaves were treated with 5 μM (±) ABA for 1 h and then stained with NBT. (A) Appearance of the NBT-stained leaves of different genotypes. Shown are representative leaves from three independent experiments. (B) Quantitative estimation of ROS levels in leaves of different genotypes. Relative ROS-staining intensities, estimated by scanning the staining profiles, were normalized relative to the ROS-staining intensity of Col (taken as 100%). Each value is the mean ± SE of three independent biological determinations.

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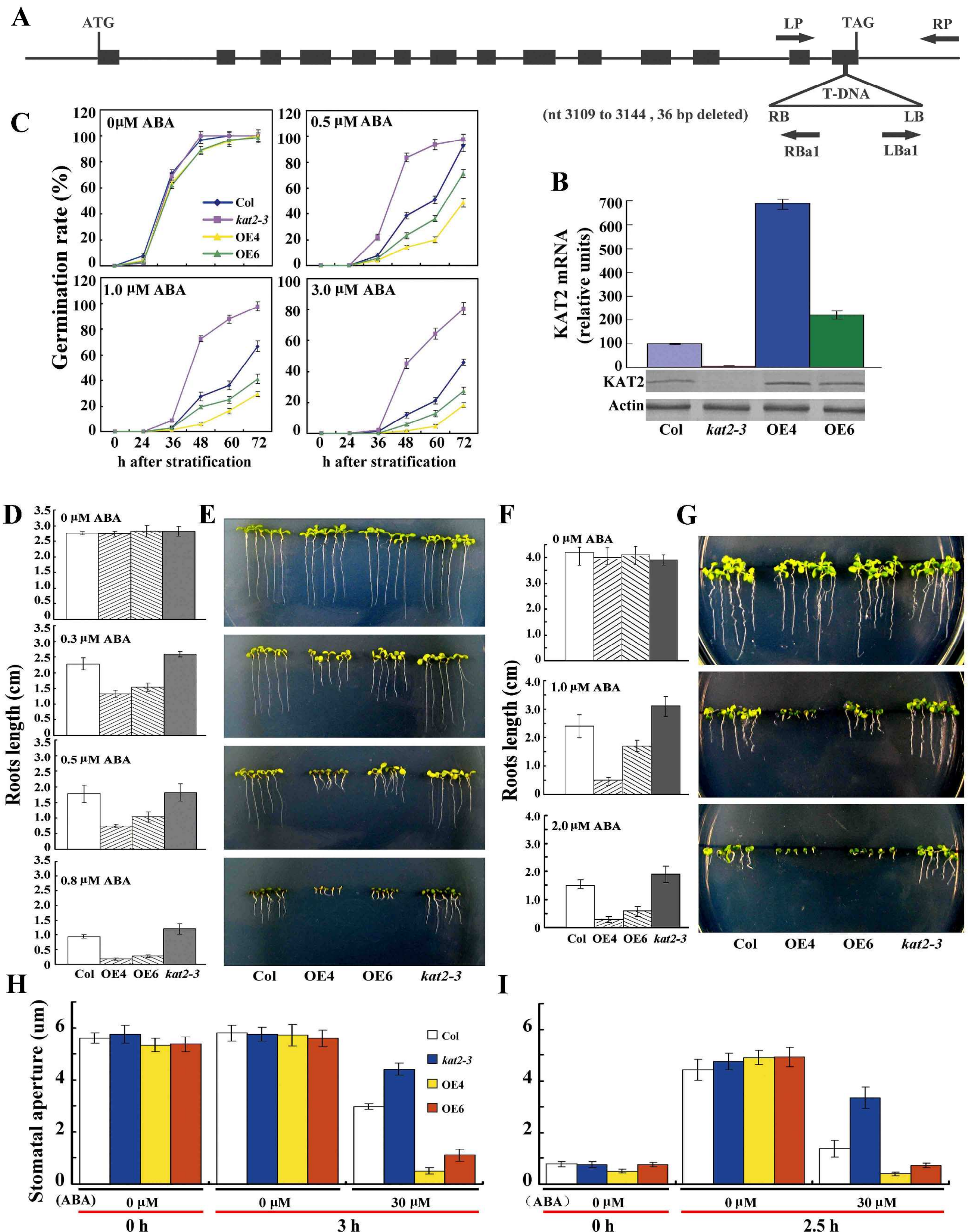

