INDETERMINATE SPIKELET1 Recruits Histone Deacetylase and a Transcriptional Repression Complex to Regulate Rice Salt Tolerance[1][OPEN]

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Perception and transduction of salt stress signals are critical for plant survival, growth, and propagation. Thus, identification of components of the salt stress-signaling pathway is important for rice (*Oryza sativa*) molecular breeding of salt stress resistance. Here, we report the identification of an apetala2/ethylene response factor transcription factor INDETERMINATE SPIKELET1 (IDS1) and its roles in the regulation of rice salt tolerance. By genetic screening and phenotype analysis, we demonstrated that IDS1 conferred transcriptional repression activity and acted as a negative regulator of salt tolerance in rice. To identify potential downstream target genes regulated by IDS1, we conducted chromatin immunoprecipitation (ChIP) sequencing and ChIP-quantitative PCR assays and found that IDS1 may directly associate with the GCC-box-containing motifs in the promoter regions of abiotic stress-responsive genes, including *LEA1* (*LATE EMBRYOGENESIS ABUNDANT PROTEIN1*) and *SOS1* (*SALT OVERLY SENSITIVE1*), which are key genes regulating rice salt tolerance. IDS1 physically interacted with the transcriptional corepressor topless-related 1 and the histone deacetylase HDA1, contributing to the repression of *LEA1* and *SOS1* expression. Analyses of histone H3 acetylation status and RNA polymerase II occupation on the promoters of *LEA1* and *SOS1* further defined the molecular foundation of the transcriptional repression activity of IDS1. Our findings illustrate an epigenetic mechanism by which IDS1 modulates salt stress signaling as well as salt tolerance in rice.

Abiotic stresses such as soil salinity, drought, cold, and heat can influence plant growth and development. Plants have evolved several strategies to cope with salt stress, including the salt overly sensitive (SOS) pathway, transcriptional cascade, and responsive gene expression (Ji et al., 2013; Zhu, 2016; Yang and Guo, 2018). Rice (*Oryza sativa*) is the dominant food crop in Asia. A common factor affecting rice grain yield is soil salinity. Many genes conferring salt stress tolerance in rice have been isolated, such as genes involved in signal transduction and transcription regulation (Dubouzet et al., 2003; Hu et al., 2006; Huang et al., 2009), ion transporters (Ren et al., 2005; Senadheera et al., 2009), and metabolic pathways (Garg et al., 2002). Among these genes, *APETALA37* (*AP37*), *ZINC FINGER PROTEIN179* (*ZFP179*), and *LATE EMBRYOGENESIS ABUNDANT PROTEIN1* (*LEA1*) are key regulators that positively modulate rice salt tolerance (Oh et al., 2009; Sun et al., 2010; Duan and Cai, 2012).

Transcriptional factors (TFs), which regulate gene expression, rely on a DNA-binding domain (DBD) that recognizes a specific DNA sequence (Mitsuda and Ohme-Takagi, 2009). In plants, several TFs regulating salinity tolerance in rice have been identified, such as APETALA2/ethylene response factors (AP2/ERFs), NAMATAF1/2 and CUC TFs (NACs), and zinc finger proteins (ZFPs; Hu et al., 2006; Liu et al., 2012; Schmidt et al., 2013; Shen et al., 2017). AP2/ERF TFs have a conserved function domain (AP2 domain) that recognizes and binds cis-elements, i.e. the GCC-box (AGCCGCC) and dehydration responsive element/C-repeat, RCCGCC element, in the promoter regions of target genes throughout the genome. This superfamily,

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T.C. and R.H. conceived and designed the experiments; X.C. designed and performed most of the experiments, analyzed the results, and wrote the manuscript; S.Z. and X.Z. characterized stress phenotypes of the transgenic plants; W.T. and L.P. helped with the analysis of the sequencing data; H.Z. screened the rice EAR proteins and generated artificial microRNA transgenic rice; J.L. and J.S. performed the EMSA experiments; T.C. finalized the manuscript.

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containing more than 100 members in rice, consists of AP2/ERF proteins and regulates plant development, propagation, and stress response. For example, a rice AP2/ERF type TF, SERF1 (SALT-RESPONSIVE ERF1), responds to salt stress and participates in regulation of grain filling and gibberellin-meditated seedling establishment (Schmidt et al., 2014). Some AP2/ERF proteins, like GmRAV-03, JcERF011, and ERF109 are involved in the regulation of salt tolerance in plants (Bahieldin et al., 2016; Tang et al., 2016b; Zhao et al., 2017). Rice INDETERMINATE SPIKELET1 (IDS1), a typical AP2 type TF, has been reported to play roles in inflorescence architecture and the establishment of floral meristems (Lee and An, 2012).

TFs may act as transcriptional activators or repressors. Transcriptional repressors can further be classified as either active or passive repressors. Passive repressors are proteins that indirectly affect transcription, for example, by steric interference of the transcriptional activator. Active repressors, which normally possess a functional repression domain, confer repressive activity through recruiting the transcriptional repression components (Licausi et al., 2013). For repression domains, a typical motif calling the ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif, which bears the consensus sequence pattern of either LxLxLx or DLNxxP, is one of the most predominant repressive motifs identified in plants (Kagale and Rozwadowski, 2011). In Arabidopsis (*Arabidopsis thaliana*), the EAR motifs recruit some transcriptional repression complex members, such as the corepressors TOPLESS/TOPLESS-LIKE PROTEIN (TPL/TPR) or the SIN3A ASSOCIATED PROTEIN18 (AtSAP18). TPL/TPR proteins are widely involved in the regulation of hormone signaling, such as auxin, jasmonic acid, abscisic acid (ABA), ethylene, and strigolactones, and affect plant embryogenesis, leaf development, morphogenesis, drought response, and ripening (Szemenyei et al., 2008; Pauwels et al., 2010; Jiang et al., 2013; Tao et al., 2013; Han et al., 2016; Tang et al., 2016a; Liu et al., 2017).

In this study, we focused on the identification of functional EAR-motif-containing TFs that may participate in the modulation of rice salt tolerance. We identified one AP2 type transcription factor, IDS1, as a key negative regulator of rice salt tolerance. According to our results, IDS1 bound to the promoter regions of *LEA1* and *SOS1*, which are positive modulators of rice salt tolerance and repressed their expression. More importantly, we showed that IDS1 could physically interact with the corepressor TPR1 and the histone deacetylase HDA1 through its EAR motif and form an IDS1-TPR1-HDA1 transcriptional repression complex, contributing to the transcriptional repression activity of IDS1. These results revealed an epigenetic mechanism for TPL- and HDAC-dependent regulation of salt-responsive genes as well as rice salt tolerance under stress conditions.

RESULTS

Identification of *IDS1* from Rice EAR-Containing TFs by Salt Tolerance Analysis

Many studies have confirmed the important role of the EAR motif in transcriptional regulation. Thus, in this study, we searched for EAR-motif-containing (DLNxxP or LxLxLx) TFs in rice to identify new TFs involved in salt tolerance regulation. Based on PatMatch1.2 (www.arabidopsis.org), 374 EAR motif TFs were identified from the database (Plant Transcription Factor Database v2.0; http://planttfdb.cbi.pku.edu.cn). These TFs belong to different TF families, including AP2/ERF, ARF, B3, BES1, C2H2, Dof, EIL, HD-ZIP, MYB, NAC, bHLH, and WRKY. Next, we conducted gene expression analysis using the rice database (http://www.ricearray.org/). Results showed that 57 TFs were either up- or down-regulated after salt treatment, indicating that these 57 TFs might be relevant to rice salt stress signaling. To experimentally confirm the potential function of these TFs in the regulation of salt resistance, we constructed artificial microRNA (amiRNA) interfering transgenic seedlings using rice ssp. *japonica* cv Nipponbare (NIP) as the background, in which the 57 TFs were separately knocked down at the transcriptional level. We then evaluated their survival rates and yield traits in high saline and alkali soil. In total, 20 of the TF RNAi seedlings showed substantially increased or decreased salt tolerance compared with the wild-type background material (Fig. 1A). Among these seedlings, *IDS1* RNAi lines (RI-1, RI-2, RI-3) showed greatly enhanced salt tolerance (Fig. 1, B and C; Supplemental Figs. S1 and S2). Under salt stress, plants rely on ion transport and compartmentation, and synthesis and accumulation of osmotic agents for survival (Fedina et al., 2002). We measured the amounts of Pro and malondialdehyde (MDA) in *IDS1* RNAi lines and found that after salt treatment, Pro accumulated while MDA decreased in these lines when compared with wild type. These results indicated better osmotic tolerance and membrane integrity after the knockdown of *IDS1*, supporting the hypothesis that *IDS1* may weaken the rice tolerance to salt stress (Fig. 1, D and E).

