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ABO3, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in Arabidopsis

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SUMMARY

The biological functions of WRKY transcription factors in plants have been widely studied, but their roles in abiotic stress are still not well understood. We isolated an ABA overly sensitive mutant, *abo3*, which is disrupted by a T-DNA insertion in *At1g66600* encoding a WRKY transcription factor AtWRKY63. The mutant was hypersensitive to ABA in both seedling establishment and seedling growth. However, stomatal closure was less sensitive to ABA, and the *abo3* mutant was less drought tolerant than the wild type. Northern blot analysis indicated that the expression of the ABA-responsive transcription factor *ABF2/AREB1* was markedly lower in the *abo3* mutant than in the wild type. The *abo3* mutation also reduced the expression of stress-inducible genes *RD29A* and *COR47*, especially early during ABA treatment. ABO3 is able to bind the W-box in the promoter of *ABF2 in vitro*. These results uncover an important role for a WRKY transcription factor in plant responses to ABA and drought stress.

Keywords: WRKY transcription factor, abscisic acid, Arabidopsis, drought stress.

INTRODUCTION

Drought stress is one of the most severe environmental culprits that greatly restrict plant distribution and crop production (Zhu, 2002). Drought stress induces the accumulation of the plant hormone abscisic acid (ABA), which leads to stomatal closure for maintaining water status in plant cells under water-deficit conditions. The increased ABA interacts with the ABA receptors PYR/PYLs of START proteins, which interact with PP2C proteins and release the inhibition of PP2Cs on SnRK2 protein kinases. The activated SnRK2s phosphorylate downstream transcriptional factors such as ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2 (ABF2) and ABI5 (ABA insensitive 5, which is a bZIP protein) to regulate the expression of ABA response genes (Fujii and Zhu, 2009; Ma *et al.*, 2009; Nakashima *et al.*, 2009; Park *et al.*, 2009). Various genes are up- or downregulated at the transcriptional level by both drought stress and ABA treatment. Analyzing the promoters of ABA-inducible genes has identified some conserved *cis*-elements, one of which is ABRE (ABA-responsive element, PyACGTGGC) (Guiltinan *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1990; Iwasaki *et al.*, 1995; Shen *et al.*, 1996). Transcription factors such as AREB (ABRE binding protein)/ABFs, and ABI5 could bind ABRE and regulate the expression of ABA-responsive genes (Uno *et al.*, 2000; Carles *et al.*, 2002; Casaretto and Ho, 2003). The transcripts of ABFs, including *ABF1*, *ABF2*/*AREB1*, *ABF3* and *ABF4*/*AREB2*, are also highly induced by the application of exogenous ABA (Choi *et al.*, 2000; Uno *et al.*, 2000). Plants overexpressing *ABF3* and *ABF4* showed increased ABA sensitivity in seed germination and seedling growth, reduced transpiration and more drought tolerance than wild-type plants (Kang *et al.*, 2002). However, constitutive overexpression of *ABF2*/*AREB1* did not increase the expression of downstream ABA-responsive genes, because the activation of ABF2/AREB1 needs ABA-triggered protein phosphorylation (Fujita *et al.*, 2005). An ABA-activated 42-kDa kinase can phosphorylate and activate ABF2/AREB1 (Fujita *et al.*, 2005;

Furihata *et al.*, 2006). When the phosphorylatable Ser/Thr residues were substituted with Asp to mimic phosphorylation, the resultant active form of AREB1 induced the expression of many downstream ABA-responsive genes, even without ABA treatment (Furihata *et al.*, 2006).

The DRE (the dehydration-responsive element)/CRT (C-repeat) element in drought and cold-responsive genes can be bound by the AP2 domain transcriptional activators CBFs (CRT binding factors)/DREB1s (DRE binding protein 1s) and DREB2s (Stockinger *et al.*, 1997; Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998). *ABI4* from Arabidopsis is another AP2 domain transcription factor that could bind the CE1 element CACCG found in ABA-responsive genes (Finkelstein *et al.*, 1998; Niu *et al.*, 2002). AtHB6 and AtERF7 can bind to other non-ABRE/DRE promoter *cis*-elements to regulate the expression of stress and/or ABA-responsive genes (Himmelbach *et al.*, 2002; Song *et al.*, 2005). Other transcription factors such as MYB, MYC, ABI3/VP1 (a multidomain transcription factor) and NAC proteins are also found to regulate the expression of ABA- or drought-responsive genes (McCarty *et al.*, 1991; Abe *et al.*, 2003; Suzuki *et al.*, 2003; Fujita *et al.*, 2004; Lu *et al.*, 2007).

The WRKYs comprise a large family of transcription factors conserved from lower plants, such as mosses, to higher plants (Eulgem *et al.*, 2000). WRKY proteins contain a conserved WRKY DNA-binding domain of approximately 60 amino acids, followed by a C_2H_2 or C_2HC zinc-finger motif (Eulgem *et al.*, 2000). The WRKY transcription factors show high binding affinity to the W-box sequence (C/T)TGAC(T/C) (Ulker and Somssich, 2004). In the Arabidopsis genome, 74 *WRKY* genes were identified (Ulker and Somssich, 2004). The transcripts of many WRKYs are strongly induced by pathogens, salicylic acid or H_2O_2 (Dong *et al.*, 2003; Ulker and Somssich, 2004). Senescence and abiotic stresses such as drought, heat and cold stress also induce the expression of *WRKY* genes in plants (Robatzek and Somssich, 2001, 2002; Rizhsky *et al.*, 2002; Seki *et al.*, 2002; Mare *et al.*, 2004; Lee *et al.*, 2005). The physiological functions of WRKYs have been established in pathogen defense, sugar signaling, senescence, trichome development, root growth and phosphate acquisition (Eulgem *et al.*, 1999, 2000; Johnson *et al.*, 2002; Robatzek and Somssich, 2002; Sun *et al.*, 2003; Miao *et al.*, 2004; Ulker and Somssich, 2004; Devaiah *et al.*, 2007; Ulker *et al.*, 2007). In rice, ABA positively or negatively regulates the transcripts of some WRKYs (Xie *et al.*, 2005). The physiological roles of WRKYs in abitotic stress have also been reported recently (Miller *et al.*, 2008; Jiang and Deyholos, 2009; Wu *et al.*, 2009; Zhang *et al.*, 2009).

In this study, we isolated an ABA-hypersensitive mutant, *abo3* (*ABA overly sensitive 3*), which is caused by a T-DNA insertion in an ABA-upregulated *WRKY* gene. Although the knock-out of *ABO3* renders plants hypersensitive to ABA in seed germination and seedling growth, *abo3* mutant plants showed reduced ABA sensitivity in guard cells, lost water faster and were more sensitive to drought stress than wild-type plants. We found that *abo3* mutation impaired the expression of *ABF2* and downstream genes such as *RD29A* and *COR47* during early ABA treatment. Gel shift analysis revealed that ABO3 protein binds to the W-box elements localized in the *ABF2* promoter. However, transgenic plants overexpressing *ABO3* showed no ABA or *ABF2* expression phenotypes, which suggests that ABO3 might need co-factor(s) or modifications for transactivating downstream target genes.

