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Activated Expression of WRKY57 Confers Drought Tolerance in *Arabidopsis*

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ABSTRACT **Drought is one of the most serious environmental factors that limit the productivity of agricultural crops worldwide. However, the mechanism underlying drought tolerance in plants is unclear. WRKY transcription factors are known to function in adaptation to abiotic stresses. By screening a pool of WRKY-associated T-DNA insertion mutants, we isolated a gain-of-function mutant, *acquired drought tolerance* (*adt*), showing improved drought tolerance. Under drought stress conditions, *adt* accumulated higher levels of ABA than wild-type plants. Stomatal aperture analysis indicated that *adt* was more sensitive to ABA than wild-type plants. Molecular genetic analysis revealed that a T-DNA insertion in *adt* led to activated expression of a WRKY gene that encodes the WRKR57 protein. Constitutive expression of *WRKY57* also conferred similar drought tolerance. Consistently with the high ABA content and enhanced drought tolerance, three stress-responsive genes (*RD29A*, *NCED3*, and *ABA3*) were up-regulated in *adt*. ChIP assays demonstrated that WRKY57 can directly bind the W-box of *RD29A* and *NCED3* promoter sequences. In addition, during ABA treatment, seed germination and early seedling growth of *adt* were inhibited, whereas, under high osmotic conditions, *adt* showed a higher seed germination frequency. In summary, our results suggested that the activated expression of *WRKY57* improved drought tolerance of *Arabidopsis* by elevation of ABA levels. Establishment of the functions of *WRKY57* will enable improvement of plant drought tolerance through gene manipulation approaches.**

Key words: abiotic stress; drought tolerance; abscisic acid (ABA); WRKY transcription factor; *acquired drought tolerance* (*adt*).

INTRODUCTION

Drought stress is one of the most severe environmental factors that greatly restrict plant distribution and crop production (Zhu, 2002). To reduce the adverse effects of drought stress, plants have evolved multifaceted strategies, including morphological, physiological, and biochemical adaptations (Ingram and Bartels, 1996; Xiong et al., 2002; Zhu, 2002; Shinozaki et al., 2003; Bohnert et al., 2006). Some of these strategies aim to avoid dehydration stress by increasing water uptake or reducing water loss, whereas other strategies seek to protect plant cells from damage when water is depleted and tissue dehydration becomes inevitable (Verslues et al., 2006). Moreover, the adaptive response to drought must be coordinated at the molecular, cellular, and whole-plant levels (Yu et al., 2008).

Abscisic acid (ABA) is an important phytohormone that regulates many essential processes, including inhibition of germination, maintenance of seed dormancy, regulation of stomatal behavior, and adaptive responses to a variety of environmental stresses (Finkelstein et al., 2002). *NCED3* encodes a key enzyme in the ABA synthesis pathway of *Arabidopsis* (Nambara and Marion-Poll, 2005) and is strongly induced by drought (Luchi et al., 2001). Drought causes increased biosynthesis and accumulation of ABA, which can be rapidly catabolized following the relief of stress (Koornneef et al., 1998; Cutler and Krochko, 1999; Liotenberg et al., 1999; Taylor et al., 2000).

Transpirational water loss through the stomata is a key determinant of drought tolerance (Xiong et al., 2002). The closing and opening of the stomata are mediated by a turgor-driven change in volume of the two surrounding guard cells (Yu et al., 2008). Guard cell turgor change is influenced by many factors, such as light, phytohormones, potassium ions, calcium ions, malate, NO, and H_2O_2 (Assmann and Wang, 2001; Schroeder et al., 2001; Assmann, 2003; Nilson and Assmann, 2007; Shimazaki et al., 2007). The guard cells

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sense and integrate environmental signals to modulate stomatal aperture in response to drought stress.

The molecular mechanisms underlying plant tolerance to drought stress are still not fully understood because of the complex nature of the response to drought stress. The study of stress-responsive transcription factors has been one of the foci in studies on drought stress tolerance. Using transcription factor to improve tolerance of crops to abiotic, such as drought, is a promising strategy because of the ability of a TF to regulate an entire set of genes in a stress-response pathway (Yang et al., 2010). The transcription factors DREB1A/CBF3 and DREB2A specifically interact with the *cis*-acting dehydration-responsive element/C-repeat (DRE/CRT) involved in cold and drought stress-responsive gene expression in *Arabidopsis* (Sakuma et al., 2006). DRIP1 and DRIP2, which are both C3H4 RING domain-containing proteins, interact with the DREB2A protein in the nucleus and act as novel negative regulators in drought-responsive gene expression by targeting DREB2A to 26S proteasome proteolysis (Qin et al., 2008). ATGPX3 might play dual and distinctive roles in H_2O_2 homeostasis, acting as a general scavenger and specifically relaying the H_2O_2 signal as an oxidative signal transducer in ABA and drought stress signaling (Yu et al., 2006). AREB1 regulates novel ABRE-dependent ABA signaling that enhances drought tolerance in vegetative tissues (Yasunari et al., 2005). NFYA5 is important for drought resistance, and its induction by drought stress occurs at both the transcriptional and posttranscriptional levels, which implies existence of a previously unknown drought tolerance mechanism (Li et al., 2008). In addition, SRK2C, a SNF1-related protein kinase2, improves drought tolerance by controlling stress-responsive gene expression in *Arabidopsis* (Taishi et al., 2004). The recent discovery of microRNAs and small interfering RNAs involved in stress responses revealed a new layer of regulation of stress tolerance (Sunkar and Zhu, 2004; Sunkar et al., 2005). A U-box E3 ubiquitin ligase, AtPUB19, negatively regulates abscisic acid and drought responses in *Arabidopsis* (Liu et al., 2011). These studies indicate the potential for improvement of drought tolerance of crops through genetic engineering.

WRKY transcription factors play important roles in biotic and abiotic stress responses (Eulgem and Somssich, 2007; Miller et al., 2008). The WRKY family in *Arabidopsis thaliana* consists of 74 members and is subdivided into three groups on the basis of the number of WRKY (WRKYGQK) domains, as well as the features of their zinc finger-like motif (Eulgem et al., 2000). A number of *WRKY* genes are involved in the response to biotic stress (Yu et al., 2001; Xu et al., 2006; Kim et al., 2008; Xing et al., 2008; Chen et al., 2010). Increasing evidence suggests that many *WRKY* genes are also involved in the response to various sources of abiotic stress (Miller et al., 2008; Chen et al., 2012). For example, *WRKY63*/*ABO3* mediates plant responses to ABA and drought tolerance in *Arabidopsis* (Ren et al., 2010). Overexpression of the stress-induced *OsWRKY45* enhances drought tolerance in *Arabidopsis* (Qiu and Yu, 2008). Heterologous expression of *OsWRK23* enhances dark-induced leaf senescence in *Arabidopsis* (Jing et al., 2009). *AtWRKY2* mediates seed germination and post-germination arrest of development by ABA (Jiang and Yu, 2009). Male gametophyte-specific *WRKY34* mediates cold sensitivity of mature pollen in *Arabidopsis* (Zou et al., 2010). *AtWRKY22* participates in the dark-induced senescence signal transduction pathway (Zhou et al., 2011). In addition, *WRKY25*, *WRKY26*, *WRKY33*, and *WRKY39* play important roles in response to heat stress in *Arabidopsis* (Li et al., 2009, 2010, 2011).

In the present study, we isolated an *adt* mutant with improved drought tolerance from a pool of WRKY-associated T-DNA insertion mutants. Further analysis revealed that a T-DNA, localized in the putative promoter region of *WRKY57*, caused elevated *WRKY57* expression in *adt*. Both *adt* and *WRKY57* transgenic plants displayed characteristics related to drought tolerance with elevation of their ABA contents and up-regulation expressive levels of stress-response genes (*RD29A*, *ABA3*, and *NCED3*). Overall, our results indicate that activated expression of *WRKY57* confers the adaption of *Arabidopsis* to drought tolerance by elevation of ABA levels and positively regulating the expression of stress-responsive genes.