IDS1 Negatively Regulates Salt Resistance

To further confirm our observations that *IDS1* knockdown led to compromised salt tolerance, we used an *IDS1* overexpression (*IDS1*-OE) transgenic rice line and a T-DNA insertion mutant line *ids1-1* to test salt tolerance phenotypes. According to immunoblot and reverse transcription quantitative PCR (RT-qPCR) assays, IDS1 was highly accumulated in *IDS1*-OE plants (Supplemental Fig. S3), and the expression of *IDS1* was blocked in the *ids1-1* mutant (Supplemental Fig. S4). Under salt stress treatment, the survival rate of *IDS1*-OE was significantly lower than that in wild-type. In

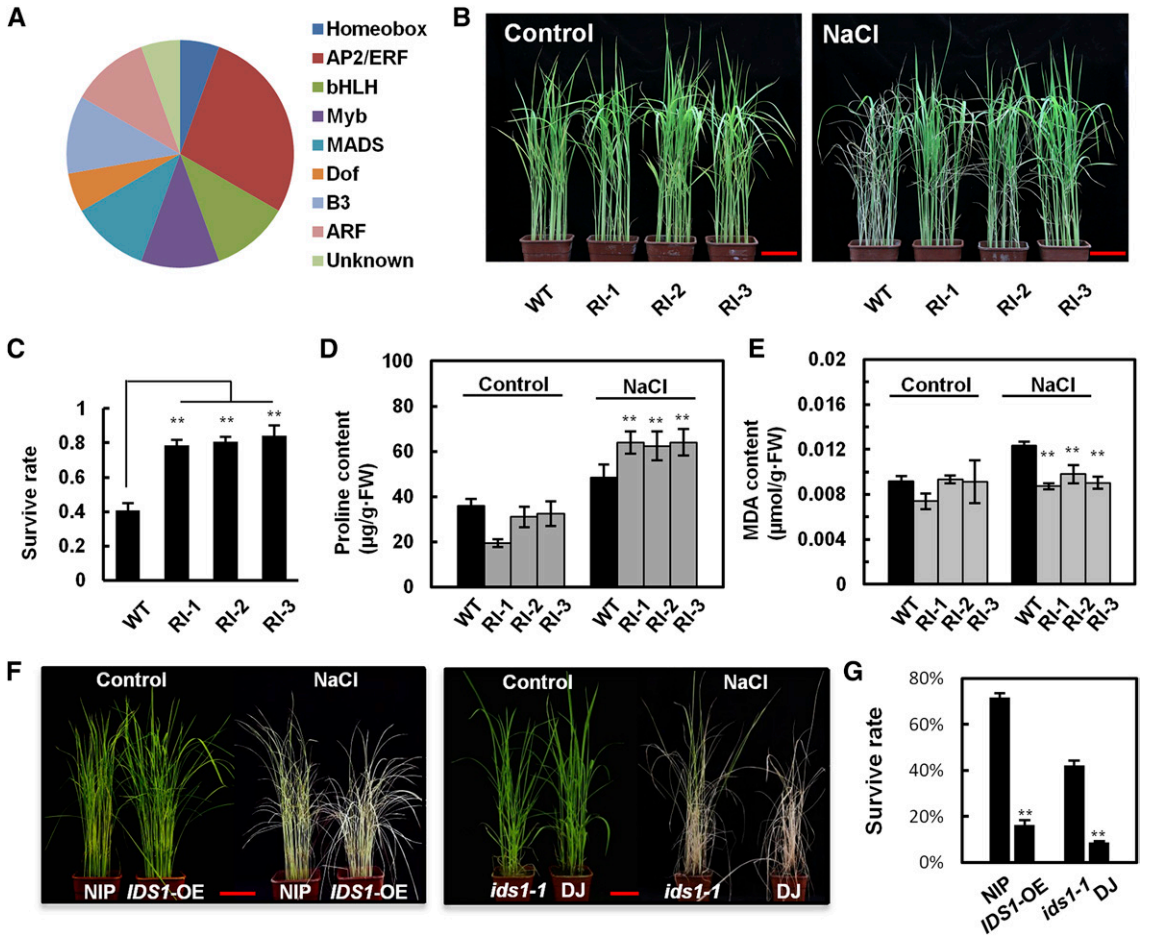

Figure 1. Identification of an EAR-motif-containing transcription factor IDS1 in rice using a salt tolerance assay. A, Pie chart showing different types of EAR-motif-containing TFs with increased or decreased salt tolerance in their amiRNA interference seedlings. B, Salt tolerance phenotype of *IDS1*-knockdown rice transgenic plants. The seedlings of wild-type (WT) and amiRNA-mediated *IDS1*-knockdown lines RI-1, RI-2, and RI-3 were separately treated with or without (control) 120 mM NaCl for 10 d ($n \geq 30$). C, The statistical quantification of survival rates of wild-type NIP and the three independent *IDS1* knockdown transgenic lines ($n \geq 30$). D and E, Determination of the Pro and MDA contents in wild-type NIP and IDS1-knockdown transgenic lines with or without NaCl treatment. Error bars represent SDs ($n \geq 30$). F, Salt tolerance phenotypes of *IDS1* overexpression (*IDS1*-OE), and *ids1-1* mutant rice plants. G, Survival rates of wild-type NIP, *IDS1*-OE, and *ids1-1* mutant seedlings as well as wild-type DJ seedlings treated with or without (control) 120 mM NaCl for 14 d ($n \geq 30$). Error bars represent SDs among three independent replicates. $^{**}P < 0.01$ (Student's *t* test). Scale bar, 5 cm.

contrast, the *ids1-1* mutant had a higher survival rate when compared to wild-type plants (Fig. 1, F and G). Meanwhile, the expression levels of abiotic stress-responsive genes were significantly increased in *ids1-1* seedlings compared to wild-type (Supplemental Fig. S5). These results indicate that *IDS1* plays a negative role in rice salt tolerance.

To examine the effects of abiotic stress on the transcriptional regulation of *IDS1* expression, we first examined the expression level of *IDS1* in rice seedlings after 120 mM NaCl treatment within 24 h using RT-qPCR. *IDS1* expression was induced by NaCl 4 h posttreatment (Supplemental Fig. S6A). To analyze the spatiotemporal expression pattern of IDS1, we generated *ProIDS1*:*GUS* transgenic rice line. GUS staining was stronger in salt-treated seedlings compared to the control group (Supplemental Fig. S6B). These results suggested that *IDS1* was transcriptionally induced by salt stress.

IDS1 Confers Transcriptional Repression Activity

IDS1 is predicted to be an AP2/ERF type TF. Subcellular localization of IDS1 was detected using the IDS1-GFP-fused protein, which was transiently expressed in rice protoplasts. The fluorescent signal of IDS1-GFP co-localized with nuclear localization signal NLS-CFP, indicating that IDS1 is a nuclear protein (Fig. 2A). Since a canonical EAR motif (DLDLDL) is located in the C-terminal region of IDS1 (Fig. 2B), we predicted that

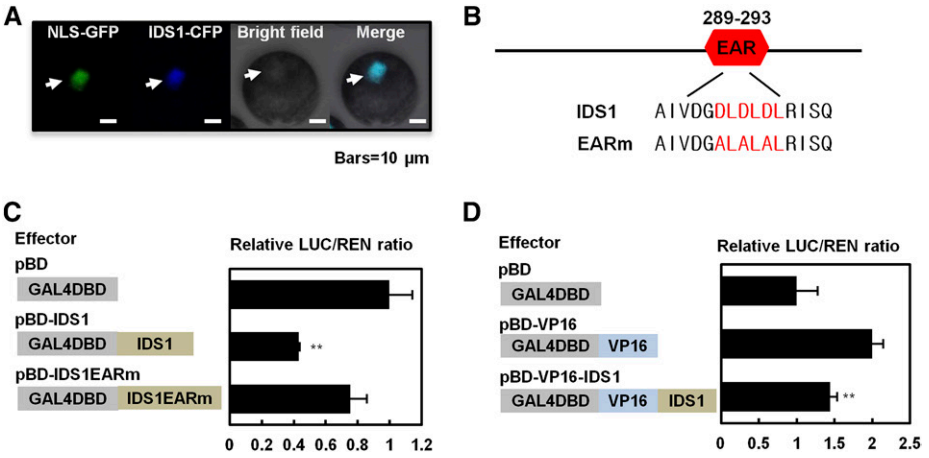

Figure 2. IDS1 confers transcriptional repression activity. A, Subcellular localization of IDS1. Arrows mark the nuclei. NLS, nuclear localization sequence. Scale bars, 10 μm. B, Wild-type and loss-of-function mutated amino acid sequence of IDS1 EAR motif. C and D, Transient dual-luciferase expression assays illustrating the transcriptional repression activity of IDS1. The reporters luciferase (LUC) and renilla luciferase (REN) and the effectors (pBD, pBD-IDS1, pBD-VP16, and pBD-IDS1-VP16) were separately constructed as shown in the left columns of C and D. The activities of LUC and REN were determined 16 h after transformation, and the relative LUC/REN ratios represent the transcriptional activation activities. Error bars represent SDs among three independent replicates. $^{**}P < 0.01$ (Student's *t* test).

