The RING Finger Ubiquitin E3 Ligase SDIR1 Targets SDIR1-INTERACTING PROTEIN1 for Degradation to Modulate the Salt Stress Response and ABA Signaling in Arabidopsis

Huawei Zhang, Feng Cui, Yaorong Wu, Lijuan Lou, Lijing Liu, Miaomiao Tian, Yuese Ning, Kai Shu, Sanyuan Tang, and Qi Xie

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

The plant hormone abscisic acid (ABA) regulates many aspects of plant development and the stress response. The intracellular E3 ligase SDIR1 (SALT- AND DROUGHT-INDUCED REALLY INTERESTING NEW GENE FINGER1) plays a key role in ABA signaling, regulating ABA-related seed germination and the stress response. In this study, we found that SDIR1 is localized on the endoplasmic reticulum membrane in Arabidopsis thaliana. Using cell biology, molecular biology, and biochemistry approaches, we demonstrated that SDIR1 interacts with and ubiquitinates its substrate, SDIRIP1 (SDIR1-INTERACTING PROTEIN1), to modulate SDIRIP1 stability through the 26S proteasome pathway. SDIR1 acts genetically downstream of SDIR1 in ABA and salt stress signaling. In detail, SDIRIP1 selectively regulates the expression of the downstream basic region/leucine zipper motif transcription factor gene ABA-INSENSITIVES, rather than ABA-RESPONSIVE ELEMENTS BINDING FACTOR3 (ABF3) or ABF4, to regulate ABA-mediated seed germination and the plant salt response. Overall, the SDIR1/SDIRIP1 complex plays a vital role in ABA signaling through the ubiquitination pathway.

INTRODUCTION

As sessile organisms, plants differ from animals as they might encounter various environmental challenges, including biotic and abiotic stresses. Abiotic stresses, such as drought and high salinity, severely affect plant growth and development and can impair crop production worldwide. Plants have evolved several strategies to adapt to such challenges. Stress signals are transduced to activate stress response genes and alter metabolic rates and ion channel permeability (Hasegawa et al., 2000; Zhu, 2002). Among these strategies, abscisic acid (ABA) plays a major role by retarding seed germination, cotyledon greening, and plant growth, reducing the transpiration rate, and modulating cellular water potential (Zhu, 2002; Tuteja, 2007).

Since the identification of ABA receptors, considerable progress has been made in understanding ABA perception and the signal transduction pathway (Raghavendra et al., 2010; Umezawa et al., 2010; Guo et al., 2011). Under normal conditions, PP2Cs (PROTEIN PHOSPHATASES TYPE 2C) keep SnRKs (SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASEs) in the inactive form via dephosphorylation (Guo et al., 2011). A high level of ABA builds up under stress conditions (Nambara and Marion-Poll, 2005) and binds to the PYR/PYL/RCARs (PYRABACTIN RESISTANCE 1/PYR-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORs), changing their conformational structures to facilitate their interaction with PP2Cs. This interaction can inhibit the phosphorylation activity of PP2Cs and release SnRKs from PP2C inhibition (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009). In turn, SnRKs phosphorylate downstream ion channels (Geiger et al., 2009; Lee et al., 2009; Sato et al., 2009) and transcription factors (Fujii et al., 2009) to trigger the ABA response.

The ubiquitin/26S proteasome pathway plays a very important role in hormone signaling, including the perception of auxin (Dharmasiri et al., 2005; Kepinski and Leyser, 2005), jasmonates (Sheard et al., 2010), and gibberellins (Murase et al., 2008), and in signal transduction in the ABA and ethylene pathways (Santer and Estelle, 2009).

E3 (ubiquitin ligase), which assists in the transfer of ubiquitin from the E2 (ubiquitin-conjugating enzyme) ubiquitin intermediate to the target protein, is the key factor that defines substrate specificity (Hare et al., 2003; Vierstra, 2003; Moon et al., 2004; Smalle and Vierstra, 2004; Vierstra, 2009). The Arabidopsis thaliana genome encodes over 1400 different E3s, including 470 REALLY INTERESTING NEW GENE (RING)-type E3s (Stone et al., 2005; Vierstra, 2009). Increasing evidence has shown that RING-type E3-mediated protein degradation by the ubiquitin-26S proteasome system plays an essential role in ABA pathways (Lee and Kim, 2011). AIP2 (ABA-INSENSITIVE3 [ABI3] INTERACTION PROTEIN2) serves as a negative regulator of ABA on germination by promoting the B3 domain transcription factor ABI3 for proteolysis (Zhang et al., 2005). KEEP ON GOING is a bifunctional E3 ligase, mediating both ABI5 ubiquitination and autoubiquitination of itself (Liu and Stone, 2009).

In previous studies, we isolated a RING-type E3, SDIR1 (SALT- AND DROUGHT-INDUCED RING FINGER1), which is involved in ABA-related high salinity and drought response (Zhang et al., 2007, 2008). SDIR1 expression is upregulated by high salinity...
and drought. SDIR1 overexpression leads to hypersensitivity to ABA and high salinity and ABA-related hyposensitivity to drought by limiting the rate of transpiration. Biochemical and genetic evidence indicates that SDIR1 acts upstream of the basic region/leucine zipper motif (bZIP)-type transcription factors ABF3 (ABA-RESPONSIVE ELEMENTS BINDING FACTOR3), ABF4, and ABF5, which are key regulators in the ABA pathway (Zhang et al., 2007).

Here, we show that SDIR1 is an endoplasmic reticulum (ER) membrane-localized protein, with its C terminus facing the cytoplasm. We identified a substrate of SDIR1, SDIRIP1 (SDIR1-INTERACTING PROTEIN1). Complementary to a previous report (Naponelli et al., 2008), we found that SDIRIP1 is localized not only in the chloroplast but also in the cell periphery and nucleus. SDIRIP1 interacts with the C terminus of SDIR1 in the cytoplasm. SDIR1 acts upstream of ABI5, ABF3, and ABF4 by promoting their gene expression (Zhang et al., 2007). However, SDIRIP1 negatively affecting SDIRIP1 stability.

**RESULTS**

**Identification of SDIRIP1 as an SDIR1 Interaction Protein**

The RING-type E3 ligase SDIR1 plays important roles in the ABA signaling pathway (Zhang et al., 2007). To identify targets and other interacting proteins of SDIR1, a yeast two-hybrid screen was performed with mutated SDIR1ΔH234Y,ΔTM as the bait, in which two transmembrane domains were deleted and His-234 was changed to Tyr-234 (Figure 1A). The H234Y point mutation, which disrupts the RING domain and ubiquitin ligase activity of SDIR1, prevents SDIR1-mediated degradation of its substrates and facilitates substrate isolation. The transmembrane domain deletion ensures that the GAL4 fusion protein is localized to the nucleus of the yeast. Among 45 positive clones, seven were characterized as containing the same protein (encoded by At5g51110), designated SDIRIP1 (Figure 1B).

