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SDIR1 Is a RING Finger E3 Ligase That Positively Regulates Stress-Responsive Abscisic Acid Signaling in *Arabidopsis* [W]

Yiyue Zhang,[a,b,c,1] Chengwei Yang,[b,1,2] Yin Li,[b] Nuoyan Zheng,[b] Hao Chen,[a,b,c] Qingzhen Zhao,[a,c] Ting Gao,[a,c] Huishan Guo,[d] and Qi Xie[a,3]

[a] State Key Laboratory of Plant Genomics, National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

[b] State Key Laboratory for Biocontrol, School of Life Sciences, Sun Yat-sen (Zhongshan) University, Guangzhou 510275, China

[c] Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

[d] State Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China

Ubiquitination plays important roles in plant hormone signal transduction. We show that the RING finger E3 ligase, *Arabidopsis thaliana* SALT- AND DROUGHT-INDUCED RING FINGER1 (SDIR1), is involved in abscisic acid (ABA)-related stress signal transduction. *SDIR1* is expressed in all tissues of *Arabidopsis* and is upregulated by drought and salt stress, but not by ABA. Plants expressing the Pro*SDIR1*–β-glucuronidase (*GUS*) reporter construct confirmed strong induction of GUS expression in stomatal guard cells and leaf mesophyll cells under drought stress. The green fluorescent protein–SDIR1 fusion protein is colocalized with intracellular membranes. We demonstrate that SDIR1 is an E3 ubiquitin ligase and that the RING finger conservation region is required for its activity. Overexpression of *SDIR1* leads to ABA hypersensitivity and ABA-associated phenotypes, such as salt hypersensitivity in germination, enhanced ABA-induced stomatal closing, and enhanced drought tolerance. The expression levels of a number of key ABA and stress marker genes are altered both in *SDIR1* overexpression and *sdir1-1* mutant plants. Cross-complementation experiments showed that the *ABA-INSENSITIVE5* (*ABI5*), *ABRE BINDING FACTOR3* (*ABF3*), and *ABF4* genes can rescue the ABA-insensitive phenotype of the *sdir1-1* mutant, whereas *SDIR1* could not rescue the *abi5-1* mutant. This suggests that *SDIR1* acts upstream of those basic leucine zipper family genes. Our results indicate that SDIR1 is a positive regulator of ABA signaling.

INTRODUCTION

Plants are sessile organisms capable of adapting to various environmental conditions, such as drought, cold, and high salt content in soil. When they encounter these stressful conditions, plant cells reprogram their cellular processes by triggering a network of signaling events. The process starts with stress signal perception and ends with a cellular response, such as gene expression in the nucleus and metabolic pathway changes that enable the plant to adapt to new environments. Normally silent genes that are activated under stress conditions often are referred to as stress genes. Researchers have worked hard to determine the genes that control the perception of environmental stresses and the subsequent activation of gene expression contributing to adaptation (Hasegawa et al., 2000; Bressan et al., 2001; Kawasaki et al., 2001; Zhu, 2002). Abscisic acid (ABA) plays a major role in transducing stress responses (Knight and Knight, 2001).

ABA is required for plant adaptation to environmental stress by affecting different plant tissues, developmental stages, and physiological processes. In particular, these include changes in seed dormancy and germination, seedling and plant growth, and stomatal function (Leung and Giraudat, 1998). For example, during water deficit, ABA induces stomatal closure, minimizing water loss through transpiration (Schroeder et al., 2001). The ABA-controlled process is vital for plant survival, and ABA-deficient and ABA-responsive mutants are susceptible to water stress (Kang et al., 2002).

Underlying the ABA-mediated stress response is the transcriptional regulation of stress-responsive gene expression (Giraudat et al., 1994; Busk and Pages, 1998). Numerous genes have been reported to be upregulated under stress conditions in vegetative tissues. Among the products of these genes are enzymes catalyzing the synthesis of osmoprotectants or antioxidants, late embryogenesis abundant proteins, chaperones and heat shock proteins, lipid desaturases, water channels, and ion transporters (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). In general, these gene products are considered to have protective or adaptive roles under stress conditions. In addition, the expression of many regulatory genes, including various kinase/phosphatase and transcription factor genes, is also induced by abiotic stresses. Most stress-inducible genes are also responsive to exogenous ABA, and in many cases their

[1] These authors contributed equally to this work.

[2] Current address: School of Life Science, South China Normal University, Guangzhou 510631, China.

[3] To whom correspondence should be addressed. E-mail qxie@genetics.ac.cn; fax 86-10-64889351.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Qi Xie (qxie@genetics.ac.cn).

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induction is impaired in ABA-deficient mutants, such as *RD29A* and *P5CS1* genes (Strizhov et al., 1997; Chak et al., 2000).

Using exogenously applied ABA at concentrations capable of inhibiting the germination of wild-type *Arabidopsis thaliana* seeds, several ABA-insensitive (ABI) mutants have been isolated, and the affected loci for *ABI1* to *ABI5* have been identified as encoding two protein phosphatase 2Cs (*ABI1* and *ABI2*) as well as B3 (*ABI3*), AP2 (*ABI4*), and basic domain/Leu zipper (*ABI5*) transcription factors (Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Merlot et al., 2001). The protein phosphatase 2Cs (PP2Cs) encoded by *ABI1* and *ABI2* also control stomatal responses to ABA (Allen et al., 1999). *ABI3* and *ABI5* encode a putative acidic domain transcription factor (Giraudat et al., 1992) and a basic leucine zipper (bZIP) transcription factor (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2002), respectively. ABI3 is vital to desiccation tolerance and dormancy during zygotic embryogenesis (Koornneef et al., 1989; Finkelstein, 1994; Parcy et al., 1994; Parcy and Giraudat, 1997). Previous work using a two-hybrid screen found that ABI3 interacted with the RING finger protein AIP2 (Kurup et al., 2000). Recently, AIP2 was demonstrated to function as a ubiquitin-protein ligase (E3) promoting ABI3 degradation (Zhang et al., 2005). ABI5 acts downstream of ABI3 (Lopez-Molina et al., 2002), and its role in postgermination developmental arrest has also been demonstrated (Lopez-Molina et al., 2001). An ABI5-interacting protein, AFP, was found to be a negative regulator of ABA signaling by promoting ABI5 through ubiquitin-mediated degradation in nuclear bodies (Lopez-Molina et al., 2002). Recently, a new RING finger protein, KEG, was also found to promote ABI5 degradation (Stone et al., 2006).

The ubiquitin/26S proteasome pathway has been implicated in diverse aspects of eukaryotic cell regulation because of its ability to rapidly remove intracellular proteins (Hershko and Ciechanover, 1998; Callis and Vierstra, 2000). Proteins designated for degradation are covalently modified by attachment of a ubiquitin polymer and are then degraded by the 26S proteasome. A ubiquitin-activating enzyme (E1) catalyzes the ATP-dependent formation of a thioester bond between ubiquitin and itself and transfers the activated ubiquitin to a ubiquitin-conjugating enzyme (E2). Formation of an isopeptide bond between ubiquitin and a substrate is facilitated by an E3 ligase that can bind both the E2-ubiquitin complex and the substrate. Ubiquitin-dependent protein degradation has been shown to play an important role in hormone regulation, embryogenesis, photomorphogenesis, circadian rhythms, floral homeosis, senescence, and pathogen defense (Callis and Vierstra, 2000; Hellmann and Estelle, 2002; Xie et al., 2002; Devoto et al., 2003; Zeng et al., 2004). Recent data suggest that ubiquitination may also play an important role in plant tolerance against abiotic stresses. *Arabidopsis* contains a CHIP homolog that functions as an E3 ligase in vitro, which is upregulated by certain stress conditions. Overexpression of *ATCHIP* rendered *Arabidopsis* more sensitive to both low and high temperatures (Yan et al., 2003). Recently, the same group demonstrated that At CHIP may function upstream of PP2A in stress-responsive signal transduction pathways under conditions of low temperature or in the dark (Luo et al., 2006). *Arabidopsis* HOS1 encodes a variant RING finger protein that has been implicated as an E3 ubiquitin ligase that negatively regulates transcription factor ICE1 and modulates downstream cold-responsive gene transcription (Lee et al., 2001; He et al., 2006). These results suggest a linkage between protein ubiquitination and stress responses in plants. A functional genomic study of RING-H2 type RING finger proteins from *Arabidopsis* and rice (*Oryza sativa*) found one insertion mutant, *atl43*, that showed an ABA-insensitive phenotype, suggesting a role in ABA response, but no experimental data were presented (Serrano et al., 2006).

To gain further insight into the molecular mechanisms of stress signaling, we isolated stress response RING finger genes identified in published microarray data and studied their function. Here, we present a molecular and genetic characterization of the *Arabidopsis* RING finger gene *SALT- AND DROUGHT-INDUCED RING FINGER1* (*SDIR1*) and the null mutant *sdir1*. Our results indicate that SDIR1 is a positive regulator of the ABA-dependent stress signaling pathway.

RESULTS

Identification of the *SDIR1* Gene

In order to identify stress-related *Arabidopsis* single subunit E3 ligase genes, in silico gene expression of *Arabidopsis* RING finger genes was analyzed in several publicly available stress-related microarray studies (Kosarev et al., 2002; Kreps et al., 2002; Seki et al., 2002; Leonhardt et al., 2004; Mudgil et al., 2004). Among 387 RING finger proteins (including the PHD family) and 40 U-box subfamily proteins analyzed from *Arabidopsis*, *SDIR1*, a C3H2C3 RING finger gene (At3g55530), attracted our interest. Microarray data indicated that its transcript was induced to high levels after salt and drought stress treatment, and this was later confirmed by RNA gel blot analysis (Figure 1A). The microarray results suggested that *SDIR1* was involved in stress response.