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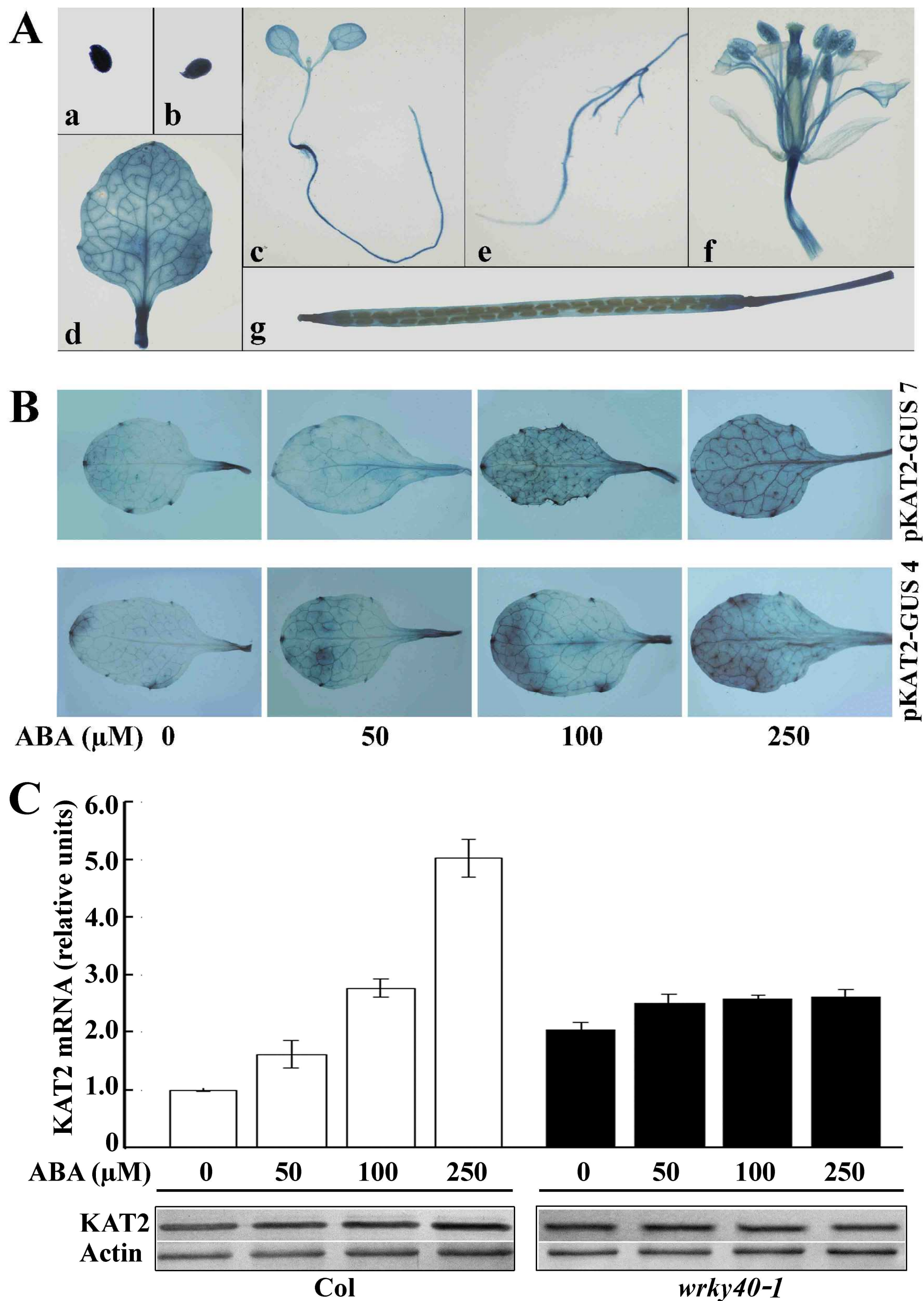

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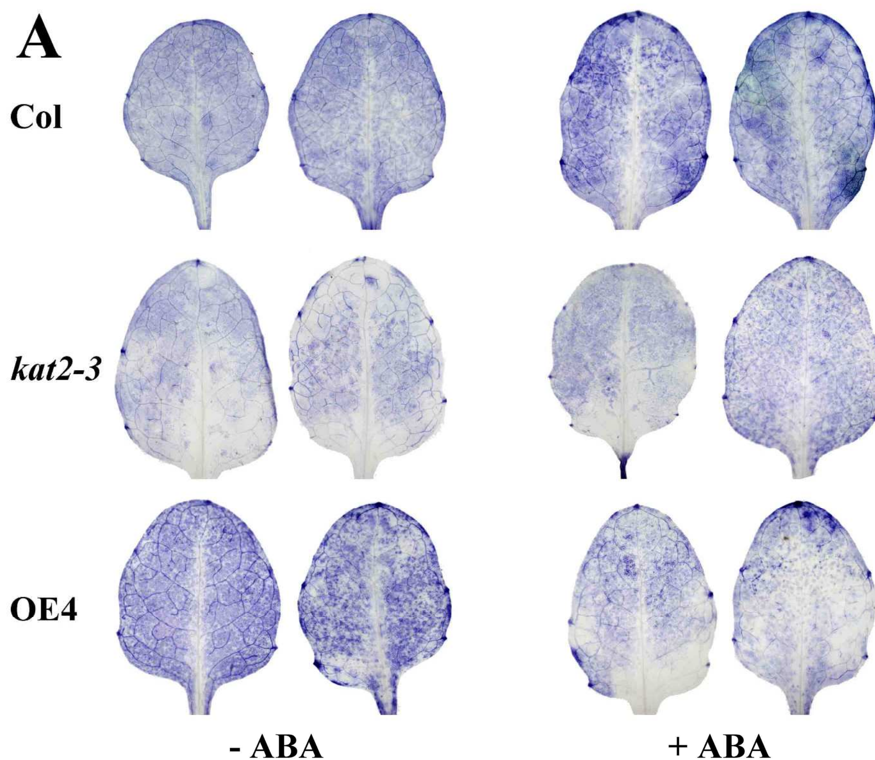

