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F-box Protein DOR Functions as a Novel Inhibitory Factor for ABA-induced Stomatal Closure under Drought Stress in *Arabidopsis thaliana*

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ABSTRACT

Guard cells, which form stoma in leaf epidermis, sense and integrate environmental signals to modulate stomatal aperture in response to diverse conditions. Under drought stress, plants synthesize abscisic acid (ABA), which in turn induces a rapid closing of stoma, to prevent water loss by transpiration. However, many aspects of **the** molecular mechanism for ABA-mediated stomatal closure are still not understood**.** Here we report a novel negative regulator of guard cell ABA signaling, DOR, in *Arabidopsis thaliana*. The *DOR* gene encodes a putative F-box protein, a member of AtSFL (S-locus F-box-Like) family related to AhSLF-S_2 and specifically interacting with ASK14 and Cul1. A null mutation in *DOR* resulted in a hypersensitive ABA response of stomatal closing and a substantial increase of drought tolerance; in contrast, the transgenic plants overexpressing *DOR* were more susceptible to the drought stress. The *DOR* is strongly expressed in guard cells and suppressed by ABA treatment, suggesting a negative feedback loop of *DOR* in ABA responses. Double-mutant analyses of *dor* with ABA-insensitive mutant *abi1-1* showed that *abi1-1* is epistatic to *dor* , but no apparent change of PLDα1 was detected between the wild type and *dor*. Affymetrix GeneChip analysis showed that DOR likely regulates ABA biosynthesis under drought stress. Taken together, our results demonstrate that DOR acts independent of PLDα1 in an ABA signaling pathway to inhibit the ABA-induced stomatal closure under drought stress.

INTRODUCTION

Guard cells, which form stoma in leaf epidermis, control the uptake of CO_2 for photosynthesis and the loss of water through transpiration. The closing and opening of the stomata is mediated by a turgor-driven volume change of the two surrounding guard cells. The guard cells sense and integrate environmental signals to modulate stomatal aperture in response to diverse conditions. Under drought stress, the endogenous level of ABA increases and, through its complex signaling cascade, results in stomatal closure to prevent transpirational water loss (Blatt, 2000). ABA acts directly on guard cell and induces stomatal closure via the efflux of potassium and anions from guard cell and the removal of osmolytes (MacRobbie, 1998; Schroeder et al., 2001).

So far, many components involved in ABA signaling in Arabidopsis guard cells have been characterized. The type 2C protein phosphatase ABI1 is a central regulator of ABA responses (Gosti et al., 1999; Merlot et al., 2001). The dominant *abi1-1* mutations render *Arabidopsis thaliana* plants insensitive to ABA in seed germination, root growth, stomatal closure, and gene regulation (Koornneef et al., 1984; Leung et al., 1997). Intragenic loss-of-function revertants of the *abi1-1* mutant and functional inactivation of the PP2C genes encoded by *ABI1* and *HAB1* by T-DNA insertions resulted in an ABA-hypersensitive phenotype (Gosti et al., 1999; Saez et al., 2004; Yoshida et al., 2006a), indicating their functions as negative regulators of the ABA response. Subsequently, two new recessive loss-of-function alleles of *ABI1*, *abi1-2* and *abi1-3*, were identified in *Arabidopsis* and showed enhanced responses to ABA both in seed and vegetative tissues (Saze et al., 2006), confirming that the ABI1 functions as a negative regulator of ABA signaling. Recessive mutations leading to the ABA-hypersensitive stomatal closing have revealed additional negative regulators of ABA signaling in guard cells. These include the farnesyltransferase β-subunit ERA1 (Cutler et al., 1996; Pei et al., 1998), the mRNA cap binding protein ABH1 (Hugouvieux et al., 2001), and the Sm-like snRNP protein SAD1 (Xiong et al., 2001). In contrast, the protein kinase OST1 (Mustilli et al., 2002), the heterotrimeric G protein (Wang et al., 2001) and the recessive *gca2* mutation (Pei et al., 2000) are characterized as the positive regulators of ABA signaling in guard cells. In addition, recent studies have indicated that phospholipase D (PLD) acts as a positive regulator for

ABA-mediated stomatal movement through the lipid metabolite phosphatidic acid (PA) binds to ABI1 and inhibits its activity (Zhang et al., 2004; Mishra et al., 2006). The plasma membrane protein SLAC1 (SLOW ANION CHANNEL-ASSOCIATED 1) was identified as a guard cell plasma membrane protein that mediates anion channel activity, and the stomata of *slac1* mutants showed a strong insensitivity to ABA (Vahisalu et al., 2008; Negi et al., 2008).

Although much progress has been made on the guard cell ABA signaling and a current working model suggests that more than 20 components have been shown to participate in ABA-induced stomatal closure (Li et al., 2006), however, many aspects of **the** molecular mechanism for ABA-mediated stomatal closure are still not understood**.** Recently, the identification of plant ABA-binding proteins marked a major advance in understanding ABA perception and ABA signaling in plants. The RNA-binding protein FCA, a homologue of an ABA-binding protein ABAP1, was identified as an ABA receptor in the regulation of flowering time (Razem et al., 2006). The Arabidopsis ABAR (ABA-binding protein)/CHLH (H subunit of Mg-chelatase), which specifically binds ABA and mediates ABA signaling as a positive regulator in seed germination, post-germination growth and stomatal movement, was also identified as an ABA receptor (Shen et al., 2006). The G Protein Coupled Receptor2 (GCR2) also was identified as a putative plasma-membrane-localized ABA receptor (Liu et al., 2007), but its function remains a controversial issue (Gao et al., 2007; Johnston et al., 2007).

It is known that cellular ABA levels fluctuate constantly to allow plants to adjust to the changing physiological and environmental condition. For example, endogenous ABA level can be rapidly increased when plants are subjected to osmotic stress and decreased when rescued from the stress condition. These physiological processes controlled by ABA are primarily regulated by bioactive ABA pool size, which is thought to be maintained through fine-tuning the rates of de novo biosynthesis and catabolism (Nambara and Marion-Poll, 2005). Previous studies showed that a number of steps may be regulated in the ABA biosynthesis pathway in higher plants. Among them, production of xanthoxin from epoxycarotenoids is a key step in ABA synthesis (Nambara and Marion-Poll, 2005), in which NCED3 (9-cis-epoxycarotenoid dioxygenase) is the key regulatory enzyme in the ABA biosynthesis pathway (Burbidge et al., 1999). Under drought stress, *NCED3* is one of the most strongly induced seven *NCED* genes in *Arabidopsis* (Luchi et al., 2001). On the other hand, ABA

breakdown is mediated by the cytochrome P450, named CYP707As, which is a key enzyme in the oxidative catabolism of ABA (Saito et al., 2004). Furthermore, recent research on ABA metabolism showed AtBG1 and the RING-H2 protein XERICO are involved in regulating ABA homeostasis in plant cell (Lee et al., 2006; Ko et al., 2006). Thus, the regulation of endogenous ABA level is crucial for plants to adapt environmental challenges (e.g. drought stress).

F-box proteins, as a subunit of SCF (Skp1/Cullin or CDC53/F-box protein) E3 ubiquitin ligases (Deshaies et al., 1999), have been shown to play essential roles in plant growth and development, including multiple phytohormone-signaling pathways, such as auxin, gibberellin, and ethylene (Moon et al., 2004; Smalle and Vierstra, 2004).The *Arabidopsis* genome encodes more than 700 putative F-box proteins (Risseeuw et al., 2003; Gagne et al., 2004). SCF^{TIR1} was the first SCF complex identified in plants and has been shown to be involved in the auxin signaling (Gray et al., 1999). In addition, Arabidopsis F-box proteins COI1 and SLEEPY1 and the rice GID2, EBF1 and EBF2 have been shown to exist as components of SCF E3 complexes involved in jasmonic acid, gibberellin and ethylene signaling pathways, respectively (Xu et al., 2002; McGinnis et al., 2003; Sasaki et al., 2003; Guo and Ecker, 2003; Potuschak et al., 2003). However, it is unclear if there is a direct link between an F-box protein and the guard cell ABA signaling. Here we report a novel negative regulator of guard cell ABA signaling, DOR, in *Arabidopsis*. The *DOR* gene encodes a putative F-box protein, a member of AtSFL (*S*-locus-F-box-like) family related to AhSLF-S_2 (Wang et al., 2004) and specifically interacting with ASK14 and Cul1. A null mutation in *DOR* resulted in a hypersensitive ABA response of stomatal closing after drought stress. These findings suggest that DOR inhibits the ABA-induced stomatal closure under drought stress, revealing a novel mechanism in guard cell ABA signaling.

RESULTS

Identification of the *dor* Mutant

F-box proteins are known to play important roles in various aspects of plant growth and development via the ubiqutin/26S proteasome pathways (Vierstra, 2003). To examine possible

functions of the Arabidopsis *AtSFL* gene family related to AhSLF-S_2 (Wang et al., 2004), which controls the pollen function of S-RNase-based self-incompatibility (Lai et al.,2002; Qiao et al.,2004a; Qiao et al.,2004b), we obtained a total of 61 T-DNA insertion lines corresponding to 40 individual genes (Wang et al., 2004). Most of the T-DNA mutations produced no obvious morphological phenotypes (Wang et al., 2004; Dong et al., 2006). We reasoned that some of them could produce conditional phenotypes under various environmental stresses given the fact that several of these genes were inducible under various abiotic stresses (data no shown). Thus, we first performed a drought treatment of these T-DNA mutants. Water was withheld from the T-DNA insertion mutant seedlings grown in a growth room for 2 weeks. Putative mutants with drought response phenotypes were scored by the degree of leaf wilting compared with that of wild type plants. In this way, we identified a loss-of-function mutant which showed a significant increase in drought tolerance after the water withdrawal experiment, thus the gene was subsequently named as *DOR (DrOught tolerance Repressor)* (*At2g31470*).

The *dor* mutant plants did not show any obvious morphological or developmental abnormalities under normal growth conditions (Fig. 1A). However, when subjected to the drought stress, as shown in Figure 1A, the *dor* plants were turgid and their leaves remained green, whereas the wild-type plants showed severe wilting and chlorosis of the rosette leaves, indicating the *dor* mutation enhanced the plant tolerance to drought stress.