Expression Pattern of *SDIR1* and the Salt and Drought Responses

To verify the microarray *SDIR1* expression results and to check whether *SDIR1* expression is regulated by other abiotic stresses, 2-week-old *Arabidopsis* seedlings were subjected to different stress treatments and RNA was prepared for RNA gel blot analysis. As shown in Figure 1A, the transcript level of *SDIR1* was upregulated by drought and NaCl, but not by ABA. *SDIR1* expression increased by approximately fivefold at the 12-h point of the drought treatment course. RT-PCR analysis detected *SDIR1* in all tissues of *Arabidopsis*, including leaves, stems, roots, siliques, and flowers (Figure 1B). To determine the expression pattern in detail, we analyzed the expression of the *SDIR1* promoter–β-glucuronidase (GUS) fusion. This consisted of a 1.3-kb DNA fragment upstream of the *SDIR1* ATG start codon fused with the GUS gene, transforming the construct into wild-type *Arabidopsis* (Columbia). Histochemical staining revealed GUS activity at all developmental stages tested, from seed germination to flowering (Figure 1C). This confirmed that *SDIR1* is expressed at all developmental stages and throughout the *Arabidopsis* plant. GUS expression was first detected in 1-d-old

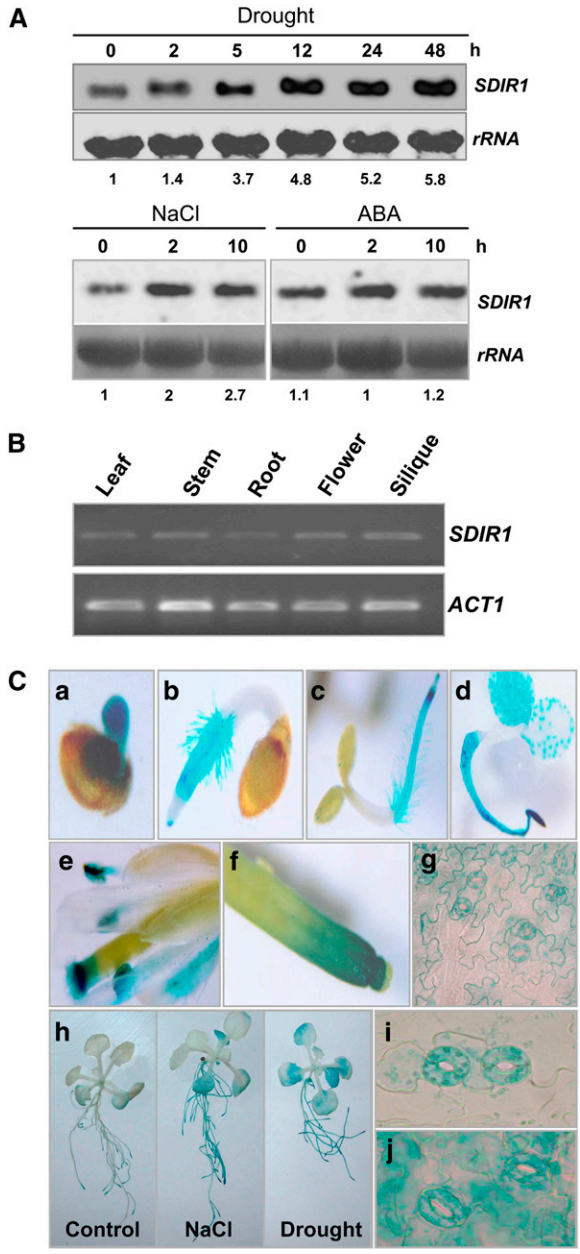

Figure 1. Expression Patterns of *SDIR1*.

(A) Expression patterns of *SDIR1* gene transcripts in response to drought, 300 mM NaCl, and 50 μM ABA treatment. Twelve micrograms of total RNA from each sample was hybridized with α-^{32}P-labeled *SDIR1* probe. The 28s rRNA is shown as a loading control, and numbers below each lane indicate the relative expression ratio.

(B) Expression of the *SDIR1* gene in different tissues of *Arabidopsis* plants. Total RNA was isolated from various tissues (root, leaf, stem, flower, and silique) of 4-week-old wild-type plants grown under long-day growth conditions. RT-PCR was performed with either *SDIR1*-specific primers (top gel) or actin-specific primers (bottom gel).

(C) *SDIR1* promoter–GUS expression pattern in transgenic *Arabidopsis* plants. (a) One-day-old germinating seed. (b) Two-day-old germinating seedling. (c) Three-day-old seedling. (d) Four-day-old seedling. (e) Flowers. (f) Silique. (g) Guard cells. (h) Tissue localization of enhanced GUS expression in *SDIR1* promoter–GUS transgenic seedlings treated with 300 mM NaCl for 5 h and drought for 5 h. (i) and (j) Details of GUS expression in guard cells under drought treatment. (i) Control without drought treatment. (j) Samples treated with drought for 3 h.

germinated seeds with stronger GUS staining in the emerging radicle (Figure 1Ca). GUS expression was also detected at the root tip and in the root hair differentiation zone of 2- and 3-d-old seedlings (Figures 1Cb and 1Cc). At 4 d, GUS expression was detected throughout the plant, with the exception of the hypocotyl (Figure 1Cd). GUS activity was also observed in the flowers, with stronger GUS staining observed in pollen grains (Figures 1Ce and 1Cf). Interestingly, strong GUS staining was also observed in guard cells of both young (data not shown) and mature leaves under normal growth conditions (Figure 1Cg).

Once we determined that *SDIR1* is upregulated by drought and NaCl (Figure 1A), we next tested whether the *SDIR1* promoter is responsible for this upregulation. The *SDIR1* promoter–GUS reporter construct provided a useful tool for identifying the promoter required for the response to drought and NaCl. GUS activity increased throughout the plant and especially in the roots after 5 h in drought conditions and 3 h of 300 mM NaCl treatment (Figure 1Ch). A substantial increase in GUS activity was also detected not only in guard cells but also in entire leaves after drought treatment (Figures 1Ci, control, and 1Cj, drought-treated).

SDIR1 Is Associated with the Intracellular Membrane

SDIR1 encodes a protein of 273 amino acids with a predicted molecular mass of 30.18 kD (Figure 2A). Two putative transmembrane domains were predicted by the SMART program (http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1). The two domains were located at the N terminus of the SDIR1 protein, between 35 and 52 amino acids and 62 and 81 amino acids, respectively. To determine whether two putative transmembrane domains predicted in SDIR1 are functional, the myc-SDIR1 full-length protein and a truncated form, myc-SDIR1ΔTM, with the transmembrane domain deleted (ΔTM; Figure 2A) were transiently expressed in *Nicotiana benthamiana* leaf cells. Cell fractionation and protein gel blot assays showed that both myc-SDIR1 and myc-SDIR1ΔTM are present in total cell extract (Figure 2B). The full-length myc-SDIR1 was detected exclusively in the microsomal fraction but not in the soluble fraction. The major part of myc-SDIR1ΔTM was detected in the soluble fraction. However, a small amount was detected in the membrane fraction, which could be due to a trace of unground cell/tissue contamination in the microsomal fraction (Figure 2B). The association of myc-SDIR1 with the intracellular membranes was further confirmed by resuspension in different detergent-free and detergent-containing buffers for extensive cell fractionation analysis. SDIR1 was always detected only in the microsomal fractions and removed from the microsomal fractions by detergent treatment (Figure 2C). Therefore, we concluded that SDIR1 is associated with the intracellular membranes.

To confirm the association of SDIR1 with the intracellular membranes, we checked the subcellular localization of the green

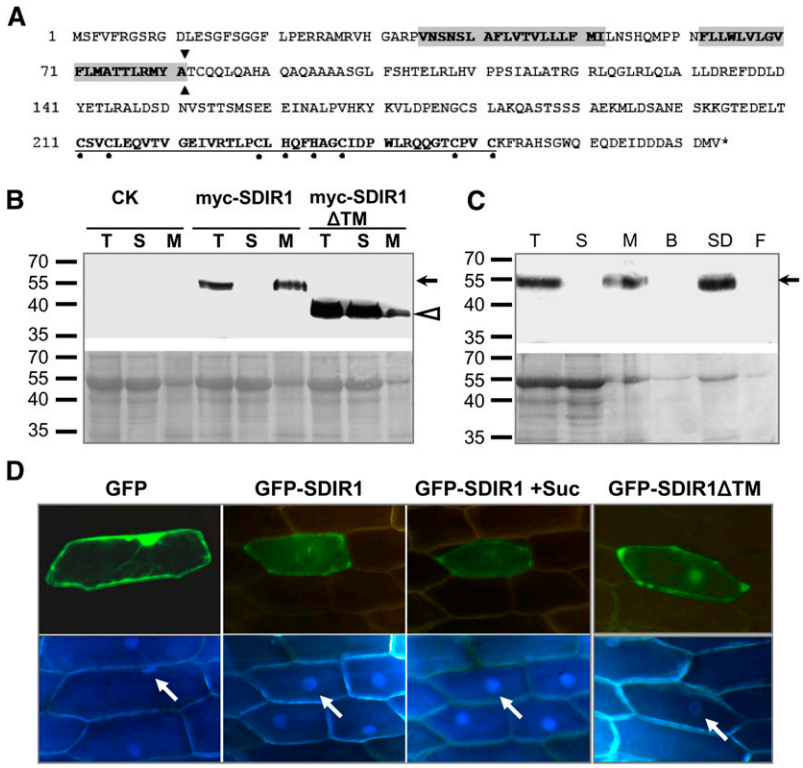

Figure 2. Intracellular Membrane Localization of the SDIR1 Protein.

(A) Amino acid sequence of SDIR1. Two putative transmembrane domains predicted by the protein domain analysis program SMART are shaded. The highly conserved RING finger domain is underlined, and asterisks indicate conserved Cys and His residues. Closed triangles indicate the transmembrane domain deletion positions.

(B) Cell fractionation assays of SDIR1 and SDIR1 transmembrane domain deletion (SDIR1ΔTM) proteins. Total extract (T) of *N. benthamiana* leaf cells expressing a myc-vector control (CK) and myc-SDIR1 and myc-SDIR1ΔTM fusions was fractionated into soluble (S) and microsomal (M) fractions, and the myc fusion proteins were detected using an anti-myc antibody (top panel). The arrow indicates myc-SDIR1, and the triangle indicates myc-SDIR1ΔTM. Ponceau S staining of the transferred membrane is displayed as a loading control (bottom panel).

(C) Further fractionation analysis for membrane association. T, total extract; S, soluble fraction; M, membrane fraction; B, buffer-extracted fraction; SD, SDS-extracted fraction; F, final membrane fraction. The antibody and loading controls are the same as in **(B)**.

(D) Subcellular localization of SDIR1 protein by GFP fusion expression in onion epidermal cells. Cells were analyzed by fluorescence microscopy and photographed after 16 h of incubation following bombardment. Panels from left to right: GFP control, GFP-SDIR1, GFP-SDIR1 treated with 1 M sucrose for plasmolysis, and GFP-SDIR1ΔTM (top panels). Samples were stained with 4′,6-diamidino-2-phenylindole; arrows indicate positions of nuclei (bottom panels).

fluorescent protein (GFP)–SDIR1 fusion protein. We constructed a *GFP-SDIR1* fusion under the control of a cauliflower mosaic virus (CaMV) *35S* promoter. To verify whether the GFP tag affects the function of the SDIR1 protein, both the *GFP-SDIR1* fusion and *SDIR1* were introduced into *Arabidopsis*. Both constructs exhibited the same phenotypes, such as ABA hypersensitivity, demonstrating that the GFP-SDIR1 fusion is a functional protein (see Supplemental Figure 1 online). Transient expression in onion (*Allium cepa*) epidermal cells demonstrated that GFP alone was found in both the cytosol and the nucleus, whereas GFP-SDIR1 was found densely localized in intracellular membranes, probably (plasma) membrane-bound in cells, as GFP-SDIR1ΔTM accumulates in the nucleus compared with GFP-SDIR1 (Figure 2D). The plasmolysis results indicated that GFP-SDIR1 localized in intracellular components rather than in cell walls. This is consistent with the predicted transmembrane domains in the N terminus of SDIR1 (Figure 2A).

