The Arabidopsis C3H2C3-Type RING E3 Ubiquitin Ligase AtAIRP1 Is a Positive Regulator of an Abscisic Acid-Dependent Response to Drought Stress1[C][W][OA]

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Ubiquitination is a eukaryotic posttranslational protein modification that is mediated by the cascade of E1, E2, and E3 ubiquitin (Ub) ligases and is involved in regulating numerous cellular functions. In this study, we obtained 100 different Arabidopsis (Arabidopsis thaliana) T-DNA insertion mutant plants in which RING E3 Ub ligase genes were suppressed and monitored their phenotypes in the presence of exogenous abscisic acid (ABA), a plant stress hormone. One of these loss-of-function mutants displayed ABA-insensitive phenotypes at the germination stage and was named atairp1 (for Arabidopsis ABA-insensitive RING protein 1). AtAIRP1 encodes a cytosolic protein containing a single C3H2C3-type RING motif with in vitro E3 Ub ligase activity. AtAIRP1 was significantly induced by ABA and drought stress. In contrast to atairp1 mutant plants, AtAIRP1-overexpressing transgenic plants (35S:AtAIRP1-sGFP) were hypersensitive to exogenous ABA in terms of radicle emergence, cotyledon development, root elongation, and stomatal closure. Ectopic expression of AtAIRP1-sGFP in atairp1 effectively rescued the loss-of-function ABA-insensitive phenotype. Both 35S:AtAIRP1-sGFP and atairp1/35S:AtAIRP1-sGFP plants accumulated higher amounts of hydrogen peroxide in response to exogenous ABA than did wild-type and atairp1 mutant plants. AtAIRP1 overexpressors were markedly tolerant to severe drought stress, as opposed to atairp1, which was highly susceptible. The levels of drought stress-related genes and basic leucine zipper transcription factor genes were up-regulated in the 35S:AtAIRP1-sGFP lines relative to wild-type and atairp1 mutant plants in response to ABA. Overall, these results suggest that AtAIRP1, a C3H2C3-type RING E3 Ub ligase, is a positive regulator in the Arabidopsis ABA-dependent drought response.

Ubiquitination is a posttranslational protein modification widely found in eukaryotic cells (Dye and Schulman, 2007; Hunter, 2007). In higher plants, ubiquitinated proteins are involved in diverse cellular processes, including abiotic or biotic stress responses, circadian rhythms, cell cycles, and differentiation (Moon et al., 2004; Smalle and Vierstra, 2004; Dreher and Callis, 2007). Ubiquitin (Ub), a highly conserved 76-amino acid polypeptide, is first activated by an E1 Ub-activating enzyme in an ATP-dependent manner and is transferred to an E2 Ub-conjugating enzyme. The Ub-E2 complex then binds an E3 Ub ligase that catalyzes the formation of an isopeptide bond between a Lys residue of the target proteins and the C-terminal Gly of Ub (Glickman and Adir, 2004; Smalle and Vierstra, 2004). The Lys-48 and Lys-63 residues of Ub are used to form a polyubiquitin chain. The most abundant Lys-48-linked polyubiquitinated target proteins are rapidly degraded by the 26S proteasome complex, while Lys-63-linked polyubiquitination confers nonproteolytic functions, such as DNA repair and protein trafficking (Jacobson et al., 2009). Interaction with other proteins, lipidation, subcellular localization, and protein activity can also be altered by monoubiquitination or multiubiquitination (Mukhopadhyay and Riezman, 2007).

The Arabidopsis (Arabidopsis thaliana) genome contains over 1,400 genes encoding E3 Ub ligases (Vierstra, 2009). Ub ligases can be classified into two groups. One class consists of RING (for Really Interesting New Gene)/U-box and HECT (for Homology to E6-AP Carboxyl Terminus) E3 enzymes that act as a single subunit. The other class, which includes SCF (for Skp1-Cullin-F-box) and APC (for Anaphase-Promoting Complex), functions as a multisubunit complex. There are about 477 RING motif-containing E3 Ub ligases, which constitute the third largest gene family in Arabidopsis (Kraft et al., 2005; Stone et al., 2005; Vierstra, 2009). Recently, a number of Arabidop-
sis RING E3 Ub ligases were shown to be involved in various cellular processes, such as auxin and abscisic acid (ABA) signaling, seed germination, early seedling development, adaptive pathway to nitrogen limitation, and sugar responses (Xie et al., 2002; Zhang et al., 2005; Stone et al., 2006; Peng et al., 2007; Bu et al., 2009; Huang et al., 2010). In particular, RING proteins play a key role in the response to environmental stimuli. For example, they participate in photomorphogenesis as a negative regulator (Hardtke et al., 2002; Seo et al., 2004), cold stress responses (Lee et al., 2001; Dong et al., 2006), defense mechanisms against salt and osmotic stress via increased ABA biosynthesis (Ko et al., 2006), and the ABA-dependent drought signal transduction pathway (Zhang et al., 2007). In addition, DRIP-RING E3 Ub ligase acts as a negative regulator in response to water stress by ubiquitinating the drought-induced drought-responsive element-binding protein 2A (DREB2A) transcription factor (Qin et al., 2008), whereas the endoplasmic reticulum-localized Rma1H1 RING E3 works as a positive regulator through the ubiquitination and 26S proteasome-dependent degradation of a water channel protein, aquaporin PIP2;1 (Lee et al., 2009). These recent studies suggest that diverse isoforms of RING E3 Ub ligases are critically involved not only in normal growth and development but also in the abiotic stress responses in Arabidopsis.

In our recent report, hot pepper (Capsicum annuum; Rma1H1) and Arabidopsis (Rma1) RING E3 Ub ligases were shown to function as positive regulators in water stress responses in transgenic Arabidopsis plants (Lee et al., 2009). The mode of action of these RING E3 enzymes appeared to be independent of the phytohormone ABA. Because ABA is a well-known plant stress hormone (Xiong et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Tuteja, 2007), we are interested in elucidating the functional relationship between ABA and RING E3 Ub ligases in response to dehydration stress in Arabidopsis.