To examine the physiological mechanism of the drought tolerance of the *dor* plants, we investigated the mutation on water loss and transpiration rate during drought stress. First, we measured the water loss from the wild type and the *dor* rosette leaves after incubation at 22℃ in the light. As shown in Fig. 1B, detached leaves of the *dor* mutant lost water more slowly than did those of the wild-type plants. But the water loss showed no apparent difference between the wild type and the *dor* in the first 2 hours, owing to the *dor* plants had a lower relative water content (RWC) level compared to wild type plants prior the treatment, with the RWC of *dor* and wild type leaves at approximately 82% and 84% ($p<0.05$) (Fig. 1C), respectively. To estimate whole-plant transpiration under drought stress, we examined the RWC level of wild type and the *dor* plants after the cessation of irrigation. Transpirational water loss, as determined by the RWC measurements after 8 and 12 days from the start of the drought treatment, was greatly

reduced in the mutant compared to the wild type ($p<0.001$) (Fig. 1C). The higher RWC level in the *dor* plants during drought stress indicates that the *dor* plants could limit the water loss and maintain a higher RWC resulting in an enhanced drought tolerance.

DOR Acts as a Negative Regulator of Drought Tolerance

DOR encodes a putative F-box protein, *AtSFL35*, a member of the Class C of *AtSFL* gene family (Wang et al., 2004). The *dor* mutant was found to have a T-DNA inserted 3 bp downstream the translational start of the coding region (Supplemental Fig. S1A). DNA-blot hybridization showed that a single T-DNA copy was inserted into the genome (Supplemental Fig. S1B), and the *DOR* is a single-copy gene in the *Arabidopsis* genome (Supplemental Fig. S1C). No *DOR* transcripts were detected in the homozygous *dor* plants, indicating a complete loss of the gene function (Fig. 2A). To further confirm the *dor* was indeed a null allele**,** we analyzed the cDNA fragment downstream of the insertion site and the result showed that no DOR transcripts were detected in the homozygous *dor* plants (Supplemental Fig. S1D).

To further test whether the drought tolerance of *dor* was the result of *DOR* gene disruption, we introduced a 35S::*DOR* wild-type genomic region construct (Fig. 2B) into the *dor* plants. Six independent transgenic lines were fully complemented showing the wild-type phenotype, indicating a full complementation of the null allele (one line, Rescued *dor-1S*, is shown in Fig. 2C). To further confirm the identity of *DOR*, the *dor* mutant was transformed with the *DOR* genomic region under its own promoter (Fig. 2C，Rescued *dor-1E*). There was no discernable difference observed between the two types of the rescued plant phenotypes, and all the following analyses were thus performed with the *dor* plants rescued with the *DOR::TAPa* under the *35S* promoter. In addition, the transgenic lines were analyzed by DNA blot to confirm their molecular phenotypes (Supplemental Fig. S2), and the results of quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) indicated that *DOR* expression was markedly increased at the mRNA level in the rescued lines compared with the wild type (Fig. 2D). This latter finding was consistent with the fact that the over-expression lines were more susceptible to the drought stress (Fig. 2C，Rescued *dor-1S* and Rescued *dor-1E*).

Infrared thermography can be used as a proxy indicator of stomatal function because plants

with stomata that are more closed lose less thermal energy by evaporative cooling and, therefore, register as being warmer by thermography. Conversely, plants whose stomata are more open should be cooler (Wang et al., 2004). Our infrared thermal imaging revealed that the leaf surface temperature of the over-expression line Rescued *dor-1S* were about 0.5^{O}C cooler than that of the wild type plants, whereas the *dor* plants was approximately 0.5^{O}C warmer than that of the wild type (Supplemental Fig. S3A). These results suggest that the Rescued *dor-1S* plants resulted in more-open stomata, so as to lose more water by transpiration under drought stress. In addition, the results of the water loss for the Rescued *dor-1S*, wild type and the *dor* plants also proved that the *dor* plant lost water more slowly during drought stress than those of the Rescued *dor-1S* and wild type plants (Supplemental Fig. S3B). Taken together, these results demonstrate that the *DOR* encodes a negative regulator of drought tolerance.

The Stomata Closing in the *dor* Plant is Hypersensitive to ABA

Stomata are known to close in response to drought stress to limit water loss by transpiration. During this process, ABA is synthesized and plays a role in closing stomata. To examine this possibility, we first measured the endogenous ABA in *dor* and the wild type using an immunoassay. The content of ABA, in the wild type and *dor* did not show a significant difference in the absence of the stress treatment (Fig. 3). But after the drought treatment, the endogenous ABA was increased more in the *dor* mutant than that in the wild type (Fig. 3) (P<0.001), consistent with the higher RWC level in the *dor* plants (Supplemental Fig. S4), suggesting that the *dor* plant had an elevated endogenous ABA level under the drought stress. To further investigate whether the *dor* affected the sensitivity of guard cells to ABA, we measured the changes in stomatal aperture after ABA treatment. We exposed wild type and the *dor* plants to strong light and high humidity to induce a full stomatal opening, and epidermal peels from these plants were used to analyze stomatal responses to ABA (Pei et al., 1997). In response to ABA treatment at different concentrations, stomata in the *dor* plants were almost completely closed with 5μM ABA treatment for 2 h, but the stomata did not completely close even with 20 μM ABA in wild-type plants for the same treatment duration (Fig. 4, A and B). These results indicate that the *dor* showed an ABA hypersensitivity of the stomatal closing

compared with the wild type. However, other well-characterized responses to ABA, such as the inhibition of seed germination (Fig. 4C) and the reduction of vegetative growth (data not shown but see Fig. 1A), were not significantly altered in the *dor* plants. These results show that the DOR plays a role in controlling the ABA sensitivity of the guard cells.

The *DOR* is Preferentially Expressed in the Guard Cells

As a first step to look into reason as why the *DOR* specifically acts in the guard cells, we used qRT-PCR to analyze the *DOR* expression in five major tissues, including silique, root, leaf, seedling and flower. As shown in Figure 5A, *DOR* mRNA was detected in flower, seeding and rosette leaves, but its expression in the root and silique was extremely low. In addition, *DOR* mRNA was generally of a very low abundance and hardly detectable in northern blots of these samples (data not shown). To gain more insight into the cell type expression pattern of *DOR*, we generated the transgenic plants harboring a transcriptional fusion of the *β-glucuronidase* (*GUS*) reporter gene and a putative *DOR* promoter. The result indicated that a GUS activity was clearly visible in the stoma of the leaves (Fig. 5B). No GUS signals were detected in tissues devoid of stoma, suggesting that the *DOR* expression is preferentially expressed in the guard cells. This was consistent with the finding that the *DOR* gene was expressed in the guard cells compared with the mesophyll cells by a previous microarray analysis (Leonhardt et al., 2004).

Furthermore, we examined whether ABA treatment induced a change in the *DOR* transcript level. As shown in Figure 5C, the *DOR* expression was rapidly reduced during early application of exogenous ABA in the wild type plants. Similarly, the *DOR* expression was reduced by drought stress (Fig. 5D). These results suggested that DOR acts as a negative regulator of the guard cell ABA signaling but its own expression is suppressed by ABA, revealing a negative feedback loop for ABA response.

ABA-Induced Stomatal Closure is Defective in *dor*/*abi1-1* Double Mutant

As a guard cell-preferential gene, how is *DOR* involved in ABA signaling? We found that the ABA-induced reduction of the *DOR* expression observed in the wild type plants (see Fig. 5C) was completely abolished in an *abi1-1* background (Fig. 5E), suggesting that ABI-1 is essential

for this ABA repression of *DOR*. In addition, the *DOR* expression was elevated in *abi1-1*, suggesting that ABI1 negatively regulates the *DOR* expression. To further examine whether *DOR* acts exclusively through the ABI-1-mediated ABA signaling pathway, we generated a homozygous double mutant of *dor*/*abi1-1*. The *dor*/*abi1-1* double mutant showed the ABA insensitivity in seed germination, the impairment in ABA-induced stomatal closing and the wilty phenotype similar to those of *abi1-1* single mutant (Fig. 6, A ,B and C) (Supplemental Fig. S5), indicating that the *abi1-1* is epistatic to *dor* and the *dor* locus acts upstream of the *abi1-1* locus, and, together with the increased expression of *DOR* in *abi1-1*, they also formed a feed back negative regulatory loop for ABA signaling.

DOR Acts Independently of PLDα1 Pathway

Recently, Mishra *et al*. reported the PLDα1 and PA act upstream of GPA1 and ABI1 in a pathway regulating the stomatal responses to ABA signals (Mishra et al., 2006). We next examined whether DOR and PLDα1 protein function in the same pathway by probing immunoblots with an antibody against PLDα1, and the results indicated that PLDα1 protein did not show any obvious change in the wild type and the *dor* plants under the normal and drought stresses (Fig. 7). However, we found that PLDα1 proteins in WT and the *dor* were both drastically decreased by drought treatment, and this may be related to other signal transduction pathway(s) that the PLDα1 protein involves. In addition, the ABA responses of the double mutant *dor*/*pldα1* resembled the wild type (Supplemental Fig. S6), confirming that they function independently. These results suggested that DOR does not act through PLDα1 in its negative regulation of ABA responses in the guard cells and possibly represents a new pathway.

DOR Interacts with ASK14 and CUL1 Proteins

To confirm that *DOR* actually encodes an F-box protein (Fig. 8A), we examined whether DOR interacts with ASK proteins by a yeast two-hybrid analysis. We isolated 17 ASK genes from *Arabidopsis* and tested the interaction between them and DOR. The result indicated that the DOR specifically interacts with ASK14 protein (Fig. 8B). To further confirm that DOR forms the SCF complex, we performed a pull-down assay using an antibody against AtCUL1, which

is a known subunit of several SCF complexes. CUL1 proteins were detected in the pull-down product of the *DOR-TAPa* transgenic plants and wild type, whereas no protein was detected in the negative control *TAPa* transgenic plants (Fig. 8C). In general, it was observed that conjugation of RUB1 occurred on WT Cul1 protein, but such a modification did not occur on the C-terminal TAPa-tagged DOR (DOR-TAPa), which could be explained by the finding that the N-terminal and C-terminal TAPa fusions could have different functional properties (Rubio et al., 2005). Taken together, these results indicate that DOR is associated with the two known subunits of SCF complex and likely forms a bona fide SCF^{DOR} complex in *A. thaliana*.