SDIR1 Is a Functional E3 Ligase

Previous research showed that RING finger–containing proteins can function as E3 ligases (Xie et al., 2002; Seo et al., 2003). The C terminus of SDIR1 contains a conserved C3H2C3-type RING domain (211 to 251 amino acids) comprising conserved Cys and His residues (Figures 2A and 3A). Therefore, we speculated that SDIR1 also had E3 ligase activity. To test this hypothesis, we produced SDIR1 in *Escherichia coli* as a fusion protein with maltose binding protein (MBP) and affinity-purified MBP-SDIR1 from the soluble fraction. In the presence of wheat (*Triticum aestivum*) E1 and a human E2 (UBCh5b), ubiquitination activity was observed

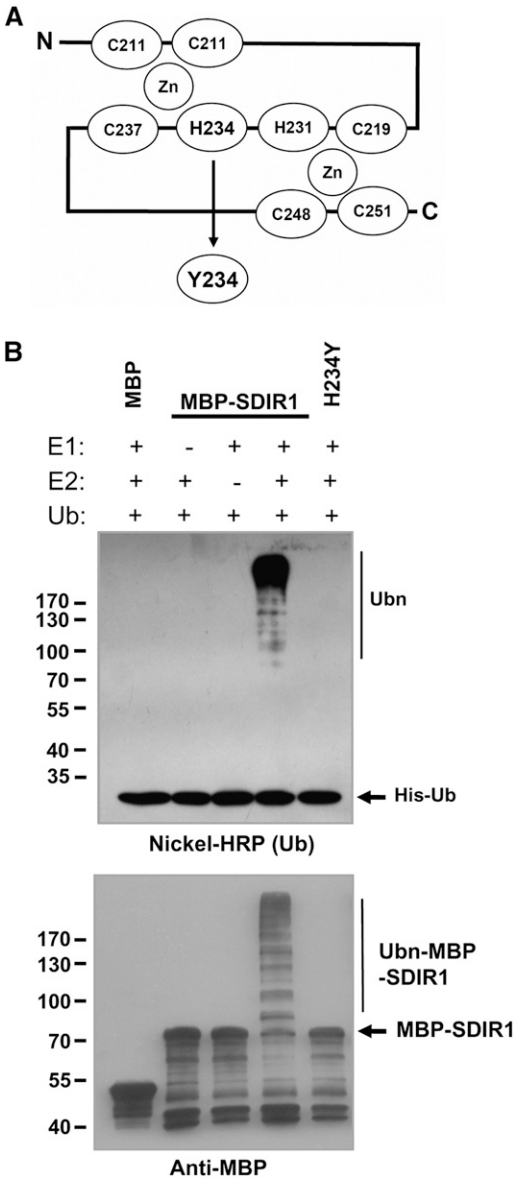

Figure 3. E3 Ubiquitin Ligase Activity of SDIR1 and the RING Mutant Variant.

(A) Scheme of SDIR1 RING finger composition and the mutated amino acid in the RING finger.

(B) E3 ubiquitin ligase activity of SDIR1. MBP-SDIR1 and its mutant form MBP-SDIR1 (H234Y) fusion proteins were assayed for E3 activity in the presence of E1 (from wheat), E2 (UBCh5b), and 6xHis tag ubiquitin (Ub). The numbers at left denote the molecular masses of marker proteins in kilodaltons. MBP itself was used as a negative control. Samples were resolved by 8% SDS-PAGE. The nickel–horseradish peroxidase was used to detect His tag ubiquitin (top panel), and the anti-MBP antibody was used for maltose fusion proteins (bottom panel).

in the presence of purified MBP-SDIR1 (Figure 3B). Due to the presence of crude extract of E1 and E2 in the reaction, the ubiquitination ladders detected by nickel–horseradish peroxidase (for detecting His-ubiquitin) could be any proteins, including SDIR1 and MBP tag, in the reaction mixture. Furthermore, the anti-MBP blot analysis indicated that at least MBP-SDIR1 was ubiquitinated. However, in the absence of E1 and E2 (Figure 3B, second and third lanes from the left), no polyubiquitination was detected, indicating that SDIR1 has E3 ligase activity.

The RING motif is essential for the E3 ligase activity of RING finger proteins (Xie et al., 2002). Therefore, we tested whether an intact RING finger domain was required for SDIR1 E3 ligase activity. A single amino acid substitution allele was produced by mutagenizing His-234 to Tyr (H234Y), as this mutation might disrupt the RING domain (Figure 3A). An in vitro ubiquitination assay indicated that the E3 ligase activity was completely abolished in the H234Y mutant of SDIR1 (Figure 3B, first lane from the right), demonstrating that an intact RING domain is required for SDIR1 E3 ligase activity.

Growth Phenotypes of the *sdir1* Mutant and *SDIR1* Overexpression Plants

Analysis of the full-length cDNA and the genomic sequences revealed that *SDIR1* is composed of eight exons and seven introns (Figure 4A). The gene is located on chromosome III of the *Arabidopsis* genome. To discover the in vivo functions of SDIR1, we applied reverse genetics and overexpression approaches. First, the loss-of-function mutant of the *SDIR1* gene, two independent T-DNA insertion lines, SALK_052702 (named *sdir1-1*) and SALK_114361 (named *sdir1-2*), were identified from the ABRC seed stock center. The T-DNA insertion positions are illustrated in Figure 4A, and homozygous mutants were verified by diagnostic PCR using *SDIR1* gene-specific and T-DNA border primers (Figure 4B). Both *sdir1-1* and *sdir1-2* null alleles were confirmed by RT-PCR and RNA gel blot analysis for loss of *SDIR1* expression (Figures 4C and 4D). On Murashige and Skoog (MS) growth medium, no obvious differences were observed between the wild type and the two *SDIR1* mutants at the germination stage (data not shown). Both *sdir1-1* and *sdir1-2* showed identical phenotypes, exhibiting a longer primary root at later growth stages compared with wild-type plants but similar to the wild type in the number of lateral roots and aerial parts (Figure 4D). The difference in primary root length between the wild type and mutants was observed over the entire growth time course (Figure 4E). To confirm whether the phenotypes of the *sdir1* mutants were indeed due to lost *SDIR1* function, *35S-SDIR1* was only overexpressed in the *sdir1-1* mutant, since both *sdir1-1* and *sdir1-2* are null alleles with the same phenotype. All phenotypes, such as root length and NaCl and ABA insensitivity, were rescued in complementation lines (see below for details and Supplemental Figure 2 online). Thus, the *sdir1-1* null mutant was used for all further phenotype comparisons and cross-complementary analysis.

The function of SDIR1 was further investigated by analysis of *SDIR1*-overexpressing phenotypes. The coding region of *SDIR1* was introduced into the transgenic vector under the control of the CaMV *35S* promoter and into wild-type *Arabidopsis* (Columbia) to produced transgenic lines overexpressing *SDIR1*. Five independent T3 homozygous lines were recovered, and lines with high levels of *SDIR1* expression confirmed by RNA gel blot analysis showed similar phenotypes (data not shown). Similar to the *sdir1* mutants, no obvious differences were observed

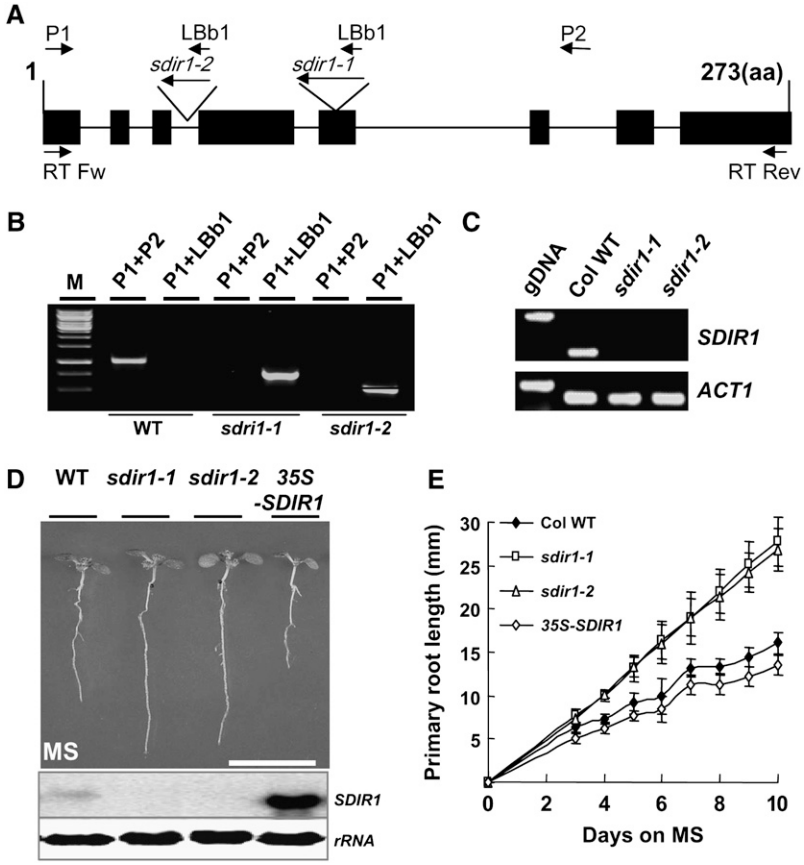

Figure 4. *SDIR1* Structure, T-DNA Insertion Diagnostic PCR, and Phenotypes of *SDIR1* Overexpression and Mutant Plants.

(A) Schematic diagram of *SDIR1* structure and T-DNA diagnostic PCR and RT-PCR. Closed boxes represent exons, and lines between closed boxes represent introns. P1, forward primer; P2, reverse primer; LBb1, primer specific to the T-DNA left border. RT Fw and RT Rev are primers used for RT-PCR analysis. aa, amino acids.

(B) Diagnostic PCR of the T-DNA inserted in two different loci of *SDIR1*. DNA from homozygous insertion lines of *sdir1-1* and *sdir1-2* were used. M, molecular mass markers. Primers used for PCR are indicated above each lane.

(C) RT-PCR analysis of the *SDIR1* transcripts in wild-type and T-DNA insertion mutant seedlings. The primer pairs used for RT-PCR are shown in **(A)**. *ACTIN1* was used as an internal control.

(D) Root phenotype of representative seedlings grown on vertical MS plates for 7 d (top panel). Bar = 1 cm. RNA blot and phenotype analysis of wild-type, two mutant, and *35S-SDIR1* plants. Ten micrograms of total RNA were loaded in each lane. 28S rRNA was used as an RNA-loading control (bottom panel).

(E) Quantitative analysis of primary root length of wild-type, two mutant, and *35S-SDIR1* plants on MS medium at vertical growth position. The values are means ± SD ($n = 30$).

between the wild type and *35S-SDIR1* at the germination stage on MS medium. The *35S-SDIR1* plants exhibited shorter primary roots and slightly smaller aerial parts than wild-type plants at seedling growth stages on plates (Figures 4D and 4E), while no obvious differences in aerial parts were observed on soil-grown plants. The opposite phenotypes of *sdir1* and *SDIR1* overexpression plants indicate that *SDIR1* is involved in root growth control under normal growth conditions.

