

A Nucleus-Localized Long Non-Coding RNA Enhances Drought and Salt Stress Tolerance^[OPEN]

Tao Qin,^a Huayan Zhao,^{b,c} Peng Cui,^c Nour Albeshar,^c and Liming Xiong^{a,c,d,1}

^aTexas A&M AgriLife Research Center, Dallas, Texas 75252

^bApplied Biotechnology Center, Wuhan Institute of Bioengineering, Wuhan 430415, China

^cDivision of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal 23955, Saudi Arabia

^dDepartment of Horticultural Sciences, Texas A&M University, College Station, Texas 77843

ORCID IDs: 0000-0003-1217-2187 (H.Z.); 0000-0001-8099-0806 (L.X.).

Long noncoding RNAs (lncRNAs) affect gene expression through a wide range of mechanisms and are considered as important regulators in many essential biological processes. A large number of lncRNA transcripts have been predicted or identified in plants in recent years. However, the biological functions for most of them are still unknown. In this study, we identified an *Arabidopsis* (*Arabidopsis thaliana*) lncRNA, *DROUGHT INDUCED lncRNA (DRIR)*, as a novel positive regulator of the plant response to drought and salt stress. *DRIR* was expressed at a low level under nonstress conditions but can be significantly activated by drought and salt stress as well as by abscisic acid (ABA) treatment. We identified a T-DNA insertion mutant, *drir^D*, which had higher expression of the *DRIR* gene than the wild-type plants. The *drir^D* mutant exhibits increased tolerance to drought and salt stress. Overexpressing *DRIR* in *Arabidopsis* also increased tolerance to drought and salt stress of the transgenic plants. The *drir^D* mutant and the overexpressing seedlings are more sensitive to ABA than the wild type in stomata closure and seedling growth. Genome-wide transcriptome analysis demonstrated that the expression of a large number of genes was altered in *drir^D* and the overexpressing plants. These include genes involved in ABA signaling, water transport, and other stress-relief processes. Our study reveals a mechanism whereby *DRIR* regulates the plant response to abiotic stress by modulating the expression of a series of genes involved in the stress response.

Drought and high soil salinity are major abiotic stresses that can significantly limit plant productivity. Plants respond to these stresses by changing their metabolism, physiology, and development, which, to a certain extent, could mitigate the negative impact of the stresses on their growth and reproduction. Underlying many of these changes is the dramatic reprogramming of gene expression in response to these stresses. Since both drought stress and salt stress cause cellular dehydration or osmotic stress, gene regulation under drought and salt stress shares some overlapping mechanisms. This is partly attributed to the activation of the biosynthesis and accumulation of the stress hormone abscisic acid (ABA) under both drought and salt stresses (Xiong and Zhu, 2003; Nambara and Marion-Poll, 2005; Barrero et al., 2006). ABA and dehydration-derived primary

and secondary signals activate many stress-responsive genes through an interconnected ABA signaling and ABA-independent signaling network that involves sensing, signal transduction, and gene activation (Yamaguchi-Shinozaki and Shinozaki, 2006; Munemasa et al., 2015; Zhu, 2016)

In the core ABA signaling pathway, ABA is perceived by the PYRABACTIN RESISTANCE1/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS (PYR/PYL/RCAR) group of receptors that, upon binding with ABA, sequester type 2C protein phosphatases and release their inhibition on downstream components, including the SUCROSE NONFERMENTING1-RELATED PROTEIN KINASES SUBFAMILY2 (SnRK2s; Ma et al., 2009; Park et al., 2009; Cutler et al., 2010). These protein kinases subsequently phosphorylate and activate various downstream targets including, among others, transcriptional factors (Cutler et al., 2010; Munemasa et al., 2015; Zhu, 2016). Among these transcription factors, the ABA-responsive element-binding factor class of bZIP transcription factors is well studied. These transcription factors bind the conserved ABA-responsive element cis-element in the promoters of many ABA- and stress-responsive genes and regulate their expression to enhance plant stress tolerance (Furihata et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006; Cutler et al., 2010). SnRK2s also could activate other targets such as NADPH oxidases, SLOW ANION CHANNEL1, and aquaporins

¹ Address correspondence to liming.xiong@ag.tamu.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Liming Xiong (liming.xiong@ag.tamu.edu).

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(Sirichandra et al., 2009; Grondin et al., 2015; Munemasa et al., 2015). These signaling molecules and channels are involved directly in stomatal closure to conserve water under drought stress (Kwak et al., 2003; Furihata et al., 2006; Geiger et al., 2009; Brandt et al., 2015; Grondin et al., 2015).

Many stress-responsive genes contain another conserved cis-element, the C-REPEAT/DEHYDRATION RESPONSIVE ELEMENT, which can be recognized by the DEHYDRATION-RESPONSIVE ELEMENT-BINDING/C-REPEAT-BINDING FACTOR class of transcription factors in response to cold, drought, or salt stress (Shinozaki and Yamaguchi-Shinozaki, 2000; Agarwal et al., 2006; Thomashow, 2010). These genes include, for example, *RESPONSIVE TO DEHYDRATION29A* (*RD29A*), *RD17*, *KIN1*, and *EARLY RESPONSE TO DEHYDRATION10*. The proteins encoded by these stress-responsive genes may help the plants to reduce stress-caused cellular damage and enhance the ability of the plants to withstand the stress.

Prior research on stress gene regulation focused mainly on protein-encoding genes. In recent years, non-protein-coding transcripts are emerging as important regulators of gene expression. Among them, long noncoding RNAs (lncRNAs) represent diverse classes of transcripts longer than 200 nucleotides without or with little protein-coding potential. The lncRNAs have been considered important regulators of many essential biological processes by functioning as precursors of microRNAs (miRNAs) and other small RNAs or as miRNA target mimics. They also may affect alternative splicing of pre-mRNA and regulate chromatin state and chromatin loop dynamics (Ariel et al., 2015; Liu et al., 2015a). In plants, lncRNAs are transcribed by the plant-specific RNA polymerases Pol IV and Pol V as well as by Pol II and Pol III (Wierzbicki et al., 2008; Dinger et al., 2009) and are considered potential regulators of the plant response to the environment. For example, overexpression of the lncRNA *INDUCED BY PHOSPHATE STARVATION1* results in reduced shoot inorganic phosphate content (Franco-Zorrilla et al., 2007). Down-regulation of *CIS-NATURAL ANTISENSE RNA* impairs the transfer of phosphate from roots to shoots and decreases seed yield in rice (*Oryza sativa*; Jabnourne et al., 2013). Transgenic overexpression of *asHSFB2a*, an antisense RNA of *HSFB2a*, affects the plant response to heat stress (Wunderlich et al., 2014). Several reports also show that drought or salt stress could alter lncRNA expression in plants. For example, 19, 664, and 98 drought-responsive lncRNAs were identified in foxtail millet (*Setaria italica*), maize (*Zea mays*), and rice, respectively (Qi et al., 2013; Zhang et al., 2014a; Chung et al., 2016). Salt stress and dehydration stress also alter the accumulation of eight and six lncRNAs, respectively, in *Arabidopsis thaliana* (Ben Amor et al., 2009). Nonetheless, no lncRNA has been characterized to regulate the plant response or tolerance to drought or salt stress.

In this study, we identified and characterized the function of a novel lncRNA, *DRIR* (for *DROUGHT*

INDUCED lncRNA). The expression of *DRIR* was induced by drought and salt stress. Increasing *DRIR* expression enhanced plant sensitivity to ABA and increased tolerance to drought and salt stress. In situ hybridization analysis revealed that *DRIR* is localized mainly in the nucleus. Transcriptome sequencing and real-time PCR analysis revealed that *DRIR* modulates the expression of genes involved in the stress response, including ABA response, water transport, and transcription, that may collectively contribute to an enhanced abiotic stress tolerance.

RESULTS

The Expression of *DRIR* Is Induced by Drought and Salt Stress

We are interested in the potential role of lncRNA in plant stress responses. We hypothesized that if lncRNAs of this nature are present, they may be induced by abiotic stress. To identify these lncRNAs, we conducted RNA sequencing (RNA-seq) of salt-treated (300 mM NaCl, 3 h) *Arabidopsis* seedlings (Ding et al., 2014) and searched for transcripts longer than 200 nucleotides but with no recognized open reading frames that were induced significantly by the stress treatment. Among dozens of putative lncRNAs identified, a transcript of 755 nucleotides, which we referred to as *DRIR*, was chosen for further study. *DRIR* does not seem to encode a protein, although a short reading frame encoding 41 amino acids that does not show homology to any known peptide could be predicted. The middle part of the sequence has limited similarity to transposon elements, but overall, the sequence does not show clear homology to other genes. Like many other lncRNAs, *DRIR* does not have homologs in other plants.

Initially identified as salt stress inducible in our RNA-seq studies, the regulation of *DRIR* by stress was investigated further. Total RNA was extracted from the wild-type seedlings that were treated with dehydration or 150 mM NaCl. The expression level of *DRIR* was examined using real-time PCR. Consistent with our RNA-seq data, higher levels of *DRIR* transcripts were found to accumulate in salt-treated seedlings relative to untreated seedlings. The expression level of *DRIR* was 168-fold that in untreated seedlings (Supplemental Fig. S1). In addition, the expression level of *DRIR* in dehydrated seedlings was 59-fold that in untreated seedlings (Supplemental Fig. S1). To confirm these data, a construct was made in which the GUS reporter gene was placed under the control of a 2-kb *DRIR* promoter. This construct (P_{DRIR} :GUS) was introduced into Columbia-0 plants using *Agrobacterium tumefaciens*-mediated transformation. Transgenic lines were treated with dehydration or 150 mM NaCl, and the P_{DRIR} :GUS activities were analyzed. As shown in Supplemental Figure S1, the P_{DRIR} :GUS activities were increased significantly under dehydration and salt stress conditions relative to the control conditions, confirming that drought and salt stress strongly up-regulate the expression of *DRIR*.

The Expression Pattern and Subcellular Localization of *DRIR*

Data from the Affymetrix AG and ATH1 GeneChip arrays in the Genevestigator database (<https://genevestigator.com/gv/>) indicate that *DRIR* is expressed at a low level in plants and is expressed mainly in the root, inflorescence, embryo, shoot, and leaf. To further examine the expression pattern of *DRIR*, different parts of P_{DRIR} :GUS transgenic lines were stained for GUS activity, and the results revealed that GUS signal was detected predominantly in roots, cotyledons, and stems in 6-d-old seedlings (Fig. 1B). In adult plants, strong GUS signals were detected in rosette leaves, guard cells, and flower petals (Fig. 1, C, D, and F). None or only weak GUS signal was detected in the embryo, axillary leaf, and silique (Fig. 1, A, E, and F). To verify these results, total RNA was extracted from different tissues and real-time PCR was performed. Consistent with the GUS staining data, real-time PCR showed that *DRIR* was highly expressed in

roots and rosette leaves and to a lesser extent in stems, axillary leaves, inflorescences, and siliques (Fig. 1G).

