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Correspondence: Dr. Woo Taek Kim

Department of Systems Biology
College of Life Science and Biotechnology
Yonsei University
Seoul 120-749
Korea
Tel: 82-2-2123-2661 (O), 82-2-313-2661 (L)
Fax: 82-2-312-5657
E-Mail: wtkim@yonsei.ac.kr

Research Category: Systems and Synthetic Biology
The Arabidopsis RING E3 Ubiquitin Ligase AtAIRP3/LOG2 Participates in Positive Regulation of High Salt and Drought Stress Responses

Jong Hum Kim and Woo Taek Kim*

Department of Systems Biology, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea (J.H.K., W.T.K.)

1This work was supported by grants from the National Research Foundation (Project No. 2010-0000782 funded by the Ministry of Education, Science, and Technology, Republic of Korea) and the National Center for GM Crops (Project No. PJ008152 of the Next Generation BioGreen 21 Program funded by the Rural Development Administration, Republic of Korea) to W.T.K.

*Corresponding author; e-mail wtkim@yonsei.ac.kr; fax 82-2-312-5657.
RING E3 ubiquitin (Ub) ligases have been implicated in cellular response to the stress hormone abscisic acid (ABA) as well as to environmental stresses in higher plants. Here, an atairp3 (Arabidopsis thaliana ABA insensitive RING protein 3) loss-of-function mutant line was isolated due to its hyposensitivity to ABA during its germination stage as compared to wild-type plants. AtAIRP3 contains a single C3HC4-type RING motif, a putative myristoylation site, and a DAR2 domain. Unexpectedly, AtAIRP3 was identified as LOG2 (Loss of GDU2), which was recently shown to participate in an amino acid export system via interaction with GDU1 (GLUTAMINE DUMPER1). Thus, AtAIRP3 was renamed as AtAIRP3/LOG2. Transcript levels of AtAIRP3/LOG2 were upregulated by drought, high salinity, and ABA, suggesting a role for this factor in abiotic stress responses. The atairp3/log2-2 knock-out mutant and 35S:AtAIRP3-RNAi knock-down transgenic plants displayed impaired ABA-mediated seed germination and stomata closure. Co-suppression and complementation studies further supported a positive role for AtAIRP3/LOG2 in ABA responses. Suppression of AtAIRP3/LOG2 resulted in marked hypersensitive phenotypes toward high salinity and water deficit relative to wild-type plants. These results suggest that Arabidopsis RING E3 AtAIRP3/LOG2 is a positive regulator of the ABA-mediated drought and salt stress tolerance mechanism. Using yeast two-hybrid, in vitro and in vivo immunoprecipitation, cell-free protein degradation, and in vitro ubiquitination assays, RD21 (Responsive to Dehydration 21) was identified as a substrate protein of AtAIRP3/LOG2. Collectively, our data suggest that AtAIRP3/LOG2 plays dual functions in ABA-mediated drought stress responses and in an amino acid export pathway in Arabidopsis.
INTRODUCTION

The ubiquitin (Ub)-26S proteasome system is an indispensable mechanism that regulates many crucial eukaryotic cellular processes, such as cell cycle progression, cell signaling, DNA repair, protein trafficking, and biotic and abiotic stress responses (Welchman et al., 2005; Dreher and Callis, 2007; Vierstra, 2009; Sadanandom et al., 2012). The ubiquitination pathway proceeds through a cascade of three enzymatic reactions catalyzed by E1 Ub-activating enzymes, E2 Ub-conjugating enzymes, and E3 Ub ligases (Guerra and Callis, 2012; Sadanandom et al., 2012). The presence of multi-isoforms of E3 Ub ligases in this system indicates that E3s determine the specificity of target proteins and facilitate the diverse functions of the ubiquitination system in many cellular processes (Vierstra, 2009; Sadanandom et al., 2012).

The E3 Ub ligases are divided into two groups. The first group is comprised of really-interesting-new-gene (RING)/U-box and homology to E6-AP carboxyl terminus (HECT) E3s, which act as a single subunit (Dreher and Callis, 2007; Yee and Goring, 2008; Vierstra, 2009). The second group is composed of multi-subunit Ub ligases, including the Skp1-cullin-F-box (SCF) complex and the anaphase-promoting complex (APC) (Vierstra, 2009; Park et al., 2011; Sadanandom et al., 2012; Seo et al., 2012). In the Arabidopsis genome, at least 477 genes encode putative RING E3 Ub ligases, and thus, these ligases are the most abundant E3s among single subunit-type Ub ligases and the third largest gene family in Arabidopsis (Kraft et al., 2005; Stone et al., 2005; Vierstra, 2009). This abundance is characteristic of plants, suggesting a potential correlation between diversity of RING E3s and the sessile life cycle of plants (Vierstra RD, 2009).

Recent reports indicate that RING E3s play roles in the cellular responses to the stress hormone ABA and to environmental stresses in higher plants (Lyzenga and Stone, 2012; Sadanandom et al., 2012). In particular, RING Ub ligases are involved in the drought stress responses. SDIR1 enhanced drought stress response by positively regulating ABA signaling at the upstream of the ABF transcription factors in Arabidopsis (Zhang et al., 2007). CaRma1H1, which is an ER-localized RING E3, increased dehydration stress tolerance through down-regulation of water channel protein aquaporin levels in transgenic Arabidopsis plants (Lee et al., 2009). Arabidopsis RING RHA2a/2b and AtRDUF1/2 function as positive
regulators in both ABA signaling and water stress responses (Bu et al., 2009; Li et al., 2011; Kim et al., 2012). On the other hand, DRIP-RING E3 serves as a negative regulator in drought stress response in Arabidopsis (Qin et al., 2008). It ubiquitinates and induces proteasomal degradation of drought-induced transcription factor DREB2A. Arabidopsis RGLG2 and rice OsDSG1, OsRDCP1, and OsDIS1 were recently reported as either negative or positive regulators in response to dehydration stress (Park et al., 2010; Bae et al., 2011; Ning et al., 2011; Cheng et al., 2012). These studies are in agreement with the notion that different RING E3 Ub ligases participate in the defense mechanism against drought conditions in a positive or negative manner in both monocot and dicot model plants.

The subgroups of the abovementioned RING E3 genes are either upregulated or down-regulated by water deficit. In addition, the protein stability and subcellular localization of the RING E3s are also affected by environmental stresses (Lyzenga and Stone, 2012). Without ABA treatment, KEG1 is auto-ubiquitinated, and thereby, its cellular levels are regulated depending on the presence or absence of ABA (Liu and Stone, 2010). The RGLG2-GFP fusion protein translocates from the plasma-membrane to the nucleus following salt stress treatment in Arabidopsis roots (Cheng et al., 2012). These observations suggest that the modes of action of plant RING E3s are modulated at various levels of cellular processes. Although the physiological roles of different RING E3s in response to environmental stresses have been elucidated, large numbers of RING Ub ligases still remain uncharacterized in higher plants.

In our previous studies, we collected 100 different Arabidopsis T-DNA-inserted loss-of-function mutants of RING E3 Ub ligases and investigated their phenotypes with respect to the ABA sensitivity of the germination stage. In these studies, the C3H2C3-type AtAIRP1 and C3HC4-type AtAIRP2 RING E3s were found to play positive and combinatory roles in ABA-mediated drought stress responses (Ryu et al., 2010; Cho et al., 2011).

In this report, atairp3 (Arabidopsis thaliana ABA insensitive RING protein 3), a line with the At3g09770 locus knocked out, was isolated and investigated. Akin to atairp1 and atairp2, atairp3 progeny displayed significantly reduced ABA responses during germination and in post-germination stages. AtAIRP3 was a C3HC4-type RING E3 Ub ligase and identified as LOG2 (Loss of GDU2), which was previously shown to be involved in amino acid export via interaction with GDU1 (GLUTAMINE DUMPER1) (Pratelli et al., 2012). Transcript levels of AtAIRP3/LOG2 were upregulated by drought, high salinity, and exogenous ABA
treatments. The atairp3/log2-2 loss-of-function mutant lines exhibited impaired ABA-mediated stomata closure and hypersensitive phenotypes in response to high salinity and drought. Using yeast two-hybrid, in vitro and in vivo immunoprecipitation (IP), cell-free protein degradation, and in vitro ubiquitination assays, we identified RD21 (Responsive to Dehydration 21) as a substrate protein of AtAIRP3. Collectively, our data indicate that the Arabidopsis C3HC4-type RING E3 Ub ligase AtAIRP3/LOG2 is a positive regulator of the ABA-mediated drought and salt stress tolerance mechanism via ubiquitination of RD21. These results further suggest that AtAIRP3/LOG2 plays dual functions in drought stress response and amino acid transport in Arabidopsis.

RESULTS

ABA-induced RING E3 Ub Ligase AtAIRP3 Was Identified as the Recently Isolated LOG2 (Loss of GDU2)

Previously, 100 different Arabidopsis T-DNA-inserted loss-of-function mutant lines of RING E3 Ub ligases were collected and examined in terms of ABA sensitivity. In those studies, the C3H2C3-type AtAIRP1 and C3HC4-type AtAIRP2 RING E3s were identified as positive regulators in ABA-mediated drought stress responses (Ryu et al., 2010; Cho et al., 2011). In this study, atairp3 (Arabidopsis thaliana ABA insensitive RING protein 3) was isolated and investigated. Similar to atairp1 and atairp2, the atairp3 progeny exhibited reduced sensitivity toward ABA. Figure 1A shows that the percentage of atairp3 seedlings (49.3%) that germinated was markedly higher than that of wild-type seedlings (6.7%) in the presence of 0.5 μM ABA for 5 days. The germination percentage of atairp3 with ABA was comparable to those of atairp1 (52.0%) and atairp2 (45.3%) (Fig. 1A).