GeneChip Analysis of *dor* Plants

To find further functional clues to *DOR*, we performed an Affymetrix GeneChip analysis to investigate the effect of the *DOR* gene disruption on global gene expression under drought stress. Subsequently, we found among the transcripts up-regulated in the *dor* mutant (Table 1), the *NCED3* gene encoding a key enzyme, 9-cis-epoxycarotenoid dioxygenase in ABA biosynthesis (Qin et al., 1999) was significantly enhanced in the *dor* plants after drought treatment, suggesting that more synthesis and accumulation of endogenous ABA occurred in the *dor* plant. Moreover, the ABA- and desiccation-inducible genes (i.e., *RD29A , RD29B, XERO2,* and *RAB18*) (Yamaguchi-Shinozaki and Shinozaki, 1993; Welin et al., 1994; Lang and Palva, 1992) were also highly expressed in the *dor* plants (Table 1). The high-level expression of ABA-biosynthetic and ABA responsive genes in the *dor* plants are consistent with the fact that the disruption of the *DOR* gene slightly increased the cellular ABA levels but with the dramatic increase after the drought stress (see Fig. 3). In addition, we did drought treatment for the detached leaves from WT and *dor* mutant, and harvested the samples at two time points, including 2 hours and 6 hours, and then did qRT-PCR for the *NCED3, RAB18* and *RD29A* expression profiles and the results showed that the *NCED3* expression was significantly enhanced in the *dor* plants after drought treatment in the first 2 hours, and the *RAB18* and *RD29A* were strongly enhanced at 6 hours after drought treatment (Supplemental Fig. S8). Taken together, these results show that the DOR, as an F-box protein, is likely involved in the regulation of ABA biosynthesis under drought stress though other possible roles

in regulating the ABA breakdown and/or signaling could not be completely excluded at this stage.

DISCUSSION

In this study, we have identified an F-box protein DOR that is important for drought response and provided evidence for a connection between ABA guard cell signaling and the ubiquitin/26S-mediated proteolysis pathway, revealing a novel inhibitory pathway for modulating the ABA-induced stomatal closure under the drought condition in *Arabidopsis*.

DOR Acts as a Novel Negative Regulator of the Guard Cell ABA Response in *Arabidopsis*

ABA signal transduction in guard cells is a highly complex process (Li et al., 2006). The type 2C protein phosphatase ABI1 acts as an important regulator of ABA responses in guard cell (Leung et al., 1994; Meyer et al., 1994; Nilson and Assmann, 2006) and plays a negative role in the ABA-induced stomatal closure. Some of the signaling components functioning upstream and downstream of ABI1 have been identified. To our knowledge, most of them function downstream of ABI1, such as OST1 and ERA1. OST1 kinase activation in response to ABA is suppressed in the dominant *abi1-1* mutant (Mustilli et al., 2002), indicating that the ABI1 negatively regulates the ABA signal transduction upstream of OST1. Partial suppression of the ABA-insensitive phenotypes of the *abi1* and *abi2* mutants by *ERA1* deletion suggests that the target of the *ERA1* FTase may function downstream or in parallel to these ABI protein phosphatases (Pei et al., 1998).

However, the early events of ABA signal transduction remains largely unknown, especially those acting upstream of ABI1. At the present time, very few factors functioning upstream of ABI1, such as PLD, have been identified in Arabidopsis. The PLD generates phosphatidic acid (PA), which is reported to bind to ABI1 and thereby abolishes the ABI1 inhibition of the ABA-induced stomatal closing (Mishra et al., 2006). In this study, we have found the F-box protein DOR plays a negative role in the ABA-induced stomatal closing (Fig. 4A and B). The *dor*/*abi1-1* double mutant analysis indicated that the *abi1-1* is epistatic to *dor* and the *dor* locus acts upstream of the *abi1-1* locus. Although PLDα1 functions in the upstream of ABI1, our results suggest that DOR does not act through PLDα1 in its negative regulation of ABA

responses in the guard cells. So we deduce that the DOR represents a novel regulator implicated in the ABA signaling and is negatively regulated by ABI1 in the guard cells.

More recently, ABAR was identified as an ABA receptor to regulate a series of the components involved in the ABA signaling (Shen et al., 2006). Interestingly, we found that the *ABAR* expression was down-regulated in the *dor* mutant under drought stress (data not shown), suggesting a positive regulation of *DOR* by *ABAR*. Further investigation on the relationship between them could provide a clue to the function of *DOR*. In addition, the role of *DOR* in the guard cell ABA responses is consistent with the fact that *DOR* is mainly expressed in the guard cells because we did not found any other well-characterized responses to ABA, such as the inhibition of seed germination and reduction of vegetative growth in the *dor* plants. Furthermore, *DOR* may also play a role in regulating the pollen development and/or pollination since its expression also was detected in pollen (Supplemental Fig. S9).

DOR is likely Involved in the Regulation of ABA Biosynthesis under Drought Stress

Plants are subjected to constant environmental and physiological changes and accordingly need to fine-tune the ABA level in relation to the severity and duration of the stresses. The current model indicates that an increase in ABA levels occurs primarily through de novo biosynthesis under various abiotic stress conditions (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005). ZEP (ABA1), NCED3, ABA3 and AAO3, act as key enzymes regulating the ABA biosynthesis pathway in *Arabidopsis* and other plant species, whose expression are generally upregulated by the physiological needs or environmental stresses (Audran et al., 1998; Cutler and Krochko, 1999; Luchi et al., 2000; Nambara and Marion-Poll, 2005). The increased transcription of these genes might lead to an increase in ABA levels, which, in turn, would induce a positive feedback regulation of *NCED3*, *AAO3* and *ABA1* gene expression (Xiong et al. 2002). In this scenario, *NCED3* is considered the most responsive gene to the newly synthesized ABA. Our GeneChip analysis also confirmed that *NCED3* is the ABA biosynthetic gene most responsive to the drought stress in the *dor* plants (Table 1). However, the transcriptional regulatory mechanism did not appear to be the only one to regulate the ABA biosynthesis. The activation of inactive ABA pools by polymerized AtBG1 is a mechanism by which plants rapidly adjust ABA levels and respond to changing environmental conditions (Lee

et al., 2006). In addition, Ko et al. (2006) also reported the upregulation of XERICO, a RING-H2-type zinc-finger protein, substantially increased cellular ABA levels. In this study, we found that the stress-induced ABA reduces the *DOR* expression and could in turn enhance the ABA biosynthesis. Thus, they could form a negative feedback regulatory loop for ABA in the guard cells under drought stress. In this way, the ABA production by its biosynthesis regulated by *DOR* could be controlled precisely and rapidly to meet the plant requirements under water stress. Indeed, the content of ABA in the wild type and *dor* did not show a significant difference in the absence of the stress. But after the drought treatment, the *dor* had an elevated endogenous ABA level higher than that detected in the wild type (Fig. 3). The enhanced ABA content in the *dor* plants correlated directly with the rapid induced-stomatal closing, and thus increases the plant tolerance to the drought stress. These results highlight the importance of the F-box protein DOR in providing a requisite ABA level for the cellular responses to drought stress. Further examination of *dor* with mutants involved in the ABA synthesis as well as using its inhibitor could help reveal how these factors are related to each other.

Previous studies have shown that most F-box proteins are associated with the ubiquitin/26S proteasome-mediated protein degradation. *DOR* encodes one of AtSFL proteins and specifically interacts with ASK14 and Cul1 based on our yeast two-hybrid and pull-down assays (Fig. 8), indicating that it may function as a subunit of SCF E3 ligase in the ubiquitin/26S-mediated proteolysis pathway. Incidentally, we detected a similar expression pattern of *ASK14* to *DOR* (data not shown). Functional analysis of *ASK14* could provide further insights into the *DOR* action in the guard cells.

In conclusion, we have shown that the *DOR* is perceived by the guard cell as a signal that triggers a response to inhibit the ABA-induced stomatal closure under drought stress. However, it is not clear how exactly *DOR* is involved in regulating the guard cell ABA signaling. As an F-box protein, we propose that DOR could target a factor (s) directly involved in the ABA signaling for degradation via the ubiquitin/26S-mediated proteolysis pathway under drought stress. Modulation of DOR or its target(s), specifically in the guard cells, could provide an avenue to genetically modify the plant tolerance to drought stress.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used. The *dor* mutant was obtained from the SALK T-DNA insertion collection (http://signal.salk.edu). The original line was Salk-050074. The *abi1-1* mutant seeds were a gift from Xiangdong Fu. *Arabidopsis* plants were grown on MS medium containing 2% (W/V) sucrose and 0.8% (W/V) agar or grown in soil under a long-day condition (16 hr light; 8 hr dark) at 22^{o}C, except where otherwise stated.

