A Nucleus-localized Long Non-Coding RNA Enhances Drought and Salt Stress Tolerance

ONE SENTENCE SUMMARY: A long non-coding RNA enhances Arabidopsis tolerance to drought and salt stress by modulating the expression of stress-related genes.
ABSTRACT

Long non-coding 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 thaliana* lncRNA, *Drought induced IncRNA (DRIR)*, as a novel positive regulator of plant response to drought and salt stress. *DRIR* was expressed at a low level under non-stress 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 plant response to abiotic stress by modulating the expression of a series of genes involved in stress response.

INTRODUCTION

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 share 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 (Barrero et al., 2006; Xiong and Zhu, 2003, Nambara and Marion-Poll, 2005). 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 (PP2Cs) and release their inhibition on downstream components, including the Sucrose nonfermenting 1-related protein kinases subfamily 2 (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 (ABRE) binding factor (ABF/AREB) class of bZIP transcription factors are well studied. These transcription factors bind the conserved ABRE cis element in the promoter of many ABA- and stress-responsive genes and regulate their expression to enhance plant stress tolerance (Cutler et al., 2010; Furihata et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). SnRK2s could also activate other targets such as NADPH oxidases, slow anion channel 1 (SLAC1), and aquaporins (Munemasa et al., 2015; Sirichandra et al., 2009; Grondin et al., 2015). These signaling molecules and channels are directly involved 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 (CRT)/dehydration responsive element (DRE), which can be recognized by the Dehydration-responsive element-binding/C-repeat-binding factor (DREB/CBF)
class of transcription factors in response to cold, drought or salt stress (Thomashow, 2010; Shinozaki and Yamaguchi-Shinozaki, 2000; Agarwal et al., 2006). These genes include, for example, *responsive to dehydration* (RD29A), RD17, KIN1 and *Early response to dehydration* (ERD10). 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 mainly focuses on protein-encoding genes. In recent years, non-protein coding transcripts are emerging as important regulators of gene expression. Among them, long non-coding RNAs (lncRNAs) represent diverse classes of transcripts longer than 200 nt without or with little protein-coding potential. lncRNAs have been considered important regulators of many essential biological processes by functioning as precursors of miRNAs and other small RNAs, or as miRNA target mimics. They may also 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 plant response to the environment. For example, overexpression of the lncRNA *INDUCED BY PHOSPHATE STARVATION 1* results in reduced shoot Pi content (Franco-Zorrilla et al., 2007). Downregulation of *cis-natural antisense RNA* impairs the transfer of phosphate from roots to shoots and decrease seed yield in rice (Jabnoune et al., 2013). Transgenic overexpression of *asHSFB2a*, an antisense RNA of *HSFB2a*, affects plant response to heat stress (Wunderlich et al., 2014). Several reports also show that drought or salt stress could alter lncRNAs expression in plants. For example, 19, 664 and 98 drought-responsive lncRNAs were identified in foxtail millet, maize 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 8 and 6 lncRNAs respectively in *Arabidopsis* (Ben Amor et al., 2009). Nonetheless, no lncRNA has been characterized to regulate plant response or tolerance to drought or salt stress.
In this study, we identified and characterized the function of a novel IncRNA DRIR (for Drought induced IncRNA). 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 mainly localized in the nucleus. Transcriptome sequencing and real-time PCR analysis revealed that DRIR modulates the expression of genes involved in stress response including ABA response, water transport, and transcription that may collectively contribute to enhanced abiotic stress tolerance.

RESULT

The Expression of DRIR Is Induced by Drought and Salt Stress

We are interested in the potential role of IncRNA in plant stress responses. We hypothesized that if IncRNAs of this nature are present, they may be induced by abiotic stress. To identify these IncRNA, we conducted a RNA-seq of salt-treated (300 mM NaCl, 3 h) Arabidopsis seedlings (Ding et al., 2014) and searched for transcripts longer than 200 nt but with no recognized open-reading frames that were significantly induced by the stress treatment. Among dozens of putative IncRNA identified, a transcript of 755 nt, we referred it as to Drought induced IncRNA (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 IncRNAs, DRIR does not have homologs in other plants either.