RESULTS

Disruption of *ABO3* gene in a T-DNA insertion mutant increases ABA sensitivity

Different concentrations of ABA can inhibit both seed germination and seedling growth of Arabidopsis. To study ABA and drought-tolerance mechanisms, we used a root-bending assay to search for mutants in which root growth is more sensitive or insensitive to ABA (Yin *et al.*, 2009). As the root growth of the Columbia accession is more insensitive to ABA than some other accessions, such as Landsberg and C24, we have usually used 20–30 μM ABA or more to test the root growth sensitivity (Yin *et al.*, 2009). Five-day-old seedlings grown on MS medium were transferred with roots upside down onto vertical agar plates containing MS medium supplemented with 20–30 μM ABA (Yin *et al.*, 2009). One of the ABA-hypersensitive mutants, *abo3*, was isolated from the T-DNA collection of SALK lines (Salk_007496). The T-DNA insertion site is located in the third exon of *At1g66600* encoding a WRKY transcription factor, AtWRKY63 (Figure 1a). The insertion would be expected to completely disrupt the expression of *At1g66600*. RT-PCR analysis confirmed that there were *At1g66600* transcripts in the wild type but not in the *abo3* mutant (Figure 1b). Northern blot analysis did not detect the transcripts of *AtWRKY63/ABO3* in our conditions, probably because of its low expression level. There is no information on microarray data for this gene. Real-time RT-PCR analysis revealed that *ABO3* expression is upregulated by ABA treatment in either Columbia or Landsberg (Figure 1c). We further checked the *ABO3* expression in different *abi* mutants. The transcriptional induction of *ABO3* by ABA was impaired in *abi1-1* (Leube *et al.*, 1998), *abi2-1* (Rodriguez *et al.*, 1998) (two dominant-negative mutations in Landsberg), *abi3-1* (Parcy *et al.*, 1997) (in Landsberg) or *abi5* (a point mutation in the fifth nucleotide before the first putative ATG, detail information about this mutant can be found in TAIR; in Columbia) mutants, suggesting that ABI1 and ABI2 negatively, and ABI3 and ABI5 positively, regulate *ABO3* expression. However, *ABO3* transcripts were still induced in the *abi4-1* mutant (Finkelstein *et al.*, 1998), indicating that ABI4 did not regulate *ABO3* expression.

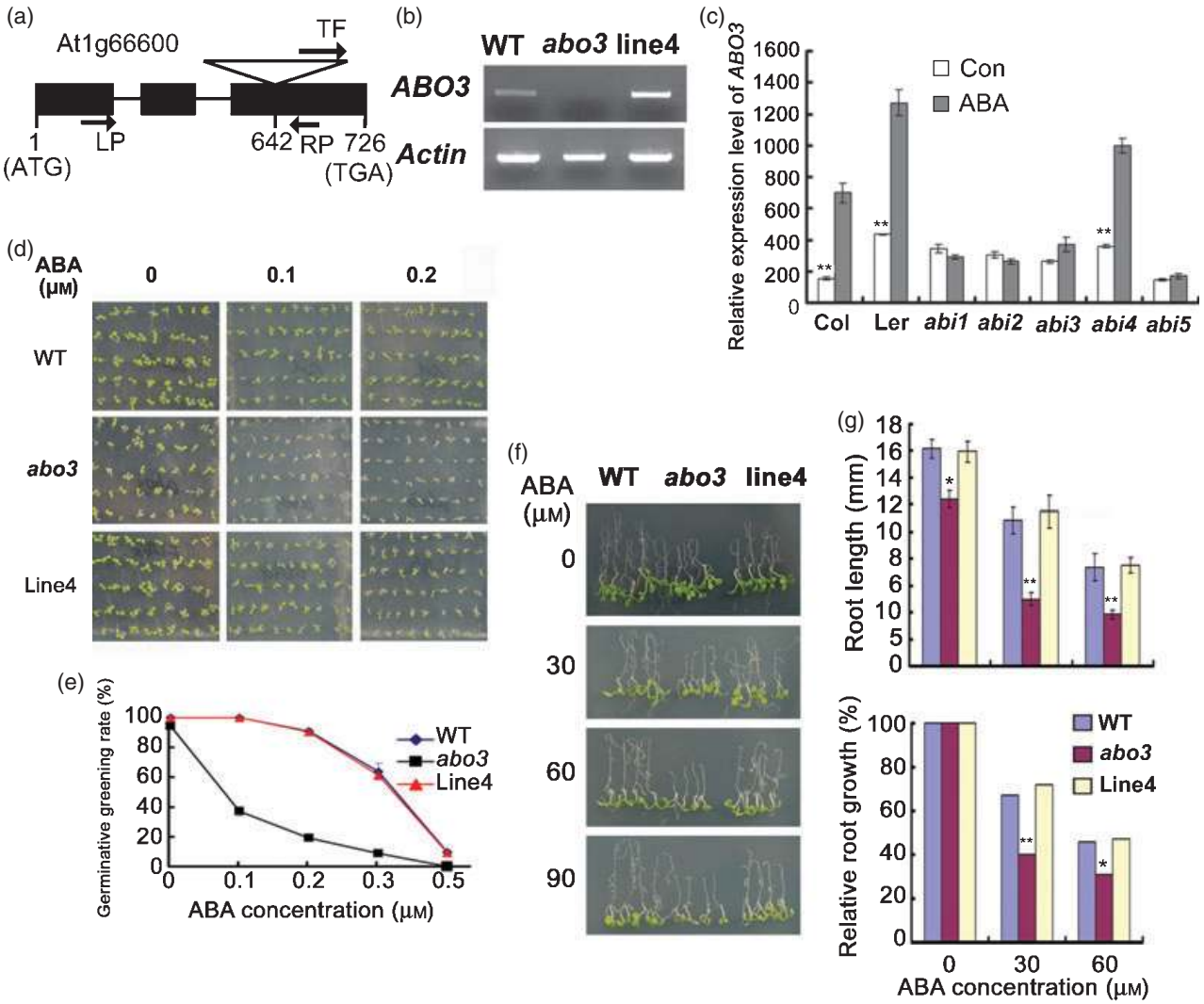


Figure 1. Phenotypic characterization of the *abo3* mutant.
(a) Diagram of the structure of the *ABO3* gene (*At1g66600*). The position of the T-DNA insertion is shown. Exons (black boxes) and introns (lines) are indicated. Two pairs of primers (LP and RP; TF and RP) were used to check the T-DNA insertion, where indicated (please see Experimental procedures for primer details).
(b) Expression of *ABO3* in the wild type, and in *abo3* and *abo3* overexpressing *ABO3* (line 4, *35S-ABO3* in *abo3* for complementation), by RT-PCR. Total RNA extracted from 10-day-old seedlings was used for cDNA synthesis. RT-PCR was performed by using the corresponding cDNA. The amplified product of *ABO3* is about 180 bp. No products were amplified from the *abo3* mutant. *Actin* was used as internal control.
(c) The expression of *ABO3* was induced by ABA treatment in different *abi* mutants and wild-type plants. Total RNAs isolated from 10-day-old seedlings treated with or without 100 μM ABA for 2 h were used for real-time RT-PCR. The relative levels of *ABO3* transcripts in ABA treatment were obtained by comparing with no ABA treatment (Con, control). Three independent experiments were performed, each with triple replicates. Data are means ± SE. (Student's *t*-test; **statistically significant difference $P < 0.01$).
(d) and (e) Comparison and quantitative evaluation of seed germination on MS, or MS supplemented with different concentrations of ABA, between *abo3* and the wild type (WT), and between *abo3* and *abo3* overexpressing *ABO3* (line 4). Three independent experiments were performed, and about 100 seeds were counted for each experiment. Data are means ± SEs.
(f) The root-bending assay of *abo3*, wild-type and complementation line-4 plants in MS plates containing different concentrations of ABA.
(g) Comparison of root growth and relative root growth between the *abo3* mutant, the wild type and the complementation plant on MS plates with different ABA concentrations. Four independent experiments were done with similar results, each with about 40 seedlings. Data are means ± SE from one experiment (*$P < 0.05$; **$P < 0.01$).

We investigated ABA sensitivity during seedling establishment. As Arabidopsis seedling establishment is more sensitive to ABA than root growth, we used low concentrations of ABA in seedling establishment and high ABA concentrations in root growth. One week after seed germination on MS media containing 0.1–0.5 μM ABA, wild-type and *abo3* mutant seedlings with green cotyledons were compared. As shown in Figure 1d,e, the *abo3* mutant was more sensitive to ABA than the wild type during seedling establishment.

Root growth of the *abo3* mutant was slower than that of the wild type on MS medium (Figure 1f,g), which suggests that the *abo3* mutation influences root development. Adding different concentrations of exogenous ABA to the MS medium inhibited both root and shoot growth of *abo3* and the wild type, but with more inhibition in *abo3* than in the wild type (Figure 1f,g).