RT-PCR (reverse transcription-polymerase chain reaction) Analysis

Total RNA was prepared as previously described (Lai et al., 2002) and was digested with DNase I (TaKaRa, Dalian, China). Reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was used to synthesize the first strand cDNA. Specific primers of *DOR* used for RT-PCR were as follows: 5'-ACCCTATTCTTATATCGTTCC-3' (forward) and 5'- AGGGTTACATATCGCCGAGAC -3' (reverse). Specific primers of *DOR* used for RT-PCR of the downstream sequence of the T-DNA insertion site were as follows: 5'- CCAGAATTTGTCTCGGCGAT -3' (forward) and 5'- CCTCTTCCTTGTCTTAGGTTTTGG -3' (reverse). Specific primers of *TUBULIN* used for RT-PCR were as follows: 5'-TTTGGAGCCTGGGACTATGGAT-3' (forward) and 5'-ACGGGGGAATGGGATGAGAT -3' (reverse)

qRT-PCR (quantitative real-time reverse transcription-polymerase chain reaction) Analysis

Reverse transcription was performed using TaqMan Reverse Transcription Regents Kit (Applied Biosystems, CA, USA). The cDNA samples were diluted to 10, 5, and 1ng/μl. Triplicate quantitative assays were performed on 1μl of each cDNA dilution using the SYBR Green Master mix with an ABI 7900 sequence detection system according to the manufacture's protocol (Applied Biosystems). Gene-specific primers were designed by using PRIMEREXPRESS 1.0 software (Applied Biosystems). The relative quantization method

(ΔΔCT) was used to evaluate quantitative variation between the replicates examined. The amplification of 18S *rRNA* was used as an internal control to normalize all data. Specific primers of *DOR* used for qRT-PCR were as follows: 5'- CCAGAATTT GTCTCGGCGAT -3' (forward) and 5'- CCTCTTCCTTGTCTTAGGTTTTGG -3' (reverse). Specific primers of 18S *rRNA* used for qRT-PCR were as follows: 5'-CGGCTACCACATCCAAGG AA-3' (forward) and 5'- TGTCACTACCTCCCCGTGTCA -3' (reverse).

Plasmid Construction and Arabidopsis Transformation

The *DOR::GUS* (*DOR:: β-Glucuronidase)* construct: Genomic DNA sequence corresponding to1858 bps upstream the predicted ATG codon of the *DOR* ORF was cloned into *pCAMBIA1391* binary vector between *Sma*I and *Sal*I sites. Junction sequences of the resulting construct *DOR::GUS* were sequenced for verification. The PCR primers used were as follows: 5' ATGTCGACGTGTGGCTAACTCTTCTCTGTC3' (forward) and 5'ATCCCGGGGTTGCAGTAAT CAAATTCTTTTGGAAC3' (reverse). The sense sequence of the *DOR* gene were introduced into the *pC-TAPa* vector (see Figure 1C). The PCR primers used were as follows: 5'-CACCACAAA ATGAAATCACGGCGA CAGAATGTG-3' (forward) and 5'-TATAAGCTTCACATCCTCTACATG-3' (reverse).

The *DORp-DOR* construct: Genomic DNA sequence corresponding to1858 bps upstream the predicted ATG codon of *DOR* and the full length genomic sequence of *DOR* was cloned into *pCAMBIA1300* binary vector between *Sma*I and *Sal*I sites. The PCR primers used were as follows: 5'-ATGTCGACGTGTGGCTAACTCTTCTCTGTC-3'(forward) and 5'-TATAAGCTTC ACATCCTCTACATG-3' (reverse). Transgenic *Arabidopsis* plants were obtained by *Agrobacterium*-mediated transformation using the floral dip method (Bechtold et al., 1998). Analyses of transgenic lines were performed on homozygous T_3 progeny plants.

ABA and Water Stress Treatments

For ABA treatments, 3-week-old wild-type and *abi1-1* seedling were grown in solid MS medium with 2%w/v sucrose and transferred to liquid MS medium supplemented with 100μM ABA. For drought stress, both the wild-type and *dor* plants were grown under normal watering conditions for 24 days and then stressed by completely depriving of irrigation for 11 days. For

water loss measurement, rosette leaves of wild type and the *dor* mutant were detached as described above. Loss in fresh weight was monitored at indicated time points. Relative water content (RWC) was measured after drought stress. The leaves were detached and weighted for the initial fresh weight (FW), then were submerged in water overnight to measure saturated weight (SW), followed by drying in an oven at 80^{o}C for 24 h for dry weight (DW). RWC was expressed as (FW-DW)/(SW-DW)x100%.

Affymetrix GeneChip Analysis

RNA isolation and purification: *Arabidopsis* rosette leaves samples were homogenized in liquid nitrogen prior to RNA isolation. Total RNA was extracted using Qiagen Rneasy kit (Valencia, CA, USA). For Affymetrix GeneChip ATH1 analysis, 8 μg of total RNA was used for making biotin-labeled cRNA targets. All the process about cDNA and cRNA synthesis, cRNA fragmentation, hybridization, washing and staining, and scanning, followed the GeneChip Standard Protocol (Eukaryotic Target Preparation). In this experiment, we applied Poly-A RNA Control Kit and the One-Cycle cDNA Synthesis kit. We used Affymetrix developed GCOS software to do data collection and normalization so that signals from different arrays were comparable. The overall intensity of all probe sets of each array was scaled to 500 so hybridization intensity of all arrays was equivalent, and each probe set was assigned “P”, “A” and “M” and also with p value from algorithm in GCOS. To find differentially expressed genes between wild-type and the mutant, or between control and drought stress, log2 transformed signal ratio of each gene was calculated by applying GCOS baseline tool.

ATH1 GeneChip was used for gene expression analysis of wild-type plants and *dor* mutant under drought treatment (both the wild-type and *dor* plants were grown under normal watering conditions for 24 days and then stressed by completely depriving of irrigation for 10 days). Two biological repeat experiments were conducted and the raw data was analyzed by applying Affymetrix GCOS software. A large number of genes showed significant up- and down-regulations in *dor*-drought *vs*. CK, and WT-drought *vs.* CK, separately. We further did a comparison for difference between *dor* mutant and WT under drought condition. The fold change for *dor*-drought/CK vs. WT-drought/CK was calculated. First, ABA biosynthesis and signal transduction pathway related genes expression in the GeneChip were investigated.

Second, we found that some of the significantly changed genes were also identified in the guard cells under an ABA treatment experiment (Leonhardt et al. 2004). We listed the top candidate genes in Table 1. The ATH1 GeneChip data for the *dor* mutant analysis has been submitted to the public microarray data base under the accession number GSE10643 (http://www.ncbi.nlm.nih.gov/projects/geo/).

Thermal Imaging

Thermal imaging of drought-stressed plantlets was performed as described previously (Merlot et al., 2002). In brief, plantlets were first grown under well-watered conditions (21°C, 60 to 70% RH, 16-h photoperiod) for approximately 1 week. Drought stress then was initiated by withholding watering and transferring the pots to a drier atmosphere (24°C, 50% RH, 16-h photoperiod). Thermal images were obtained using a Thermacam PM250 infrared camera (Inframetrics, North Billerica, MA). Images were saved on a Personal Computer Memory Card International Association card and were analyzed subsequently on a Macintosh computer using version 1.56 of the public domain image-analysis program NIH Image (http://rsb.info.nih.gov/nih-image/).

Stomatal Aperture Bioassays

Stomatal closing assays were conducted as described previously (Pei et al., 1997). Rosette leaves were floated in a solution containing 50μM $CaCl_2$, 10 mM KCl, 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid]-Tris, pH 6.15, and exposed to light (150 μmol m^{-2} s^{-1}) for 2 h. Subsequently, ABA was added to the solution to assay for stomatal closing. After ABA treatment for 2 h, stomatal apertures were measured as described previously (Pei et al., 1997). Values are means ± standard errors (SE) (*n* =50).

Histochemical Analysis of GUS Activity

Histochemical location of GUS activities in the transgenic plants was analyzed after incubating tissues in X-gluc buffer (50mM sodium phosphate buffer, pH 7.0, 10mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferrocyanide, and 2mg/ml 5-bromo-4-chloro-3-indolyl glucuronide[X-gluc]) at 37°C for 12h and subsequently cleared in a transparent liquid medium

(85% lactic acid, chloral hydrate, phenol, clove oil, xylene) (weight ratio is 2:2:2:2:1). GUS images were taken with an Olympus BX-RFA fluorescence microscope.

ABA Content Analysis

Drought stress was treated by the method of Lee et al. (2006). In brief, plants grown on MS plates for 21 days and were exposed to 30% RH (relative humidity) for 10 hr. The leaves of drought stress and unstressed control were frozen in liquid nitrogen and ground into powder. One gram of the tissue was suspended in 10 ml of extraction solution containing 80% methanol, 100 mg/L butylated hydroxytoluene, and 0.5 g/L citric acid monohydrate. The suspension was stirred overnight at 4^{o}C and centrifuged at 1000*g* for 20 min. The supernatant was transferred to a new tube and dried under vacuum. The dry residue was dissolved with 500 ul of Tris-buffered saline (50 mM Tris, 0.1 mM MgCl2·6H2O, and 0.15 M NaCl, pH7.8). ABA concentration in the solution was then determined using the Phytodetek ABA immunoassay kit (Idetek, Inc., Sunnyvale, CA, USA).

Isolation of *dor*/*abi1-1* Double Mutant

We obtained the *dor*/*abi1-1* homozygous mutant lines by crossing *abi1-1* into *dor*. F_2 plants were screened by PCR amplification with the *DOR*-specific primers of 5'-CAAGCAATGCTTC AAAGCAGAGGG ATGG-3' and 5'-AGGGTTACATATCGCCGAGAC-3', the segregation ratio was about 9:3:4. The homozygous *dor* plants, which had been confirmed by DNA blot (Supplemental Fig. S1), were further confirmed by direct sequencing of the *ABI1* loci. The primers of *abi1-1* were 5'-GGAATCAGCAGCTGCTGATATAGTCGT-3' and 5'-TCTC CGAGT CAACTC TCAGGAACGAG-3'.

Immunoblot Analysis

21-day-old seedlings of wild type and *dor* (treated with or without 30% PEG 6000 for 5 hours) were homogenized with extraction buffer (0.2 M Tris-HCl, pH 6.8, 1mM DTT, 4% SDS, and 25% glycerol). The samples were boiled and analyzed by immunoblotting with the PLDα1 Antibody (a gift from Wenhua Zhang) and a mouse monoclonal anti-tubulin Ab (Sigma, St. Louis, MO, USA) as described previously (Qiao et al., 2004a).

Plasmid construction and yeast transformation assay

The coding sequence of the *DOR* gene was introduced to *pGBKT7* bait plasmid (Clontech, CA, USA) to produce a fusion protein with the GAL4 DNA binding domain, and 17 ASK cDNA (ASK1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18 and 19) were inserted into *pGADT7* prey plasmid containing the GAL4 activation domain (Wang et al., 2004). Yeast transformation, growth conditions, and assays for β-galactosidase activity were performed according to the manufacturer's instructions (Clontech, CA, USA).

