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The basic helix-loop-helix transcription factor gene, *OsbHLH38*, plays a key role in controlling rice salt tolerance[∞]

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ABSTRACT

The plant hormone abscisic acid (ABA) is crucial for plant seed germination and abiotic stress tolerance. However, the association between ABA sensitivity and plant abiotic stress tolerance remains largely unknown. In this study, 436 rice accessions were assessed for their sensitivity to ABA during seed germination. The considerable diversity in ABA sensitivity among rice germplasm accessions was primarily reflected by the differentiation between the *Xian* (*indica*) and *Geng* (*japonica*) subspecies and between the upland-*Geng* and lowland-*Geng* ecotypes. The upland-*Geng* accessions were most sensitive to ABA. Genome-wide association analyses identified four major quantitative trait loci containing 21 candidate genes associated with ABA sensitivity of which a basic helix-loop-helix transcription factor gene, *OsbHLH38*, was the most important for ABA sensitivity. Comprehensive functional analyses using knockout and overexpression transgenic lines revealed that *OsbHLH38* expression was responsive to multiple abiotic stresses. Overexpression of *OsbHLH38* increased seedling salt tolerance, while knockout of *OsbHLH38* increased sensitivity to salt stress. A salt-responsive transcription factor, *OsDREB2A*, interacted with OsbHLH38 and was directly regulated by OsbHLH38. Moreover, *OsbHLH38* affected rice abiotic stress tolerance by mediating the expression of a large set of transporter genes of phytohormones, transcription factor genes, and many downstream genes with diverse functions, including photosynthesis, redox homeostasis, and abiotic stress responsiveness. These results demonstrated that *OsbHLH38* is a key regulator in plant abiotic stress tolerance.

Keywords: abscisic acid, genome-wide association analysis, rice, salt tolerance, seed germination

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INTRODUCTION

Rice is a salt-sensitive crop that is most susceptible to salinity stress during the seedling and reproductive stages (Qin et al., 2020a). Previous research on rice revealed a set of genes/quantitative trait loci (QTLs) associated with salt tolerance on the basis of an integrative mapping analysis (Singh et al., 2021). In addition, functional genomics research

has characterized many genes encoding transcription factors (TFs) that positively or negatively regulate rice salt tolerance (Ganie et al., 2019). The basic helix-loop-helix (bHLH) TFs are among the largest families of TFs encoded in the rice genome (Carretero-Paulet et al., 2010). Several bHLH TFs have been confirmed to contribute to salt tolerance in rice. For example, overexpression of the wild rice gene *OrbHLH2* leads to increased salt tolerance of *Arabidopsis thaliana* in an abscisic acid (ABA)-independent manner (Zhou et al., 2009). Overexpression of *OrbHLH001* results in the up-regulated expression of *OsAKT1* (an inward-rectifying K^+ channel) and the maintenance of the ionic balance in rice plants under salt stress (Li et al., 2010; Chen et al., 2013). However, the functions and regulatory roles of bHLH TFs associated with rice salt tolerance require more thorough characterization.

ABA is a phytohormone that is crucial for plant growth and adaptation to environmental stresses (Finkelstein et al., 2002). In growing plants, the endogenous ABA content increases in response to adverse environmental conditions. More specifically, ABA plays an important role in plant responses to abiotic stresses (e.g., high salinity and water deficit) because it modulates stomatal movement to prevent water loss through transpiration (Schroeder et al., 2001). Under normal growth conditions, ABA homeostasis is maintained via the controlled biosynthesis and catabolism of the phytohormone (Yoshida et al., 2019). Exogenous application of ABA affects plant growth and abiotic stress responses. Several studies indicate that sensitivity to exogenous ABA is associated with plant tolerance to environmental stresses (Xu et al., 2002; Kurahashi et al., 2009; Lehisa and Takumi, 2012). A genome-wide association study (GWAS) of the ABA sensitivity of natural populations revealed that many genes at the loci associated with ABA sensitivity are involved in abiotic stress tolerance instead of ABA signaling pathways (Peng et al., 2021). These results imply that ABA sensitivity may be useful for identifying genetic resources pertinent to abiotic stress tolerance.

Different regional rice populations often represent locally adapted, geographically or ecologically distinct populations, which are expected to contain unique allelic variants responsible for their adaptations to specific environments. For example, upland rice accessions represent a unique rice ecotype adapted to upland aerobic conditions of south and southeast Asia where drought stress frequently occurs. Thus, upland rice accessions are expected to carry novel natural genetic variation, underlying their adaptation to the upland aerobic conditions and conferring drought tolerance, which can be readily discovered using a GWAS approach (Negrão et al., 2013; Zhang et al., 2017; Mao et al., 2019; Xu et al., 2020). However, allelic mining efforts have been hindered by the difficulty and high costs in phenotyping large numbers of accessions for abiotic stress tolerance. In the present study, we aimed to exploit the valuable natural variation in abiotic stress tolerance among upland rice accessions by assessing their ABA sensitivity during the seed germination stage using GWAS, which resulted in identification of several QTLs and candidate genes for ABA sensitivity. One gene encoding a bHLH TF (Os08g0432800; OsbHLH38) was functionally characterized and revealed to be involved in ABA-dependent salt tolerance of rice during the seedling stage.

RESULTS

Genetic structure and phenotypic variation analyses

On the basis of 3.1 million filtered single nucleotide polymorphisms (SNPs) (Figure S1A), a principal component analysis was performed to investigate the population structure. The 436 rice accessions included in this study could be divided into three major subpopulations, namely *Xian* (*indica*), *Geng* (*japonica*), and *admix* (Figure 1A). More specifically, the upland accessions were classified in the upland-*Geng* (139 accessions), upland-*Xian* (89 accessions), and upland-*admix* (22 accessions) subgroups (Figure S1D). Coleoptile emergence of 2 mm to indicate seed germination (Figure 1B) was used as an indicator for ABA sensitivity of the rice accessions. Different rice accessions varied considerably in their responses to ABA treatment at the germination stage (Figure 1C). The average seed germination percentage (SGR) at 1, 2, and 3 d under the control (CKG) were significantly higher than that under the ABA treatment (ABAG), indicating that exogenous ABA significantly inhibited seed germination. However, the sensitivity of different rice accessions to ABA varied substantially between the two rice subspecies and between the two subpopulations, lowland-*Geng*, and upland-*Geng* accessions at 1 d after ABA treatment (Figure 1D–F). The SGR was significantly higher for lowland rice than for upland rice under the CKG and ABAG conditions (Figure 1D). In contrast, no significant differences in SGR were detected between the lowland-*Xian* and upland-*Xian* subgroups under the CKG condition. However, the upland-*Xian* accessions had significantly lower SGR than the lowland-*Xian* accessions at 1 d under the ABAG condition (Figure 1E). Furthermore, the SGR of the upland-*Geng* subgroup was much lower than that of the lowland-*Geng* subgroup at 1 d under the CKG and ABAG conditions (Figure 1F). These results indicated that the SGR was significantly lower for the *Geng* subspecies than the *Xian* subspecies in response to ABA treatment. Moreover, upland rice accessions, especially the upland-*Geng* accessions, were more sensitive to ABA than lowland rice.