To investigate the subcellular localization of *DRIR*, fluorescence in situ hybridization in roots was performed with Alexa Fluor 488-labeled probe specific to *DRIR*. Fluorescence signal could be seen in nuclei of root cells and root hair cells that were hybridized with the Alexa Fluor 488-labeled *DRIR* probe but not in cells hybridized with the unlabeled *DRIR* probe or a labeled control probe (Fig. 2). To confirm this, nucleus was stained with 4',6-diamino-phenylindole, and the signal of Alexa Fluor 488-labeled *DRIR* probe and the signal of 4',6-diamino-phenylindole were found to colocalize in nuclei of root hairs (Supplemental Fig. S2). In addition, we isolated nuclei from a *DRIR*-overexpressing line (A12; see below) and extracted RNA from nuclei. One microgram of cDNA from nuclear RNA or total RNA was used to perform reverse transcription (RT)-PCR. A random mRNA (*CAR4*) was used as a control. The results show that a *DRIR* fragment could be amplified

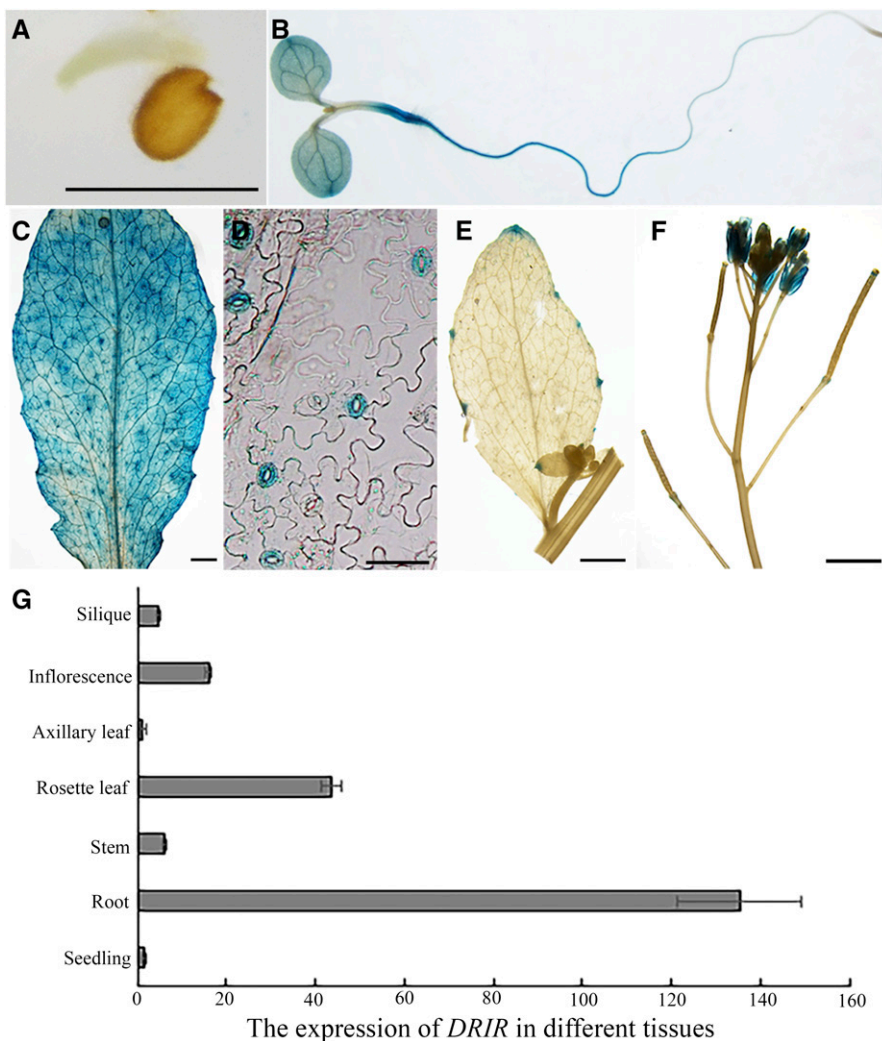
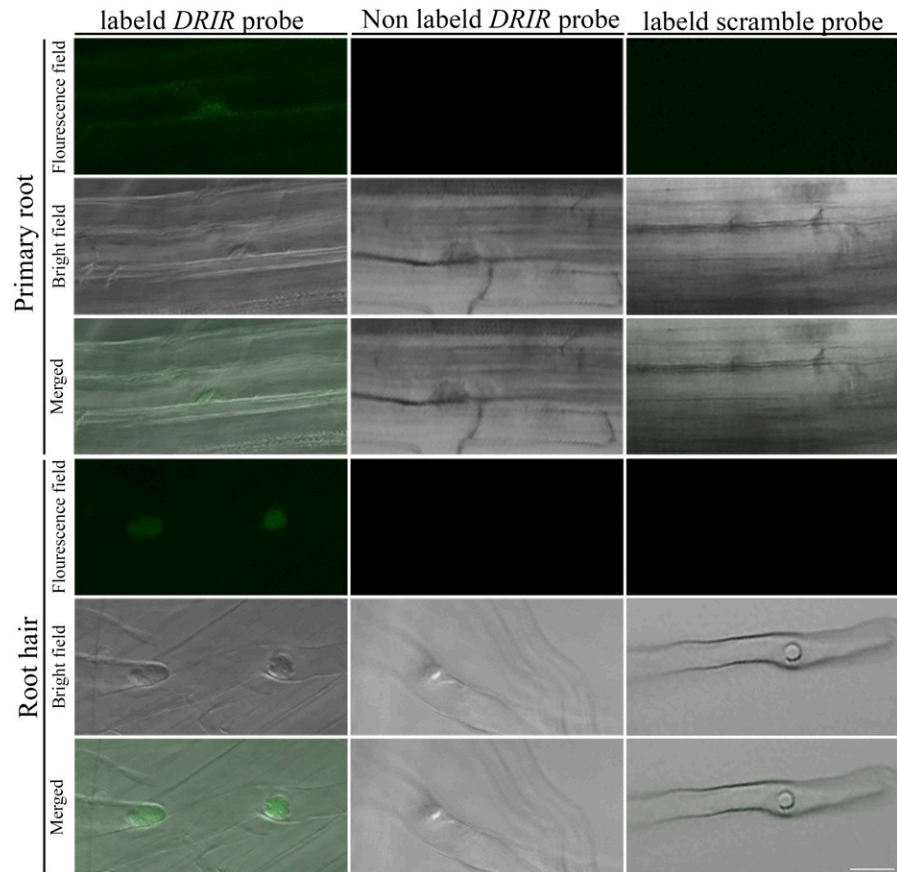


Figure 1. Expression pattern of *DRIR* in seedlings. Shown are *DRIR* promoter-GUS activities in germinating embryo (A), 6-d-old seedling (B), rosette leaf (C), guard cells in rosette leaf epidermis (D), axillary leaf (E), and inflorescence (F). Bars = 1 mm (A, B, C, E and F) and 50 μ m (D). G shows transcript abundance of *DRIR* in different tissues as determined by quantitative RT-PCR. Values shown are means \pm SD from three biological replicates.

Figure 2. Subcellular localization of the *DRIR* transcripts. Fluorescence in situ hybridization with Alexa Fluor 488-labeled antisense *DRIR* probe was performed in 7-d-old Columbia-0 root. Hybridization with unlabeled probe and Alexa Fluor 488-labeled scramble probe was used as a negative control. Bar = 20 μm .



from nuclear RNA and that the gel signal strength was similar to that amplified from total RNA. In contrast, the *CAR4* fragment could only be amplified from total RNA but not from nuclear RNA. These data suggest that *DRIR* transcripts are localized mainly in nuclei (Supplemental Fig. S3).

***DRIR* Has a Function in Response to Drought and Salt Stress**

Since the expression of *DRIR* was up-regulated by drought and salt stress, the functions of *DRIR* in plant responses to drought and salt stress were analyzed. A T-DNA insertion mutant of *DRIR*, SAIL_813_G12, was obtained. The mutant contained a T-DNA insertion at 417 bp downstream of the gene At1g21529 (i.e. *DRIR*) and 467 bp upstream of the gene At1g21528 that encodes a hypothetical protein (Fig. 3A). The expression of *DRIR* in the mutant was significantly higher than that in the wild type (Fig. 3B). However, there was no significant difference in the expression of At1g21528 between the mutant and the wild type (Supplemental Fig. S4). Due to the activation nature of the mutation, we referred to this mutant as *drir^D*. To test the drought tolerance of the mutant, 3-week-old seedlings of the

wild type and *drir^D* in soil were treated with drought stress by stopping watering for 20 d. All leaves of wild-type seedlings became totally withered and dry, but *drir^D* leaves withered to a lesser extent and none was totally dry (Fig. 3D). Two days after rewatering, the wild-type seedlings were still withered; in contrast, *drir^D* leaves became green and turgid again (Fig. 3D), indicating that the *drir^D* mutant is more tolerant to drought stress. We examined transpirational water loss rates of fifth and sixth leaves of 4-week-old soil-grown plants. The water loss rate of *drir^D* detached leaves was much slower than that of the wild-type leaves. *drir^D* leaves lost 18%, 22%, 25%, 27%, and 30% of fresh weight at 40, 60, 80, 100, and 120 min after detachment, respectively, whereas wild-type leaves lost 29%, 32%, 34%, 36%, and 39% of fresh weight at the same times (Fig. 3E). These data demonstrate that *DRIR* is important for drought stress tolerance.