In this study, we obtained 100 Arabidopsis T-DNA insertion mutant plants in which different RING E3 Ub ligase genes were suppressed and monitored their phenotypes in the presence or absence of exogenous ABA. One of these loss-of-function mutant plants displayed ABA-insensitive phenotypes during the germination stage. This mutant was referred to as atairp1 (for Arabidopsis ABA-insensitive RING protein 1). AtAIRP1 encodes an E3 Ub ligase containing a single C3H2C3-type RING motif. The AtAIRP1 gene was significantly activated by ABA and abiotic stresses, including drought, low temperature, and high salinity. In contrast to the atairp1 mutant plants, AtAIRP1-overexpressing transgenic Arabidopsis plants (35S:AtAIRP1-sGFP) showed hypersensitive phenotypes to exogenous ABA in terms of germination rate, root elongation, and stomatal closure. In addition, ectopic expression of AtAIRP1 in atairp1 effectively complemented the loss-of-function insensitive phenotype of the mutant plants to ABA. Both 35S:AtAIRP1-sGFP and atairp1/35S:AtAIRP1-sGFP transgenic plants accumulated higher amounts of reactive oxygen species (ROS) as compared with wild-type and atairp1 mutant plants in response to exogenous ABA. AtAIRP1 overexpressors were markedly tolerant to drought stress. Overall, the data presented in this study indicate that AtAIRP1, a C3H2C3-type RING E3 Ub ligase, plays a role as a positive regulator in the ABA-dependent drought response in Arabidopsis.

**RESULTS**

Identification of atairp1 T-DNA Insertion Mutant Plants

To understand the functional relationship between RING E3 Ub ligases and the stress hormone ABA within the water stress signaling pathway, we first selected 100 RING E3 Ub ligase genes that appeared to be up-regulated by abiotic stresses based on a microarray database (http://www.genevestigator.com). We next obtained T-DNA insertion loss-of-function mutants of the selected genes from the Arabidopsis Biological Resource Center.

Wild-type and 100 various knockout mutant seeds were plated and incubated on half-strength Murashige and Skoog (MS) medium for 7 d in the presence or absence of 0.5 μM ABA, and their germination rates were monitored. As shown in Figure 1A, germination rates in terms of cotyledon greening of wild-type and most mutant seeds (e.g. nos. 12, 20, and 80) were markedly reduced in the presence of ABA (from 100% to 25%–30%). The cotyledons failed to green and remained unexpanded during the 7-d incubation period. In contrast, the germination rate of one of the RING mutant plants (no. 78) was only slightly inhibited by exogenously applied ABA. Approximately 80% of the sample 78 mutant plants developed normal green and expanded cotyledons with 0.5 μM ABA 7 d after germination. Thus, this mutant was insensitive to ABA at the germination stage and was named atairp1. The AtAIRP1 gene (GenBank accession no. NM_118474) consists of 1,904 bp with five exons and four introns. The T-DNA insertion was mapped to the third exon in AtAIRP1, which is located on chromosome 4 (Fig. 1B). Homozygous atairp1 mutant plants were verified by genotyping PCR using LB_6313R, FW1, and RV1 primers (Fig. 1C). Reverse transcription (RT)-PCR with forward primer FW1 and reverse primer RV1 showed that full-length AtAIRP1 mRNA was not detected in atairp1 mutant seedlings (Fig. 1D). In addition, no AtAIRP1 transcripts were detectable with a primer set (FW2 and RV1) that amplified a region downstream of the T-DNA insertion site in AtAIRP1. However, significant levels of AtAIRP1 mRNA were observed with primers (FW1 and RV2) for the upstream region of the insertion site. Thus, it is most likely that, although the partial transcript is still present, atairp1 loss-of-function mutant plants lack functional full-length AtAIRP1 mRNA.
Sequence Analysis of AtAIRP1

The AtAIRP1 gene encodes a 153-amino acid protein with a predicted molecular mass of 16.9 kD (Fig. 2A). AtAIRP1 shares a relatively low degree of amino acid sequence identity with other Arabidopsis RING proteins (29% identical to At5g41350, 24% to At4g00335, and 18% to At5g38895) for which cellular functions are unknown. This is consistent with the view that the RING proteins are encoded by a large and divergent gene family (Kraft et al., 2005; Stone et al., 2005; Vierstra, 2009). In addition, AtAIRP1 is 18% to 30% identical to putative RING proteins from rice (Oryza sativa), poplar (Populus trichocarpa), maize (Zea mays), and grape (Vitis vinifera; Fig. 2, B and D). The functions of these proteins are also unknown. AtAIRP1 contains a single RING domain in its C-terminal region that is 65% to 80% identical to the corresponding region of other plant RING proteins. Because the Cys-X$_2$-Cys-X$_{14}$-Cys-X$_2$-His-X$_2$-His-X$_2$-Cys-X$_{10}$-Cys-X$_2$-Cys sequence is well conserved in the 41-amino acid RING motif (Fig. 2C), AtAIRP1 is a C3H2C3-type RING protein (Stone et al., 2005).

AtAIRP1 Is Up-Regulated by Abiotic Stresses and ABA Treatment

An in silico database (http://www.genevestigator.com) showed that AtAIRP1 is induced by ABA and various environmental stresses. To confirm the expression profiles of AtAIRP1 mRNA in response to abiotic stresses and ABA treatment, we performed RT-PCR and real-time quantitative (q)RT-PCR experiments. Total RNA was prepared from light-grown, 10-d-old
Arabidopsis seedlings that had been treated with different abiotic stresses. As shown in Figure 3A, AtAIRP1 transcripts were significantly up-regulated in response to cold (4°C for 6–12 h), drought (1–2 h), and high-salinity (300 mM NaCl for 1.5–3 h) stresses. In addition, AtAIRP1 was highly activated within 1.5 h of ABA (100 μM) treatment (Fig. 3A). Quantitative (q)RT-PCR results demonstrated that AtAIRP1 mRNA showed 2- to 4-fold induction by cold and drought stress, whereas the transcript levels were elevated 10- to 100-fold in response to high-salt and ABA treatments, respectively (Fig. 3B). The RD29A and RAB18 genes were used as positive controls for abiotic stress and ABA, respectively. The magnitude of stress induction of RD29A was greater than that of AtAIRP1, while induction of AtAIRP1 in response to ABA treatment was higher than that of RAB18. Collectively, the data in Figure 3 indicate that expression of AtAIRP1 is subject to control by a broad spectrum of abiotic stresses and also by the stress hormone ABA. These results raise the possibility that AtAIRP1 is involved in the ABA-dependent stress signaling pathway in Arabidopsis.