IDS1 may possess a transcriptional repression activity. To confirm this prediction, we performed a transcriptional activity assay using an effector-reporter system by comparing luciferase (LUC) expression levels after the accumulation of GAL4 DNA binding domain (BD), BD-IDS1 fusion protein and BD-IDS1 with a mutated EAR motif (BD-IDS1EARm, ALALAL) (Fig. 2, B and C). In the assay, Renilla luciferase (*REN*) driven by the 35S promoter was used as an internal control. As shown in Figure 2C, luciferase activity was significantly reduced in BD-IDS1 compared with that in BD, suggesting that IDS1 may confer transcriptional repression activity. Nevertheless, the LUC activity in BD-IDS1m showed no obvious difference with that in the BD control (Fig. 2C), indicating that the transcriptional repression activity of IDS1 may largely depend on its functional EAR motif. To further confirm the repression activity of IDS1, the IDS1 protein was fused with the activator VP16 to generate VP16-IDS1. According to our results, IDS1 significantly suppressed the activation activity of VP16 since the VP16-IDS1-accumulated sample exhibited relatively lower luciferase activity when compared to the VP16-expressing positive control (Fig. 2D). These results suggested that IDS1 may function as a transcriptional repressor.

IDS1 Binding Profiles in Rice Seedlings

To further understand how IDS1 regulates plant salt tolerance, we comprehensively identified IDS1 target genes by investigating global binding profiles of IDS1. Chromatin immunoprecipitation sequencing (ChIP-seq) was performed using *IDS1-OE* transgenic seedlings with three technical replicates (rep1, rep2, rep3; Supplemental Fig. S7). Each two replicates shared a large number of target genes that covered more than 50% of one set (Fig. 3A; Supplemental Data Set 1), indicating high sequencing quality. Further genome distribution analysis revealed that the IDS1 binding sites were highly enriched in the promoter regions, where they accounted for 30% of all the peaks. This was much higher than in the gene body regions (Fig. 3B). To investigate the detailed IDS1 binding profile in the entire genome, the distance between each peak and its nearest gene was calculated. The line chart of these distances in the ±2-kb region around the transcription start site (TSS) confirmed that the IDS1 binding sites were strongly enriched in the promoter regions of target genes and reached one peak at about 250 bp upstream of TSSs (Fig. 3C). These distribution patterns indicated that IDS1 acted as a TF.

To identify the IDS1 binding motifs, the ±100-bp flanking sequences around peak summits were submitted to Multiple EM for Motif Elicitation (MEME) to calculate the statistically predominant motifs de novo (Egea et al., 2018). Two similar motifs were found with conserved sequences TCCTCC and GCCGCC. Thus, they could be combined as one motif, xCCxCC, which is known as GCC- or GCC-like-box (Fig. 3D). Therefore, we further focused on the GCC- or GCC-like-box motifs for further analysis. Motif alignment with peaks database has been conducted with MAST (Dong et al., 2015). By calculating the occurrence plots of the GCC- and GCC-like-box within all peaks, the motifs were observed to be enriched precisely in the peak summits and to decrease to the background level in the flanking ±500-bp sites (Fig. 3E).

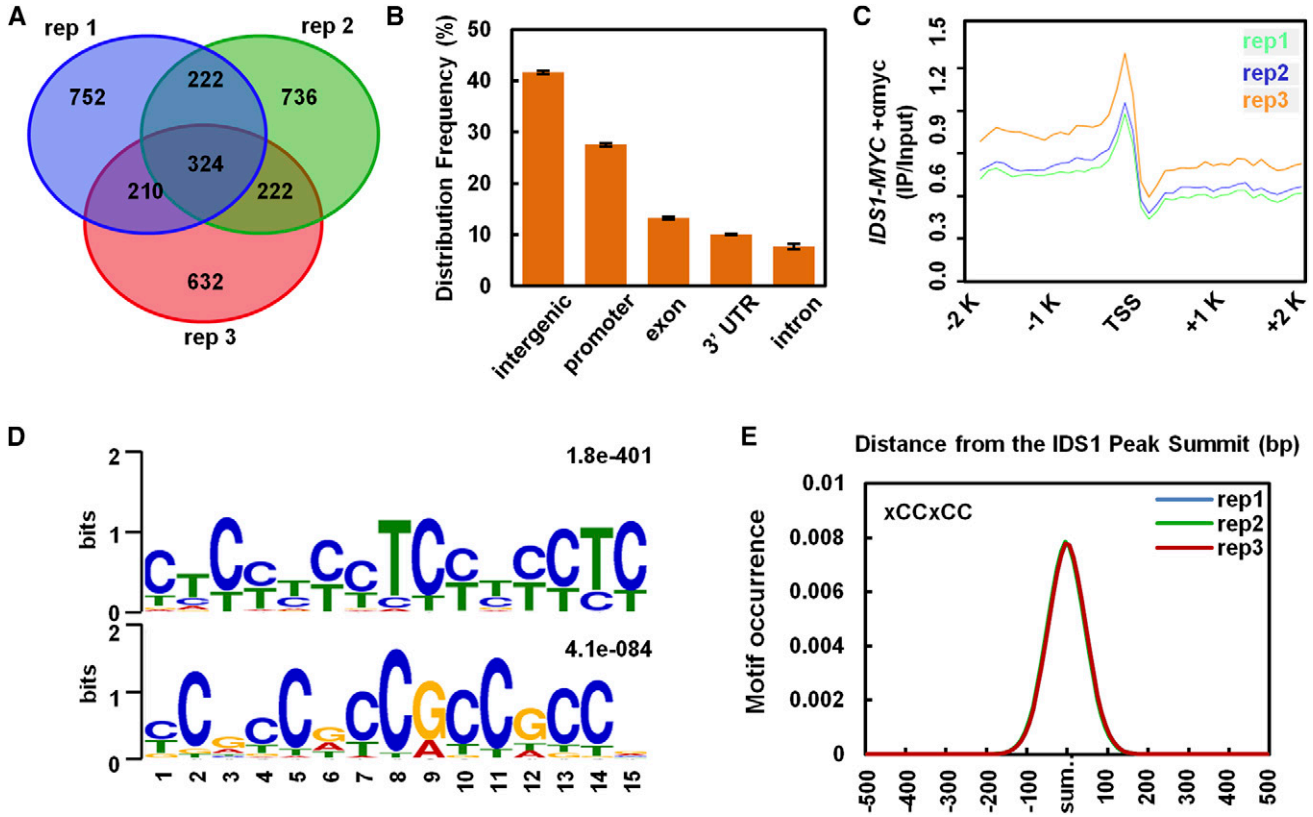

Figure 3. IDS1 binding site analysis in the rice genome. A, Venn diagram depicting genes reproducibly associated with IDS1 binding peaks in the ChIP-seq analysis of three replicates. B, Relative distribution of IDS1 binding peaks in the rice genome. Error bars represent SDs among three independent replicates. C, IDS1 binding peaks highly enriched in −2,000 to 0 bp of promoter regions in seedlings. D, MEME motif search identifying two dominant IDS1-binding motifs, defined as GCC-box or GCC-box-like (xCCxCC) motifs. E, Distribution of IDS1 binding motifs in the 200-bp regions surrounding the IDS1 binding peak summits.

Genes Associated with IDS1 Binding Sites Are Enriched in the Abiotic Stress-Responsive Pathway

We then performed a gene ontology enrichment analysis to investigate in which biological processes IDS1-targeted downstream genes may be involved. IDS1 participated in diversified pathways that affect not only plant growth and development but also stress responses (Fig. 4A). Several important plant stress tolerance-related genes were identified (Supplemental Data Set 2). Among these genes, *AP37*, *ZFP179*, and *LEA1* positively regulate rice salt tolerance (Oh et al., 2009; Sun et al., 2010; Duan and Cai, 2012). According to the ChIP-seq assay, the peak summits for IDS1 binding sites were located within 2 kb upstream of the *AP37*, *ZFP179*, and *IDS1* TSS (Fig. 4B). ChIP-qPCR further confirmed that IDS1 could indeed associate with the promoter regions of these genes in vivo (Fig. 4C).