Initially identified as salt stress-inducible in our RNA-seq studies, the regulation of DRIR by stress was further investigated. Total RNA was extracted from the wild type (WT) 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 Figure 1). In addition, the expression level of *DRIR* in dehydrated seedlings was 59-fold that in untreated seedlings (Supplemental Figure 1). To confirm these data, a construct was made in which the β-glucuronidase (GUS) reporter gene was placed under the control of a 2.0-kb *DRIR* promoter. This construct (P_{DRIR}:GUS) was introduced into Col-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 1, the P_{DRIR}:GUS activities were significantly increased 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 (www.genevestigator.ethz.ch) indicate that *DRIR* is expressed at a low level in plants and is mainly expressed 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 predominantly detected in roots, cotyledons and stems in 6-day-old seedlings (Figure 1B). In adult plants, strong GUS signals were detected in rosette leaves, guard cells and flower petals (Figure 1C, Figure 1D and Figure 1F). None or only weak GUS signal was detected in the embryo, axillary leaf and siliques (Figure 1A, Figure 1E and Figure 1F). To verify these results, total RNA was extracted from different tissues and real-time PCR was performed. Consistent with data of the GUS staining, real-time PCR showed that *DRIR* was highly expressed in roots and rosette leaves, and to a lesser extent, in stems, axillary leaves, inflorescence and siliques (Figure 1G).
To investigate the subcellular localization of DRIR, fluorescence in situ hybridization in roots was performed with Alexa Fluor 488-labelled 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-labelled DRIR probe but not in cells hybridized with the unlabeled DRIR probe or a labelled control probe (Figure 2). To confirm this, nucleus was stained with DAPI and the signal of Alexa Fluor
488-labelled DRIR probe and the signal of DAPI were found to co-localize in nuclei of root hairs (Supplemental Figure 2). In addition, we isolated nuclei from a DRIR-overexpressing line (A12, see below) and extracted RNA from nuclei. One µg of cDNA from nuclear RNA or total RNA was used to perform RT-PCR. A random mRNA (CAR4) was used as a control. The results shown that DRIR fragment could be amplified from nuclear RNA and 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 mainly localized in nuclei (Supplemental Figure 3).

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

Since the expression of DRIR was upregulated by drought and salt stress, the functions of DRIR in plant response 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 (Figure 3A). The expression of DRIR in the mutant was significantly higher than in the wild type (WT) (Figure 3B). However, there was no significant difference in the expression of At1g21528 between the mutant and the WT (Supplemental Figure 4). Due to the activation nature of the mutation, we referred this mutant as drirD. To test drought tolerance of the mutant, 3-week-old seedlings of WT and drirD in soil were treated with drought stress by stopping watering for 20 days. All leaves of WT seedlings became totally withered and dry, but drirD leaves withered to a lesser extent and none was totally dry (Figure 3D). Two days after re-watering, the WT seedlings were still withered, in contrast, drirD leaf became green and turgid again (Figure 3D), indicating that the drirD 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 drirD detached leaves was much slower than that of the WT leaves. drirD leaves lost 18, 22, 25, 27, and 30% fresh weight at 40, 60, 80, 100 and 120 min after detachment, respectively, whereas WT leaves lost 29, 32, 34, 36 and 39% of fresh weight at the same time (Figure 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 Col-0 and
more than twenty independent transgenic lines were obtained. Two overexpressing lines (A12 and A14) were randomly selected for detailed phenotype analysis. The expression level of DRIR in both transgenic lines was higher than in WT (Figure 3C). These lines were tested for their drought tolerance along with the drirD mutant. It was found that the two overexpressing lines, similar to drirD, were more tolerant to drought stress (Figure 3D). The water loss speed of detached leaves of the transgenic
plants was also slower than that of the WT leaves but was similar to that of $drir^D$ (Figure 3E). These data indicate that 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 Arabidopsis response to abiotic stress, seeds of WT, $drir^D$, A12 and A14 were sown onto 1/2 MS medium plates containing 150 mM or 200 mM NaCl. As shown in Figure 4A and Figure 4B, in contrast to seedlings on 1/2 MS media without NaCl, many seedlings were bleached and dead at 8 days after germination and growth on 1/2 MS media containing different concentrations of NaCl. Comparing with 36% survival rate of the WT seedlings, the survival rates of $drir^D$, A12 and A14 seedlings were 48%, 60%, and 62% on media containing 150 mM NaCl, respectively (Figure 4A and Figure 4B). To distinguish the effect of salt stress on seed germination and on seedling growth, 4-day-old seedlings of WT, $drir^D$, A12 and A14 grown on 1/2 MS were transferred to 1/2 MS media containing different concentrations of NaCl and allowed to grow for additional 4 days. Similar to plants with seeds directly sown onto media supplemented with salt, the transferred seedlings of $drir^D$ and overexpressing lines were also more tolerant to the salt stress than WT. 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 WT seedlings survived under 150 mM or 200 mM NaCl, respectively (Supplemental Figure 5). 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 WT seedlings were dead (Figure 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 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 significantly increased by ABA treatments. However, the effect of indole acetic acid (IAA), zeatin (ZT), 24-Epibrassinolide (EBL) and gibberellin (GA) on the expression of DRIR was not as profound as that of ABA (Supplemental Figure 6). We also analyzed GUS activities in
P_{DRIR}:GUS transgenic plants under ABA treatment. As shown in Supplemental Figure 6, stronger 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 \textit{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 and 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 WT and \textit{drir}^D or overexpressing lines. The width to length ratio for all these genotypes was about 0.6 (Figure 5A and Figure 5B). However, the stomata aperture of \textit{drir}^D or overexpressing lines was much smaller than that of WT with the ABA treatment. The stomata width to length ratio of \textit{drir}^D or the two overexpressing lines decreased from about 0.6 to 0.27, 0.22 and 0.25 respectively, whereas that of WT merely decreased to 0.46 (Figure 5A and Figure 5B). These data indicate that stomata of \textit{drir}^D and the overexpressing lines were more responsive to ABA-induced closure than those of WT.