GWAS analysis and identification of candidate genes

Analyses of the population structure and genetic relationships on the basis of filtered SNPs revealed considerable genetic diversity in the natural population, which was used for the following association analysis (Figure S1). Because of the obvious differences in germination between the CKG and ABAG treatments at the 24-h time-point (Figure 2E–G), the SGR of the samples under the CKG and ABAG conditions as well as the relative germination percentage (REG) after 1 d of germination were used for the association mapping analysis. The GWAS was performed using the EMMAX mixed linear

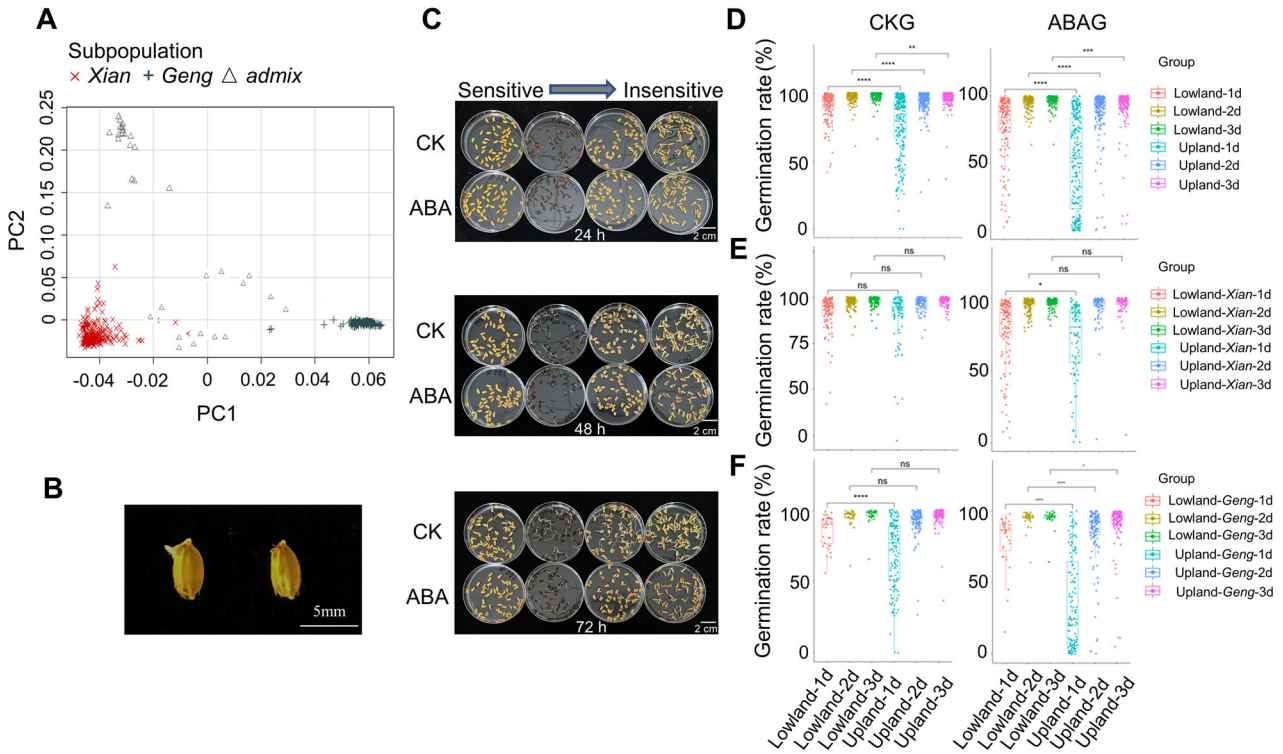


Figure 1. Population structure analysis and phenotypic variation in abscisic acid (ABA) sensitivity during seed germination in different rice populations

(A) Principal component analysis of 436 rice accessions, including *Xian*, *Geng*, and *admix* subspecies. **(B)** Seed germination (left, germinated; right, ungerminated). **(C)** Typical germination phenotypes of four accessions at 24, 48, and 72 h after ABA treatment. (**D**–**F**) Boxplots of the germination percentage of lowland and upland rice varieties **(D)**, lowland-*Xian* and upland-*Xian* rice varieties **(E)**, and lowland-*Geng* and upland-*Geng* rice varieties **(F)** under the control (left) and ABA treatment (right) conditions for 1–3 d. Significant differences, which were determined by Student's *t*-tests, are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; NS, not significant).

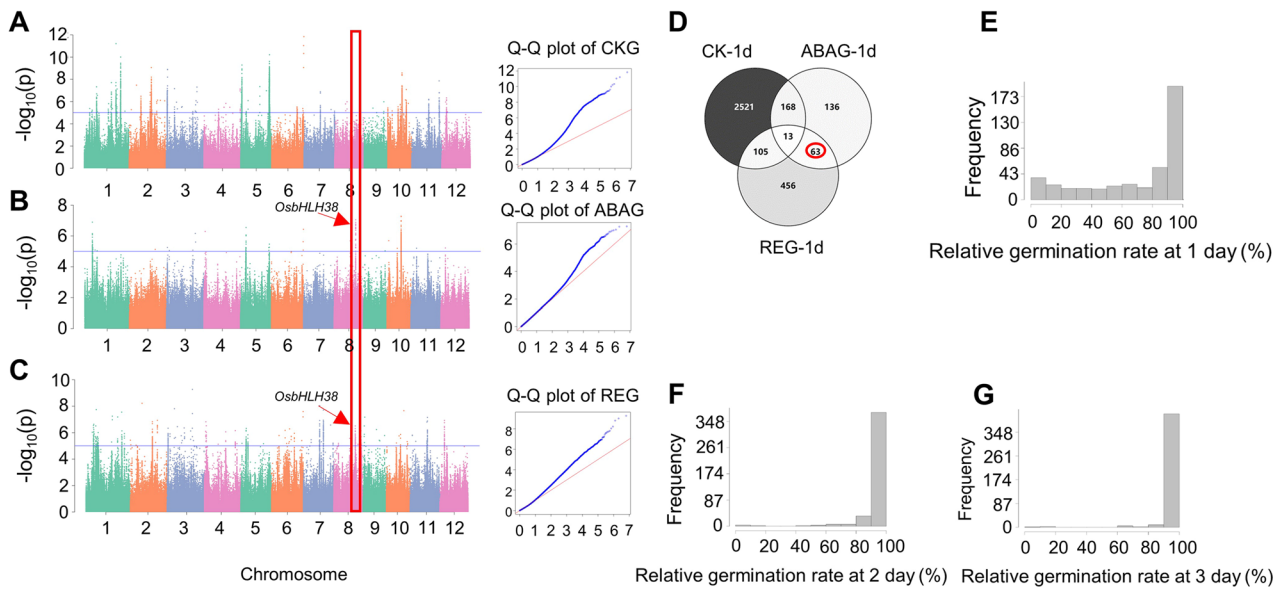


Figure 2. Genome-wide association analysis of seed germination percentage under the control (CKG) and abscisic acid treatment (ABAG) conditions as well as the relative germination percentage (REG) (ABAG/CKG)

(A) Manhattan plots and Q–Q plots for the CKG and ABAG **(B)** conditions and REG **(C)**. The horizontal line in each Manhattan plot represents the suggestive threshold ($P = 1.0 \times 10^{-5}$). **(D)** Venn diagram of the overlapping significant single nucleotide polymorphisms (SNPs) detected under CKG, ABAG, and REG at 1 d after germination. The unique overlapping significant SNPs between ABAG and REG are highlighted. **(E**–**G)** Distribution of REG after 1–3 d.

model (Zhou and Stephens, 2012) and the filtered SNPs. A total of 2,807, 380, and 637 significant SNPs for CKG, ABAG, and REG, respectively, were detected by the GWAS at a significance level of $-\log_{10}(P) > 5$ (Figure 2A–C). To minimize the number of SNPs involved in the ABA response, only the overlapping SNPs between ABAG and REG were selected. Thus, 63 SNPs within four QTL regions were analyzed further. Finally, 21 candidate genes corresponding to the SNPs were identified (Figure 2D, Table S3), but none of them were associated with ABA signaling pathways or metabolism based on the functional annotation (https://www.ricedata.cn/gene/). These candidate genes included *OsIAA3*, *OsCKI1*, *OsbHLH173*, *OsbHLH174*, and *OsSUT3*, which are reportedly involved in plant development, stress responses, or transcriptional regulation (Li et al., 2006; Nakamura et al., 2006; Dong et al., 2018). One candidate gene (Os08g0432800) encoded a bHLH TF (*OsbHLH38*) with an unknown function; its expression was highly responsive to ABA and other abiotic stresses in previous transcriptomic analyses (https://tenor.dna.affrc.go.jp/) and it showed the maximum differentiated effects on ABA sensitivity between the two subspecies and between the two rice ecotypes (see the next section).

Haplotype analysis of the candidate gene *OsbHLH38*

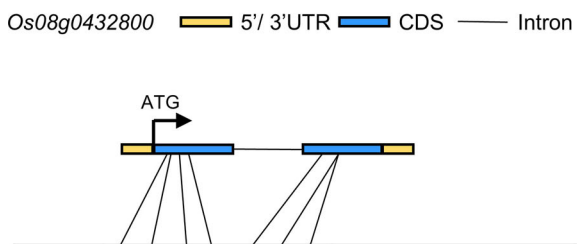
Gene expression profiles (https://tenor.dna.affrc.go.jp) and bioinformatics data (https://www.rmbreeding.cn/index.php) were combined to perform a haplotype analysis of the non-synonymous SNPs in the *OsbHLH38* coding region, which revealed four major haplotypes, of which haplotype 1 was detected only in the *Xian* subpopulation, whereas haplotypes 2, 3, and 4 were exclusive to the *Geng* subpopulation (Figure 3A). We also detected significant differences in REG among the haplotypes (Figure 3A). There was no significant difference in REG among the lowland subgroup (Figure 3A), but there were significant differences in REG between haplotypes 2 and 3 with the other two haplotypes in the upland subgroup (Figure 3A). Based on the detected SNPs within *OsbHLH38*, 40 accessions with extreme ABA-sensitive and ABA-insensitive phenotypes were selected for an additional haplotype analysis, which indicated that the distribution of SNPs within *OsbHLH38* varied among the accessions (Figure 3B). These findings suggested that changes in the *OsbHLH38* coding region might affect ABA sensitivity.