AtAIRP1 Is a Cytosolic RING E3 Ub Ligase

RING motif-harboring proteins have been shown to work as E3 Ub ligases (Kraft et al., 2005; Stone et al., 2005). We conducted in vitro self-ubiquitination assays to examine whether AtAIRP1 possesses E3 Ub ligase enzymatic activity. Maltose-binding protein (MBP)-tagged AtAIRP1 recombinant protein was expressed in *Escherichia coli* and purified by amylase resin affinity chromatography. Purified MBP-AtAIRP1 was incu-
bated at 30°C for 2 h in the presence of Ub, ATP, E1 (Arabidopsis UBA1), and E2 (Arabidopsis UBC8). Previous studies showed that UBA1 and UBC8 worked effectively as E1 and E2 enzymes, respectively, in Arabidopsis U-box and RING E3 Ub ligase enzyme assays (Cho et al., 2008; Son et al., 2009, 2010). Samples were separated by SDS-PAGE, and ubiquitinated proteins were detected by immunoblot analysis using anti-MBP and anti-Ub antibodies. As shown in Figure 4A, high-molecular-mass ubiquitinated bands were produced by AtAIRP1, indicating that AtAIRP1 had E3 Ub ligase activity in vitro.

We next investigated the E2 specificity of AtAIRP1. In this experiment, various Arabidopsis E2s, including UBC5, UBC8, UBC10, and UBC13, were individually incubated with MBP-AtAIRP1 in a mixture of E1 (UBA1), Ub, and ATP. Reactions were assayed as described above. The results revealed that MBP-AtAIRP1 could self-ubiquitinate in the presence of UBC8 and UBC10 to similar levels, while it failed to give rise to ubiquitination products with UBC5 and UBC13. To further test the specificity of the in vitro ubiquitination assay, we constructed an AtAIRP1 mutant with a substitution of His-127 for Tyr-127 in the RING motif. In contrast to wild-type MBP-AtAIRP1, MBP-AtAIRP1H127Y was unable to produce ubiquitinated bands (Fig. 4B). This indicated that partial destruction of the RING structure resulted in the loss of E3 Ub ligase activity of AtAIRP1. Therefore, bacterially expressed AtAIRP1 has E3 Ub ligase activity in vitro.

To investigate cellular localization of AtAIRP1, we performed an in vivo subcellular targeting experiment. 35S:AtAIRP1-sGFP and 35S:sGFP constructs were transformed into Arabidopsis leaf protoplasts using a polyethylene glycol (PEG)-mediated method. Fusion protein localization was visualized by fluorescence microscopy. As shown in Figure 5, the fluorescence signal of the AtAIRP1-sGFP fusion protein was predominantly found in the cytosolic fraction, which

Figure 3. AtAIRP1 is up-regulated by abiotic stresses and ABA. Total RNA was prepared from light-grown, 10-d-old Arabidopsis seedlings, which had been treated with cold (4°C for 6–12 h), drought (1–2 h), high salinity (300 mM NaCl for 1.5–3 h), or 100 μM ABA (1.5–3 h). Induction patterns of AtAIRP1 were investigated by RT-PCR (A) or real-time qRT-PCR (B). The RD29A and RAB18 genes were used as positive controls for abiotic stress and ABA, respectively. Ubiquitin10 (UBC10) transcript levels were used as loading controls. Data represent means ± SD from three independent experiments. [See online article for color version of this figure.]

Figure 4. In vitro self-ubiquitination of AtAIRP1. A, Purified MBP-AtAIRP1 was incubated at 30°C for 2 h with different Arabidopsis E2s (UBC5, UBC8, UBC10, or UBC13), Arabidopsis E1 (UBA1), Ub, and ATP. Samples were separated by SDS-PAGE, and ubiquitinated proteins were detected by immunoblot analysis using anti-MBP (left) and anti-Ub (right) antibodies. B, Wild-type MBP-AtAIRP1 and single-amino acid substitution mutant MBP-AtAIRP1H127Y were incubated at 30°C for 2 h in the presence of Ub, ATP, E1, and E2 and analyzed by protein gel blotting using anti-MBP (left) and anti-Ub (right) antibodies.
was similar to the localization pattern of sGFP. Arabidopsis ABA response element-binding protein 1 (AREB1; Yoshida et al., 2010) was employed as a specificity control. Consistent with a previous finding, AtAREB1 was localized in the nuclei (Fig. 5, bottom row). Taken together, we concluded that AtAIRP1 is a cytosolic RING E3 Ub ligase.

Overexpression of AtAIRP1-sGFP in Wild-Type and atairp1 Loss-of-Function Mutant Arabidopsis Plants

We obtained only one allele of the atairp1 loss-of-function mutant plants (Fig. 1). Thus, before conducting detailed phenotypic analyses of atairp1, we constructed transgenic Arabidopsis plants, in which AtAIRP1 is ectopically expressed under the control of the cauliflower mosaic virus 35S promoter. The 35S:AtAIRP1-sGFP or 35S:sGFP chimeric genes were introduced into Arabidopsis plants by means of an Agrobacterium tumefaciens-mediated transformation method. Several independent T0 transformants were selected due to resistance to the herbicide BASTA (glufosinate ammmonium), and T3 transgenic plants were used for subsequent experiments (Supplemental Fig. S1). As shown in Supplemental Figure S2A, transgenic lines 1 and 7, in contrast to wild-type and 35S:sGFP control plants, constitutively expressed AtAIRP1 mRNA without stress treatments. In addition, protein gel blot analysis using an anti-sGFP antibody revealed that 35S:AtAIRP1-sGFP lines effectively expressed the AtAIRP1-GFP protein (Supplemental Fig. S2B).