To verify the interaction between SDIRIP1 and the full-length SDIR1, an in vitro pull-down assay was performed. [35S]Methionine-labeled SDIRIP1 was generated through in vitro transcription and translation. MBP (MALTOSE BINDING PROTEIN)-SDIR1 expressed in *Escherichia coli* was used as the bait, with MBP as the negative control. MBP-SDIR1 was able to pull down [35S]methionine-labeled SDIR1, but not MBP, suggesting that SDIR1 might interact directly with SDIRIP1 (Figure 1C). We next assessed this interaction in plants by coimmunoprecipitation (Co-IP). As SDIR1 could not be easily detected in transgenic *Arabidopsis* plants, we performed a Co-IP assay in *Nicotiana benthamiana*. 35S:GFP (green fluorescent protein)-SDIR1 and 35S:SDIRIP1-MYC were transiently expressed in *N. benthamiana* leaves by agroinfiltration.

Leaves infiltrated with *Agrobacterium tumefaciens* suspension buffer (10 mM MgCl₂) were used as the negative control (mock samples) (Liu et al., 2010). Total protein was extracted from leaf tissues, and equal amounts of proteins from GFP-SDIR1 samples were mixed with the proteins extracted from the SDIRIP1-MYC samples and mock samples. Co-IP assays were performed, and the anti-MYC antibody was able to coimmunoprecipitate GFP-SDIR1 (Figure 1D). These results indicated that SDIR1 could interact with SDIRIP1 in plants.

**SDIR1 Interacts with SDIR1 in Vivo**

The SDIRIP1 gene encodes a plant-specific type 2 PTERIN-4-α-CARBOXYLAMINE DEHYDRATASE (PCD), but without the PCD activity (Naponelli et al., 2008). The function of SDIRIP1 remained unknown. In mammals, the single copy of PCD functions as a bifunctional protein, as both a metabolic enzyme and a nuclear cofactor in transcriptional regulation (Suck and Ficner, 1996). It has been reported that in *Arabidopsis* protoplasts, SDIRIP1-GFP was localized exclusively to the chloroplast (Naponelli et al., 2008). As the environment of protoplasts might affect protein localization, we aimed to determine the localization of SDIRIP1 in intact plant cells. 35S:SDIRIP1-GFP was expressed transiently in *N. benthamiana*. To our surprise, in addition to the chloroplast, SDIRIP1-GFP also existed in the cell periphery and nucleus-like structures (Figure 2A). Coexpression of 35S:SDIRIP1-GFP with 35S:HY5-RFP (red fluorescent protein), encoding the nucleus-localized transcription factor HY5, demonstrated that part of SDIRIP1 was also localized to the nucleus (Figure 2B). To confirm this result, we isolated nuclear and chloroplastic fractions from total protein samples. SDIRIP1-GFP could be detected in the nuclear fraction, which was indicated by the anti-histone H3 antibody, and in the chloroplastic fraction, which was indicated by the anti-PsbA (Photosystem II Reaction Center Protein A) antibody (Supplemental Figure 1). These results showed that SDIRIP1 was localized not only to the chloroplast but also to the cell periphery and nucleus.

The subcellular localization of SDIRIP1 prompted us to examine the SDIR1-SDIRIP1 interaction at the intracellular level. It has been reported that SDIR1 is an intracellular membrane-localized protein (Zhang et al., 2007). We first examined the subcellular localization of SDIR1 in detail. 35S:GFP-SDIR1 was transiently expressed in *N. benthamiana* leaves and *Arabidopsis* leaf protoplasts. Green fluorescence was detected in a net-like compartment, which is similar to the ER. A coexpression assay showed that GFP-SDIR1 was colocalized with RFP-HDEL, an ER localization marker, demonstrating that GFP-SDIR1 is at least located on the ER membrane, although SDIR1 localization on other types of intracellular membranes cannot be excluded (Figures 2C and 2D).

According to the yeast two-hybrid results, the C terminus of SDIR1 behind the second transmembrane domain can interact with SDIRIP1 (Figures 1A and 1B). To deeply investigate the subcellular localization of this interaction, it is essential to check the orientation of SDIR1 across the membrane. As shown in Supplemental Figure 2, there are two possibilities. The C terminus of SDIR1 exists in the cytoplasm or ER lumen. To address this, we utilized a fluorescence protease protection (FPP) assay (Lorenz et al., 2006a, 2006b, 2008). We transiently coexpressed 35S:RFP-HDEL and 35S:SDIR1-GFP in *Arabidopsis* protoplasts. RFP-HDEL, which resides in the ER lumen, was used as a control. We treated these protoplasts with 50 μM digitonin for 5 min; digitonin can permeabilize the plasma membrane but not the ER membrane. Subsequently, protoplasts were exposed to 4 mM trypsin. The ER membrane can protect RFP-HDEL from digestion by trypsin. However, proteins in the cytoplasm would be...
digested. Images were taken before and after trypsin treatment. After trypsin treatment, the fluorescence from SDIR1-GFP quickly vanished, while the fluorescence from RFP-HDEL did not change (Figure 2E). This result indicated that the C terminus of SDIR1 faces the cytoplasm.

Next, we performed a bimolecular fluorescence complementation (BiFC) assay (Waadt et al., 2008) to check the interaction between SDIRIP1 and SDIR1. The fluorescent protein VENUS was spliced into the N-terminal fraction (VYNE) and the C-terminal fraction (VYCE). VYNE was fused to the N terminus of SDIR1 to

Figure 1. SDIRIP1 Interacts with SDIR1.

(A) Schematic illustration of the structures of SDIR1 and SDIR1ΔTM (containing an 81-amino acid deletion on the N terminus). TM, transmembrane domain.

(B) Interaction between SDIRIP1 and SDIR1ΔH234YΔTM verified by yeast two-hybrid assays. In SDIR1ΔH234YΔTM, His-234 of SDIR1 was mutated to Tyr-234 and the transmembrane domains of SDIR1 were deleted. BD, bait vector pGBK7; AD, prey vector pGADT7; BD/SDIR1ΔH234YΔTM, SDIR1ΔH234YΔTM fused to pGBK7 as the bait; AD/SDIRIP1, SDIRIP1 fused to pGADT7 as the prey. Yeast was grown on synthetic dropout medium lacking leucine and tryptophan (SD-L-W) and synthetic dropout medium lacking leucine, tryptophan, and histidine and containing 2 mM 3-aminotriazole (SD-L-W-H + 2 mM 3-AT) (selective medium).

