Abstract

Ubiquitination is an important post-translational protein modification that is known to play critical roles in diverse biological processes in eukaryotes. The RING E3 ligases function in ubiquitination pathways, and are involved in a large diversity of physiological processes in higher plants. The RING domain-containing E3 ligase AtRDUF1 was previously identified as a positive regulator of ABA-mediated dehydration stress response in Arabidopsis. In this study, we report that AtRDUF1 is involved in plant responses to salt stress. AtRDUF1 expression is upregulated by salt treatment. Overexpression of AtRDUF1 in Arabidopsis results in an insensitivity to salt and osmotic stresses during germination and seedling growth. A double knock-out mutant of AtRDUF1 and its close homolog AtRDUF2 (atrduf1 atrduf2) was hypersensitive to salt treatment. The expression levels of the stress-response genes RD29B, RD22, and KIN1 are more sensitive to salt treatment in AtRDUF1 overexpression plants. In summary, our data show that AtRDUF1 positively regulates responses to salt stress in Arabidopsis.

Introduction

Ubiquitination is a mechanism of post-translational regulation. The ubiquitination cascade is catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin protein ligase (E3). There are >1300 predicted E3 ligases in the Arabidopsis genome, including >450 RING type E3s [1,2]. The vast majority of E3 ubiquitin ligases in the Arabidopsis genome facilitate the identification of specific substrates and their subsequent ubiquitination [1,3]. The E3 ubiquitin ligases are a huge and varied family of proteins and protein complexes which contain either a HECT domain or a U-box/RING domain. The HECT domain subfamily of E3s is large in Arabidopsis. The RING domain subfamily of E3s is relatively small in Arabidopsis. The RING domain subfamily of E3s is large in Arabidopsis and can be further divided into single subunit RING E3s, such as Constitutive Photomorphogenesis1 (COP1) [4], SEVEN IN ABSENTA IN ARABIDOPSIS THALIANA 5 (SINAT5) [5], and Arm Repeat-Containing 1 (ARC1) [6], and multisubunit RING E3s including the SCF, CUL3-BTB, and ACP complexes [7]. The RING E3s typically contain a cross-brace structure formed of eight Cys and His residues that coordinates two zinc ions [8,9,10]. E3 ligases are involved in various aspects of plant biological processes, including growth, development, and protection from biotic and abiotic stresses [11,12].

To adapt to stressful conditions such as drought, cold, and salinity, plants have developed redundant and sophisticated response strategies which function throughout their life cycle [13,14,15]. Plants subjected to stress often accumulate abscisic acid (ABA), an important phytohormone that can protect plants from damage induced by drought, salinity, and pathogenic attack [16,17]. The accumulation of compatible osmolytes such as proline under dehydration conditions allow cells to maintain osmotic balance with the extracellular space and help to protect the activities of the enzyme activity [18].

AtRDUF1 and AtRDUF2 are homologous proteins with a domain-of-unknown-function (DUF) 1117 motif in their C-terminal regions. Both proteins were identified as ABA-, salt-, and drought-inducible RING finger domain-containing E3 ligases [19]. A study using knock-out mutations revealed that AtRDUFs are positive regulators of ABA response and drought tolerance [19]. Here, through the use of overexpression and knock-out materials, we show that AtRDUF1 positively participates in the response of plants to salt stress.

Results

Characterization of AtRDUF1 protein

In order to identify stress-related genes, we analyzed several publicly available databases of Arabidopsis microarray experiments. A gene family with three genes (At5g59550, At3g46620 and At2g39720) that encode DUF1117-containing RING finger proteins from stress-specific expression profiles in Genevestigator [20] attracted our interest. The microarray data showed that the expression of these genes was up-regulated by several kinds of abiotic stresses. Of particular interest were the data showed that
At5g59550 and At3g46620 were up-regulated 5.5 and 2.9-fold, respectively, after salt treatment for 6–24 h. At5g59550 and At3g46620 were previously designated as AtRDUF2 and AtRDUF1, respectively [19].