IDS1 Binds to *LEA1* and *SOS1* Promoters in Vivo and in Vitro

LEA1 and *SOS1* play important roles in regulating rice and Arabidopsis salt tolerance, respectively (Duan and Cai, 2012). In this study, we also found that the *LEA1*-OE line exhibited a more energetic status and a significantly higher survival rate than the background material NIP after NaCl treatment, supporting the previously reported conclusion (Fig. 5A). Considering that the sequential GCC-boxes exist in the *LEA1* and *SOS1* promoters (approximately 800 bp and 1200 bp upstream from the *LEA1* or *SOS1* TSS, respectively), we hypothesized *LEA1* and *SOS1* as two potential downstream target genes of IDS1. First, we performed ChIP-qPCR to test our prediction. According to the ChIP-qPCR assay, IDS1 was indeed enriched on the promoter regions of *LEA1* and *SOS1* in vivo (Fig. 5B). Next, we conducted electrophoretic mobility shift assays (EMSAs) to test the direct association of IDS1 with *LEA1* and *SOS1* promoters in vitro. Results show that IDS1 could combine exclusively with the wild-type probes of *LEA1* and *SOS1* promoters (*LEA1* wt and *SOS1* wt), which contain the intact GCC-box, but not the probes with a mutated GCC-box (*LEA1* mut and *SOS1* mut, in which the GCC-boxes were replaced by AAAAAA), further proving that IDS1 could directly associate with *LEA1* and *SOS1* promoters (Fig. 5C). These results together confirmed that *LEA1* and *SOS1*

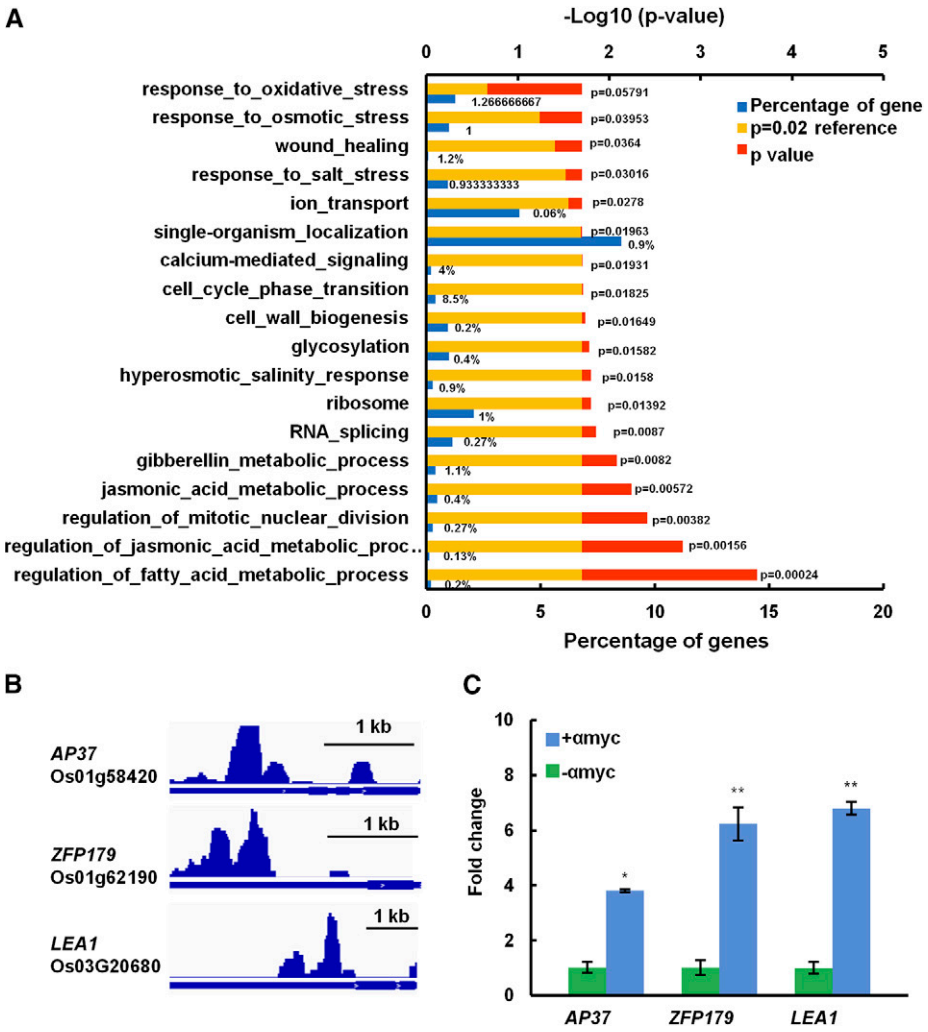

Figure 4. Genes associated with IDS1 binding sites. A, Enriched pathways of genes associated with IDS1 binding sites. B, IDS1 binding profiles in the promoter regions of abiotic stress-responsive genes. C, Validation of IDS1 binding sites in the promoter regions of genes in B by ChIP-qPCR analysis. The fold enrichment was normalized against the promoter of *Actin*. αmyc, antibodies against myc tag. Error bars represent SDs for three independent replicates. $*P < 0.05$. $**P < 0.01$ (Student's *t* test).

were two downstream target genes that are directly regulated by IDS1.

IDS1 Represses Expression of Target Genes at the Transcriptional Level

Since IDS1 has transcriptional repression activity, we hypothesized that the increased resistance to salt tolerance in the *ids1-1* mutant may be due to the elevated expression levels of the IDS1-targeted salt-responsive genes. As shown in Figure 6A and Supplemental Figure S8, the expression levels of *LEA1*, *SOS1*, *AP37*, and *ZFP179* were all significantly increased in the *ids1-1* mutant compared to in the wild type. Consistently, the expression levels of *LEA1* and *SOS1* were downregulated in *IDS1*-OE plants (Fig. 6A). These results were further supported by the transcriptional activity assays in *Nicotiana benthamiana*. Our results showed that *IDS1-GFP* specifically repressed the expression of the *LUC* gene driven by native promoters of *LEA1* and *SOS1*, but not by those with mutated GCC-boxes (Fig. 6, B and C). In summary, our data supported the conclusion that IDS1, as a transcriptional repressor, may negatively regulate the expression of its downstream target genes, such as *LEA1* and *SOS1*.

IDS1 Interacts with Transcriptional Corepressor TPR1

The EAR motif can recruit transcriptional corepressors such as TPL family proteins or AtSAP18 in Arabidopsis to form a transcriptional repression complex. To determine if IDS1 may interact with rice corepressor homologs, interactions between TPR1 and IDS1 and SAP18 and IDS1 were detected using several biological approaches. In yeast two-hybrid (Y2H), pull–down, and firefly LUC complementation imaging (LCI) assays, IDS1 notably interacted with TPR1 in vitro and in vivo (Fig. 7, A–C), but not with SAP18 (Supplemental Fig. S9). The bimolecular fluorescence complementation (BiFC) assay further showed that the interaction between IDS1 and TPR1 occurred specifically in the nucleus (Fig. 7D). In addition, according to the LCI and BiFC assays, mutation in the IDS1 EAR motif eliminated the interaction between IDS1 and TPR1, indicating