The AtAIRP3 gene (GenBank accession number NC_003074), which is located on chromosome 3, was comprised of 1,922 bp with 3 exons and 2 introns. The atairp3 mutant line (Sail_729_A08) contained double T-DNA insertions in the first intron after nucleotides 873 and 887 in an antisense orientation (Fig. 1B). Genotyping PCR verified homozygous atairp3 progeny, and RT-PCR further confirmed that, although partial mRNAs were still detectable, full-length AtAIRP3 transcripts were absent in the homozygous line (Fig. 1C). The predicted protein encoded by AtAIRP3 contains 388 amino acid residues with a molecular
mass of 42.85 kDa (Supplemental Fig. S1). AtAIRP3 harbors a single C3HC4-type RING motif in its C-terminal region, a putative myristoylation site in the N-terminal region, and a DAR2 domain in the central region (Fig. 1D). To examine whether AtAIRP3 contains an E3 Ub ligase activity, the MBP-AtAIRP3 fusion construct was expressed in *E. coli*. The full-length MBP-AtAIRP3 was highly insoluble in bacterial cells under our experimental conditions. The MBP-AtAIRP3\textsuperscript{101-388}, an N-terminal partial deleted form, was expressed in the soluble fraction and used for an in vitro self-ubiquitination assay. As expected, MBP-AtAIRP3\textsuperscript{101-388} displayed in vitro Ub ligase activity in the presence of ATP, E1, and E2 (Supplemental Fig. S2). Search of the Arabidopsis database (The Arabidopsis Information Resource, http://www.arabidopsis.org) revealed that AtAIRP3 is identical to LOG2 (Loss of GDU2), which was recently reported to be involved in amino acid export (Pratelli et al., 2012). LOG2 interacted with GDU1 (GLUTAMINE DUMPER1) and the LOG2-GDU1 complex was proposed to play a significant role in the regulation of amino acid export. The identity of AtAIRP3 as LOG2 was unexpected as AtAIRP3 was highly inducible by the stress hormone ABA (see Figure 2), while LOG2 was identified as an interacting partner of a subunit of amino acid exporter complex (Pratelli et al., 2012). AtAIRP3 was renamed as AtAIRP3/LOG2.

**AtAIRP3/LOG2 was Induced by Drought, High Salinity, and ABA Treatment**

The fact that the *atairp3/log2-2* mutant seedlings were hyposensitive to the stress hormone ABA (Fig. 1A) raised the possibility that *AtAIRP3/LOG2* is a stress-related gene. To follow-up on this possibility, RT-PCR and real-time qRT-PCR experiments were conducted. The steady state levels of *AtAIRP3/LOG2* mRNAs were slightly enhanced by drought (1.3-1.6-fold) and high salinity (1.2-2.0-fold) (Fig. 2A and B). In addition, gene expression was elevated 3.5- and 4.5-fold after 1.5 and 3.0 h of treatment with ABA (100 \( \mu \)M), respectively. The magnitude of induction of *AtAIRP3/LOG2*, however, was less than those of known marker genes, *RD29A* for drought and salt and *RAB18* for ABA (Fig. 2A and B).

A promoter-GUS assay demonstrated that *LOG2* was mainly expressed in vascular tissues in vegetative organs and in the style and pollen grains in reproductive organs (Pratelli et al., 2012). In addition to these basal levels of expression, *AtAIRP3/LOG2* promoter activity
increased in both leaves and roots in response to drought, salt, and ABA treatments (Fig. 2C). These results suggest that *AtAIRP3/LOG2* is controlled at the transcriptional level in response to stress and ABA. Thus, we speculated that *AtAIRP3/LOG2* is involved not only in the amino acid export system (Pratelli et al., 2012) but also in the ABA-mediated stress response (Fig. 1A and Fig. 2) in Arabidopsis.

**Suppression of *AtAIRP3/LOG2* Resulted in Hyposensitivity to ABA with Respect to Germination Rates and Stomatal Movement**

To examine the role of *AtAIRP3/LOG2* in ABA and stress responses, the phenotype of the *atairp3/log2-2* knock-out line was tested. First, wild-type and mutant seeds were germinated in the absence or presence of ABA (0, 0.2, 0.5, or 1 μM), and germination percentages were counted in terms of radicle emergence and cotyledon greening after 3 and 5 days, respectively (Fig. 3A). In the presence of 0.5 μM ABA, 79.8% and 88.2% of the wild-type and *atairp3/log2-2* seeds yielded radicles, respectively (upper panel, Fig. 3B). In the presence of 1.0 μM ABA, only 19.7% of the wild-type seeds were able to germinate, while 47.9% of the mutant seeds germinated. Differences between the wild-type and mutant seeds were even more distinguishable in cotyledon greening. With 0.5 μM ABA, only approximately 2% of wild-type seeds developed normal green cotyledons (lower panel, Fig. 3B). In contrast, 56.3% of *atairp3/log2-2* seeds displayed functional green cotyledons. These results, along with those in Figure 1A, indicate that the *atairp3/log2-2* knock-out seeds were hyposensitive to ABA during germination as compared to wild-type seeds.

Secondly, ABA-mediated stomatal behavior of wild-type and *atairp3/log2-2* plants was monitored. Light-grown, 4-week-old mature leaves were treated with ABA for 2 h, and stomatal movement profiles were measured as the ratio of width to length (Fig. 3C). Average stomatal apertures of wild-type leaves were concurrently reduced as concentrations of ABA increased (0, 0.1, 1.0, and 10 μM) from 0.19±0.025 to 0.13±0.015, 0.1±0.013, and 0.07±0.01, respectively (Fig. 3D). However, decreases in the stomatal apertures of *atairp3/log2-2* mutant leaves in response to same range of ABA concentrations appeared to be less evident. They were reduced from 0.19±0.025 to 0.16±0.016, 0.12±0.01, and 0.11±0.016.

To further unearth the effects of *AtAIRP3/LOG2*-2 suppression, RNAi-mediated knock-
down transgenic lines (35S:AtAIRP3-RNAi) were constructed (Supplemental Fig. S3). The level of AtAIRP3/LOG2 mRNAs was significantly reduced in independent T4 35S:AtAIRP3-RNAi lines #1 and #2 as determined by RT-PCR (Fig. 1B). As shown in Figure 3, phenotypes of the 35S:AtAIRP3-RNAi knock-down plants in response to ABA were intermediate between wild-type and atairp3/log2-2 plants with respect to both germination percentages and stomatal behaviors. These results indicate that ABA sensitivity during germination and post-germination growth is positively correlated with the expression level of AtAIRP3/LOG2.

Co-suppression and Complementation Experiments

To obtain more detailed evidence for the roles of AtAIRP3/LOG2 in the response of Arabidopsis to ABA, we generated transgenic Arabidopsis plants that overexpressed AtAIRP3/LOG2 under the control of the CaMV 35S promoter. Several independent T4 transgenic progeny (35S:AtAIRP3/LOG2) were obtained and used for phenotypic assays. In contrast to what we expected, the 35S:AtAIRP3/LOG2 seedlings (lines #1 and #2) displayed hyposensitivity toward ABA in germination stage. Their germination percentages (cotyledon greening) were similar (28.7% for line #1 and 30.0% for line #2) to those of atairp3/log2-2 (27.3%) in the presence of 0.5 μM ABA (Fig. 4A). Under the same ABA concentration, less than 4.0% of the wild-type seeds successfully germinated. We repeated the over-expression experiments and obtained similar results. This led us to hypothesize that ectopic expression of AtAIRP3/LOG2 with the CaMV 35S promoter may cause co-suppression in Arabidopsis. RT-PCR analysis indeed demonstrated that the AtAIRP3/LOG2 transcripts were significantly reduced in the 35S:AtAIRP3/LOG2 lines relative to those in wild-type plants (Fig. 4B).

We next carried out complementation experiment. A genomic fragment that contained the 5’ upstream region (1.35 kbp in length) and the entire coding region (1.85 kbp in length) of AtAIRP3/LOG2 was transformed into atairp3/log2-2 mutant plant (Fig. 4C). Independent T3 complementation transgenic lines (atairp3/AtAIRP3) were subsequently obtained. RT-PCR analysis showed that AtAIRP3/LOG2 mRNA levels were effectively restored in atairp3/AtAIRP3 transgenic plants (Fig. 4D). In addition, atairp3/AtAIRP3 transgenic seeds displayed increased sensitivity to ABA in germination as compared to the atairp3/log2-2 seeds (Fig. 4E). The ABA sensitivities of the complementation lines were intermediate between those exhibited by wild-type and atairp3/log2-2 seeds. Thus, introduction of the
functional *AtAIRP3/LOG2* gene into *atairp3/log2-2* mutant plants partially rescued the loss-of-function ABA-insensitive phenotype. Collectively, the results of the co-suppression and complementation studies (Fig. 4) in combination with the results of the knock-out experiments (Fig. 3) strongly suggest that AtAIRP3/LOG2 is positively involved in the response of Arabidopsis to ABA.

*atairp3/log2-2* Mutant and *35S:AtAIRP3-RNAi* Knock-down Transgenic Plants Were Hypersensitive to High Salinity

We went on to estimate the capacity of the *atairp3/log2-2* mutant and the *35S:AtAIRP3-RNAi* transgenic (lines #1 and #2) plants to respond to high salinity. Wild-type, mutant, and *RNAi* seedlings were grown for 5 days under normal growth conditions and then transported into medium supplemented with 150 mM NaCl. Their morphological changes were monitored after 14 days. In the presence of NaCl, growth of wild-type plants was significantly retarded, and their leaves accumulated brown precipitates (Fig. 5A). The *atairp3/log2-2* mutant plants showed even more evident developmental anomalies after salt treatment. Their leaves became pale-green and yellowish with markedly reduced growth (Fig. 5A). The degree of aberrant growth of the *RNAi*-knock-down leaves was intermediate between the wild-type and knock-out plants. Consistently, the average chlorophyll content in the mutant leaves was approximately 30% (75.4±23.52 μg/g fresh weight) of that in wild-type leaves (239±23.50 μg/g fresh weight) upon salt stress (Fig. 5B). Under the same salt conditions, the average chlorophyll levels in the *RNAi* leaves were 113.5±45.8 to 133.7±28.1 μg/g fresh weight.

In addition to leaf growth, root growths of *atairp3/log2-2* mutant and *35S:AtAIRP3-RNAi* knock-down plants were also severely impaired by high salinity as compared to wild-type plants. Before salt treatment, morphology of roots in wild-type, knock-out, and knock-down plants was similar. Elongation of wild-type roots was significantly inhibited by 100-150 mM NaCl treatments for 14 days (Fig. 5C). While elongation of mutant roots appeared to be somewhat similar to that of wild-type roots, lateral root formation in *atairp3/log2-2* and *RNAi* plants was reduced more dramatically by high salinity than that in wild-type plants (Fig. 5C). Thus, both the leaf and root tissues of *atairp3/log2-2* mutant and *35S:AtAIRP3-RNAi* knock-down plants exhibited hypersensitive phenotypes in response to high salinity.
Some of the *atairp3/log2-2* mutant leaves were severely discolored and became whitish or dark brown (indicated by red and brown arrows, respectively, in Fig. 5A). To further evaluate the sensitivity of leaf tissue to high salinity, salt-treated leaves were subjected to Evans blue staining, which assesses cell death. Mature, healthy leaves of wild-type and *atairp3/log2-2* plants were incubated with 300 mM NaCl for 6-12 h and subsequently treated with Evans blue staining solution. The degree of cell death was determined by the strength of the dark blue color (Watanabe and Lam, 2006). Upon salt treatment, cell death was significantly induced in wild-type leaves. In addition, more dramatic cell death was clearly detected in *atairp3/log2-2* leaves (Fig. 5D), indicating that *atairp3/log2-2* leaves were more susceptible to NaCl treatment than wild-type leaves. Thus, suppression of *AtAIRP3/LOG2* resulted in increased sensitivity to high salinity.