The AtRDUF1 protein contains a conserved C3H2C3-type RING domain, which shows similarity with many known proteins in Arabidopsis (Figure 1A), including several proteins known to be involved in ABA and/or stress signaling pathways, such as ABI3-interacting protein 2 (AIP2) [21], SALT- AND DROUGHT-INDUCED RING FINGER 1 (SDIR1) [22] and ATLs [23,24]. One of these homologous proteins, RING finger protein for embryogenesis (RIE1), is required for normal development of seeds [25].

Subcellular studies using a 35S::AtRDUF1:GFP fusion protein in Arabidopsis leaf protoplast cells showed that the fusion protein was mainly found in the cytosol and in the nuclei (Figure 1B). We examined whether AtRDUF1 is an E3 ligase using in vitro methods. As shown in Figure 1C, in the presence of E1 and E2, autoubiquitination of MBP:AtRDUF1 can be detected in the presence of E1 and E2 by both nickel-horseradish peroxidase as well as by anti-MBP antibody assay, indicating that AtRDUF1 is an active E3 ligase. A mutant allele with substitution of metal ligand positions Cys-3, His-4, and His-5 of the RING motif with Tyr (CH/Y) was completely inactive (Figure 1C), indicating that an intact RING motif is essential for the E3 ligase activity of AtRDUF1.

Expression pattern of AtRDUF1
To investigate the tissue-specific expression pattern of AtRDUF1, a fusion gene comprising the native AtRDUF1 promoter, a 1.3-kb fragment upstream of the start codon of AtRDUF1 CDS, and the β-glucuronidase (GUS) gene [26] coding sequence as the reporter gene were constructed and transformed into wild-type Arabidopsis. Histochemical staining showed that AtRDUF1 expressed abundantly in seeds, but was also locally detectable in flowers, hypocotyls, leaves and roots (Figure 2). The staining was strong in immature seeds (Figure 2A), whereas in intact desiccated seeds, the GUS expression was only detectable at the funiculus attachment region (Figure 2B). In broken seeds, GUS staining was uniformly present throughout the seed (Figure 2C), indicating that the limitation of GUS staining in intact seeds was due to blocking by the seed coat. To exclude possible false observations caused by the diffusion of the soluble intermediate of GUS substrates [27,28], seeds were dissected and stained separately. The GUS staining signal could be observed uniformly throughout the embryo, but only in the funiculus attachment region of the seed coat (Figure 2D). During germination, a reduction in GUS staining was detectable early on (Figure 2E–H), which is consistent with the decrease of AtRDUF1 expression detected by real-time qRT-PCR (Figure S2 in File S1). In 4-d-old seedlings, the GUS expression was mainly detected in the junction of the root and hypocotyl, in leaf tips, and around the meristem (Figure 2I). In 2-week old seedlings, the GUS staining was only detectable in leaf tips and root tips (Figure 2J). In reproductive tissue,
AtRDUF1::GUS activity was observed at the junction of carpels and pedicels, as well as in stigma, anthers, and pollen, and low levels were detected in the vascular tissues of sepals and petals (Figure 2K–M).

AtRDUF1 positively regulates plant salt and osmotic stress responses

To investigate the function of AtRDUFs, mutants with T-DNA insertions within the exons of the AtRDUFs were obtained and verified (Figure S1A–B in File S1). Expression of the mutant genes was not detected by RT-PCR with primers spanning the T-DNA in the respective homozygous mutants (Figure S1C in File S1). The mutants for AtRDUF1 and AtRDUF2 were named atrduf1-2 and atrduf2-1, respectively (hereinafter called “atrduf1” and “atrduf2”). The double mutant atrduf1atrduf2 was generated by crossing.

We generated transgenic Arabidopsis plants with constitutive expression of AtRDUF1, driven by cauliflower mosaic virus (CaMV) 35S promoter. Six transgenic lines of AtRDUF1 were obtained (Figure S1D and S1E in File S1). The 35S::AtRDUF1 plants showed a wild-type growth phenotype under normal conditions.