To determine whether the *abo3* phenotypes are caused by the disruption of *AtWRKY63*, we amplified *AtWRKY63* cDNA by RT-PCR and overexpressed it under the control of the constitutive CaMV *35S* promoter in *abo3* mutant plants (Figure 1d). We obtained eight independent transgenic lines, and randomly picked up three lines. All of the three lines complemented the growth and ABA-sensitive phenotype of the *abo3* mutant in T_3 homozygous plants. Here, we took line 4 as an example for detailed analysis. RT-PCR revealed that the *AtWRKY63* transcript was overexpressed in line 4 (Figure 1b). We analyzed line 4 on MS plates supplemented with different concentrations of ABA or no ABA for both seed germination and root growth. Transgenic line 4 displayed a wild-type phenotype on MS medium containing different concentrations of ABA, or no ABA, in both seed germination (Figure 1d,e) and root growth assays (Figure 1f,g). We further performed drought-tolerance and stomatal movement tests (Figure 2a–e) and northern blot analysis for the expression of *ABF2* (a putative target of *AtWRKY63*; Figure 5b, see detailed explanation later), which further confirmed that the *abo3* mutant phenotypes were completely rescued by *35S- AtWRKY63*. Therefore, the disruption of *AtWRKY63* is responsible for the phenotypes observed in the *abo3* mutant.

abo3 mutation impairs ABA-induced stomatal closure, and the *abo3* mutant is more sensitive to drought stress than the wild type

Plant drought tolerance is influenced by a combination of multiple molecular and cellular pathways. Some ABA-hypersensitive mutants, such *era1* (Pei *et al.*, 1998) and

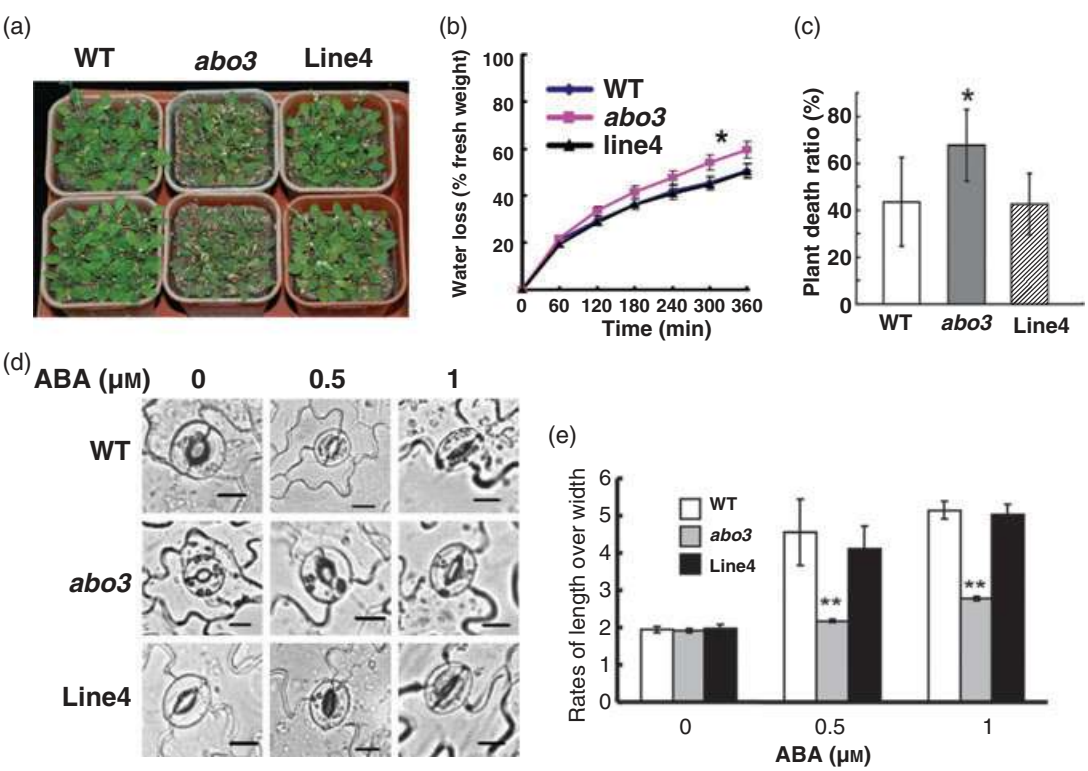


Figure 2. The *abo3* mutant was more sensitive to drought stress.
The wild type, *abo3* mutant and *abo3* overexpressing ABO3 (line 4) were compared.
(a) Reduced drought tolerance of the *abo3* mutant. Three-week-old plants in soil were exposed to dehydration by withholding water for 2 weeks. Three independent assays were performed with similar results.
(b) Water loss from detached leaves. The water loss of 0.5 g detached leaves from different plants was measured at different times with triple replicates. Five independent experiments were performed with similar results. Data are means ± SEs.
(c) Rate of plant death after re-watering for 7 days. Plants in (A) were continually treated for two more weeks without watering, and then re-watered. After 1 week, the number of surviving plants was counted. Three independent experiments were performed, each with about 108 plants. Data are means ± SEs.
(d) Comparison of stomatal aperture in response to ABA treatment.
(e) Ratios of stomatal aperture length to width. Three independent experiments were performed with similar results. Data were from one experiment with 60 stomata cells from leaves of three different plants with triple replicates. Data are means ± SEs.

abo1 (Chen *et al.*, 2006), are more tolerant to drought stress, whereas the *sad1* mutant is more sensitive to both ABA and drought stress (Xiong *et al.*, 2001). Plants overexpressing *AtHD2C* are insensitive to ABA but show increased drought tolerance (Sridha and Wu, 2006). Because *abo3* mutants are more sensitive to ABA in both seedling growth and seed germination, we tested the drought-tolerance phenotype of the *abo3* mutant. Plants were grown for 3 weeks and then exposed to dehydration by withholding water for 2 weeks, *abo3* mutant plants showed a less resistant phenotype to drought than wild-type plants or complementation line 4 (Figure 2a). After drought treatment for two more weeks, plants were re-watered and continually cultured for 1 week to count the plant survival rate. The *abo3* mutant displayed a higher death rate when compared with the wild type or with line 4 (Figure 2c). We further evaluated water loss rates with detached leaves. Detached leaves of *abo3* lost water more quickly than wild-type or line-4 leaves (Figure 2b), consistent with the increased drought sensitivity of the mutant.

Because water loss mainly depends on stomatal regulation, we then compared stomatal apertures under treatment with different concentrations of ABA. The epidermal peels of *abo3* and wild-type or complementation line-4 plants were incubated in a buffer solution under strong light conditions for 12 h to fully open the stomata. Then, the peels were treated with different concentrations of ABA for 2 h. The ratio of stomatal length to width indicates the degree of stomatal closure. *abo3* and wild-type or line-4 plants showed the same ratio of length to width of fully opened stomata without ABA treatment, but wild-type or line-4 plants showed higher ratios of length to width of stomata than *abo3* mutant plants after treatment with ABA (Figure 2d,e). These results suggest that stomata closure in *abo3* is less sensitive to ABA than that in the wild type or line 4. The drought sensitivity of the *abo3* mutant as well as its impaired stomatal closure under exogenous ABA treatment suggests that ABO3 functions in ABA-mediated drought stress response pathways.

To test the potential impact of *ABO3* overexpression on plant responses to ABA and drought, we introduced the *35S-AtWRKY63* construct to wild-type plants, and obtained 11 independent transgenic lines. Through northern blot, we selected two homozygous T_3 lines (OE8 and OE11) with different *ABO3* expression levels for further phenotype analysis (Figure 3c). However, we did not observe any ABA-related phenotype either in seed germination or in root bending of the overexpression lines (Figure 3b–d). Drought treatment and stomata closure experiments also lacked any visible phenotype (data not shown). These results suggest that ABO3 protein may need other components or ABA or drought-induced post-translational modifications to activate downstream genes in plant stress responses.