Functional annotation of *OsbHLH38*

A previous bioinformatics analysis confirmed that *OsbHLH38* encodes a bHLH TF (Li et al., 2006). The present subcellular localization analysis revealed that OsbHLH38 is a nuclear protein (Figure S2).

To investigate the effect of different abiotic stresses on *OsbHLH38* expression, a quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using total RNA extracted from the leaves and roots collected from 2-week-old rice seedlings exposed to H_2O_2, salt, ABA, jasmonic acid (JA), cold, and polyethylene glycol (PEG) stresses.

A



Hap	*Os08g0432800* CDS Physical position (bp)							Whole				Upland		Lowland	
	20974572	20974578	20974627	20974693	20976715	20976811	20976885	*GENG* Num.	REG (%)	*XIAN* Num.	REG (%)	Num.	REG (%)	Num.	REG (%)
Hap1	c	g	c	c	t	G	t	0	-	131	84.5(a)	55	78.7(a)	79	88.1(a)
Hap2	G	g	c	G	t	G	t	36	48.6(b)	0	-	32	43.0(b)	6	85.2(a)
Hap3	c	g	c	c	t	t	t	36	44.0(b)	0	-	30	35.0(b)	7	90.2(a)
Hap4	c	C	T	c	C	t	C	12	85.5(a)	0	-	9	81.5(a)	3	97.5(a)

B

Gene	SNP Position(bp)	$-\log_{10}(P)$	Allele types	ABA insensitive	Allele rates	ABA sensitive	Allele rates
Os08g0432800	20974361	6.12	T/C	C	17/20	T	16/20
	20974973	5.81	C/T	T	16/20	C	15/20
	20975478	6.14	T/G	G	18/20	T	14/20
	20975934	5.84	A/C	C	18/20	A	13/20
	20976317	6.28	G/A	A	18/20	G	14/20
	20976478	6.07	G/T	T	16/20	G	15/20
	20976811	7.04	T/G	G	16/20	T	15/20
	20977227	5.88	C/G	G	16/20	C	15/20

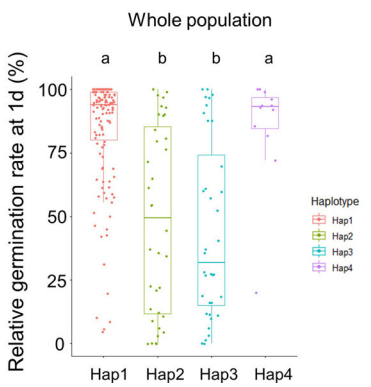


Figure 3. Haplotype analysis of the candidate gene (*Os08g0432800*) in the whole population and different subgroups

(A) Haplotype analysis of *Os08g0432800* in different subspecies and subgroups. Red numbers indicate the key positions where non-synonymous amino acid substitutions occurred among major haplotypes. Statistical analysis of relative germination percentage (REG) among the four major haplotypes at *Os08g0432800*. Statistical analysis of REG among four major haplotypes in the whole population, lowland subgroups, and upland subgroups. Significant differences, which were determined by Tukey's multiple comparison test (significance is indicated by different letters; $P < 0.05$). **(B)** Key single nucleotide polymorphism (SNPs) (identified by genome-wide association studies) in the candidate gene among 40 accessions with extreme abscisic acid responses.

The *OsbHLH38* expression level increased, especially in the leaves, in response to all treatments, although the expression patterns varied among the treatments. The *OsbHLH38* expression level peaked after 1 h of salt or PEG treatment, and after 3 h of ABA or cold treatment. In addition, the *OsbHLH38* expression level was highest in the leaves after 1 h of H_2O_2 or JA treatment, whereas it was highest in the roots after 6 and 24 h of H_2O_2 and JA treatment, respectively (Figure 4). These results led us to conclude that *OsbHLH38* expression was stimulated by multiple abiotic stresses, including salt stress.

To investigate the biological function of OsbHLH38 in response to abiotic stresses, we constructed *OsbHLH38* overexpressing (OE) and clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) knockout (KO) lines. After identifying the genotypes, two homozygous OE and KO lines were used to evaluate ABA sensitivity at the seed germination stage. No phenotypic differences were observed among the OE, KO, and wild-type (WT) plants at the seed germination stage under the CKG condition (Figure 5A). However, compared with that in the WT control, seed germination was inhibited and enhanced in the OE and KO lines, respectively, under the ABAG condition (Figure 5D). Under the saline condition, the OE lines were more tolerant of salt stress than the WT plants. The survival rates were higher for the OE seedlings than for the WT seedlings after a 10-d recovery period. In contrast, compared with the WT plants, the KO lines were more sensitive to salt stress and showed significantly lower percentage survival (Figure 5B, C). These results suggested that *OsbHLH38* was involved in the transcriptional regulation of salt tolerance-related genes in rice.

To investigate the physiological effects of OsbHLH38 in response to salt stress, we measured the sodium, potassium, proline, and malondialdehyde (MDA) contents and examined the activities of reactive oxygen species (ROS)-scavenging enzymes, including peroxidase (POD) and superoxide dismutase (SOD), in the KO and WT seedlings under control and salt-stress conditions. No major difference was detected between the KO and WT plants under the control condition, but the KO plants had an increased Na^+/K^+ ratio compared with the WT plants after exposure to salt stress (Figure 6A). The MDA and proline contents were significantly higher and lower, respectively, in the KO seedlings than in the WT seedlings under the saline condition (Figure 6B–E). The SOD and POD activities were significantly lower in the KO lines than in the WT control under the salt-stress condition (Figure 6C, D). The ABA content was significantly lower and higher in KO lines before and after salt stress, respectively (Figure 6F). These observations indicated that KO of *OsbHLH38* led to decreased salt tolerance because of the increased accumulation of harmful ions, disruption to osmotic regulation, and decreased ROS-scavenging enzyme activities in rice plants.

Identification of proteins that interact with OsbHLH38

To investigate the molecular mechanisms underlying the contribution of OsbHLH38 to salt tolerance, a yeast two-hybrid assay was conducted to identify proteins able to interact with OsbHLH38. Based on the observed α-galactosidase activity, a TF (OsDREB2A, Os01g0165000) was identified as potential interacting partners of OsbHLH38.