RESULTS

Isolation of *adt* Mutant

During the past decade, a majority of studies have demonstrated that *Arabidopsis* WRKY proteins play key roles in regulation of transcriptional reprogramming associated with plant defense responses (Eulgem et al., 2007; Rushton et al., 2010; Ishihama and Yoshioka, 2012). Moreover, our lab and other independent groups have shown that WRKY factors play important positive or negative roles in abiotic stress responses in *Arabidopsis* (Chen et al., 2012). However, few studies were focused on their function in drought stress. To investigate the functions of *Arabidopsis* WRKY transcription factors in drought tolerance, 43 WRKY-associated T-DNA insertion lines (either gain-of-function or loss-of-function) from the Arabidopsis Biological Resource Center (ABRC) were performed for screening mutants that exhibit tolerance to drought stress in a natural drought assay (water was withheld) (Alonso et al., 2003). Drought tolerance of these mutants was compared with that of wild-type plants. Finally, an *acquired drought tolerance* (*adt*) mutant was isolated and used for further characterization.

The *adt* mutant and wild-type seeds were germinated simultaneously and then planted in soil. Four weeks after germination, the plants were treated with natural drought (water was withheld) (Figure 1). The wild-type plants showed wilting symptoms 3 d in advance compared with the *adt* mutants. After 2 weeks without watering, the *adt* plants showed only mild drought stress symptoms, whereas the wild-type plants

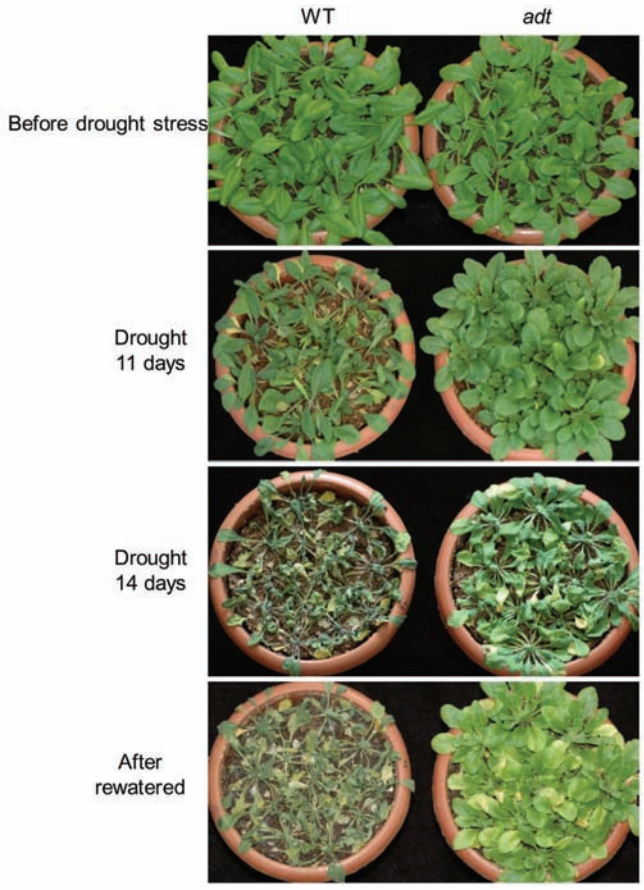

Figure 1. Improved Drought Tolerance of the *adt* Mutant. Drought stress was imposed on 4-week-old seedlings for 14 d. Drought experiments were repeated four times and at least 35 plants for each individual line were used in each repeated experiment and one representative picture was shown.

exhibited severe drought symptoms. When plants were re-watered, none of the wild-type plants survived, but all of the mutant plants were still alive. These results suggested that *adt* plants were comparatively more resistant to drought stress than wild-type plants.

Phenotypes of *adt*

Transpirational water loss is one of the most important factors related to drought tolerance. To assess the rate of water loss by *adt*, rosettes were detached and their fresh weight changes were measured over a 200-min period. The *adt* leaves showed a slower rate of water loss than wild-type leaves (Figure 2A). The reduced rate of water loss is attributable to the increased drought tolerance in *adt*.

Drought stress can cause increased accumulation of ABA, which plays crucial roles in the drought response. To determine whether ABA levels were affected, we quantified the ABA content in *adt* mutants. Under normal conditions, the ABA content of *adt* is 1.2-fold higher than the wild-type. When exposed to 10% polyethylene glycol (PEG) 6000, a stress treatment commonly used to mimic drought tolerance in the laboratory, *adt* accumulated 1.6-fold higher ABA content than the wild-type (Figure 2B). These results suggest that the elevated ABA levels contribute to drought tolerance in *adt*.

In response to drought stress, stomata often close to limit water loss by transpiration. During this process, ABA plays a role in stomata closure. Given that endogenous ABA was up-regulated in *adt*, we further investigated whether *adt* affects the sensitivity of guard cells to ABA. Epidermal peels of *adt* and wild-type 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 (Pei et al., 1997). The ratio of stomatal width to length indicated the degree of stomatal closure. *adt* and wild-type plants showed the same stomatal width:length ratio of fully opened stomata without ABA treatment, but *adt* plants showed a lower stomatal width:length ratio than wild-type plants after treatment with ABA (Figure 2C and 2D). These results suggest that stomata closure in *adt* is more sensitive to ABA than in the wild-type, which may be critical for *adt* mutants to adapt to drought stress.

Tagged Gene *WRKY57* Is Activated in *adt*

According to the information provided by ABRC, the *adt* mutant contains a T-DNA insertion in the promoter region of *WRKY57* (Figure 3A). We further confirmed the insertion site (159 bp from the translation start site) by PCR with primers specific to the *WRKY57* gene and the T-DNA left border. Real-time RT–PCR and Northern blot analysis indicated that, in all tissues examined, *WRKY57* expression was significantly increased in *adt* compared with wild-type plants (Figure 3B and 3C). This result suggests that *WRKY57* is activated, which may cause the drought tolerance phenotypes of *adt*.

To confirm whether the activated expression of *WRKY57* causes the drought tolerance phenotypes of the *adt* mutant, recapitulation experiments were conducted. *WRKY57* cDNA was driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter in wild-type *Arabidopsis*. *WRKY57* expression levels of transgenic plants were analyzed by Northern blot analysis, then 35S::*WRKY57*-L3, 35S::*WRKY57*-L12, and 35S::*WRKY57*-L14 were selected for further analysis (Figure 4A). The *WRKY57* overexpression lines (L3, L12, and L14) showed significantly improved drought tolerance compared with the wild-type plants (Figure 4B). After 2 weeks of drought stress, the wild-type plants displayed severe wilting symptoms, whereas the transgenic plants did not show any wilting symptom (Figure 4B). All transgenic lines were still alive after re-watering, whereas none of the wild-type seedlings survived (Figure 4B). Compared with the wild-type plants, the transgenic plants also showed a slower rate of water loss in leaves (Figure 4C). The ABA content of these transgenic plants was similar to the *adt* mutant and was significantly higher than that of the wild-type (Figure 4D). These results suggest that overexpression of *WRKY57* can recreate the drought