ABO3 is a nuclear protein with transcriptional activation activity

To explore the spatial expression pattern of *ABO3*, we obtained 14 independent T_3 transgenic Arabidopsis lines that harbored an *ABO3* promoter::GUS reporter construct, and six lines were analyzed by GUS staining with similar expression patterns. We found the expression of the *ABO3* promoter::GUS fusion gene very low and detectable only after staining for a long time (2 days) (Figure 4a; i, whole seedling; ii, root; iii, stem; iv, leaf; v, flower). The GUS expression appeared higher in roots and flowers than in stems and leaves. A very low level of GUS staining was detected in the early development of seedlings (24–72 h after seed germination; Figure 4bi–iii), or only after ABA treatment in guard cells (Figure 4bv). Consistent with this GUS staining pattern, RT-PCR results also indicated relatively higher expression in roots and flowers (Figure 4avi). Interestingly, the GUS expression in the leaves was concentrated in trichomes (Figure 4iv, Leaf, biv). The low expression of *ABO3* is consistent with public microarray data, in which no expression data are available for *AT1G66600/ABO3*.

To examine the subcellular distribution of the ABO3 protein, we fused ABO3 with GFP (*35S::ABO3-GFP*). Confocal imaging showed the ABO3-GFP fusion protein localized exclusively in the nuclei of onion (*Allium cepa*) epidermal cells in a transient expression assay (Figure 4c). As a control, the GFP protein was found in both the nucleus and cytoplasm. The nuclear localization of ABO3 is consistent with its predicted function as a transcription factor.

The WRKY proteins comprise a superfamily of transcription factors that can be divided into three subgroups according to the conserved WRKY DNA-binding domain sequences (Wu *et al.*, 2005). ABO3/AtWRKY63 belongs to the third subgroup of WRKY proteins (Wu *et al.*, 2005). To investigate whether ABO3 possesses transcriptional activation activity, we assayed the GAL4 DNA binding domain-ABO3 fusion protein in yeast for its ability to activate transcription of the GAL4 upstream sequence-driven His and LacZ reporter gene expression. The yeast growth on the His-deficient medium, LacZ staining and relative quantitative assay of β-galactosidase activity in Figure 4D indicate that the transactivation sites of ABO3 are located in the W domain and C-terminal region of ABO3.

***ABO3* positively regulates the expression of the *ABF2* transcription factor**

The drought and ABA response phenotypes of the *abo3* mutant suggest that the expression of some ABA-responsive genes might be altered in the mutant. To verify this possibility, we selected various stress-inducible marker genes, including *ABF2*, *ABF3*, *RD29A*, *COR47*, *DREB2A*, *RD22* and *KIN1*, and analyzed their expression under treatments

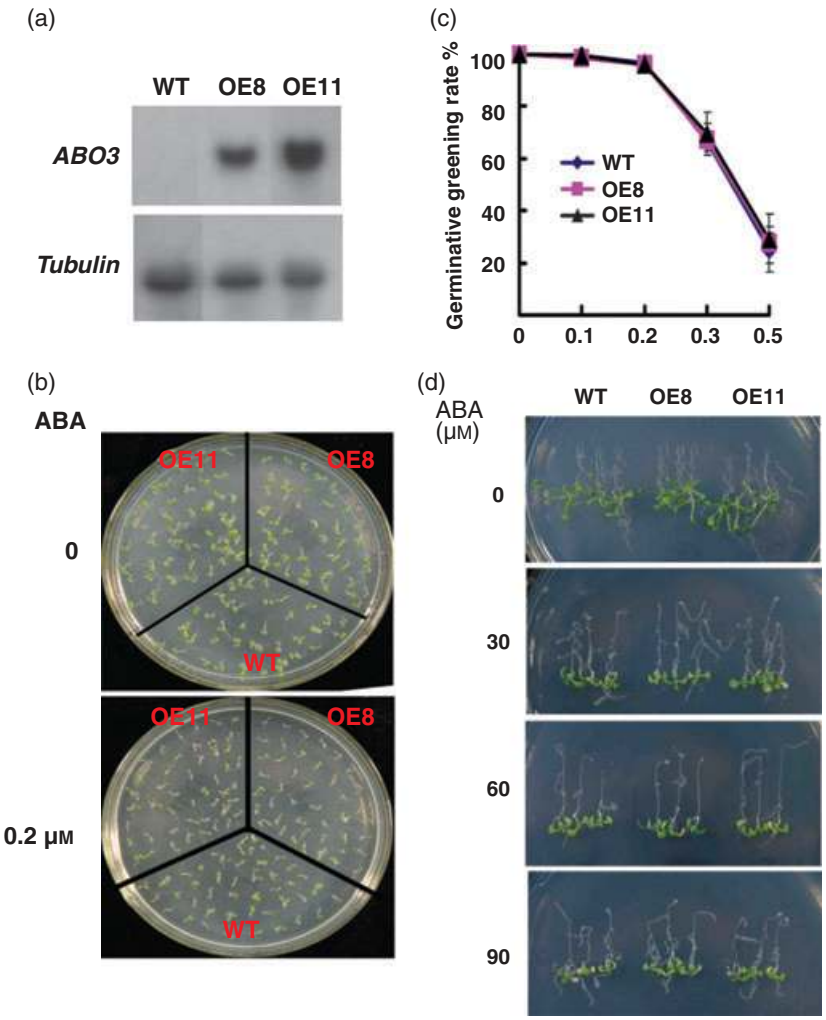


Figure 3. Overexpression of *ABO3* in Arabidopsis didn't cause the ABA-sensitive phenotype.
(a) Northern blot analysis of *ABO3* transcript in two wild-type lines (OE8 and OE11) overexpressing *ABO3*.
(b) Comparison and (c) quantitative evaluation of seed germination on MS, or MS medium supplemented with different concentrations of ABA, between two overexpression (OE) lines and the wild type (WT). Three independent experiments were performed, and about 100 seeds were counted for each experiment. Data are means ± SEs.
(d) The root bending assay of two OE lines and wild-type plants in MS plates containing different concentrations of ABA.

with 20 and 100 μM ABA by northern blots. *RD29A*, *COR47* and *KIN1* are stress-responsive genes with a presumed protective function against stress damage, and part of their stress induction is dependent on ABA (Kurkela and Franck, 1990; Lin *et al.*, 1990; Guo *et al.*, 1992; Yamaguchi-Shinozaki and Shinozaki, 1993a, 1994; Wang and Cutler, 1995); expression of another stress-responsive gene, *RD22*, is induced by salt, drought and ABA through a pathway dependent on ABA-induced *MYC* and *MYB* genes (Yamaguchi-Shinozaki and Shinozaki, 1993b). ABF2, ABF3 and DREB2A are ABA-responsive transcription factors (Liu *et al.*, 1998; Uno *et al.*, 2000; Kang *et al.*, 2002; Kim *et al.*, 2004). As shown in Figure 5a, all of the tested genes were induced by different concentrations of ABA treatments after 2 and 5 h. The levels of *ABF3*, *DREB2A*, *RD22* and *KIN1* did not differ between *abo3* and wild-type plants. However, the expression of *ABF2* was greatly reduced in *abo3* compared with wild-type plants, especially at 2 h. The transcripts of *RD29A* and *COR47* were reduced in the *abo3* mutant compared with the wild type upon treatment after 2 h, but not after 5 h. These results indicate that ABO3 is critical for the expression of some ABA-inducible genes at early time points during treatment.

We further monitored the expression patterns of *ABF2*, *RD29A* and *COR47* during a 2-h, 20-μM ABA treatment (Figure 5b). The transcript of *ABF2* was detected at 30 min in the wild type but not in the *abo3* mutant, and was less abundant in the *abo3* mutant than in the wild type at 60 and 120 min. The transcript levels of *RD29A* and *COR47* were lower in the *abo3* mutant than in the wild type at all three time points. Here, we also included *abo3* plants transformed with *35S-AtWRKY63* to show that the construct restored the expression of *ABF2*, *RD29A* and *COR47* to the wild-type levels at 120 min.