To confirm that the drought-tolerant phenotype of the T-DNA insertion mutant is attributable to increased expression of *DRIR*, a construct consisting of the *DRIR* gene driven by the cauliflower mosaic virus 35S promoter was transferred to Columbia-0, and more than 20 independent transgenic lines were obtained. Two overexpressing lines (A12 and A14) were randomly selected for detailed phenotypic analysis. The expression

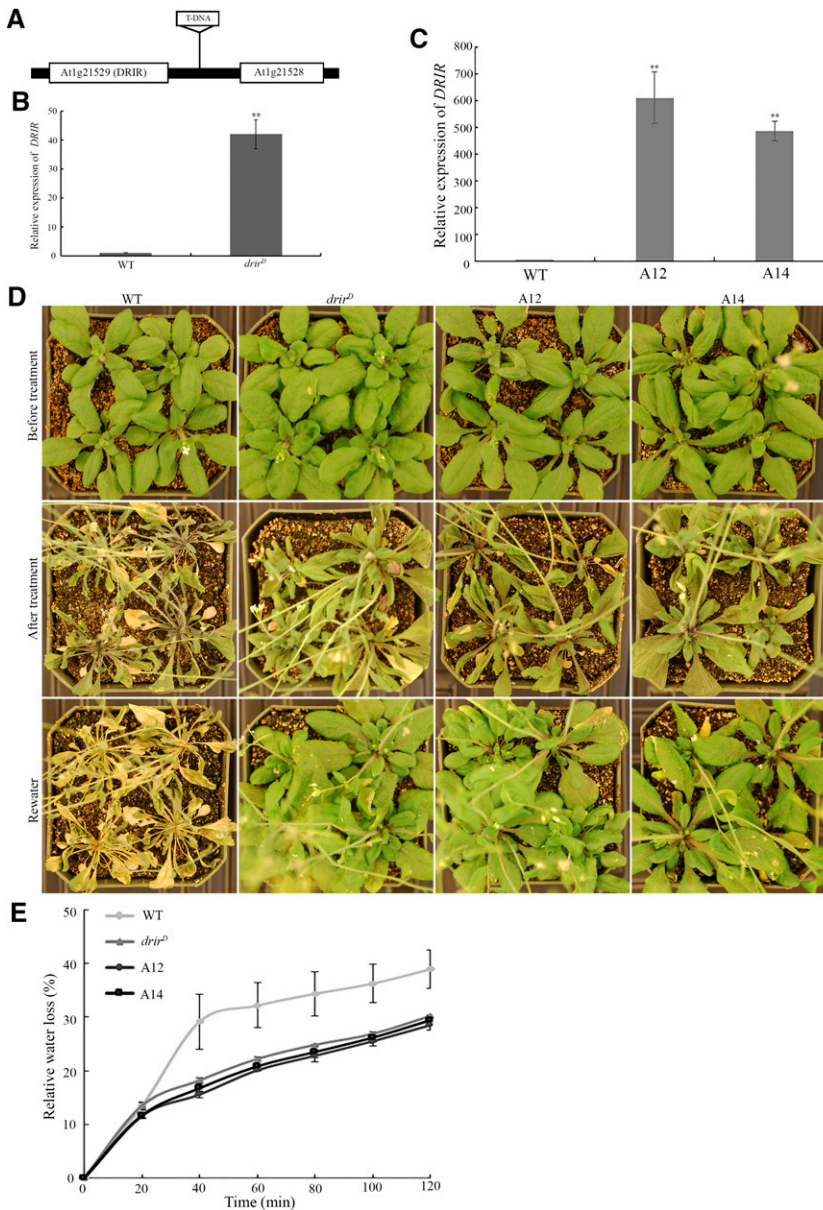


Figure 3. The *drir^D* mutant and overexpressing lines are more tolerant to drought stress. A, The Arabidopsis *DRIR* locus. The position of a T-DNA insertion in the *drir^D* mutant (SAIL_813_G12) is shown. B, Relative transcript levels of *DRIR* in the wild type (WT) and *drir^D*. **, $P < 0.01$ by Student's *t* test. Data represent means \pm SD. C, Relative transcript levels of *DRIR* in the wild type and two overexpressing lines, A12 and A14. **, $P < 0.01$ by Student's *t* test. Data represent means \pm SD. D, Morphology of seedlings before and after drought stress treatment. Three-week-old seedlings were drought stressed by stopping watering for 20 d before rewatering. Photographs were taken before (top row) and after (middle row) the drought treatment and 2 d after rewatering (bottom row). E, Transpirational water loss rates of detached leaves at the indicated times after detachment. Values shown are means \pm SD from three replicates.

level of *DRIR* in both transgenic lines was higher than in the wild type (Fig. 3C). These lines were tested for their drought tolerance along with the *drir^D* mutant. It was found that the two overexpressing lines, similar to *drir^D*, were more tolerant to drought stress (Fig. 3D). The water loss speed of detached leaves of the transgenic plants also was slower than that of the wild-type leaves but was similar to that of *drir^D* (Fig. 3E). These data indicate that the drought tolerance seen in the *drir^D* mutant is caused by the increased expression of *DRIR* and that *DRIR* plays a positive role in limiting transpirational water loss and increasing drought tolerance.

To further elucidate the role of *DRIR* in the Arabidopsis response to abiotic stress, seeds of the wild type, *drir^D*, A12, and A14 were sown onto one-half-strength Murashige and Skoog (1/2 MS) medium plates containing

150 or 200 mM NaCl. As shown in Figure 4, A and B, in contrast to seedlings on 1/2 MS medium without NaCl, many seedlings were bleached and dead at 8 d after germination and growth on 1/2 MS medium containing different concentrations of NaCl. Compared with the 36% survival rate of the wild-type seedlings, the survival rates of *drir^D*, A12, and A14 seedlings were 48%, 60%, and 62% on medium containing 150 mM NaCl, respectively (Fig. 4, A and B). To distinguish the effect of salt stress on seed germination and on seedling growth, 4-d-old seedlings of the wild type, *drir^D*, A12, and A14 grown on 1/2 MS were transferred to 1/2 MS medium containing different concentrations of NaCl and allowed to grow for an additional 4 d. Similar to plants with seeds sown directly onto medium supplemented with salt, the transferred seedlings of *drir^D* and overexpressing lines also were more tolerant to

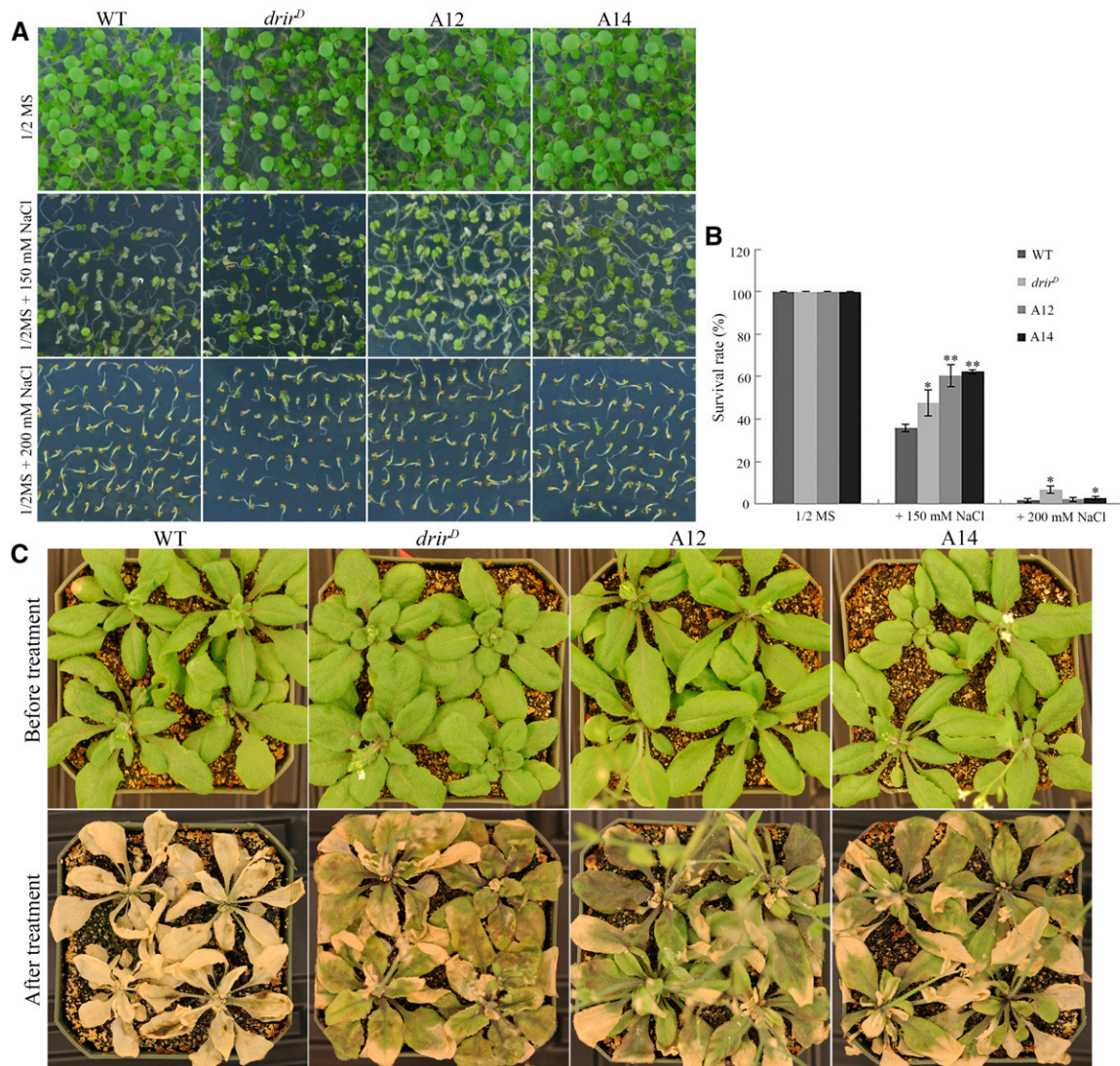


Figure 4. The *drir^D* mutant and *DRIR*-overexpressing lines are more tolerant to salt stress. **A**, Seeds of the wild type (WT), *drir^D*, A12, and A14 were sown onto 1/2 MS agar medium plates supplemented with 0, 150, or 200 mM NaCl. Photographs were taken 8 d after the plates were incubated in the growth chamber for germination and growth. **B**, Seedling survival rate. At least 300 seedlings for each treatment per genotype were scored. *, $P < 0.05$ and **, $P < 0.01$ by Student's *t* test compared with the wild type. Data represent means \pm SD. **C**, Morphology of 4-week-old seedlings irrigated with 200 mM NaCl. Photographs were taken before and 2 weeks after the salt stress treatment.