***TAPa* purification procedure**

DOR-TAPa and *TAPa* seedlings (5g, fresh weight) grown in agar under a long-day condition (16 hr light; 8 hr dark) at 22°C were ground in liquid nitrogen, thawed in 2 volumes of extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Tween-20, 1 mM PMSF, and 1x complete protease inhibitor cocktail; Roche), filtered through four layers of cheesecloth, and centrifuged at 12 000*g* for 10 min at 4°C. The protein concentration in the supernatant was determined by Bradford assay (Bio-Rad, USA). Extracts containing similar amounts of total protein were incubated with 500µl IgG beads for 6 h at 4°C with gentle rotation. Elution from the IgG beads was performed by the protocol of IgG Sepharose™ 6 Fast Flow (Amersham Biosciences, Sweden). All the steps in the purification procedure were carried out at 4°C. Proteins in each fraction were separated on a 12%SDS-PAGE gel. Protein bands were visualized, independently, by immunoblotting using the *Arabidopsis* Cul1 antibody (Liu et al., 2002).

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Figure Legends

Figure 1. Increased tolerance of the *dor* plants under drought stress.

A**,** Phenotype of the wild type and the *dor* plants after drought treatment. Both the wild-type and the *dor* plants were grown under normal watering conditions for 24 days (a) and then stressed by completely depriving of irrigation for 11 days (b).

B, Time courses of the water loss from the detached leaves of the *dor* and WT plants. The water loss is expressed as a percentage of the initial fresh weight at indicated intervals. Each point indicates the mean of 6 measurements with standard errors

C, Relative water content (RWC) in leaves from the wild type and the *dor* plants after drought stress. RWC was measured 0, 8 and 12 day after the drought stress. The leaves were detached and weighted for the initial fresh weight (FW), saturated weight (SW) and dry weight (DW). RWC was expressed as (FW-DW)/(SW-DW)x100%. Values are means ± SD (n=3) from three independent experiments.

Figure 2. Complementation of the *dor* drought tolerance phenotype by the wild-type *DOR* gene.

A**,** RT-PCR analysis of the *DOR* expression in the homozygous *dor* plants. No detectable *DOR* transcripts were found in the mutant. The expression of *Tubulin* is used as the control. "+" and "-" indicate whether M-MLV reverse transcriptase was present or absent in the process of reverse transcription reaction.

B**,** Schematic presentation of the *DOR:TAPa* construct. The *DOR* is driven by two copies of the cauliflower mosaic virus 35S promoter (2X35S) and a tobacco mosaic virus (TMV) U1 X translational enhancer. The TAPa tag consists of two copies of the protein A IgG binding domain (2XIgG-BD), an eight amino acid sequence corresponding to the 3C protease cleavage site (3C), a six histidine stretch (6XHis), and nine repeats of the MYC epitope (9Xmyc). The vector contains the GATEWAY cloning sites (attR1::Cmr::ccdB::attR2). A Nos terminator (*Noster*) sequence is located downstream of the expression cassette.

C**,** Phenotypes of the transgenic Rescued *dor-1S* and Rescued *dor-1E* plants. The wild-type, Rescued *dor-1S*, Rescued *dor-1E* and *dor* plants were grown under normal watering

conditions for 24 days, and then stressed by completely depriving of irrigation for 11 days. The upper panel shows the plants with regular watering and the lower with the drought treatment. Rescued *dor-1S* and Rescued *dor-1E* indicate the *dor* plants harboring the *CaMV 35S-DOR:TAPa* and *DORp:DOR*, respectively.

D**,** qRT-PCR analysis of the *DOR* expression in the seedlings from the *dor*, the rescued transgenic lines and the wild type plants. The amplification of 18S *rRNA* was used as an internal control to normalize all data.

Figure 3. The *dor* plant has an increased cellular ABA level.

ABA contents were measured from the leaves of WT and the *dor* plants grown for 21 days, and then subjected to the drought treatment for 10 hr (see Materials and Methods). The ABA amount is expressed as ng per g of fresh weight leaf tissue. Error bars, mean ±SD from three independent experiments.

Figure 4. Stomatal behavior of the wild-type and the *dor* plants in response to ABA.

A-B**,** Stomatal closure in the *dor* plants is hypersensitive to ABA. Data represent the means ± SE from 50 stomata measured for each data point, from three independent experiments. Stomatal aperture ABA (0)= (100%) corresponded to average stomatal apertures of 2.94 ±0.12 µm (wild type) and 2.88 ± 0.18 µm in the *dor*. Scale bar=10µM. Stomata were opened by exposing to light for 2 hr and after 2 hr of the treatment with different concentrations of ABA.

C, Effect of ABA on the *dor* seed germination. Seeds of the wild type and the *dor* were plated on agar plates with or without 0.75µM ABA (upper plate: control; lower plate: 0.75µM ABA). The germination was scored at day 7 after being incubated at 22^{o}C. Seeds were incubated at 4^{o}C for 3 days prior to incubation at 22^{o}C for germination.

Figure 5. *DOR* is preferentially expressed in the guard cells.

A, qRT-PCR analysis of the expression of *DOR* in different tissues. Si, siliques; R, roots; L, leaves; Se, seedling; F, flower.

B, The *DOR promoter::GUS* expression in the guard cells. Blue color indicates the GUS expression. Scale bar=10µM

C, qRT-PCR analysis of the *DOR* expression in response to 100µM ABA in the wild type

plants.

D, qRT-PCR analysis of the expression of *DOR* in response to desiccation in the wild type plants.

E, qRT-PCR analysis of the *DOR* expression in response to 100μM ABA in the *abi-1* mutant.

The amplification of 18S *rRNA* was used as an internal control to normalize all data in the qRT-PCR analysis.

Figure 6. Analysis of *dor/abi1-1* double mutant characteristics.

A, Effect of ABA on the *dor/abi1-1* seed germination. Seeds of the wild type(Ler), wild type(Col), *dor, dor/abi1-1*and *abi1-1* were plated on agar plates with or without 0.75μM ABA (left: control; right: 0.75μM ABA). The germination was scored at day 7 after incubation at 22^{o}C. Seeds were incubated at 4^{o}C for 3 days prior to incubation at 22^{o}C for germination.

B, Comparison of the stomatal closing responses induced by ABA (10μM) in the wild type (Ler), wild type (Col), *dor, dor/abi1-1*and *abi1-1* plants. Stomatal aperture ABA (0)= (100%) corresponded to average stomatal apertures of 2.96 ±0.16 μm wild type(Ler), 2.98±0.08 μm wild type(Col) and 2.80 ± 0.13 μm in *dor*, 3.07±0.12 μm in *dor/abi1-1* and 3.12±0.05 μm in *abi1-1*. Data presented are the means of 50 stomatal apertures ± SEM.

C, Phenotype of the *dor/abi1-1* plants after drought treatment. The wild type(Ler), wild type(Col), *dor, dor/abi1-1*and *abi1-1* plants were grown under normal watering conditions for 24 days and then stressed by completely depriving of irrigation for 11 days .

Figure 7. PLDα1 levels in the wild-type and the *dor* plants.

Immunoblot analysis of PLDα1 was done with 10μg protein of crude extracts of the wild type and the *dor* with or without the drought stress. The proteins also were analyzed by immunoblot hybridization using an anti-tubulin antibody for loading control in both plants.

Figure 8. DOR interacts with ASK14 and CUL1 proteins.

A**,** Schematic representation of the DOR domain structure. Besides the F-box motif, conserved domains in the C-terminal regions contain C1, C2, C3 and C4 domains (Wang et al., 2004).

B,Yeast cells containing various combinations of BD and AD fusions were tested for their growth on SD/-Ade/-His/-Leu/-Trp media; *pGBKT7-53* with *pGADT-7* was used as positive

control and *pGBKT7* with *pGADT* as negative control (upper panel). The strains were grown further to test for the expression of the beta-galactosidase reporter gene (lower panel).

C, A pull-down assay for DOR and CUL1 proteins. Protein extracts from the *DOR-TAPa* and the negative control *TAPa* transgenic plants were subjected to the TAPa purification. WT was used as a positive control. Proteins corresponding to each fraction obtained were separated on a 12% SDS-PAGE gel. The Arabidopsis CUL1 antibody was used for immunoblotting.

Table 1. *Top candidate genes differentially expressed between the wild-type and dor leaves under drought stress using ATH1 GeneChip*

Affy Probe Set ID	Description	AGI Number	*dor*-dr/ck log2 ratio [a]	P-value [b]	Fold Change [c]	WT-dr/ck log2 ratio [a]	P-value [b]	Fold Change [c]
257280_at	NCED3* ♣	At3g14440	5.15	0.00002	35.5	2.2	0.000836	4.6
263570_at	AAO3 ♣	At2g27150	3.05	0.000025	8.3	1.4	0.002744	2.6
247957_at	ABI2 ♣	At5g57050	2.9	0.000194	7.5	1.75	0.000366	3.4
247095_at	RAB18*	At5g66400	6.1	0.00002	68.6	4.65	0.00002	25.1
248337_at	RD29A*	At5g52310	5	0.00002	32.0	1	0.165514	2.0
248352_at	RD29B	At5g52300	6.5	0.00002	90.5	3.95	0.001028	15.5
252102_at	XERO2	At3g50970	6.7	0.00002	104.0	3.05	0.00002	8.3
259231_at	AtPP2CA	At3g11410	2.85	0.00002	7.2	1.9	0.00002	3.7
259570_at	COR47	At1g20440	4.65	0.00002	25.1	2.3	0.000025	4.9
262128_at	LEA	At1g52690	7.7	0.00002	207.9	4.7	0.000044	26.0
258347_at	LEA-like	At3g17520	10.35	0.00002	1305.2	6.05	0.00002	66.3
265216_at	MAP kinase	At1g05100	3.9	0.00002	14.9	2.45	0.201916	5.5
264005_at	AGP	At2g22470	1.95	0.000229	3.9	0.5	0.631018	1.4
267080_at	unknown	At2g41190	5.5	0.00002	45.3	4.4	0.00002	21.1
263881_at	unknown	At2g21820	6.3	0.00002	78.8	5.7	0.00002	52.0
264524_at	tat-binding	At1g10070	4.95	2.15E-05	30.9	3.2	2.85E-05	9.2