*atairp3/log2-2* Knock-out Mutant and 35S:*AtAIRP3-RNAi Knock-down Transgenic Plants Were More Susceptible to a Water Deficit than Wild-type Plants

Because the *atairp3/log2-2* and 35S:*AtAIRP3-RNAi lines were hyposensitive to ABA (Fig. 3) and susceptible to high salinity (Fig. 5), we speculated that suppression of *AtAIRP3/LOG2* alters the drought stress responses. Therefore, wild-type, *atairp3/log2-2*, and RNAi plants were subjected to dehydration stress. These plants were grown for 3 weeks in pots under the normal growth conditions and grown for another 14 days without watering. Survival of these water-stressed plants was monitored 3 days after re-watering. Under these experimental conditions, 54.2% (26 of 48) of wild-type plants resumed their growth after re-watering (Fig. 6A); however, only 13.6% (6 of 44) of the *atairp3/log2-2* plants resumed growth after re-watering. In addition, survival of 35S:*AtAIRP3-RNAi plants was also very low (12.5% [6 of 48] for line #1 and 11.1% [5 of 45] for line #2). Thus, suppression of *AtAIRP3/LOG2* resulted in a greatly reduced tolerance to dehydration stress.

Furthermore, mutant and RNAi transgenic leaves lost their water content more rapidly than wild-type leaves. After a 2-h incubation at room temperature, detached wild-type and mutant leaves lost approximately 23.8% and 27.3% - 30.4% of their fresh weight, respectively (Fig. 6B). After 4 h of incubation, the fresh weight of wild-type leaves decreased approximately 34.7%, while that of mutant and knock-down leaves was reduced by 39.1% - 40.9% (Fig. 6B). These results indicate that *atairp3/log2-2* knock-out and 35S:*AtAIRP3-
RNAi knock-down plants were more susceptible to a water deficit than wild-type plants. Overall, our phenotypic analyses suggest that suppression of AtAIRP3/LOG2 results in hyposensitivity to ABA (Fig. 3) and markedly reduces tolerance not only to high salinity (Fig. 5) but also to drought (Fig. 6) in Arabidopsis.

**AtAIRP3/LOG2 Interacts with RD21**

To uncover the cellular mechanism by which AtAIRP3/LOG2 participates in drought stress responses, we performed yeast two-hybrid screening using a cDNA library prepared from 3-day-old etiolated seedlings and a full-length AtAIRP3/LOG3 cDNA as bait. Several positive colonies that grew in the presence of AtAIRP3/LOG2 in the three-minus selection medium (SD/-Trp/-Leu/-His) with 10 mM 3-amino-1,2,4,-triazole (3-AT) were identified. One of the positive yeast cells contained a partial cDNA encoding RD21 (Responsive to dehydration 21; At1g47128) (Fig. 7A). Arabidopsis RD21 was initially identified as a drought-inducible cysteine proteinase of the papain family (Koizumi et al., 1993). It is produced as a 57-kDa pre-proprotein and post-translationally cleaved to a 30-33-kDa mature protein (Yamada et al., 2001; Gu et al., 2012). The intermediate and mature forms of RD21 localize to the ER body and lytic vacuole in senescing Arabidopsis leaves (Hayashi et al., 2001; Yamada et al., 2001; Lampl et al., 2013).

To confirm the interaction between AtAIRP3/LOG3 and RD21 in yeast cells, we constructed four different truncated forms of RD21 (Fig. 7B) and repeated the yeast two-hybrid assay using RD21 as prey and AtAIRP3/LOG3 as bait under the four-minus (SD/-Leu/-Trp/-His/-Ade) growth conditions. Full-length RD21 was unable to interact with AtAIRP3/LOG2. In contrast, RD21 ΔN (the intermediate form) that contains the protease, proline-rich, and granulin domains and mRD21 (the mature form) that consists of only the protease domain interacted with AtAIRP3/LOG2 (Fig. 7C). These results indicate that the intermediate and mature forms of RD21 interact with AtAIRP3/LOG2 in yeast cells.

To corroborate the association of AtAIRP3/LOG2 and RD21, an in vitro IP experiment was carried out. AtAIRP3/LOG2-flag and mRD21-myc fusion proteins were expressed in *E. coli*. Purified recombinant proteins were co-incubated in the presence of an anti-flag affinity gel matrix. After extensive washing, the bound proteins were eluted with glycine (100 mM) buffer and subjected to immuno-blot analysis using anti-flag and anti-myc antibodies. The
results of Figure 7D revealed that mRD21A-myc protein was co-immunoprecipitated with AtAIRP3/LOG2-flag by anti-flag antibody. In contrast, mRD21-myc alone failed to bind the anti-flag affinity matrix. These results indicate that AtAIRP3/LOG2 and mRD21 interact in vitro.

Interaction between AtAIRP3/LOG2 and RD21 was further substantiated by an in vivo IP assay. The AtAIRP3/LOG2-flag and mRD21-myc gene constructs were expressed in tobacco (*Nicotiana benthamina*) leaves using an *Agrobacterium*-mediated transient expression method. Leaf crude extracts (1 mg protein) were immunoprecipitated with anti-flag affinity gel matrix. The bound proteins were eluted, separated by SDS-PAGE, and detected using anti-flag and anti-myc antibodies. AtAIRP3/LOG2-flag and mRD21-myc were co-immunoprecipitated by anti-flag antibody in leaf crude extracts expressing AtAIRP3/LOG2-flag and mRD21-myc fusion genes (Fig. 7E); however, mRD21-myc was not immunoprecipitated with the anti-flag antibody in tobacco leaf extracts expressing mRD21-myc fusion gene alone. These yeast two-hybrid and IP assays suggest that RD21 is one of the target proteins of AtAIRP3/LOG2.

**Ubiquitination of RD21 by AtAIRP3/LOG2**

The interaction between AtAIRP3/LOG2 and RD21 raised the possibility that the cellular level of RD21 is subject to control by the Ub-26S proteasome pathway. To test this possibility, an in vitro cell-free degradation assay was performed. Bacterially-expressed mRD21-myc protein was incubated for different time periods (0, 1.5, and 3 h) with crude extracts prepared from salt-treated (300 mM NaCl) 10-day-old wild-type seedlings. The protein levels were monitored over time by immuno-blotting with anti-myc antibody. The mRD21-myc level was rapidly reduced with wild-type cell-free extracts. After 1.5 h of incubation, approximately 90.1% of the protein was already degraded (Fig. 8A). However, mRD21-myc was stable during incubation with the wild-type crude extracts in the presence of MG132, an inhibitor of the proteasome complex. After 3 h of incubation with 50 μM MG132, only 5% of mRD21-myc was degraded (lane 3 h-M in Fig. 8A). These results revealed that MG132 hinders reduction of the mRD21-myc level, indicating proteasome-dependent degradation of RD21 with cell-free extracts. An identical experiment was conducted with crude extracts prepared from salt-treated *atairp3/log2-2* mutant seedlings. After a 1.5-h incubation, more than 40% of
mRD21-myc was still detected in the reaction mixture (Fig. 8A). Thus, degradation of mRD21-myc was significantly slowed down with *atairp3/log2-2* cell-free extracts as compared to wild-type extracts. This finding suggests that stability of RD21 is dependent, at least in part, on AtAIRP3/LOG2. Again, degradation of mRD21-myc was strongly hampered by MG132 with *atairp3/log2-2* extracts. RGA1 and Rubisco proteins were used for control experiments. RGA1 is regulated by the Ub-26S proteasome pathway (Dill et al., 2004; Lee et al., 2010), whereas Rubisco served as a loading control. The results showed that the proteasome-dependent fluctuation patterns of the RGA1-flag protein were very similar in the wild-type and *atairp3/log2-2* cell-free extracts, indicating that its degradation was independent of AtAIRP3/LOG2. As expected, the level of Rubisco remained constant in all the reaction mixtures examined (Fig. 8A). Overall, these results suggest that stability of RD21 is subject to control by the 26S proteasome complex in an AtAIRP3/LOG2-dependent manner.

We next performed an in vitro ubiquitination assay. Recombinant mRD21-myc and AtAIRP3/LOG2-flag were co-incubated in the presence or absence of Ub, ATP, E1 (Arabidopsis UBA1), and E2 (Arabidopsis UBC8) at 30°C for 1 h. The reaction mixture was analyzed by immuno-blotting using anti-myc antibody. Co-incubation of mRD21-myc and AtAIRP3/LOG2-flag gave rise to a high-molecular-mass band (left panel, Fig. 8B). Exclusion of E1 or E2 from the reaction mixture abrogated the ubiquitinated band. In addition, the AtAIRP3/LOG2-flag<sup>C319S</sup> derivative, in which the conserved Cys residue in the RING motif was replaced by Ser, was unable to ubiquitinate mRD21-myc (right panel, Fig. 8B). Taken together, the data presented in Figure 8, together with those in Figure 7, further support the view that RD21 is one of the substrate proteins of AtAIRP3/LOG2 in Arabidopsis.

**DISCUSSION**

As sessile organisms, land plants encounter diverse environmental stresses, including drought, high salinity, heavy metals, and extreme temperatures, during their life cycle (Boyer, 1982; Cushman and Bohnert, 2000). The prompt and appropriate responses of plants to these adverse growth conditions are inevitable factors that determine whether the plants survive and flourish. With respect to agricultural facet, the defense mechanisms by which plants retaliate to detrimental environmental stresses are closely tied with crop yield (Ahuja et al.,
Thus, examination of the cellular stress responses and development of stress-tolerant transgenic crops have steadily increased (Verslues and Juenger, 2011; Deikman et al., 2012).

Considerable evidence indicates that plant RING E3 Ub ligases mediate cellular responses to abiotic stresses (Lyzenga and Stone, 2012; Sadanandom et al., 2012). Modes of action of RING E3s appear to be diverse. These ligases work as either positive or negative factors and/or in either an ABA-dependent or -independent manner. RING E3s also function individually or in combination with other homologous RING E3s. Furthermore, these proteins localize to different cellular sites within plant cells.