the salt stress than the wild type. The survival rates of *drir^D*, A12, and A14 were 38%, 48%, and 42% under 150 mM NaCl treatment and 17%, 4%, and 8% under 200 mM NaCl treatment, respectively. In contrast, only 27% and 2% of the wild-type seedlings survived under 150 or 200 mM NaCl, respectively (Supplemental Fig. S5). To evaluate the role of *DRIR* in plant salt tolerance in soil, 4-week-old seedlings in soil were irrigated with 200 mM NaCl. Several days later, damage to leaves was observed. Two weeks later, leaves of *drir^D*, A12, and A14 showed chlorosis but none died. However, nearly all wild-type seedlings were dead (Fig. 4C). These data indicate that *drir^D* and the overexpressing lines are more tolerant to salt stress.

drir^D and Overexpressing Lines Are More Sensitive to ABA

Since phytohormones, in particular ABA, play critical roles in the plant response to abiotic stress (Yamaguchi-Shinozaki and Shinozaki, 2006; Waadt et al., 2014; Munemasa et al., 2015), the possible regulation of *DRIR* by phytohormones was analyzed. We found that the expression level of *DRIR* was increased significantly by ABA treatment. However, the effects of indole acetic acid, zeatin, 24-epibrassinolide, and GA on the expression of *DRIR* were not as profound as that of ABA (Supplemental Fig. S6). We also analyzed GUS activities in P_{DRIR} :GUS transgenic plants under ABA treatment. As shown in Supplemental Figure S6, stronger

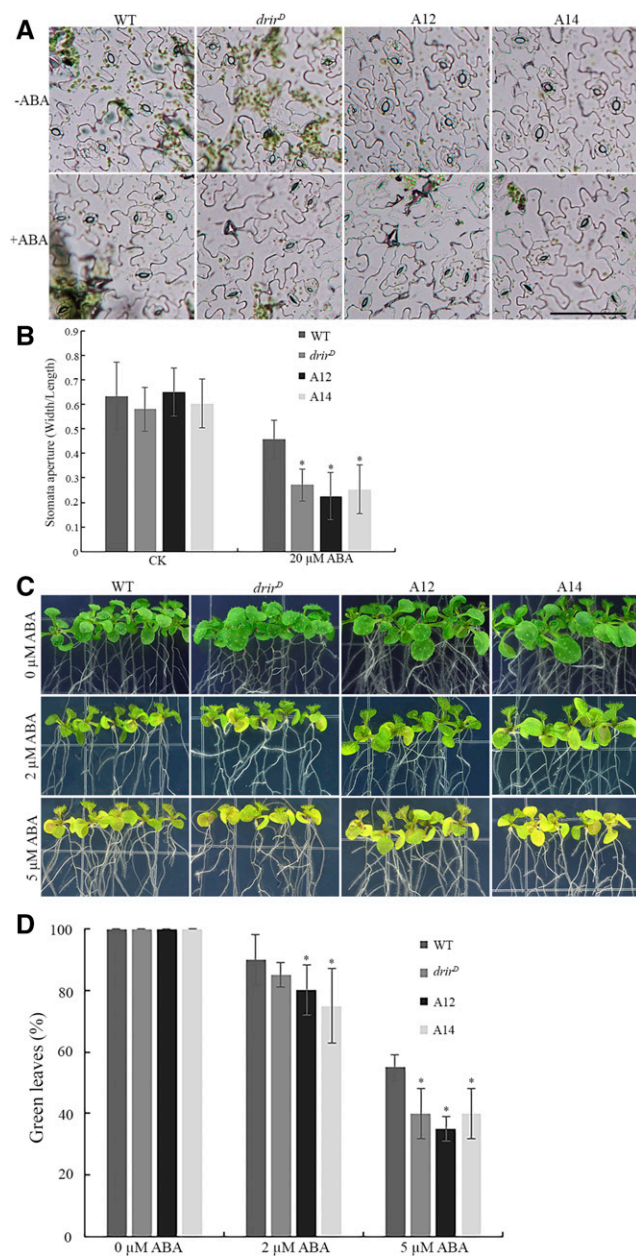


Figure 5. *drir^D* and overexpressing lines are more sensitive to ABA. A and B, Stomata of *drir^D* and the overexpressing lines are more sensitive to ABA-induced stomata closure. A, Representative images of stomata on leaf epidermis in response to ABA treatment. The fifth and sixth rosette leaves of 4-week-old plants were incubated in a stomata open solution for 2 h and then transferred to the open solution supplemented with 20 μM ABA. Images were taken before and 2 h after ABA treatment. Bar = 100 μm . B, Stomata aperture (measured by width over length) in response to the ABA treatment in different genotypes. At least 500 stomata from 30 leaves of each genotype were measured. CK, control with no ABA treatment. *, $P < 0.05$ by Student's *t* test compared with the wild type (WT). Error bars represent SD. C, Seedling sensitivity to ABA. Four-day-old seedlings of the wild type, *drir^D*, A12, and A14 on 1/2 MS plates were transferred to 1/2 MS plates supplemented with 0, 2, or 5 μM ABA. Representative images show the morphology of seedlings 8 d after growth on ABA medium plates. D, Green leaf percentage of each genotype. At least 100 seedlings for each treatment were scored. *, Significant between the sample and the wild type at $P < 0.05$ by Student's *t* test. Data represent means \pm SD.

P_{DRIR} :GUS activities could be observed in ABA-treated seedlings compared with mock-treated seedlings, indicating that the expression of *DRIR* is induced by ABA.

Since ABA increases the expression of *DRIR*, we investigated the responses of *drir^D* and overexpressing lines to ABA. We first examined the sensitivity of stomata movement to exogenous ABA. The fifth and sixth leaves of 4-week-old plants were detached, submerged in stomata open solution, and incubated in a growth chamber for 2 h. The leaf samples were then transferred to a similar open solution with 20 μM ABA to induce stomata closure as described (Brandt et al., 2015). After incubation in the open solution for 2 h, stomata were widely open, and there was no significant difference in stomata aperture between the wild type and *drir^D* or overexpressing lines. The width-to-length ratio for all these genotypes was about 0.6 (Fig. 5, A and B). However, the stomata aperture of *drir^D* or overexpressing lines was much smaller than that of the wild type with the ABA treatment. The stomata width-to-length ratio of *drir^D* and the two overexpressing lines decreased from about 0.6 to 0.27, 0.22, and 0.25, respectively, whereas that of the wild type merely decreased to 0.46 (Fig. 5, A and B). These data indicate that stomata of *drir^D* and the overexpressing lines were more responsive to ABA-induced closure than those of the wild type.

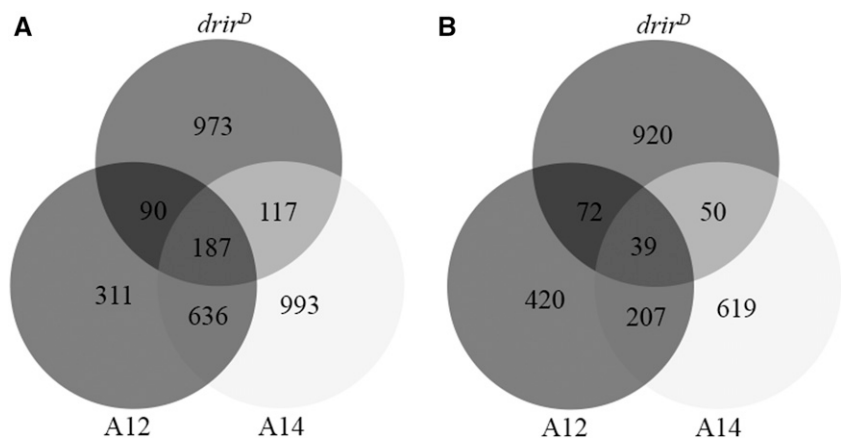
To exclude the possibility that *DRIR* affects the plant response to drought stress by regulating stomatal density, stomatal densities of the fifth and sixth leaves of 4-week-old plants were examined, but no significant difference was found among these genotypes (Supplemental Fig. S7), suggesting that *DRIR* does not regulate the morphogenesis and differentiation of stomatal cells.

We further examined the sensitivity of *drir^D* and overexpressing seedlings to ABA. Four-day-old seedlings of the wild type, *drir^D*, A12, and A14 grown on 1/2 MS agar plates were transferred to 1/2 MS agar plates supplemented with different concentrations of ABA. Eight days later, more seedlings of *drir^D* and overexpressing lines were found to have etiolated leaves compared with the wild type (Fig. 5C). While almost 90% of leaves of wild-type seedlings were green, less than 85% of leaves of *drir^D* and overexpressing seedlings were green under 2 μM ABA treatment. Although the percentage of green leaves of wild-type seedlings decreased to 55% under 5 μM ABA treatment, less than 40% of green leaves could be found in *drir^D* and overexpressing seedlings (Fig. 5D), indicating that *drir^D* and overexpressing seedlings are more sensitive to ABA than the wild type.

***DRIR* Regulates the Expression of Genes Involved in the Stress Response**

To elucidate the molecular mechanism of *DRIR* function in the plant response to drought stress, 10-d-old seedlings

Figure 6. Number of up- and down-regulated genes in dehydration-treated *drir^D* and overexpressing lines in transcriptome sequencing analysis compared with dehydration-treated wild-type seedlings. A, Number of genes up-regulated more than 2-fold in dehydration-treated *drir^D* and overexpressing seedlings. B, Number of genes down-regulated more than 2-fold in dehydration-treated *drir^D* and overexpressing seedlings.



treated with dehydration were used for transcriptome sequencing analysis. Compared with the wild type, there were 1,367, 1,224, and 1,933 genes up-regulated and 1,081, 738, and 915 genes down-regulated more than 2-fold in *drir^D*, A12, and A14, respectively (Fig. 6; Supplemental Table S1). Among them, 187 genes were up-regulated and 39 genes were down-regulated both in *drir^D* and the two overexpressing lines (Fig. 6; Supplemental Table S2). Among those genes up-regulated both in *drir^D* and the overexpressing lines, 28 genes have been suggested to be involved in the plant response to drought stress, salt stress, or ABA (Table I).