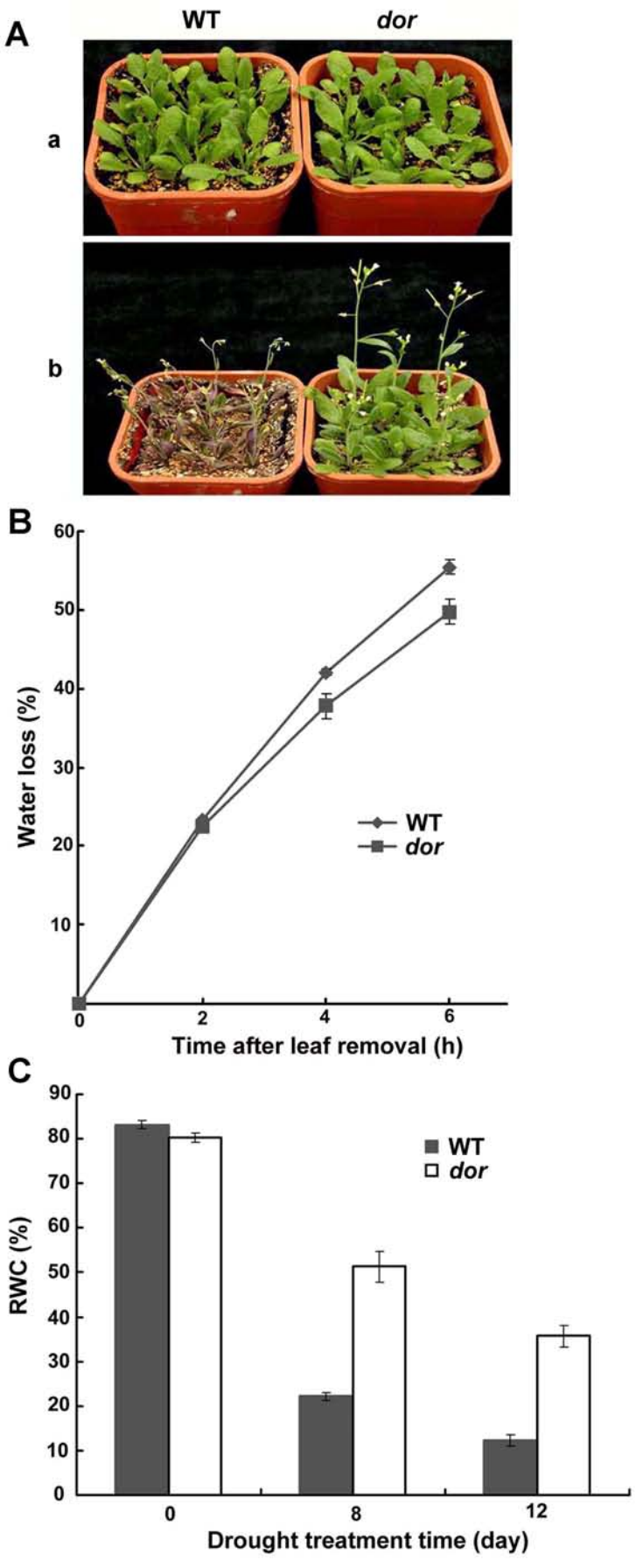
[a] $\log_2$ ratio measures the change in expression level for each probe set between drought treatment (dr) *vs*. control (ck). This change is expressed as the $\log_2$ ratio. A $\log_2$ ratio of 1 is the same as a fold change of 2. Here we averaged the $\log_2$ ratio for two biological replicates.

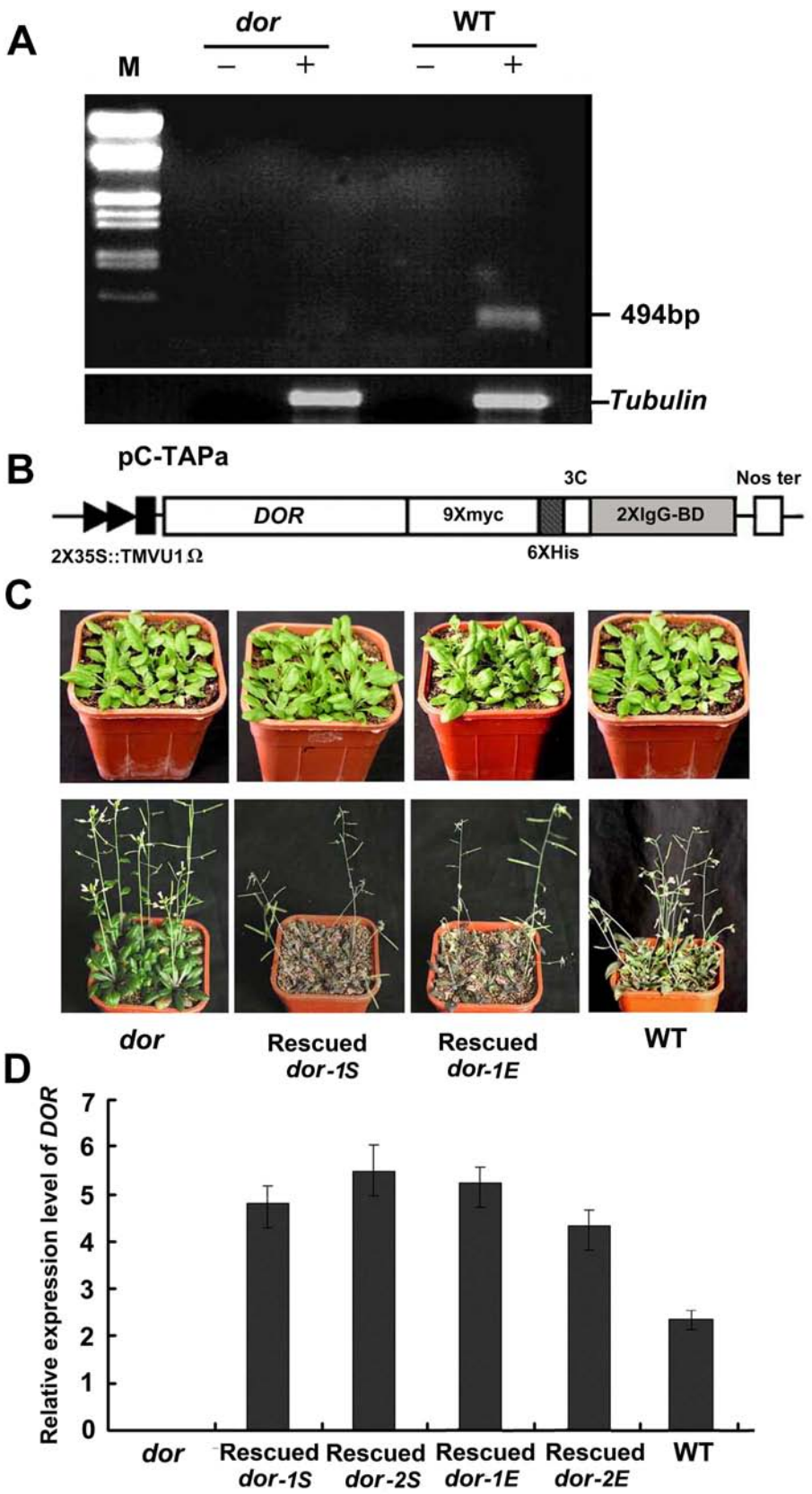
[b] Change P-value, which measures the probability that the expression levels of each probe set between drought treatment *vs*. control are the same. Here we averaged the Change P-value for two biological replicates.

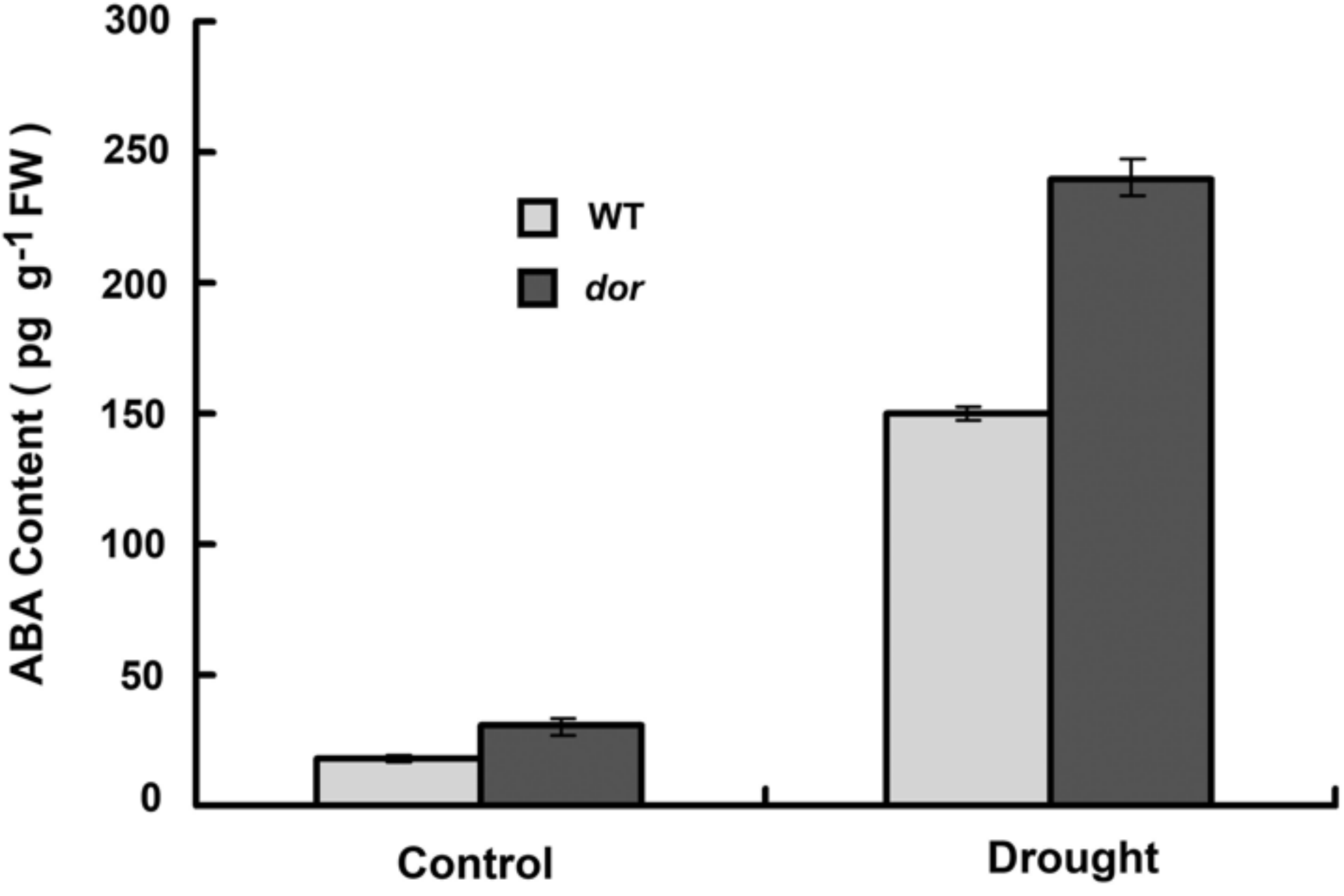
[c] Fold change is calculated using the signal log ratio.