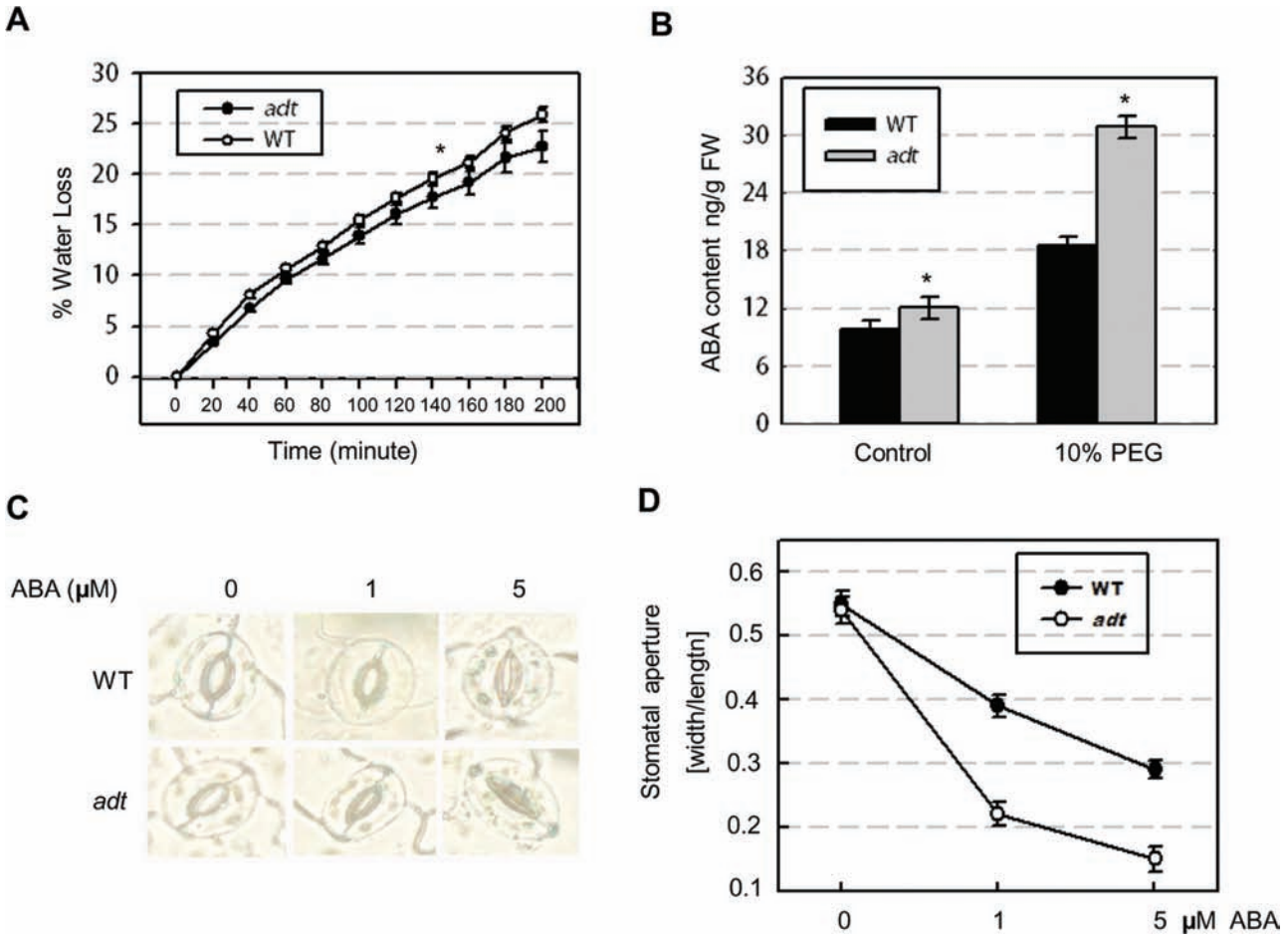

Figure 2. Quantification of Endogenous Abscisic Acid (ABA) Content and Analysis of Water Loss Rate and Stomatal Behavior.

(A) Rate of water loss by detached leaves from wild-type and *adt* plants. Values are the mean ± SE ($n = 6$ plants, * $P < 0.05$).

(B) ABA content as determined by ELISA. Values are the mean ± SE ($n = 3$ experiments, * $P < 0.05$). FW, fresh weight.

(C, D) Stomatal closure in response to ABA treatment. The data represent the mean ± SE from 30 stomata measured for each time point, from three independent experiments. Stomatal aperture ABA (0) = (100%) corresponded to average stomatal apertures of 2.86 ± 0.22 μm (wild-type) and 2.79 ± 0.31 μm (*adt*). Scale bar = 10 μm.

tolerance phenotypes of *adt*, which strongly supports our hypothesis that the drought tolerance phenotypes of *adt* were caused by activated *WRKY57* expression.

Identification of *WRKY57* Loss-of-Function Allele

To understand the function of *WRKY57*, real-time RT–PCR analysis was conducted to examine whether *WRKY57* is responsive to drought and other abiotic stresses. *WRKY57* expression was up-regulated in response to mannitol, ABA, PEG, NaCl, or dehydration treatments (Figure 5A), which implied that *WRKY57* plays a role in abiotic stress. To clarify the underlying role of *WRKY57* in drought tolerance, a loss-of-function mutant *wrky57* (SALK_076716) was obtained. In the *wrky57* mutant, a T-DNA was inserted into the first exon of *WRKY57* (Figure 5B), which resulted in no *WRKY57* transcripts (Figure 5C). Unexpectedly, the *wrky57* mutant had similar drought sensitivity to wild-type plants. We proposed that a homolog of WRKY57 exists and is able to compensate for the function loss of WRKY57 in *Arabidopsis*. WRKY48 is a homologous protein of WRKY57 with high amino acid sequence similarity (63%), and also belongs to the subgroup IId of the WRKY family (Dong et al., 2003). To confirm our hypothesis, we obtained a homozygous *wrky48* mutant (Salk_066438, Figure 5B and 5C), and constructed *wrky48 wrky57* double mutants. The single (*wrky57* and *wrky48*) and double (*wrky48 wrky57*) mutants were subjected to the drought tolerance assay. Unexpectedly, the single and double mutants showed no apparent difference in drought sensitivity compared with the wild-type (Figure 5D). However, we cannot discount the existence of other genes that can complement the function loss of WRKY57.

WRKY57 Up-Regulates the Expression of Stress-Responsive Genes

Plants have evolved some mechanisms to adapt to the stress environments, such as drought, salinity, and cold. Some stress-responsive genes can be induced to help plants to

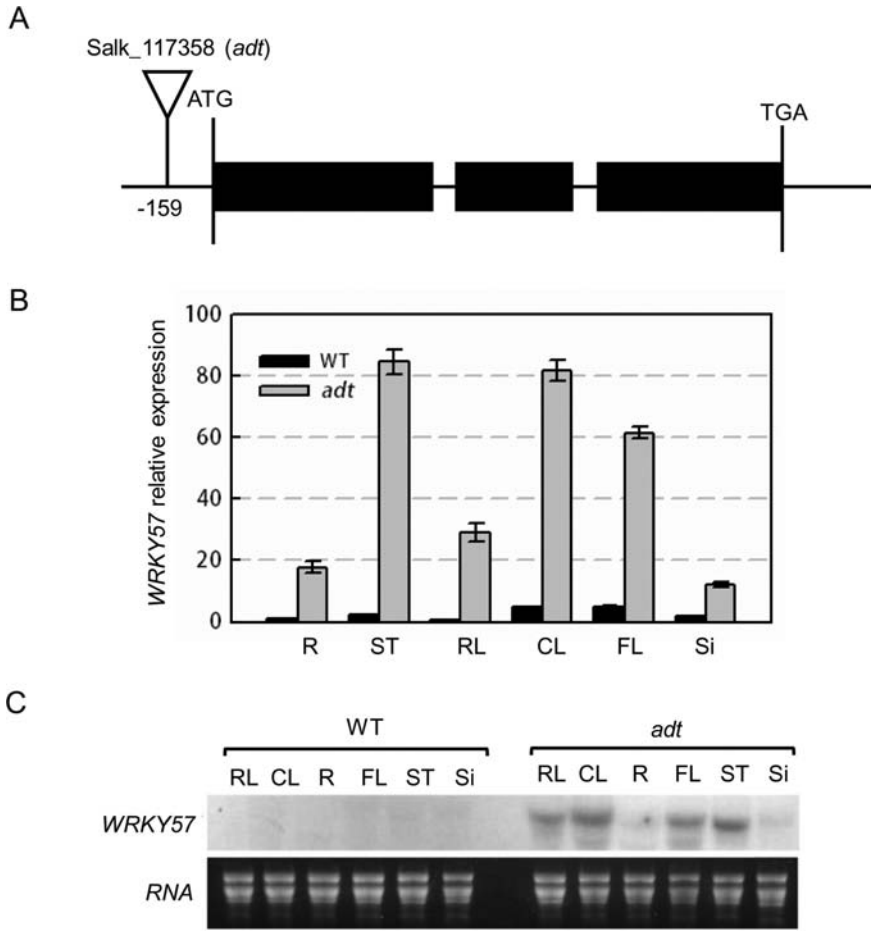

Figure 3. Identification of the T-DNA Tagged Locus and Activated Expression of *WRKY57*.