To further validate the expression of those genes that are known to be critical in the plant response to stress, total RNA was extracted from 10-d-old *drir^D* and overexpression seedlings treated with dehydration stress, and real-time PCR was performed to analyze their expression. As shown in Figure 7, the expression of *Arabidopsis thaliana respiratory burst oxidase B (AtrbohB)*, an NADPH oxidase gene, increased more than 15-fold in dehydration-treated *drir^D* and the overexpressing lines, whereas less than 2-fold increase was found in dehydration-treated wild-type seedlings (Fig. 7E). The expression of *FUCOSYLTRANSFERASE4 (FUT4)* increased 19-, 16-, and 20-fold in dehydration-treated *drir^D* and the A12 and A14 overexpressing lines, respectively, but only 4-fold increase was observed in wild-type seedlings (Fig. 7F). Whereas not significantly up-regulated by dehydration stress in the wild type, *NOD26-LIKE INTRINSIC PROTEIN1 (NIP1)*, a member of the NIP aquaporin subfamily, was expressed about 2-fold in dehydrated *drir^D* and the overexpressing lines compared with the untreated wild type (Fig. 7G). The expression level of another aquaporin protein gene, *TONOPLAST INTRINSIC PROTEIN4 (TIP4)*, increased 24-fold in dehydration-treated wild-type seedlings, but it increased more than 40-fold in *drir^D* and the overexpressing lines (Fig. 7H). The expression of an Arabidopsis annexin gene, *ANNAT7*, increased more than 8-fold in dehydration-treated *drir^D* and the overexpressing lines, while it increased only 2-fold in wild-type seedlings (Fig. 7I). Two transcription factor genes, *NAM*, *ATAF*, *AND CUC PROTEIN3 (NAC3)* and *WRKY PROTEIN8*

(*WRKY8*), also were expressed significantly higher in dehydrated *drir^D* and the overexpressing lines than in wild-type seedlings (Fig. 7, J and K).

In our RNA-seq analysis, we also found that several other ABA-signaling or ABA-inducible genes were expressed at a higher level in dehydration-treated *drir^D* and the overexpressing seedlings than in wild-type seedlings, although the increasing levels were less than 2-fold (Supplemental Table S1). These genes include, for example, *ABSCISIC ACID-INSENSITIVE5 (ABI5)*, Δ^1 -*PYRROLINE-5-CARBOXYLATE SYNTHETASE1 (P5CS1)*, *RD29A*, and *RD29B*. Real-time PCR analysis was performed to validate the expression levels of these genes. Compared with untreated seedlings, the expression of these genes was increased significantly in dehydration-treated wild-type seedlings, but the extent of increase was much smaller than in *drir^D* or overexpressing seedlings. The expression of *ABI5* increased more than 90-fold in dehydration-treated *drir^D* and overexpressing seedlings, while it increased only 6-fold in dehydrated wild-type seedlings (Fig. 7A). The expression of *P5CS1* increased 4-, 42-, 35-, and 33-fold in dehydration-treated wild-type, *drir^D*, A12, and A14 seedlings, respectively (Fig. 7B). The expression of *RD29A* and *RD29B* also increased more than 160- and 1,400-fold in dehydration-treated *drir^D* and overexpressing seedlings, respectively, while it increased only 37- and 86-fold in dehydration-treated wild-type seedlings (Fig. 7, C and D). These data indicate that *DRIR* could regulate plant tolerance to drought stress by modulating the expression of genes involved in ABA signaling or stress responses.

We further investigated the regulation of these stress-related genes by *DRIR* in response to salt stress. Ten-day-old seedlings were treated with 200 mM NaCl for 3 h, and real-time PCR was performed to analyze the expression of the above-mentioned genes. The results showed that the expression of *P5CS1*, *RD29A*, *RD29B*, *AtrbohB*, *FUT4*, *ANNAT7*, and *NAC3* increased dramatically in salt-treated *drir^D* and overexpressing seedlings, whereas their expression increased to a much lesser extent in salt-treated wild-type seedlings (Fig. 8), indicating that *DRIR* also regulates the expression of these genes under salt stress. Nonetheless, no significant difference

Table 1. Genes reported to be involved in the plant response to drought stress, salt stress, or ABA that are up-regulated both in *drir^D* and in the overexpressing lines

Gene Identifier	Gene Name	Description	Reference
AT1G02920	<i>ATGST11</i>	Glutathione transferase	Yang et al. (1998)
AT1G09090	<i>AtrbohB</i>	NADPH oxidase <i>AtrbohB</i>	Kwak et al. (2003)
AT1G65970	<i>TPX2</i>	Thioredoxin-dependent peroxidase2	Kumar et al. (2015)
AT1G67760		TCP-1/cpn60 chaperonin family protein	Gong et al. (2001)
AT1G73260	<i>ATK11</i>	Trypsin inhibitor	Chini et al. (2004)
AT1G74080	<i>ATMYB122</i>	Putative transcription factor	Ding et al. (2013)
AT1G79840	<i>GL2</i>	Homeodomain protein	Wang and Li (2008)
AT2G15390	<i>FUT4</i>	α -(1,2)-Fucosyltransferase	Tryfona et al. (2014)
AT2G18550	<i>ATHB21</i>	Homeodomain Leu zipper class I (HD-Zip I) protein	Ding et al. (2013)
AT2G25810	<i>TIP4</i>	Tonoplast intrinsic protein4;1	Alexandersson et al. (2005); Regon et al. (2014)
AT2G26290	<i>ARSK1</i>	Root-specific kinase1 (ARSK1)	Hwang and Goodman (1995)
AT2G29090	<i>CYP707A2</i>	Protein with ABA 8'-hydroxylase activity	Arc et al. (2013); Sasaki et al. (2015)
AT2G38905		Low temperature- and salt-responsive protein family	Medina et al. (2007)
AT3G09940	<i>ATMDAR3</i>	Member of the monodehydroascorbate reductase gene family	Brini et al. (2011)
AT3G29035	<i>NAC3</i>	Protein with transcription factor activity	Nakashima et al. (2012)
AT3G45700	<i>NPF2.4</i>	Member of the NAXT NPF subfamily	Li et al. (2016a)
AT3G54770	<i>ARP1</i>	Putative RNA-binding protein	Jung et al. (2013)
AT4G19030	<i>NIP1</i>	Aquaporin	Alexandersson et al. (2005)
AT4G25820	<i>XTR9</i>	Xyloglucan endotransglycosylase	Li et al. (2008)
AT4G28520	<i>CRU3</i>	12S seed storage protein	Nambara et al. (1995)
AT4G40090	<i>AGP3</i>	Arabinogalactan protein3 (AGP3)	Ma and Bohnert (2007)
AT5G01550	<i>LECRKA4.2</i>	Member of the lectin receptor kinase subfamily A4	Xin et al. (2009)
AT5G06630		Pro-rich extensin-like family protein	Dinneny et al. (2008)
AT5G10230	<i>ANNAT7</i>	Calcium-binding protein annexin (ANNAT7)	Cantero et al. (2006)
AT5G13080	<i>WRKY75</i>	Transcription factor induced during inorganic phosphate deprivation	Ding et al. (2013)
AT5G44610	<i>MAP18</i>	Protein with seven repeated VEEKK motifs	Kato et al. (2010)
AT5G46350	<i>WRKY8</i>	Member of the WRKY transcription factors	Hu et al. (2013)
AT5G48010	<i>THAS</i>	Oxidosqualene cyclase	de Silva et al. (2011)

was found in the expression of *ABI5* and *NIP1* between salt-treated wild-type and *drir^D* or overexpressing seedlings (Supplemental Fig. S8, A and B). Furthermore, the expression of *TIP4* and *WRKY8* in salt-treated wild-type seedlings was even higher than in *drir^D* and overexpressing seedlings (Supplemental Fig. S8, C and D). These results suggest that *DRIR* may differentially regulate stress-related genes through complex mechanisms.

***DRIR* Affects the Accumulation of Pro and Reactive Oxygen Species**

Pro is an important osmolyte with cellular protection functions in plants. Abiotic stress conditions as well as ABA treatment could promote the accumulation of Pro by inducing *P5CS1* expression (Abrahám et al., 2003; Sharma et al., 2011). Since *drir^D* and the overexpressing lines are more sensitive to ABA and the expression of *P5CS1* in *drir^D* and the overexpressing lines is increased dramatically when treated with dehydration and salt stress, we analyzed the contents of Pro in 10-d-old seedlings treated with dehydration or salt stress. As shown in Supplemental Figure S9, the content of Pro increased from 10 to 30 $\mu\text{g g}^{-1}$ fresh weight in wild-type seedlings when treated with dehydration stress, while it increased to more than 60 $\mu\text{g g}^{-1}$ fresh weight in *drir^D*

and the overexpressing lines. When treated with salt stress, the content of Pro increased to 30 $\mu\text{g g}^{-1}$ fresh weight in wild-type seedlings, yet it increased to more than 42 $\mu\text{g g}^{-1}$ fresh weight in *drir^D* and the overexpressing lines. These results suggest that the synthesis of Pro was likely enhanced in *drir^D* and the overexpressing lines under drought and salt stress.

Reactive oxygen species (ROS) are critical second messengers in the ABA regulation of stomata closure, and their synthesis is catalyzed by NADPH oxidases (Kwak et al., 2003; Watkins et al., 2014). Since stomata closure of *drir^D* and the overexpressing lines was more sensitive to ABA and the expression of the NADPH oxidase catalytic subunit gene *AtrbohB* was dramatically up-regulated in dehydration- and salt-treated *drir^D* and overexpressing seedlings, we examined whether *DRIR* affects ROS accumulation in guard cells during ABA regulation of stomata closure. Peeled epidermises from leaves treated with or without ABA were stained by 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$) and examined with a confocal microscope. As shown in Figure 9, the intensity of the fluorescence signal of $\text{H}_2\text{DCF-DA}$ in guard cells of leaves without ABA treatment was very low but increased dramatically after ABA treatment, indicating that ROS accumulation was increased significantly in ABA-treated leaves. Interestingly, the fluorescence signal of $\text{H}_2\text{DCF-DA}$ in ABA-treated *drir^D*