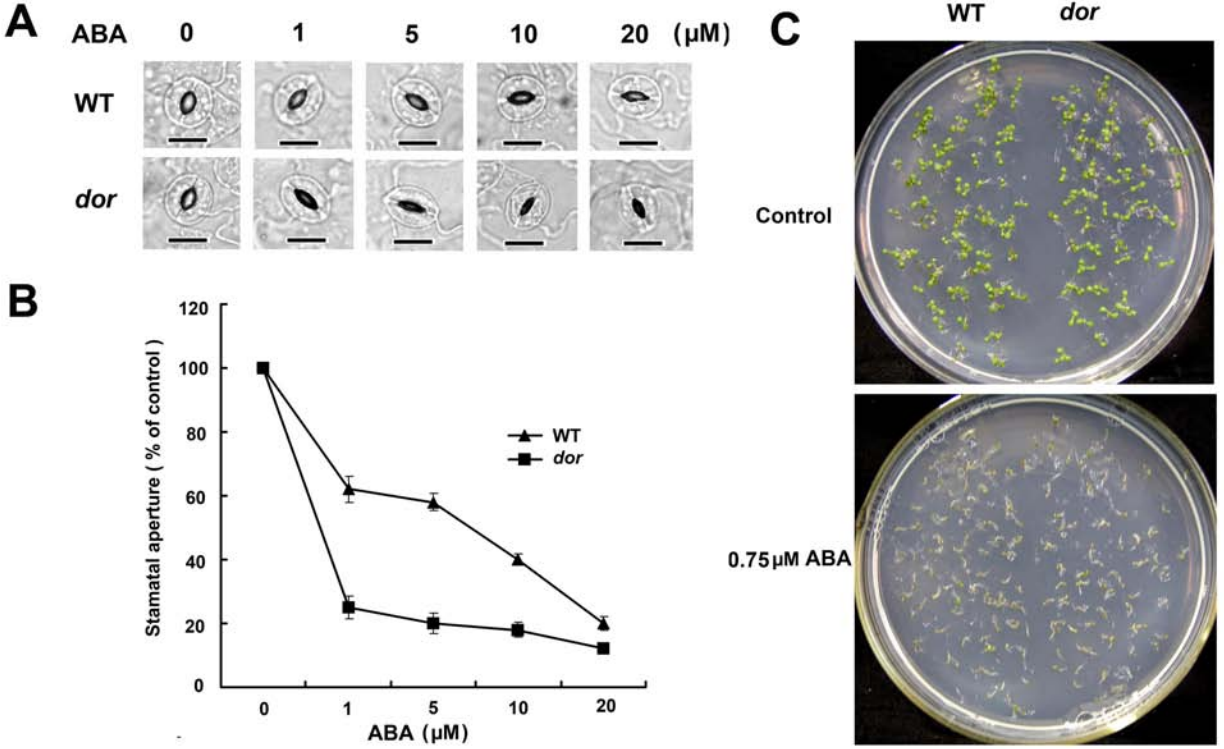
* indicates that the gene expression level was independently validated by Q-RT-PCR, shown in Supplemental Fig. S7.

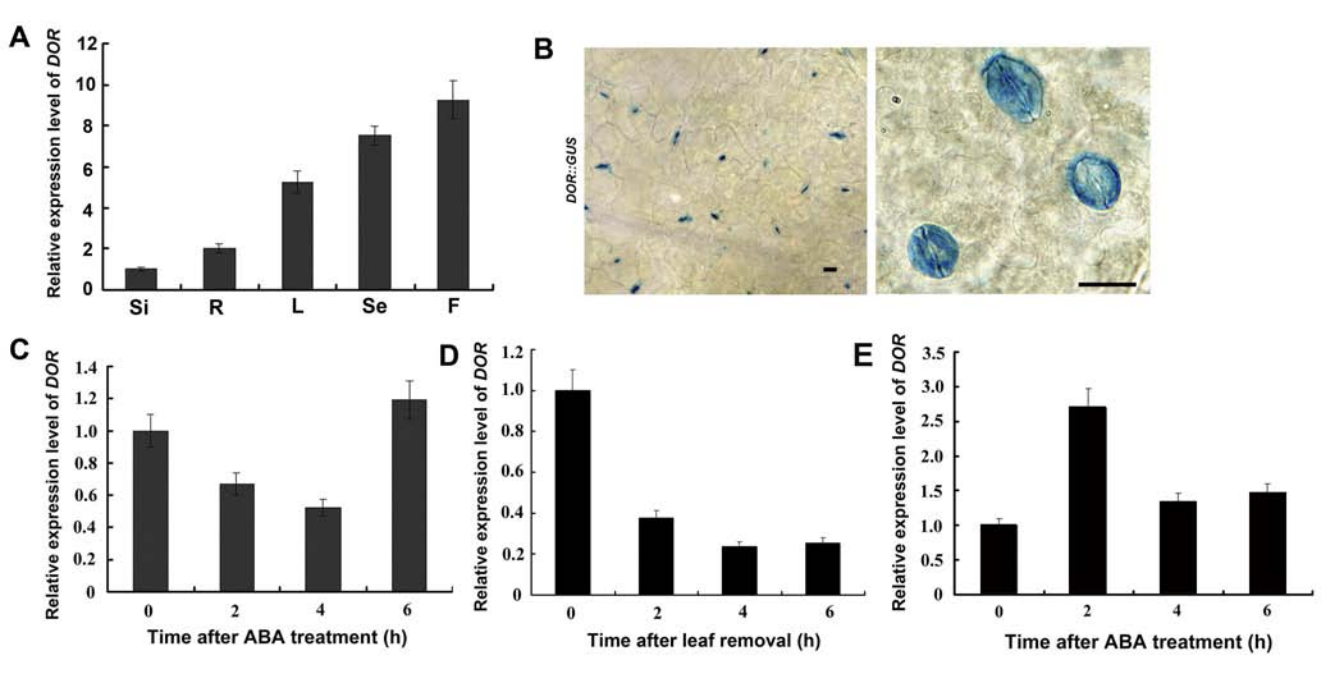
♣ indicates some key genes for ABA biosynthesis and signal transduction pathway, but was not shown in the Affymetrix 8k chip used previously (Leonhardt et al., 2004).