(C) Pull-down assays for analyzing the interaction between SDIRIP1 and SDIR1 in vitro. MBP-SDIR1 was used as the bait to pull down [35S]methionine-labeled SDIRIP1. The MBP tag was used as the negative control. An equal amount of SDIRIP1 was used in pull-down assays and as the input control. Input and bound forms of SDIRIP1 were detected by autoradiography. The relative amount of bound SDIRIP1 was normalized by using input as a control. Immobilized MBP and MBP-SDIR1 were detected by Coomassie blue staining.

(D) Co-IP assays of SDIR1 with SDIRIP1. SDIRIP1-MYC and GFP-SDIR1 were transiently expressed in N. benthamiana leaves. Co-IP assays were performed at 4°C, and protein gel blot analysis was performed using anti-MYC antibody. The asterisk indicates the modified forms of SDIRIP1-MYC. The top panel shows the input control.
generate VN(R)-SDIR1, and VYCE was fused to the C terminus of SDIRIP1 to generate SDIRIP1-VC. However, no reconstituted fluorescence was detected (Figure 3A; Supplemental Figures 3A and 3B). It is possible that SDIRIP1 was degraded when associated with SDIR1. To prove this hypothesis, we treated the VN(R)-SDIR1/SDIRIP1-VC samples with the 26S proteasome inhibitor MG132. After MG132 treatment, the reconstituted fluorescence was observed to be colocalized with RFP-HDEL (Figure 3B). We previously reported that the SDIR1 E3 ligase activity was completely abolished in the H234Y mutant of SDIR1 (Zhang et al., 2007). This mutation did not disrupt the SDIR1-SDIRIP1 interaction in the yeast two-hybrid assays (Figures 1A and 1B). Meanwhile, GFP-SDIR1<sup>H234Y</sup> was still localized to the ER (Figure 2D). In addition, the BiFC assays showed that reconstituted fluorescence was only detected in the VN(R)-SDIR1<sup>H234Y</sup>/SDIRIP1-VC samples (Figures 3C and 3D; Supplemental Figure 3C), indicating that SDIR1<sup>H234Y</sup> can interact with SDIRIP1 in vivo. Importantly, the reconstituted fluorescence was not colocalized with the chloroplast but with RFP-HDEL (Figures 3C and 3D).

Meanwhile, two other assays were performed. First, 35S:GFP-SDIR1 or pVIP (vector control) was coexpressed with 35S:SDIRIP1-MYC and 35S:RFP (internal control). SDIRIP1-MYC could not be detected at all when coexpressed with 35S:GFP-SDIR1, while MG132 treatment increased the protein level of SDIRIP1. No significant difference was observed between the SDIRIP1 RNA levels of the three samples (Figure 3E). Together with the yeast two-hybrid results, we came to the conclusion that the C terminus of SDIR1 in the cytoplasm interacts with SDIRIP1. Because SDIR1 is localized on the ER membrane, the BiFC fluorescence was colocalized with the ER marker RFP-HDEL. Therefore, SDIR1 and SDIRIP1 interacted in the cytosol but appeared to be associated with the ER.

SDIRIP1 Is Unstable and Degraded Partially by the 26S Proteasome

Because ATP is essential for ubiquitination and 26S proteasome-dependent degradation (Smalle and Vierstra, 2004), we examined the influence of ATP on the stability of SDIRIP1. Total protein was extracted from a <i>N. benthamiana</i> sample transiently expressing 35S:SDIRIP1-MYC and incubated at room temperature with or without ATP. During incubation, SDIRIP1 shifted to higher molecular weight forms (Supplemental Figure 4A), and adding ATP promoted the degradation of SDIRIP1 (Supplemental Figure 4A). To further demonstrate the function of the 26S proteasome on the degradation of SDIRIP1, we checked whether MG132 can attenuate the degradation of SDIRIP1. Because the SDIRIP1 level in the former sample was too high, we chose another sample with a lower SDIRIP1 level. Total protein was extracted from this <i>N. benthamiana</i> sample, which transiently expressed 35S:SDIRIP1-MYC, and the protein was incubated at room temperature with MG132 or its solvent DMSO as the control. Nearly all SDIRIP1-MYC shifted to higher molecular weight forms after a 15-min incubation and then disappeared quickly after a 30/60-min incubation. MG132 could delay the rate of SDIRIP1-MYC degradation, suggesting that SDIRIP1 is degraded at least partially by the 26S proteasome (Supplemental Figure 4B). To identify the features of the modified form of SDIRIP1-MYC, we immunoprecipitated SDIRIP1-MYC and MYC-GFP from transiently expressed samples using anti-MYC antibody. Using anti-ubiquitin antibody, we were able to detect the ubiquitinated form of SDIRIP1-MYC but not MYC-GFP in the immunoprecipitates (Supplemental Figure 4C), indicating that SDIP-MYC could be ubiquitinated in cell extracts.
To investigate the stability of SDIRIP1 in Arabidopsis, we generated 35S:SDIRIP1-MYC transgenic Arabidopsis plants in both Columbia-0 (Col-0) and sdir1-1 backgrounds. As SDIR1 expression levels are very low under normal growth conditions and this gene can be induced by NaCl treatment (Zhang et al., 2007), we then checked the stability of SDIRIP1 with or without NaCl treatment in the presence of cycloheximide (CHX), which can block new protein synthesis. Figure 4A shows that SDIRIP1 was slightly unstable under normal growth conditions and that NaCl treatment can dramatically increase the degradation of SDIRIP1.

Meanwhile, MG132 could delay but not completely inhibit the degradation of SDIRIP1 (Figure 4B). This result indicates that the 26S proteasome is one but not the only pathway for SDIRIP1 degradation. It has been reported that autophagy plays an important role in the degradation of chloroplast proteins (Wada et al., 2009; Dong and Chen, 2013). E64d, an inhibitor of lysosomal/vacuolar hydrolases, can dramatically inhibit autophagy (Bassham, 2007). Therefore, we also examined the influence of E64d on the stability of SDIRIP1 under NaCl treatment. Figure 4C shows that E64d can efficiently decrease the degradation rate of SDIRIP1, indicating that SDIRIP1 might also be degraded through the autophagy pathway.

As SDIR1 also participates in the ABA signaling pathway, we then checked the stability of SDIRIP1 with or without ABA treatment in the presence of CHX. To our surprise, ABA treatment could inhibit the degradation of SDIRIP1 (Supplemental Figure 5A). However, the mechanism remains unclear.