In this report, 100 different Arabidopsis T-DNA-inserted loss-of-function mutants of RING E3 Ub ligases were screened with regard to ABA sensitivity during germination. The atairp3 mutant was isolated due to its hyposensitivity to ABA (Fig. 1). AtAIRP3 gene activity was upregulated by high salinity, drought, and ABA treatment, suggesting a role for this factor in abiotic stress responses (Fig. 2). AtAIRP3 is a RING E3 Ub ligase with a single C3HC4-type RING motif, a putative myristoylation site, and a DAR2 domain. Unexpectedly, AtAIRP3 was turned out to be identical to LOG2 that is involved in amino acid export at the plasma-membrane (Pratelli et al., 2012). LOG2 interacts and ubiquitinates GDU1, which is considered as a subunit of the amino acid export complex. Association of LOG2 with GDU1 was proposed to stabilize GDU1 localization at the plasma-membrane (Pratelli et al., 2012). Therefore, AtAIRP3 was renamed AtAIRP3/LOG2.

Detailed phenotypic analyses revealed that atairp3/log2-2 and 35S:AtAIRP3-RNAi plants exhibited markedly reduced sensitivity to ABA for both germination rates and stomatal movements (Fig. 3). Co-suppression and complementation data were also in good agreement with those of the knock-out and RNAi experiments (Fig. 4). In addition, suppression of AtAIRP3/LOG2 resulted in decreased tolerance to high salinity (Fig. 5) and drought conditions (Fig. 6). Therefore, AtAIRP3/LOG2 most likely regulates water stress responses and serves as a positive regulator in an ABA-dependent fashion. Based on these results, we speculate that AtAIRP3/LOG2 has dual functions in Arabidopsis: this protein participates in the ABA-mediated drought stress responses and in an amino acid export pathway.

Pratelli et al. (2012) showed that AtAIRP3/LOG2 was localized to the membrane fractions, particularly to the plasma-membrane. Their results indicated that myristoylation was important for the localization of AtAIRP3/LOG2 to the plasma-membrane and facilitated...
the association of AtAIRP3/LOG2 with GDU1. The AtAIRP3/LOG2-GDU1 complex may play a role in the regulation of amino acid export (Pratelli et al., 2012). With these in mind, we hypothesized that mechanism by which AtAIRP3/LOG3 regulates drought stress response may be also linked to the plasma-membrane. Membrane-associated RING E3s have recently been implicated in stress responses in plants (Guerra and Callis, 2012). For example, SDIR1 is an endomembrane-localized RING E3 that functions positively in ABA-associated drought stress tolerance (Zhang et al., 2007). ER-localized RING E3 CaRma1H1 enhances dehydration stress tolerance by ubiquitinating the water channel protein aquaporin PIP2;1 isoform in transgenic Arabidopsis plants (Lee et al., 2009). The mode of action of CaRma1H1 appeared to be ABA-independent. KEG RING E3 exhibits subcellular localization at both the endosomes and the nucleus. At the endosomes, it is involved in programmed cell death (PCD) in response to pathogen invasion (Gu and Innes, 2011), whereas, at the nucleus, this protein down-regulates the ABA-responsive transcription factor ABI5 (Stone at al., 2006; Liu and Stone, 2010). More recently, RGLG2 RING E3 was shown to localize to the plasma-membrane under normal growth conditions, but this protein moved into the nucleus in response to high salinity, where it negatively modulates salt-stress responses (Cheng et al., 2012).

Yeast-two-hybrid screening identified RD21 as a protein that interacts with AtAIRP3/LOG2. Subsequently, mRD21, the 30-kDa mature form of RD21, was shown to physically bind AtAIRP3/LOG2 on the basis of the yeast two-hybrid and in vitro and in planta IP assays (Fig. 7). The mRD21 protein was degraded by a 26S proteasome complex in an AtAIRP3/LOG2-dependent manner in cell-free degradation assay (Fig. 8A). Moreover, mRD21 was ubiquitinated in vitro by AtAIRP3/LOG2 (Fig. 8B). Notably, RD21 was previous identified as an ubiquitinated protein via MALDI-TOF + LC/MS proteomic analysis of Arabidopsis proteins (Manzano et al., 2008). Taking these results into account, it seems highly likely that RD21 is one of the target proteins of AtAIRP3/LOG2.

RD21 was initially isolated as a drought-induced cysteine proteinase (Koizumi et al., 1993). It localizes to the ER body and lytic vacuoles in senescing leaves and may act to aid in the cell death process under stress conditions and during leaf senescence by facilitating nitrogen recycling (Yamada et al., 2001; Hayashi et al., 2001). Protein disulfide isomerase-5 (PDI) is concomitantly targeted with RD21 from the ER through the Golgi to vacuoles and inhibits the proteinase activity of RD21 in the endothelial cells of developing Arabidopsis.
seeds (Ondzighi et al., 2008). Loss of PDI, which in turn resulted in increased RD21 proteinase activity, initiated premature PCD of the endothelium in developing seeds, suggesting a role for RD21 in PCD. C14 is the tomato homolog of RD21. Ectopic expression of C14 hindered Phytophthora infestans infection at the apoplast in tobacco (Bozkurt et al., 2011). All these results are consistent with the view that RD21 may be functional under stress conditions (e.g., drought, senescence, PCD, or pathogen attack) to remobilize the building blocks of macromolecules.

Currently, however, the functional relationships between AtAIRP3/LOG2 and RD21 in drought stress tolerance remain to be solved. Because RD21 contains cysteine proteinase activity, excessive release of RD21 from the vacuole may be harmful to cells. In this aspect, we are tempted to propose that AtAIRP3/LOG2 ubiquitinates and thus leads to proteasomal degradation of RD21 in response to drought to ward off the undesirable degradation of cellular proteins facilitated by RD21. AtAIRP3/LOG2 is a plasma-membrane-bound protein through its myristoylation without membrane-spanning domains (Pratelli et al., 2012). Hence, AtAIRP3/LOG2 may meet RD21 released from the vacuole in response to water stress at the cytosolic surfaces of the plasma-membrane. In fact, recent report suggested that RD21 that was sequestered in the ER body and vacuole is released to the cytoplasmic fractions during plant-pathogen (necrotrophic fungi) interactions (Lampl et al., 2013). RD21 is then associated with cytoplasmic AtSerpin1, a protease inhibitor. The AtSerpin-RD21 complex may limit the cellular damage caused during cell death. These results are in concordance with our current scenario of functional correlations of AtAIRP3/LOG2 and RD21 in drought stress responses. Alternatively, AtAIRP3/LOG2 and RD21 may interact with each other during the trafficking processes to the plasma-membrane and vacuole, respectively, under drought stress conditions. Yamada et al. (2001) showed that the intermediate (the protease + proline-rich + granulin domains) and mature (the protease domain) forms of RD21 were present in the vacuoles. AtAIRP3/LO2 is able to interact with the intermediate and mature forms but not with the pro-protein form of RD21 (Fig. 7B and 7C). Thus, interaction between AtAIRP3/LOG2 and RD21 during their trafficking pathway appears to be unlikely. Therefore, additional studies are required to uncover how AtAIRP3/LOG2 and RD21 are functionally correlated to expedite defense mechanism against drought conditions.

The 35S:AtAIRP3-RNAi knock-down plants exhibited phenotypes that were intermediate between those of wild-type and atairp3/log2-2 knock-out mutant plants in terms of ABA-
mediated inhibition of seed germination (Fig. 3A and 3B) and salt stress responses (Fig. 5). However, it should be noted that the RNAi and knock-out plants displayed comparable insensitivities in ABA-mediated stomatal closure (Fig. 3C and 3D). In addition, the RNAi plants showed greatly reduced tolerance to drought stress, and their survival rates (Fig. 6A) and leaf-water-loss rates (Fig. 6B) were very similar to those of knock-out mutant plants. These may indicate that AtAIRP3/LOG2 is more intimately associated with drought stress responses than with either ABA or salt stress responses.

The roles of AtAIRP3/LOG2 in exporting amino acids and in degrading RD21 do not seem to be unrelated with regard to the regulation of intra-cellular free amino acid levels. AtAIRP3/LOG2, along with GDU1, promotes amino acid export at the plasma-membrane, and AtAIRP3/LOG2 ubiquitinates RD21, which increases amino acid levels through its proteinase activity. Thus, two different functions of AtAIRP3/LOG2 may be inter-connected. In conclusion, our data presented in this report suggest that AtAIRP3/LOG2 plays dual functions in ABA-mediated drought stress response and in the amino acid export system in Arabidopsis.

MATERIALS AND METHODS

Plant Materials

*Arabidopsis thaliana* (ecotype Columbia-0) wild-type and T-DNA inserted *atairp3/log2*-2 loss-of-function mutant seeds were collected from the Arabidopsis Biological Resource Center (ABRC). The T-DNA inserted mutation was confirmed by genotyping PCR and RT-PCR. Seeds were treated with 30% bleach solution and washed 10 times with sterilized water to remove residual bleach solution. Seedlings were grown in medium containing Murashige and Skoog (MS) salt (Duchefa Biochmie, Haarlem, The Netherlands) and sucrose (1-3%) in phytoagar (0.8%) or in soil (Sunshine mix5; Sun Gro Horticulture, Agawam, MA, USA) at 22°C under long-day conditions (16 h light/8 h dark).

RT-PCR and Real-time Quantitative RT-PCR Analysis
Light-grown 10-day-old seedlings were subjected to dehydration by opening the lid of plant culture plate at growth chamber (22°C) for 1.5, 3 and 6 h. For NaCl and exogenous ABA treatment, seedlings were transferred to half strength MS liquid medium (Duchefa Biochmie) containing 300 mM NaCl or 100 μM ABA (Sigma-Aldrich, St. Louis, MO, USA) for 1.5 and 3 h.

Total RNA was isolated from stress- or ABA-treated 10-day-old wild-type, knock-out mutant, and RNAi knock-down seedlings with an Easy Spin plant total RNA extraction kit (Intron Biotechnology, Seoul, Korea) according to the manufacturer’s protocol. The cDNA was synthesized using an M-MLV cDNA synthesis kit (Enzynomics, Seoul, Korea) with 1.5 μg total RNA. RT-PCR was performed according to the established protocol described in the previous study (Seo et al., 2012). The DNA sequences of the primers used in this study are listed in Supplemental Table S1. PCR products were separated with agarose gel electrophoresis and visualized with Bio Rad Gel-doc (Bio-Rad Laboratories, Hercules, CA, USA). Real-time qRT-PCR was performed using an IQ5 light cycler (Bio-Rad Laboratories) as described by Ryu et al. (2010). The relative levels of the transcripts were normalized to the glyceraldehyde-3-phosphate dehydrogenase C subunit (GAPDHC) mRNA level, which was included as an internal control.