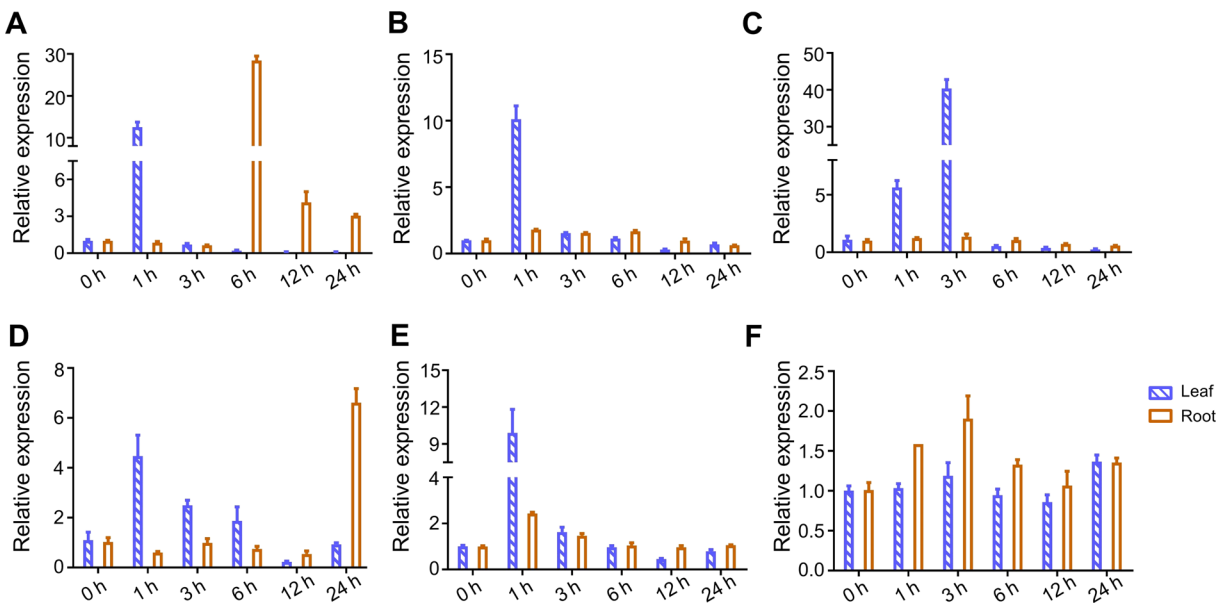


Figure 4. *OsbHLH38* expression patterns in rice seedlings exposed to different abiotic stresses
Two-week-old rice seedlings were treated with 20 mmol/L H_2O_2 **(A)**, 120 mmol/L NaCl **(B)**, 100 μmol/L abscisic acid (ABA) **(C)**, 100 μmol/L jasmonic acid (JA) **(D)**, 20% polyethylene glycol (PEG) **(E)**, or 4°C **(F)**. Total RNA was isolated at the indicated time-points after each treatment. Error bars indicate the *SD* based on three replicates. *Ubiquitin* was used as the internal reference gene for normalization.

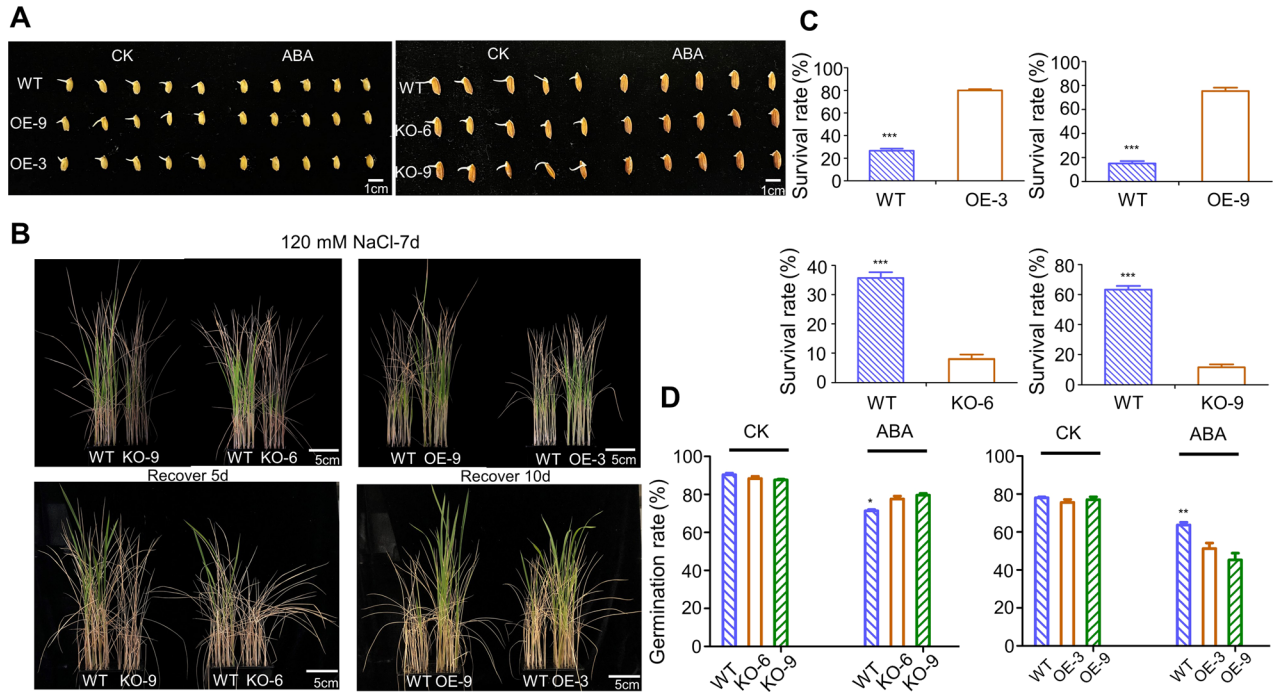


Figure 5. Phenotypes of the *OsbHLH38* transgenic lines and wild-type (WT) plants in response to abscisic acid (ABA) treatment and exposure to salt stress

(A) Seed germination after 2 d of the 5 μmol/L ABA treatment. **(B)** Seedlings after 7-d salt-stress treatment and a 5- or 10-d recovery period. **(C)** Survival percentages were calculated after a 5- or 10-d recovery period in the nutrient solution. **(D)** Germination percentage after 2 d of ABA treatment. The error bar indicates the *SD* based on three replicates. Significant differences, which were determined by Student's *t*-tests, are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

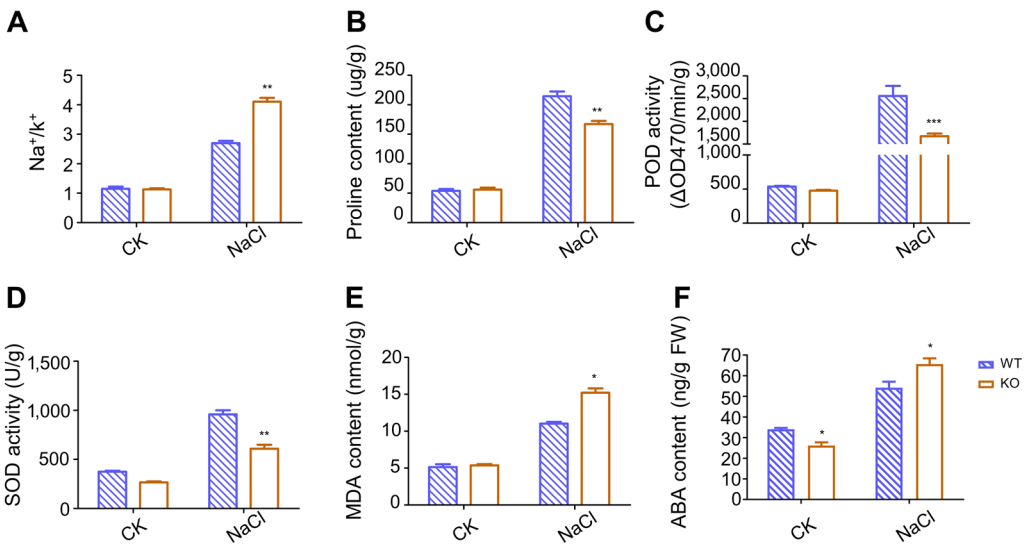


Figure 6. Physiological and abscisic acid (ABA) content analysis of the knockout (KO) and wild-type (WT) plants under the control (CK) and salt-stress conditions

(A) Na^+/K^+ ratio; **(B)** proline (Pro) content; **(C)** peroxidase (POD) reactive oxygen species (ROS)-scavenging activity; **(D)** superoxide dismutase (SOD) ROS-scavenging activity; **(E)** malondialdehyde (MDA) content; **(F)** ABA content before and after salt stress. Each column represents the mean ± *SD* (three replicates). Significant differences, which were determined by Student's *t*-tests, are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

To confirm that OsbHLH38 can interact with OsDREB2A, we constructed the BD-OsbHLH38 and AD-OsDREB2A recombinant plasmids for cotransformation of yeast cells with the BD and AD recombinant plasmid pairs. The subsequent analysis of the transformants confirmed that OsbHLH38 interacts with OsDREB2A (Figure 7A). A bimolecular fluorescence complementation (BiFC) assay performed to further verify this interaction revealed that OsbHLH38 interacts with

OsDREB2A in the nucleus (Figure 7B). The luciferase complementation imaging (LCI) and pull-down analyses provided further evidence for the interaction between OsbHLH38 and OsDREB2A (Figure 7C, D).