To exclude the possibility that DRIR affects plant response to drought stress by regulating stomata density, stomata density of the fifth and sixth leaves of 4-week-old plants were examined but no significant difference was found among these genotypes (Supplemental Figure 7), suggesting that DRIR does not regulate the morphogenesis and differentiation of stomata cells.

We further examined the sensitivity of \textit{drir}^D and overexpressing seedlings to ABA. Four-day-old seedlings of WT, \textit{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 \textit{drir}^D and overexpressing lines were found to have etiolated leaves compared to the WT (Figure 5C). While almost 90% of leaves of WT seedlings were green, less than 85% of leaves of \textit{drir}^D
and overexpressing seedlings were green under 2 µM ABA treatment. Although the percentage green leaves of WT decreased to 55% under 5 µM ABA treatment, less than 40% of green leaves could be found in *drrD* and overexpressing seedlings (Figure 5D), indicating that *drrD* and overexpressing seedlings are more sensitive to ABA than WT.

**DRIR Regulates the Expression of Genes Involved in Stress Response**
To elucidate the molecular mechanism of *DRIR* function in plant response to drought stress, 10-day-old seedlings treated with dehydration were used for transcriptome sequencing analysis. Compared with the WT, there were 1367, 1224 and 1933 genes up-regulated and 1081, 738 and 915 genes down-regulated more than two-fold in *drirD*, A12 and A14, respectively (Figure 6 and Supplemental Table 1). Among them, 187 genes were up-regulated and 39 genes were down-regulated both in *drirD* and the two overexpressing lines (Figure 6 and Supplemental table 2). Among those genes up-regulated both in *drirD* and overexpressing lines, 28 genes have been suggested to be involved in plant response to drought stress, salt stress or ABA (Table 1).

To further validate the expression of those genes that are known to be critical in plant response to stress, total RNA was extracted from 10-day-old *drirD* 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*), a NADPH oxidase gene, increased more than 15-fold in dehydration treated *drirD* and overexpressing lines whereas less than 2-fold increase was found in dehydration treated WT seedlings (Figure 7E). The expression of *fucosyltransferase4* (*FUT4*) increased 19-fold, 16-fold and 20-fold in dehydration treated *drirD*, the A12 and A14 overexpressing lines, respectively, but only 4-fold increase was observed in WT seedlings (Figure 7F). Whereas not significantly up-regulated by dehydration stress in the wild type, *NOD26-like intrinsic protein1* (*NIP1*), a member of the NIPs aquaporin subfamily was expressed about 2-fold in dehydrated *drirD* and overexpressing lines compared with untreated WT (Figure 7G). The expression level of another aquaporin proteins gene, *tonoplast intrinsic protein4* (*TIP4*), increased 24-fold in dehydration treated WT seedlings, but it increased more than 40-fold in *drirD* and overexpressing lines (Figure 7H). The expression of *Arabidopsis* annexin gene, *ANNAT7*, increased more than 8-fold in dehydration treated *drirD* and overexpressing lines while it increased only 2-fold in WT seedlings (Figure 7I). Two transcription factor genes, *NAM, ATAF and
CUC protein3 (NAC3) and WRKY protein8 (WRKY8), were also expressed significantly higher in dehydrated drirD and overexpressing lines than in WT seedlings (Figure 7J to Figure 7K).