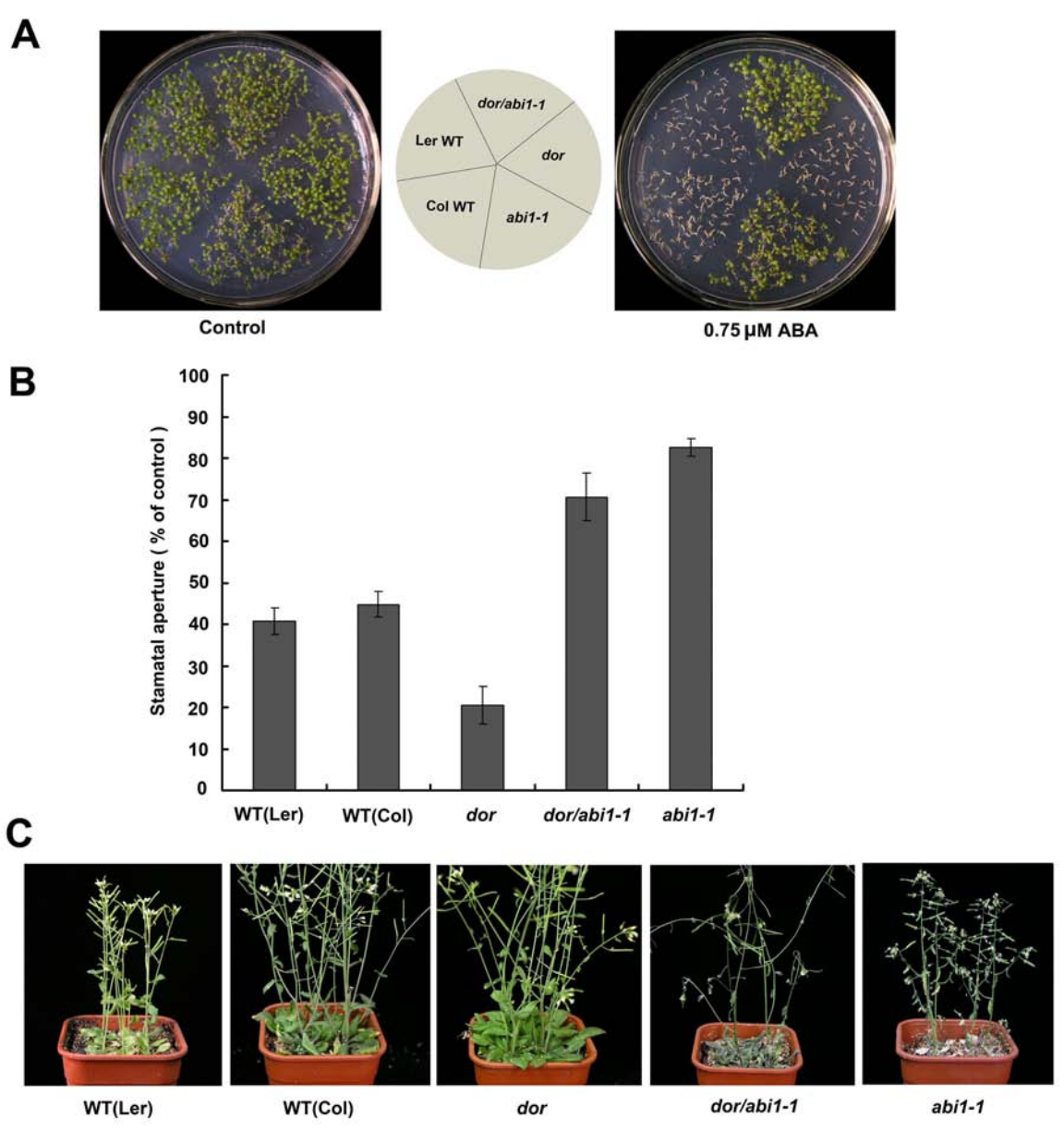

A
WT
dor
a
b
B
Water loss (%)
60
50
40
30
20
10
0
2
4
6
Time after leaf removal (h)
WT
dor
C
RWC (%)
90
80
70
60
50
40
30
20
10
0
0
8
12
Drought treatment time (day)
WT
dor

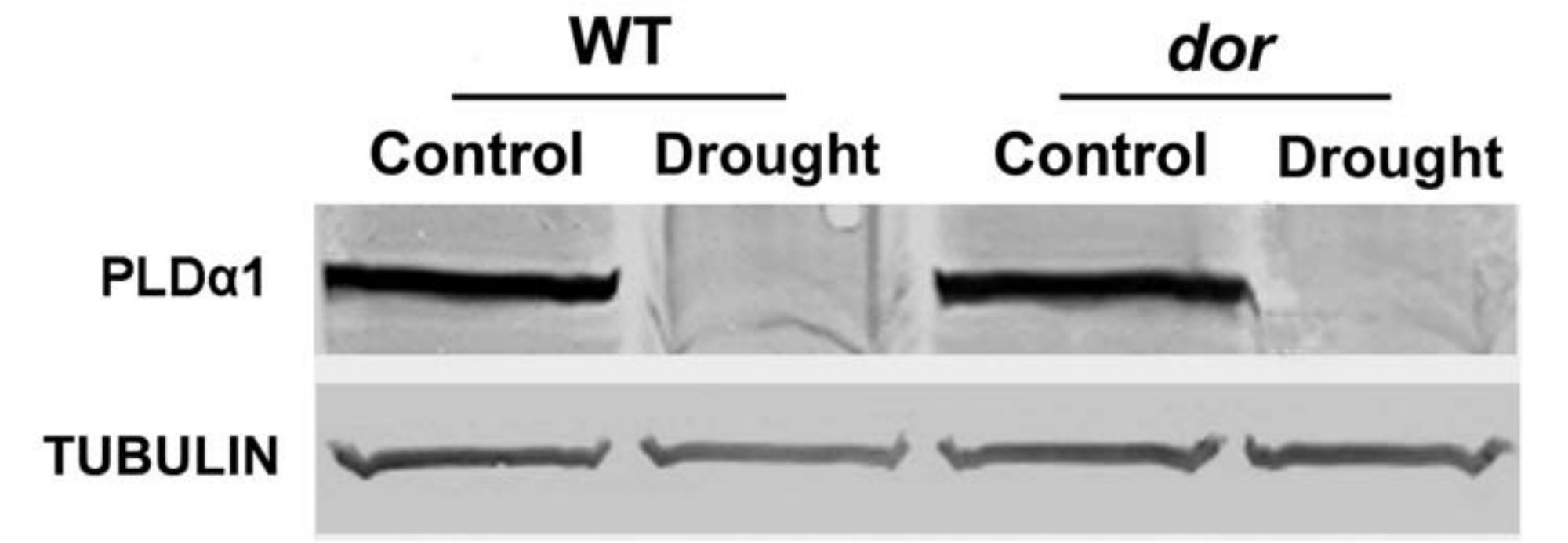

A
dor
WT
M
–
+
–
+
494bp
Tubulin
B
pC-TAPa
3C
Nos ter
DOR
9Xmyc
2XIgG-BD
2X35S::TMVU1 Ω
6XHis
C
dor
Rescued dor-1S
Rescued dor-1E
WT
D
Relative expression level of DOR
0
1
2
3
4
5
6
7
dor
Rescued dor-1S
Rescued dor-2S
Rescued dor-1E
Rescued dor-2E
WT

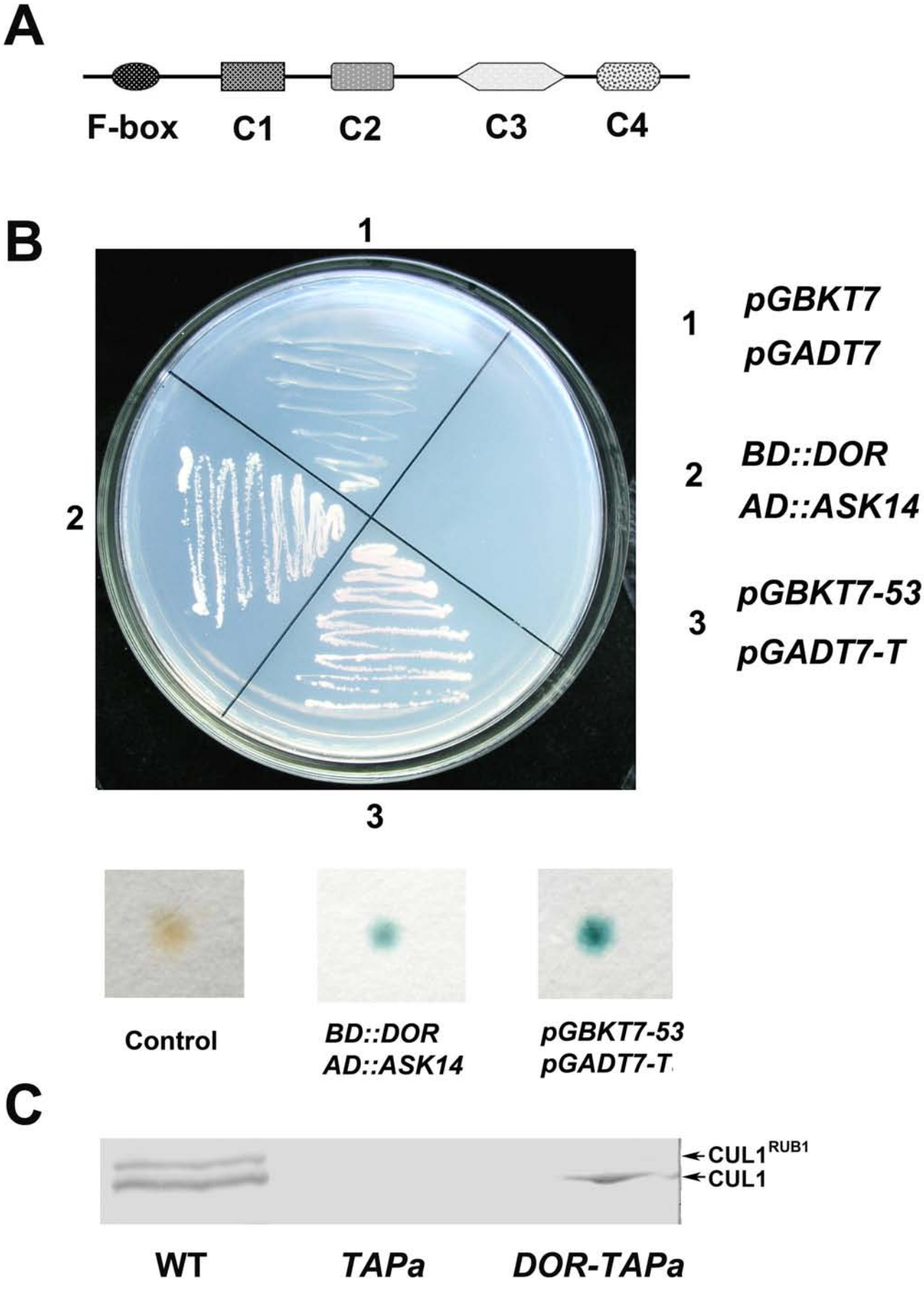

300
250
200
150
100
50
0
ABA Content (pg g-1 FW)
WT
dor
Control
Drought


A
ABA
0
1
5
10
20
(μM)
WT
dor
B
Stamatal aperture (% of control)
120
100
80
60
40
20
0
WT
dor
0
1
5
10
20
ABA (μM)
C
WT
dor
Control
0.75μM ABA


A
Relative expression level of DOR
12
10
8
6
4
2
0
Si
R
L
Se
F
B
DOR::GUS
C
Relative expression level of DOR
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
Time after ABA treatment (h)
D
Relative expression level of DOR
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
Time after leaf removal (h)
E
Relative expression level of DOR
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
2
4
6
Time after ABA treatment (h)


A
dor/abi1-1
Ler WT
dor
Col WT
abi1-1
Control
0.75 μM ABA
B
Stamatal aperture (% of control)
100
90
80
70
60
50
40
30
20
10
0
WT(Ler)
WT(Col)
dor
dor/abi1-1
abi1-1
C
WT(Ler)
WT(Col)
dor
dor/abi1-1
abi1-1


WT
dor
Control
Drought
Control
Drought
PLDα1
TUBULIN


A
F-box
C1
C2
C3
C4
B
1
2
3
1
pGBKT7
pGADT7
2
BD::DOR
AD::ASK14
3
pGBKT7-53
pGADT7-T
Control
BD::DOR
AD::ASK14
pGBKT7-53
pGADT7-T
C
CUL1RUB1
CUL1
WT
TAPa
DOR-TAPa