For a complementation experiment, the 35S:AtAIRP1-sGFP construct was transformed into atairp1 knockout mutant plants and levels of AtAIRP1-sGFP transcripts, as well as its translational products, were examined by RT-PCR and immunoblot analysis in independent transgenic lines (Supplemental Fig. S3). The results demonstrated that high levels of both AtAIRP1 mRNA and protein were found in atairp1/35S:AtAIRP1 transgenic plants (lines 1 and 2), indicating that the expression of AtAIRP1 was effectively restored in atairp1 mutant plants (Supplemental Fig. S4).

Expression Levels of AtAIRP1 Are Closely Correlated with Development at the Germination Stage and Postgermination Stage in the Presence of ABA

Since the atairp1 mutant was selected based on the ABA-insensitive phenotype (Fig. 1), we compared seed germination rates of wild-type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants in the presence or absence of ABA. To measure germination rates, approximately 100 seeds were plated on full-strength solid MS medium supplemented with different concentrations (0.1, 0.5, or 1 μM) of ABA. After 3 d of stratification, germination rates were determined as a percentage of radicle emergence (Fig. 6A). Wild-type, atairp1, 35S:AtAIRP1-sGFP (lines 1 and 7), and atairp1/35S:AtAIRP1-sGFP (lines 1 and 2) seeds fully germinated on MS medium without ABA. On the ABA-containing growth medium, germination rates of wild-type seeds were concomitantly reduced as concentrations of ABA increased. In the presence of 0.5 μM ABA, 87% of wild-type seeds failed to germinate at 3 d after germination (Fig. 6B). As expected, the atairp1 knockout mutant seeds displayed a hypersensitive phenotype toward ABA as compared with wild-type seeds. More than 25% of mutant seeds germinated normally with 0.5 μM ABA, while approx-
Approximately 15% of the mutants were still able to germinate on medium containing 1 μM ABA. In contrast, both 35S:AtAIRP1-sGFP and atairp1/35S:AtAIRP1-sGFP transgenic seeds exhibited hypersensitivity to ABA. More than 50% of these transgenic seeds were unable to germinate in the presence of a low ABA concentration (0.1 μM), and less than 5% of the transgenic seeds germinated with 0.5 μM ABA (Fig. 6B). Thus, AtAIRP1 efficiently rescued the ABA-insensitive phenotype of the loss-of-function atairp1 mutant.

In addition, at 7 d after germination, approximately 35% of wild-type and 75% of atairp1 mutant seedlings developed true green cotyledons in the presence of 0.5 μM ABA (Fig. 6C). In contrast, under the same concentration of ABA, the growth of 82% to 93% of 35S:AtAIRP1-sGFP and atairp1/35S:AtAIRP1-sGFP transgenic seedlings was completely arrested and true green cotyledons failed to develop 7 d after germination (Fig. 6C). Thus, AtAIRP1-overexpressing plants were hypersensitive to ABA in terms of both radicle emergence and cotyledon development, demonstrating that the atairp1 mutant and AtAIRP1 overexpressors had opposite phenotypes in response to ABA.
Ten days after germination, the effects of ABA on root growth were identifiable in wild-type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants (Fig. 6D). Whereas the growth of atairp1 mutant roots was generally unaffected by 0.1 μM ABA, elongation of AtAIRP1-overexpressing roots was significantly inhibited by a low concentration (0.1 μM) of ABA, indicating enhanced sensitivity to ABA. In the presence of 0.5 μM ABA, growth of most AtAIRP1-overexpressing roots was severely retarded in contrast to the atairp1 loss-of-function roots, which were still able to elongate under the same conditions. The phenotype of wild-type roots was intermediate between atairp1 and AtAIRP1 overexpressors (Fig. 6D). Thus, atairp1 mutant plants were insensitive to ABA with regard to root growth. In contrast, both 35S:AtAIRP1-sGFP (lines 1 and 7) and atairp1/35S:AtAIRP1-sGFP (lines 1 and 2) transgenic roots displayed ABA hypersensitivity. Collectively, these results indicate that expression levels of AtAIRP1 are closely related to development during the germination (Fig. 6, A and B) and postgermination (Fig. 6, C and D) stages in the presence of ABA in Arabidopsis plants. On the other hand, the phenotypes of wild-type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants were indistinguishable in the absence of ABA in both germination and postgermination stages.

**AtAIRP1 Participates Positively in ABA-Induced Stomatal Closure and ROS Production**

To further examine the role of AtAIRP1 in relation to the ABA response, we examined ABA-dependent stomatal movement phenotypes of wild type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants. Leaves of 4-week-old plants were submerged in stomatal opening solution (Lemichez et al., 2001) and then treated with various concentrations of ABA (0, 0.1, 1.0, and 10 μM) for 2 h. Stomatal apertures were measured in the focal planes of the outer edges of guard cells in epidermal strips. None of the plants exhibited altered stomatal movement in the absence of ABA, as evidenced by the fact that all of the guard cells examined were fully opened in the stomatal opening solution without ABA (Fig. 7, A and B). However, in the presence of 0.1 to 10 μM ABA, stomatal closure in atairp1 mutant leaves was markedly impaired as compared with that in wild-type leaves. As shown in Figure 7, A and B, most mutant guard cells remained open in the presence of 10 μM ABA, with average stomatal apertures (the ratio of width to length) of 0.09 ± 0.01. In contrast, 35S:AtAIRP1-sGFP plants displayed strong ABA-induced stomatal closure at 0.1, 1, and 10 μM ABA relative to wild-type plants. The average stomatal aperture in AtAIRP1-overexpressing leaves (lines 1 and 7) was approximately 0.02 ± 0.01 at 10 μM ABA, which was approximately 25% of that in atairp1 mutant leaves (Fig. 7B). In addition, the stomatal movement of atairp1/35S:AtAIRP1-sGFP plants (lines 1 and 2) was highly sensitive to ABA and was comparable to that of 35S:AtAIRP1-sGFP transgenic plants, indicating that the mutant phenotype was sufficiently complemented by the expression of AtAIRP1 (Fig. 7, A and B). Thus, these stomatal behavior studies revealed that AtAIRP1 positively participated in the ABA-dependent stomatal movement.