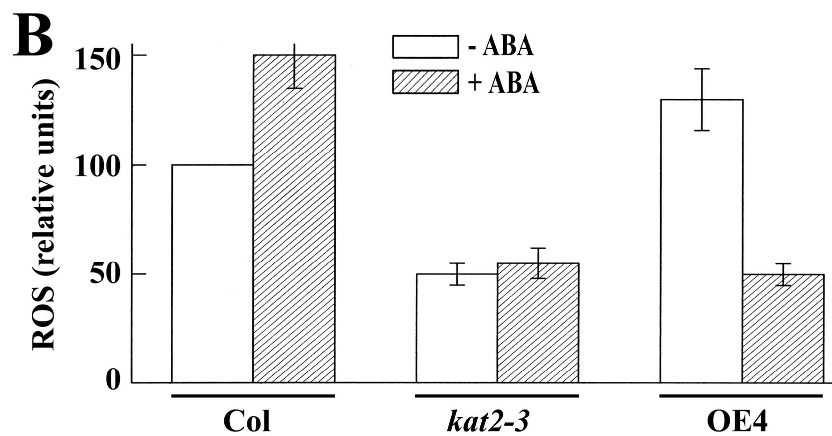

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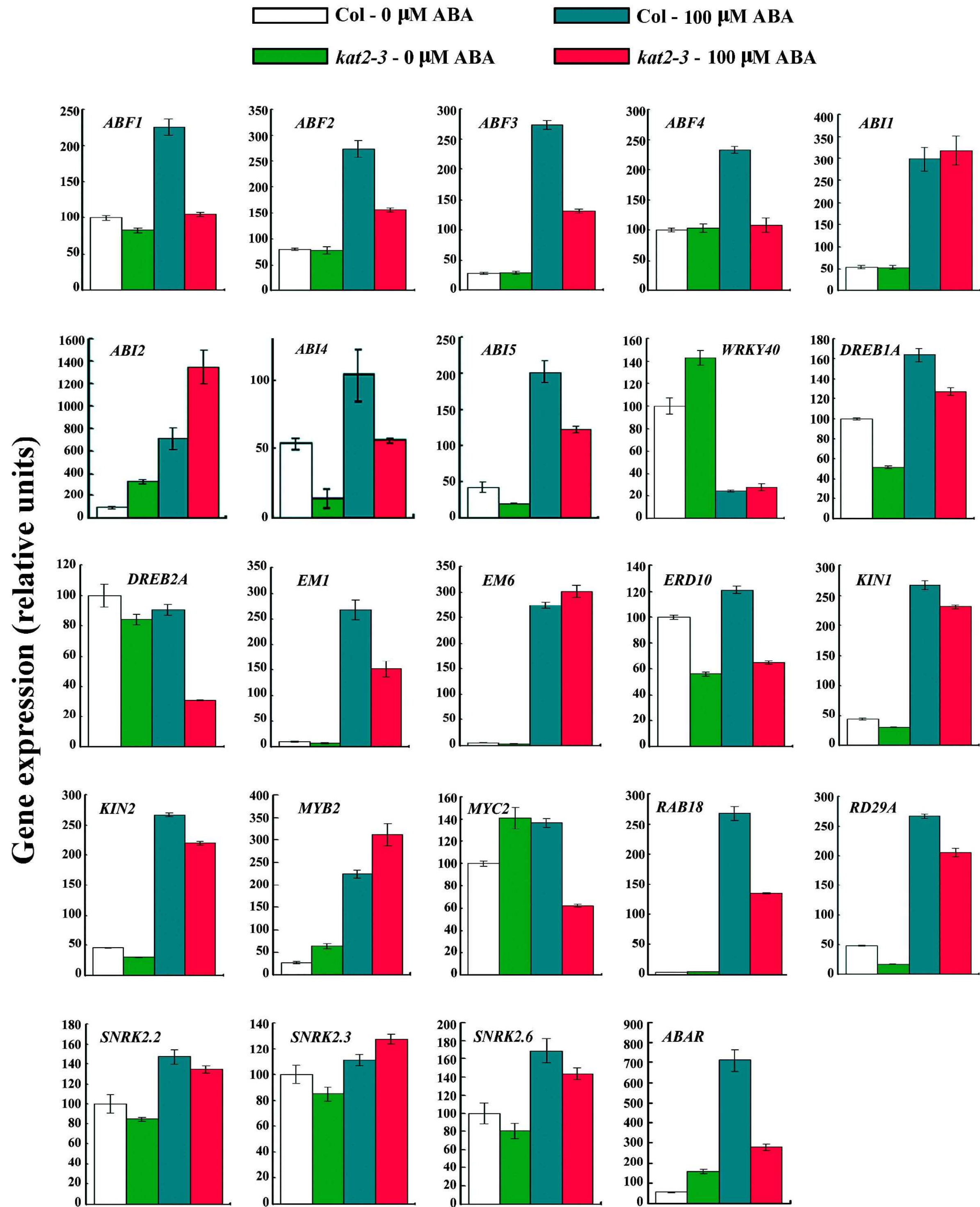

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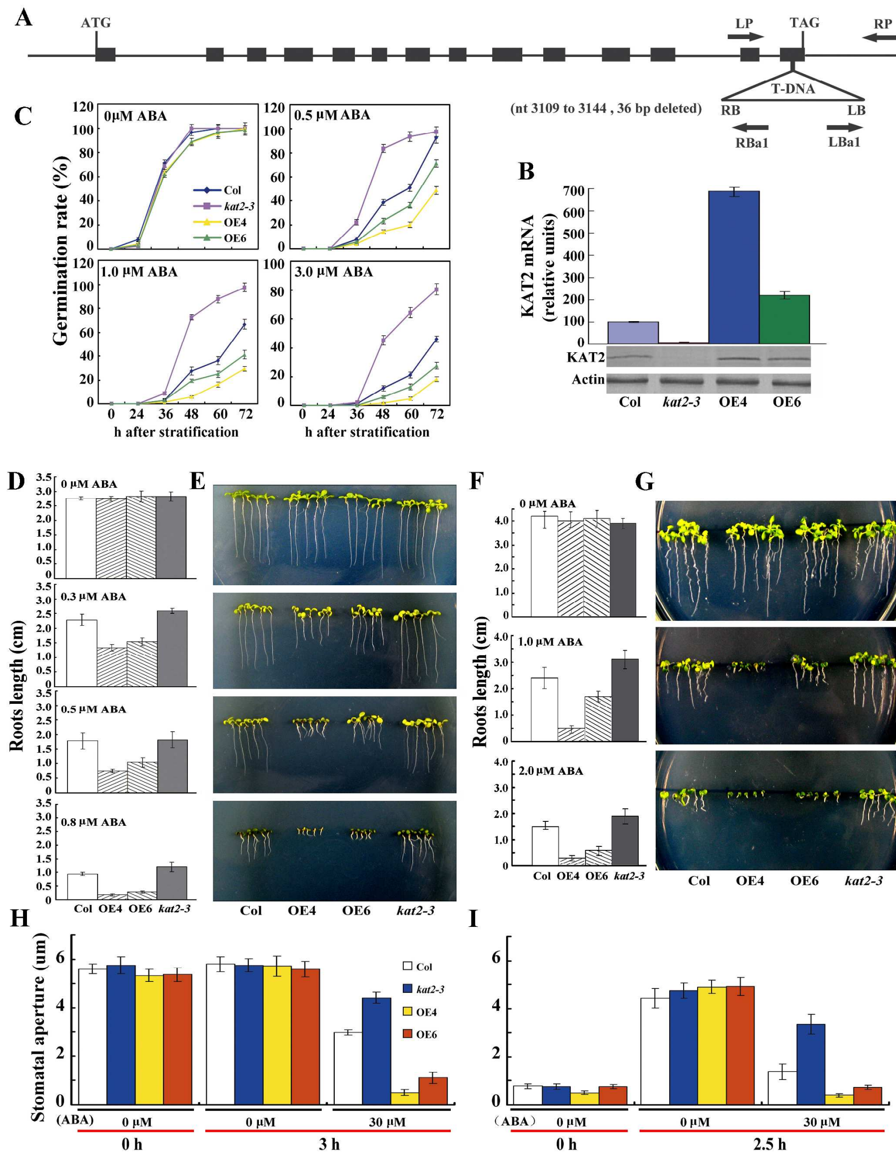