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 drirD and overexpressing seedlings than in WT seedlings although the increasing level were
less than 2-fold (Supplemental Table 1). These genes include, for example: \textit{ABSCISIC ACID-INSENSITIVE5 (ABI5)}, \textit{Δ1-PYRROLINE-5-CARBOXYLATE SYNTHETASE1 (P5CS1)}, \textit{Response-to-Dehydration 29A (RD29A)} and \textit{Response-to-Dehydration 29B (RD29B)}. Real-time PCR analysis was performed to validate the expression level of these genes. Compared with untreated seedlings, expression of these genes was significantly increased in dehydration treated WT seedlings, but the extent of increase
was much smaller than in *drir*<sup>D</sup> or overexpressing seedlings. The expression of *ABI5* increased more than 90-folds in dehydration-treated *drir*<sup>D</sup> and overexpressing seedlings while it only increased 6-fold in dehydrated WT seedlings (Figure 7A). The expression of *P5CS1* increased 4, 42, 35, and 33-fold in dehydration treated WT, *drir*<sup>D</sup>, A12 and A14 seedlings, respectively (Figure 7B). The expression of *RD29A* and *RD29B* also increased more than 160-fold and 1400-fold in dehydration treated *drir*<sup>D</sup> and overexpressing seedlings, respectively, while it only increased 37-fold and 86-fold in dehydration treated WT seedlings (Figure 7C and Figure 7D). 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* dramatically increased in salt treated *drir*<sup>D</sup> and overexpressing seedlings whereas their expression increased to a much lesser extent in salt treated WT seedlings (Figure 8), indicating that *DRIR* also regulates the expression of these genes under salt stress. Nonetheless, no significant difference in the expression of *ABI5* and *NIP1* between salt treated WT and *drir*<sup>D</sup> or its overexpressing seedlings was found (Supplemental Figure 8A and Supplemental Figure 8B). Furthermore, the expression of *TIP4* and *WRKY8* in salt treated WT seedlings was even higher than in *drir*<sup>D</sup> and overexpressing seedlings (Supplemental Figure 8C and Supplemental Figure 8D). These results suggest that *DRIR* may differentially regulate stress-related genes through complex mechanisms.

**DRIR Affects Accumulation of Proline and ROS**

Proline is an important osmolyte with cellular protection functions in plants. Abiotic stress conditions as well as ABA treatment could promote the accumulation of proline by inducing *P5CS1* expression (Abraham et al., 2003; Sharma et al., 2011).
Since *drir*<sup>D</sup> and overexpressing lines are more sensitive to ABA and the expression of *P5CS1* in *drir*<sup>D</sup> and overexpressing lines are dramatically increased when treated with dehydration and salt stress, we analyzed the contents of proline in 10-day-old seedlings treated with dehydration or salt stress. As shown in Supplemental Figure 9, the content of proline increased from 10 µg per gram fresh weight to 30 µg per gram fresh weight in WT seedlings when treated with dehydration stress, while it increased...
to more than 60 µg per gram fresh weight in \textit{drir}^D and overexpressing lines. When treated with salt stress, the content of proline increased to 30 µg per gram fresh weight in WT seedlings, yet it increased to more than 42 µg per gram fresh weight in \textit{drir}^D and overexpressing lines. These results suggest that the synthesis of proline was likely enhanced in \textit{drir}^D and overexpressing lines under drought and salt stress.

Reactive oxygen species (ROS) are critical second messengers in 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 \textit{drir}^D and the overexpressing lines was more sensitive to ABA and the expression of NADPH oxidase catalytic subunit gene \textit{AtrbohB} was dramatically up-regulated in dehydration and salt treated \textit{drir}^D and overexpressing seedlings, we examined whether \textit{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 H$_2$DCF-DA and examined with a confocal microscope. As shown in Figure 9, the intensity of fluorescence signal of H$_2$DCF-DA in guard cells of leaves without ABA treatment was very low, but increased dramatically after ABA treatment, indicating that ROS accumulation was significantly increased in ABA treated leaves. Interestingly, the fluorescence signal of H$_2$DCF-DA in ABA treated \textit{drir}^D and overexpressing guard cells were much higher than that in ABA treated WT guard cells (Figure 9A). The relative fluorescence intensity in guard cells of ABA treated WT leaves was 11.7, while that of \textit{drir}^D and overexpressing leaves were 24.5, 21.6 and 23.3, respectively (Figure 9B). These data demonstrate that more ROS accumulated in guard cells of \textit{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 IncRNA 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 stress response.

Figure 9. More ROS accumulated in guard cells of *drir* and overexpressing leaves in response to ABA treatment. A, Representative images of ROS production stained by the fluorescent dye H$_2$DCF-DA in guard cells without (left) or with ABA treatment (right). Upper panel, fluorescence images; middle panel, bright field images; lower panel, merged images. Bar = 20 μm. B, Relative fluorescence intensity of H$_2$DCF-DA. More than 100 guard cells from 10 leaves of each genotype were measured. * P < 0.05 by t test compared with the untreated or ABA-treated WT. Error bars represent SD.
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 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 lncRNA is still challenging. This may not only be due 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 due to the fact that plant lncRNAs are not evolutionarily conserved in general (Liu et al., 2012; Liu et al., 2015a; Liu et al., 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. identified 584 lncRNAs in foxtail millet. Among them, 17 lincRNAs and 2 NATs are drought-responsive (Qi et al., 2013). Chung et al., also found 98 drought responsive lncRNAs in rice by using RNA-seq (Chung et al., 2016). By genome-wide analysis of full-length cDNA databases, Ben Amor et al. identified 76 *Arabidopsis* lncRNAs and found that the accumulation of 22 lncRNAs was altered by abiotic stress. These lncRNAs include 3 up-regulated and 5 down-regulated under salt treatment and 4 up-regulated and 2 down-regulated under dehydration treatment. Transgenic analyses showed that overexpressing *npc536* increases lateral root number under salt treatment (Ben Amor et al., 2009). 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 plant response to drought or salt stress tolerance has been detailed characterized.