(A) T-DNA insertion position in *adt* mutants (Salk_117358).

(B) Real-time RT–PCR analysis of *WRKY57* expression in different tissues. Total RNA was isolated from roots (R), rosette leaves (RL), cauline leaves (CL), flowers (FL), inflorescence stem (ST), and siliques (Si). Values are the mean ± SE ($n = 3$ experiments).

(C) Northern blot analysis of WRKY57 expression using the same RNA in (B).The experiment was repeated three times, and a typical RNA gel blot is presented.

survive under drought conditions. To explore the molecular mechanisms of *WRKY57* function in drought tolerance, we determined whether WRKY57 regulates the expression of stress-responsive genes. *RD29A* is a stress-responsive marker (Yamaguchi-Shinozaki and Shinozaki, 1993) used as a control for stress treatments. Both *NCED3* and *ABA3* encode key enzymes in the ABA synthesis pathway (Chernys and Zeevaart, 2000; Xiong et al., 2001). As expected, these three genes were up-regulated in *adt* under dehydration treatment (Figure 6). In contrast, their expression decreased in *wrky57* null mutant compared with that in wild-type. These results indicate that *WRKY57* positively regulates these stress-responsive genes, which may account for the enhanced drought tolerance in *adt*.

WRKY57 Can Bind to the W-Box Motifs on the Promoters of *RD29A* and *NCED3*

It is well known that WRKY transcription factors function by binding to the typical *cis*-element W-box of the target gene promoter. Several W-box motifs were identified in the putative promoter regions of three stress-responsive genes (*RD29A*, *NCED3*, and *ABA3*) (Figure 7A). To determine whether these three genes are directly regulated by WRKY57, chromatin immunoprecipitation (ChIP) experiments were conducted. 35S::*myc-WRKY57* transgenic plants were used for ChIP assays. The primers used for PCR amplification of different promoters are listed in Supplemental Table 1. PCR amplification was performed using 36 cycles for all promoter fragments. Aliquots of the PCR products were resolved by electrophoresis on 2% agarose gel. The results presented were obtained from at least three independent experiments. To quantify WRKY57-DNA (target promoters) binding, real-time RT–PCR analysis was performed in accordance with a procedure described previously with the *ACTIN2* 3′ untranslated region as the endogenous control (Mukhopadhyay et al., 2008). The relative quantity value is presented as DNA binding ratio (differential site occupancy). The same primers as for the above-mentioned

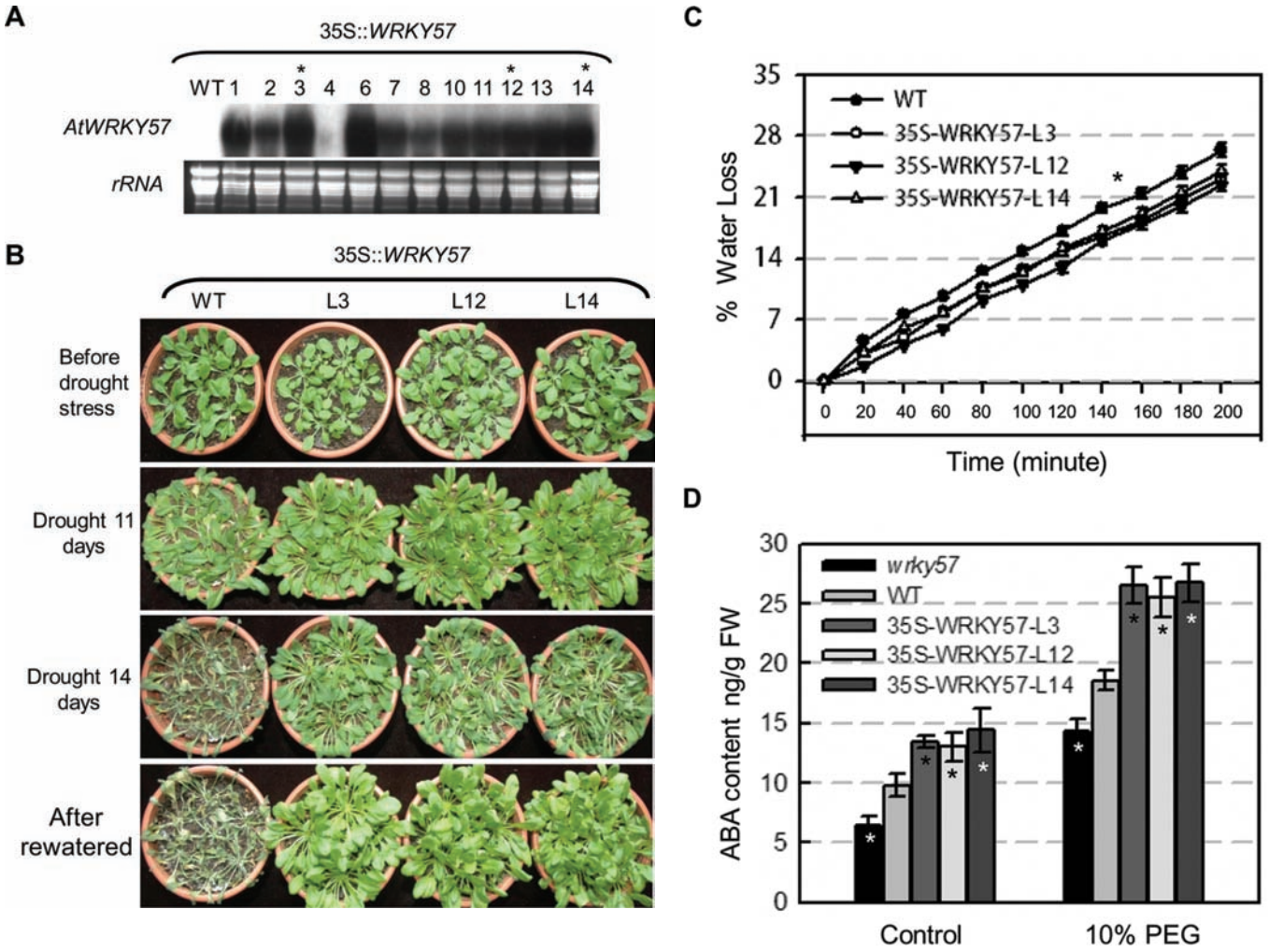

Figure 4. *WRKY57* Transgenic Plants Improve Drought Tolerance.
(A) Northern blot analysis of transgenic lines. The experiment was repeated three times, and a typical RNA gel blot is presented.
(B) Drought tolerance assay of transgenic plants expressing *WRKY57*. T_2 transgenic seedlings of the three transgenic lines (L3, L12, and L14) were selected for drought stress.
(C) Comparison of the rate of water loss from detached rosettes between transgenic and control plants. Values are the mean ± SE ($n = 10$ plants).
(D) ABA content. Values are the mean ± SE ($n = 3$ experiments, * $P < 0.05$). FW, fresh weight.

PCR analysis were used for the real-time RT–PCR. A fragment of the *ACTIN2* promoter was used as a negative control. The results presented were obtained from at least three independent experiments. Results from PCR and quantitative real-time RT–PCR analyses showed that WRKY57 binds to the promoters of *RD29A* and *NCED3* via the core W-box sequence (Figure 7B and 7C).