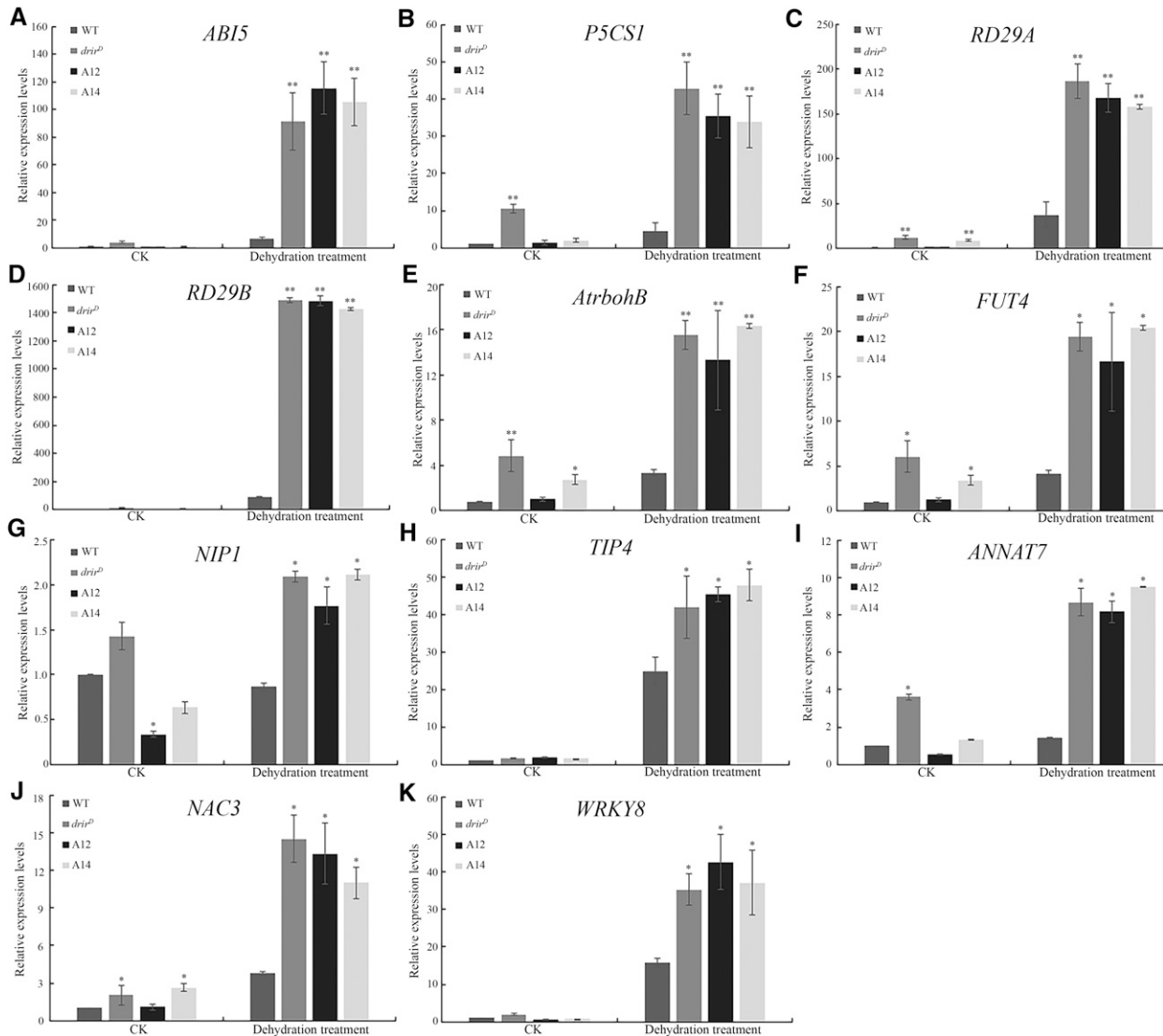


Figure 7. Relative expression levels of selected genes in dehydration-treated seedlings. RNA was extracted from 10-d-old seedlings that were dehydrated to lose about 40% fresh weight, and gene expression level was measured by RT-quantitative PCR normalized against the *18S rRNA* gene. Relative expression levels are shown for *AB15* (A), *P5CS1* (B), *RD29A* (C), *RD29B* (D), *AtrbohB* (E), *FUT4* (F), *NIP1* (G), *TIP4* (H), *ANNAT7* (I), *NAC3* (J), and *WRKY8* (K). CK, controls without dehydration treatment. *, $P < 0.05$ and **, $P < 0.01$ by Student's *t* test compared with untreated (CK) or dehydration-treated wild-type (WT) seedlings. Data represent means \pm sd.

and overexpressing guard cells was much higher than that in ABA-treated wild-type guard cells (Fig. 9A). The relative fluorescence intensity in guard cells of ABA-treated wild-type leaves was 11.7, while those of *drir^D* and overexpressing leaves were 24.5, 21.6, and 23.3, respectively (Fig. 9B). These data demonstrate that more ROS accumulated in guard cells of *drir^D* and overexpressing leaves during ABA induction of stomata closure.

DISCUSSION

In this study, we identified and functionally characterized a novel abiotic stress-related lncRNA from

Arabidopsis, *DRIR*. Our experimental data demonstrate that *DRIR* positively regulates plant tolerance to drought and salt stress by modulating the expression of genes critical to the stress response.

DRIR Is a Novel lncRNA Regulating Plant Tolerance to Drought and Salt Stress

Plant lncRNAs are expected to play important roles in plant development and the response to environment conditions, and thousands of lncRNAs have been identified in plants (Liu et al., 2012; Li et al., 2014; Shuai et al., 2014; Wang et al., 2014; Zhang et al., 2014b; Chen et al., 2015). However, revealing the functions of plant

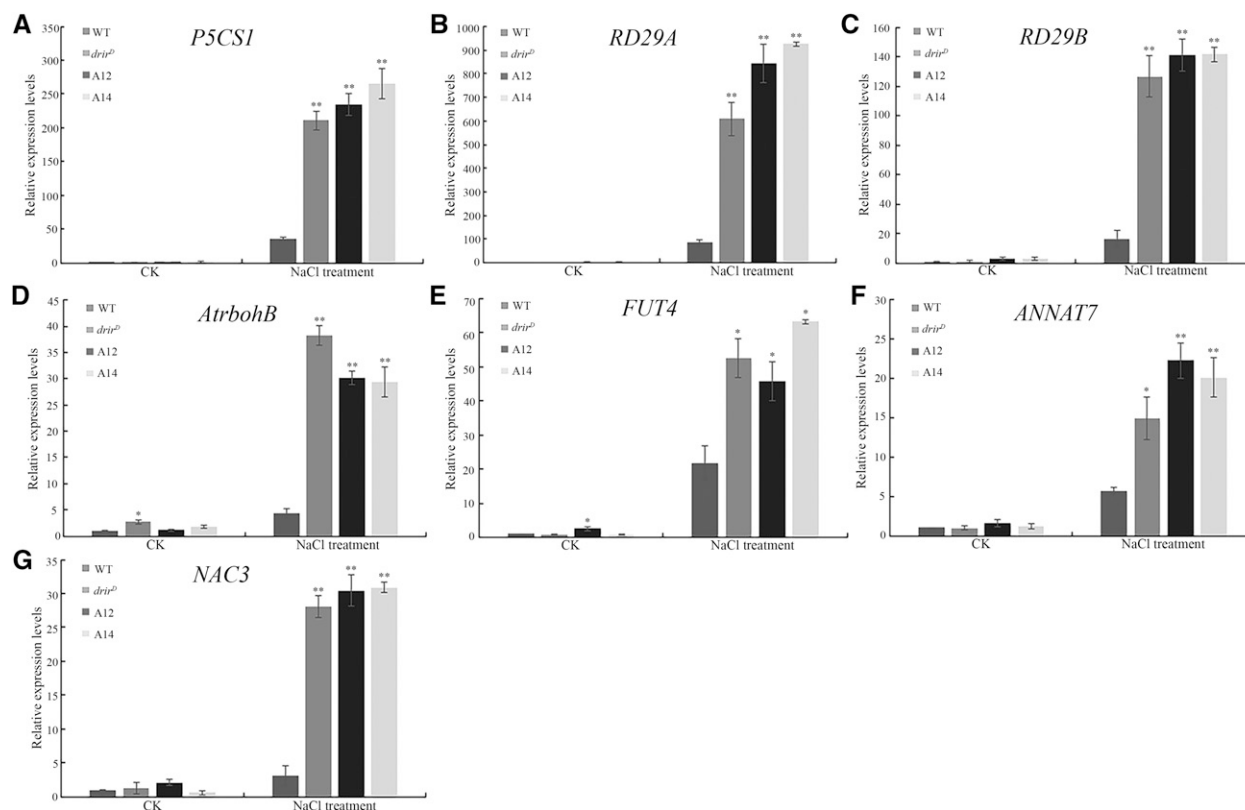


Figure 8. Relative expression levels of selected genes in salt stress-treated seedlings. RNA was extracted from 10-d-old seedlings that were treated with 200 mM NaCl for 3 h, and gene expression level was measured by RT-quantitative PCR normalized against the *18S rRNA* gene. Relative expression levels are shown for *P5CS1* (A), *RD29A* (B), *RD29B* (C), *AtrbohB* (D), *FUT4* (E), *ANNAT7* (F), and *NAC3* (G). CK, controls without NaCl treatment. *, $P < 0.05$ and **, $P < 0.01$ by Student's *t* test compared with untreated (CK) or salt-treated wild-type (WT) seedlings. Data represent means \pm sd.

lncRNA is still challenging. This may not be due only to the fact that most plant lncRNAs are expressed at low levels and their expression may be confined to specific cell types or specific conditions, but also to the fact that plant lncRNAs are not evolutionarily conserved in general (Liu et al., 2012, 2015a, 2015b).

Among the lncRNAs identified so far, some of them are probably responsive to drought and salt stress. By using a deep transcriptome sequencing approach, Qi et al. (2013) identified 584 lncRNAs in foxtail millet. Among them, 17 lncRNAs and two NATs are drought responsive. Chung et al. (2016) also found 98 drought-responsive lncRNAs in rice by using RNA-seq. By genome-wide analysis of full-length cDNA databases, Ben Amor et al. (2009) identified 76 Arabidopsis lncRNAs and found that the accumulation of 22 lncRNAs was altered by abiotic stress. These lncRNAs include three up-regulated and five down-regulated under salt treatment and four up-regulated and two down-regulated under dehydration treatment. Transgenic analyses showed that overexpressing *npc536* increases lateral root number under salt treatment. Using RNA-seq, 664 drought-responsive lncRNAs were identified in maize. Among them, 567 lncRNAs were up-regulated and 97 lncRNAs were down-regulated in drought-

stressed leaves of maize (Zhang et al., 2014a). Nonetheless, no lncRNA that functions in the plant response to drought or salt stress tolerance has been characterized in detail.

In this study, we identified a lncRNA whose length is 755 nucleotides and does not seem to encode a protein. The transcriptional locus of *DRIR* is initiated and terminated between two genes, and *DRIR* should be an intergenic lncRNA. We showed that the *drir^D* activation mutant and *DRIR*-overexpressing lines are more tolerant to drought and salt stress. Three-week-old *drir^D* and overexpressing seedlings were able to survive 20 d of drought stress treatment, whereas wild-type seedlings were killed by the stress. Leaves of the *drir^D* mutant and overexpressing lines also had slower transpirational water loss rate than the wild type. This is likely due to *DRIR*'s role in regulating the transpiration rate, since leaf stomata closure of *drir^D* and overexpressing lines was more sensitive to ABA. Furthermore, growth assays either on culture medium or in soil showed that the *drir^D* mutant and overexpressing seedlings are more tolerant to salt stress than the wild type. These results indicate that *DRIR* regulates the plant response to both drought and salt stress.