Amino Acid Sequence Alignment

The predicted amino acid sequences for AtAIRP3/LOG2 and its homologues were obtained from The Arabidopsis Information Resources (http://www.arabidopsis.org). Amino acid alignment was performed using ClustalX2.0 software (http://www.clustal.org), and the results were edited using the Genedoc software (http://www.nrbsc.org/gfx/genedoc). Functional domains in the predicted AtAIRP3/LOG2 were analyzed as described by Stone et al. (2005) and Marchler-Bauer et al. (2011).

Construction of AtAIRP3-promoter:GUS, 35S:AtAIRP3-RNAi Knock-down, 35S:AtAIRP3 Co-suppression, and atairp3/AtAIRP3 Complementation Transgenic Plants

For the promoter-GUS assay, the upstream region (1.5 kb) of AtAIRP3/LOG2 was
amplified from genomic DNA by PCR. The PCR product was ligated into the pJET blunt vector (Fermentas, Vilnius, Lithuania) and subcloned into the pCAMBIA1381 vector using the BamHI and NcoI sites.

For the RNAi suppression study, the C-terminal region (from 1126 bp to 1557 bp) of the AtAIRP3/LOG2 cDNA was amplified by PCR using RNAi primer sets (Supplemental Table S1), and the product was ligated into the pENTR vector (Invitrogen, Carlsbad, CA, USA), which harbored a GUS intron. The RNAi construct was inserted into the pEarleyGate100 vector (Earley et al., 2006), which contains the CaMV 35S promoter, using an LR clonase II enzyme (Invitrogen) reaction. For the 35S:AtAIRP3 co-suppression study, a full-length AtAIRP3/LOG2 cDNA was obtained by RT-PCR using gene-specific primers (Supplemental Table S1). The PCR product was cloned into the pJET blunt cloning vector (Fermentas). After confirming the correct reading frame of the gene construct by DNA sequencing, the plasmid containing a full-length AtAIRP3/LOG2 cDNA was ligated into the pENTR vector using the BamHI restriction enzyme. The cDNA clone was again subcloned into the pEarleyGate100 vector using the LR clonase II enzyme.

For the complementation study, genomic DNA that contained the 5’ upstream region (1.35 kbp) and the entire coding region (1.85 kbp) of AtAIRP3/LOG2 was amplified by gene-specific primers (Supplemental Table S1) and ligated into the pTOP blunt vector (Enzynomics). The construct was ligated into the pENTR vector and re-inserted into the pMDC163 vector (Curtis and Grossniklaus, 2003) via an LR reaction.

The constructed plasmids were transformed into Arabidopsis plants by the floral dipping method (Clough and Bent 1998) using Agrobacterium tumefaciens. Glufosinate-ammonium (BASTA; 25 μg/ml; Duchefa Biochmie) and hygromycin (35 μg/ml; Duchefa Biochmie) were used for the selection of transgenic lines. RT-PCR and genomic Southern blot analyses were performed to confirm the expression and copy number of the transgene in transgenic plants.

**Germination Assay and Stomata Aperture Measurement**

Seeds of wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3-RNAi (independent transgenic lines #1 and #2) plants were sterilized with a 30% bleach solution and sown on MS growth medium with various concentrations (0, 0.1, 0.5, or 1.0 μM) of ABA (Sigma-
Aldrich). Percentages of radicle emergence and cotyledon greening were counted at 3 and 5 days after sowing, respectively.

For stomata aperture analysis, rosette leaves of 4-week-old wild-type, \textit{atairp3/log2-2}, and 35S:AtAIRP3-RNAi (transgenic lines #1 and #2) plants were detached and incubated for 4 h in stomata opening solution (10 mM MES [pH 6.1], 100 µM CaCl$_2$, and 10 mM KCl) and transferred into the ABA-containing solution (0, 0.1, 1.0, or 10 µM) for 2 h. The adaxial side of the leaf epidermis was peeled off using 3M tape, mounted on slide glasses, and observed with an Olympus CX41 microscope (Olympus, Tokyo, Japan). Stomata images were photographed with a JUJAK560 CCD camera (Dixi Optics, Seoul, Korea) and analyzed using Photoshop CS4 software (Adobe Systems, San Jose, CA, USA). At least 35 stomata were analyzed for each experimental group.

**Phenotype Analysis in Response to High Salinity and Drought**

Wild-type, mutant, and \textit{RNAi} (independent transgenic T4 lines #1 and #2) seedlings were grown for 5 days under normal growth conditions and transferred into medium supplemented with 100 or 150 mM NaCl. Morphological abnormalities were observed after 14 days.

Light-grown, 3-week-old wild-type, \textit{atairp3/log2-2}, and 35S:AtAIRP3-RNAi (transgenic lines #1 and #2) plants were grown for 14 days without irrigation as described by Kim et al. (2010). After 3 days of irrigation, survival rates of drought-stressed plants were counted. To analyze the leaf-water-loss rates, the aerial parts of the plants were detached from the roots and incubated for 4 h on the bench at room temperature. Fresh weights of detached plants were measured by electric microbalance at various time points. In each assay, at least 10 plants were used. The experiments were performed four times with four different replicates.

**Quantification of Chlorophyll Content, Evans Blue Staining, and GUS Staining**

Quantification of chlorophyll content was performed based on the method described by Page et al. (2001). Salt-treated leaves of wild-type, \textit{atairp3/log2-2} mutant, and 35S:AtAIRP3/LOG2-RNAi plants were pulverized in liquid nitrogen using Retsch MM301
mixin mill homogenizer. Total chlorophyll was extracted with 80% (v/v) acetone and analyzed with a spectrophotometer (model DU800; Beckman Coulter, Brea, CA, USA). Chlorophyll a was measured at 663.6 nm, and chlorophyll b was measured at 646.6 nm. Total chlorophyll content was calculated by the following formula: chlorophyll (μg/ml) = 17.34 x A_{663.6} + 17.76 x A_{646.6}. The absorbance was normalized by measurement at A_{750}.

Evans blue staining was performed by the method of Watanabe et al. (2006). Light-grown, 4-week-old rosette leaves were incubated with 300 mM NaCl for 6 or 12 h. The salt-treated leaves were incubated with 0.1% Evans blue solution overnight. Leaf chlorophyll was eliminated with a de-staining solution (95% EtOH:lactophenol = 2:1). The degree of cell death was determined by the strength of the dark blue color.

The histochemical GUS staining was performed as previously described by Cho et al. (2011). AtAIRP3-promoter:GUS T3 transgenic plants were incubated with a GUS staining solution (2 mM X-GlcA [cyclohexylammonium salt; Duchefa Biochemie], 0.5 mM K_{3}Fe(CN)_{6}, and 0.5 mM K_{4}Fe(CN)_{6} in 50 mM Tris buffer [pH 7.5]) for 1 h at 37°C. To remove the chlorophyll, tissues were treated with 70% ethanol for 5 h.

**Yeast Two-hybrid Screening**

The Arabidopsis Lambda-ACT two-hybrid cDNA library was obtained from ARBC (stock No. CD4-22). AtAIRP3/LOG2 was cloned into the pGBKT7 vector. The cDNA clones in the pACT vector and the AtAIRP3/LOG2-pGBKT7 construct were co-transformed into yeast strain AH109 (Clontech, Mountain View, CA, USA) as recently described (Bae and Kim, 2013) and allowed to grow for 5 days at 30°C. Approximately 5.0 × 10^{5} transformants were screened on the SD/-Trp/-Leu/-His growth medium supplemented with 10 mM 3-AT. To check the interaction between AtAIRP3/LOG2 and various forms of RD21, RD21-pGADT7 and AtAIRP3/LOG2-pGBK7T constructs were co-transformed into yeast AH109 cells. Yeast cells were grown on the four-minus (SD/-Leu/-Trp/-His/-Ade) growth medium supplemented with p-nitrophenyl-α-d-galactoside at 30°C for 3 days. In addition, p53 (murine p53^{72-390}) + T-antigen (SV40 large T-antigen^{87-708}) were used as a positive control. Lambda (human lamin C^{66-230}) + T-antigen were used as a negative control.

**In Vitro and In Vivo Co-IP Experiments**
The in vitro co-IP experiment was conducted as described by Cho et al. (2008) with modifications. Bacterially-expressed AtAIRP3/LOG2-2xflag and mRD21-6xmyc recombinant proteins were co-incubated in 50 μl anti-flag M2 affinity gel matrix (Sigma-Aldrich) for 2 h at 4°C in IP buffer (phosphate buffered saline, 0.5 M EDTA, 0.2 M phenylmethylsulfonyl fluoride, protease inhibitor cocktail VI [AG Scientific, San Diego, CA, USA], and 0.5% Triton X-100). The affinity gel was washed three times with IP buffer, and bound proteins were eluted with 0.1 M glycine buffer, pH 2.3. The eluted proteins were resolved by 8% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with anti-flag (1:10,000 dilution, Sigma-Aldrich) and anti-myc (1:4,000 dilution, Applied Biological Materials, Richmond, BC, Canada) antibodies.

The in vivo co-IP experiment was performed as described by Son et al. (2010) with modifications. The 35S:p19, 35S:AtAIRP3/LOG2-flag, and 35S:mRD21-myc constructs were transformed into Agrobacterium tumefaciens (GV3101) and cultured overnight at 28°C in YEP medium containing 50 μg/ml kanamycin (Sigma-Aldrich) and 50 μg/ml rifampicin (Sigma-Aldrich). The cultured bacterial cells were harvested and re-suspended in tobacco infiltration medium (10 mM 2-[N-morpholine]-ethanesulfonic acid [MES] pH 5.7, 10 mM MgCl₂, and 0.5 mM acetosyringone [MB cell, Pasadena, CA, USA]) at a final O.D.=1.0. Light-grown, 3-week-old tobacco (Nicotiana benthamiana L.) leaves were infiltrated with Agrobacterium suspension mixtures (35S:p19 + 35S:mRD21-myc and 35S:p19 + 35S:AtAIRP3/LOG2-flag + 35S:mRD21-myc) and incubated for 4 days at 25°C. Infiltrated leaves were ground in a mixer mill, and total protein was extracted in extraction buffer (50 mM Tris [pH 7.5], 100 mM NaCl, 0.5% Triton X-100, 2 mM EDTA, 1 mM MgCl₂, protease inhibitor cocktail VI [AG Scientific], 1 mM PMSF, 20 mM sodium fluoride, and 10% glycerol) in the presence of 10 μM MG132 (AG Scientific). Leaf extract (1 mg of total proteins) was incubated with anti-flag gel matrix for 2 h, and precipitated proteins were detected with immuno-blot analysis using anti-flag (1:10,000 dilution; Sigma-Aldrich) and anti-myc (1:4,000 dilution; Applied Biological Materials) antibodies.