To avoid the artificial interactions resulting from constitutive expression, a construct containing the *WRKY57* cDNA fused with MYC tag driven by *WRKY57* promoter were induced into *wrky57* mutant. The transgenic plants were also used for ChIP analysis. Although the DNA binding ratio in transgenic plants driven by *WRKY57* promoter were lower than in transgenic plants driven by 35S promoter, results also showed that WRKY57 can bind to the promoters of *RD29A* and *NCED3* via the core W-box sequence (Figure 7D and 7E).

Overall, ChIP analysis showed that WRKY57 directly up-regulated expression of *RD29A* and *NCED3* under drought stress.

WRKY57 Affects Seed Germination under ABA, Osmotic, and Salt Stress Conditions

Our results revealed that *WRKY57* expression was induced by abiotic stresses, such as ABA, dehydration, and mannitol (Figure 5A). Further analysis revealed that WRKY57 up-regulated *NCED3* and *ABA3* (Figure 6) and caused elevation of ABA levels (Figure 2B and 4D). Given the function of ABA in seed dormancy, we explored the functions of *WRKY57* in seed

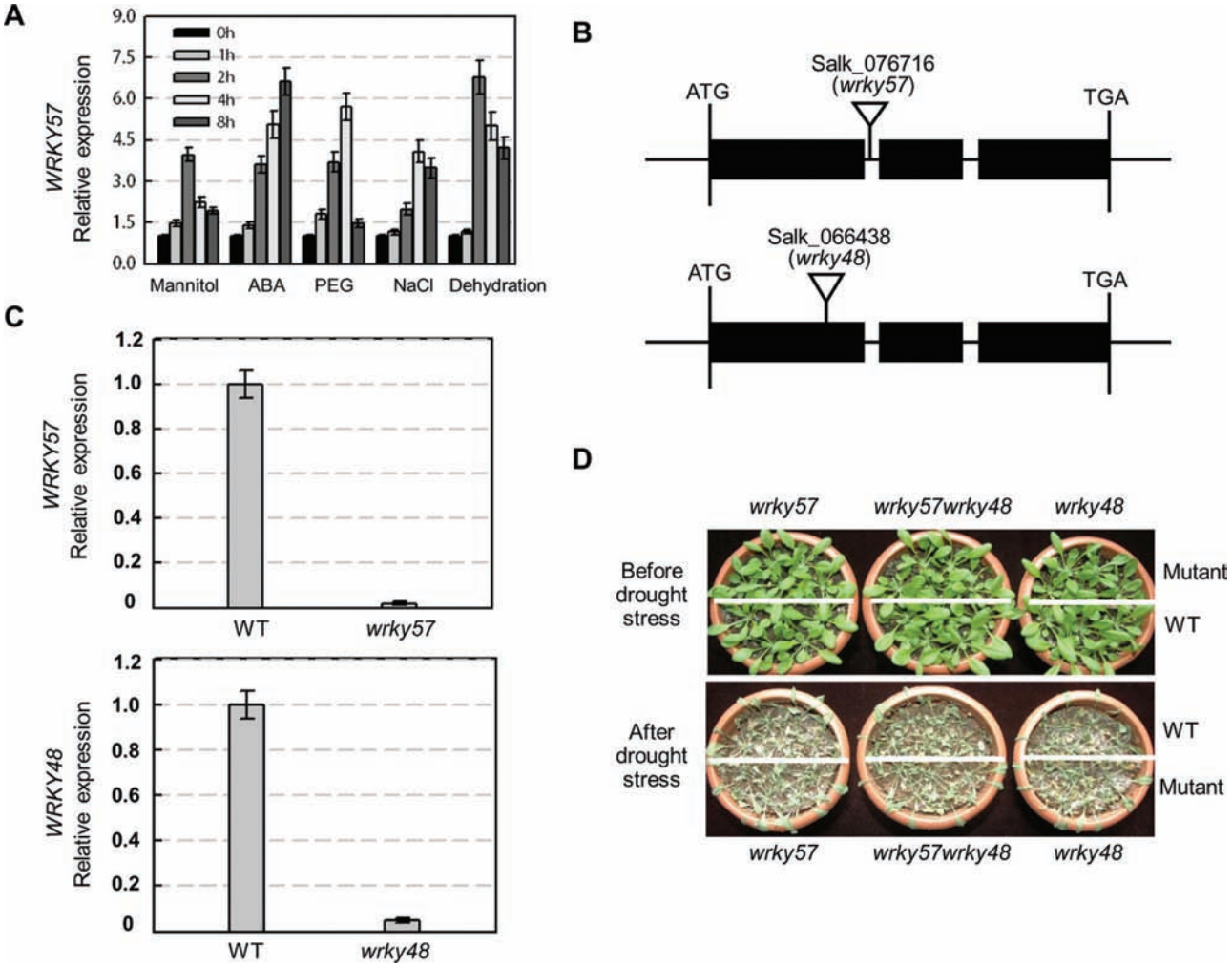

Figure 5. Expression of *WRKY57* under Abiotic Stress Treatments.
(A) Real-time RT–PCR analysis of the *WRKY57* transcript level in response to stress treatment. Total RNA was isolated from 3-week-old wild-type seedlings treated with 300 mM mannitol, 100 μM ABA, 10% PEG, 175 mM NaCl, and dehydration.
(B) T-DNA insertion position in *wrky57* (Salk_076716) and *wrky48* (Salk_066438) mutants.
(C) Real-time RT–PCR analysis of the *WRKY57* and *WRKY48* transcript level in the wild-type and mutants.
(D) Drought tolerance assay. Drought stress was imposed on 4-week-old single (*wrky48* and *wrky57*) and double (*wrky48 wrky57*) mutants for 2 weeks.

germination. We tested the germination frequency of wild-type, *adt*, and *wrky57* mutant seeds on Murashige and Skoog (MS) medium supplemented with different concentrations of ABA. The *wrky57* seeds displayed a higher germination frequency than wild-type seeds, whereas the seed germination frequency of *adt* was significantly lower than that of the wild-type (Figure 8A). Moreover, early seedling growth of *adt* was also more sensitive to ABA than the wild-type and *wrky57* (Figure 8D). When seedlings were grown on basal MS medium for 3 d and then grown on MS medium supplemented with 20 μM ABA, no significant difference in growth was observed between *adt*, wild-type, and *wrky57* seedlings (Supplemental Figure 2). It is suggested that WRKY57 only affected seed germination and post-germination early growth.

ABA also plays an important role in adaptation to osmotic and salt stresses (Yamaguchi-Shinozaki and Shinozaki, 1993). Since *adt* plants exhibited increased ABA sensitivity in both germination and early seedling growth, we investigated the response of *adt* to osmotic stress. Wild-type, *wrky57*, and *adt* seeds were sown on MS medium supplemented with 300 mM mannitol. Seed germination and early seedling growth of *adt* were less sensitive to mannitol treatments compared with the wild-type, whereas the *wrky57* mutant displayed the opposite results (Figure 8B and 8D). When subjected to salt treatment, *adt* was also less sensitive compared with wild-type (Figure 8C and 8D). These results suggest that *WRKY57* promotes tolerance to osmotic and salt stresses in *Arabidopsis*.