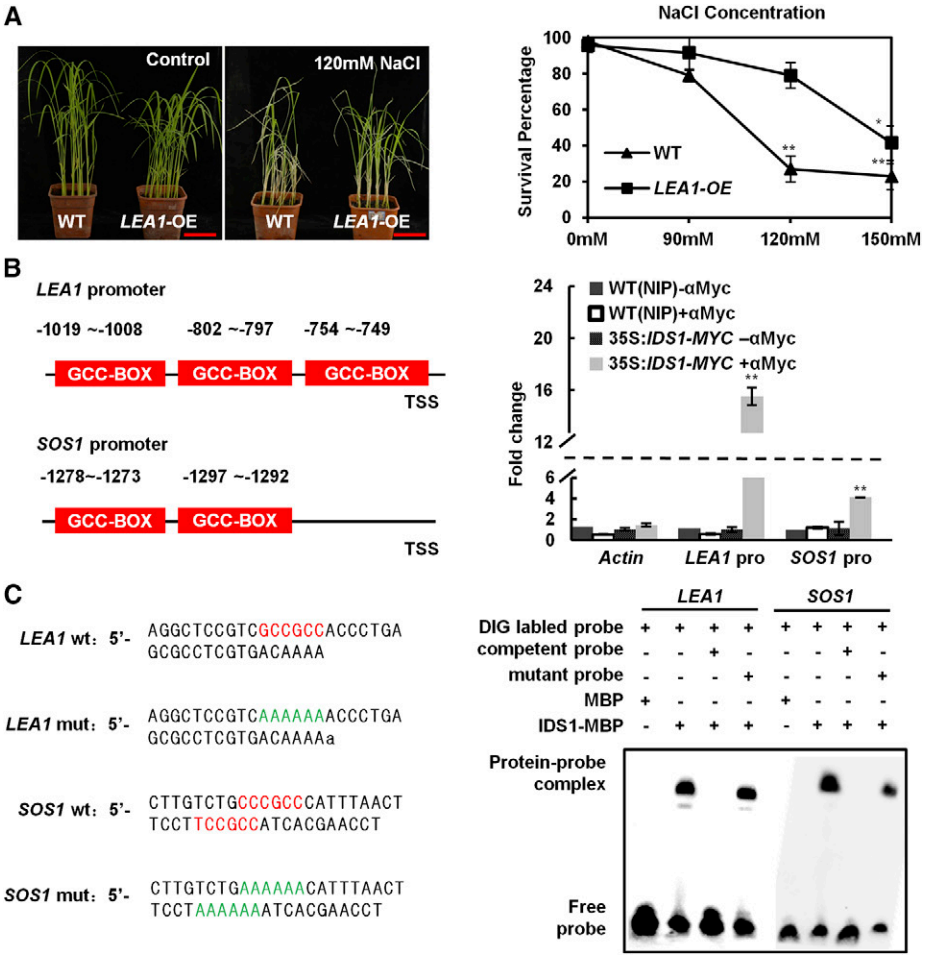

Figure 5. IDS1 directly binds to the *LEA1* promoter. A, Improvement of salt tolerance in *LEA1*-OE compared to wild-type (WT) seedlings under NaCl stress. Left, salt treatment phenotype; right, the statistics of survival percentage. The seedlings of wild-type and *LEA1*-OE lines were separately treated with or without (control) NaCl for 10 d ($n \geq 30$). Scale bar, 5 cm. Error bars represent SDs among three independent replicates. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test). B, ChIP-qPCR assay detected IDS1 enrichment level on the *LEA1* and *SOS1* promoters. Schemes illustrate the 2-kb promoters of *LEA1* and *SOS1* (left). The red boxes show the positions of GCC-box motifs. Validation of IDS1 direct binding sites in the *LEA1* and *SOS1* promoters by ChIP-qPCR analysis (right). Three biological replicates were performed. Error bars represent SDs among three independent replicates. $^{**}P < 0.01$ (Student's *t* test). C Direct binding of IDS1 to the *LEA1* and *SOS1* promoters in EMSAs. The wild-type probes containing GCC-box motifs (red color) were derived from *LEA1* and *SOS1* promoters, while *LEA1* mut and *SOS1* mut show probes with mutated GCC-box motifs (AAAAAA, green color). Competition was performed with 125-fold wild-type or mutated cold probes. MBP protein was used as a negative control.

that the IDS1-TPR1 association may depend on the IDS1 EAR motif (Fig. 7, E and F). To map the interacting domain of TPR1 with IDS1, we divided the TPR1 into four truncated parts, i.e. NT (N terminus, including the LiSH and C-terminal to lissencephaly homology domains), MD (middle domain), CT (C terminus, containing repeated WD40 domains), and the ΔNT (NT region deletion) (Fig. 7G). BiFC, pull-down, and Y2H assays all showed that NT of TPR1 mediated the physical interaction with IDS1 (Fig. 7, H–J). To experimentally confirm the corepressor function of TRP1,we coexpressed *35S:IDS1* and *35S:TPR1* in *N. benthamiana* with a *LUC* gene reporter driven by promoters of IDS1 targeting genes. As shown in Supplemental Figure S10, TPR1 enhanced the transcriptional repression activity of IDS1.

IDS1 Directly Interacts with Rice Histone Deacetylase HDA1

To identify new IDS1-interacting proteins besides TPR1 in rice, we screened a rice Y2H library using IDS1 as bait. HDA1, a histone deacetylase, was finally identified as a candidate. As shown in Figure 8A, AD-IDS1 and BD-HDA1 were coexpressed in yeast cells, and the transformed cells could grow on medium lacking Leu, His, and Trp, indicating the interaction between IDS1 and HDA1. Moreover, the LCI assay also showed that IDS1 could interact with HDA1 in planta (Fig. 8B). Similarly, the in vitro pull-down assay showed HDA-GST could exclusively interact with IDS1-maltose binding protein (MBP), but not MBP, further confirming the direct interaction between IDS1 and HDA (Fig. 8C). These results demonstrated that IDS1 could directly interact with HDA1 in vitro and in vivo.

TPR1 Associates with HDA1 to Form a Transcription Repression Complex

Several studies demonstrate that TPL/TPR proteins may interact with histone deacetylase family members and regulate embryogenesis, circadian transcription, and stress responses (Long et al., 2006; Szemenyei et al., 2008; Wang et al., 2013; Tang et al., 2016a). Thus, we detected the physical interaction between TPR1 and HDA1. As expected, the Y2H assay proved that TPR1 and HDA1 could indeed interact with each other in yeast cells (Fig. 8D), and this interaction was further confirmed by an LCI assay in *N. benthamiana* (Fig. 8E). To define the interaction domains of TPR1 and HDA1, the NT, MD, and CT of TPR1 were fused with the N-terminal part of LUC and separately coexpressed

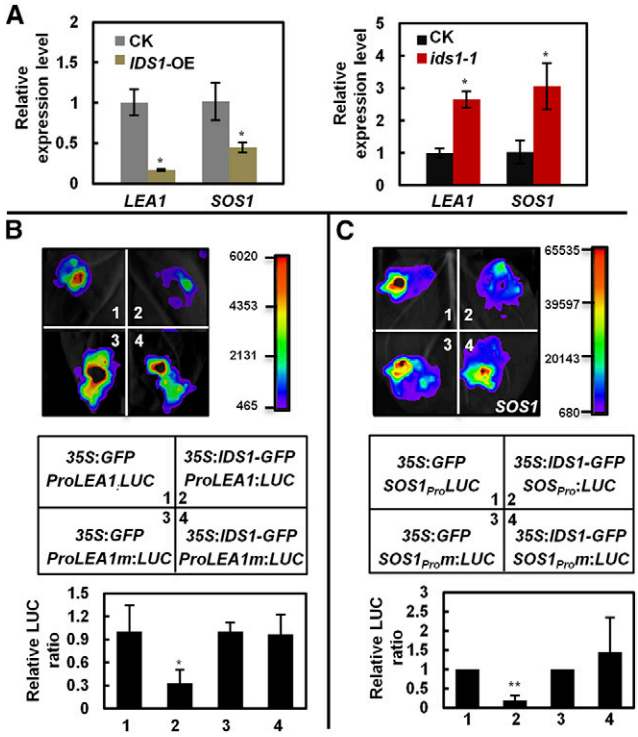

Figure 6. IDS1 negatively regulates the expression of *LEA1* and *SOS1* in rice. A, Transcription levels of *LEA1* and *SOS1* in *IDS1*-OE (left) and *ids1-1* (right) rice materials. *ACTIN* was the internal control. Error bars represent SDS among three independent replicates. B and C, Transient expression assays illustrating the transcriptional repression of *LEA1* (B) and *SOS1* (C) by IDS1. The 2-kb promoters of *LEA1* and *SOS1* containing the wild-type or mutated GCC-box motifs were separately used to drive the LUC gene expression and were coexpressed with 35S:GFP or 35S:IDS1-GFP in *N. benthamiana* leaves. LUC activities were determined 48 h postinfiltration. Quantification of the relative luminescence intensities ($n = 4$) is shown in the bottom of B and C. The mean values of each combination were normalized against controls. Error bars represent SDS among three independent replicates. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test).

with HDA1, which was fused with the C-terminal part of LUC. The LCI results showed that the strongest interaction signal occurred between the TPR1-NT and CT-HDA1, demonstrating that the N-terminal part of TPR1 is not only responsible for the interaction with IDS1 but also mediated the association with HDA1 (Fig. 8F). Besides the physical interactions among IDS1, TPR1, and HDA1, the *IDS1*, *TPR1*, and *HDA1* genes also showed similar expression patterns in rice roots, stems, and leaf tissues (Supplemental Fig. S10). These results indicate that the three members may function together and may form a transcriptional repression complex in vivo (Supplemental Fig. S11). Interestingly, the EARm version of IDS1 could still interact with HDA1, implying that the EAR motif is dispensable in the interaction between IDS1 and HDA1 (Supplemental Fig. S12).

IDS1 Mediates Transcriptional Repression by Chromatin Remodeling

Histone acetylation is a marker of gene transcription activation status. HDA1 plays an important role in controlling gene expression by changing histone acetylation levels. We tried to clarify whether this mechanism is involved in the transcriptional repression of IDS1-regulated target genes in vivo. To this end, histone acetylation levels in the *IDS1*-OE, *ids1-1* mutant, and the wild type were checked using ChIP-qPCR with an antibody against acetylated histone H3. H3 acetylation levels on the promoter regions of *LEA1* and *SOS1* were increased in *ids1-1* mutants and accordingly decreased in *IDS1*-OE seedlings compared to wild type (Fig. 8G). These results were consistent with the changes of expression levels of IDS1-targeting genes in the *ids1-1* mutant and *IDS1*-OE. In NIP protoplasts, the transcription levels of *LEA1* and *SOS1* were increased upon exposure to the HDAC inhibitor trichostatin A (TSA) compared to the sample without TSA treatment, confirming that HDACs may potentially participate in the transcriptional repression of *LEA1* and *SOS1* (Supplemental Fig. S13). Previous studies have shown that nucleosomes with H3me and H3Ac modifications together with RNA polymerase II (Pol II) occupy the promoters of most protein-coding genes and represent a hallmark of transcription initiation and elongation (Guenther et al., 2007). Transcription initiation requires the assembly of the preinitiation complex, the mediator complex, and Pol II with an unphosphorylated CTD (C-terminal domain). However, active transcription requires highly phosphorylated CTD with Ser-2 and Ser-5 positions of conserved YSPTSPS heptapeptide repeats. The Ser-5 phosphorylation peaks emerge around the TSS (Baugh et al., 2009). Thus, a ChIP-qPCR assay, using anti-Pol II CTD YSPTSPS S5P antibody, was performed using *ids1-1* mutants, *IDS1*-OE, and wild-type plants to detect the enrichment levels of active Pol II on the TSS of promoter regions of IDS1 target genes. The results showed that Pol II proteins significantly concentrate on the TSS of *LEA1* and *SOS1* in *ids1-1* mutants (Fig. 8H). Together, the above results demonstrated that IDS1 may antagonize the histone acetylation and active Pol II occupation levels on the promoters of IDS1-targeting genes.