The expression of the AtRDUF1 had been shown to be induced by salt treatment [19]. We therefore tested whether AtRDUF1 plays a role in plant responses to salt. Seeds of wild-type (WT), atrduf1atrduf2, atrduf1, and AtRDUF1 overexpression lines were germinated vertically in 1/2 MS medium. 3-day-old seedlings were transferred to 1/2 MS medium supplemented with 110, 120 or 150 mM NaCl. In the control condition, no significant difference in the length of primary roots was observed among any of the materials (Figure 3A and 3D). Under salt stress conditions, the primary roots of 35S::AtRDUF1 seedlings grew faster than did wild-type seedlings, and the atrduf1atrduf2 double mutants showed inhibited growth (Figure 3A and 3D).

Germinated atrduf1atrduf2, atrduf1 and 35S::AtRDUF1 lines were transplanted to soil for two weeks, and watered with 200 mM

Figure 2. Histochemical localization of GUS activity in AtRDUF1::GUS transgenic plants. (A) Developing seed at 12 days after pollination. (B) Desiccated mature seed. (C) Broken mature seed. (D) Dissected seed after imbibition. (E–H) Germinating seedlings at 1-day (E), 2-days (F), 3-days (G), and 4-days (H) after germination. (I) Leaf. (J) Root. (K) Flower. (L) Pollen. (M) Siliques. Bars represent 0.25 mm in A-D; 0.1 mm in J; 10 μm in L; and 1 mm in E-I, K, and M.

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NaCl solution for 15 days to induce salt stress. 35S::AtRDUF1 seedlings exhibited higher survival rates than did WT seedlings. Contrastingly, the survival rate of the salt treated atrduf1atrduf2 double mutant seedlings was lower than the WT control seedlings (Figure 3B and 3E). After imbibition on 1/2 MS medium containing NaCl and transfer to a growth chamber for 10 days, the germination rates of 35S::AtRDUF1 seeds were higher than that of WT seeds (Figure 3C and 3F). Therefore, the germination ability of AtRDUF1 overexpression seeds is insensitive to salt treatment. In our salt tolerance tests, no significant difference was observed between WT and the atrduf1 mutant.

Salinity causes ionic and osmotic stresses in plant cells. Germinated transgenic seedlings were transferred to MS medium supplemented with mannitol, a nonmetabolizable sugar, which is known to be used as an osmotic agent in some studies [29,30,31]. The root growth of 35S::AtRDUF1 seedlings was less severely inhibited by mannitol than that of WT seedlings (Figure 4A and 4B). Therefore, the tolerance of 35S::AtRDUF1 seedlings to salt treatment is at least partly osmotic in nature.

In contrast with the accelerated water loss in detached leaves of the atrduf1 mutant [19], the water loss of rosette leaves in the overexpression lines was slower than that of WT leaves (Figure 4C). The overexpression plants also showed a sensitive response to ABA in terms of primary root length (Figure S3 in File S1). Our results support that AtRDUF1 positively participates in ABA-mediated dehydration stress responses.

We investigated the expression profiles of several stress-responsive genes in the AtRDUF1 overexpression lines grown under salt stress conditions. 10-day-old seedlings grown on 1/
2 MS agar plates were sprayed with 200 mM NaCl solution, the seedlings were harvested after 1 h and 2 h, for extraction of total RNA. The result showed that in RDUF1-L1 seedlings, the transcription levels of RD29B, RD22, and KIN1 increased more than those of the WT after 2 h of salt treatment (Figure 5A), suggesting that AtRDUF1 may directly or indirectly interact with known abiotic stress response pathways. When the AtRDUF1::GUS transgenic plants were subjected to salt treatment, the GUS staining was enhanced compared with control plants (Figure 5Ba–5Bd), which is consistent with the real-time qRT-PCR results (Figure 5Be).