Several bHLH TF-binding sites in the *OsDREB2A* promoter region were detected in a bioinformatic analysis. Thus, a transient dual-luciferase assay was performed to test whether OsbHLH38 can bind to the *OsDREB2A* promoter and regulate its expression. The assay results indicated the expression of green fluorescent protein (GFP)-OsbHLH38 in tobacco protoplasts induced the expression of the reporter gene under the control of the *OsDREB2A* promoter (Figure 8A, B). A yeast one-hybrid assay indicated that OsbHLH38 binds to a site 1,000–2,000 bp upstream of *OsDREB2A* (Figure 8C). Next, the glutathione S-transferase (GST)-OsbHLH38 fusion protein was purified for an electrophoretic mobility shift assay (EMSA), which was performed using biotin-labeled probes that were synthesized from promoter segments of varied lengths. From the results, possible binding sites for OsbHLH38 were identified (Figure 8D). In addition, we designed three primer pairs (P1–P3) to perform a chromatin immunoprecipitation (ChIP)-qPCR assay and observed that OsbHLHL38 bound to the *OsDREB2A* promoters in the P1 and P2 regions (Figure 8E). Gene expression analyses revealed that the messenger RNA (mRNA) level of *OsDREB2A* was correlated with the *OsbHLH38* expression level in *OsbHLH38*-OE and *OsbHLH38*-KO lines (Figure S8). These results indicated that OsbHLH38 can bind to the *OsDREB2A* promoter and enhance its expression.

Transcriptome analysis of KO and WT plants under control and salt-stress conditions

To further clarify the molecular basis of the effect of *OsbHLH38* on rice salt tolerance, a global transcriptome sequencing analysis was performed using the KO and WT plants treated with the control and salt-stress conditions (for 24 and 48 h).

We first compared the transcriptome data between KO and WT plants under the control condition, which revealed 143 and 64 genes that were expressed at significantly higher and lower levels, respectively, in the KO plants than in the WT plants (Table S4). The down-regulated genes in the KO lines were associated with the oxidation–reduction process, response to stress, and metal ion binding, suggesting that KO of *OsbHLH38* led to inhibition of the expression of genes associated with the intrinsic tolerance to abiotic stresses.

At 24 h under the salt-stress condition, the expression levels of 59 and 189 genes were significantly higher and lower, respectively, in the KO lines than in the WT control (Table S5). The down-regulated genes were involved in photosynthesis as well as transferase and oxidoreductase activities (Figure 9A). At the 48-h time-point of the salt

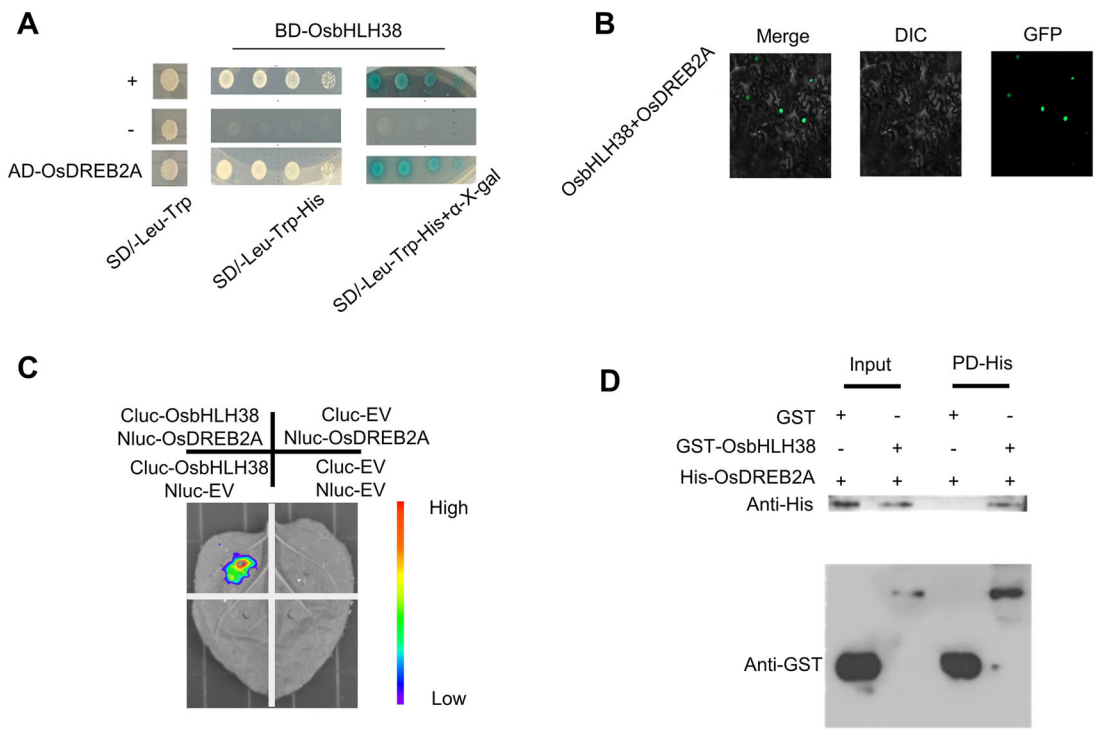


Figure 7. Identification of proteins that interact with OsbHLH38

(A) Yeast two-hybrid assay results for the *in vivo* interaction between OsbHLH38 and OsDREB2A. The pGBKT7-53/pGADT7-T and pGBKT7-Lam/pGADT7-T recombinant plasmids were used as the positive and negative controls, respectively. **(B)** Verification of the interactions between OsbHLH38 and OsDREB2A in the bimolecular fluorescence complementation (BiFC) assay. **(C)** Results of the luciferase complementation imaging (LCI) assay involving *Nicotiana benthamiana* leaves confirming that OsbHLH38 can interact with OsDREB2A. **(D)** Pull-down assay results providing additional evidence that OsDREB2A can interact with OsbHLH38.