**Cell-free Degradation and In Vitro Ubiquitination Assays**

Cell-free crude extracts were prepared from salt (300 mM NaCl)-treated 10-day-old
wild-type and \textit{atairp3/log2-2} seedlings using extraction buffer (25 mM Tris-HCl [pH 7.5], 10 mM MgCl$_2$, 5 mM DTT, 0.1% Triton X-100, 10 mM ATP, and 10 mM NaCl). The mRD21-6xmyc and RGA1-2xflag recombinant proteins were incubated with cell-free crude extracts (50 μg of total proteins) for 1.5 and 3 h in the presence or absence of 50 μM MG132 (AG Scientific). SDS sample buffer was added to the reaction mixture. The protein degradation profiles were analyzed by immuno-blotting using anti-flag and anti-myc antibodies. RGA1 was used as a positive control for proteasome-dependent degradation (Lee et al., 2010). Rubisco served as the loading control.

An in vitro self-ubiquitination assay was conducted as described by Cho et al. (2006). MBP-AtAIRP3/LOG2$_{101-388}$ recombinant protein was incubated for 1 h in the presence or absence of E1 (Arabidopsis UBA1), E2 (Arabidopsis UBC2), ATP, and Ub. The reaction products were subjected to immuno-blotting using anti-MBP and anti-Ub antibodies. For the in vitro mRD21 ubiquitination assay, AtAIRP3/LOG2-flag and mRD21-myc proteins were co-incubated as described above. The reaction products were analyzed using an anti-myc antibody.

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Figure Legends

Figure 1. Isolation of atairp3/log2-2 T-DNA-inserted loss-of-function mutant line. A, Screening of an ABA-hyposensitive RING E3 Ub ligase mutant in the germination stage. Wild-type and knock-out mutant seeds were germinated on MS growth medium in the absence (upper panel) or presence (lower panel) of 0.5 μM ABA. Germination rates with respect to cotyledon greening were determined after 5 days. The atairp1 and atairp2 mutants served as positive controls for ABA-insensitive phenotypes, while the #99 RING mutant served as a negative control to demonstrate the wild-type phenotype in response to ABA. Scale bars = 1 cm. B, Schematic structure of the atairp3/log2-2 mutant line (Sail_729_A08). Grey bars represent the 5’- and 3’-untranslated regions, dark bars show the coding regions, and solid lines indicate the introns of AtAIRP3/LOG2 (GenBank accession number NC_003074). The atairp3/log2-2 mutant contains double T-DNA insertions in the first intron after nucleotides 873 and 887 in an antisense orientation. Open boxes depict T-DNA insertions. Primers used for genotyping PCR and RT-PCR are shown with arrows. Nucleotide sequences of primers are listed in Supplemental Table S1. C, Genotyping and RT-PCR analyses of the atairp3/log2-2 knock-out mutant and 35S:AtAIRP3-RNAi knock-down transgenic plants. Left panel, genotyping PCR of wild-type and atairp3/log2-2 plants. Primers used for genomic PCRs are shown on the right side of the agarose gel. Middle panel, RT-PCR of wild-type and atairp3/log2-2 plants. Primers used for RT-PCRs are shown on the right side of the gel. The level of Arabidopsis AtUBC10 (E2 ubiquitin-conjugating enzyme) transcripts was used as a loading control. Right panel, RT-PCR of wild-type, atairp3/log2-2, and 35S:AtAIRP3-RNAi (independent transgenic T4 lines #1 and #2) plants. Primers used for RT-PCRs are shown on the right. AtUBC10 was used as a loading control. D, Schematic structure of the AtAIRP3/LOG2 gene and its predicted protein. Grey bars represent 5’- and 3’-untranslated regions, dark bars show coding regions, and solid lines indicate introns. A putative N-terminal myristoylation site, a DAR2 domain, and a single C3HC4-type RING motif are indicated. Primers used for RT-PCR are shown with arrows.

Figure 2. Induction of AtAIRP3/LOG2 in response to drought, high salinity, and ABA. A, RT-PCR analysis of AtAIRP3/LOG2. Light-grown, 10-day-old Arabidopsis seedlings were

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subjected to drought (0, 1.5, 3, and 6 h), 300 mM NaCl (0, 1.5, and 3 h), or 100 μM ABA (1.5 and 3 h). Total RNA prepared from the treated tissues was analyzed by RT-PCR using gene-specific primer sets. RD29A served as a positive control for drought and salt treatments, whereas RAB18 served as a positive control for ABA induction. AtUBC10 was used as a loading control. B, Real-time qRT-PCR analysis of AtAIRP3/LOG2. Total RNA was isolated from the treated tissues and used for real-time qRT-PCR. Fold induction of AtAIRP3/LOG2 was normalized to levels of glyceraldehyde-3-phosphate dehydrogenase C subunit (GAPDHC) mRNA, which served as an internal control. Error bars indicate the mean ± SD from three independent experiments. C, AtAIRP3/LOG2 promoter-GUS assay. Light-grown, 10-day-old AtAIRP3-promoter:GUS transgenic T3 plants were grown under drought conditions (4 h), with 300 mM NaCl (4 h), or with 100 μM ABA (4 h). Histochemical localization of GUS activities in leaf and root tissues were visualized by X-GlcA staining for 1 h. Scale bars = 50 μm.

Figure 3. ABA-insensitive phenotypes of atairp3/log2-2 knock-out mutant and 35S:AtAIRP3/LOG2-RNAi knock-down transgenic plants with respect to germination rates and stomatal movements. A, ABA-mediated inhibition of germination. Wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3-RNAi (T4 lines #1 and #2) seeds were germinated on MS growth medium supplemented with different concentrations (0, 0.2, 0.5, and 1.0 μM) of ABA. Germination rates were determined after 3-5 days. Photographs were taken at 5 days after germination. Scale bars = 1 cm. B, Germination percentages of wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3-RNAi (T4 lines #1 and #2) seeds in response to ABA. Upper panel, germination percentages were determined with respect to radicle emergence at 3 days after ABA treatment. Lower panel, germination percentages were measured in terms of cotyledon greening at 5 days after ABA treatment. Error bars indicate the mean ± SD (n = 110) from three independent experiments. C, ABA-mediated stomatal closure. Light-grown, 4-week-old rosette leaves of wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3-RNAi (T4 lines #1 and #2) plants were immersed in stomatal opening solution for 4 h and in ABA solutions (0, 0.1, 1.0, and 10 μM) for another 2 h. Bright-field microscopy was used to photograph the guard cells. Scale bars = 10 μM. D, Measurement of stomatal aperture after ABA treatment. Stomatal apertures of wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3-RNAi (T4 lines #1 and #2) leaves were measured as the ratio of
width to length after ABA treatment. Error bars indicate the mean ± SD (n≥35).

**Figure 4.** Co-suppression and complementation experiments. A and B, Arabidopsis plants that expressed AtAIRP3/LOG2 under the control of the CaMV 35S promoter were constructed. Several independent T4 transgenic plants (35S:AtAIRP3/LOG2) were obtained and used for phenotypic assays. A, Inhibition of germination by ABA in wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3/LOG2 transgenic T4 plants (independent lines #1 and #2). Freshly harvested seeds of each plant were germinated on MS growth medium supplemented with 0.5 μM ABA. Percentages of green cotyledon development were counted after 5 days. B, AtAIRP3/LOG2 transcript levels of wild-type and 35S:AtAIRP3/LOG2 transgenic plants. AtUBC10 is used as an equal loading control. C-E, Generation and phenotypic analysis of atairp3/AtAIRP3 complementation transgenic plants. C, Cartoon of AtAIRP3/LOG2 complementation construct. The plasmid construct (3.2 kb in-length) is composed of 1.35 kb upstream region and 1.85 kb coding region. The gray bar indicates the upstream region. The black bars indicate coding regions and white bars indicate 5’ untranslated region. Solid lines represent introns. D, Transcript levels of wild-type, atairp3/log2-2, and atairp3/AtAIRP3 T3 complementation transgenic plants. Total RNAs were isolated as indicated and subjected to RT-PCR analysis using gene-specific primer sets (Supplemental Table S1). E, Complementation of atairp3/log2-2 by AtAIRP3/LOG2 transgene. Seeds of wild-type, atairp3/log2-2, and atairp3/AtAIRP3 complementation T3 plants (independent lines #1, #2, #3, and #4) were germinated on MS medium containing 0.5 μM ABA. Percentages of green cotyledon development of wild-type, atairp3/log2-2, and atairp3/AtAIRP3 complementation T3 plants were determined after 5 days. Error bars are the mean ± SD (n = 72) from three independent experiments.

**Figure 5.** Hypersensitive phenotypes of atairp3/log2-2 knock-out mutant and 35S:AtAIRP3/LOG2-RNAi knock-down transgenic plants in response to high salinity. A, NaCl-sensitive phenotypes of atairp3/log2-2 and 35S:AtAIRP3/LOG2-RNAi plants as compared to wild-type plants. Wild-type, mutant, and RNAi (independent T4 lines #1 and #2) seedlings were grown for 5 days under the normal growth conditions and placed in medium...
supplemented without (upper panel) or with (lower panel) 150 mM NaCl. The morphological abnormalities were observed after 14 days. Some of the atairp3/log2-2 mutant leaves were severely discolored and became whitish or dark brown as indicated by red and brown arrows, respectively. Scale bars = 1 cm. B, Chlorophyll content of leaves from wild-type, mutant, and RNAi transgenic plants after salt treatments. Wild-type, atairp3/log2-2, and 35S:AtAIRP3/LOG2-RNAi plants were identically treated as described above, and total leaf chlorophyll levels were measured. Error bars indicate the mean ± SD from three independent experiments. C, Root growth analysis of wild-type, atairp3/log2-2, and 35S:AtAIRP3-RNAi plants in response to different concentrations (0, 100, and 150 mM) of NaCl. Wild-type, mutant, and RNAi (independent T4 lines #1 and #2) seedlings were grown for 5 days under normal growth conditions and placed in medium supplemented without (left panel) or with (right panel) 100-150 mM NaCl. The root growth profiles were observed after 14 days. Note that lateral root formation in atairp3/log2-2 and RNAi plants was greatly reduced by high salinity relative to wild-type plants. D, Evans blue staining. Mature, healthy leaves from wild-type and atairp3/log2-2 mutant plants were incubated with 300 mM NaCl for 6-12 h. Salt-treated leaves were subsequently treated with Evans blue staining solution. The degree of cell death was determined by the strength of dark blue color. Scale bar = 1 cm.