Salt and Osmotic Responses of *35S-SDIR1* and *sdir1* Plants

Salts inhibit germination and seedling growth in a concentration-dependent manner (Xiong et al., 2002). Since *SDIR1* is a salt-induced gene (Figures 1A and 1Ch), it is probable that *SDIR1* plays a role in plant responses to salt. The *35S-SDIR1* transgenic and *sdir1* mutant seeds were germinated on medium containing 100 mM NaCl, and differences were observed at both germination and postgerminative growth stages. Seeds of both mutants germinated much earlier than in the wild type. After 2 d, only 29% of wild-type seeds germinated, but ~70% of *sdir1-1* and 66% of *sdir1-2* seeds germinated (Figure 5A). Seedlings of both mutants also grew faster than wild-type seedlings, with longer primary roots, bigger cotyledons, and earlier true leaf emergence in 97 and 96%, respectively, of *sdir1-1* and *sdir1-2* seedlings (Figure 5B). Germination of *35S-SDIR1* seeds was much delayed; at 3 d, only 52% of *35S-SDIR1* seeds germinated, but nearly 100% of wild-type seeds germinated (Figure 5A). In fact, cotyledon greening and expansion, as well as root growth of *35S-SDIR1*, were also

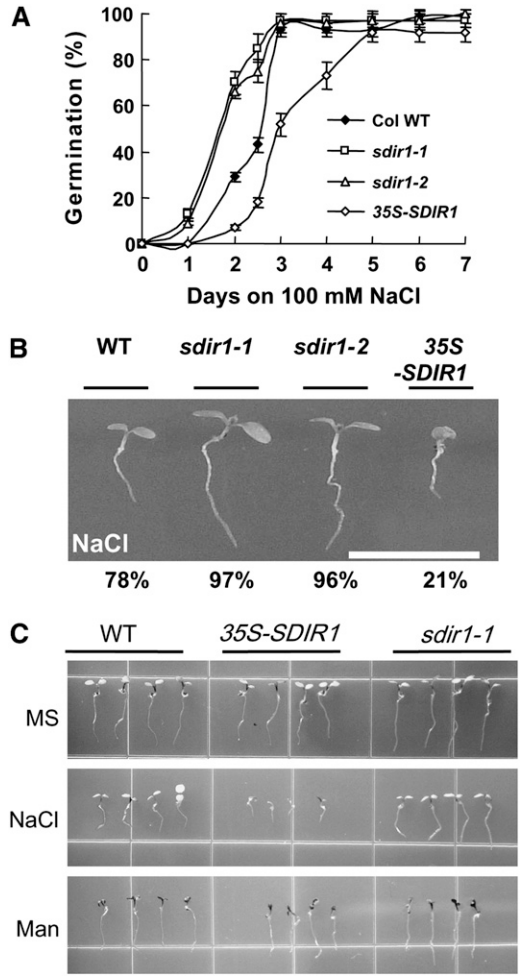

Figure 5. Salt and Osmotic Sensitivity of *35S-SDIR1* and *sdir1-1* Plants.

(A) Quantitative analysis of germination on 100 mM NaCl. Germination (radical emergence) of wild-type, two mutant, and *35S-SDIR1* plants on MS medium containing 100 mM NaCl. Percentages are means ($n = 60$ to 90 each) of three repeats ± SD.

(B) Growth phenotype of transgenic and mutant plants on MS medium containing 100 mM NaCl. Seeds were germinated and grown for 7 d. Numbers below the panel indicate the percentage of seedlings with green cotyledons in total seedlings ($n = 60$ to 90).

(C) Osmotic effects on newly germinated seedling growth. Seeds of three different genotypes were germinated for 4 d on MS medium and MS medium containing 100 mM NaCl or 200 mM mannitol (Man), transferred to the same type of medium, and grown in a vertical position for an additional 1 d. Representative seedlings are shown.

inhibited at the postgermination stage, and at 7 d, only 21% of the seedlings had small green cotyledons (Figure 5B). Similar results were observed when plants were grown in medium with 100 mM KCl, while no obvious difference was detected when 5, 10, and 15 mM LiCl was added (see Supplemental Figure 3 online).

To distinguish whether *SDIR1* is involved in salt-specific or general osmotic effects, *35S-SDIR1* transgenic and *sdir1-1* mutant plants were germinated and grown on MS medium with or without 100 mM NaCl and 200 mM mannitol (an osmotic agent; double concentration of NaCl tested) (Figure 5C). The growth of *35S-SDIR1* plants was strongly inhibited compared with wild-type plants and *sdir1-1* mutants upon NaCl treatment (Figure 5C). However, no different effects among *35S-SDIR1*, wild-type, and *sdir1-1* mutant plants were observed on plates containing mannitol (Figure 5C). These results suggest that the response to salt is ionic rather than osmotic.

Drought Response of *35S-SDIR1* and *sdir1* Plants

Because *SDIR1* is induced by drought (Figures 1A and 1C), it is expected that the overexpression and mutant plants have altered responses to water deficit conditions. To test this, 1-week-old *35S-SDIR1* and *sdir1-1* mutant plants, as well as wild-type control plants, were transplanted to growth on soil for an additional 2 weeks. Thereafter, plants were not watered for 18 d to induce drought stress. The plants were then rehydrated and photographed after 1 d (Figure 6A). Before rewatering, most of the wild-type plants and all *sdir1-1* mutant plants were withered, but the *35S-SDIR1* plants exhibited continued survival and growth. After rewatering, the *35S-SDIR1* plants exhibited a high survival rate (95%), whereas the corresponding survival rate was 50% for wild-type plants, and none of the *sdir1-1* mutant plants survived (Figure 6A). Overexpression of *SDIR1* enhanced drought tolerance, while *SDIR1* gene knockout reduced drought tolerance. Thus, these results suggest that *SDIR1* plays an important role in plant drought response.

The altered drought tolerance of *SDIR1* overexpression and *sdir1-1* plants could be attributed, at least in part, to changes in transpiration rate. Stomata control gas exchange and water evaporation. Leaves from *35S-SDIR1*, wild-type, and *sdir1-1* plants grown in soil were examined to determine stomatal aperture status. Stomatal apertures were smaller on *35S-SDIR1* leaves compared with wild-type leaves, while stomata on *sdir1-1* leaves were almost fully open (Figures 6B and 6C). As shown in Figure 6B, 67% of wild-type stomata and 89% of *sdir1-1* stomata but only 13% of *35S-SDIR1* stomata were open (width:length ratio of stomatal pore > 0.2 is considered open). Consistent with these results, the fresh weight loss of detached rosette leaves in *35S-SDIR1* transgenic plants was <25%, as opposed to 42 and 65% for wild-type and *sdir1-1* plants, respectively (Figure 6D). This suggests that during dehydration, the stomata of *35S-SDIR1* plants can respond to water deficit better than wild-type or *sdir1-1* plants.

Stomatal closure is a key ABA-controlled process that determines the rate of transpiration under water deficit conditions (Leung and Giraudat, 1998). To investigate whether SDIR1 is involved in ABA-related stomatal closure, we treated leaves of three genotypes with ABA to analyze stomatal aperture. Indeed, treating the leaf epidermis of *35S-SDIR1* plants with ABA caused complete closure of stomata; the effect was not so pronounced in wild-type and *sdir1-1* plants (Figure 6E). The *sdir1-1* mutant guard cells clearly have an impaired response to ABA, whereas the guard cells of *35S-SDIR1* plants show a remarkable increase in response to ABA. Thus, SDIR1 may play a crucial role in ABA-mediated guard cell control.

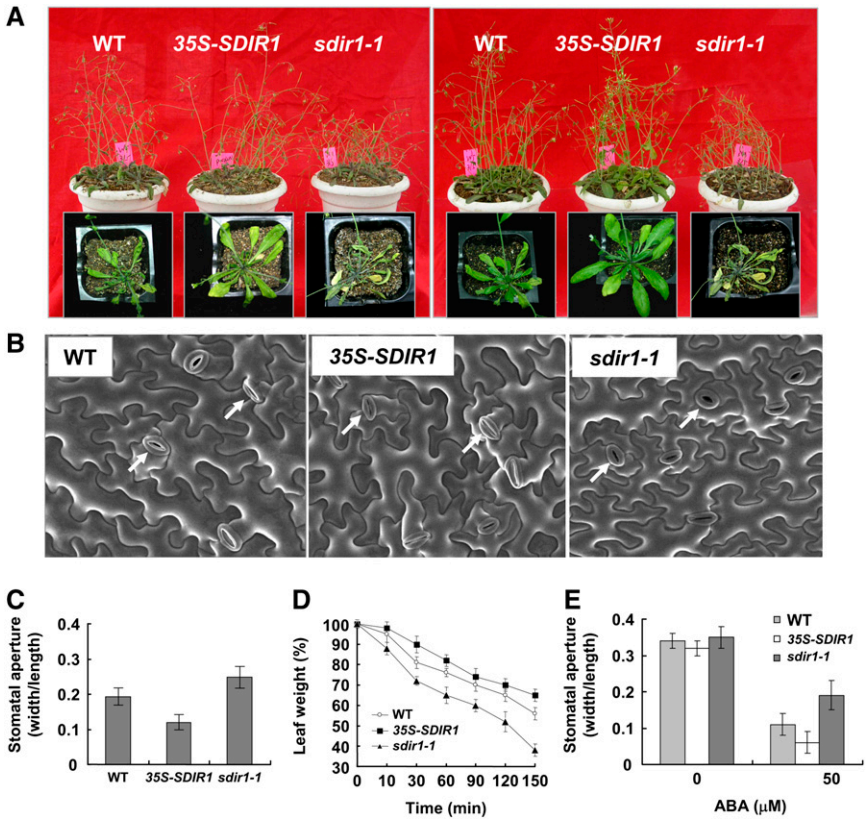

Figure 6. Responses to Drought of *35S-SDIR1*, Wild-Type, and *sdir1-1* Plants.

(A) Drought tolerance assay of 3-week-old plants. Plants were grown in soil in the same container, withheld from water for 18 d (left), and then rewatered (right). The photographs were taken 1 d after rewatering. Representative plants from each treatment group are enlarged for better visualization.
(B) Stomatal apertures of wild-type plants (left), *35S-SDIR1* transgenic plants (center), and *sdir1-1* plants (right). Stomatal guard cells were observed in the middle of the watering period by liquid nitrogen–coupled scanning electron microscopy.
(C) Measurement of stomatal aperture on transgenic and mutant seedlings corresponding to **(B)**. Data are mean ratios of width to length ± SE of three independent experiments ($n = 30$ to 40).
(D) Transpiration rates. Leaves of the same developmental stages were excised and weighed at various time points after detachment. Each data point represents the mean of duplicate measurements. Error bars represent SE ($n = 8$ each).
(E) Effects of ABA on stomatal aperture in wild-type, *35S-SDIR1*, and *sdir1-1* plants. Epidermal peels from plants were kept for 12 h in the dark, incubated under light in buffer for 3 h, and then treated with 0 and 50 μM ABA for 4 h before aperture measurements. Data are mean ratios of width to length ± SE of three independent experiments ($n = 30$ to 40).