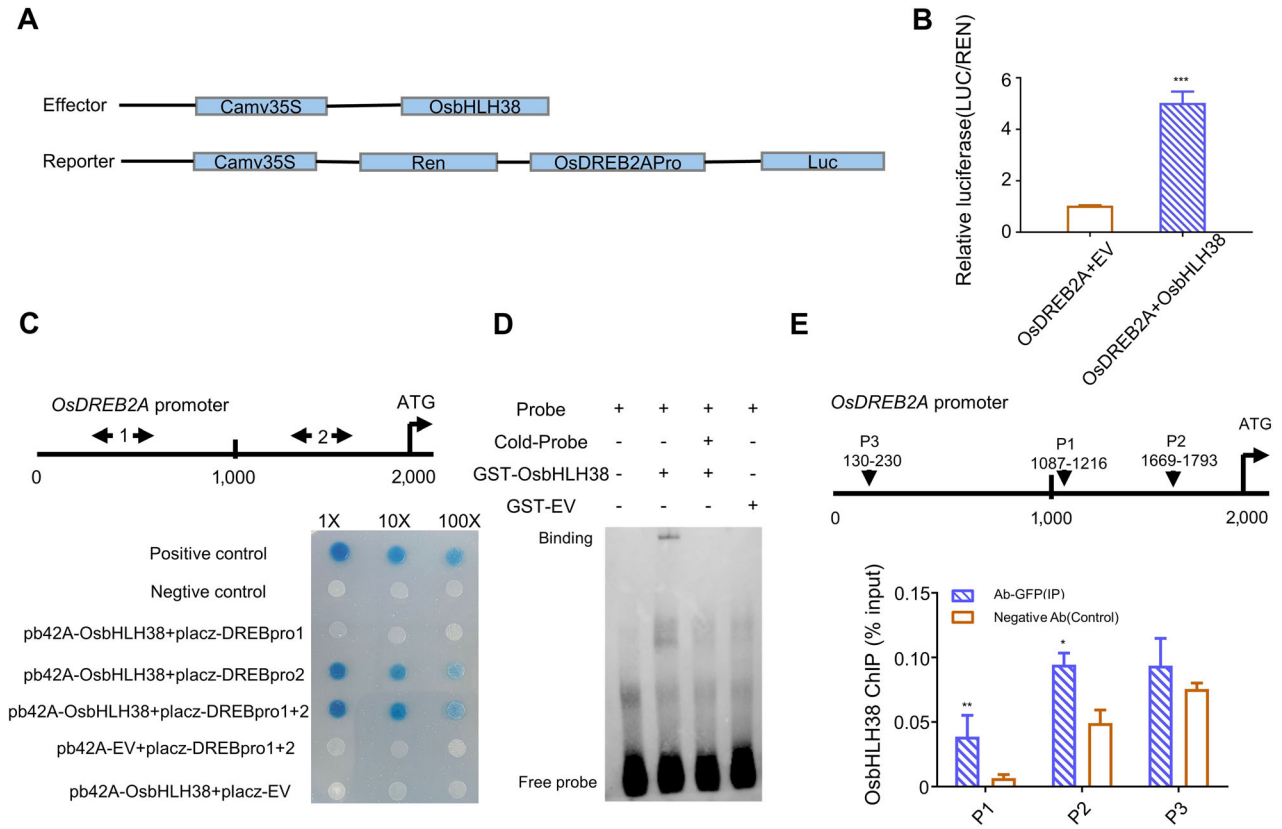


Figure 8. Binding of OsbHLH38 to the *OsDREB2A* promoter region

(A) Constructs for the dual-luciferase assay. The *OsDREB2A* promoter was inserted into the pGreenII0800-LUC vector (reporter construct), whereas the *OsbHLH38* sequence was inserted into the pAN580 vector (effector construct). **(B)** Ratios of the firefly luciferase (LUC) and *Renilla* luciferase (REN) activities in the absence or presence of OsbHLH38. **(C)** Yeast one-hybrid assay results demonstrating the interaction between OsbHLH38 and the *OsDREB2A* promoter. The pb42A-ABI19/placz-RY element was used as the positive control, whereas pb42A-EV and placz-EV served as negative controls. **(D)** Electrophoretic mobility shift assay (EMSA) results indicating that the glutathione S-transferase (GST)-OsbHLH38 fusion protein can bind directly to the *OsDREB2A* promoter. The GST-EV protein was used as the negative control. Non-biotin-labeled probes were used as the cold-probe competitor. **(E)** Chromatin immunoprecipitation – quantitative polymerase chain reaction (ChIP-qPCR) assays of OsbHLH38 binding to the promoters of *OsDREB2A* (3-week-old seedling leaves, 6 h after salt stress. P1, P2, and P3 indicate different regions). Significant differences, which were determined by Student's *t*-tests, are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

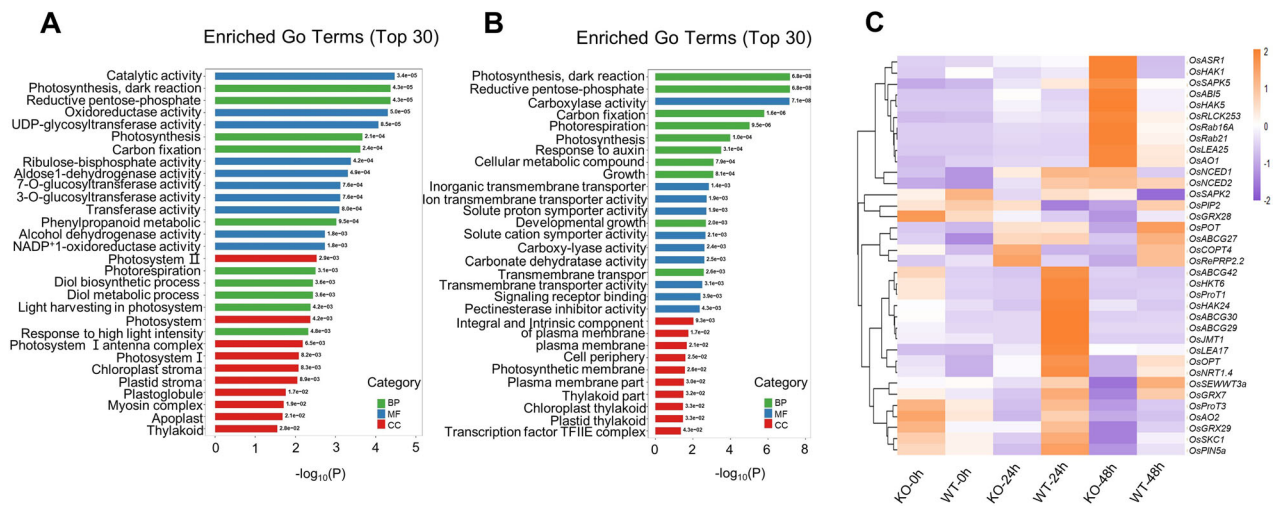


Figure 9. Results of the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the differentially expressed genes between the *OsbHLH38*-KO (knockout) and wild-type (WT) plants under the salt-stress condition

(A, B) The 30 most highly enriched GO terms among the uniquely down-regulated genes in the KO lines after 24 h **(A)** and 48 h **(B)** of salt stress. **(C)** Results of the hierarchical cluster analysis and the expression levels of OsbHLH38-dependent genes in the KO lines after 24 and 48 h of salt stress.

treatment, 1,298 and 368 genes were expressed at higher and lower levels, respectively, in the KO plants than in the WT plants (Table S6). The down-regulated genes were associated with photosynthesis, transmembrane transport, and ion transport (Figure 9B).

The further functional annotation of the differentially expressed genes in the salt-stressed KO lines detected genes involved in transporter activities and ABA signaling pathways that were differentially regulated between the KO and WT plants under salt stress (Figure 9C). Genes encoding transporters (i.e., *OsPOT*, *OsSKC1*, *OsHKT6*, *OsProT1*, *OsProT3*, *OsABCG29*, *OsABCG30*, *OsABCG42*, *OsSWEET3a*, *OsJMT1*, and *OsPIN5a*) had significantly down-regulated expression levels in the KO lines after 24 h under the salt-stress condition (Figure 9C; Table S5). In contrast, the expression levels of ABA synthesis and signaling pathway genes (e.g., *OsAO1*, *OsABI5*, *OsRab16A*, *OsASR1*, and *OsNCED3*) and the potassium ion transporter genes *OsHAK1* and *OsHAK5* were significantly up-regulated in the KO lines after 48 h under the salt-stress condition. Notably, the expression levels of a set of TF genes were clearly higher in the KO lines than in the WT control after 48 h of salt stress. These genes encoded 23 OsWRKY TFs, 12 OsNAC TFs, six bZIP TFs, and four bHLH TFs (Table S6). They may be involved in the *OsbHLH38*-mediated salt stress response. To verify the accuracy of the RNA-seq data, the expression levels of nine genes associated with ion transport and the ABA response were quantified by qRT-PCR. The qRT-PCR results were consistent with the RNA-seq data (Figure S3).

DISCUSSION

Substantial research has been conducted to identify the genes conferring abiotic stress tolerance in plants on the basis of QTL mapping and GWAS results. However, identifying the QTLs/genes associated with natural variation in abiotic stress tolerance remains challenging because of the polygenic nature of this type of trait and the difficulty in quantifying phenotypic differences of large numbers of accessions. ABA is a stress hormone closely associated with responses to abiotic stresses, including drought and salinity (Mehrotra et al., 2014; Sah et al., 2016). In the present study, we used ABA sensitivity at the germination stage as a phenotypic indicator to exploit natural variation in abiotic stress tolerance in 436 rice accessions. We showed that rice sensitivity to ABA varied considerably in the examined accessions, particularly between the two rice subspecies and between the upland and lowland ecotypes within the *Geng* subspecies. *Geng* accessions were more sensitive to ABA than those in the *Xian* and *admix* subgroups (Figure 1). Moreover, upland-*Geng* accessions were more sensitive to ABA than lowland-*Geng* accessions. With distinct geographic and ecological distributions, the rice subspecies *Xian* and *Geng* are well known for their differential adaptation to abiotic stresses (Kumar et al., 2017; Schläppi et al., 2017; Shi et al., 2017; Hoang et al., 2019). Accordingly, ABA sensitivity may be an important property associated with the adaptation of the *Geng* subspecies to its environment. This is not surprising given that previous investigations also detected the association between ABA sensitivity and drought tolerance in wheat (Kurahashi et al., 2009; Lehisa and Takumi, 2012) and clover (Xu et al., 2002). Additional experiments are needed to verify the correlation between ABA sensitivity and tolerance of other abiotic stresses in rice and other plants.

GWASs have been performed to identify the QTLs/genes affecting complex agronomic traits in crops (Liu and Yan, 2019). In the current study, our GWAS experiment detected four major QTLs influencing rice ABA sensitivity at the seed germination stage. Surprisingly, we did not locate any candidate genes involved in ABA signaling pathways or metabolism in the four QTL regions (Table S3); most of the candidate genes encode putative expressed proteins with unknown functions. Nevertheless, several candidate genes, such as *OsIAA3*, *OsCKI1*, *OsbHLH173*, and *OsbHLH174*, in the identified QTL regions are reportedly responsive to abiotic stresses (Liu et al., 2003; Li et al., 2006; Nakamura et al., 2006; Dong et al., 2018), implying they might be involved in ABA-dependent plant responses to abiotic stresses.