ROS are involved in the ABA signal transduction pathway in guard cells (Wang and Song, 2008; Cho et al., 2009). Therefore, we investigated the accumulation of hydrogen peroxide (H₂O₂) in response to ABA in wild-type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants. Leaves of 4-week-old plants were incubated with 0 or 50 μM ABA for 2 h and treated with 3,3′-diaminobenzidine (DAB) that could interact with H₂O₂ in the presence of endogenous peroxidase (Thordal-Christensen et al., 1997). Figure 7C shows that higher levels of ABA-induced H₂O₂ production were clearly detected in both 35S:AtAIRP1-sGFP and atairp1/35S:AtAIRP1-sGFP leaves in comparison with wild-type and atairp1 mutant leaves. Taken together, the findings presented in Figure 7 strongly suggest that AtAIRP1 is involved in ABA-promoted stomatal closure and ROS production as a positive regulator in Arabidopsis.

**Overexpression of AtAIRP1 Enhances Tolerance to Drought Stress in Arabidopsis**

Overexpression of RING E3 Ub ligase Rma1H1 conferred strong tolerance to drought stress in an ABA-independent manner in Arabidopsis (Lee et al., 2009). On the other hand, the aforementioned results concerning RT-PCR assay of AtAIRP1 (Fig. 3) and ABA-related phenotypic analyses of the atairp1 mutant and AtAIRP1-overexpressing plants (Figs. 6 and 7) led us to hypothesize that AtAIRP1 is involved in the ABA-dependent response to water deficit. To test this possibility, the effects of altered AtAIRP1 expression on plant responses to drought stress were estimated. Wild-type and atairp1 mutant plants were grown for 2 weeks in pots under normal growth conditions and further grown for 10 d without watering. During this dehydration condition, most wild-type and mutant plants wilted (Fig. 8A). These plants were then rewatered and their phenotypes were compared. After 3 d of rewatering, 84% (42 of 50) of the wild-type plants were able to resume their growth and survived, whereas only 30% (15 of 50) of the mutant plants survived after rewatering (Fig. 8A), indicating that loss-of-function mutant plants were more susceptible to water deficit than wild-type plants.

We next investigated the capacity of 35S:AtAIRP1-sGFP (lines 1 and 7) and atairp1/35S:AtAIRP1-sGFP (lines 1 and 2) plants to respond to severe drought stress. In this experiment, 2-week-old wild-type and transgenic plants were grown for another 12 d without watering to completely dry the soil. These plants were rewatered and their survival rates were determined. As shown in Figure 8B, the morphology of wild-type plants was seriously wilted and impaired after dehy-
hydration stress, and after rewatering for 3 d, the survival rate was 14.4% (13 of 90). On the other hand, both 35S:AtAIRP1-sGFP and atairp1/35S:AtAIRP1-sGFP lines appeared relatively healthy after this severe drought condition. After 3 d of rewatering, survival rates of 35S:AtAIRP1-sGFP and atairp1/35S:AtAIRP1-sGFP plants were 75% (30 of 40 for line 1) to 82.5% (33 of 40 for line 7) and 63.3% (57 of 90 for line 1) to 65.5% (59 of 90 for line 2), respectively (Fig. 8B). This strongly suggested that AtAIRP1-overexpressing transgenic plants, as opposed to atairp1 mutant plants, were highly tolerant to severe water stress. In addition, AtAIRP1 effectively rescued the loss-of-function susceptible phenotype of atairp1.

To further evaluate the responses to drought stress, cut rosette water loss rates (Bouchabke et al., 2008) of the plants were estimated. The detached rosette leaves of 2-week-old wild-type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants. Leaves from 4-week-old plants were treated with 0 or 50 μM ABA for 2 h and transferred to 100 μg mL⁻¹ DAB solution overnight. The presence and level of H₂O₂ in the leaves were visualized as a dark brown color. [See online article for color version of this figure.]
Figure 8. Overexpression of AtAIRP1 enhanced tolerance to drought stress in Arabidopsis. A, Drought sensitivity of wild-type (WT) and ataip1 loss-of-function mutant plants. Light-grown, 2-week-old wild-type and ataip1 mutant plants were further grown for 10 d without watering. Plants were rewatered, and surviving plants were counted 3 d after rewatering. B, Drought tolerance of wild-type, 35S:AtAIRP1-sGFP, and ataip1/35S:AtAIRP1-sGFP transgenic plants. Light-grown, 2-week-old plants

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more slowly than those of wild-type and atairp1 mutant plants. During the first 0.5 to 1 h of incubation, cut rosette water loss rates of AtAIRP1-overexpressing plants were already lower than those of wild-type and atairp1 mutant plants. After 5 h of incubation, fresh weights of AtAIRP1 overexpressors were approxi-
mately 60% of the starting weights (Fig. 8C). These results support the conclusion that, while atairp1 loss-of-function plants were hypersensitive to water stress, AtAIRP1-overexpressing gain-of-function transgenic plants displayed highly tolerant phenotypes to severe drought stress. Taken together, expression levels of AtAIRP1 are intimately tied with responses to water deficit, strongly suggesting that AtAIRP1 is a positive regulator of ABA-dependent responses to drought stress in Arabidopsis.