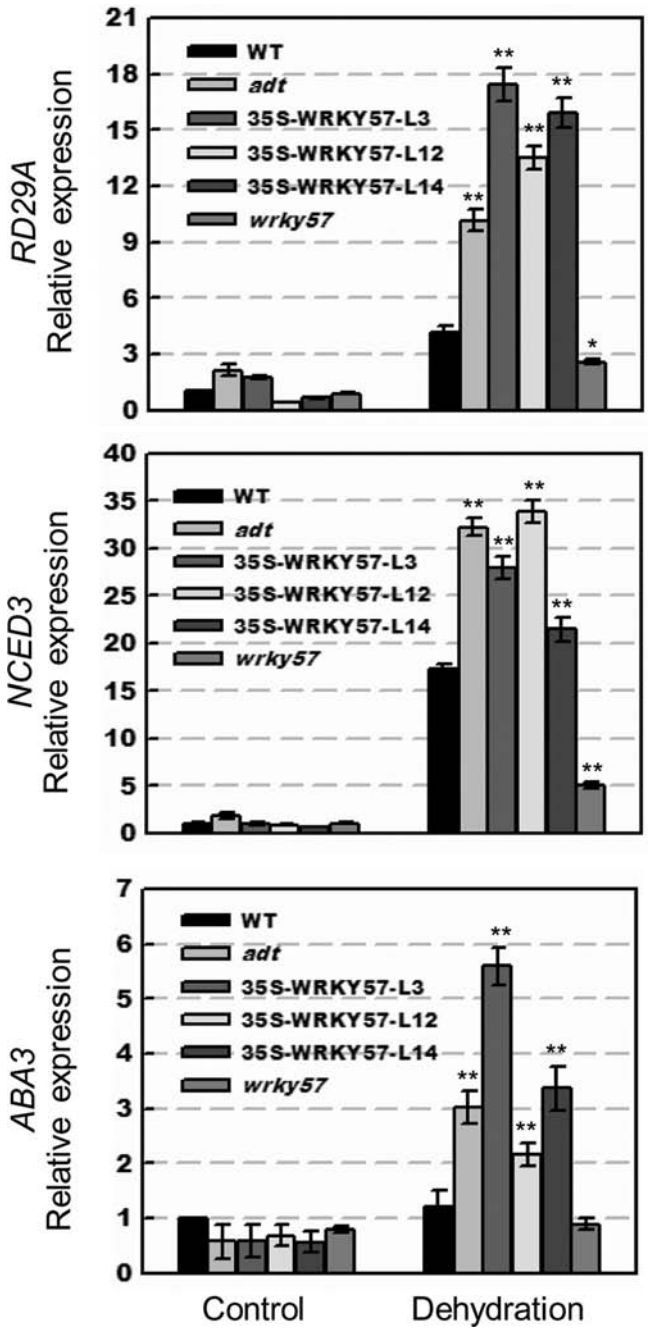

Figure 6. Expression of Stress-Responsive Genes in the *adt* Mutant. Relative expression levels of *RD29A, NCED3* and *ABA3*. The above-ground shoot of 3-week-old plants was detached from the root system for dehydration treatment. Values are the mean ± SE ($n = 3$ experiments, * $P < 0.05$, ** $P < 0.01$).

DISCUSSION

Drought, an abiotic factor, directly affects the productivity of crops. WRKY transcription factors are known to regulate a multiplicity of biotic and abiotic stress responses (Eulgem and Somssich, 2007; Miller et al., 2008; Chen et al., 2012). Members of the WRKY transcription factor family are implicated in the regulation of genes involved in pathogen-induced stress, as well as drought, cold, and salinity stresses (Seki et al., 2002; Dong et al., 2003; Chen et al., 2012). Some WRKY proteins have diversified from the WRKY gene family at relatively high rates under evolutionary pressure in order to acquire unique biological functions (Dong et al., 2003). From a collection of WRKY-associated T-DNA insertion mutants, we obtained one mutant, *adt*, with enhanced tolerance to drought. Further experiments revealed that *adt* is a gain-of-function mutant of *WRKY57*. Detailed functional analysis showed that activated *WRKY57* expression confers drought tolerance in *Arabidopsis*. First, *adt* plants reduced water loss, which is necessary for plants to survive under drought conditions. Second, *adt* accumulated higher quantities of ABA than wild-type plants. The accumulation of high levels of ABA not only contributed to the reduced rate of water loss in *adt* leaves, but also may have enhanced the stress-response capacity. Third, the higher transcript levels of some stress-responsive genes suggested that *adt* enhanced the response to stress signaling.

The *RD29A* gene is induced by abiotic stresses such as drought, high salinity, and low temperature (Kasuga et al., 1999; Narusaka et al., 2003) and is also involved in signaling pathways downstream of WRKY transcription factors (Rushton et al., 2012). Our results demonstrated that the promoter of *RD29A* could be bound by WRKY57 and its expression was up-regulated in *adt* upon drought stress.

Drought can dramatically stimulate *de novo* ABA biosynthesis, and ABA is closely involved in drought stress responses (Bray, 1994; Ingram and Bartels, 1996). Many genes are involved in ABA biosynthesis, of which *ABA3* is a key regulator of ABA biosynthesis (Xiong et al., 2001). *ABA3* is expressed ubiquitously in different plant parts and is up-regulated by drought stress. Recently, *NCED3* was reported to be expressed in both leaf and root tissues (Tan et al., 2003) and can affect both constitutive and stress-induced ABA levels in shoot tissues (Luchi et al., 2001; Ruggiero et al., 2004), which implies *NCED3* has a complex role in the regulation of ABA levels. Overexpression of *NCED3* results in plants with increased ability to survive severe desiccation stress (Luchi et al., 2001). The present results revealed that WRKY57 directly activated *NCED3* expression and up-regulated *ABA3* expression in an indirect manner, which was consistent with the elevated ABA contents under drought stress conditions.

ABA is not only involved in stomatal movement and improves drought tolerance, but also increases the capacity of osmotic regulation in plants. Our germination assays showed that *adt* is more sensitive to ABA but exhibits resistance to osmotic stress caused by mannitol and salt stress by NaCl. Additionally, the ABA levels in *adt* leaves were higher than that of wild-type (Figure 2B) and, on the contrary, the ABA levels in *wrky57* leaves were lower than those of wild-type in leaves (Figure 4D). These results indicated that *WRKY57* was involved not only in ABA accumulation, but also in ABA responses. In addition, the stress-responsive gene *RD29A*,