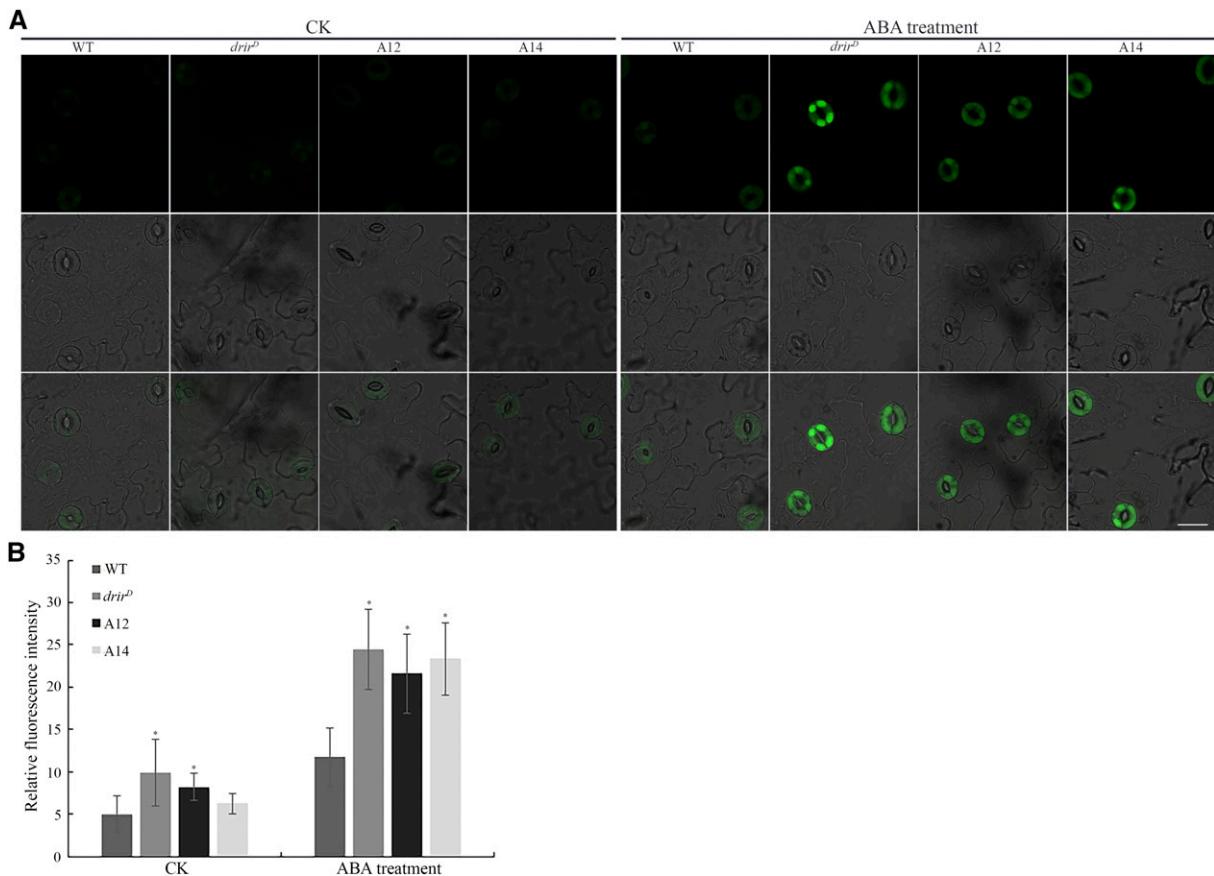


Figure 9. More ROS accumulated in guard cells of *drir^D* and overexpressing leaves in response to ABA treatment. A, Representative images of ROS production stained by the fluorescent dye H₂DCF-DA in guard cells without (left) or with (right) ABA treatment. Top row, Fluorescence images; middle row, bright-field images; bottom row, merged images. Bar = 20 μ m. B, Relative fluorescence intensity of H₂DCF-DA. More than 100 guard cells from 10 leaves of each genotype were measured. CK, controls without ABA treatment. *, $P < 0.05$ by Student's *t* test compared with untreated or ABA-treated wild type (WT) seedlings. Error bars represent SD.

DRIR Regulates ABA-Mediated Responses to Drought and Salt Stress

It has been reported that some plant lncRNAs may participate in a phytohormone-mediated response to the environment. The Arabidopsis lncRNA *APOLO* is transcribed by RNA polymerases Pol II and Pol V in response to auxin from a locus located about 5 kb upstream of *PINOID* (*PID*), which is a key regulator of polar auxin transport. The dual transcription of *APOLO* regulates the formation of a chromatin loop encompassing the promoter of *PID* and, thus, affects auxin transport (Ariel et al., 2014). Liu et al. (2015b) suggested that lncRNAs may participate in ABA-induced complex assembly and the relocalization of RNA-BINDING PROTEIN. However, lncRNAs involved in the ABA-mediated stress response are still unknown.

In this study, *DRIR* may regulate ABA-mediated drought and salt stress responses. There appears to exist a positive feed-forward mechanism for *DRIR* regulation of plant tolerance to drought and salt stress. The expression of *DRIR* is induced by ABA, and the resulting increased *DRIR* level further enhances the

sensitivity of the plants to ABA. This was shown by increased etiolated leaves in *drir^D* and overexpressing seedlings under ABA treatment as well as by increased sensitivity to ABA in stomata closure in these plants relative to the wild-type plants. At the molecular level, ABA-signaling or ABA-responsive genes were expressed at higher levels in *drir^D* and overexpressing plants. For example, *ABI5* is a transcription factor in ABA signaling and has a function in the plant response to abiotic stress (Tanaka et al., 2012; Hopper et al., 2016). *P5CS1* is involved in ABA modulation of the stress response in Arabidopsis by controlling Pro accumulation (Abrahám et al., 2003; Sharma et al., 2011). *RD29A* and *RD29B* play roles in the plant response to drought or salt stress, and their expression is controlled by ABA (Nakashima et al., 2006). *AtrbohB* is a member of the NADPH oxidase catalytic subunit genes, and the enzyme catalyzes the synthesis of the second messengers ROS in ABA signaling (Kwak et al., 2003). The expression of these genes in *drir^D* and overexpressing lines is much higher than in the wild type when treated with dehydration. Consistent with the increased gene expression, increased accumulation of Pro

and ROS was observed in *drir^D* and overexpressing lines under dehydration stress or ABA treatment. Since ROS are critical second messengers in the ABA regulation of stomata closure (Kwak et al., 2003; Watkins et al., 2014), the increased sensitivity of stomata closure to ABA likely resulted from enhancing ABA-induced ROS accumulation in guard cells. This increased guard cell responsiveness to ABA, rather than alteration in stomatal density, should be responsible for the reduced transpirational water loss seen in the mutant and overexpressing lines (Fig. 3E). Thus, together with increased Pro accumulation and the enhanced expression of other ABA- and stress-responsive genes, *DRIR* significantly improves plant drought tolerance.

Under salt stress conditions, the expression levels of *P5CS1*, *RD29A*, *RD29B*, and *AtrbohB* are notably increased in *drir^D* and overexpressing lines as well. These data suggest that *DRIR* regulates plant tolerance to drought and salt stress at least partly by mediating the expression of those genes involved in ABA signaling and ABA-regulated stress tolerance. The expression levels of other stress-related genes, including *FUT4*, *NIP1*, *TIP4*, *ANNAT7*, *NAC3*, and *WRKY8*, also are increased significantly in *drir^D* and overexpression lines under dehydration stress. *FUT4* has been reported to affect plant sensitivity to salt stress by coding a fucosyltransferase that catalyzes the synthesis of fucosylated AGPs in leaves and roots (Tryfona et al., 2014). *TIP4* and *NIP1*, coding for two aquaporin proteins, play important roles in water uptake (Alexandersson et al., 2005; Regon et al., 2014). *ANNAT7* encodes a calcium-binding protein annexin and likely has a function in salt and dehydration stress response (Cantero et al., 2006). The *NAC3* and *WRKY8* transcription factors also function in drought or salt stress response (Nakashima et al., 2012; Hu et al., 2013). The regulation of these many stress-related proteins by *DRIR* suggests that its mechanism in regulating plant tolerance to drought or salt stress is complex. For example, *DRIR* could regulate plant tolerance to drought stress by enhancing water transport, increasing the accumulation of stress-relief proteins, and sensitizing the stomata response to ABA. *DRIR* also could regulate plant tolerance to salt stress by affecting the activity of fucosyltransferase or *NAC3* transcription factor or by regulating redox status. The difference between gene expression in dehydration-treated and salt-treated seedlings is likely due to different mechanisms of *DRIR* in regulating plant tolerance to drought and salt stress. For example, the expression levels of *NIP1* and *TIP4* were increased to much higher levels in *drir^D* and overexpressing seedlings than in wild-type seedlings when treated with dehydration, but the difference was not as significant when treated with salt stress. This could suggest that aquaporins participate in the *DRIR* regulation of drought stress tolerance but not in salt stress tolerance.

The lncRNAs may execute their functions through a multitude of mechanisms. Cytoplasm-localized lncRNAs could function as target mimics of miRNAs and, thus, promote its target mRNA translation (Ariel et al., 2015).

The lncNATs usually trigger their complementary mRNA degradation by binding with them or promote their translation through recruitment to polysomes (Zubko and Meyer, 2007; Jabnourne et al., 2013). lncRNAs commonly function to regulate target RNA alternative splicing or affect chromatin topology and regulate neighboring gene transcription (Ariel et al., 2014; Bardou et al., 2014). In addition, lncRNAs could function as precursors of miRNAs and other small RNAs (Pant et al., 2008). In this study, *in situ* hybridization showed that *DRIR* is localized mainly in the nucleus, suggesting that *DRIR* may function mainly in nuclear processes such as transcription but may not function as a target mimic of miRNAs, as do other cytoplasm-localized lncRNAs. Furthermore, no known miRNA in Arabidopsis could be found either at the *DRIR* locus or to match well with the *DRIR* sequence. Thus, *DRIR* may not be a precursor of miRNAs. The observation that the expression of many stress-responsive genes was altered in *drir^D* and the overexpressing lines suggests that *DRIR* may function at or upstream of the stage of gene transcription in the stress or ABA signal transduction pathways. One intriguing observation is that constitutive expression of *DRIR* did not significantly increase the expression levels of most stress-responsive genes under normal conditions. Rather, the expression of these genes was potentiated significantly by the overexpression of *DRIR* only under stress conditions (Figs. 7 and 8). As *DRIR* is transcribed at the chromatin locus between two genes, it is unlikely that *DRIR* functions by binding directly with cognate RNA and modulating their degradation or promoting their translation, as lncNATs do. However, the molecular mechanisms of how *DRIR* exerts its functions are still an open question.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) plants and materials used in this study were in the Columbia-0 ecotype background. Unless stated otherwise, seeds were sterilized and planted on 1/2 MS medium (Sigma-Aldrich) plates containing 0.8% (w/v) agar and 1% (w/v) Suc. Plates were moved to 22°C chambers with a 16-h-light/8-h-dark cycle for germination and growth after stratification at 4°C for 3 d. Ten days later, seedlings were transferred to soil and placed in a growth room at 22°C with a 16-h-light/8-h-dark cycle.