Lev els of ABA Induction of Water Stress-Related Genes and Basic Leu Zipper Transcription Factor Genes Are Up-Regulated in 35S:AtAIRP1-sGFP Transgenic Plants Relative to Wild-Type and atairp1 Mutant Plants

Because the expression level of AtAIRP1 is positively correlated with both ABA sensitivity and drought resistance, we next asked whether AtAIRP1 affects the expression profiles of ABA-induced drought stress-related genes. Light-grown, 3-week-old wild-type, atairp1 mutant, and 35S:AtAIRP1-sGFP transgenic plants were treated with ABA (100 μM) for 5 h. Total leaf RNA was analyzed by real-time qRT-PCR using various gene-specific primers (Supplemental Table S1). The degree of ABA induction of RD20, RD26, RD29A, RD29B, RAB18, and KIN2, which are drought-inducible genes (Fujita et al., 2009), were significantly higher in 35S:AtAIRP1-sGFP lines and lower in atairp1 mutants than in wild-type plants (Fig. 9). The magnitude of ABA induction of these marker genes was two to nine times different between atairp1 and 35S:AtAIRP1-sGFP plants depending on the individual genes, with the biggest difference in RD29B expression. One exception was RD22, which was similarly induced by ABA in wild-type, atairp1 mutant, and AtAIRP1 overexpressors for unknown reasons (Fig. 9). In addition, ABA induction of ABF3 and ABI5, which encode ABA-responsive basic leucine zipper (bZIP) transcription factors (Finkelstein et al., 2002), was also up-regulated in 35S:AtAIRP1-sGFP transgenic plants as compared with wild-type and atairp1 mutant plants, indicating that the effects of AtAIRP1 were upstream of these ABA-responsive nuclear proteins (Fig. 9). Taken together, our expression data suggest that AtAIRP1 acts positively on ABA induction of drought stress-related genes.

DISCUSSION

Water deficit greatly inhibits plant growth and causes global reductions in crop yields. Higher plants have developed a number of defense strategies to minimize the negative effects of such detrimental growth conditions (Fujita et al., 2006; Yoo et al., 2009). The stress hormone ABA participates in dehydration stress responses as a key modulator. Particularly, ABA promotes stomatal closure to prevent undesirable water loss through transpiration and induces diverse sets of genes that encode defense mechanism-associated proteins (Xiong et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Tuteja, 2007).

Although plant RING E3 Ub ligases have recently attracted much interest, as they are involved in diverse cellular processes, there are only a few reports on the functional relationships between RING E3 ligases and ABA-mediated drought stress responses. Overexpression of XERICO, encoding an Arabidopsis RING-H2 E3, resulted in drought tolerance through enhanced synthesis of ABA (Ko et al., 2006). Zhang et al. (2007) reported that a salt- and drought-induced RING E3 ligase, SDIR1, participates in ABA-related stress signal transduction. Overexpression of SDIR1 led to increased drought tolerance, whereas sdirt1 mutant plants were hypersensitive to water stress. Thus, it was proposed that SDIR1 is a positive regulator of ABA signaling in stress responses and acts upstream of ABA-responsive bZIP transcription factors. KEG, a RING E3 ligase, was shown to be involved negatively in ABA and Glc signaling (Stone et al., 2006). Most recently, Liu and Stone (2010) reported that KEG E3 ligase ubiquitinates ABI5 transcription factor and thus inhibits ABA signaling. However, its role in ABA-mediated abiotic stress responses was not addressed.

Therefore, in this study, we attempted to investigate the functional relationship between ABA and RING E3 Ub ligases in response to dehydration stress in Arabidopsis. The screening of 100 different loss-of-function mutant plants in which RING E3 Ub ligases were suppressed resulted in the isolation of a mutant (atairp1) that was insensitive to exogenous ABA during the germination stage (Fig. 1). AtAIRP1 encodes a cytosolic C3H2C3-type RING protein with E3 Ub ligase activity in vitro (Figs. 2, 4, and 5). We considered the possibility that AtAIRP1 participates in an ABA-dependent water stress response in Arabidopsis based on the following reasons. First, AtAIRP1 is rapidly induced by water stress and in response to ABA (Fig. 3). Second, the loss-of-function atairp1 mutant and AtAIRP1-overexpressing transgenic plants exhibited
opposite sensitivities to ABA in both germination and postgermination growth (Fig. 6). The AtAIRP1 overexpressors were hypersensitive to ABA in terms of radicle emergence, cotyledon development, and ROS production. Third, AtAIRP1 positively regulates ABA-promoted stomatal closure, which may reduce transpirational water loss in response to dehydration stress (Fig. 7). Phenotypic analysis indeed demonstrated that AtAIRP1-overexpressing transgenic plants were highly resistant to severe water stress, as they were able to survive without watering for 12 d (Fig. 8B). This phenotype was in sharp contrast to that of the loss-of-function atairp1 mutant line that was susceptible to the stress and lost leaf water faster than the wild-type plants (Fig. 8, A and C). In addition, ABA induction of water stress-related genes was more evident in 35S:AtAIRP1 plants in comparison with the atairp1 knockout mutant plants (Fig. 9). Overall, these results led us to propose that the RING E3 ligase AtAIRP1 is a positive regulator of an ABA-dependent response to drought stress in Arabidopsis.

It was previously shown that HOS1, a functional RING protein with E3 ligase activity, ubiquitinates ICE1, a transcription factor that activates the expression of C-repeat binding factors (Dong et al., 2006). Similarly, DRIP1 and DRIP2, C3HC4-type RING ligases, ubiquitinated and inactivated DREB2A, which resulted in the down-regulation of drought-induced genes (Qin et al., 2008). Thus, HOS1 and DRIPs are negative regulators of plant cold and drought responses, respectively. In contrast, SDIR1 (Zhang et al., 2007) and AtAIRP1 RING E3s positively modulate dehydration stress responses (Fig. 8). It is worth noting that DRIPs are nuclear E3 ligases, while SDIR1 and AtAIRP1 are mainly found in intracellular membranes and cytosolic fractions, respectively (Fig. 5). Therefore, these results raise the tantalizing possibility that plant drought stress responses are mediated by a functional balance between positive and negative RING E3 Ub ligases at different subcellular locations. Arabidopsis has at least 477 RING motif-containing proteins (Vierstra, 2009). This suggests that the defense mechanism by which

Figure 9. Real-time qRT-PCR analysis of ABA induction of drought-inducible genes. Light-grown, 3-week-old wild-type, atairp1, and 35S:AtAIRP1-sGFP plants were treated with 100 μM ABA for 5 h. Total RNA was obtained from treated plants and analyzed by qRT-PCR using the gene-specific primers listed in Supplemental Table S1. The graphs indicate the induction fold of the RD20, RD26, RD29A, RD29B, RAB18, KIN2, ABF3, and ABI5 genes in response to ABA (100 μM) as compared with the control treatment (0 μM ABA). The mean value of three technical replicates was normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase C subunit mRNA, an internal control. [See online article for color version of this figure.]
plants adjust their cellular responses to abiotic stresses, such as water deficits, is regulated by a complex network in which diverse RING E3 Ub ligase isoforms act as negative or positive factors. This functional network may allow plants to fine-tune their responses to dehydration, one of the most serious abiotic stresses plants are exposed to during their life span.