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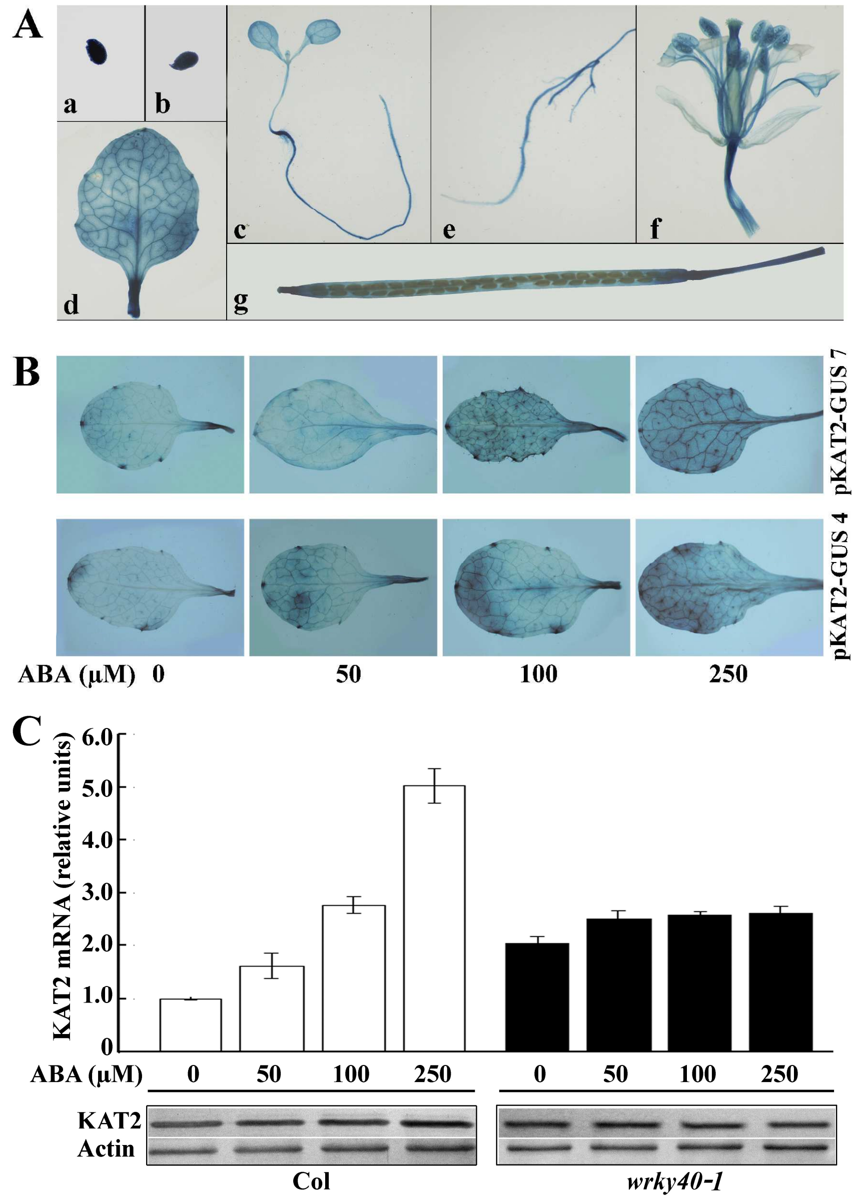

Fig. 2 Analysis of KAT2 expression.
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