Several powerful approaches, including linkage mapping and reverse genetics, have been used to validate the association between genetic loci detected by GWAS and specific traits (Liu and Yan, 2019). In the present study, we identified *OsbHLH38* as a candidate gene by conducting gene KO and overexpression experiments. A haplotype analysis detected variability in seed germination among four major haplotypes following ABA treatment. In addition, REG was highest for haplotype 4 of the *Geng* varieties, especially those in the upland-*Geng* subgroup, implying that the diversity in the candidate gene sequence is strongly associated with ABA sensitivity. Moreover, KO or overexpression of *OsbHLH38* resulted in major changes to seed germination under the ABAG condition (Figure 5), reflecting the ABA-dependent regulation of *OsbHLH38* expression.

The *OsbHLH38* gene encodes a functionally uncharacterized bHLH TF (Li et al., 2006). We showed that *OsbHLH38* expression is highly responsive to abiotic stresses, especially salt stress, during the seedling stage. The increased and decreased survival percentages of the transgenic OE and KO lines, respectively, under the saline condition, were indicative of the positive effect of OsbHLH38 on the salt tolerance of rice plants. Several bHLH TF genes involved in salt tolerance have been identified, including *OsbHLH35*, which contributes to the regulation of seed germination under salt stress (Chen et al., 2018), and *OsbHLH68*, which enhances salt tolerance and flowering (Chen et al., 2017). KO of *OsbHLH38* can adversely affect osmotic regulation and ROS homeostasis, further verifying the role of OsbHLH38 in rice salt tolerance.

A yeast two-hybrid assay identified OsDREB2A as proteins that interact with OsbHLH38. These interactions were confirmed by BiFC, LCI, and pull-down assays (Figure 7).

The *OsDREB2A* gene, which encodes an AP2 TF with a dehydration-responsive element (DRE)-binding domain, was previously observed to help mediate salt and drought tolerance (Dubouzet et al., 2003; Cui et al., 2011; Zhang et al., 2013). In the current study, OsbHLH38 was revealed to bind to the *OsDREB2A* promoter and activate transcription. Moreover, the expression of *OsDREB2A* in WT and OE lines showed different patterns under salt treatment (Figure S6). Global transcriptome profiling detected a unique set of genes with decreased expression levels in the *OsbHLH38* KO plants under the control and salt-stress conditions. The functional annotation of differentially expressed genes indicated the oxidation–reduction process was enriched among the down-regulated genes in the KO lines under the control and salt-stress conditions (24-h time-point). This result is consistent with the observed decrease in ROS-scavenging enzyme activity in the KO plants, suggesting that *OsbHLH38* is important for the maintenance of redox homeostasis in rice plants under control and saline conditions. Moreover, genes with functions associated with photosynthesis and transporter activities were prevalent among the down-regulated genes in the KO lines at 24 and 48 h after initiating the salt-stress treatment, which was in accordance with the observed delayed growth and accumulation of harmful ions in KO plants under the salt-stress condition (Figure S7). Genes encoding transporters, including an iron ion transporter (*OsPOT*) (Bashir et al., 2015), sodium ion transporters (*OsSKC1* and *OsHKT6*) (Jabnoune et al., 2009; Kobayashi et al., 2017), proline transporters (*OsProT1* and *OsProT3*) (Lin et al., 2019), ABA transporters (*OsABCG29*, *OsABCG30*, and *OsABCG42*) (Kuromori et al., 2010; Borghi et al., 2015; Kang et al., 2015), a sugar and gibberellin (GA) transporter (*OsSWEET3a*) (Morii et al., 2020), and an auxin transporter (*OsPIN5a*) (Wang et al., 2009), as well as a JA carboxyl methyltransferase gene (*OsJMT1*) (Qi et al., 2016) were expressed at considerably lower levels in the KO plants than in the WT plants under salt-stress conditions. These results indicated that OsbHLH38 may mediate the translocation of ions, nutrients, proline, and phytohormones (ABA, GA, and JA) to mediate abiotic stress tolerance. Notably, the expression of a unique set of TF genes was up-regulated in the KO lines following salt treatment. Of these genes, *OsWRKY53*, *OsWRKY62*, *OsWRKY55*, *OsWRKY77*, *OsWRKY70*, *OsbZIP52*, and *OsMYB30* were previously identified as genes encoding negative regulators of abiotic stress tolerance (Xie et al., 2005; Liu et al., 2012; Hu et al., 2015; Zhang et al., 2015; Lv et al., 2017; Huang et al., 2021; Xu et al., 2022). Thus, *OsbHLH38* may modulate salt-stress tolerance by negatively regulating the expression of these TF genes.

In conclusion, a GWAS was performed to analyze the genetic loci controlling the ABA sensitivity of 436 rice accessions at the seed germination stage. A total of 21 candidate genes within four QTLs associated with ABA sensitivity were identified in the examined population. The gene encoding the OsbHLH38 TF was subsequently revealed to influence rice salt tolerance by interacting with OsDREB2A. A transcriptome analysis demonstrated that a loss-of-function mutation to *OsbHLH38* negatively affects the transcription of genes associated with abiotic stress tolerance. These results provide a basis for future comprehensive investigations of the genetic mechanism underlying ABA sensitivity associated with abiotic stress tolerance in rice.

MATERIALS AND METHODS

Rice materials and evaluation of ABA sensitivity at the seed germination stage

A panel comprising 436 rice genotypes was analyzed in this study. The genotypes, which were selected from the core collection of 3,000 rice accessions used for the 3000 Rice Genomes Project (Wang et al., 2018a), included 186 lowland and 250 upland ecotypes (Table S1) to ensure the GWAS had sufficient power to detect allelic differences between the two major ecotypes. All rice plants were grown and harvested at the field experimental station of the Institute of Crop Sciences at Sanya (China) in 2020. Seeds were incubated at 50°C in an oven for 3 d to break dormancy and then disinfected with 10% sodium hypochlorite for 30 min. For each accession, 100 surface-sterilized seeds were placed on two layers of filter paper moistened with sterile distilled water (control) or 100 mg/L ABA solution for 24 h in 12-cm-diameter Petri dishes. All control and ABA-treated seeds were incubated at 28°C in darkness. Coleoptile emergence of 2 mm was used as the indicator of seed germination as previously described (Fujino et al., 2008; Wang et al., 2018b). The seed germination percentage was calculated on d 1, 2, and 3 after the seeds were sown. The REG = (number of germinated seeds/total number of germinated seeds) × 100. The experiments were repeated three times and the average REG of each accession was used as input data in the following GWAS analysis.

Genome-wide association and haplotype analyses

Genotypic data were derived from the set of 4.8 million SNPs in the rice SNP-Seek database (http://snpseek.irri.org/). A total of 3,148,899 high-quality SNPs were obtained after filtering on the basis of the following criteria using the Plink software (http://zzz.bwh.harvard.edu/plink/data.shtml): >5% minor allele frequency and <20% missing data. A principal component analysis was performed using the Plink software and R package (Purcell et al., 2007). A population structure analysis was conducted using the ADMIXTURE 1.3 software, with *K* values of 2–10 (Alexander et al., 2009) A phylogenetic tree was constructed based on 404K Core SNP (http://snpseek.irri.org/). An association analysis was conducted using the mixed linear models of the EMMAX software to assess the effect of SNPs on phenotypes (Zhou and Stephens, 2012). Suggestive significance thresholds of association by the GEC software (Li et al., 2012) and the Bonferroni correction method were calculated for $P = 5.05E-05$. Complete DNA sequences of potential candidate genes were

downloaded from the RFGB database (https://www.rmbreeding.cn/index.php) and used for a haplotype analysis of the 436 rice genotypes. The significance of the differences in the haplotype phenotypes was assessed with one-way analysis of variance and multiple comparison tests.