In this study, we identified a lncRNA whose length is 755 nt 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 lincRNA. We showed that the *drir<sup>D</sup>* 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 days of drought stress treatment whereas WT seedlings were killed by the stress. Leaves of the $drir^D$ mutant and overexpressing lines also had slower transpirational water loss rate than WT. 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 on either culture media or in soil showed that the $drir^D$ mutant and overexpressing seedlings are more tolerant to salt stress than WT. These results indicate that $DRIR$ regulates plant response to both drought and salt stress.

**$DRIR$ Regulates ABA-mediated Responses to Drought and Salt Stress**

It has been reported that some plant lncRNAs may participate in phytohormone-mediated response to the environment. *Arabidopsis* lincRNA *APOLO* is transcribed by RNA polymerases II and 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. suggested that lncRNAs may participate in ABA induced complex assembly and relocalization of RNA-BINDING PROTEIN (Liu et al., 2015b). However, lncRNAs involved in ABA-mediated stress response is still unknown.

In the current 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 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 have a function in plant response to abiotic stress (Tanaka et al., 2012; Hopper et al., 2016). P5CS1 is involved in ABA modulation of stress response in Arabidopsis by controlling proline accumulation (Abraham et al., 2003; Sharma et al., 2011). RD29A and RD29B play roles in 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 drirD and overexpressing lines are much higher than in WT when treated with dehydration. Consistent with the increased gene expression, increased accumulation of proline and ROS was observed in drirD and overexpressing lines under dehydration stress or ABA treatment. Since ROS are critical second messengers in ABA regulation of stomata closure (Kwak et al., 2003; Watkins et al., 2014), 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 stomata density, should be responsible for the reduced transpirational water loss seen in the mutant and overexpressing lines (Figure 3E). Together with increased proline accumulation and enhanced expression of other ABA and stress-responsive genes, DRIR thus significantly improves plant drought tolerance.

Under salt stress conditions, the expression levels of P5CS1, RD29A, RD29B and AtrbohB are notably increased in drirD 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 WREKY8 are also significantly increased in drirD 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 stomata response to ABA. DRIR could also regulate plant tolerance to salt stress by affecting the activity of fucosyltransferase or NAC3 transcription factor, or 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 drirD and overexpressing seedlings than in WT seedlings when treated with dehydration, but the difference was not as significant when treated with salt stress. This could suggest that aquaporins participate in DRIR regulation of drought stress tolerance but not in salt stress tolerance.

lncRNAs may execute their functions through a multitude of mechanisms. Cytoplasm-localized lncRNAs could function as target mimicry of miRNAs and thus promotes its target mRNAs translation (Ariel et al., 2015). LncNATs usually trigger their complementary mRNA degradation by binding with them or promote their translation through recruitment to polysomes (Zubko and Meyer, 2007; Jabnoune 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 sRNAs (Pant et al., 2008). In the current study, in situ hybridization showed that DRIR is mainly localized in the nucleus, suggesting that DRIR may mainly function in nuclear processes such as transcription but may not function as a target mimicry 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 \textit{drir}^D and the overexpressing lines suggests that \textit{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 \textit{DRIR} did not significantly increase the expression levels for most of stress-responsive genes under normal conditions. Rather, the expression of these genes was significantly potentiated by overexpression of \textit{DRIR} only under stress conditions (Figure 7 and Figure 8). As \textit{DRIR} is transcribed at the chromatin locus between two genes, it is unlikely that \textit{DRIR} function by directly binding with cognate RNA and modulating their degradation or promoting their translation as what IncNATs do. However, the molecular mechanisms of how \textit{DRIR} exert its functions are still an open question.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

All \textit{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 half-strength Murashige and Skoog (MS) medium (Sigma-Aldrich) plates containing 0.8\% agar and 1\% sucrose. Plates were moved to 22°C with a 16-h-light/8-h-dark cycle chambers for germination and growth after stratification at 4°C for 3 days. Ten days later, seedlings were transferred to soil and placed in a growth room at 22°C with 16-h-light/8-h-dark cycle.