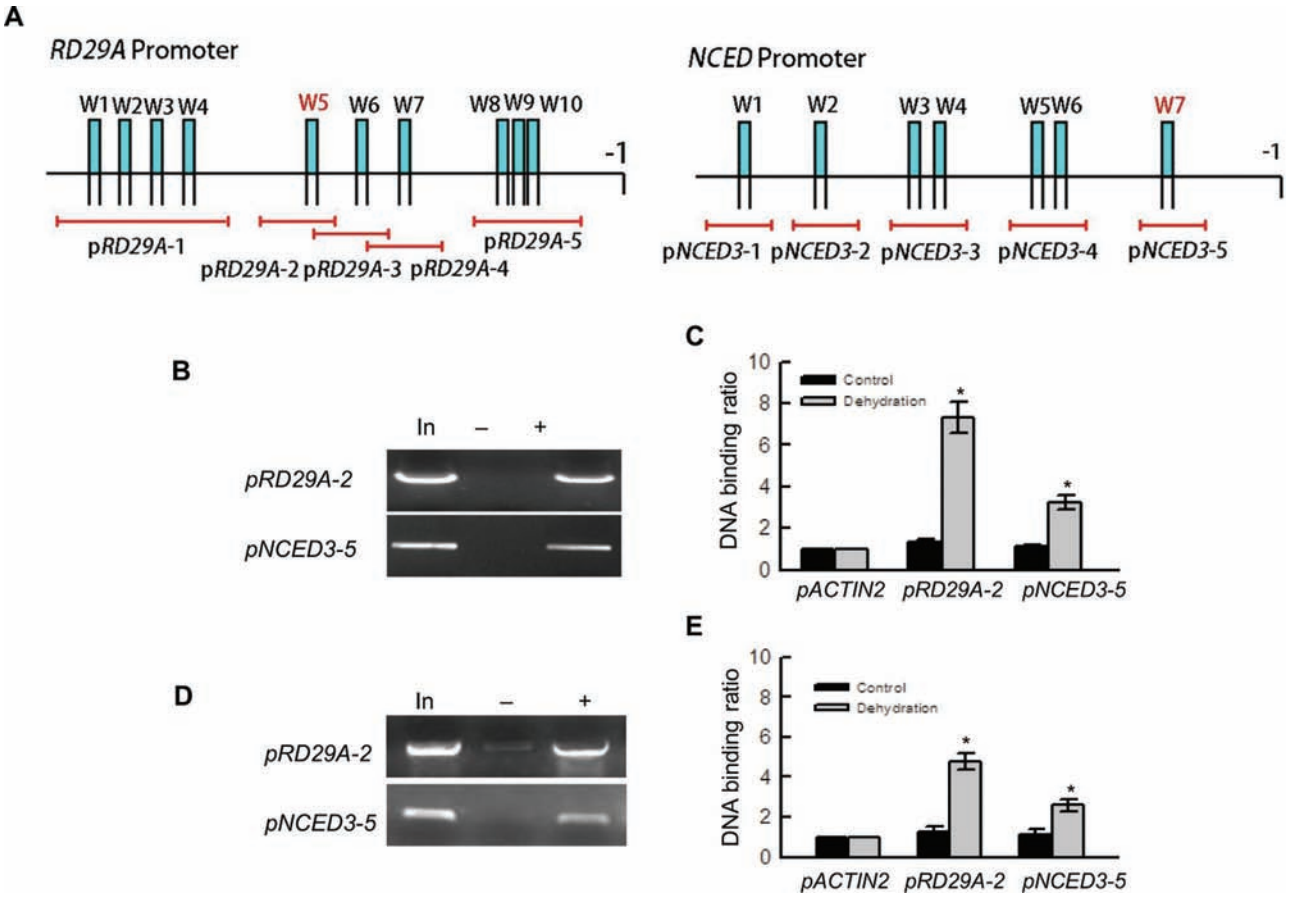

Figure 7. WRKY57 Binding to the W-Box Motif on the Promoters of *RD29A* and *NCED3*.

(A) The promoter structure of *RD29A* and *NCED3* genes. W1, W2 … denote each W-box numbered from left to right with sequence sites relative to the start code. Red lines indicate the sequences detected by ChIP assays described in Supplemental Figure 1.

(B, D) WRKY57, driven by 35S and WRKY57 promoter, respectively, interacts with the promoters of *RD29A* and *NCED3*. PCR data from the ChIP assay with the antibody against MYC. In the promoter fragment names, the prefix 'p' indicates promoter. The sequences for each promoter fragment are indicated in (A) and listed in detail in Supplemental Table 1. In, PCR product from the chromatin DNA; –, PCR product from ChIP with preimmune serum (as a negative control); +, PCR product from ChIP with the antibody against MYC.

(C, E) WRKY57, driven by 35S and WRKY57 promoter, respectively, interacts with the promoters of *RD29A* and *NCED3*. Real-time RT–PCR data from ChIP assay with the antibody against MYC with the *Actin* promoter (p*Actin*) as a negative control.

and the ABA biosynthesis gene *NCED3* and ABA3, were up-regulated in *adt* but down-regulated in *wrky57* when compared with wild-type (Figure 4D), which suggested that *WRKY57* acts as a positive regulator hyperosmotic stress responses in *Arabidopsis*.

It is well known that mutations in regulatory sequences play a predominant role in evolution (Carroll, 2005). The T-DNA is located in a 159-bp region upstream of the *WRKY57* translation start site in *adt*. It is possible that some negative regulatory elements of *WRKY57* are disrupted by the insertion, or new positive regulatory elements are introduced. Furthermore, the present results confirmed that it was the activated expression of *WRKY57* that conferred the drought tolerance phenotype in *adt*. *WRKY57* was expressed at a very low level in wild-type tissues (Figure 3B). Although abiotic stress expressive pattern analysis suggested that *WRKY57* was induced by ABA and drought (Figure 5A), neither single mutants (*wrky57* or *wrky48*) nor double mutants (*wrky48 wrky57*) showed any difference in drought tolerance (Figure 5D) compared with the wild-type (Figure 5D). Therefore, *WRKY57* maybe plays a minor role in drought stress response in wild-type plants. These results also suggested that the activated expression of the *WRKY57* gene allowed WRKY57 to gain a function in drought tolerance. This viewpoint is consistent with the hypothesis concerning the predominant role of regulatory mutations on organism evolution (King and Wilson, 1975) and is similar to a previous finding (Yu et al., 2008) that activated expression of an *Arabidopsis* HD-START protein confers drought tolerance in *Arabidopsis* and tobacco plants. Our present results may reveal a mechanism that plants evolve to adapt to drought tolerance. Establishment of *WRKY57* functions will enable

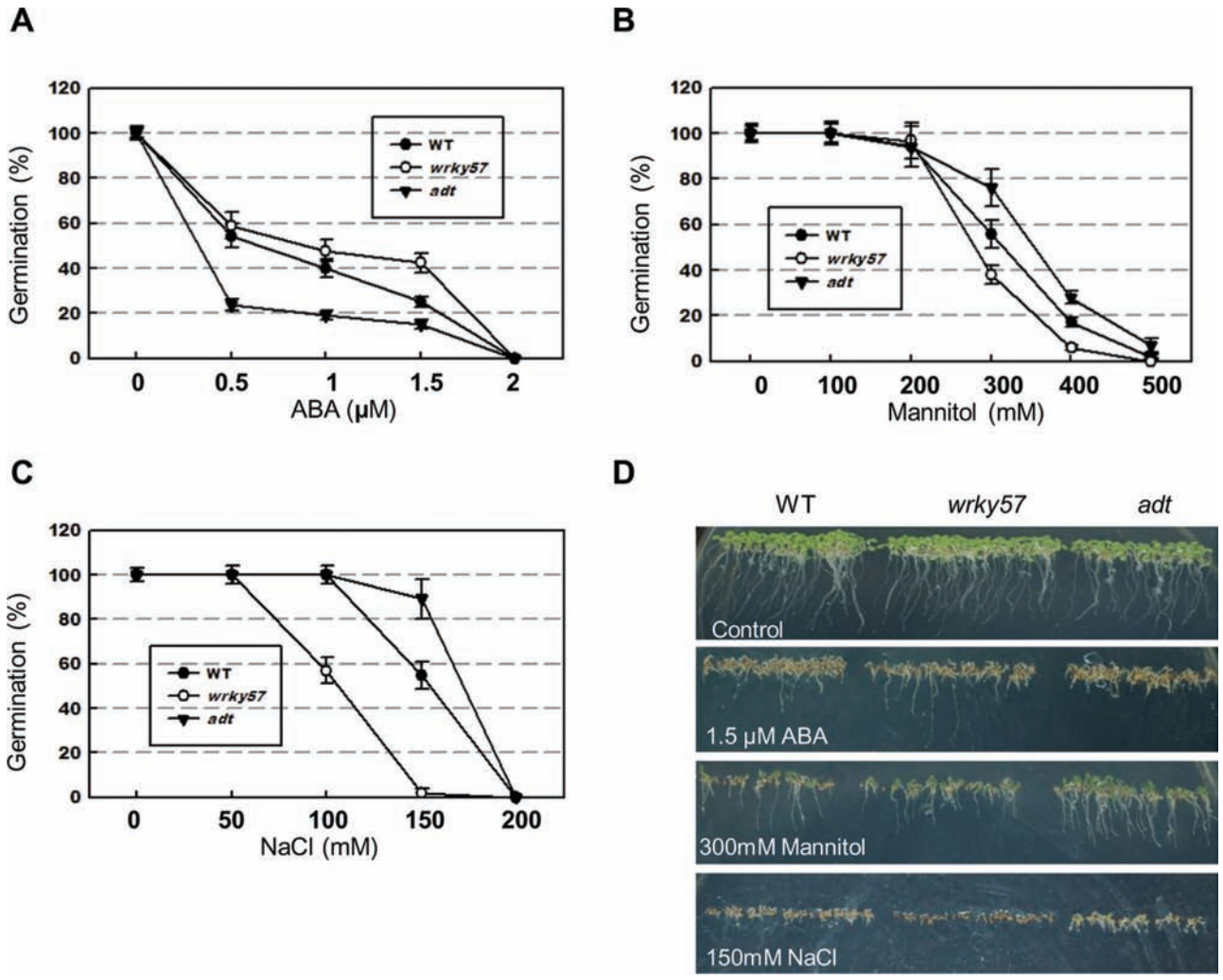

Figure 8. Seed Germination and Post-Germination Early Growth.