**Figure 6.** Phenotypic analysis of atairp3/log2-2 knock-out mutant and 35S:AtAIRP3/LOG2-RNAi knock-down transgenic plants in response to drought stress. A, Hypersensitive phenotypes of atairp3/log2-2 and 35S:AtAIRP3/LOG2-RNAi plants in response to water deficit. Wild-type, mutant, and RNAi (independent T4 transgenic lines # 1 and # 2) plants were grown for 3 weeks in pots under normal growth conditions. The plants were grown for another 14 days without watering. Survival of these water-stressed plants was monitored after 3 days after re-watering. B, Measurement of leaf-water-loss rates. The aerial parts of 4-week-old wild-type, atairp3/log2-2, and 35S:AtAIRP3/LOG2-RNAi plants were incubated at room temperature for different time periods. Changes in fresh weight of leaves were measured at given time points. Error bars indicate the mean ± SD of 10 plants. Similar results were obtained from four biologically independent replicates.

**Figure 7.** AtAIRP3/LOG2 interacts with RD21. A, Yeast two-hybrid screening using a cDNA library prepared from 3-day-old etiolated seedlings and a full-length AtAIRP3/LOG3
cDNA. Arabidopsis cDNAs were cloned into the pACT2 vector (prey), and AtAIRP3/LOG2 was cloned into the pGBK7 vector (bait). The cDNA-pACT2 and AtAIRP3/LOG2-pGBK7 plasmids were co-transformed into yeast AH109 cells. Yeast cells were plated on the three-minus (SD/-His/-Trp/-Leu) medium that contained 10 mM 3-AT and allowed to grow for 5 days at 30°C. The yeast cells containing RD21-pACT2 + AtAIRP3/LOG2-pGBK7 grew efficiently in the three-minus medium in the presence of 3-AT. p53 + T-antigen were used as the positive control. Lambda + T-antigen and p53 + RD21 were used as the negative control. B, Schematic representation of full-length (RD21 full), intermediate (RD21 ∆N and RD21 ∆C), mature (mRD21), and deletion (cRD21) forms of RD21. S, signal peptide; P, proline-rich domain. C, Yeast two-hybrid assay. Different forms of RD21 were cloned into pGADT7. The RD21-pGADT7 and AtAIRP3/LOG2-pGBK7 constructs were co-transformed into yeast AH109 cells. Yeast cells were grown in the four-minus (SD/-Leu/-Trp/-His/-Ade) growth medium at 30°C for 3 days. p53 + T-antigen were used as the positive control. Lambda + T-antigen were used as the negative control. D, In vitro immunoprecipitation (IP) assay. Bacterially-expressed AtAIRP3/LOG2-flag and mRD21-myc fusion proteins were co-incubated in the presence of an anti-flag affinity gel matrix. After extensive washing, the bound proteins were eluted with glycine (100 mM) buffer and subjected to immuno-blot analysis using anti-flag and anti-myc antibodies. E, In vivo IP assay. The AtAIRP3/LOG2-flag and mRD21-myc fusion genes were transiently expressed in tobacco (Nicotiana benthamina) leaves. Leaf crude extracts (1 mg protein) were immunoprecipitated with anti-flag affinity gel matrix. The bound proteins were eluted, separated by SDS-PAGE, and detected with anti-flag or anti-myc antibody.

**Figure 8.** AtAIRP3/LOG2-dependent degradation of RD21. A, Cell-free degradation assay for mRD21. The mRD21-myc protein was incubated for 0-3 h with crude extracts of salt-treated (300 mM NaCl) 10-day-old wild-type or atairp3/log2-2 mutant seedlings in the absence (lanes labeled 0 h, 1.5 h, and 3 h) or presence (lanes labeled 3 h-M) of 50 μM MG132. The time-dependent protein levels were examined by immuno-blotting with anti-myc antibody. RGA1, which is known to be regulated by the Ub-26S proteasome pathway, was used as a positive control for proteasome-dependent degradation. The RGA1-flag protein was detected with an anti-flag antibody. Rubisco served as a loading control and was detected
by Ponceau S staining. The protein levels were quantified using Multi Gauge Version 3.1 software (Fuji film).  B, In vitro ubiquitination of mRD21 by AtAIRP3/LOG2. Recombinant AtAIRP3/LOG2-flag or the AtAIRP3/LOG2-flagC319S derivative were co-incubated with mRD21-myc in the presence or absence of Ub, ATP, E1 (Arabidopsis UBA1), and E2 (Arabidopsis UBC8) at 30°C for 1 h. The reaction mixture was analyzed by immuno-blotting with an anti-myc antibody. A close circle indicates shifted high-molecular-mass ubiquitinated protein band.
A

WT | atairp1 | atairp2 | atairp3/log2-2 | #99

mock

ABA 0.5 μM

Scale bars: 1 cm

B

atairp3/log2-2 knock-out mutant
(Sail_729_A08)

LBP3

LP1 + SP1

LP1 + RP1

LBP3 + RP1

C

Genotyping PCR

WT | atairp3/log2-2

RT-PCR

WT | atairp3/log2-2

RT-PCR

35S:AtAIRP3-RNAi

WT | atairp3/log2-2 | #1 | #2

LP1 + RP1

LP1 + SP1

SP2 + SP3

AtUBC10

D

AtAIRP3/LOG2 (At3g09770)

AtAIRP3/ATLOG2 (C3HC4 type)

G

DAR2 domain

RING domain

N-myristoylation site

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**Figure 1.** Isolation of atairp3/log2-2 T-DNA-inserted loss-of-function mutant line. A, Screening of an ABA-hyposensitive RING E3 Ub ligase mutant in the germination stage. Wild-type and knock-out mutant seeds were germinated on MS growth medium in the absence (upper panel) or presence (lower panel) of 0.5 μM ABA. Germination rates with respect to cotyledon greening were determined after 5 days. The atairp1 and atairp2 mutants served as positive controls for ABA-insensitive phenotypes, while the #99 RING mutant served as a negative control to demonstrate the wild-type phenotype in response to ABA. Scale bars = 1 cm.  
B, Schematic structure of the atairp3/log2-2 mutant line (Sail_729_A08). Grey bars represent the 5’- and 3’-untranslated regions, dark bars show the coding regions, and solid lines indicate the introns of AtAIRP3/LOG2 (GenBank accession number NC_003074). The atairp3/log2-2 mutant contains double T-DNA insertions in the first intron after nucleotides 873 and 887 in an antisense orientation. Open boxes depict T-DNA insertions. Primers used for genotyping PCR and RT-PCR are shown with arrows. Nucleotide sequences of primers are listed in Supplemental Table S1.  
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A

|          | drought | mock | NaCl (300 mM) | mock | ABA (100 µM) |
|----------|---------|------|---------------|------|--------------|
| Time (h) | 0       | 1.5  | 3             | 0    | 1.5          |
|          | 1.5     | 3    | 6             | 1.5  | 3            |

**AtAIRP3/LOG2**
**RD29A**
**AtUBC10**

B

|          | Drought | NaCl | ABA |
|----------|---------|------|-----|
| Time (h) | 0       | 1.5  | 3   |
|          | 1.5     | 3    | 3   |

**AtAIRP3/LOG2**
**RD29A**
**RAB18**

C

Mock

Drought 4 h

NaCl (300 mM) 4 h

ABA (100 µM) 4 h

Scale bars: 50 µm
**Figure 2.** Induction of *AtAIRP3/LOG2* in response to drought, high salinity, and ABA.  

A, RT-PCR analysis of *AtAIRP3/LOG2*. Light-grown, 10-day-old Arabidopsis seedlings were subjected to drought (0, 1.5, 3, and 6 h), 300 mM NaCl (0, 1.5, and 3 h), or 100 μM ABA (1.5 and 3 h). Total RNA prepared from the treated tissues was analyzed by RT-PCR using gene-specific primer sets. *RD29A* served as a positive control for drought and salt treatments, whereas *RAB18* served as a positive control for ABA induction. *AtUBC* was used as a loading control.  

B, Real-time qRT-PCR analysis of *AtAIRP3/LOG2*. Total RNA was isolated from the treated tissues and used for real-time qRT-PCR. Fold induction of *AtAIRP3/LOG2* was normalized to levels of glyceraldehyde-3-phosphate dehydrogenase C subunit (*GAPDH-C*) mRNA, which served as an internal control. Error bars indicate the mean ± SD from three independent experiments.  

C, *AtAIRP3/LOG2* promoter-GUS assay. Light-grown, 10-day-old *AtAIRP3-promoter*::GUS transgenic T3 plants were grown under drought conditions (4 h), with 300 mM NaCl (4 h), or with 100 μM ABA (4 h). Histochemical localization of GUS activities in leaf and root tissues were visualized by X-GlcA staining for 1 h. Scale bars = 50 μm.
AB

Kim and Kim, Fig. 3

A

ABA (µM) WT atairp/log2-2 35S:AtAIRP3-RNAi #1 #2

0

0.2

0.5

1.0

Scale bars: 1 cm

B

ABA (µM)

WT atairp/log2-2 RNAi #1 RNAi #2

0

0.2

0.5

1

Radicle emergence (%)

Green cotyledon (%)

C

ABA (µM) 0 0.1 1.0 10

WT

atairp3/log2-2

35S:AtAIRP3-RNAi #1

35S:AtAIRP3-RNAi #2

Scale bars: 10 µm

D

ABA (µM)

WT atairp3/log2-2 RNAi #1 RNAi #2

0

0.1

1

10

Stomata aperture (width/length)
Figure 3. ABA-insensitive phenotypes of atairp3/log2-2 knock-out mutant and 35S:AtAIRP3/LOG2-RNAi knock-down transgenic plants with respect to germination rates and stomatal movements. A, ABA-mediated inhibition of germination. Wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3-RNAi (T4 lines #1 and #2) seeds were germinated on MS growth medium supplemented with different concentrations (0, 0.2, 0.5, and 1.0 μM) of ABA. Germination rates were determined after 3-5 days. Photographs were taken at 5 days after germination. Scale bars = 1 cm. B, Germination percentages of wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3-RNAi (T4 lines #1 and #2) seeds in response to ABA. Upper panel, germination percentages were determined with respect to radicle emergence at 3 days after ABA treatment. Lower panel, germination percentages were measured in terms of cotyledon greening at 5 days after ABA treatment. Error bars indicate the mean ± SD (n = 110) from three independent experiments. C, ABA-mediated stomatal closure. Light-grown, 4-week-old rosette leaves of wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3-RNAi (T4 lines #1 and #2) plants were immersed in stomatal opening solution for 4 h and in ABA solutions (0, 0.1, 1.0, and 10 μM) for another 2 h. Bright-field microscopy was used to photograph the guard cells. Scale bars = 10 μM. D, Measurement of stomatal aperture after ABA treatment. Stomatal apertures of wild-type, atairp3/log2-2 mutant, and 35S:AtAIRP3-RNAi (T4 lines #1 and #2) leaves were measured as the ratio of width to length after ABA treatment. Error bars indicate the mean ± SD (n≥35).
Kim and Kim, Fig. 4