Because overall phenotypes of atairp1 knockout mutants and AtAIRP1-overexpressing plants were indistinguishable without ABA treatment and under normal growth conditions (Figs. 6–8), it is unlikely that constitutive expression of AtAIRP1 conferred drought stress tolerance via increased ABA production. Alternatively, drought-induced AtAIRP1 may ubiquitinate negative regulators of components of the ABA action and/or signal transduction pathways. In this scenario, the ubiquitinated negative regulators are rapidly degraded by a 26S proteasome complex, which in turn results in an enhanced sensitivity of plants to ABA-promoted cellular processes and drought stress responses. Overexpression of AtAIRP1 resulted in the up-regulation of ABA induction of bZIP transcription factors as well as most ABA-inducible downstream markers (Fig. 9). With this in mind, we speculate that AtAIRP1 likely acts upstream of ABA-responsive bZIP transcription factors and ubiquitinates and inactivates negative regulator(s) that inhibit the expression of the bZIP genes. In addition, analysis of recent microarray data from Fujita et al. (2009) suggests that AtAIRP1 is not induced by ABA in a snrk2d/e/f triple mutant in which three SnRK2 genes are knocked out. SnRK2s are protein kinases that act upstream of ABA-responsive bZIP transcription factors (Cutler et al., 2010; Raghavendra et al., 2010); thus, ABA induction of AtAIRP1 appears to be under the control of SnRK2s. In contrast, ABA reduces the level of KEG RING E3 ligase, a negative regulator of ABA signaling, by promoting self-ubiquitination and subsequent proteasomal degradation (Liu and Stone, 2010). This suggests that different types of RING E3s are inversely regulated by ABA depending on their modes of action. Finally, we could not rule out the possibility that AtAIRP1 may exert its effects indirectly on the plant responses to ABA and dehydration stress through the elevation of overall cellular metabolism and/or nonspecific adaptive capacities to avoid or tolerate unfavorable growth conditions.

ABA increases H$_2$O$_2$ production in guard cells, which is a prerequisite step for stomatal closure (Wang and Song, 2008; Cho et al., 2009; Cutler et al., 2010). AtAIRP1-overexpressing plants produced more H$_2$O$_2$, in response to ABA than did wild-type and atairp1 mutant plants (Fig. 7C), indicating that AtAIRP1 is a positive regulator in ABA-induced ROS production. Among 10 NADPH oxidase catalytic subunits, AtbboH and AtbboHf function in ABA-triggered ROS production in guard cells (Kwak et al., 2005). It was suggested that OST1 (SnRK2E) protein kinase acts upstream of ROS production in stomatal closure (Mustilli et al., 2002; Cho et al., 2009). At present, however, the mechanism by which AtAIRP1 mediates enhanced H$_2$O$_2$ production remains to be determined. Therefore, to elucidate a more detailed mode of action of AtAIRP1, it is essential to identify its target proteins. We are currently making an effort to isolate AtAIRP1-interacting proteins by means of yeast two-hybrid screening and matrix-assisted laser-desorption ionization time of flight mass spectrometry proteomic approaches. The results of these experiments will provide a better understanding of the cellular functions of RING E3 Ub ligases with regard to drought stress responses in higher plants.

**MATERIALS AND METHODS**

**Plant Materials and Stress Treatments**

Arabidopsis (Arabidopsis thaliana) ecotype Columbia was used in this study. Columbia seeds were soaked in a 30% bleach solution for 10 min and then rinsed 10 times with sterilized water. Seedlings were grown in MS medium (Duchefa Biochemie) containing 1% to 3% Suc and 0.8% phytoagar (pH 5.7) or in soil (sunshine mix 5; SunGro) in a growth chamber at 22°C with a 16-h-light/8-h-dark cycle. The atairp1 T-DNA insertion mutant (SALK_110094) was obtained from the Arabidopsis Biological Resource Center. Wild-type, atairp1 mutant, 3SS:AtAIRP1-sGFP, and atairp1/3SS:AtAIRP1-sGFP plants were subjected to drought, cold, high-salinity, and ABA treatments as described previously (Cho et al., 2006b, 2008).

**Sequence Homology and Phylogenetic Analyses**

Protein homology searches were performed with the BLASTP program (http://www.ncbi.nlm.nih.gov/BLAST/). Selected amino acid sequences were aligned using ClustalW software (http://workbench.sdsc.edu). The multiple alignment results were edited in the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/). Phylogenetic trees were generated using the neighbor-joining method (Tamura et al., 2007) in MEGA software version 4. Bootstrap values were supported from 10,000 replicates. Branch lengths indicated divergence distance, which was calculated using the scale at the bottom of Figure 2D. Numbers on the branches designate percentage bootstrap support.

**RT-PCR and Real-Time qRT-PCR Analyses**

Total RNA was extracted from light-grown, 10-d-old seedlings that had been subjected to stress and ABA treatments by an Easy-spin total RNA extraction kit (Intron Biotechnology) following the manufacturer’s manual. Each cDNA was synthesized using 2 μg of total RNA with the RevertAid first-strand cDNA synthesis kit (Fermentas). RT-PCR was performed as described previously (Joo et al., 2006). Primers used in RT-PCR analyses are listed in Supplemental Table S1. RT-PCR products were separated on 1% agarose gels by electrophoresis and imaged on a Gel-Doc XR (Bio-Rad). qRT-PCR was performed on an iQ5 light cycler (Bio-Rad) using SYBR Premix Ex Taq II (Takara). Data were obtained using the iQ5 optical system software (Bio-Rad). Data were normalized to the IQ5 light system software (Bio-Rad). Data were normalized to the glyceraldehyde-3-phosphate dehydrogenase C subunit mRNA levels.