**Analysis of \textit{DRIR} Promoter:GUS Activity**

The \textit{DRIR} promoter fragment (2.0 Kb) was amplified and inserted into pMDC162 vector using the GATEWAY cloning technology. After sequence confirmation, the construct was transformed into \textit{Arabidopsis} using \textit{Agrobacterium tumefaciens} (strain GV3101). The GUS staining procedure was performed as previously described (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 under 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-day-old Columbia-0 seedlings and were 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% Tween 20, 80 mM EGTA, 5% paraformaldehyde, and 10% DMSO). The samples were gently shaken 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% paraformaldehyde. The samples were post fixed in the fixation buffer for 30 min at the room temperature, rinsed twice with fixation buffer without 5% 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 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'-CTCCAAACTCCTTTATTTCTTAACCAAAAGTTACAATTTCATGAGAAGATGATCTAGAACATCATTTCTAGACTCATCTTCTAAATCTCACACACGAGATTGT
TTACACAAATTGCTAATAAGCTTCTCTCTTTAAACATGAGTACCTCAGAATATCTTCTTC-3',
```

The sequence of scramble probe is:

```
5'-GTGTAACACGTCTATACGCCACGTGAACACGTCTTACGCCACGTGAACACGTCTTACGCCCAGTGTAACACGTCTCTATACGC

TACGCCCAGTGTAACACGTCTTACGCCACGTGAACACGTCTCTATACGC
```
GTGTAACACGTCTATACGCCCAGTGTAACACGTCTATACGCCCAGT-3’.

Probes were labelled with or without Alexa Fluor 488 by using ULYSiS Nucleic Acid Labelling kit (Molecular Probes company; 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), 0.1% SDS at 50°C and once for 20 min in 0.2× SSC, 0.1% SDS at 50°C in darkness. The samples were observed immediately using a Zeiss LSM 710 confocal microscope with 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 g of one-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 ice-cold NIB buffer (10 mM Tris–HCl, 80 mM KCl, 10 mM EDTA, 1 mM spermidine, 1 mM spermine, 0.15 % mercaptoethanol, 0.5 M sucrose, 10 µl RNase Inhibitor, pH 9.5) was added to suspend the powder. After being gently agitated on ice for 6 min, the mixture was filtered through a folded four-layer Miracloth into a new 2-ml tube and centrifuged at 1,100 × g for 10 min at 4 °C. After the supernatant was decanted as much as possible, 1 ml of NIB buffer with additional 0.5 % Triton X-100 was added to resuspend the pellet and the tube was subsequently centrifuged at 1,100 × g for 10 min at 4 °C. The supernatant was discarded and the above step was repeated for three times. The final pellet was used to extract nuclear RNA by using the Plant RNeasy Kit (Qiagen).

Stress Treatment

For drought tolerance test, 3-week-old well-watered seedlings were withheld watering for 20 days, and then re-watered to allow recovery for 2 days. The plants before and after the treatment were photographed and surveyed as previously described (Li et al., 2016). To detect the rate of water loss, detached leaves from 4-week-old plants were
exposed to air at the room temperature and weighed at the indicated times as previously described (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 days. The plates were then incubated in a growth chamber for germination and growth for 8 days before scoring the phenotypes. Alternatively, 4-day-old seedlings on 1/2 MS plates were transferred to 1/2 MS plates containing different concentrations of NaCl and grown for 4 more days 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-day-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 previously described (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 added with 20 µM ABA. After incubation under cool-white light for 2 h, leaf epidermis peels were prepared and imaged under a BX52M microscope. Apertures were measured using the ImageJ software.

**Transcriptome Sequencing Analysis**

Ten-day-old seedlings were dehydrated on dry filter paper in Petri dishes till 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 an Illumina Whole Transcriptome Analysis Kit following the standard protocol (Illumina, HiSeq system) and sequenced on the HiSeq 2000 platform. The gene expression levels (FPKM) were calculated with Cufflinks (2.0.2) as previously described (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 3.

**Proline and ROS Content**

For free proline measurement, 10-day-old seedlings of WT, *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 proline 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 H2O with or without 20 µM ABA for 1 h. Epidermises were then peeled and stained with 2.5 µM H2DCF-DA (Sigma) for 30 min. After being washed three times with H2O, guard cells were examined using 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 the accession numbers DRIR, At1g21529. RNA-seq data from this article can be found in the SRA database (NCBI) under accession number SRP113651.

**SUPPLEMENTAL DATA**

Supplemental Figure 1. Expression of DRIR was induced by dehydration and salt stress.

Supplemental Figure 2. Localization of DRIR RNA in the nucleus.

Supplemental Figure 3. The DRIR fragment amplified from total RNA or nuclear RNA by RT-PCR. A random mRNA (CAR4) was used as a control.