**SDIRIP1 Is a Substrate of SDIR1**

As SDIR1 is an active RING finger ligase and SDIRIP1 degradation is partially dependent on the 26S proteasome pathway, we investigated whether SDIR1 affected the stability of SDIRIP1 in Arabidopsis. We selected one Col-0/35S:SDIRIP1-MYC line and one sdir1-1/35S:SDIRIP1-MYC line with comparable SDIRIP1-MYC protein levels for a half-life study of SDIRIP1 in the presence of CHX. Figure 4D and Supplemental Figure 5B show that under both normal growth conditions and NaCl treatment, the SDIR1 null mutation could efficiently delay SDIRIP1 degradation. These data suggested that SDIR1 negatively regulated the stability of SDIRIP1. Together with the coexpression assay results in N. benthamiana mentioned above, we come to the conclusion that SDIR1 can promote the degradation of SDIRIP1 by the 26S proteasome.

To verify our speculation that SDIRIP1 is a substrate of SDIR1, we conducted an in vitro ubiquitination assay. SDIRIP1 was expressed in E. coli as a fusion protein with MYC and GST (GLUTATHIONE S-TRANSFERASE) tags, and SDIR1 was expressed with an MBP tag. Figure 4E and Supplemental Figure 6
Figure 4. SDIRIP1 Is a Substrate of SDIR1.

(A) to (D) Protein gel blot results of one experiment. Anti-PAG1 (20S proteasome α-subunit G1) was used as the loading control; SDIRIP1-MYC protein level was normalized. Three independent biological repeats were performed and analyzed. Fourteen-day-old seedlings were used in these assays. Error bars represent SD.
show that SDIRIP1 was ubiquitinated by SDIR1 in the presence of E1, E2, and ubiquitin. When any of these proteins in the reaction was omitted, the ubiquitinated form of SDIRIP1 was not found. These data directly demonstrated that SDIRIP1 is a substrate of SDIR1.

**SDIRIP1 Is Involved in the Plant Response to Salt Stress and ABA Signaling**

In a previous study, we demonstrated that the sdir1 mutant alleles are less sensitive to salt stress and ABA treatment than the wild type, whereas higher sensitivity to drought stress and SDIR1 overexpression can render opposite phenotypes (Zhang et al., 2007). To answer whether SDIRIP1 works with SDIR1 in the germination rate of Col-0/35S:SDIRIP1, we gene-matched plants and selected two 35S:SDIRIP1-RNAi transgenic lines among all available stocks. Two 35S:SDIRIP1 lines (Col-0/35S:SDIRIP1 20.1 and Col-0/35S:SDIRIP1 12.3) and two 35S:SDIRIP1-RNAi lines (Col-0/35S:SDIRIP1-RNAi 4.8 and Col-0/35S:SDIRIP1-RNAi 1.5) were selected for further study. The SDIRIP1 transcript level in the transgenic lines was much higher in the overexpression lines and lower in the RNA interference (RNA) lines than in wild-type plants (Supplemental Figure 7A). The seeds of wild-type and transgenic plants were germinated on half-strength Murashige and Skoog (MS) medium containing 0, 100, 125, or 150 mM NaCl or 1 or 2 μM ABA.

In the presence of 100 mM NaCl, none of the Col-0/35S:SDIRIP1-RNAi plants developed true leaves at 14 d, whereas all of the Col-0/35S:SDIRIP1 plants developed true leaves (Figure 5A). In the presence of 100 and 125 mM NaCl, germination of Col-0/35S:SDIRIP1 plants was less sensitive than that of wild-type plants. In the presence of 100 mM NaCl, after 2 d, only 7.8% of Col-0/35S:SDIRIP1-RNAi 4.8 and 12.6% of Col-0/35S:SDIRIP1-RNAi 1.5 seeds germinated, but 19.6% of wild-type, 23.1% of Col-0/35S:SDIRIP1 20.1, and 40.1% of Col-0/35S:SDIRIP1 12.3 seeds germinated (Supplemental Figure 7B). In the presence of 150 mM NaCl, after 3 d, only 31.4% of Col-0/35S:SDIRIP1-RNAi 4.8 and 34.8% of Col-0/35S:SDIRIP1-RNAi 1.5 seeds germinated, but 64.4% of wild-type, 65.7% of Col-0/35S:SDIRIP1 20.1, and 69.4% of Col-0/35S:SDIRIP1 12.3 seeds germinated (Supplemental Figure 7B). At this concentration, the germination rate of Col-0/35S:SDIRIP1 plants was similar to that of wild-type plants. The reason could be that high salt concentrations might promote SDIRIP1 degradation by inducing the high expression of SDIR1 in plants. To examine the salt sensitivity of the plants in soil, one RNAi line and one overexpression line were chosen. Seeds were stratified in darkness for 4 d at 4°C and then sown in soil with or without different concentrations of NaCl. After 24 d of growth in soil, photographs were taken (Supplemental Figure 8A). NaCl treatment can dramatically inhibit plant growth, and SDIRIP1 RNAi plants were more sensitive to salt stress than wild-type and SDIRIP1 overexpression plants (Supplemental Figure 8B). These results indicated that SDIRIP1 could negatively regulate the sensitivity of plants to salt stress.

We also examined the ABA sensitivity of these plants. Compared with wild-type plants, Col-0/35S:SDIRIP1 plants were less sensitive to ABA, whereas Col-0/35S:SDIRIP1-RNAi plants were more sensitive (Supplemental Figure 7). In the presence of 1 μM ABA, after 4 d, only 16.8% of Col-0/35S:SDIRIP1-RNAi 4.8 and 16.9% of Col-0/35S:SDIRIP1-RNAi 1.5 seeds germinated, but 39.1% of wild-type, 62.3% of Col-0/35S:SDIRIP1 20.1, and 68.8% of Col-0/35S:SDIRIP1 12.3 seeds germinated (Supplemental Figure 7B). In the presence of 1 μM ABA, after 10 d, only 43.6% of Col-0/35S:SDIRIP1-RNAi 4.8 and 57.3% of Col-0/35S:SDIRIP1-RNAi 1.5 plants had small green cotyledons, but 67.4% of wild-type, 87.1% of Col-0/35S:SDIRIP1 20.1, and 95.7% of Col-0/35S:SDIRIP1 12.3 plants had small green cotyledons (Supplemental Figure 7C). These results indicated that SDIRIP1 could negatively regulate the sensitivity of plants to ABA.

**SDIRIP1 Acts Genetically Downstream of SDIR1 in ABA and Salt Stress Responses**

To analyze the genetic relationship between SDIR1 and SDIRIP1, we generated sdir1-1/35S:SDIRIP1-RNAi transgenic plants and selected two SDIRIP1 knockdown lines for further studies (sdir1-1/35S:SDIRIP1-RNAi 8.3 and sdir1-1/35S:SDIRIP1-RNAi 9.10) (Supplemental Figure 9A). Col-0, sdir1-1, and sdir1-1/35S:SDIRIP1-RNAi plants were germinated on half-strength MS medium containing 0, 100, 125, or 150 mM NaCl or 1 or 2 μM ABA. Figure 5B and Supplemental Figure 9B show that the knockdown of SDIRIP1 expression could rescue the ABA- and salt-insensitive phenotype of Col-0, sdir1-1, and sdir1-1/35S:SDIRIP1-RNAi plants germinated under ABA or NaCl treatment. Compared with wild-type plants, sdir1-1 was less sensitive to ABA and salt stress (Figure 5B; Supplemental Figures 9B and 9C). In the presence of 100 mM

---

**Figure 4.** (continued).

(A) Seedlings were pretreated in half-strength MS medium with or without 300 mM NaCl for 3 h and then incubated in the same medium with 50 μM CHX. Samples were collected 0, 2, 4, and 6 h after treatment.