ABA Response of *35S-SDIR1* and *sdir1* Plants

ABA plays an important role in regulating plant responses to different stresses (Finkelstein et al., 2002). Salt and drought responses in plants are triggered (at least in part) by increased levels of the phytohormone ABA, which leads to the activation of a series of ABA-dependent responses (Zhu, 2002). Nearly all ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutants exhibit salt insensitivity during germination and are susceptible to drought because of impaired stomatal aperture regulation (e.g., *abi1* and *abi2*), while plants overexpressing the ABA response pathway gene, such as *ABRE BINDING FACTOR3* (*ABF3*) and *ABF4*, are tolerant to drought and hypersensitive to salt (Leon-Kloosterziel et al., 1996; Murata et al., 2001; Kang et al., 2002). Inhibitory experiments of seed germination have provided useful insights into components of ABA signaling (Giraudat, 1995). To determine whether *SDIR1* overexpression and *sdir1-1* affect ABA response, *35S-SDIR1* transgenic and *sdir1-1* plants were germinated and grown on MS medium with ABA to determine sensitivity. In the presence of 1 μM ABA, cotyledon greening/expansion and root growth were severely inhibited in *35S-SDIR1* transgenic plants (28% of their cotyledons expanded and turned green). By contrast, 88% of the *sdir1-1* mutant plants showed expanded and green cotyledons. Wild-type plants showed an intermediate phenotype, with 62% of the plants having expanded and green cotyledons (Figure 7A). To test whether the effect was dosage-dependent, various concentrations of ABA were added to the medium. The ABA-hypersensitive response of *35S-SDIR1* occurred at concentrations as low as 0.5 μM ABA (Figures 7B to 7D). When the ABA concentration was >2 μM, the growth of *35S-SDIR1* plants was arrested completely after germination and none of the transgenic seedlings developed

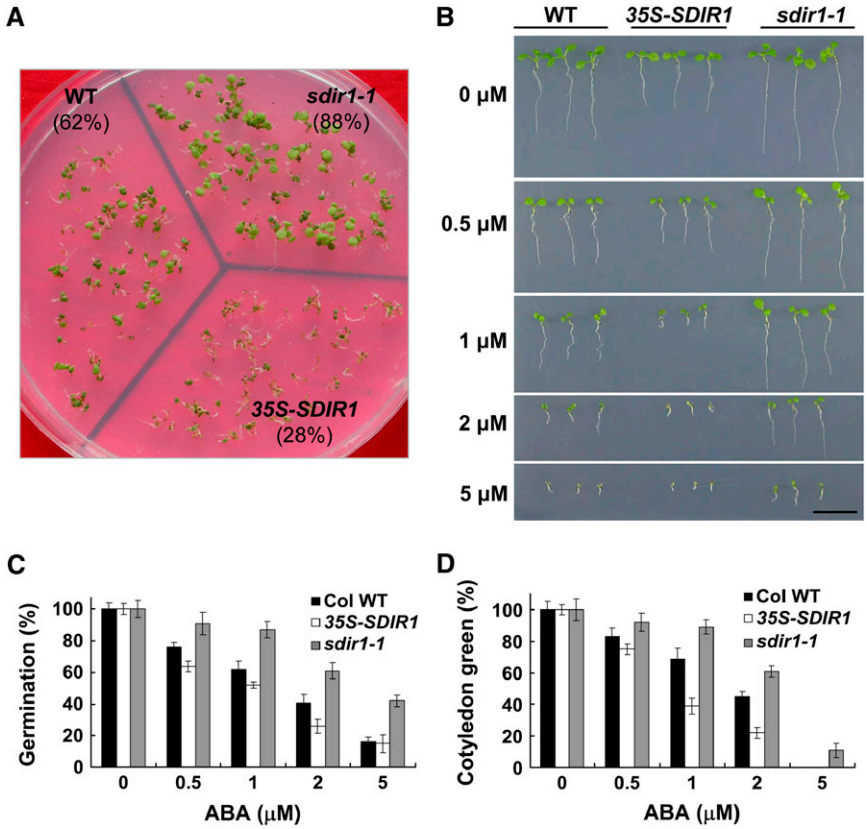

Figure 7. ABA Sensitivity of *35S-SDIR1* and *sdir1-1* Plants.

(A) Growth of different genotypes of plants on MS medium containing 1 μM ABA. Seeds were germinated and grown for 8 d. The percentages shown indicate seedlings with cotyledon expansion in total germinated seeds.

(B) Growth of transgenic and mutant plants on MS medium containing a range of concentrations (0, 0.5, 1, 2, and 5 μM) of ABA. Seeds were germinated for 8 d on MS medium with or without ABA, and representative plants are shown. Bar = 1 cm.

(C) ABA dose-response analysis of germination. Seeds were germinated for 4 d on plates containing different amounts of ABA. The germination percentage without ABA was considered to be 100%, and the germination frequency in ABA for these lines was normalized based on this value. Error bars represent SE (triplicate measurements; $n = 60$).

(D) ABA dose-response analysis of postgerminative growth (cotyledon greening/expansion). Results were scored at 10 d after plating (triplicate measurements; $n = 60$). The data analysis is the same as in **(C)**.

true leaves at 8 d (Figure 7B). At all concentrations tested, *35S-SDIR1* transgenic plants were hypersensitive to ABA at both germination and postgerminative growth stages (Figures 7C and 7D). The *sdir1-1* mutant plants were less sensitive to ABA than the wild-type plants, and the insensitivity could be reversed by SDIR1 overexpression (see Supplemental Figure 2C online). The sensitivity occurred in a dosage-dependent manner (Figures 7B to 7D). No obvious differences were observed when all three groups of plants were germinated on MS plates without ABA in early growth stages (Figures 7B to 7D). It can be concluded that *35S-SDIR1* plants are hypersensitive, while *sdir1-1* plants possess reduced sensitivity to ABA. These results indicate that *SDIR1* is involved in the ABA response.

Expression of Stress-Responsive Genes in *35S-SDIR1* and *sdir1* Plants

Downstream genes directly or indirectly regulated by SDIR1 may be responsible for ABA sensitivity in *35S-SDIR1* and *sdir1-1* plants. To investigate the roles of SDIR1 in transcriptional regulation in plants, *35S-SDIR1* and *sdir1-1* plants were treated or mock treated with ABA to determine the expression of several genes in different groups responsive to ABA signaling. For group I, the *PLDα1* gene encodes a phospholipase Dα, and PLDα1 and its product, phosphatidic acid, regulate either ABI1 or GPA1 to control stomatal closure and opening in the ABA signaling pathway (Zhang et al., 2004; Mishra et al., 2006). When plants were treated with ABA, the induction pattern showed no obvious changes for the *PLDα1* gene or the *GPA1* gene among the three types of plants, while the expression of the *ABI1* gene, a negative regulator of the ABA response pathway in *Arabidopsis*, did not show any clear change in *SDIR1* overexpression plants but was reduced threefold in the *sdir1-1* mutant compared with the wild type after a 10-h treatment. Two other *PP2C* family members that encode phosphatase, At*PP2C* and *ABI2*, have been detected, but no obvious differences were found in either overexpression or mutant plants (Sheen, 1998) (group I in Figure 8). We also tested

the nuclear genes, including the ABRE binding transcription factors *ABI5*, *ABF3*, and *ABF4* (group II in Figure 8). After the 10-h treatment with ABA, *ABI5* and *ABF4* levels increased only slightly, 1.5- and 1.3-fold, respectively, in *35S-SDIR1* plants, while almost no change was observed in *ABF3* expression. However, *ABI5* and *ABF4* clearly decreased by ~3- and 10-fold, respectively, in the *sdir1-1* plants. In contrast with *ABI5* and *ABF4*, *ABF3* levels in *sdir1-1* decreased only slightly, 1.3-fold, after 10 h of treatment with ABA. The most notable reduction occurred in *ABF4* expression (Figure 8). This suggests that SDIR1 plays a role, directly or indirectly, in controlling the transcription of ABA signaling pathway components.

For group III (Figure 8), the *RD29A* gene is a drought-, cold-, and ABA-inducible gene that contains dehydration-responsive elements (DREs) and ABA-responsive elements in its promoter region (Shinozaki and Yamaguchi-Shinozaki, 1997). However, we could not find any significant differences in *RD29A* gene expression between *35S-SDIR1* and wild-type plants. Only a slight reduction, 0.7-fold compared with the wild type, was observed in *sdir1-1* plants after a long (10-h) treatment. Similar expression patterns were also detected in the other dehydration-responsive gene, *RD22* (decreased by 0.7-fold at 4 h and by 0.6-fold at 10 h compared with the wild type), which encodes a protein similar to the nonstorage seed protein, USP, of *Vicia faba*. Another ABA-responsive gene, *RAB18*, encodes a dehydrin, a member of a large family of proteins induced in response to low temperature and drought in both vegetative tissues and seeds, and was also slightly reduced in *sidr1-1* (decreased by 0.7-fold at both 4 and 10 h compared with the wild type) (Lang and Palva, 1992). Expression of Δ1-pyrroline-5-carboxylate synthase (P5CS1), a rate-limiting enzyme in the biosynthesis of Pro, whose mRNA is induced by drought, salinity, and ABA, is reduced by ~50% in *abi1-1* compared with the wild type (Strizhov et al., 1997). Likewise, in our study, the *sdir1-1* plants had reduced levels of *P5CS1* transcript, especially at later treatment stages (decreased by 0.7-fold at 4 h and by 0.6-fold at 10 h compared with the wild type). Different expression patterns were detected for *ADH1*, which was downregulated in *35S-SDIR1* overexpression plants (decreased by 0.8-fold at both 4 and 10 h compared with the wild type). *ADH1* encodes an alcohol dehydrogenase, and its expression is induced by ABA (de Bruxelles et al., 1996). This result is consistent with the reduction of *ADH1* expression in plants overexpressing two bZIP transcription factors, ABF3 and ABF4 (Kang et al., 2002). Our results indicate that *SDIR1* acts upstream of bZIP family transcription factors that mediate ABA signaling.

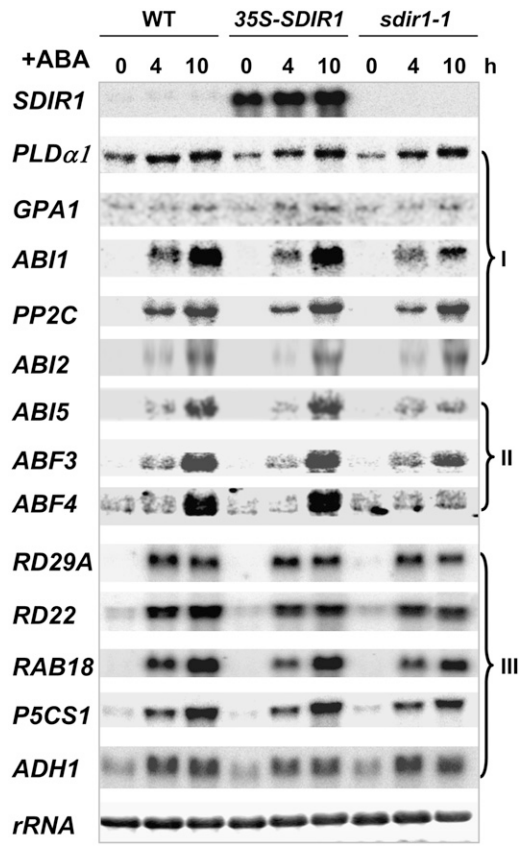

Figure 8. Expression of ABA and Stress-Responsive Genes in Wild-Type, *35S-SDIR1*, and *sdir1-1* Plants Induced by 100 μM ABA at Different Times.

Total RNA was extracted from whole seedlings after different treatment times, as indicated. Numbers at top indicate the treatment period (hours). Total RNA (10 μg) was loaded in each lane and analyzed by RNA gel blots hybridized to gene-specific probes. The top panels show hybridization with the *SDIR1* probe. The bottom panels show methylene blue–stained total RNA gels as a loading control. Group I genes were known as upstream genes in the ABA signaling pathway. Group II genes were ABA-responsive bZIP transcription factors. Group III showed the other stress- or ABA-responsive genes.

ABI5, *ABF3*, and *ABF4* Can Rescue the ABA Insensitivity of the *sdir1* Mutant

Transgenic plants overexpressing *SDIR1* are hypersensitive to ABA, while *sdir1-1* plants are ABA-insensitive. These phenotypes are similar to *ABI5* overexpression and *abi5-1* mutant plants (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001). Similar phenotypes were also observed in overexpression and mutant plants for two other ABRE binding factors, ABF3 and ABF4 (Kang et al., 2002; Kim et al., 2004). To test whether SDIR1 acts in the same or a different pathway to ABI5, ABF3, and ABF4, we generated *sdir1-1* transgenic lines expressing *ABI5*, *ABF3*, and *ABF4* under the control of a *35S* promoter. The expressed transgenes were confirmed by RNA gel blot analysis (see Supplemental Figure 4A online).