ABO3 is able to bind the W-box in the *ABF2* promoter *in vitro*

Plant WRKY proteins recognize various W-box elements with a TGAC core sequence in the promoters of many defense-related genes (Yu *et al.*, 2001). The promoter region

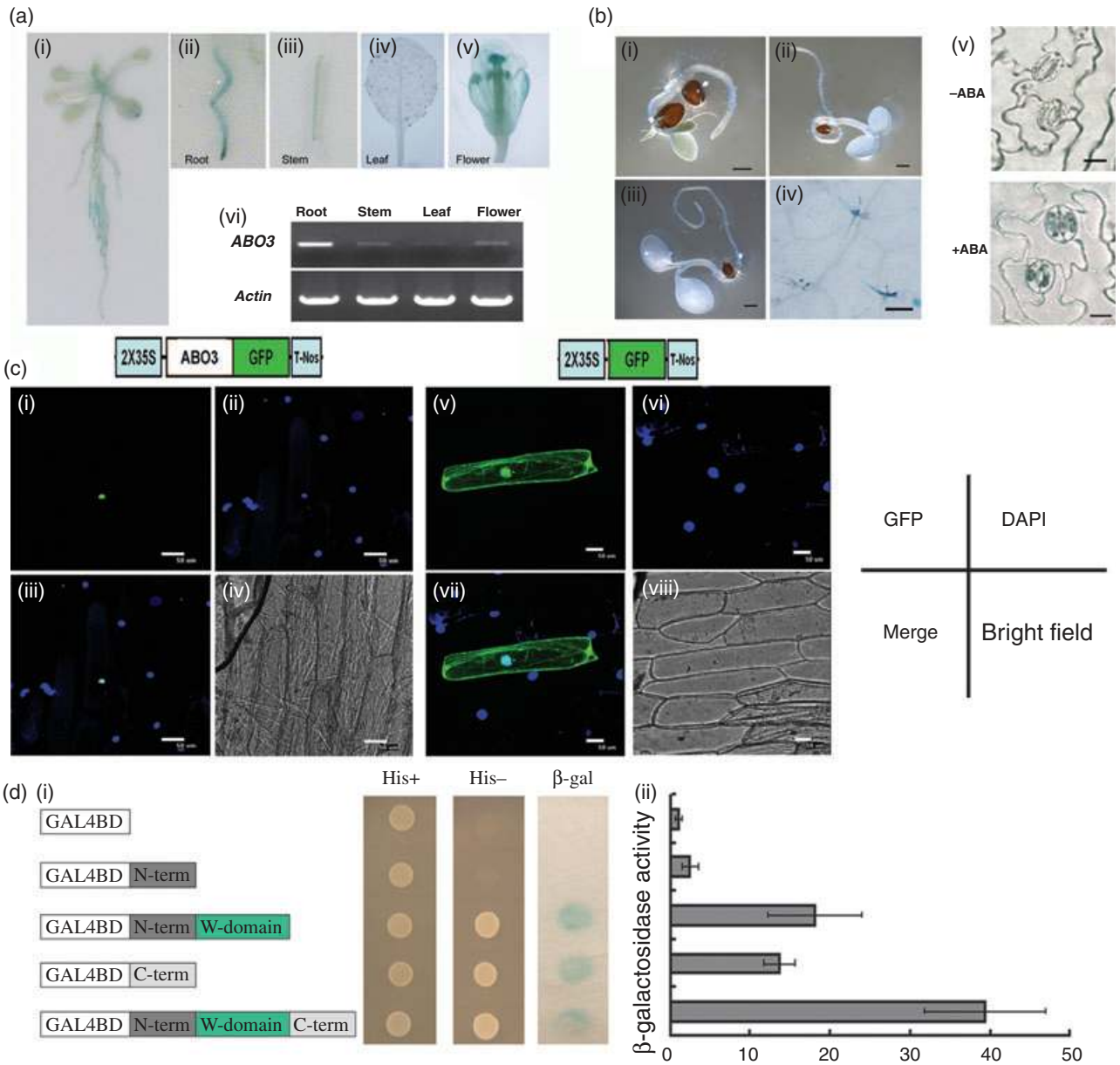


Figure 4. Transcription activation activity, subcellular localization and gene expression of *ABO3*.
(a) *ABO3* promoter-driven GUS expression in different tissues: i, a whole seedling; ii, root; iii, stem; iv, leaf; v, flower; vi, total RNA was extracted from 10-day-old seedlings and reverse transcribed to cDNA. RT-PCR analysis of *ABO3* transcript levels in the different tissues under internal control *Actin* was also displayed. The size of the amplified fragment is 180 bp for *ABO3* and 220 bp for *Actin*.
(b) *ABO3* promoter-driven GUS expression in the early development of seedlings, trichomes and guard cells. GUS expression in a seedling after seed germination for 24 (i), 48 (ii) or 72 h (iii). iv, GUS expression in trichomes. v, GUS expression in guard cells before and after 100 μM ABA treatment for 4 h. Scale bar: 10 μM.
(c) Subcellular localization of ABO3. An ABO3-GFP translational fusion construct was introduced into onion epidermal cells by particle bombardment: i, fluorescence image of ABO3-GFP; ii, the corresponding DAPI staining for the nucleus; iii, merged fluorescence and DAPI image; iv, the corresponding bright field; v, fluorescence image of GFP control; vi, the corresponding DAPI staining for the nucleus; vii, merged fluorescence and DAPI image; viii, the corresponding bright field. Scale bars: 50 μm.
(d) GAL4 DNA binding domain-ABO3 fusion analysis for transactivation activity and β-galactosidase quantitative assay. i, GAL4 DNA binding domain was fused with different parts of ABO3 and transformed into yeast strain AH109 containing the *His3* and *LacZ* reporter genes. Three independent experiments were performed and each showed similar patterns. ii, Quantitative analysis of β-galactosidase activity of the relative yeast strains in liquid culture. Values are the means of data taken from three independent experiments. Error bars indicate the standard deviation. The labeling of the *x*-axis indicates the relative fold change of β-galactosidase activity between samples and the empty vector.

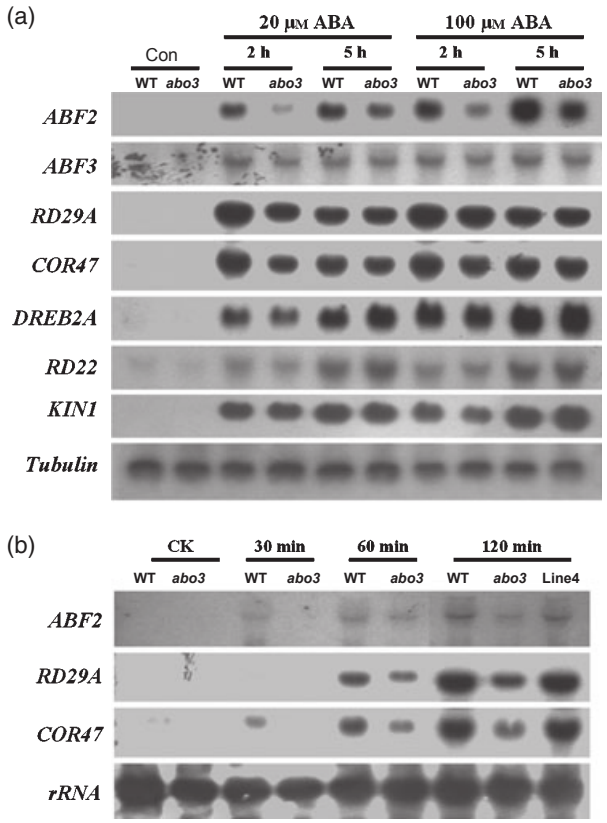


Figure 5. Northern blot analysis of ABA-inducible genes.
Total RNA (20 μg) was extracted from 10-day-old seedlings grown in plates and used for northern blot.
(a) Expression of *ABF2*, *ABF3*, *RD29A*, *COR47*, *DREB2A*, *RD22* and *KIN1* induced by 20 and 100 μM ABA for 2 or 5 h. *TUBULIN* was used as a loading control; Con, control (without ABA treatment).
(b) Expression of *ABF2*, *COR47* and *RD29A* induced by 20 μM ABA for up to 120 min. At 120 min, *abo3* overexpressing ABO3 (line 4) was also included. Three independent experiments were performed with similar results.