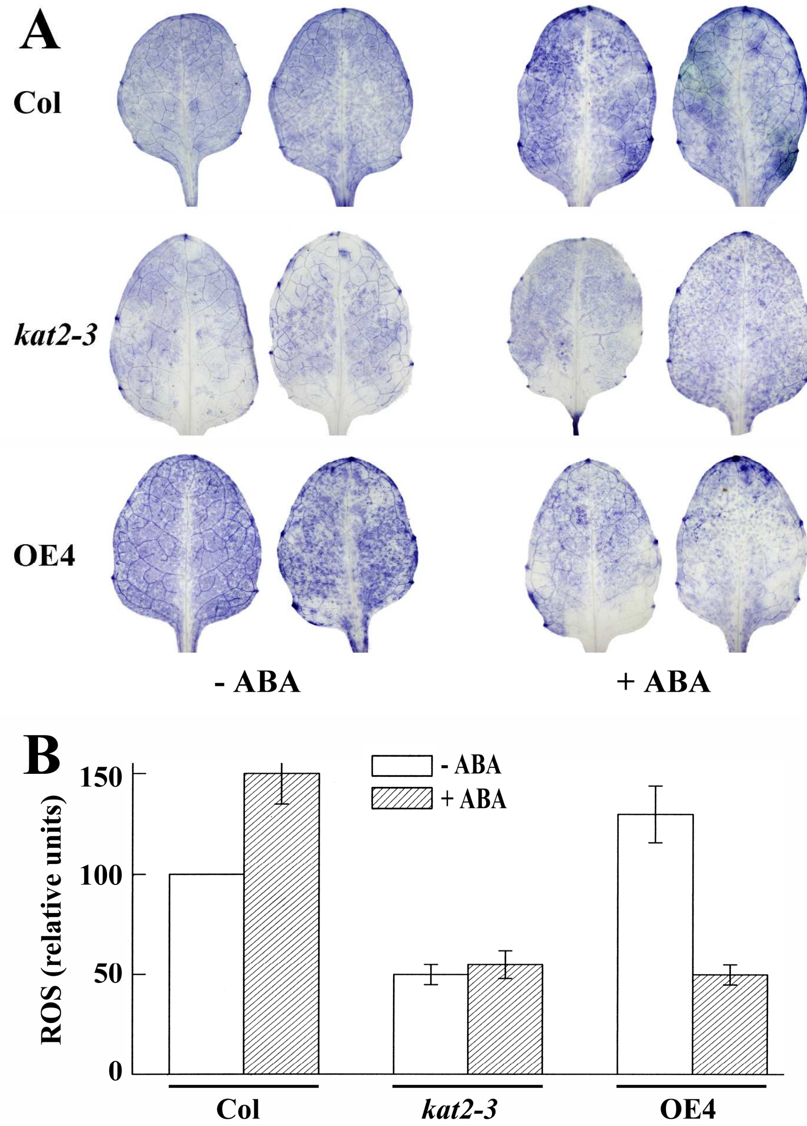

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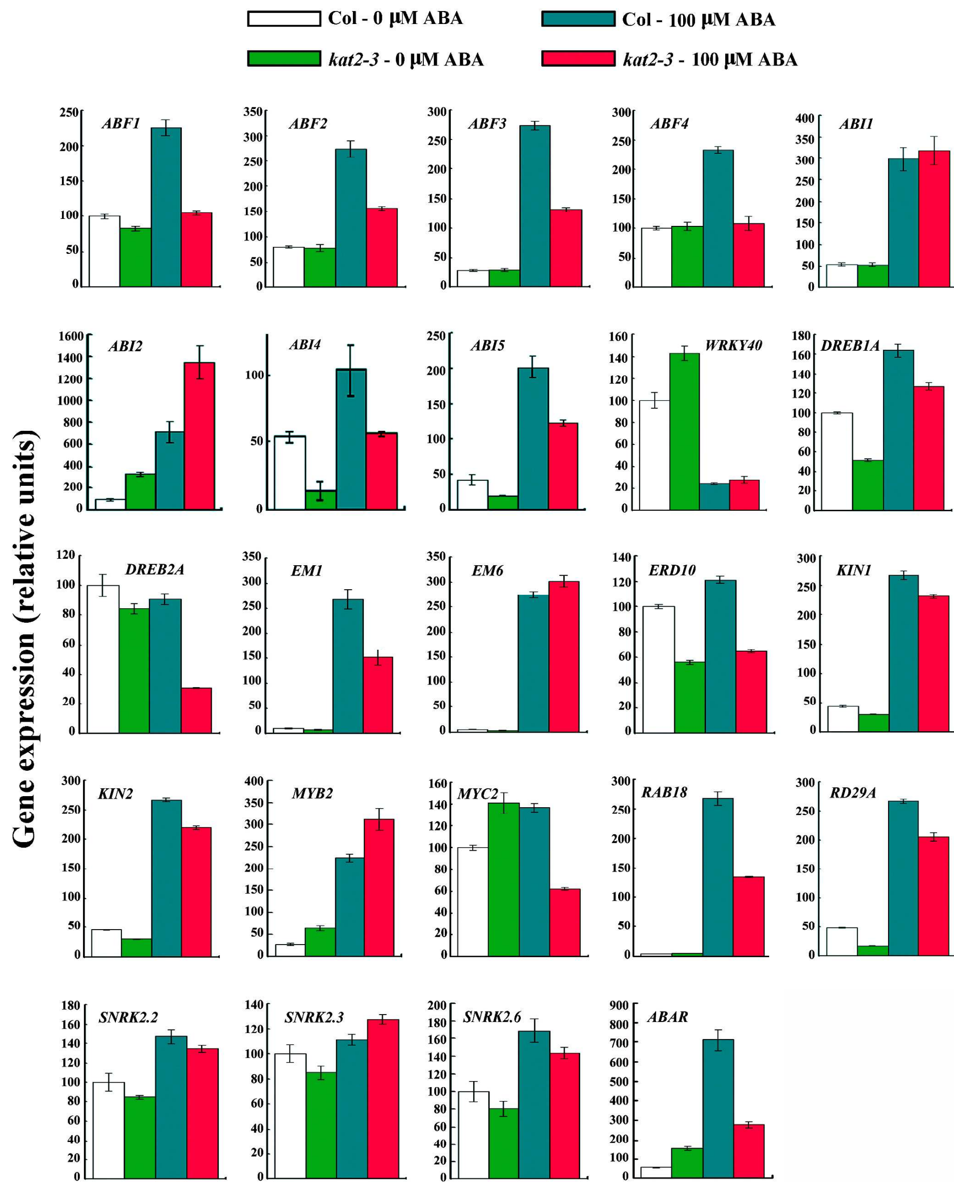

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