The *sdir1-1* mutant plants are insensitive to ABA at both germination and the postgerminative growth stage when placed on ABA-infused plates. Overexpression of the *ABI5* gene in the *sdir1-1* mutant background resulted in different levels of rescue with the ABA-insensitive phenotype. Germination and postgerminative growth varied across different lines. Some were similar to the wild type, having incomplete expansion of green cotyledons and short main roots, indicative of ABA-sensitive recovery. Others showed arrested growth at the seedling stage, suggesting more sensitivity to ABA than wild-type plants (Figure 9A; see Supplemental Figures 4B and 4C online). This may be due to

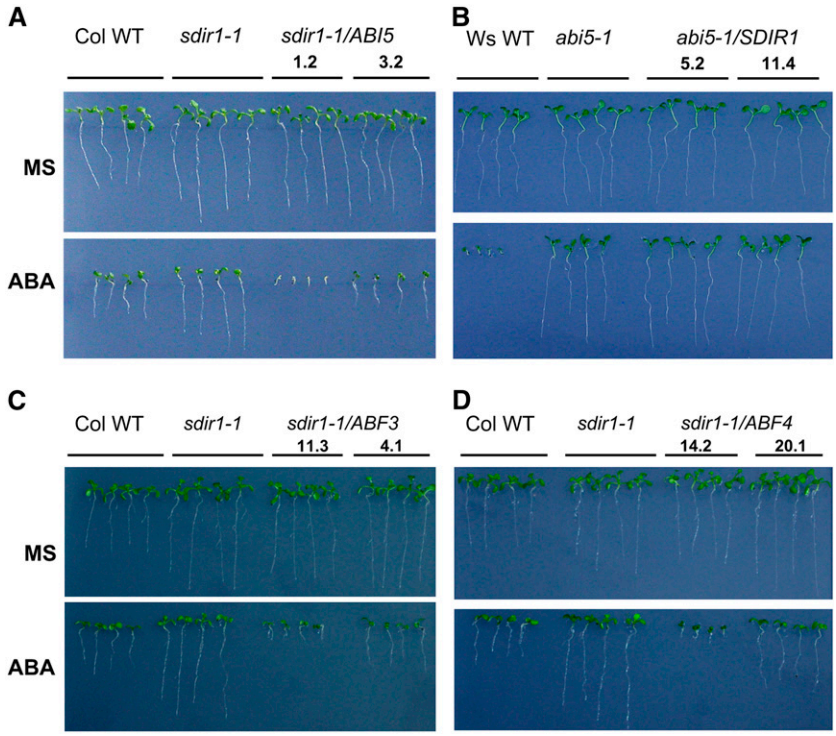

Figure 9. Complementation Experiment.

Numbers indicate different T3 homozygous transgenic lines. Photographs were taken after 10 d of germination. MS, control plate; ABA, MS plate supplied with 1 μM ABA.

(A) Complementation of *ABI5* in *sdir1-1* mutant.

(B) Complementation of *SDIR1* in *abi5-1* mutant.

(C) Complementation of *ABF3* in *sdir1-1* mutant.

(D) Complementation of *ABF4* in *sdir1-1* mutant.

overexpression of the gene under the control of the 35S promoter. The different expression levels of transgenes may contribute to the variation in response to ABA. Significant differences in *ABI5* transcript levels in *sdir1-1/ABI5* lines 1.2 and 3.2 were detected. Line 1.2 had greater sensitivity to ABA as well as higher *ABI5* transcript levels compared with line 3.2 (see Supplemental Figure 4A online). Similar variations were observed in different *sdir1-1/ABF3* and *sdir1-1/ABF4* transgenic lines (Figures 9C and 9D; see Supplemental Figure 4A online), but after analyzing >10 lines of each construct, no severe arrest transgenic lines were observed that were similar to *sdir1-1/ABI5* line 1.2. It seems that *ABI5* overexpression in *sdir1-1* confers more ABA sensitivity at the postgerminative growth stage than at the germination stage. For *ABI5* overexpression in *sdir1-1* lines, at 2 μM ABA, the germination ratio was not obviously decreased compared with *sdir1-1*, while postgerminative growth, such as cotyledon greening/expansion and the development of true leaves, was inhibited more severely (Figure 9A; see Supplemental Figures 4B and 4C online). Nevertheless, *ABF3* and *ABF4* overexpression in *sdir1-1* is involved in both germination and postgermination stages, since the obvious inhibition of germination and postgerminative growth can be detected on both 1 and 2 μM ABA (Figure 9A; see Supplemental Figures 4B and 4C online). This is consistent with previous reports that those bZIP transcription factors have both redundant and distinct functions in ABA response (Finkelstein et al., 2005). However, under normal growth conditions, neither *ABI5* and *ABF3* nor *ABF4* can totally restore the root phenotype of the *sdir1-1* mutant (Figures 9A, 9C, and 9D), which indicates that SDIR1 may affect root length through pathways other than those controlled by bZIP genes. Therefore, we surmise that *ABI5*, *ABF3*, and *ABF4* can rescue the ABA insensitivity of *sdir1-1* in different aspects.

SDIR1 Cannot Rescue the ABA Insensitivity of *abi5-1*

Overexpression of *ABI5*, *ABF3*, and *ABF4* can rescue the ABA insensitivity of *sdir1-1*, which indicates that *SDIR1* may act upstream of these bZIP transcription factors. We conducted further studies to exclude the possibility that SDIR1 functions in parallel with *ABI5*, *ABF3*, and *ABF4*. To do this, we transformed *abi5-1* mutant plants with a *35S-SDIR1* construct and generated *abi5-1* transgenic lines carrying a *35S-SDIR1* transgene and confirmed them by RNA gel blot analysis (see Supplemental Figure 4D online). All *abi5-1* transgenic lines showed the same phenotype as *abi5-1* mutants on MS plates and remained insensitive to 1 μM ABA, overcoming the ABA inhibition phenotype

(Figure 9B). The effect was similar with 2 and 3 μM ABA treatment (data not shown). This demonstrated that *ABI5* acted downstream, rather than in parallel with *SDIR1*. We also created *SDIR1* overexpression transgenic plants for *abf3* or *abf4* mutant backgrounds. Since the ABA phenotypes of both mutants are not obvious under our experimental conditions, it is difficult to analyze the cross-complementation phenotype, and the data are not presented.

DISCUSSION

Ubiquitination has been shown to play an important role in the perception and signal transduction of various internal (hormone) and external environmental signals (Hellmann and Estelle, 2002; Hare et al., 2003). Our data suggest that SDIR1 is involved in stress-responsive ABA signaling and is a positive regulator of ABA signal transduction. Its overexpression conferred several ABA-associated phenotypes, such as ABA hypersensitivity, salt hypersensitivity, and enhanced drought tolerance. Its mutant altered the expression of ABA/stress-regulated genes and displayed reduced sensitivity to ABA and salt and was susceptible to water deficit stress.

SDIR1 Is a Salt- and Drought-Induced Gene and Encodes a RING Finger Protein with Functional E3 Ligase Activity

Substantial work has been done to analyze the functions of key stress response genes, including receptors, kinases, transcription factors, and other signal molecules. Most studies have focused on the expression and regulation of gene function, but protein turnover has attracted attention recently due to the discovery of a number of key mutants, such as *hos1* (Dong et al., 2006). Several studies of protein interaction and functional genomics accelerated understanding of the role of protein turnover in stress signaling. It has been suggested that ubiquitination also plays an important role in plant defense against abiotic stresses. Recent work on At *CHIP1*, *HOS1*, *AFP*, and *AIP2*, as well as *KEG* genes, supports this claim (Lee et al., 2001; Lopez-Molina et al., 2003; Yan et al., 2003; Dong et al., 2006; Luo et al., 2006; Stone et al., 2006). These results suggest a linkage between protein ubiquitination and stress responses in plants. The existence of a large number of RING finger proteins may reflect the need to selectively target many different substrate proteins for degradation (Callis and Ling, 2005). To identify whether there are other RING finger type E3 ligases involved in salt and drought stress responses, we analyzed publicly available microarray studies and identified several different RING finger type E3 ligases that respond to drought and/or salt stress. Detailed functional analysis showed that SDIR1 is involved in drought and salt stress signaling pathways in an ABA-dependent manner. In silico analysis of the promoter with the PLACE promoter analysis program identified at least three MYC recognition sites (CANNTG) at −37 to 42, −268 to 273, and −439 to 444 positions from ATG and one MYB binding site (TAACTG) at the −448 to 453 position from ATG in the promoter region of *SDIR1* (Prestridge, 1991; Higo et al., 1999). These boxes are also present in the promoter region of several dehydration-responsive genes, including At *MYB2* and At *MYC2*, which are known transcriptional activators in ABA signaling (Abe et al., 2003). Promoter expression analysis of *SDIR1* provided further support for its role in stress tolerance (Figure 1C); GUS gene expression was enhanced by drought and NaCl, and it was observed throughout the entire leaf after drought treatment, including in the guard cells (Figure 1C).

Although SDIR1 contains all of the canonical amino acids (Figure 2A) present in other RING finger proteins, we needed to establish whether SDIR1 is a ubiquitin E3 ligase. To confirm this, we overexpressed and purified MBP-tagged SDIR1 from *E. coli* and performed in vitro ubiquitination assays. SDIR1 was able to conjugate ubiquitin moieties to *E. coli* expressed fusion proteins, clearly indicating that it is a ubiquitin E3 ligase (Figure 3). Furthermore, mutating the conserved His-234 to Tyr (H234Y) within the RING domain completely abolished ubiquitin E3 ligase activity (Figure 3). This effect was seen in a number of other RING domain E3 ligases (Dong et al., 2006), illustrating that SDIR1 functions as an E3 ubiquitin ligase.

SDIR1 Overexpression Affects Drought Tolerance

ABA is an essential mediator in triggering plant responses to most of the common abiotic stresses, including drought, salinity, high temperature, oxidative stress, and cold (Finkelstein et al., 2002; Xiong et al., 2002). Nevertheless, high levels of ABA inhibit plant growth by affecting cell division and elongation (Finkelstein et al., 2002). The smaller aerial part of *SDIR1*-overexpressing plants in vitro may be due to the constitutive activation of ABA signal transduction cascades, which enhances the ABA effect on the inhibition of plant growth. Stomatal closure is a key ABA-controlled process in dealing with water deficit conditions. ABA-insensitive mutants (i.e., *abi1* and *abi2*) are very susceptible to water deficit because of impaired stomatal aperture regulation (Schroeder et al., 2001). Our results indicated that SDIR1 was involved in stomatal regulation. Its overexpression resulted in lower transpiration and enhanced drought tolerance (Figure 6). The stomatal openings of *35S-SDIR1* transgenic plants were smaller than those of wild-type plants under normal light and growth in soil (Figures 6B and 6C). Furthermore, stomatal closure in the epidermis of *35S-SDIR1* plants was more sensitive than in wild-type and *sdir1-1* plants after ABA treatment (Figure 6E). The highest promoter activity was observed in roots and guard cells, which is consistent with their roles during stress response (Figure 1C). Thus, our results suggest that SDIR1 may play an active role in ABA-mediated guard cell control and the regulation of drought response.