Supplemental Figure 4. Transcript level of the DRIR neighboring gene At1g21528 in the wild type and drir<sup>D</sup> mutant.

Supplemental Figure 5. drir<sup>D</sup> and overexpressing lines are more tolerant to salt stress.

Supplemental Figure 6. Expression of DRIR was induced by ABA.

Supplemental Figure 7. Stomata density of rosette leaves of WT, drir<sup>D</sup> and overexpressing lines.

Supplemental Figure 8. Relative expression levels of selected genes in response to salt stress treatment.

Supplemental Figure 9. Free proline in dehydration and salt treated seedlings.

Supplemental Table S1. Results of transcriptome sequencing analysis.

Supplemental Table 2. List of genes up-regulated or down-regulated for more than 2-fold in drir<sup>D</sup> or overexpressing lines compared with WT in transcriptome sequencing analysis.

Supplemental Table 3. Primers used in Real-time PCR analysis.
seedlings treated with dehydration or 150 mM NaCl. **P < 0.01 by \( t \) test. Data are means ± SD of three biological replicates. B, \( DRIR \) promoter-GUS activities in dehydration and salt treated seedlings. Bar = 1 mm.

Supplemental Figure 2. Localization of \( DRIR \) RNA in the nucleus. A, In situ hybridization with Alexa Fluor 488 labelled antisense \( DRIR \) probe (green) in root hairs; B, Nucleus staining with DAPI (blue). C, Differential interference contrast (DIC) bright field of root hairs. D, Merged image of (A) to (C). Bar = 20 \( \mu \)m.

Supplemental Figure 3. The \( DRIR \) fragment amplified from total RNA or nuclear RNA by RT-PCR. A random mRNA (\( CAR4 \)) was used as a control.

Supplemental Figure 4. Transcript level of the \( DRIR \) neighboring gene At1g21528 in the wild type and \( drirD \) mutant.

Supplemental Figure 5. \( drirD \) and overexpressing lines are more tolerant to salt stress. A, Morphology of seedlings under salt stress treatment. Four-day-old seedlings grown on 1/2 MS medium were transferred to 1/2 MS media supplemented with 0, 150 or 200 mM NaCl, respectively. Photographs were taken 4 days after the transfer. B, Percentage of seedlings survived with at least one green leaf. At least 100 seedlings for each treatment were counted. * and ** denote significant difference by \( t \) test between the genotype and WT at the \( P < 0.05 \) and \( P <0.01 \) level, respectively. Data represent means ± SD.

Supplemental Figure 6. Expression of \( DRIR \) was induced by ABA. A, Transcription level of \( DRIR \). RNA was extracted from 10-day-old seedlings treated with 1 \( \mu \)M of the indicated phytohormones for 3 h. Mock, no hormone treatment; IAA, indole 3-acetic acid; ZT, zeatin; eBL, 24-epibrassinolide; GA, gibberellin; ABA, abscisic acid. Data represent means ± SD. **P <0.01 by \( t \) test. Data are means ± SD of three biological replicates. B, \( DRIR \) promoter-GUS activities in ABA treated seedlings. Bar
Supplemental Figure 7. Stomata density of rosette leaves of WT, *drir*<sup>D</sup> and overexpressing lines. A, Representative images show the stomata density of the fifth or sixth rosette leaves of 4-week-old plants. Bar = 100 µm. B, Stomata density. At least 500 stomata from each leaf were counted. More than 30 leaves of each genotype were measured. Data represent means ± SD.

Supplemental Figure 8. Relative expression levels of selected genes in response to salt stress treatment. RNA was extracted from 10-day-old seedlings that were treated with 200 mM NaCl for 3 h. 18S rRNA was used as a control to calculate the relative expression level of *ABI5* (A), *NIP1* (B), *TIP4* (C), and *WRKY8* (D). ** denotes significant difference by *t* test between the indicated genotype and the WT at the P <0.01 level. Data represent means ± SD.

Supplemental Figure 9. Free proline in dehydration and salt treated seedlings. Ten-day-old seedlings of each genotype grown on 1/2 MS plates were allowed to lose 40% fresh weight or were incubated in 1/2 MS solution supplemented with 200 mM NaCl for 3 h before being sampled for proline measurement. * and ** denote significant difference by *t* test between the indicated genotype and WT at the P <0.05 and P <0.01 level, respectively. Data represent means ± SD from three replicates.

Supplemental Table S1. Results of transcriptome sequencing analysis.

Supplemental Table 2. List of genes up-regulated or down-regulated for more than 2-fold in *drir*<sup>D</sup> or overexpressing lines compared with WT in transcriptome sequencing analysis.

Supplemental Table 3. Primers used in Real-time PCR analysis.
ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center for providing the T-DNA insertion lines.

TABLES

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

<table>
<thead>
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<th>Gene Identifier</th>
<th>Gene Name</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>AT1G09090</td>
<td>AtrbohB</td>
<td>NADPH-oxidase AtrbohB</td>
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<td>TPX2</td>
<td>Thioredoxin-dependent peroxidase 2</td>
<td>(Kumar et al., 2015)</td>
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<td>TCP-1/cpn60 chaperonin family protein</td>
<td>(Gong et al., 2001)</td>