(B) Seedlings were pretreated in half-strength MS medium with 300 mM NaCl for 3 h and then incubated in the same medium with or without 75 μM MG132 in the presence of 300 mM NaCl and 50 μM CHX. Samples were collected 0, 2, 4, and 6 h after treatment.

(C) Seedlings were pretreated in half-strength MS medium with 300 mM NaCl for 3 h and then incubated in the same medium with or without 10 μM EB44d in the presence of 300 mM NaCl and 50 μM CHX. Samples were collected 0, 2, 4, and 6 h after treatment.

(D) Col-0/35S:SDIRIP1-MYC and sdir1-1/35S:SDIRIP1-MYC seedlings were pretreated in half-strength MS medium with 300 mM NaCl and then incubated in the same medium with 50 μM CHX. Samples were collected 0, 3, and 6 h after treatment.

(E) Ubiquitination of SDIRIP1 by SDIR1 in vitro. The protein gel blot was analyzed using anti-MYC antibody.
SDIR1 Destabilizes SDIRIP1 in ABA Signaling

Because two Col-0/35S:SDIRIP1 lines behaved similarly under salt stress and ABA treatment, as did the Col-0/35S:SDIRIP1-RNAi lines, we selected Col-0/35S:SDIRIP1 12.3 and Col-0/35S:SDIRIP1-RNAi 4.8, respectively, for further study. We previously reported that SDIR1 acted upstream of ABF3, ABF4, and ABI5 by positively regulating their expression under ABA treatment (Zhang et al., 2007). To investigate the function of SDIR1 in the ABA signaling pathway, we treated Col-0, Col-0/35S:SDIRIP1, and Col-0/35S:SDIRIP1-RNAi plants with 50 μM ABA for 0, 1, 2, and 3 h and measured ABF3, ABF4, and ABI5 expression. ABI5 was induced after ABA treatment in all lines. A significant difference in ABI5 levels was observed after ABA treatment. Compared with the wild type, the ABI5 mRNA level was lower in Col-0/35S:SDIRIP1 but higher in the Col-0/35S:SDIRIP1-RNAi transgenic line. By contrast, the expression patterns of ABF3 and ABF4 were similar among these different lines (Figure 6A). These data indicated that ABA induction of ABI5 was attenuated by SDIRIP1 specifically and that SDIRIP1 has no significant effects on ABF3 and ABF4 expression.

To investigate whether SDIRIP1 indeed acts upstream of ABI5, we generated abi5-1/35S:SDIRIP1-RNAi transgenic plants (Supplemental Figure 10A). Wassilewskija (Ws) wild-type, abi5-1, and abi5-1/35S:SDIRIP1-RNAi seeds were germinated on half-strength MS medium containing 0, 1, or 2 μM ABA and 150 mM NaCl. The abi5-1 plants were insensitive to ABA and salt stress, whereas SDIRIP1-RNAi produced ABA- and salt-sensitive phenotypes in wild-type plants (Figure 5A; Supplemental Figure 7B). However, compared with Ws, two abi5-1/35S:SDIRIP1-RNAi lines were more insensitive to ABA and NaCl treatment, and no significant difference was observed between the two abi5-1/35S:SDIRIP1-RNAi lines (abi5-1/35S:SDIRIP1-RNAi 3.3 and abi5-1/35S:SDIRIP1-RNAi 8.4) and abi5-1 (Figure 6B; Supplemental Figure 10B). For example, in the presence of 2 μM ABA, after 10 d, only 26% of Ws wild-type plants had green cotyledons, but all abi5 and abi5/35S:SDIRIP1-RNAi plants had green cotyledons (Supplemental Figure 10C). ABA and salt sensitivity, which was enhanced by 35S:SDIRIP1-RNAi, was rescued by the ABI5 mutation, indicating that SDIRIP1 acts upstream of ABI5.

Previously, we found that SDIR1 overexpression could enhance the drought tolerance ability of plants by reducing the rate of leaf water loss (Zhang et al., 2007). Therefore, we measured the rate of water loss in detached rosette leaves from Col-0/35S:SDIRIP1, Col-0/35S:SDIRIP1-RNAi, and wild-type plants. However, no obvious difference was observed (Supplemental Figure 11A). To confirm this observation, we checked whether SDIRIP1 could influence the closure of stomata under ABA treatment. Again, no obvious difference was observed (Supplemental Figure 11B). These data suggest that SDIRIP1 might not be involved in the regulation of the stomatal response to ABA. This result is correlated to the different effects of SDIRIP1 on different bZIP transcription factors. SDIRIP1 has little influence on the expression of both ABF3 and ABF4, which are known to play major roles in the plant drought response. However, the detailed mechanism is unclear.

<table>
<thead>
<tr>
<th>A</th>
<th>35S:SDIRIP1</th>
<th>WT</th>
<th>35S:SDIRIP1-RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl, after 2 d, 98.9% of sdrl-1 seeds germinated, but 69.2% of wild-type, 72.2% of sdrl-1/35S:SDIRIP1-RNAi 9.10, and 32.9% of sdrl-1/35S:SDIRIP1-RNAi 8.3 seeds germinated (Supplemental Figure 9B). In the presence of 1 μM ABA, after 3 d, 97.8% of sdrl-1 seeds germinated, but 66.7% of wild-type, 70.8% of sdrl-1/35S:SDIRIP1-RNAi 9.10, and 4.3% of sdrl-1/35S:SDIRIP1-RNAi 8.3 seeds germinated (Supplemental Figure 9B). After 10 d, all sdrl-1 plants had small green cotyledons, while 85.2% of wild-type, 70.1% of sdrl-1/35S:SDIRIP1-RNAi 9.10, and 30.3% of sdrl-1/35S:SDIRIP1-RNAi 8.3 plants had small green cotyledons (Supplemental Figure 9C). ABA and salt sensitivity, which were reduced in the sdrl-1 mutant, were rescued by 35S:SDIRIP1-RNAi transformation, indicating that SDIRIP1 acts downstream of SDIR1. Together, the biochemical and genetic evidence above further confirmed that SDIRIP1 is a substrate of SDIR1.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

Previously, we demonstrated that SDIR1 is an intracellular membrane-localized ubiquitin ligase (E3) that acts upstream of many transcription factors, including ABF3, ABF4, and ABI5, in the ABA pathway and is involved in plant responses to salt and drought stress (Zhang et al., 2007). In this work, we identified the chloroplast/nuclear protein SDIRIP1, which acts with SDIR1 in the ABA signaling pathway.