Analysis of *DRIR* Promoter:GUS Activity

The *DRIR* promoter fragment (2 kb) was amplified and inserted into the pMDC162 vector using the Gateway cloning technology. After sequence confirmation, the construct was transformed into Arabidopsis using *Agrobacterium tumefaciens* (strain GV3101). The GUS staining procedure was performed as described previously (Chen et al., 2013). Samples were stained in GUS staining buffer at 37°C overnight followed by decoloring with 70% ethanol and 30% acetic acid. Samples were observed and photographed using a microscope.

RNA *In Situ* Hybridization

RNA *in situ* hybridization was performed as described (Gong et al., 2005) with minor modifications. Roots were taken from 7-d-old Columbia-0 seedlings and fixed in a glass vial by adding 10 mL of fixation buffer (120 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, 2.7 mM KCl, 0.1% [v/v] Tween 20, 80 mM EGTA, 5% [v/v] paraformaldehyde, and 10% [v/v] dimethyl sulfoxide). The samples were shaken gently for 2 h at room temperature. After dehydration twice for

5 min each in absolute methanol and three times for 5 min each in absolute ethanol, the samples were incubated for 30 min in 1:1 ethanol:xylene and then washed twice for 5 min each with absolute ethanol, twice for 5 min each with absolute methanol, and once for 5 min with 1:1 methanol:fixation buffer without 5% (v/v) paraformaldehyde. The samples were postfixed in the fixation buffer for 30 min at room temperature and rinsed twice with fixation buffer without 5% (v/v) paraformaldehyde and once with 1 mL of perfect HybPlus hybridization buffer (Sigma-Aldrich; H-7033). Each glass vial was then added with 1 mL of hybridization buffer and prehybridized in an incubator for 1 h at 50°C. After prehybridization, 5 pmol of probe specific to *DRIR* or scramble probe was added into the vial and hybridized at 50°C in darkness for more than 8 h. The sequence of probe specific to *DRIR* is 5'-CTCCAAACTCCTTTATTTCTTAACCAAAAGTTA-CAATTCATGAGAAGATGATCTAGAACATCATTTCTAGACTCATCTCTAA-ATCTCACACAGGATTTGTTACACAAAATTCATAAAGCTCTCTAAACAA-TGAGATACCTATTATAACCAAAAAGCAGTAAAGATAGATGCGGATA-TTACCTCAGAATATCTTC-3', and the sequence of scramble probe is 5'-GTGTAACACGTCTATACGCCAGTGTAACACGTCTATACGCCAGTG-TAACACGTCTATACGCCAGTGTAACACGTCTATACGCCAGTGTAACA-CGTCTATACGCCAGTGTAACACGTCTATACGCCAGTGTAACACGTCTA-TACGCCAGTGTAACACGTCTATACGCCAGTGTAACACGTCTATACGC-CCAGT-3'. Probes were labeled with or without Alexa Fluor 488 by using the ULYSIS Nucleic Acid Labeling kit (Molecular Probes; U21650). After hybridization, the samples were washed once for 60 min in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 50°C and once for 20 min in 0.2× SSC and 0.1% (w/v) SDS at 50°C in darkness. The samples were observed immediately using a Zeiss LSM 710 confocal microscope with a 488-nm excitation laser.

Nuclear RNA Extraction

Nuclei of the *DRIR*-overexpressing line A12 were isolated as described (Zhang and Jiang, 2015) with minor modifications. One gram of 1-week-old A12 seedlings was ground to fine powder in liquid nitrogen. The ground powder was transferred into a 2-mL ice-cold centrifuge tube, and 1 mL of ice-cold NIB buffer (10 mM Tris-HCl, 80 mM KCl, 10 mM EDTA, 1 mM spermidine, 1 mM spermine, 0.15% [v/v] mercaptoethanol, 0.5 M Suc, and 10 μL of RNase inhibitor, pH 9.5) was added to suspend the powder. After being agitated gently on ice for 6 min, the mixture was filtered through a folded four-layer Miracloth into a new 2-mL tube and centrifuged at 1,100g for 10 min at 4°C. After the supernatant was decanted as much as possible, 1 mL of NIB buffer with the addition of 0.5% (v/v) Triton X-100 was added to resuspend the pellet, and the tube was centrifuged subsequently at 1,100g for 10 min at 4°C. The supernatant was discarded, and the above step was repeated three times. The final pellet was used to extract nuclear RNA by using the Plant RNeasy Kit (Qiagen).

Stress Treatment

For the drought tolerance test, 3-week-old well-watered seedlings had watering withheld for 20 d and then were rewatered to allow recovery for 2 d. The plants before and after the treatment were photographed and surveyed as described previously (Li et al., 2016b). To detect the rate of water loss, detached leaves from 4-week-old plants were exposed to air at room temperature and weighed at the indicated times as described previously (Xiong et al., 2001).

For NaCl treatment, seeds were sown onto 1/2 MS medium plates containing different concentrations of NaCl and were stratified at 4°C for 3 d. The plates were then incubated in a growth chamber for germination and grown for 8 d before scoring the phenotypes. Alternatively, 4-d-old seedlings on 1/2 MS plates were transferred to 1/2 MS plates containing different concentrations of NaCl and grown for an additional 4 d before scoring the phenotypes. Seedling survival rate referred to the percentage of seedlings with at least one green leaf among total seedlings treated. For NaCl treatment of soil-grown seedlings, 4-week-old seedlings were watered with 200 mM NaCl and photographs were taken 2 weeks later.

ABA Treatment

For ABA sensitivity analysis, 4-d-old seedlings on 1/2 MS plates were transferred to 1/2 MS plates supplemented with different concentrations of ABA. Eight days later, photographs were taken and green leaves of each seedling were counted. ABA-induced stomata closure assays were performed as described previously (Brandt et al., 2015) with minor modifications. The fifth and sixth rosette leaves of 4-week-old plants were detached and immersed in a

stomata open solution (5 mM KCl, 50 μM CaCl₂, and 10 mM MES-Tris, pH 5.6) and incubated under cool-white light for 2 h. To induce stomata closure, leaves were transferred to the stomata open solution with 20 μM ABA added. After incubation under cool-white light for 2 h, leaf epidermis peels were prepared and imaged with a BX52M microscope. Apertures were measured using ImageJ software.

Transcriptome Sequencing Analysis

Ten-day-old seedlings were dehydrated on dry filter paper in petri dishes until loss of 40% fresh weight and then incubated for 2 h in sealed plastic bags to prevent further water loss. Total RNA was extracted using the RNeasy Mini Kit (Invitrogen), and DNA was cleaned by DNase I (New England Biolabs). About 2 to 4 μg of cleaned total RNA was used to construct RNA-seq libraries by using the Illumina Whole Transcriptome Analysis Kit following the standard protocol (Illumina HiSeq system) and sequenced on the HiSeq 2000 platform. The gene expression levels (fragments per kilobase of transcript per million mapped reads, FPKM) were calculated with Cufflinks (2.0.2) as described previously (Trapnell et al., 2010; Chen et al., 2013). Two biological replicates were performed.

Real-Time Quantitative PCR

Total RNA was extracted from seedlings by using the Plant RNeasy Kit with DNase I treatment (Qiagen). cDNAs were synthesized from total RNA by using SuperScript III reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed on the ABI 7900HT Fast Real-Time PCR System using 18S rRNA as a control. Primers used in this study are presented in Supplemental Table S3.

Pro and ROS Content

For free Pro measurement, 10-d-old seedlings of the wild type, *drir^D*, A12, and A14 grown on 1/2 MS plates were transferred to empty plates to lose 40% fresh weight or transferred to 1/2 MS solution with 200 mM NaCl for 3 h. Free Pro was assayed using the ninhydrin assay as described (Bates et al., 1973).

ROS content in guard cells was measured as described (Watkins et al., 2014) with minor modifications. Detached leaves from 4-week-old seedlings were immersed in water with or without 20 μM ABA for 1 h. Epidermises were then peeled and stained with 2.5 μM H₂DCF-DA (Sigma-Aldrich) for 30 min. After being washed three times with water, guard cells were examined using a Zeiss LSM 710 confocal microscope with excitation at 488 nm and emission at 525 nm.

Accession Numbers

Sequence data described in this article can be found in the Arabidopsis Genome Initiative under accession number At1g21529 (*DRIR*). RNA-seq data from this article can be found in the Short Read Archive database (National Center for Biotechnology Information) under accession number SRP113651.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Expression of *DRIR* was induced by dehydration and salt stress.

Supplemental Figure S2. Localization of *DRIR* RNA in the nucleus.

Supplemental Figure S3. The *DRIR* fragment amplified from total RNA or nuclear RNA by RT-PCR.

Supplemental Figure S4. Transcript levels of the *DRIR* neighboring gene At1g21528 in the wild type and the *drir^D* mutant.

Supplemental Figure S5. *drir^D* and overexpressing lines are more tolerant to salt stress.

Supplemental Figure S6. Expression of *DRIR* was induced by ABA.

Supplemental Figure S7. Stomata density of rosette leaves of the wild type, *drir^D*, and overexpressing lines.

Supplemental Figure S8. Relative expression levels of selected genes in response to salt stress treatment.

Supplemental Figure S9. Free Pro in dehydration- and salt-treated seedlings.

Supplemental Table S1. Results of transcriptome sequencing analysis.

Supplemental Table S2. List of genes up-regulated or down-regulated more than 2-fold in *drir^D* or overexpressing lines compared with the wild type in transcriptome sequencing analysis.

Supplemental Table S3. Primers used in real-time PCR analysis.

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