of *ABF2* contains two potential W-box elements within a 1-kb region upstream of the transcription initiation site: one reverse sequence, APN1, is located between −868 and −838 bp, and the other forward sequence, APN2, is located between −193 and −163 bp. A double-stranded APN1 or APN2 probe containing one typical W-box was used in an electrophoresis mobility shift assay (EMSA) to determine whether the ABO3 protein can recognize the TGAC core sequence. Recombinant ABO3-GST protein (Figure 6a) from *Escherichia coli* was incubated with [γ-^{32}P]ATP-labeled APN1/2 probes. The retarded complexes were detected with either APN1 or APN2, which indicates that ABO3 can bind to these two sequences, with the binding to APN2 being stronger (Figure 6b). To determine the binding specificity, we tested mutant probes (mAPN1/2) in which the TTGAC sequence of each W-box was changed to TTAAA. The mutant probes failed to produce retarded bands on incubation with the recombinant ABO3 protein (Figure 6b). Upon incubation with 100 times more unlabeled PNA1/2 probes, the retarded bands became weaker. This competition assay further indicates that ABO3 specifically recognizes the W-box *in vitro*. These results suggest that the interaction between DNA and ABO3 requires the WRKY-recognition sequences. Thus, ABO3 is capable of recognizing two W-boxes in the *ABF2* promoter, which is consistent with its important role *in vivo* in controlling *ABF2* expression under ABA treatment.

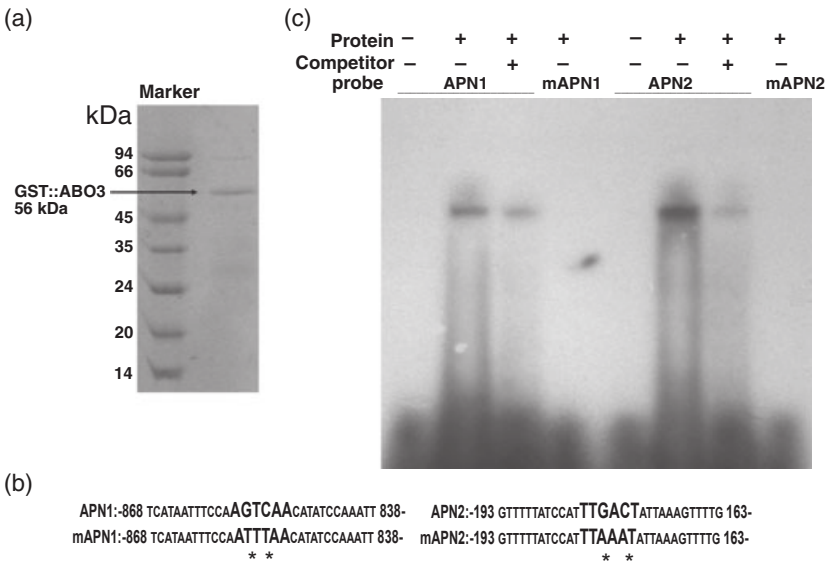
Genetic analysis of *abo3* with other ABA-responsive mutants

To further investigate the function of ABO3 in the ABA signaling pathway, we crossed *abo3* with *abi* mutants *abi1-1*, *abi2-1*, *abi3-1*, *abi4-1* and *abi5*, and obtained double mutants of *abi1-1/abo3*, *abi2-1/abo3*, *abi3-1/abo3*, *abi4-1/abo3* and *abi5/abo3*. As shown in Figure 7a,b, the double mutants of *abi1-1/abo3*, *abi2-1/abo3*, *abi3-1/abo3* and *abi5/abo3* displayed a similar ABA-resistant phenotype as their corresponding single *abi* mutants, although *abi1*, *abi2* and *abi3* are in the Landsberg background, which is more sensitive to ABA than Columbia in seed germination. However, the seed germination of the *abi4-1/abo3* double mutant was more sensitive than *abi4-1* was to ABA, which is consistent with the real-time RT-PCR analysis that upregulation of the *ABO3* transcript by ABA is not influenced by *abi4* mutation. We also compared the expression of these *ABI* genes under ABA treatment by real-time RT-PCR, and found that there was no apparent expression difference between the wild type and *abo3* (Figure 7b). These results indicate that ABO3 may act at, or upstream of, ABI1, ABI2, ABI3 and ABI5 in the ABA pathway, but might act in a different pathway with ABI4. It seems that ABO3 does not directly regulate the expression of these *ABI* genes, although they may be genetically related to each other in the ABA signaling pathway.

DISCUSSION

Our data demonstrate a critical role for a WRKY transcription factor encoded by *ABO3* in ABA response and drought tolerance. The expression of *ABF2/AREB1*, a key transcriptional activator for ABA-responsive genes is largely dependent on ABO3. ABO3 is capable of binding the core sequences in the promoter region of *ABF2/AREB1*, so *ABF2* is likely to be one target of ABO3. Interestingly, the expression of *ABO3* itself is upregulated by ABA, but overexpression of the ABO3 protein doesn't induce any ABA- or drought-related phenotype, except for mutant complementation. These observations suggest that there might be a complex network of ABA-responsive transcription factors involved in stress and ABA responses.

Previous studies found that *ABF2/AREB1* is an important mediator in glucose signaling and drought stress tolerance in vegetative tissues (Kim *et al.*, 2004; Fujita *et al.*, 2005). Overexpression of an activated form of AREB1 (AREB1ΔQT) increased the expression of some stress-inducible genes



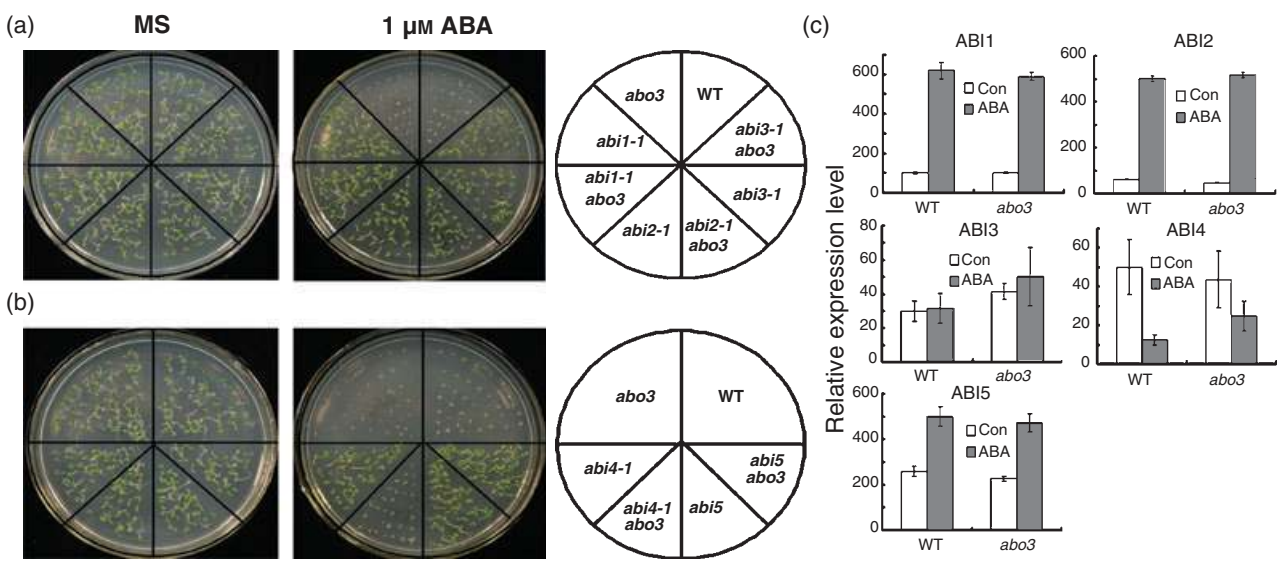


Figure 7 Phenotypic characterization of *abo3* and *abi* double mutants.
(a) and (b) Comparison of seed germination among different kinds of double mutants, *abo3* and wild-type plants in MS or MS containing 1 μM ABA. F_2 generation seedlings of crossed plants were subjected to single nucleotide polymorphism (SNP) identification to isolate *abi* mutation loci and *abo3* T-DNA insertion.
(c) Comparison of the expression of *ABI1*, *ABI2*, *ABI3*, *ABI4* and *ABI5* induced by ABA treatment between *abo3* and the wild type. Total RNAs isolated from 10-day-old seedlings treated with or without 100 μM ABA for 2 h were used for real-time RT-PCR. The relative levels of *ABI* transcripts after treatment with ABA were obtained by comparing the levels of transcripts after no treatment with ABA. Three independent experiments were performed, each with triple replicates. Data are means ± SEs (Student's *t*-test, $^{**}P < 0.01$).