(A) Seed germination frequency under ABA treatment.

(B) Seed germination frequency under mannitol treatment.

(C) Seed germination frequency under NaCl treatment.

(D) Post-germination early growth of wild-type, *wrky57*, and *adt* mutant seedlings in MS supplemented with ABA, mannitol, and NaCl.

improvement of plant drought tolerance through gene manipulation approaches.

METHODS

Plant Growth Conditions

Arabidopsis thaliana cv. Columbia was grown in soil or on half-strength Murashige and Skoog medium (1/2 MS) with 1.5% (w/v) sucrose. Seeds were surface-sterilized (with 20% (v/v) bleach for 15 min) before sowing on ½ MS medium and incubated at 4°C for 3 d. Plants were then kept in a growth cabinet at 22°C under short-day conditions (12-h light/12-h dark photoperiod) and long-day conditions (16-h light/8-h dark photoperiod). The *WRKY* mutants were obtained from the Arabidopsis Biological Resource Center.

Measurement of Stomatal Closure in Response to ABA Treatment

Stomatal closure assays were conducted as described previously (Pei et al., 1997). Rosette leaves were floated in a solution containing 50 μM $CaCl_2$, 10 mM KCl, 10 mM MES (2-(N-morpholino)ethanesulfonic acid)-Tris, pH 6.15, and exposed to light for 2 h. Subsequently, ABA was added to the solution. After ABA treatment for 2 h, stomatal apertures were measured. Each sample was replicated at least three times.

Drought Tolerance Assays

For drought treatments, seedlings from wild-type Col-0, *adt*, *wrky57*, *wrky48*, *wrky58wrky48*, and three independent 35S lines were used. Seedlings grown for 7 d on ½ MS

medium plates were transplanted in separate pots under normal watering conditions. After 3 weeks' growth, plants were treated with natural drought (water was withheld). After 14 d without watering, the drought-treated plants were re-watered, and recovery was checked after 24 h. Drought experiments were repeated four times and at least 35 plants for each individual line were used in each repeated experiment and one representative picture was shown.

The rate of water loss by the leaves was measured. The aboveground shoot of 4-week-old plants was detached from the root system and weighed immediately. The shoots were placed in a plate on a laboratory bench and weighed at designated time intervals. The proportion of fresh weight lost was calculated on the basis of the initial weight of the plant. At least three biological replicates for each sample were used for drought tolerance assays.

ABA Content

Measurement of ABA content was conducted as described by Yang et al. (2001). Briefly, 10-day-old seedlings of the *adt* mutant and wild-type plants grown on ½ MS agar plates were transferred to ½ MS liquid medium supplemented with 10% PEG 6000 and incubated at 22°C under continuous light for 2 d. One gram of seedling tissue was used for ABA quantification by the ABA immunoassay kit as described by Yang et al. (2001). At least three biological replicates per sample were used.

RT–PCR and Real-Time RT–PCR Analysis

Northern blot analysis was performed as described previously (Chen et al., 2010). For RT–PCR analysis, total RNA was extracted using TRIzol reagent (Invitrogen) and treated with RNase-free DNase I (Fermentas) in accordance with the manufacturer's instructions. Total RNA (~2 μg) was reverse-transcribed in a 20-μl reaction mixture using the Superscript II reverse transcriptase (Invitrogen). After the reaction, 1-μl aliquots were used as a template for PCR amplification. For real-time RT–PCR analysis, the PCR templates were obtained from the same procedure as the RT–PCR. SYBR Green was used to monitor the kinetics of PCR product amplification in the real-time RT–PCR. As an internal control, the *ACTIN2* transcript was used to quantify the relative transcript level of each target gene in each tissue type. Three replicate biological experiments were conducted.

Identification of the T-DNA Insertion Mutants

The *adt* (SALK_117358) and *wrky57* (SALK_076716) lines contain a T-DNA insertion in the promoter and third exon, respectively, of the *WRKY57* gene. We confirmed the T-DNA insertions by PCR using a combination of a gene-specific primer and a T-DNA border primer. Homozygous *adt* and *wrky57* mutant plants were identified by PCR using a pair of primers corresponding to sequences flanking the T-DNA insertion sites. The identity of *adt*, *wrky57*, and *wrky48* mutants was confirmed further by qRT–PCR. The *wrky48 wrky57* double mutant was identified by both gene-specific primers and T-DNA border primers, and was confirmed further by qRT–PCR.

Generation of Transgenic Lines

To generate the 35S:*WRKY57* construct, the cDNA fragment containing the full coding sequence and 3′ untranslated region of *WRKY57* was excised from a cloning plasmid and subcloned into the same restriction sites of the binary vector pOCA30 (Chen and Chen, 2002) in the sense orientation behind the CaMV 35S promoter. *Arabidopsis* transformation was performed by the floral dip procedure (Clough and Bent, 1998). Seeds were collected from the infiltrated plants and selected on ½ MS medium supplemented with 50 μg ml^{-1} kanamycin. Kanamycin-resistant plants were transferred to soil 8 d after germination and were grown in a growth chamber.

ChIP Assays

ChIP assays were performed essentially in accordance with previously described protocols (Saleh et al., 2008; Shang et al., 2010). 35S-*myc-WRKY57* and *WRKY57* P- *myc-WRKY57* transgenic plants were used for ChIP assays, respectively. The primers used for PCR amplification of different promoters are listed in Supplemental Table 1. PCR amplification was performed using 36 cycles for all promoter fragments. Aliquots of the PCR products were resolved by electrophoresis on 2% agarose gel. The results presented were obtained from at least three independent experiments. To quantify WRKY57-DNA (target promoters) binding, real-time RT–PCR analysis was performed in accordance with a procedure described previously with the *ACTIN2* 3′ untranslated region as the endogenous control (Mukhopadhyay et al., 2008). The relative quantity value is presented as DNA binding ratio (differential site occupancy) (Figure 6D). The same primers as for the above-mentioned PCR analysis were used for the real-time RT–PCR. A fragment of the *ACTIN2* promoter was used as a negative control. The results presented were obtained from at least three independent experiments.

Seed Germination and Early Seedling Growth Analysis

For the seed germination test, sterile seeds were sown on MS medium plus 1.5% sucrose supplemented with different concentrations of ABA and mannitol. Plates were incubated for 3 d in the dark at 4°C to break dormancy, and then transferred to a growth cabinet maintained at 22°C. The number of germinated seeds was counted daily. For post-germination seedling growth analysis, sterile seeds were sown on MS medium plus 1.5% sucrose supplemented with different concentrations of ABA and mannitol. Plates were incubated for 3 d in the dark at 4°C to break dormancy, and then transferred to a growth cabinet maintained at 22°C. Photographs were taken after growth for 10 d. For

seedling growth analysis, sterile seeds were sown on MS medium plus 1.5% sucrose. Plates were incubated for 3 d in the dark at 4°C to break dormancy, and then transferred to a growth cabinet maintained at 22°C. Three-day-old seedlings were transferred to MS supplemented with 20 μM ABA and 300 mM mannitol. Photographs were taken after growth for 10 d. All results presented were obtained from at least three independent experiments.

Accession Numbers

Sequence data generated in this study were lodged in the Arabidopsis Genome Initiative and GenBank/EMBL databases under the following accession numbers: *WRKY57* (AT1G38010), *WRKY48* (AT5G49520), *ACTIN2* (AT3G46520), *RD29A* (AT5G52310), *NCED3* (AT3G14440), and *ABA3* (AT1G16540).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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