DISCUSSION

IDS1 Acts as a Negative Regulator of Rice Salt Tolerance

Understanding the salt response mechanism of rice is important for molecular improvement of rice salt tolerance. Many rice salt-responsive genes have been cloned and characterized (Hu et al., 2006; Liu et al., 2012; Jin et al., 2013; Schmidt et al., 2013; Toda et al., 2013; Shen et al., 2017). Several genes have been used as molecular markers in salt tolerance breeding, which

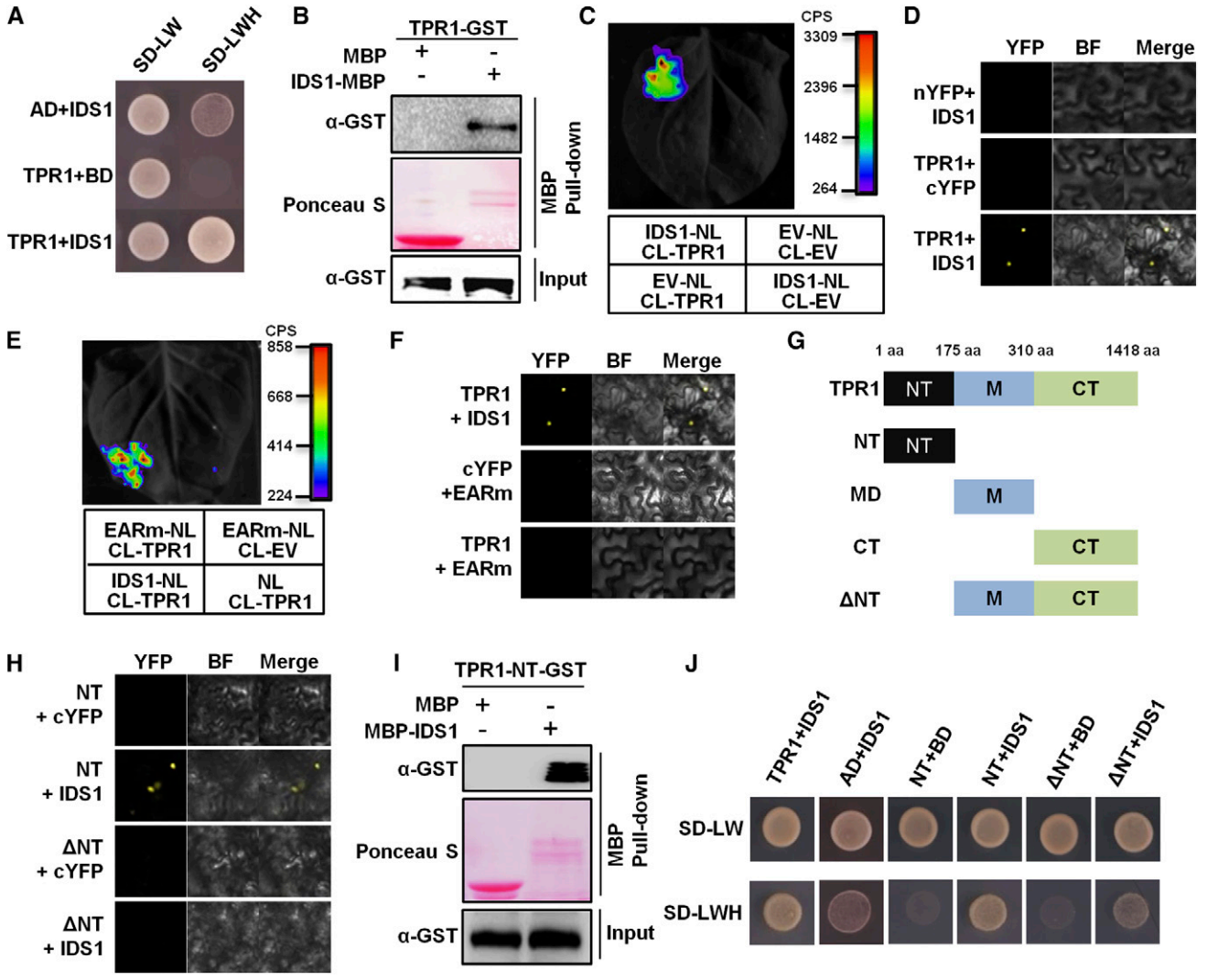

Figure 7. IDS1 directly interacts with the transcriptional corepressor TPR1. A, Y2H assay showing the physical interaction between IDS1 and TPR1. SD-LW, synthetic dextrose medium lacking Leu and Trp; SD-LWH, SD medium lacking Leu, Trp, and His. B, Pull-down assay illustrating that IDS1 directly interacts with TPR1. C, Firefly LCI assay detecting the interaction between IDS1 and TPR1. Left, a representative leaf image; right, the colored scale bar indicates the luminescence intensity (CPS). NL, N terminus of LUC; CL, C terminus of LUC; EV, empty vector. D, BiFC assay revealing the interaction of IDS1 with TPR1. BF, bright field. E and F, LCI and BiFC assays showing that the EARm version of IDS1 failed to interact with TPR1. G, Schemes showing the full-length as well as truncated versions of TPR1 protein. NT, N-terminal domain; MD, middle domain; CT, C-terminal domain; ΔNT, deletion of the NT; aa, amino acids. H, BiFC assay showing that the NT of TPR1 is sufficient for the interaction with IDS1. I, Pull-down assay showing the interaction between TPR1-NT and IDS1. J, Y2H assay confirming the interaction between TPR1-NT and IDS1.

can shorten breeding and selection. SKC1, a major QTL gene for salt tolerance, which is an HKT-type sodium transporter, was cloned by QTL mapping with a nearly isogenic line of salt-tolerant rice and a salt-susceptible rice variety (Ren et al., 2005). Hitomebore salt tolerant1), a B-type response regulator named OsRR22, was isolated by screening EMS mutagenesis lines of a local rice cultivar. The *hst1* mutant was used to breed a salt-tolerant variety (Takagi et al., 2015). Our previous studies and these data show that ERF transcription factors play a role in rice abiotic stress responses. This suggests that there may be many ERF TFs participating in salt tolerance (Wang et al., 2012; Zhang et al., 2012, 2013; Xiao et al., 2016). We have used bioinformatics analysis combined with amiRNA library screening to identify several ERF TFs involved in rice salt tolerance. Among these, IDS1 was proven to be a negative regulator of the salt response.