SDIR1 Acts Upstream and Affects Multiple ABA-Responsive Genes

To test whether SDIR1 affects the expression of ABA-related or other stress pathway genes, several groups of marker genes in the stress-responsive pathway were analyzed in *SDIR1* overexpression and *sdir1-1* mutant plants. It was recently reported that several genes interact in the ABA signaling pathway to control stomatal closure. *PLDα1* encodes a phospholipase Dα, and PLDα1 and its product (phosphatidic acid) regulate either a PP2C family member (ABI1) or a G-protein (GPA1) to control

stomatal movement (Zhang et al., 2004; Mishra et al., 2006). No changes have been detected for the *PLDα1* gene, which may indicate that it is upstream of SDIR1 or that SDIR1 is involved in a different ABA regulatory pathway than PLDα1. Similarly, no changes were detected for the *GPA1* gene, while expression of the *ABI1* gene, a negative regulator of the ABA response pathway in *Arabidopsis*, showed no clear changes in *SDIR1* overexpression plants but was reduced significantly in *sdir1-1* mutants. This demonstrated that SDIR1 might interact with ABI1 to control stomatal movement rather than to form a PLDα1 and GPA1 complex to affect ABA signaling. Two other PP2C gene family members encoding phosphatases, At *PP2C* and *ABI2* (Sheen, 1998), showed no clear changes in either *SDIR1* overexpression or mutant plants. This may be due to a specific interaction between SDIR1 and ABI1. ABI1 is known as a negative regulator in the ABA signaling pathway. It could provide feedback regulation, such as in the *sdir1-1* mutant, in which the ABA signal was reduced, and may lead to the downregulation of *ABI1* gene expression. This is consistent with the increased *ABI1* gene expression level in plants overexpressing two *SDIR1* downstream genes, *ABF3* and *ABF4* (Kang et al., 2002). The relation between *SDIR1* and *ABI1* is based on the mRNA expression level. Whether the protein level of ABI1 is also altered needs to be analyzed further.

Differing effects on bZIP transcription factor expression were also observed in *sdir1-1* mutants, especially after extended ABA treatment. Dramatic decreases in expression level were observed for both *ABI5* and *ABF4* genes, while moderate reductions were seen in *ABF3* gene expression. In contrast with the dramatic or clear reduction of all three bZIP family members in the *sdir1-1* mutant, only slightly increased expression levels were detected in *SDIR1* overexpression plants. This could be due to plant adaptation to the constitutive expression of *SDIR1* under the control of a 35S promoter. Several previous works demonstrated redundant and distinct functions of different bZIP family transcription factors. The constitutive overexpression of *ABF3* or *ABF4* in *Arabidopsis* resulted in ABA hypersensitivity and other common ABA-associated phenotypes. In addition, the transgenic plants exhibited reduced transpiration and enhanced drought tolerance. On the other hand, ABF2 overexpression promoted glucose-induced inhibition of seedling development, whereas its mutation impaired the glucose response. These results indicate distinct roles of ABF family members. Whereas ABF3 and ABF4 play essential roles in ABA/stress responses, ABF2 is required for normal glucose response (Kim et al., 2004). Furthermore, the temporal and spatial expression patterns of ABF3 and ABF4 were consistent with their suggested roles in mediating stress-responsive ABA signaling. ABI5 was identified genetically, and the most similar effects and interaction members ABF3 and ABF1 were analyzed by genetic interaction (Finkelstein et al., 2005). According to our data and published results, SDIR1 may regulate these transcription factors in different ways.

The *RD29A* gene is a drought-, cold-, and ABA-inducible gene with DRE and ABRE present in its promoter region (Shinozaki and Yamaguchi-Shinozaki, 1997). GUS analysis indicated that ABRE and DRE are interdependent in the ABA-responsive expression of *RD29A* in *Arabidopsis* (Narusaka et al., 2003). The effect of *RD29A* expression in *sdir1-1* mutants could be due to the reduction of the upstream bZIP family transcription factors. However, we do not know which of the bZIP family factors affected the expression of *RD29A*. *RD22* encodes a protein similar to a nonstorage seed protein, USP of *V. faba*. Its expression is induced by ABA, but no conserved consensus ABRE was identified from its promoter region. Instead, it contains the recognition sequences for some of the transcription factors, such as MYC, MYB, and GT-1. In *sdir1-1*, missing SDIR1 causes reduced ABA signal transduction, which might result in reduced expression of MYC, MYB, and GT-1 and finally also affect the expression of *RD22*. The expression pattern of *RAB18* is dramatically reduced in both *abi1-1* and *abi2-1* mutants and in *sdir1-1* shows reduction patterns similar to those in *RD22*. Several MYC and MYB binding elements were also found in the promoter region of the *RAB18* promoter (analyzed using the PLACE program; http://www.dna.affrc.go.jp/PLACE/signalscan.html). The ABRE element and other consensus sequences were also found in the nucleotide sequence of the *P5CS1* promoter region, including the CCAAT motif and recognition sites for MYC and MYB binding sites. This indicates that the reduction of *P5CS1* expression in the *sdir1-1* mutant may be controlled by ABRE and other indirect ABA-responsive factors. The *ADH1* gene encodes an alcohol dehydrogenase, and its expression is induced by ABA (de Bruxelles et al., 1996). *ADH1* transcription is less induced by ABA in *SDIR1* overexpression plants, which is consistent with the reduction of *ADH1* expression in plants when overexpressing the two bZIP family transcription factors ABF3 and ABF4 (Kang et al.,

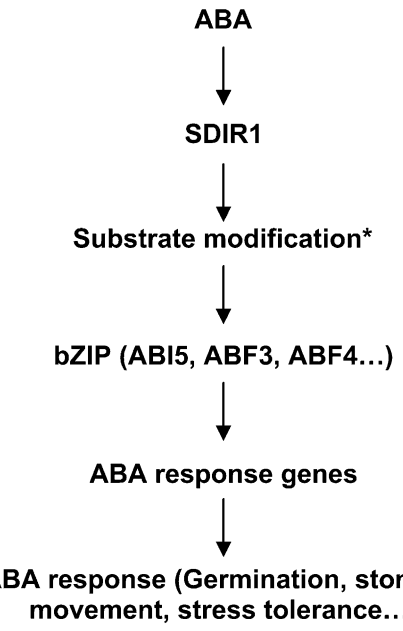

Figure 10. Proposed Model for the Role of SDIR1 in the ABA Signaling Pathway.

Substrate modification could be either degradation or monoubiquitination.

2002). It is suggested that the *SDIR1* gene may be located upstream of the bZIP family transcription factors in the ABA signaling pathway (Figure 10).

SDIR1 Is a Positive Component of ABA Signal Transduction

Not all stress-inducible genes are regulated by ABA. However, a large number of them are responsive to exogenous ABA (Kang et al., 2002). The hypersensitivity conferred by SDIR1 overexpression was very pronounced, compared with wild-type and *sdir1-1* plants, and was observed at both germination and later growth stages (Figure 7). Salt hypersensitivity was also observed in *35S-SDIR1* transgenic plants during germination (Figure 5); however, *35S-SDIR1* plants responded normally to mannitol, indicating that osmotic sensitivity was not affected. Thus, it appears that SDIR1 is involved in ionic rather than osmotic effects in the salt signaling pathway. Also, it has been reported that overexpression of two bZIP family transcription factors, *ABF3* and *ABF4*, resulted in ABA and salt hypersensitivity during seed germination and drought tolerance in vegetative growth (Kang et al., 2002). Overexpression of the other bZIP transcription factors also confers hypersensitivity to ABA and sugar (Brocard et al., 2002). Thus, our results suggest that SDIR1 may have overlapping functions with ABF3, ABF4, and ABI5 in ABA-mediated signaling. At the same time, the ABA hypersensitivity of *35S-SDIR1* plants (Figure 7) and the ABA insensitivity of *sdir1-1* mutant seedlings (Figure 7; see Supplemental Figure 2 online) demonstrated that SDIR1 may be a positive component of ABA signal transduction. Since complementation of *ABI5*, *ABF3*, and *ABF4* genes can rescue the ABA-insensitive phenotype of the *sdir1-1* mutant, whereas SDIR1 cannot rescue the ABA-insensitive phenotype of the *abi5-1* mutant, we propose that SDIR1 and bZIP family transcription factors function in the same pathway in ABA response signaling and that SDIR1 acts upstream of the bZIP family transcription factors tested. ABI5 is the positive regulator in the ABA signaling pathway (Lopez-Molina et al., 2002, 2003). Since SDIR1 can promote ABA signaling and then activate ABI5, we conclude that SDIR1 is a positive regulator in the ABA signaling pathway (Figure 10).

Action Model of SDIR1 in ABA Signaling

Based on our results, we hypothesize that SDIR1 functions as an E3 ligase that mediates the degradation of substrates through the ubiquitin-proteasome machinery (Xie et al., 2002; Zhang et al., 2005). The interaction is likely to lead to the ubiquitination of the target protein and subsequent proteasomal degradation. Our results suggest that SDIR1 represents a new class of positive regulators of the ABA signaling pathway; thus, we propose that the degraded protein is a negative regulator of ABA signaling and that removing this molecule has the effect of activating ABA signaling. We tested AFP protein levels, one of the negative factors in the ABA signal pathway (Lopez-Molina et al., 2003). AFP promotes ABI5 degradation to negatively regulate the ABA signal. No significant difference in AFP protein levels was observed in the seedling samples of wild-type, *SDIR1* overexpression, and *sdir1-1* plants (data not shown). Conversely, the predicted SDIR1 protein contains transmembrane domains, and protein localization and fractionation experiments also show that SDIR1 is associated with intracellular membranes, but not in the nucleus, where AFP occurs. Therefore, AFP could not be the substrate of SDIR1. Alternatively, SDIR1 could activate a positive regulator by monoubiquitination and stabilize some key regulators in the ABA signaling pathway, in the way that At CHIP ubiquitinates PP2A in ABA signaling (Figure 10). Thus, further functional dissection of SDIR1, its target proteins, and their interplay in ABA signaling is necessary for complete understanding of ABA signaling networks in plants.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* ecotypes Columbia and Wassilewskija were used for this study. Seeds were surface-sterilized with 10% bleach and 0.01% Triton X-100 and washed three times with sterile water. Sterile seeds were suspended in 0.15% agarose and plated on MS medium plus 1.5% sucrose. Plates were stratified in darkness for 2 to 4 d at 4°C and then transferred to a tissue culture room at 22°C under a 16-h-light/8-h-dark photoperiod. After 2 to 3 weeks, seedlings were potted in soil and placed in a growth chamber at 22°C and 70% RH under a 16-h-light/8-h-dark photoperiod. The MS medium was supplemented with 1.5% sucrose and, unless described in Results, with ABA, NaCl, and mannitol as needed. To test germination, seeds were collected at the same time they were used. For root growth measurements, plants were germinated and grown on vertical plates.

Transformation Vectors and Construction of Transgenic Plants

Transgenic plants carrying constitutively expressing transgenes were generated. To produce *35S-SDIR1* plants, an 838-bp *Eco*RI-*Spe*I fragment containing the *SDIR1* (The Arabidopsis Information Resource locus At3g55530) cDNA was cloned into the vector pBA002, in which transgene expression is under the control of the CaMV *35S* promoter. For the *SDIR1* promoter and GUS fusion construct, a 5′ flanking sequence (a 1.3-kb promoter region just upstream of the ATG start codon of *SDIR1*) was amplified from genomic DNA by PCR and verified by sequencing. The PCR fragment was cloned into the *Hin*dIII-*Bam*HI site of binary vector pBI101.1 to obtain a transcriptional fusion of the *SDIR1* promoter and the GUS coding sequence. *35S-myc-ABI5* was prepared by inserting the PCR-amplified coding region of *ABI5* fused with MYC in the N-terminus of *ABI5* to the pCAMBIA1300-221 vector under the control of the *35S* promoter. The cDNA clone of ABF was amplified by PCR, and *35S-HA-ABF3* and *ABF4* were generated by inserting the coding region of ABF fused with the hemagglutinin tag to the pCAMBIA1300-221 vector.

