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 WRKY57 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; Liottenberg 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 H₂O₂ (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 

...O. OsWRKY45 Enhances Drought Tolerance

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, increased drought tolerance of these transgenic plants displayed characteristics related to drought tolerant with elevation of their ABA contents and up-regulation expression of ABA-responsive genes (RD29A, ABA3, and NCED3). Overall, our results indicate that the activation expression of WRKY57 confers the adaption of Arabidopsis to drought tolerant 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...
Changes were measured over a 200-min period. The loss by transpiration related to drought tolerance. To assess the rate of water loss, transpirational water loss is one of the most important factors that may cause the drought tolerance phenotypes of mutants. 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 tolerance of the adt mutant.

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.

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.
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 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.
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. 3SS::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 3. Identification of the T-DNA Tagged Locus and Activated Expression of WRKY57.](http://mplant.oxfordjournals.org/)

**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.
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
We tested the germination frequency of wild-type, \textit{adt}, and \textit{wrky57} mutant seeds on Murashige and Skoog (MS) medium supplemented with different concentrations of ABA. The \textit{wrky57} seeds displayed a higher germination frequency than wild-type seeds, whereas the seed germination frequency of \textit{adt} was significantly lower than that of the wild-type (Figure 8A). Moreover, early seedling growth of \textit{adt} was also more sensitive to ABA than the wild-type and \textit{wrky57} (Figure 8D). When seedlings were grown on basal MS medium for 3 days and then grown on MS medium supplemented with 20 \mu M ABA, no significant difference in growth was observed between \textit{adt}, wild-type, and \textit{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 \textit{adt} plants exhibited increased ABA sensitivity in both germination and early seedling growth, we investigated the response of \textit{adt} to osmotic stress. Wild-type, \textit{wrky57}, and \textit{adt} seeds were sown on MS medium supplemented with 300 mM mannitol. Seed germination and early seedling growth of \textit{adt} were less sensitive to mannitol treatments compared with the wild-type, whereas the \textit{wrky57} mutant displayed the opposite results (Figure 8B and 8D). When subjected to salt treatment, \textit{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 \textit{Arabidopsis}.
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,

**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.

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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
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 CaCl₂, 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, wrky58 wrky48, 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 loss 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 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 (AT5G2310), NED3 (AT3G14440), and ABA3 (AT1G16540).

SUPPLEMENTARY DATA
Supplementary Data are available at Molecular Plant Online.

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