**Discussion**

In the course of our investigation of likely stress-related genes, we identified AtRDUFs from in silico data. Their putative proteins contain both RING finger and DUF1117 domains, and represent a novel E3 ligase family in plants. Real time qRT-PCR and promoter-GUS analyses confirmed that the transcription of AtRDUF1 was indeed salt inducible (Figure 5B). We analyzed the phenotypes of wild-type as well as overexpression and loss-of-function mutants of AtRDUFs following salt treatment. Our results showed that AtRDUF1 positively regulates plant responses to salt treatment during both germination and post-germination growth (Figure 3). Furthermore, AtRDUF1 also positively regulates plant tolerance to osmotic and dehydration stress (Figure 4).

The fluorescence of AtRDUF1::GFP fusion protein was detectable in our subcellular localization study (Figure 1B), and 35S::AtRDUF1 transgenic plants showed obvious tolerance to salt, osmotic, and water loss stresses (Figure 3 and 4). In a similar report, a fusion protein approach was used to develop transgenic plants that overexpressed AtRDUF1. However, the authors were unsuccessful in detecting significant accumulation of the fusion protein, in spite of expression of significant amounts of AtRDUF1 mRNA, so the plants were not extensively analyzed [19]. The discrepancy of detection may be attributable to distinctions in vector efficiency or differences in the sensitivity of the assay methods employed in these independent studies.

In addition to the results reported here, AtRDUF1 and AtRDUF2 are also known to be up-regulated by chitin treatment [32]. Among the homologous proteins of the AtRDUFs subfamily, the E3 ligases KEG [12] and AIP2 [21] are negative regulators of ABA signaling, acting by targeting and degrading ABI5 and ABI3, respectively. The E3 ligase SDIR1 positively regulates ABA signaling, and the sdir1 mutant is resistant to salt and drought stress [22]. Therefore, it is apparent that AtRDUFs and their homologous proteins are widely involved in plant adaptations to stress.

We were able to confirm that AtRDUF1 has E3 ligase activity by ubiquitination assays (Figure 1C). Ubiquitination has been shown to play an important role in the perception and transduction of various internal and external environmental signals [33,34]. To test whether AtRDUF1 affects the expression of known stress pathway genes, several marker genes in the stress-responsive pathways were analyzed, RD29B, RD22, and KIN1 showed a hypersensitive salt response in transgenic plants (Figure 5A). RD29B is a cold-, high salt-, and dessication-inducible gene with two ABA-responsive elements (ABREs) present in its promoter region [35]. RD22 transcription is induced by salt and ABA treatment, but no ABRE was identified in its promoter.

Water-loss rate is calculated as the ratio between water loss and plant initial fresh weight (FW), expressed in %. Data are presented as means ± SD. Asterisks indicate significance (*, P < 0.05 versus WT control). doi:10.1371/journal.pone.0071078.g004

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KIN1, which contains the C repeat/dehydration-responsive element (CRT/DRE) motif in its promoter, can be induced by cold, ABA and dehydration treatment [37]. According to our data and published results [19], AtRDUF1 may be involved in the up-regulation of stress responses in Arabidopsis seedlings, in this respect, AtRDUF1, may be similar to SDIR1 and AtSAP5, both of which have been shown to be E3 ligases and are known in promoting stress gene expression and stress tolerance [22,38,39].