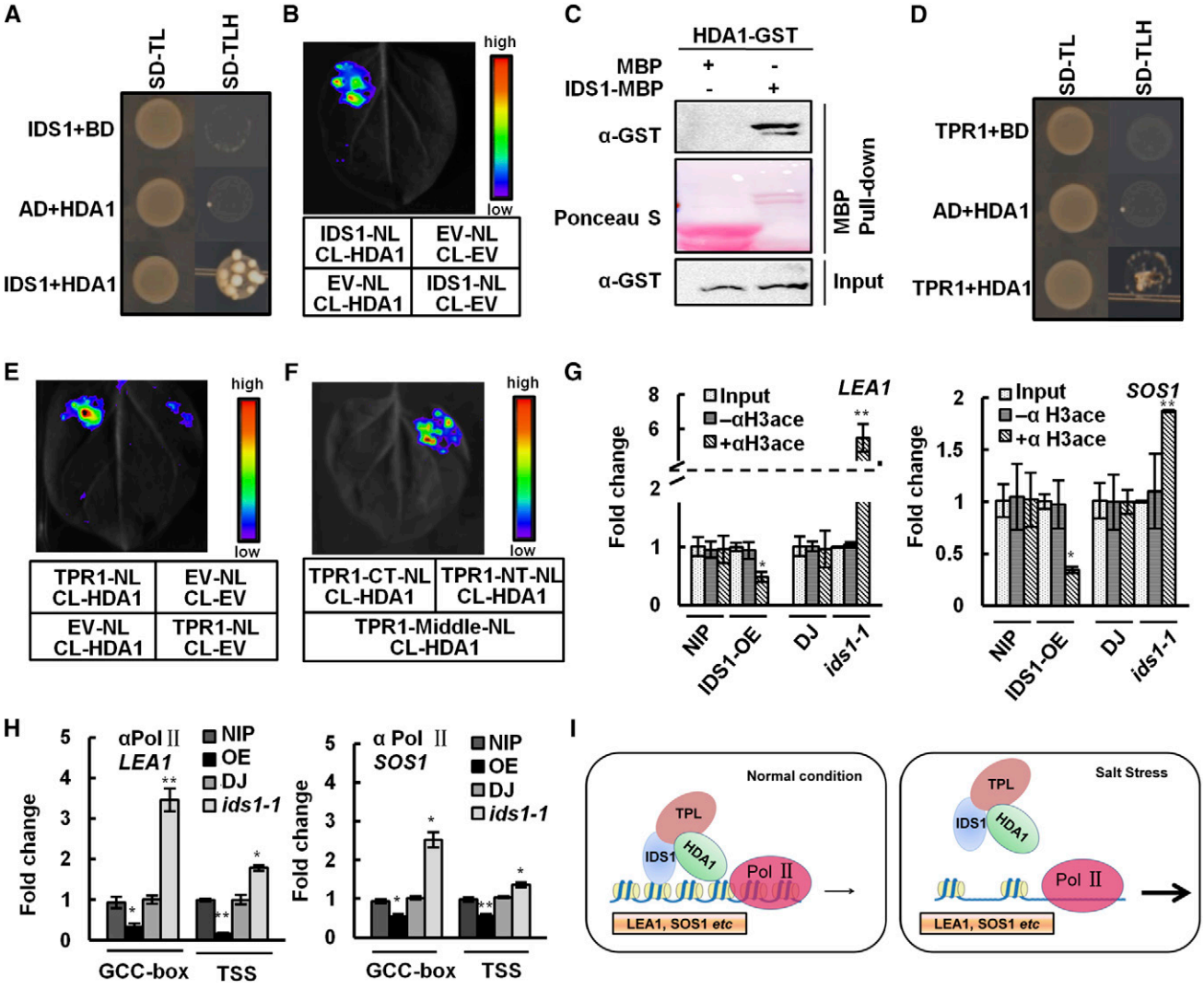

Figure 8. IDS1, TPR1, and histone deacetylase HDA1 physically interact with each other and lead chromatin remodeling in *LEA1* and *SOS1* promoter regions. A, Y2H assay showing the interaction of IDS1 and HDA1. B, LCI showing the interaction of IDS1 and HDA1. C, Pull-down assay confirming the interaction between IDS1 and HDA1. D, Y2H assay revealing the interaction between TPR1 and HDA1. E, LCI showing the interaction of TPR1 and HDA1. F, LCI showing that the N-terminal domain of TPR1 mediates its interaction with HDA1. TPR1-NT, TPR1-MD, and TPR1-CT represent truncated versions of TPR1 as shown above. ChIP assays by using the anti-acetyl-histone H3 (G) or Pol II antibodies (H) were performed to measure the histone acetylation levels (G) and Pol II assembling (H) at the promoter regions of *LEA1* and *SOS1* in the *IDS1*-OE, *ids1-1* mutant, and wild-type (NIP and DJ) seedlings. The specific amplicons were described as above. I, A model for the IDS1 function in rice salt tolerance. Error bars (in G and H) represent SDs among three independent replicates. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t* test).

The IDS1-TPR1-HDA1 Module Acts as Transcriptional Repressor Complex

Epigenetic mechanisms, including DNA/RNA methylation, histone modification, and noncoding RNA-mediated gene expression adjustment, are important mechanisms used by plants to cope with abiotic environmental stress (Kinoshita and Seki, 2014). Most of the methylated cytosine sites can persist in subsequent generations when plants are exposed to salt stress (Jiang et al., 2014). Changes of chromatin structure by histone modification can change target gene expression and help plants to adapt to and survive harsh environments. In Arabidopsis, HD2C and HDA6 regulate ABA and salt response by increasing the expression of ABA response genes *ABI1* and *ABI2* via histone deacetylation (Luo et al., 2012). In crops, the histone acetylation level of promoter regions of some cell cycle genes in maize (*Zea mays*) and peroxidize genes in beet (*Beta vulgaris*) can change under salt stress (Zhao et al., 2014; Yolcu et al., 2016). Detailed molecular mechanisms of histone modification linked with salinity response in rice require additional clarification. Our data showed that the IDS1-TPR1-HDA1 module regulated

expression levels of the salt-responsive genes *LEA1* and *SOS1*. The IDS1-mediated salt response recruited TPR1-HDA1 to inhibit target gene expression, and this can be released in rice under salt stress (Fig. 8I). Our results established a model in which IDS1 recruits TPR1 and HDA1 to target stress-specific genes participating in osmotic stress, ion transport, transcriptional cascade, and metabolic processes.

The transcription repression activity of IDS1 was endowed by its EAR motif. Much evidence indicates that the EAR motif is responsible for repression of target gene expression. In addition, EAR motif-containing TFs can activate the expression of other TFs. The function of EAR-containing TFs on target gene regulation is still unclear (Ohta et al., 2001; Kagale and Rozwadowski, 2011; Barah et al., 2016). For stress-related EAR-containing TFs, there are at least two additional functions in plant cells: suppression of the stress response genes under stress-free environments and activation of other target genes in biological processes such as the cell cycle, plant cell differentiation, and meristem development. The EAR domain of IDS1 in salt response interacts with the N terminus of TPR1 to recruit the histone deacetylation enzyme. The conserved amino acid residues, DLDLDL, are the interacting sites indicating that the epigenetic mechanism of transcription repression is conserved in plants.

The NT, containing the C terminus to lissencephaly homology domain, was considered to be a binding partner of TPL/TPRs (Szemenyei et al., 2008). Our results demonstrated that the NT of TRP1 interacts with the IDS1. The EAR motif usually mediates the interaction with corepressors like the TPL/TPR protein or the SAP18 in Arabidopsis. We showed that the EAR motif in IDS1 is indispensable for the interaction with TPR1, but not SAP18. In a transient expression system, TPR1 could facilitate IDS1 to repress the expression of *LEA1* and *SOS1*.

IDS1 Maintains the Balance of Yield and Survival of Rice

IDS1 was initially identified as an AP2 type TF in maize, which determines spikelet meristem fate and controls the number of floral meristems (Chuck et al., 1998). In rice, IDS1 is involved in inflorescence architecture, affecting yield. The *ids1* and *snb ids1* (supernumerary bract) mutants show no obvious differences compared with the wild type in vegetative growth, flowering time, tiller number, and plant height. However, the two mutants have severe reproductive defects with more bracts, including rudimentary glumes, lemma, and palea (Lee and An, 2012). These data show that IDS1 controls yield and salt response and indicates that IDS1 may have multiple roles in balancing the energy consumption of rice to develop the best strategies in response to environmental changes.

A single gene can have several different pathways to control the development of different tissues. For example, WOX11, wuschel-related homeobox 11, regulates cell proliferation of the crown root meristem by recruiting the ADA2-GCN5 histone acetyltransferase module and also controls shoot development by recruiting histone H3K27me3 demethylase (Zhou et al., 2017; Cheng et al., 2018). While IDS1 suppresses the salt response genes by recruiting TPR1 and HDA1, IDS1 activates many B, C, and E function genes such as OsMADS2, OsMADS4, OsMADS16, OsMADS3, OsMADS1, OsMADS5, OsMADS7, and OsMADS8, in panicle formation. These data indicate that IDS1 plays several important roles, involving distinct molecular mechanisms, in response to environmental stimuli and developmental signals.

CONCLUSION

This study focused on functions of IDS1 in the regulation of rice salt tolerance. IDS1 bound to the GCC-box motif of the rice genome at a global level, including GCC-boxes in the promoter regions of *LEA1*, *AP37*, *ZFP179*, and *SOS1*. IDS1 repressed expression of target genes at the transcriptional level. We demonstrated that IDS1 interacted with the transcriptional repression complex in vitro as well as in vivo and caused chromatin remodeling in the promoter regions of target genes. We propose that IDS1 modulates target gene expression at the transcriptional level to regulate rice plant salt tolerance. Additional research will be needed to determine the molecular factors that regulate *IDS1* expression under salt stress.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

To construct the *IDS1* amiRNA interference vector, 21-nt cDNA of *IDS1* was fused with the pNW55 stem-loop structure and cloned into binary pCAMBIA5300. The construct was introduced into rice (*Oryza sativa* ssp. *japonica* cv Nipponbare [NIP]) by *Agrobacterium tumefaciens*-mediated transformation. For the overexpression construct, the full-length cDNA of *IDS1* was cloned into the pENTR/D-TOPO vector (Invitrogen) and subcloned into the expression vector with a myc tag. The construct was transformed into NIP. The loss-of-function mutant *ids1-1* was ordered from the Rice T-DNA Insertion Sequence Database rice mutant library (http://cbi.khu.ac.kr/RISD_DB.html).

The rice plants were grown in the greenhouse with a 12-h-light/12-h-dark cycle at 25°C to 30°C. *Nicotiana benthamiana* plants were grown in a greenhouse at 24°C with a 16-h-light/8-h-dark cycle.

Salt Treatment

The salt tolerance of the amiRNA interference plants, overexpression plants, and T-DNA insertion mutants was compared to that of the wild type. Plants of different genotypes were germinated in petri dishes and transplanted in soil. At the four-leaf stage, salt stress treatments were conducted using different concentrations of NaCl for several days until leaf curvature was observed. Plants were allowed to recover for 10 d and then the survival rates were calculated. Plants with green leaves and regenerating shoots were considered survivors. Three replicates were performed for each experiment.