SDIRIP1 Is Localized to the Chloroplast, Cell Periphery, and Nucleus

A previous report showed that SDIRIP1 is an Arabidopsis homolog of the bifunctional protein PCD/DCoH (DIMERIZATION COFACTOR OF HEPATOCYTE NUCLEAR FACTOR1), with dual subcellular location (Naponelli et al., 2008). The sole copy of PCD/DCoH has PCD activity in the mitochondria and also acts as DCoH in the nucleus in mammals (Suck and Ficner, 1996). Plants contain two copies of PCD/DCoH homologs (Naponelli et al., 2008). Type 1 has been demonstrated to have PCD activity and is localized in the mitochondria. However, biochemical analysis showed that SDIRIP1 encodes a type 2 plant-specific PCD/DCoH without PCD activity (Naponelli et al., 2008). This suggests that the two copies of PCD/DCoH homologs in plants might possess different functions. One conserved copy might share the function of mammalian PCD/DCoH in the mitochondria. The other copy might act as a dimerization cofactor with a protein in the nucleus. The localization of the type 2 plant-specific PCD/DCoH, which was reported to be exclusive to the chloroplast (Naponelli et al., 2008), casts doubt on this premise. We were able to demonstrate that SDIRIP1 is not only localized in the chloroplast but also in the cell periphery and nucleus, supporting the hypothesis of the other conserved function of PCD/DCoH between mammals and plants. Whether SDIRIP1 acts as a dimerization cofactor in the nucleus is a question for future studies.

Several other Arabidopsis proteins have been shown to have chloroplast and nucleus dual localization (Silva-Filho, 2003;
Caplan et al., 2008; Chen et al., 2010; Shang et al., 2010; Sun et al., 2011; Krause et al., 2012). The dual localization may be essential for signal communication at the intracellular level. However, our knowledge of the dual localization mechanism is quite limited. Via a transplastic method, it has been proven that WHIRLY1 is translocated from the chloroplast to the nucleus, where it affects the expression of PATHOGENESIS-RELATED PROTEIN 1 (Isemer et al., 2012). PTM is another chloroplast envelope-bound transcription factor that possesses transmembrane domains. In response to retrograde signals, PTM is cleaved and the N terminus of PTM is transported to the nucleus to activate ABI4 transcription (Sun et al., 2011). Protein shuttles between other organelles and the nucleus have also been observed in signal transduction. A similar cleavage mechanism was found in the ER-to-nucleus translocation of ETHYLENE INSENSITIVE2 (EIN2) (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012) to activate ethylene signaling. It is also possible that a chloroplast and nucleus dual localized protein is directly targeted to the nucleus rather than to the nucleus after translation. The mechanism of the multiple subcellular localization of SDIR1 remains elusive and will be the focus of a future study.

SDIR1 Destabilizes SDIRIP1 in ABA Signaling

SDIR1 acts on the ER membrane, while SDIRIP1 is a chloroplast-, cell periphery-, and nucleus-localized protein. Where does their interaction take place? No interaction could be observed between intact SDIR1 and SDIRIP1 in the BiFC assays. This result was consistent with that of the coexpression assay, in which SDIR1 was not detected when coexpressed with SDIR1. This might have been due to the interaction between SDIR1 and SDIRIP1 quickly driving SDIRIP1 degradation via the 26S proteasome. Similarly, Ning et al. (2011) described that the colocalization of the rice (Oryza sativa) RING finger E3 ligase OsDIS1 (DROUGHT-INDUCED SEVEN IN ABSENTIA PROTEIN1) with its substrate Os-Nek6 (NEVER-IN-MITOSIS/ ASPERGILLUS-RELATED KINASE) was almost undetectable in vivo, but Os-DIS1H71Y, losing E3 activity due to a point mutation in the RING motif, was well colocalized with Os-Nek6. SDIR1H234Y, which destroyed the E3 activity of SDIR1 but remained in the same cellular localization as SDIR1, could still interact with SDIRIP1 in yeast two-hybrid assays. Hence, we performed a BiFC assay using SDIR1H234Y and SDIRIP1. Meanwhile, after treatment with MG132, we also detected the interaction between SDIR1 and SDIRIP1 by BiFC. Confocal observation showed that the fluorescence of BiFC can colocalize with RFP-HDEL. It seems that SDIR1 is recognized by SDIRIP1 on the ER membrane.

Most chloroplastic and nuclear proteins are translated in the cytoplasm in an ER-independent manner and are transported to the chloroplast and the nucleus by the transit peptide and the nuclear localization signal (Inaba and Schnell, 2008; Zybalov et al., 2008). Moreover, some proteins can be translocated from the chloroplast to the nucleus (Isemer et al., 2012), possibly via the cytoplasm. Yeast two-hybrid assays indicated that the C terminus of SDIR1 interacts with SDIRIP1, and the FPP assays demonstrated that the C terminus of SDIR1 faces the cytoplasm. Therefore, it is possible that the C terminus of SDIR1 recruits SDIRIP1 in the cytoplasm and then SDIRIP1 is degraded by the 26S proteasome. Because SDIR1 is localized on the ER membrane, the fluorescence from BiFC can colocalize with RFP-HDEL.

SDIRIP1 Is a Substrate of SDIR1

We identified SDIR1 as an SDIR1-interacting protein. MG132 retarded the degradation of SDIRIP1. In addition, we found that SDIR1 could ubiquitinate SDIRIP1 in vitro and promote its degradation in vivo, indicating that SDIRIP1 is a substrate of SDIR1. Meanwhile, phenotypic and genetic analyses showed that RNAi knockdown of the SDIR1 RNA level could rescue the hypersensitive phenotype of the sdir1 mutant under salt stress and ABA treatment.

However, SDIR1 can be degraded in other pathways. SDIRIP1 was more stable in the sdir1-1 mutant than in the wild-type plants; however, SDIRIP1 in the sdir1-1 mutant could still be degraded under salt stress. This suggests that perhaps other E3 ligase complexes, rather than SDIR1 only, participate in SDIR1 degradation or that SDIR1 is degraded by another protein turnover mechanism in addition to the 26S proteasome pathway, as MG132 could not inhibit the degradation of SDIRIP1 completely.

It has been reported that chloroplast protein could be degraded by autophagy (Wada et al., 2009; Dong and Chen, 2013). E64d, an inhibitor of autophagy, could dramatically delay the degradation of SDIR1, indicating that SDIR1 might also be degraded partially by autophagy. However, the detailed mechanism remains unknown.