and greatly enhanced drought tolerance in Arabidopsis (Fujita *et al.*, 2005). However, AREB1ΔQT-overexpressing plants did not change ABA-mediated stomatal closure, which suggests that the increased drought tolerance was attributed mainly to the elevated expression of downstream genes, such as LEA-type genes (Fujita *et al.*, 2005). Nevertheless, we found that mutation in *ABO3* impaired the sensitivity of ABA-mediated stomatal closure. Our data suggest that the drought-sensitive phenotypes exhibited by the *abo3* mutant may be attributable to both the impaired stomatal closure and lower expression of some downstream ABA-responsive genes.

Contrary to the reduced ABA sensitivity of guard-cell movement, mutation in *ABO3* resulted in an increased sensitivity of both seed germination and root growth to ABA. Furthermore, *abo3* mutant plants showed some root developmental defects. These results suggest that ABO3 functions in regulating both plant growth and stress

tolerance related to ABA response pathways. In this case, plant hypersensitivity to ABA in seed germination and seedling growth is not accompanied by higher drought tolerance. Similar results were also reported for *AtHD2C*, *CaXTH3*, *SAD1* and *AtTPS1*, and overexpression of rice *OsMYB3R-2* in Arabidopsis (Xiong *et al.*, 2001; Avonce *et al.*, 2004; Cho *et al.*, 2006; Sridha and Wu, 2006; Dai *et al.*, 2007). ABO3 appears to play a negative role in the inhibition of seed germination and root growth by ABA, but plays a positive role in ABA-mediated stomatal closure. In addition to the reduced expression of *ABF2* in the *abo3* mutant, the expression of *RD29A* and *COR47* was also reduced in the *abo3* mutant compared with the wild type, especially during early ABA treatment. ABO3 might directly regulate the expression of *RD29A* and *COR47*, or indirectly control their expression, through transcription factors such as ABF2.

Genetic analysis combining with various *abi* mutants suggests that ABO3 is one of the components in the ABA signal pathway. Double mutants of *abo3* with *abi1*, *abi2*, *abi3* and *abi5* all showed the ABA insensitivity, which is similar to the ABA-insensitive phenotype exhibited by these ABA-insensitive mutants in seed germination. ABO3 transcripts were not induced by ABA more in these *abi* mutants. Recent work revealed that ABI1 and ABI2 function redundantly in early ABA signaling after the perception of ABA by ABA receptors (Ma *et al.*, 2009; Park *et al.*, 2009). ABI3 acts as a regulator of the downstream target ABI5, which can be phosphorylated by OST1 *in vitro* (Lopez-Molina *et al.*, 2002; Umezawa *et al.*, 2009). Interestingly, only the *abi4-1/abo3* double mutant did not display an ABA-insensitive phenotype like the *abi4-1* mutant. Consistent with this observation, the induced transcripts of *ABO3* by ABA were not disturbed in the *abi4* mutant. These data suggest that ABO3 and ABI4 might operate in different ABA signaling pathways.

We did not observe any phenotypic changes in transgenic plants overexpressing ABO3. The full function of ABO3 might need some ABA-triggered post-translational modification, or ABO3 might need to cooperate with other ABA-related proteins for its capacity. Recent work indicated that ABF2 can be phosphorylated by OST1 (Fujii and Zhu, 2009). We also tried to discover whether ABO3 could be phosphorylated by OST1 *in vitro*, but got negative results (data not show). Previous work also suggests that the senescence-related WRKY53 protein may need the mitogen-activated protein kinase kinase kinase MEKK1 for its W-box binding activity (Miao *et al.*, 2007). It is speculated that ABO3 may be modified by some protein kinases other than OST1. On the other hand, our evidence indicated that WRKY18, WRKY40 and WRKY60 interact both physically and functionally in a complex for plant responses to different pathogens (Xu *et al.*, 2006). In a future study, the possibility of whether ABO3 works in a complex with other proteins needs to be explored.

EXPERIMENTAL PROCEDURES

Plant growth conditions and *abo3* T-DNA insertion

Plants were grown in 340-ml pots filled with a mixture of peat/forest soil and vermiculite (3:1) in a glasshouse at 21°C with a light intensity of 50 μmol m^{-2} sec^{-1} and 70% relative humidity (RH) under long-day conditions (16-h light/8-h dark). Seedlings were germinated and grown on MS medium (Chen *et al.*, 2005) supplemented with 3% (w/v) sucrose and 0.8% agar under the same growth conditions. The plate-grown seedlings were transferred to soil after 1 week.

For *abo3* (Salk_007496) T-DNA insertion identification, total genomic DNA was extracted from two rosette leaves. PCR was performed using primer pairs: SALK_007496LP 5′-CTTCTTCTCGAGACATGGCAG-3′ and SALK_007496RP 5′-TTGTTCCATGTTGTGAGGTTG-3′; TF 5′-GCGTGGACCGCTTGCTGCAACT-3′.

Plasmid constructs and plant transformation

RT-PCR was performed on total RNA extracted from 2-week-old *Arabidopsis thaliana* Columbia seedlings with the use of TRIzol (Invitrogen, Cat. No. 15596-026). The first-strand cDNA was synthesized with an 18-mer oligo(dT) primer. Subsequent PCR amplification of *ABO3* cDNA involved the primer pair ABO3F, 5′-ATGTTTTCAAACATCGATCACAAGGCTGTGG-3′, and ABO3R, 5′-CAACATCAGGTCTTCCGATGAAAATAGAGGAAATTCATTCC-3′. The PCR products were cloned into the CaMV *35S* promoter driving the binary vector pMDC32 (for complementation and overexpression analysis) or pMDC85 (for protein targeting; Curtis and Grossniklaus, 2003) with Gateway Technology (Invitrogen). A promoter fragment, 1544 bp of the *ABO3* gene, p*ABO3*, was amplified from Columbia genomic DNA by PCR with the primer pair 5′-TGAGCCCTCTGATCTCCTTACGACTTTACGTGTCTTTGTC-3′ and 5′-CATCGATCACAAGGCTGTGGCAGC-3′. The amplified fragment was cloned into the pCAMBIA 1391 for a transcriptional fusion of the *ABO3* promoter with the *GUS* coding region. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed by floral infiltration into wild-type Arabidopsis (Columbia accession, for gene overexpression and GUS staining assays) and *abo3* mutants (for gene complementation).

Stomatal aperture bioassays and water loss measurements

Stomatal closing assays were conducted as described by Chen *et al.* (2006). Rosette leaves were floated in solutions containing 50 μM CaCl, 10 mM KCl, 10 mM Mes-Tris, pH 6.15, and exposed to light (150 μmol m^{-2} sec^{-1}) for 2 h. Subsequently, ABA was added to the solution at 0.5 and 1 μM to assay for stomatal closing. After ABA treatment for 2 h, stomatal apertures were measured as described by Chen *et al.* (2006). Values are means ± SE, $n = 60$. Significance ($P < 0.05$) was assessed by the Student's *t*-test.