Glycerol, a compatible osmolyte, is used in defending against dehydration stresses in yeast, marine algae, insects, and amphibians [40,41,42,43]. Accumulation of intracellular glycerol was observed during the salt adaptation processes of many microorganism such as Aspergillus nidulans [44] and Aureobasidium pullulans [45]. In Arabidopsis, the results from studies in mutants with defects in storage lipid accumulation prior to seed maturation or lipid catabolism following germination [29,30,31] suggest that glycerol or glycerol-derived lipids could serve as compatible osmolytes in dehydration stress conditions in plants. As AtRDUF1 expressed primarily in embryos of matured seeds (Figure 2), we tested the correlation of AtRDUF1 expression with storage lipids (Text S1 in File S1). In young seedlings, the staining of GUS driven by the AtRDUF1 promoter partially coincided with staining of Sudan red 7B, a fat-soluble dye that stains lipids red, and the Sudan red 7B staining was darker in the 35S::RDUF1 seedlings (Figure S4A in File S1). The vegetative tissues of 35S::RDUF1 seedlings contained a higher content of triglycerides (the ester of glycerol) than did WT control tissues (Figure S4C in File S1). Finally, the oleosin maker gene OleS3 and triglycerides content change in response to salt stress (Figure S4D and S4E in File S1). We speculate that a mechanism by which AtRDUF1 increases salt tolerance is through the positively regulation of either the delayed catabolism or the increased accumulation of the storage lipids.

In conclusion, our data show that AtRDUF1 is a functional E3 ligase and a positive regulator of the Arabidopsis response to salt stress. This study contributes to our understanding of the molecular factors involved in the responses of plants to abiotic stresses.

Materials and Methods

Plant materials

All Arabidopsis plants used in this study were of the Columbia (Col-0) ecotype. T-DNA insertion lines SALK_131634 (for AtRDUF1) and N471914 (for AtRDUF2) were obtained from ABRC [46] and NASC [47], respectively. Seedlings were grown under long-day conditions (16 h light/8 h dark) at 22°C, 40 to 60% RH and 63 mE s⁻¹m⁻² light intensity.

Vector construction and Arabidopsis transformation

The cDNA of AtRDUF1 was amplified and cloned into the pSN1301 expression vector [48] driven by the CaMV 35S region [22,36]. KIN1, which contains the C repeat/dehydration-responsive element (CRT/DRE) motif in its promoter, can be induced by cold, ABA and dehydration treatment [37]. According to our data and published results [19], AtRDUF1 may be involved in the up-regulation of stress responses in Arabidopsis seedlings, in this respect, AtRDUF1, may be similar to SDIR1 and AtSAP5, both of which have been shown to be E3 ligases and are known in promoting stress gene expression and stress tolerance [22,38,39].

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promoter. The primers used for AtRDUF1 overexpression were R1BamHIF and R1KpnIR (all primer sequences used in this study are listed in Table S1 in File S1). The 1.3 kb promoter sequence of AtRDUF1 was amplified with primers R1PKpnIF and R1PBamHIR, and cloned into the pGUS1301 vector [49]. The constructed plasmid was introduced into Agrobacterium tumefaciens strain CS5. Arabidopsis was transformed using the floral dip method [50]. Transgenic plants were first screened on medium containing 40 mg/l hygromycin and subsequently transferred to soil. To produce a 35S::AtRDUF1:GFP fusion gene driven by 35S promoter, the 35S::AtRDUF1 CDS sequence with the stop codon was amplified with primers R1GXIIF and R1GXIIR, and cloned in frame into pBluB121GFP. To generate the MBP:AtRDUF1 fusion, the 35S::AtRDUF1 CDS was cloned in frame into pMAL-c2 (NEB, Beverly, MA, USA) with primers R1MBamHIF and R1MBamHIR. A mutant allele with substitution of metal ligand (NEB, Beverly, MA, USA) according to the manufacturer’s instructions. 2 μg of total RNA was used for cDNA synthesis with avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Real-Time qRT-PCR was performed as described previously [56]. Three independent experiments were performed. The relative quantification method (ΔΔCT) was used to evaluate variation in expression. Significance of differences was determined by χ2-test. The primer sets for PCR were: R1realtimeF and R1realtimeR for AtRDUF1; RD29BF and RD29BR for RD29B; RD22F and RD22R for RD22; KIN1F and KIN1R for KIN1; TubulinF and TubulinR for TUBULIN; OleS3F and OleS3R for OleS3; ACTINRF and ACTINRR for ACTIN.