We also found that ABA could stabilize SDIRIP1. The mechanism remains unclear. It is possible that there is a negative feedback loop regulated by ABA. ABA might be able to destabilize SDIR1 or inhibit SDIR1’s activity by another mechanism. It is also possible that ABA could inhibit other degradation pathways, such as autophagy.

SDIR1 Acts Upstream of ABI5 in ABA Signaling

In our previous work (Zhang et al., 2007), we found that SDIR1-regulated ABA affected seed germination, salt sensitivity, as well as ABA-dependent stomata movement. In this work, we found that the substrate SDIRIP1 only affected the expression of ABI5 but not ABF3 or ABF4, both of which function in ABA-dependent drought responses. ABI5 is a positive regulator in the ABA signaling pathway. The abi5 mutant exhibits severe hyposensitivity to ABA and salt stress (Carles et al., 2002), and overexpression of ABI5 increases drought tolerance ability by reducing the rate of transpiration (Lopez-Molina et al., 2001). Phenotypic analysis showed that, compared with wild-type plants, Col-0/3SS:SDIRIP1 plants were more resistant to salt stress and ABA, whereas Col-0/3SS:SDIRIP1-RNAi plants were more sensitive to these treatments. To our surprise, we observed no significant difference in the rate of transpiration between these transgenic and wild-type plants. One possibility is that SDIR1 overexpression did not increase the expression of ABI5 in plants enough to affect transpiration.
SDIR1 acts upstream of ABI5, ABF3, and ABF4 and participates in ABA, salt, and drought stress pathways (Zhang et al., 2007). However, SDIRIP1 selectively regulates the expression of ABI5 and only participates in the ABA and salt stress pathways. Meanwhile, several other SDIR1-interacting proteins were identified in yeast two-hybrid assays. These results strongly indicated that there might be other substrates of SDIR1 that regulate other types of bZIP transcription factors, such as ABF3 and ABF4, that function in the drought response. Indeed, different substrates have been found for one particular RING finger E3 ligase, COP1. For example, the RING finger E3 ligase COPI1 has a number of different substrates involved in different biological functions (Lau and Deng, 2012). In mammals, one RING finger E3 ligase, named XIAP (Galbán and Duckett, 2010), has more than 50 different substrates in different cellular compartments with different biological functions. The identification of additional substrates may help shed light on this process in the future.

METHODS

Plant Materials and Growth Conditions

The Arabidopsis thaliana ecotypes Col-0 and Ws were used in this study. Three mutant lines were used in this study: sdrl-1 (Salk_027002) and abf5-1 (CS8105). Arabidopsis growth and treatment were performed following the protocol described previously (Zhang et al., 2007). Wild-type Nicotiana benthamiana plants were also used as host plants for Agrobacterium tumefaciens-mediated transient expression. Plants were grown in a growth chamber at 22°C and 70% relative humidity under a 16-h-light/8-h-dark photoperiod for ~1 to 1.5 months before infiltration.

Yeast Two-Hybrid Assays

Yeast two-hybrid assays were performed following the protocol described previously (Xie et al., 1999). SDIR1H234YΔTM was fused to the GAL4 DNA binding domain in pGBKT7 as the bait construct. Prey proteins were fused to the GAL4 activation domain in pGADT7 (Clontech). Initial screens were performed with an Arabidopsis cDNA library constructed using the pGADT7 vector (Zhang et al., 2011). Bait and prey constructs were cotransformed into yeast HFC7 cells. Positive clones were identified by the ability to grow on SD medium minus histidine/leucine/tryptophan and containing 2 mM 3-aminoatrazole.

Agrobacterium-Mediated Transient Expression in N. benthamiana

Agrobacterium-mediated transient expression in N. benthamiana was performed following the protocol described previously (Liu et al., 2010).

Pull-Down and Immunoprecipitation Assays

[35S]Methionine-labeled SDIRIP1 was generated by in vitro transcription and translation with the T7/SP6-coupled TNT kit (Promega). The open reading frame of SDIR1 was cloned into the pMal-C2 vector (New England Biolabs) and expressed in Escherichia coli. Expression of MBP fusion proteins and in vitro binding experiments were performed following the protocol described previously (Xie et al., 1999).

For immunoprecipitation and co-IP assays, total proteins were extracted from leaf samples with native extraction buffer (50 mM Tris-MES, pH 8.0, 0.5 M sucrose, 1 mM MgCl2, 10 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail Complete Minitablets [Roche]). Immunoprecipitation and co-IP were performed at 4°C following the protocol described previously (Liu et al., 2010).

Chloroplast and Nucleus Isolation

Chloroplasts and nuclei were isolated from N. benthamiana leaves expressing SDIRIP1-GFP. For chloroplast isolation, 2 g of leaves was homogenized in 4 mL of grinding buffer (50 mM HEPES-KOH, pH 7.3, 0.33 M sorbitol, 0.1% BSA, 1 mM MnCl2, and 2 mM EDTA). Ground tissues were filtered through two layers of Miracloth. Chloroplasts were pelleted by centrifugation at 2600g for 5 min. The crude chloroplasts were resuspended in 0.4 mL of grinding buffer with 40% and 80% Percoll. A total of 3.2 mL of 40% (v/v) Percoll was gently layered on top of 1 mL of 80% (v/v) Percoll. Then, the resuspended chloroplast pellet was gently layered on top of the Percoll gradient and centrifuged at 1500g for 10 min. We harvested the band between the two phases, which contained intact chloroplasts.

Nucleus isolation was performed using a plant nucleus isolation kit (CellYtic kit; Sigma-Aldrich) following the protocol for highly pure preparation of nuclei in the technical bulletin.

BiFC Assays

SDIR1, SDIR1H234Y, and SDIRIP1 were fused to the N and C termini of Venus (Waadt et al., 2008) to produce VN(R)-SDIR1, VN(R)-SDIR1H234Y, and VN(R)-SDIR1-VC, respectively. VC and VN(R) were used as negative controls. VN(R)-SDIR1-VC, VN(R)-SDIRIP1-VC, VN(R)-SDIR1H234Y-VC, and VN(R)-SDIR1H234Y/SDIRIP1-VC were transiently expressed in N. benthamiana. Leaves were collected 3 d after infiltration. The fluorescence was detected using a Leica TCS SP5 confocal laser scanning microscope.

FPP Assays

Arabidopsis protoplasts were isolated from rosette leaves using the Tape-Arabidopsis Sandwich method (Wu et al., 2009). Protoplast transformation was performed following the protocol described previously (Yoo et al., 2007).