Rosette leaves of mutant and wild-type plants (about 0.5 g leaves from the same stage of different plants) growing under normal conditions for 3 weeks were detached and weighed immediately on a piece of weighting paper, and then placed on a laboratory bench (40% RH) and weighed at designated times, with three replicates. The percentage loss of fresh weight was calculated on the basis of the initial weight of the plants.

Analysis of *abo3* and *abi1-1*, *abi2-1*, *abi3-1*, *abi4-1* and *abi5* double mutants

Total genomic DNA was extracted from four rosette leaves. The oligonucleotide primers used for amplification of the *ABIs* gene fragments were as follows: ABI1dcapsF, 5′-GATATCTCCGCCGGA

GAT-3′; ABI1dcapsR, 5′-CCATTCCACTGMTCACTTT-3′; ABI2dcapsF, 5′-CATCATCTGCTATGGCAGG-3′; ABI2dcapsR, 5′-CCGGAGCATG-AGCCACAG-3′; ABI3dcapsF, 5′-CGGTTTCTCTTGCAGAAAGTCTTG-AAGCAAGTC-3′; ABI3dcapsR, 5′-TTGCCTCTAGCTCCGGCAAGT-3′; ABI4dcapsF, 5′-ATGGACCCTTTAGCTTCCCAACATC-3′; ABI4d-capsR, 5′-AGTTACCGGAACATCAGTGAGCTCG-3′; ABI5dcapsF, 5′-AGCTGAACAGGGACAAGTAACTGAAGTTTG-3′; ABI5dcapsR, 5′-CTCTGACGTCAACTTCGTTTCTCTAGTTACCATTTAT-3′. PCR reactions were performed in a 20-μl reaction system, containing 10 ng of genomic DNA, 0.5 μM of each primer, and 1 U of Taq polymerase. Amplification conditions were 94°C for 4 min, followed by 35 cycles consisting of 94°C for 15 sec, 55°C for 30 sec and 72°C for 60 sec. PCR products were digested by different restrictive enzymes and compared between wild-type and different *abi* mutants. PCR products for *abi1-1* and *abi2-1* loci were digested by *Nco*I, as described by Yin *et al.* (2009). The G → A point mutation in *abi3-1* occurs 2143 bp upstream of the start codon (Parcy *et al.*, 1997), and PCR products were digested by *Sal*I. In *abi4-1*, a G-deletion mutation was found at 619 bp that causes the early termination of translation (Finkelstein *et al.*, 1998), and PCR products were digested by *Nla*IV. A G → A point mutation occurred in the fifth nucleotide before the first putative ATG in the *abi5* mutant, and PCR products were digested by *Mse*I. The primer sequences of *ABI* genes for real-time RT-PCR are available upon request.

Protein targeting and histochemical analysis

The recombinant *ABO3-GFP* fused plasmid was introduced into onion epidermal cells by particle bombardment. GFP analysis was performed as described by Gong *et al.* (2002). Hygromycin-resistant, *ABO3* promoter–*GUS* transgenic Arabidopsis seedlings and plant parts (T_1 generation) were stained in GUS assay buffer (3 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 0.1 M Na-phosphate, pH 7, 0.1% Triton X-100, 8 mM β-mercaptoethanol) for 12 h at 37°C, followed by incubation in 70% ethanol to remove chlorophyll.

Semiquantitative and quantitative RT-PCR and RNA gel blot analysis

RNA was isolated from 200-mg tissue samples with Trizol solution (Invitrogen). First-strand cDNA was synthesized by using 5 μg of total RNA and Moloney murine leukemia virus reverse transcriptase (Promega, M5101). cDNA was used to amplify ABO3 by PCR; *Actin* was used as the internal standard. Cycling conditions were 5 min at 94°C and 20 cycles of 30 sec at 94°C, 30 sec at 58°C and 40 sec at 72°C. The volume of each cDNA pool was adjusted to give the same exponential-phase PCR signal strength for *Actin* after 20 cycles. The RT-PCR product was analyzed by electrophoresis on a 1.5% agarose gel. All PCRs were performed in triplicate.

The expression of the *ABO3* gene in ABA induction was analyzed by two-step real-time quantitative RT-PCR with use of the fluorescent intercalating dye SYBR-Green in a LightCycler detection system (Bio-Rad, Chromo 4). β-*actin* was used as a standard control. First, total RNA samples (2 μg per reaction) from 10-day-old seedlings were reversably transcribed into cDNAs by AMV reverse transcriptase, following the manufacturer's instructions (TAKARA, RR019 v.0609). Then, the cDNAs were used as templates in real-time PCR reactions with the gene-specific primers ABO3RTF, 5′-CTGT-GGCAGCACTCCTTCATGG-3′, and ABO3RTR, 5′-CACAAGACCTGC-CATGTCTCGAG-3′. The amplification of the target gene was monitored every cycle by SYBR-Green fluorescence. The cycle threshold C_t, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, was used as a measure for the starting copy numbers of the target gene. Relative quantitation of the target *ABO3* expression level involved the comparative C_t method (LightCycler; Bio-Rad). The relative expression level of the *ABO3* gene was calculated by the equation $Y = 10^{C_t/3} \times 100\%$ (C_t is the difference in C_t between the control β-*actin* products and the target *ABO3* products, i.e. $C_t = C_{tABO3} - C_{t\beta\text{-}actin}$). PCR products were confirmed on an agarose gel, and the RT-PCR data were normalized to the relative efficiency of each primer pair.

Seedlings grown on MS medium for 10 days were transferred to a solution containing 20 or 100 μM ABA, or no ABA (for control), for the described times. Total RNA (20 μg) was isolated and analyzed as previously described (Gong *et al.*, 2002). The fragments of *RD29A*, *COR47*, *RD22*, *ABI1*, *KIN1*, *DREB2A*, *ABF2* or *ABF3* were ^{32}P-labeled and used as probes for a northern blot (Chen *et al.*, 2006). *rRNA* or *tubulin* was used as a loading control.

Transcriptional activation analysis

For transcriptional activation analysis, the full-length or partial sequence of ABO3 cDNA was amplified from the ABO3-GFP plasmid fused in-frame with the GAL4 DNA binding domain in the pGBKT7 vector (Clontech), using the following primers: ABO3YF, 5′-CG-GGGATCCCGATGTTTTCAAACATCGATCACAAGGCTG-3′; ABO3-YNR, 5′-CGGCTGCAGGAAGCCGTCATCAAGGCGG-3′; ABO3YWR, 5′-CGGCTGCAGCATGCACCCCAAAGGCTTTACATG-3′; ABO3YCF, 5′-GCGAATTCGCATGTAAAGCCTTTGGGGTGCATG-3′; and ABO3-YR, 5′-CGGCTGCAGTCAAAACAA CATCAGGTCTTCCGATG-3′. The constructs were transformed into yeast strain AH109 containing the *His3* and *LacZ* reporter genes. The transformed yeast cells were grown on synthetic defined (SD) plates, with or without His, and were subjected to β-galactosidase assay. The Beyotime kit (RG0036) was used for the β-galactosidase quantitative assay.

Purification of recombinant protein and electrophoretic mobility shift assay (EMSA)

Recombinant and fusion *GST-ABO3* plasmids were transferred to the *E. coli* BL21 cell line. Under 16°C and 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for overnight incubation, the recombinant proteins were induced and purified by GST-agarose affinity.

The EMSA was performed essentially as described by Promega (http://www.promega.com/multimedia/tnt02.htm). The DNA protein binding reaction involved the incubation of 0.02 pmol of [γ-^{32}P]ATP-labeled synthesized DNA fragments APN1 and APN2 with 1 μg of purified recombinant GST-ABO3 in a total volume of 20 μl. For mutated DNA fragments, the 5′-TTGACC/T-3′ motifs of W-boxes were substituted by 5′-TTAAA-3′. The reaction products were analyzed on 5% non-denaturing polyacrylamide gels. The specificity of the DNA binding protein for the putative binding sites was established by using either the described fold excess of unlabeled or mutated [γ-^{32}P]ATP-labeled DNA fragments.

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