**Subcellular localization**

Plasmid 35S::AtRDUF1:GFP and 35S::GFP were purified by the use of Tiangen kits according to the manufacturer’s protocols. Arabidopsis protoplasts transformations were performed as described previously [52]. For detection of nuclei, samples were stained with DAPI at a final concentration of 1 μg/mL.

**E3 ubiquitin ligase activity assay**

The MBP::AtRDUF1 fusion protein was expressed in Escherichia coli strain BL21 (DE3), and subsequently purified using amylose resin (NEB). The in vitro E3 ubiquitin ligase activity assay was performed as described previously [5]. Following the assay reactions, proteins were separated by SDS-PAGE, blotted, and probed by either HisDetector Nickel-HRP (KPL company, USA) for the detection of His-tagged ubiquitin or antibody to MBP (antiserum; NEB) for detection of the MBP-tagged ArRDUF1 protein. Results were visualized using chemiluminescence as per the instructions of the manufacturer (ECL; Amersham Pharmacia, Amersham, UK).

**Stress and ABA treatment**

After surface sterilization, seeds were imibed at 4°C for 3 d in the dark. The seeds were sown on half-strength Murashige and Skoog medium [53] supplemented with 1% sucrose and 0.7% agar. 3-day-old seedlings were transferred to 1/2 MS medium containing NaCl, mannitol, or ABA. Primary root length was measured after 6 d. 4-day-old seedlings were transplanted to soil and cultured for two weeks, and treated with 200 mM NaCl for 15 days to induce salt stress. For the germination test, all seeds were harvested simultaneously and stored for 5 weeks after harvest. Germination was determined as penetration of the radicle through the seed coat 10 days after imbibition. Each assay was repeated three times. The data in the graphs (Figure 3D–F, 4B–C, 4D, 4E in File S1) were subjected to analysis of variance (ANOVA) and means were compared by t-test at the 5% level. All analyses were performed with version 13.0 of SPSS software.

The water loss assay was performed as described previously [54]. Eight rosette leaves per plant were detached from 3-week-old plants. The leaves were exposed to air and the fresh weights were measured at the indicated time points shown in Figure 4C. Water-loss rate is calculated as the ratio between water loss and plant initial fresh weight, expressed in %.

**GUS histochemical assays**

Tissues of transgenic seedlings harboring AtRDUF1::GUS at various growth stages were used for the GUS activity assays. Fixation of the tissues by acetone and incubation in staining solution was performed as described by Sieburth and Meyerowitz [55]. For staining of pollen, the pollen was isolated from stamens by vortexing in acetone and enriched using centrifugation at rcf 3000.

**Expression analysis**

Total RNA was extracted from tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and treated with RNase-free DNase (Takara, Takara, Dalian, China) according to the manufacturer’s instructions. 2 μg of total RNA was used for cDNA synthesis with avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Real-Time qRT-PCR was performed as described previously [56]. Three independent experiments were performed. The relative quantification method (ΔΔCT) was used to evaluate variation in expression. Significance of differences was determined by χ2-test. The primer sets for PCR were: R1realtimeF and R1realtimeR for AtRDUF1; RD29BF and RD29BR for RD29B; RD22F and RD22R for RD22; KIN1F and KIN1R for KIN1; TubulinF and TubulinR for TUBULIN; OleS3F and OleS3R for OleS3; ACTINRF and ACTINRR for ACTIN.

**Supporting Information**

**File S1** Text S1, lipid detection. Figure S1, verification of T-DNA insertion mutants of AtRDUF5 and AtRDUF1 overexpression lines. Figure S2, relative quantification of AtRDUF1 transcription during germination assayed by real-time qRT-PCR. Figure S3, response of AtRDUF1 overexpression plants to ABA. Figure S4, effects of AtRDUF1 and salt treatment on plant lipids. Table S1, sequences of the oligonucleotides used in this study.

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**Author Contributions**

Conceived and designed the experiments: QX KC YX. Performed the experiments: JL YH QZ CL. Analyzed the data: JL YH QZ CL QX KC YX. Wrote the paper: JL QZ KC YX.

**References**