FPP assays were performed using a modified procedure. After incubation overnight in W5 solution (2 mM MES, pH 5.7, 154 mM NaCl, 125 mM CaCl2, and 5 mM KCl), protoplasts were washed with W5 solution three times and then resuspended in W5 solution (4 mM HEPES, pH 7.5, and 20 mM KCl). To increase the permeability of the plasma membrane, digitonin was added to the medium to a final concentration of 50 μM and incubated at room temperature for 5 min. After that, the same volume of W5 solution containing 2% low-melting-point agarose was added to the protoplast solution. The protoplasts were spread onto a glass slide. Photographs were taken before trypsin treatment. Then, W5 solution containing 4 mM trypsin was added onto the slide. After 1 min of incubation, photographs were taken.

In Vitro Degradation Assays

Total protein was extracted from leaf samples with native extraction buffer (50 mM Tris-MES, pH 8.0, 0.5 M sucrose, 1 mM MgCl2, 10 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail Complete Minitablets [Roche]). Samples were incubated at room temperature (for SDIRIP1) for various time periods.

Protein Gel Blot Analysis and Image Quantification

Protein gel blot analysis was performed following the protocol described previously (Liu et al., 2010) with primary anti-MYC antibody (9E10; Santa Cruz Biotechnology) (1:1500 diluted), anti-GFP antibody (AbK9004-25-PU; Beijing Protein Innovation) (1:1500 diluted), anti-ubiquitin antibody (Liu et al., 2010) (1:3000 diluted), anti-PAS1 antibody (raised in our laboratory) (1:10,000).
diluted), anti-Abi1 Arabidopsis PbA antibody (AS01 016; Agrisera) (1:6000 diluted), and anti-Abi1 Arabidopsis histone H3 antibody (AS10 710; Agrisera) (1:6000 diluted) followed by secondary goat anti-mouse antibody or goat anti-rabbit antibody conjugated to horseradish peroxidase and visualized using ECL solution (Amersham Pharmacia). Protein gel blot pictures were scanned, and the intensity of the images was quantified by ImageJ (National Institutes of Health).

In Vitro Ubiquitination Assay

For the in vitro ubiquitination assay, crude extract containing recombinant wheat (Triticum aestivum) E1 (GI: 136632), At-UBC10 (40 ng), purified SDIR1 (1 μg) fused with the MBP tag, purified Arabidopsis ubiquitin (UBQ14; At4g02890; 2 μg) fused with the His tag, and purified GST-MYC-SDIRIP1 (2 μg) fused with both the GST and MYC tags were used for the assay. MBP was used as the negative control of MBP-SDIR1. Reactions were performed following the protocol described previously (Xie et al., 2002). The reaction was stopped by the addition of 4× protein loading sample buffer (258 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.4% Coomassie Brilliant Blue, and 0.4 M β-mercaptoethanol) and boiling for 10 min.

RT-PCR and Quantitative RT-PCR Assays

DNase I-treated total RNA was denatured and subjected to reverse transcription reaction using SuperScript II (200 units per reaction; Invitrogen) at 37°C for 1 h followed by heat inactivation of the reverse transcriptase at 70°C for 15 min. For coexpression assays, PCR was performed using the primers shown in Supplemental Table 1 for 25 cycles. Quantitative RT-PCR was performed using a SsoFast EvaGreen Supermix kit (Bio-Rad) and the primers listed in Supplemental Table 1.

Measurement of the Transpiration Rate

Plants were grown on half-strength MS medium for 7 d and then transplanted to soil to grow for 21 d. To minimize the experimental variation, whole aerial parts were detached from plants and placed on Petri dishes with the abaxial side of every leaf up. The leaves did not overlap. Samples were kept at room temperature and weighed at different times.

Measurement of Stomatal Aperture

In this assay, we used the same plants that were used to measure transpiration rates. Fresh leaves at similar developmental stages were harvested. These leaves were then incubated in a buffer (5 mM KCl, 50 mM CaCl₂, and 10 mM MES-Tris, pH 6.1) at room temperature under high light for 3 h to ensure the opening of all stomata. After that, ABA was added to the buffer to a final concentration of 5 μM. After 5 h of incubation, photographs were taken, and stomatal apertures were measured by ImageJ.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: SDIR1, At3g55530; SDIRIP1, At5g51110; HY5, At5g11260; ABI5, At2g36270; ABF3, At4g34000; ABF4, At3g19290; Actn7, At5g09810; PAG, At2g27020; PbA, AtCg00020; Histone H3, At1g01370.

Supplemental Figure Data

Supplemental Figure 1. Detection of SDIRIP1-GFP in the Nuclear Fraction by Protein Gel Blot.

Supplemental Figure 2. Schematic Illustration of the Principle behind the FPP Assay to Reveal the C-Terminal Orientation of SDIR1.

Supplemental Figure 3. Controls for BiFC Assays.

Supplemental Figure 4. SDIRIP1 Is Unstable and Degraded by the 26S Proteasome Pathway.

Supplemental Figure 5. The Stability of SDIRIP1.

Supplemental Figure 6. Ubiquitination of SDIRIP1 by SDIR1 in Vitro.

Supplemental Figure 7. Statistical Analysis of Rates of Germination and Cotyledon Greening for Col-0 Wild-Type, Col-0/3SS:SDIRIP1, and Col-0/3SS:SDIRIP1-RNAi Plants.

Supplemental Figure 8. Salt Treatment of Col-0 Wild-Type, Col-0/3SS:SDIRIP1, and Col-0/3SS:SDIRIP1-RNAi Plants in Soil.

Supplemental Figure 9. Statistical Analysis of Rates of Germination and Cotyledon Greening for Col-0 Wild-Type, sdir1-1, and sdir1-1/3SS:SDIRIP1-RNAi Plants.

Supplemental Figure 10. Statistical Analysis of Rates of Germination and Cotyledon Greening for Ws Wild-Type, abi5-1, and abi5-1/3SS:SDIRIP1-RNAi Plants.

Supplemental Figure 11. SDIRIP1 Has No Obvious Influence on Transpiration and Effects of ABA on Stomatal Aperture.

Supplemental Table 1. Primers Used in This Work.

ACKNOWLEDGMENTS

We thank Gang Zeng for technical support. This research was supported by 973 Program Grant 2011CB915402 from the National Basic Research Program of China and Grant NSFC 31030047/90717006 from the National Science Foundation of China. Y.-R.W. was supported by 973 Program Grant 2012CB114300.

AUTHOR CONTRIBUTIONS


Received November 12, 2014; revised December 17, 2014; accepted January 6, 2015; published January 23, 2015.

REFERENCES


Sun, X., Feng, P., Xu, X., Guo, H., Ma, J., Chi, W., Lin, R., Lu, C., and Zhang, L. (2011). A chloroplast envelope-bound PHD transcription...