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<td>AT1G73260</td>
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<td>Trypsin inhibitor</td>
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<td>Putative transcription factor</td>
<td>(Ding et al., 2013)</td>
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<td>GL2</td>
<td>Homeodomain protein</td>
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<td>AT2G15390</td>
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<td>THAS</td>
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**FIGUE LEGENDS**

Figure 1. Expression pattern of *DRIR* in seedlings. Shown are *DRIR* promoter-GUS activities in germinating embryo (A), 6-day-old seedling (B), rosette leaf (C), guard cells in rosette leaf epidermis (D), axillary leaf (E), and inflorescence (F). Bars in A, B, C, E and F are 1 mm. Bar in D is 50 µm. (G) Transcript abundance of *DRIR* in different tissues as determined by quantitative RT-PCR. Shown are means ± SD from three biological replicates.

Figure 2. Subcellular localization of the *DRIR* transcripts. Fluorescence in situ hybridization with Alexa Fluor 488-labelled antisense *DRIR* probe was performed in 7-day-old Columbia-0 root. Hybridization with unlabelled probe and Alexa Fluor 488-labelled scramble probe were used as negative control. Bar = 20 µm.
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 level of DRIR in WT and $drir^D$. **P <0.01 by $t$ test. Data represent means ± SD. C, Relative transcript level of DRIR in WT and two overexpressing lines, A12 and A14. **P <0.01 by $t$ test. Data represent means ± SD. D, Morphology of seedlings before and after drought stress treatment. Three-week-old seedlings were drought stressed by stopping watering for 20 days before rewatering. Photographs were taken before (upper panels) and after (middle panels) the drought treatment and 2 days after rewatering (lower panels). E, Transpirational water loss rates of detached leaves at the indicated time after detachment. Error bars represent means ± SD from three replicates.

Figure 4. The $drir^D$ mutant and DRIR overexpressing lines are more tolerant to salt stress. A, Seeds of 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 days after the plates being incubated at 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 $t$ test compared with the WT. Data represent means ± 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.

Figure 5. $drir^D$ and overexpressing lines are more sensitive to ABA. A-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. * P < 0.05 by $t$ test compared with the WT. Error bars represent SD.
Seedling sensitivity to ABA. Four-day-old seedlings of WT, \( drir^D \), A12 and A14 on 1/2 MS plates were transferred to 1/2 MS plates supplemented with 0, 2.0, or 5.0 µM ABA. Representative images show the morphology of seedlings 8 days after growth on ABA medium plates. D, Green leaves percentage of each genotype. At least 100 seedlings for each treatment were scored. * significant between the sample and WT at the P < 0.05 level by \( t \) test. Error bars represent means ± SD.

Figure 6. Number of up- and down-regulated genes in dehydration treated \( drir^D \) and overexpressing lines in transcriptome sequencing analysis compared with in dehydration treated WT. 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.

Figure 7. Relative expression levels of selected genes in dehydration treated seedlings. RNA was extracted from 10-day-old seedlings that were dehydrated to lose about 40% fresh weight and gene expression level was measured by RT-quantitative PCR normalized against 18S rRNA gene. Relative expression level of \( ABI5 \) (A), \( P5CSI \) (B), \( RD29A \) (C), \( RD29B \) (D), \( AtrbohB \) (E), \( FUT4 \) (F), \( NIP1 \) (G), \( TIP4 \) (H), \( ANNAT7 \) (I), \( NAC3 \) (J), and \( WRKY8 \) (K). * P < 0.05 and **P <0.01 by \( t \) test compared with untreated (CK) or dehydration treated WT. Data represent means ± SD.

Figure 8. Relative expression levels of selected genes in salt stress treated seedlings. RNA was extracted from 10-day-old seedlings that were treated with 200 mM NaCl for 3 h and gene expression level was measured by RT-quantitative PCR normalized against 18S rRNA gene. Relative expression level of \( P5CSI \) (A), \( RD29A \) (B), \( RD29B \) (C), \( AtrbohB \) (D), \( FUT4 \) (E), \( ANNAT7 \) (F), and \( NAC3 \) (G). * P < 0.05 and **P <0.01 by \( t \) test compared with the untreated (CK) or salt-treated WT. Data represent means ± SD.
Figure 9. More ROS accumulated in guard cells of *drin*<sup>D</sup> and overexpressing leaves in response to ABA treatment. A, Representative images of ROS production stained by the fluorescent dye H<sub>2</sub>DCF-DA in guard cells without (left) or with ABA treatment (right). Upper panel, fluorescence images; middle panel, bright field images; lower panel, merged images. Bar = 20 µm. B, Relative fluorescence intensity of H<sub>2</sub>DCF-DA. More than 100 guard cells from 10 leaves of each genotype were measured. * P < 0.05 by t test compared with the untreated or ABA-treated WT. Error bars represent SD.


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