Transformation of *Arabidopsis* was performed by the vacuum infiltration method (Bechtold and Pelletier, 1998) using *Agrobacterium tumefaciens* strains EHA105 and GV3101. For the phenotypic analysis, T3 or T4 homozygous lines were used. T3 homozygous lines were used for detailed analysis. T2 seeds were germinated on MS plates containing 6 μg/mL BASTA for pBA002 constructs, 50 μg/mL kanamycin for pBI101.1, and 20 μg/mL hygromycin for pCAMBIA1300-221, and the resistant plants were transferred to soil to obtain homozygous T3 seeds. Two independent lines of homozygous T4 plants containing a single insertion of each construct were used for detailed analysis.

Gene Expression Analysis

Two-week-old seedlings grown on agar plates were treated with NaCl, ABA, and drought. Total RNA was isolated using the Qiagen RNeasy kit,

and 10 μg (except for Figure 1, where the RNA amount is indicated in the figure legend) from each sample was separated on 1.2% (w/v) agarose formaldehyde gels and transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech). To specifically detect the *SDIR1* transcript, blots were probed with a PCR fragment encoding *SDIR1* labeled with [α-^{32}P]dCTP using a Ready-Primed labeling kit (Amersham International). The probe for *SDIR1* consisted of the entire open reading frame of this gene.

Other transcripts were labeled by random priming and were detected by hybridization to exon-specific PCR fragments corresponding to the following segments of the coding sequences: *PLDα1*, nucleotides 1003–1761; *GPA1*, nucleotides 81–733; At *PP2C*, nucleotides 546–1334; *ABI1*, nucleotides 444–1228; *ABI2*, nucleotides 36–575; *P5CS1*, nucleotides 1059–1648; *ADH1*, nucleotides 202–710; and *RD22*, nucleotides 277–757. The *ABI5* probe was prepared by *Sma*I digestion from cDNA excluding the conserved bZIP domain to generate 782 bp of the 5′ coding region. *ABF3* and *ABF4* transcripts were detected by hybridization to cDNA clones as described by Choi et al. (2000). The *RD29A* and *RAB18* probes were prepared as described previously (Gonzalez-Guzman et al., 2002).

The relative expression level of each sample was quantified by Quantity One software (Bio-Rad). Values below each blot in the figures represent the ratio of target gene to rRNA.

RT-PCR Amplification

To examine the expression of *SDIR1* by RT-PCR, DNase I–treated total RNA (5 μg) was denatured and subjected to reverse transcription reaction using SuperScript II (200 units per reaction; Invitrogen) at 42°C for 50 min followed by heat-inactivation of the reverse transcriptase at 70°C for 15 min. PCR amplification was performed using *SDIR1*-specific forward (RT Fw, 5′-ATGAGCTTTGTTTTCCGGGG-3′) and reverse (RT Rev, 5′-TCAAACCATGTCGGAAGCATC-3′) primers and 25 cycles. Expression levels of *Actin1* were monitored with forward (F, 5′-CATCAGGAAGGACTTGTACGG-3′) and reverse (R, 5′-GATGGACCTGACTCGTCATAC-3′) primers to serve as an internal control.

Subcellular Localization

For transient expression in onion (*Allium cepa*) epidermal cells, *GFP-SDIR1* and membrane domain deletion *GFP-SDIR1ΔTM* gene fusions were constructed under the control of the 35S promoter and the nopaline synthase terminator (Kost et al., 1998). After bombardment, epidermal peels were incubated with liquid MS medium for 16 h in the dark, mounted on slides, and visualized using a fluorescence microscope. For detection of nuclei, samples were stained with 1 mg/mL 4′,6-diamidino-2-phenylindole for 10 min. The plasmolysis of the onion epidermal cell was induced by 1 M sucrose treatment for 20 min.

Cell Fractionation Assay

For cell fractionation analysis, SDIR1 and the truncated form were transiently expressed in *Nicotina benthamiana* leaf cells. *A. tumefaciens* cells containing the pBA002-myc vector constructed with PCR-amplified *SDIR1* or *SDIR1ΔTM* fused with MYC at the N terminus under the control of the 35S promoter were injected into *N. benthamiana* leaves as described (English et al., 1997). After incubation for 3 d, the infiltrated parts of leaves were subjected to protein extraction followed by cell fractionation. Total protein extracts were obtained from *N. benthamiana* leaf tissue overexpressing a myc-SDIR1 or myc-SDIR1ΔTM gene fusion by grinding in liquid nitrogen and then suspended in 2 volumes of extraction buffer (50 mM Tris-MES, pH 8.0, 0.5 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, 5 mM DTT, and protease inhibitor cocktail; Roche) on ice as described with minor modifications (Kim et al., 2006). Total extract was centrifuged at 10,000*g* to separate into the soluble fraction (supernatant was recentrifuged at 100,000*g* to discard insoluble material) and the membrane fraction. The membrane fraction was further extracted with a detergent-free buffer and centrifuged at 100,000*g* for the buffer-extracted fraction, and the pellet in this step was suspended with SDS-containing buffer, then centrifuged at 100,000*g* to separate the supernatant as the SDS-extracted fraction and the pellet final membrane fraction. Aliquots of each sample were added with SDS-PAGE loading buffer for protein gel analysis. Buffers and the procedure were as described (Kim et al., 2006).

E3 Ubiquitin Ligase Activity Assay

The entire SDIR1 open reading frame (822 bp) was cloned into the pMAL-c2 vector (New England Biolabs) and expressed in *Escherichia coli*. The fusion proteins were prepared according to the manufacturer's instructions. The *sdir1* mutants that contain mutation in the RING finger domain were prepared using the Quickchange site-directed mutagenesis kit (Stratagene) according to the protocol provided by the manufacturer. The sequences of the primer pair used for the preparation of the His-234 to Tyr-234 mutant are as follows: M1F, 5′-ACCTTGTTTGCATCAGTTTTATGCAGGATGTATCGATCC-3′; M1R, 5′-GGATCGATACATCCTGCATAAAACTGATGCAAACAAGGT-3′. For the E3 ubiquitin ligase activity assay of the fusion proteins, crude extract containing recombinant wheat (*Triticum aestivum*) E1 (GI: 136632), human E2 (UBCh5b; ~40 ng), and purified E3 (~1 μg) fused with the MBP tag, and purified *Arabidopsis* ubiquitin (UBQ14, At4g02890; ~2 μg) fused with the His tag, were used for the assay. The in vitro E3 ligase assays were performed as described (Xie et al., 2002). Proteins after reaction were separated by SDS-PAGE, blotted, probed by HisDetector nickel–nitrilotriacetic acid agarose conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories) for the detection of His-tagged ubiquitin or antibody to MBP (antiserum; New England Biolabs), and visualized using chemiluminescence as instructed by the manufacturer (ECL; Amersham Pharmacia).

Verification of the SDIR1 T-DNA Insertion Mutant

The *sdir1-1* (SALK_052702) and *sdir1-2* (SALK_114361) seeds were obtained from the ABRC (Ohio State University, Columbus). Homozygous mutant was identified by PCR from genomic DNA using forward (P1, the same as the RT Fw primer mentioned above) and T-DNA left border primers (LBb1, 5′-GCGTGGACCGCTTGCTGCAACT-3′) and *SDIR1* gene-specific reverse primer (P2, 5′-CACCTTCCCCTGTTAAGGAAG-3′) and analyzed further by DNA sequencing to confirm the insertion of the T-DNA in the gene.

GUS Bioassays

Seeds and young seedlings at different developmental stages, and different parts from mature transgenic plants, were collected and used for histochemical detection of GUS expression. For general detection, materials were stained at 37°C overnight in 1 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.03% Triton X-100, and 0.1 M sodium phosphate buffer, pH 7.0. To test the induction of GUS expression by salt and drought, 10-d-old transgenic seedlings were transferred from agar plates to MS liquid medium containing NaCl for salt treatment or to a filter exposed in the air with 70% RH for drought treatment. The treated and control transgenic seedlings were stained in 1 μg/mL X-Gluc, 0.03% Triton X-100, and 20 mM HEPES buffer, pH 7.0, for 2 h for histochemical detection.

Drought Treatment and Measurement of Transpiration Rate

For RNA gel blot analysis, 2-week-old seedlings from the agar plate were transferred onto a filter paper in a covered Petri dish and subjected to drought treatment. The treatment was conducted in an environment of 70% RH. For the soil-grown plant drought tolerance test, 1-week-old

seedlings were transplanted to the soil for 2 weeks under standard growth conditions, and then plants were subjected to progressive drought by withholding water for specified times. To minimize experimental variations, the same numbers of plants were grown on the same tray. The entire test was repeated a minimum of three times. To measure the transpiration rate, detached fresh leaves were placed abaxial side up on open Petri dishes and weighed at different time intervals at room temperature. Leaves of similar developmental stages (third to fifth true rosette leaves) from 3-week-old soil-grown plants were used.

Stomatal Aperture Analysis

Ten fresh leaves from 4-week-old soil-grown plants in the middle of the watering period (3 d after watering) and light period plants at similar developmental stages (grown in 8 h of light at 22°C and 16 h of darkness at 20°C, 70% RH) were harvested in the morning, 1.5 h after giving light. Leaves were placed on slides abaxial side up and frozen immediately in liquid nitrogen, and scanning electron microscopy was performed to observe guard cells. The numbers of guard cells in randomly chosen fields were counted. Widths and lengths of stomatal pores were measured for statistical analysis (Lemichez et al., 2001).

Leaves from 4- to 5-week-old plants grown in the same conditions described above were harvested in darkness at the end of the night. Paradermal sections of abaxial epidermis obtained in dim green light were incubated in 10 mM KCl, 7.5 mM potassium iminodiacetate, and 10 mM MES-KOH, pH 6.15, at 20°C. ABA was added to the solution, and stomatal apertures were measured with an optical microscope (Optiphot-2; Nikon) fitted with a camera lucida and a digitizing table (TG1017; Houston Instrument) linked to a personal computer.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for major genes mentioned in this article are as follows: *SDIR1* (At3g55530); *PLDα1* (At3g15730); *GPA1* (At2g26300); *ABI1* (At4g26080); At *PP2C* (At3g11410); *ABI2* (At5g57050); *ABI5* (At2g36270); *ABF3* (At4g34000); *ABF4* (At3g19290); *RD29A* (At5g52310); *RD22* (At5g25610); *RAB18* (At5g66400); *P5CS1* (At2g39800), and *ADH1* (At1g77120).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The GFP-SDIR1 Fusion Protein Is Functional.

Supplemental Figure 2. *35S-SDIR1* Can Complement ABA- and NaCl-Insensitive Phenotypes of *sdir1-1*.

Supplemental Figure 3. KCl and LiCl Sensitivity of *35S-SDIR1* and *sdir1-1* Plants.

Supplemental Figure 4. RNA Gel Blot Analysis of *sdir1-1* Complementation Plants and Quantification of the ABA-Responsive Phenotype.

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