**In Vitro Self-Ubiquitination Assay**

Full-length AtAIRP1 cDNA was amplified with EcoRI-F1 and BamHI-R1 primers (Supplemental Table S1). PCR products were cleaved with BamHI and EcoRI and inserted into a pMAL-C2 vector (New England Biolabs). The AtAIRP1 mutant was constructed with substitution of the His-127 residue with Tyr in the RING motif using the QuickChange site-directed mutagenesis kit (Stratagene) and H127Y-F1 and H127Y-R1 primers (listed in Supplemental Table S1). These plasmids were expressed in E. coli. Full-length AtAIRP1 in the vector pGEX-KG was purified by affinity chromatography using amylose resin (New England Biolabs). Purified MBP-AtAIRP1 and MBP-AtAIRP1H127Y proteins (500 ng) were mixed with Arabidopsis E2 (UBC5, UBC8, UBC10, or UBC13) in ubiquitination reaction buffer (50 mM Tris-HCl, pH 7.5, 4 mM ATP, 0.5 mM dithiothreitol, and 2.5 mM MgCl$_2$) including 100 ng of Arabidopsis E1 (UBA1)
and 5 μg of Ub and incubated at 30°C for 2 h as described by Cho et al. (2006a). Reaction samples were separated by 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore) using a semidry transfer cell (Bio-Rad). Immunoblotting was performed with anti-MBP antibody (New England Biolabs) or anti-Ub antibody (Santa Cruz Biotechnology) as described by Son et al. (2010).

**Subcellular Localization**

The 35S:AtAIRP1-sGFP and 35S:sGFP plasmids were constructed using pBII21 transient expression vectors. The constructed fusion genes were transformed into wild-type Arabidopsis protoplasts by means of PEG treatment (Lee et al., 2009). The expression of AtAIRP1-sGFP and sGFP was monitored 12 h after transformation. Transformed protoplasts were placed on the slide glass and observed using a fluorescence microscope (BX51; Olympus). Images were acquired with a 1600 CCD camera (PCI) and analyzed with Image Pro Plus software (Media Cybernetics). AREB1-sGFP was used as a specific control. All primers used in this experiment are listed in Supplemental Table S1.

**Construction of 35S:AtAIRP1-sGFP and atairp1/35S: AtAIRP1-sGFP Transgenic Plants and Drought Phenotype Analysis**

The full-length AtAIRP1 cDNA was amplified by PCR using a BamHI-F1 and EcoRI-R1 primer set (Supplemental Table S1). PCR products were digested with BamHI and EcoRI and inserted into a modified pENTR vector (Invitrogen). AtAIRP1-sGFP and sGFP clones were translocated into pEYcage 100 destination vectors by a LR recombination reaction (LR Clonase II; Invitrogen) and transformed into Arabidopsis using an Agrobacterium tumefaciens-mediated floral dip method (Joo et al., 2006). To obtain independent transgenic lines, the collected seeds were selected for on half-strength MS plates containing 25 μg mL⁻¹ BASTA. The presence and expression of the transgenes were confirmed by genomic Southern blots, RT-PCR, and protein gel-blot analysis as described by Lee et al. (2009). Homozygous T3 lines were obtained by further self-crossing and used for phenotypic analysis.

Lightgrown, 2-week-old wild-type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants were subjected to drought stress by lowering the water potential for 10 to 12 d as described by Kim et al. (2010). Three days after rewatering, surviving plants were counted. For the cut root resorption loss experiment, detached rootlet leaves were placed on petri dishes at room temperature with approximately 60% humidity under dim light. The weights of rootlet leaves were measured at various times. Leaf water loss was expressed as a percentage of initial fresh weight.

**DAB Staining**

DAB staining was used to detect the accumulation of H₂O₂ (Thordal-Christansen et al., 1997). Lightgrown, 4-week-old wild-type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants were treated with 100 μM ABA for 2 h and incubated with 100 μg mL⁻¹ DAB solution. DAB-stained samples were boiled in 97% (v/v) ethanol to remove chlorophyll. The presence and levels of H₂O₂ in leaves were visualized as a dark brown color.

**Germination Assay and Root Growth Measurement**

Germination assays were performed in triplicate with 30 to 35 seeds. Surface sterilized seeds 3 d after imbibition of wild-type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants were grown on MS medium containing 0, 0.1, 0.5, or 1 μM ABA (Sigma-Aldrich) at 22°C with a 16-h-light/8-h-dark photoperiod. The percentages of radicle emergence and cotyledon greening were determined after 3 and 7 d, respectively. For root growth measurements, seeds were grown vertically for 10 d on MS medium containing appropriate concentrations of ABA. Scanned images of seedling roots were analyzed with Scion Image software (www.scioncorp.com).

**Stomatal Aperture Measurement**

Mature leaves of light-grown, 4-week-old wild-type, atairp1, 35S:AtAIRP1-sGFP, and atairp1/35S:AtAIRP1-sGFP plants were used for the observation of mature stomata. Detached leaves were incubated in stomatal opening solution containing 10 mM KCl, 100 μM CaCl₂, and 10 mM MES, pH 6.1, for 2 h and then transferred to stomatal opening solution supplemented with various concentrations of ABA (0, 0.1, 1, and 10 μM) for 2 h. Subsequently, the adaxial surface of each leaf was applied to 3M clear tape to peel off the epidermal layer. Epidermal strips were mounted on glass slides and observed with an Olympus BX51 microscope. Images were captured with a cool CCD camera (PCO 1600) and imported into Image Pro Plus software (Media Cybernetics). The ratio of width to length of the stomata was measured using Multiprobe version 3.1 software (Fuji Film). Over 60 guard cells from each sample were used to measure stomatal aperture.

**Supplemental Data**

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

**Supplemental Figure S1.** DNA gel-blot analysis of wild-type (WT) and transgenic Arabidopsis plants.

**Supplemental Figure S2.** Overexpression of AtAIRP1-sGFP in wild-type (WT) Arabidopsis plants.

**Supplemental Figure S3.** DNA gel-blot analysis of wild-type (WT) and two independent T3 atairp1/35S:AtAIRP1-sGFP transgenic Arabidopsis plants.

**Supplemental Figure S4.** Overexpression of AtAIRP1-sGFP in atairp1 loss-of-function mutant plants.

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