RNA Extraction and Gene Expression Analyses

Total RNA was extracted from the rice materials using Trizol (Invitrogen) reagent. About 2 μg of total RNA was used for further reverse transcription

using M-MLV reverse transcriptase (Promega). For determination of gene expression, SYBR Premix Ex Taq (Perfect Real Time; TaKaRa) was used for the RT-qPCR assays. Expression levels of target genes were normalized to the internal control gene *Actin1* or *Ubiquitin*. Each assay was repeated three times independently, and the statistical significance was evaluated by Student's *t* test (significance, $P < 0.05$). Nucleotide sequences of the primers used in RT-qPCR assays are listed in Supplemental Data Set 3. The RT-qPCR primers for abiotic stress responsive genes followed the information in Schmidt et al. (2013).

ChIP Assays

NIP and its transgenic lines generated from the transformation of *ProUbi:IDS1*-myc, wild-type DJ (Dongjin), and the *ids1-1* mutant were used for the ChIP assays (Lu et al., 2013). A total of 4 g of 4-week-old seedlings were harvested. Plant materials were cross linked with 1% (v/v) formaldehyde under vacuum for 10 min. The samples were subjected to nucleus isolation and sonication and then incubated with different antibodies, including anti-c-myc antibodies (Roche; 11667149001), anti-acetyl-histone H3 (Millipore; 06-599) and anti-RNA polymerase II CTD repeat YSPTSPS (phospho S5) (Abcam; ab5408), with protein G magnetic beads (Invitrogen; 10003D) together. The DNA was then precipitated and stored at −80°C.

For ChIP-seq, isolated DNA samples were fragmented and amplified for 14 cycles. Then DNA fragments of 100 to 500 bp were purified for construction of a sequencing library.

Analysis of ChIP-Seq Data

After quality control analysis, the clean reads of ChIP-seq data were mapped to Oryza_sativa.IRGSP-1.0 with Bowtie2 (Langmead and Salzberg, 2012). Then the uniquely mapped reads were used for peak identification, and the peaks were searched with MACS (Zhang et al., 2008).

Motif Search and Classification

For all peaks present in three replicates, 200 bp around the peak summits (upstream 100 bp and downstream 100 bp) were subjected to MEME (http://meme-siute.org/) to calculate motifs.

ChIP-qPCR

The prepared DNA was applied for qPCR using low rox EVER Green PCR Master Mix (Abm) with ABI QuantStudio 7 real-time PCR detection system. The enrichment levels were normalized to the input sample and fold enrichment was calculated against the *Actin* promoter. The negative control had no antibodies added. Primers are listed in Supplemental Data Set 3.

Luciferase Transient Expression Assays

To test the transcriptional repression activity of *IDS1* in *N. benthamiana* protoplasts, a dual-luciferase reporter assay was conducted. For the effectors generation, IDS1 was fused with the GAL4 DBD to generate pBD-*IDS1*. IDS1 containing a transactivating domain VP16 in its N terminus was also fused with the GAL4 DBD to generate a pBD-VP16-IDS1 effector. The GAL4 DBD with or without VP16 (pBD and pBD-VP16) was used as the positive and the negative controls, respectively. The activities of LUC and REN were determined using the Dual-Luciferase Reporter Assay System (Promega, E1910), and the relative LUC/REN ratio was calculated. For each replication, three independent transformations were conducted.

To evaluate the transcriptional repression activity of IDS1, the 2-kb *LEA1* and *SOS1* promoters were first fused with *LUC* to generate the reporter constructs *ProLEA1:LUC* or *ProSOS1:LUC*. For generation of the effectors, the *IDS1-GFP* coding sequence was cloned into the expression vector. The coding sequence of GFP was also cloned into the expression vector as the negative control. All the reporter and effector constructs were separately introduced into *A. tumefaciens* strain GV3101. *A. tumefaciens* harboring the indicated reporter or effector constructs were coinfiltrated into *N. benthamiana* leaves, and the LUC signals were detected and quantified 48 h postinfiltration using Night-SHADE LB 985 (Berthold).

EMSAs

The MBP tagged IDS1 protein was expressed in *Escherichia coli* strain Transetta-DE3 (Transgen Biotech, CD801) and purified using the amylose resin (New England Biolabs; E8021V) following manufacturer's instructions. The *LEA1* promoter probes containing the GCC-box cis-elements were synthesized and labeled with digoxigenin-11-ddUTP at the 3′ end by using the DIG gel shift kit (Roche; 03353591910). EMSAs were performed as described by Liu et al. (2017). The MBP protein alone was used as the negative control. Each experiment was independently replicated three times.

Pull-Down Assays

The indicated proteins were fused with MBP or GST tags and expressed in the *E. coli* strain Transetta-DE3 (Transgen Biotech; CD801). The supernatant containing MBP- and GST-tagged soluble proteins was mixed and incubated with 20 µL amylose resin beads (New England Biolabs; E8021V) for 2 h. The beads were then harvested and washed four times with Tris buffer. The eluted samples were analyzed by immunoblots using the indicated antibodies.

LCI and BiFC Assays

The LCI and BiFC assays for protein interaction detection were performed in *N. benthamiana* leaves using *A. tumefaciens*-mediated transient expression. For the LCI assay, the indicated genes were separately fused with the N- or C-terminal parts of the reporter gene *LUC* and then coinfiltrated into *N. benthamiana* leaves. LUC activities were analyzed 48 h postinfiltration using Night-SHADE LB 985 (Berthold). For the BiFC assay, the indicated genes were fused with the N- or C-terminal parts of the yellow fluorescent protein and coexpressed in *N. benthamiana* leaves. After 48 h postinfiltration, the yellow fluorescent protein fluorescence signals were imaged with a confocal microscope (Carl Zeiss; LSM880).

Y2H Assays

The coding sequences of indicated genes were first cloned into the pGADT7 (AD) and pGBKT7 (BD) plasmids to generate the GAL4-AD and GAL4-BD derivatives. Next, the GAL4-AD and GAL4-BD derivatives were cotransformed into the yeast (*Saccharomyces cerevisiae*) strain AH109 and grown on synthetic dextrose medium lacking Leu and Trp (SD-L/W). Then, the yeast cells were screened on synthetic dextrose selection medium lacking Leu, Trp, and His (SD-L/W/H). A total of three independent replications were performed.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: *IDS1*, LOC_Os03g60430; *TPR1*, LOC_Os03g14980; *HDA1*, LOC_Os06g38470; *LEA1*, LOC_Os06g06760; *SOS1*, LOC_Os12g44360; *SAP18*, LOC_Os02g02960; *ACTIN*, LOC_Os03g50890; *DREB2A*, LOC_Os01g07120; *LEA*, LOC_Os01g50910; *ABI5*, LOC_Os01g64000; *SNAC2*, LOC_Os01g66120; *bZIP23*, LOC_Os02g52780; *SNAC1*, LOC_Os03g60080; *LEA3*, LOC_Os05g46480; *SALT-RESPONSIVE ERF1*, LOC_Os05g34730; *ZFP182*, LOC_Os03g60560; *NAC5*, LOC_Os11g08210; *AP37*, LOC_Os01g58420; *dehydrin*, LOC_Os11g26760; *ZFP179*, LOC_ Os01g62190; *ZFP252*, LOC_Os12g39400. All the ChIP-seq data sets have been deposited in ArrayExpress under accession number E-MTAB-6504. The ChIP-seq raw data files were deposited in the SRA under accession number SRP130178.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Sequence analysis of IDS1.

Supplemental Figure S2. Determination of *IDS1* expression levels in *IDS1*-knockdown transgenic lines.

Supplemental Figure S3. Overexpression of *IDS1*.

Supplemental Figure S4. T-DNA insertion mutant of *IDS1*.

Supplemental Figure S5. Expression levels analysis of representative abiotic stress responsive genes in *ids1-1* transgenic plants by RT-qPCR.

Supplemental Figure S6. IDS1 expression responses to salt treatment in rice.

Supplemental Figure S7. Overview of ChIP assays and ChIP-seq data.

Supplemental Figure S8. Expression level analysis of IDS1-targeted genes in *ids1-1* with RT-qPCR.

Supplemental Figure S9. IDS1 does not interact with SAP18.

Supplemental Figure S10. *IDS1*, *TPR1*, and *HDA1* show similar expression patterns.

Supplemental Figure S11. EARm version of IDS1 could interact with HDA1.

Supplemental Figure S12. TPR1 could facilitate IDS1 to repress the expression of *LEA1* and *SOS1* in the *N. benthamiana* transient expression system.

Supplemental Figure S13. Determination of the transcription levels of *LEA1* and *SOS1* in NIP protoplasts with or without TSA treatment.

Supplemental Table S1. Overview of ChIP-seq results.

Supplemental Data Set 1. ChIP-seq results of NIP-IDS1 in seedlings.

Supplemental Data Set 2. Representative salt stress response genes associated with IDS1 binding sites.

Supplemental Data Set 3. Primers used in this study.

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