A

![Bar chart showing Green cotyledon (%) for different genotypes under ABA treatment.](image)

B

![Image of RT-qPCR gel for AtAIRP3 and AtUBC10 genes.](image)

C

![Diagram of AtAIRP3 genomic DNA with upstream and coding regions.](image)

D

![Image of RT-qPCR gel for AtAIRP3 and AtUBC10 genes in atairp3/AtAIRP3 genotypes.](image)

E

![Bar chart showing Green cotyledon (%) for different genotypes under ABA treatment.](image)
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A

|           | WT       | atairp3/log2-2 | 35S:AtAIRP3-RNAi #1 | 35S:AtAIRP3-RNAi #2 |
|-----------|----------|----------------|----------------------|----------------------|
| mock      |          |                |                      |                      |
| NaCl 150 mM |         |                |                      |                      |

Scale bars: 1 cm

B

![Bar graph showing chlorophyll content](image)

C

![Image showing Evans blue staining](image)

Scale bar: 1 cm
Figure 5. Hypersensitive phenotypes of *atairp3/log2-2* knock-out mutant and *35S:AtAIRP3/LOG2-RNAi* knock-down transgenic plants in response to high salinity. A, NaCl-sensitive phenotypes of *atairp3/log2-2* and *35S:AtAIRP3/LOG2-RNAi* plants as compared to wild-type plants. Wild-type, mutant, and RNAi (independent T4 lines #1 and #2) seedlings were grown for 5 days under the normal growth conditions and placed in medium supplemented without (upper panel) or with (lower panel) 150 mM NaCl. The morphological abnormalities were observed after 14 days. Some of the *atairp3/log2-2* mutant leaves were severely discolored and became whitish or dark brown as indicated by red and brown arrows, respectively. Scale bars = 1 cm. B, Chlorophyll content of leaves from wild-type, mutant, and RNAi transgenic plants after salt treatments. Wild-type, *atairp3/log2-2*, and *35S:AtAIRP3/LOG2-RNAi* plants were identically treated as described above, and total leaf chlorophyll levels were measured. Error bars indicate the mean ± SD from three independent experiments. C, Root growth analysis of wild-type, *atairp3/log2-2*, and *35S:AtAIRP3-RNAi* plants in response to different concentrations (0, 100, and 150 mM) of NaCl. Wild-type, mutant, and RNAi (independent T4 lines #1 and #2) seedlings were grown for 5 days under normal growth conditions and placed in medium supplemented without (left panel) or with (right panel) 100-150 mM NaCl. The root growth profiles were observed after 14 days. Note that lateral root formation in *atairp3/log2-2* and RNAi plants was greatly reduced by high salinity relative to wild-type plants. D, Evans blue staining. Mature, healthy leaves from wild-type and *atairp3/log2-2* mutant plants were incubated with 300 mM NaCl for 6-12 h. Salt-treated leaves were subsequently treated with Evans blue staining solution. The degree of cell death was determined by the strength of dark blue color. Scale bar = 1 cm.
Figure 6. Phenotypic analysis of *atairp3/log2-2* knock-out mutant and *35S:AtAIRP3/LOG2-RNAi* knock-down transgenic plants in response to drought stress. A, Hypersensitive phenotypes of *atairp3/log2-2* and *35S:AtAIRP3/LOG2-RNAi* plants in response to water deficit. Wild-type, mutant, and RNAi (independent T4 transgenic lines # 1 and # 2) plants were grown for 3 weeks in pots under normal growth conditions. The plants were grown for another 14 days without watering. Survival of these water-stressed plants was monitored after 3 days after re-watering. B, Measurement of leaf-water-loss rates. The aerial parts of 4-week-old wild-type, *atairp3/log2-2*, and *35S:AtAIRP3/LOG2-RNAi* plants were incubated at room temperature for different time periods. Changes in fresh weight of leaves were measured at given time points. Error bars indicate the mean ± SD of 10 plants. Similar results were obtained from four biologically independent replicates.
Kim and Kim, Fig. 7

A

| OD<sub>600</sub> | Positive control (p53 + T-antigen) | Negative control (lambda + T-antigen) | AtAIRP3/LOG2 + RD21 | Negative control (p53 + RD21) |
|------------------|------------------------------------|--------------------------------------|---------------------|-----------------------------|
| 3 days           | [Image of plates]                   | [Image of plates]                     | [Image of plates]   | [Image of plates]           |

B

| Peptidase inhibitor | Protease | Granulin |
|---------------------|----------|----------|
| RD21 full           |          |          |
| RD21 ΔN             |          |          |
| RD21 ΔC             |          |          |
| mRD21               |          |          |
| cRD21               |          |          |

C

| OD<sub>600</sub> | Positive control (p53 + T-antigen) | Negative control (lambda + T-antigen) | AtAIRP3/LOG2 + RD21 full | AtAIRP3/LOG2 + RD21 ΔN | AtAIRP3/LOG2 + RD21 ΔC | AtAIRP3/LOG2 + mRD21 | AtAIRP3/LOG2 + cRD21 |
|------------------|------------------------------------|--------------------------------------|-------------------------|------------------------|------------------------|----------------------|----------------------|
| 3 days           | [Image of plates]                   | [Image of plates]                     | [Image of plates]       | [Image of plates]      | [Image of plates]      | [Image of plates]   | [Image of plates]   |

D

| 10% input | IP product (anti-flag) |
|-----------|------------------------|
| AtAIRP3/LOG2-flag | + - + - - + |
| mRD21-myc       | - + + - + + |

E

| 10% input | IP product (anti-flag) |
|-----------|------------------------|
| AtAIRP3/LOG2-flag | - + - + + |
| mRD21-myc       | + + + + + |

[Image of Western blots]
**Figure 7.** AtAIRP3/LOG2 interacts with RD21. A, Yeast two-hybrid screening using a cDNA library prepared from 3-day-old etiolated seedlings and a full-length AtAIRP3/LOG3 cDNA. Arabidopsis cDNAs were cloned into the pACT2 vector (prey), and AtAIRP3/LOG2 was cloned into the pGBKTK7 vector (bait). The cDNA-pACT2 and AtAIRP3/LOG2-pGBKTK7 plasmids were co-transformed into yeast AH109 cells. Yeast cells were plated on the three-minus (SD/-His/-Trp/-Leu) medium that contained 10 mM 3-AT and allowed to grow for 5 days at 30°C. The yeast cells containing RD21-pACT2 + AtAIRP3/LOG2-pGBKTK7 grew efficiently in the three-minus medium in the presence of 3-AT. p53 + T-antigen were used as the positive control. Lambda + T-antigen and p53 + RD21 were used as the negative control. B, Schematic representation of full-length (RD21 full), intermediate (RD21 ∆N and RD21 ∆C), mature (mRD21), and deletion (cRD21) forms of RD21. S, signal peptide; P, proline-rich domain. C, Yeast two-hybrid assay. Different forms of RD21 were cloned into pGADT7. The RD21-pGADT7 and AtAIRP3/LOG2-pGBKTK7 constructs were co-transformed into yeast AH109 cells. Yeast cells were grown in the four-minus (SD/-Leu/-Trp/-His/-Ade) growth medium at 30°C for 3 days. p53 + T-antigen were used as the positive control. Lambda + T-antigen were used as the negative control. D, In vitro immunoprecipitation (IP) assay. Bacterially-expressed AtAIRP3/LOG2-flag and mRD21-myc fusion proteins were co-incubated in the presence of an anti-flag affinity gel matrix. After extensive washing, the bound proteins were eluted with glycine (100 mM) buffer and subjected to immuno-blot analysis using anti-flag and anti-myc antibodies. E, In vivo IP assay. The AtAIRP3/LOG2-flag and mRD21-myc fusion genes were transiently expressed in tobacco (Nicotiana benthamina) leaves. Leaf crude extracts (1 mg protein) were immunoprecipitated with anti-flag affinity gel matrix. The bound proteins were eluted, separated by SDS-PAGE, and detected with anti-flag or anti-myc antibody.
**A**

300 mM NaCl treatment

| crude extracts (50 µg protein) of wild-type seedlings | crude extracts (50 µg proteins) of atairp3/log2-2 seedlings |
|------------------------------------------------------|-------------------------------------------------------------|
| 0 h 1.5 h 3 h 3 h-M                                   | 0 h 1.5 h 3 h 3 h-M                                         |

- mRD21-myc
- RGA1-flag
- Rubisco
  (Ponceau S staining)

M: 50 µM MG132 treatment

**B**

| Protein          | 0 h 1.5 h 3 h 3 h-M | 0 h 1.5 h 3 h 3 h-M |
|------------------|---------------------|---------------------|
| ubiquitin        | ++ + + +            | + + + + +           |
| UBA1             | - + + +             | - + + + +           |
| UBC8             | + - + +             | + - + + +           |
| AtAIRP3/LOG2-flag| + + - +             | + + + + -           |
| AtAIRP3/LOG2C319S-flag | - - - -          | + + - + +           |
| mRD21-myc        | + + + +             | + + + + +           |

(kDa)

| Protein | anti-myc |
|---------|----------|
| Ubiquitin |         |
| UBA1 |         |
| UBC8 |         |
| AtAIRP3/LOG2-modified | |
| AtAIRP3/LOG2C319S-modified | |
| mRD21-myc | |

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**Figure 8.** AtAIRP3/LOG2-dependent degradation of RD21. A, Cell-free degradation assay for mRD21. The mRD21-myc protein was incubated for 0-3 h with crude extracts of salt-treated (300 mM NaCl) 10-day-old wild-type or *atairp3/log2-2* mutant seedlings in the absence (lanes labeled 0 h, 1.5 h, and 3 h) or presence (lanes labeled 3 h-M) of 50 μM MG132. The time-dependent protein levels were examined by immuno-blotting with anti-myc antibody. RGA1, which is known to be regulated by the Ub-26S proteasome pathway, was used as a positive control for proteasome-dependent degradation. The RGA1-flag protein was detected with an anti-flag antibody. Rubisco served as a loading control and was detected by Ponceau S staining. The protein levels were quantified using Multi Gauge Version 3.1 software (Fuji film). B, In vitro ubiquitination of mRD21 by AtAIRP3/LOG2. Recombinant AtAIRP3/LOG2-flag or the AtAIRP3/LOG2-flag$^{C319S}$ derivative were co-incubated with mRD21-myc in the presence or absence of Ub, ATP, E1 (Arabidopsis UBA1), and E2 (Arabidopsis UBC8) at 30°C for 1 h. The reaction mixture was analyzed by immuno-blotting with an anti-myc antibody. A close circle indicates shifted high-molecular-mass ubiquitinated protein band.