Construction of candidate gene OE and KO recombinant plasmids

The full-length candidate gene (Os08g0432800; *OsbHLH38*) sequence was amplified from the genotype with the dominant haplotype 1 using specific primers containing a *Bam*HI or *Hin*dIII linker and then cloned into the pCAMBIA3301 vector. Two fragments (ccgcctccgtcgaagtagaaggg and tcatcatcagtacgggatggagg) targeting *OsbHLH38* were selected to generate mutants using the CRISPR/Cas9 system with the pYLCRISPR/Cas9Pubi-H binary vector as previously described (Ma et al., 2015). The recombinant plasmids were inserted into *Agrobacterium tumefaciens* strain EHA105 cells for subsequent transformation of rice varieties (Nipponbare for OE, IRIS_313-10863^{Hap1} for CRISPR/Cas9).

Phenotypic analysis of transgenic lines following different treatments

For the germination assay, the WT and transgenic seeds were placed in dishes containing distilled water or 5 μmol/L ABA solution and incubated for 2 d. For the salt stress assay, 2-week-old WT and transgenic seedlings were treated with 120 mmol/L NaCl for 7 d and then transferred to Yoshida nutrient solution for a recovery period of 5 or 10 d. The seedling survival percentage was calculated and statistically analyzed. To examine the effects of different abiotic stresses, 2-week-old WT seedlings were treated with 10 μmol/L GA, 100 μmol/L ABA, 100 μmol/L JA, 20 mmol/L hydrogen peroxide (H_2O_2), 20% PEG-6000, or low temperature (4°C). Shoots and roots were sampled at 0, 1, 3, 6, 12, and 24 h post-treatment and stored at −80°C until analysis.

Physiological and endogenous ABA content analysis of the transgenic lines under saline conditions

Two-week-old WT and CRISPR/Cas9 mutant seedlings were treated with 120 mmol/L NaCl for 5 d. Leaves were collected for determination of the MDA and proline contents as well as SOD and POD activities using hydroxylamine hydrochloride and a nitroblue tetrazolium kit (JianCheng, China) as previously described (Zhao et al., 2021). The sodium and potassium concentrations were determined as follows. First, the leaf dry weight was recorded after samples were dried in an oven. Next, an acetic acid solution was added and the samples were placed in a 90°C thermostatic water bath oscillator for a 2-h extraction. The Na^+ and K^+ concentrations were determined at 589 and 766.5 nm using an iCE 3300 AAS atomic absorption spectrometer (Thermo, USA). The endogenous ABA content before and after exposure to salt stress was determined with a 5,500 QTRAP(AB SCIEX) mass spectrometer using liquid chromatography–mass spectrometry as previously described (Chen et al., 2003; Seino et al., 2021).

RNA extraction and qRT-PCR

Total RNA was isolated from plants using TRIzol Reagent (Invitrogen, USA). The complementary DNAs (cDNAs) were synthesized from 2 μg RNA using the 5× All-in-One RT MasterMix system (Applied Biological Materials Inc, Canada). The cDNA solution was diluted with water (1:5, v/v) and used as the template for the qRT-PCR analysis, which was performed using the SYBR® Premix Ex Taq Kit (TaKaRa, Japan). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A rice ubiquitin-encoding gene was selected as the internal reference control for normalization of the expression data. The qRT-PCR primers are listed in Table S2. All analyses were conducted with three biological replicates.

Subcellular localization of *OsbHLH38*

To examine the subcellular localization of *OsbHLH38*, a construct encoding the GFP-*OsbHLH38* fusion protein was prepared using the pAN580 vector, which contains the CaMV *35S* promoter. The construct was used together with the construct encoding the mCherry-labeled protein for co-transformation of tobacco leaves and protoplasts. The leaves and protoplasts were then incubated at 28°C for 48 and 18 h, respectively, then examined using a LSM980 confocal laser scanning microscope (Carl Zeiss, Germany).

Yeast two-hybrid and BiFC assays

Protein–protein interactions were analyzed using the Matchmaker Two-Hybrid System 3 kit (Clontech; http://www.clontech.com/) as previously described (Huang et al., 2018). The pGBKT7-*OsbHLH38* recombinant plasmid was used for production of the bait protein for screening an AD fusion library constructed for rice leaves in our laboratory. The pGBKT7-*OsbHLH38* and AD library plasmids were used for cotransformation of yeast strain AH109 cells, which then were spread on SD/−Ade/−His/−Leu/−Trp/X-α-gal medium in plates. The plates were incubated at 30°C until colonies appeared.

A BiFC assay was conducted as previously described (Sparkes et al., 2006). The pnYFP-*OsbHLH38* and pnCFP-*OsDREB2A* recombinant plasmids were constructed. *Agrobacterium tumefaciens* strain GV3101 cells were cotransformed with a pair of recombinant plasmids and then injected into the leaves of 3-week-old tobacco (*Nicotiana benthamiana*) plants.

Firefly LCI analysis

The pCAMBIA1300-Cluc-*OsbHLH38* and pCAMBIA1300-Nluc-*OsDREB2A* recombinant plasmids were constructed. A pair of plasmids was included in the cotransformation of *A. tumefaciens* strain GV3101 cells for infiltration of the leaves of 3-week-old *N. benthamiana* plants. Fluorescence was examined at 2 d post-infiltration to detect interactions

using the NightSHADE LB 985 *In vivo* Plant Imaging System (Berthold, Germany).

Pull-down assay

The *OsbHLH38* cDNA sequence was inserted into the pGEX-4T vector for the subsequent expression of an N-terminal GST-tagged fusion protein. The *OsDREB2A* coding sequences were inserted into the pCold-TF vector for the production of C-terminal His-tagged fusion proteins. The generated recombinant plasmids were inserted into *Escherichia coli* strain BL21 cells. The recombinant proteins expressed in the *E. coli* cells were purified and used in pull-down assays, which were performed as previously described (Qin et al., 2020b).

Transient transcription dual-luciferase assay

The 2,000 bp promoter sequences of *OsDREB2A* were cloned and inserted into the pGreenII0800-LUC vector (reporter construct), whereas the *OsbHLH38* coding sequence was amplified and inserted into the pAN580 vector (effector construct). Both constructs were incorporated into tobacco protoplasts, which were then cultured in darkness for 16 h. The Luciferase Reporter kit (Promega, USA) was used to detect the luciferase activity in the protoplasts. The LUC/REN (Renilla) ratio was used to represent the relative promoter activity.

Yeast one-hybrid assay

The cDNA of *OsbHLH38* was inserted into the pB42AD vector. Fragments of the promoter sequence of different lengths (pro1: 0–1,000, pro2: 1,001–2,000, and pro1 + 2: 1–2,000) of *OsDREB2A* were inserted in the pLacZ vector, cotransformed into yeast strain EGY48 cells, and then cultured on synthetic defined (SD)/−Trp1/−Uar3/X-α-gal medium to detect expression of the reporter gene.

EMSA

An EMSA was conducted to more precisely characterize the interaction between OsbHLH38 and the *OsDREB2A* promoter, which was revealed by the yeast one-hybrid assay. Biotin-labeled probes were synthesized for the promoter region. Unlabeled DNA oligos were used as competitors. After the probe was incubated with the purified maltose-binding protein-OsbHLH38 protein, the EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo, USA).

ChIP assays

The ChIP-qPCR assays were performed based on a previous report with minor modifications (Nelson et al., 2006). Three-week-old seedlings of OsbHLH38-GFP transgenic lines were harvested after treatment with 120 mmol/L NaCl for 6 h. Approximately 2 g leaves were fixed with 1% (v/v) formaldehyde under vacuum for 10 min, then ground in liquid nitrogen for lysis of the chromatin complexes and ultrasonically disrupted into fragments of an average size of 200–700 bp. The mAb-Magnetic Beads with anti-GFP (MBL, Japan) were used to pull down the protein–DNA complex. Decrosslinking took a minimum of 6 h. Purified DNA was used for subsequent qPCR assays. Primers were designed in the *OsDREB2A* promoter regions of P1, P2, and P3. Primer pairs for qPCR are listed in Table S1. All analyses were conducted with three biological replicates.

Transcriptome sequencing (RNA-seq) and data analysis

Leaves were collected from the 3-week-old CRISPR/Cas9 mutant and WT plants treated with 120 mmol/L NaCl for 24 and 48 h (with three replicates per sample) for transcriptome analyses. The transcriptome sequencing analysis was performed by Shanghai APT Biotechnology (http://www.aptbiotech.com/). The following criteria were used to detect differentially expressed genes: $P \leq 0.05$ and expression level fold change ≥2. Using InterProScan to find homolog sequences, the Gene Ontology (GO) terms were mapped and sequences were annotated using the Blast2GO software (http://www.blast2go.com/).

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CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

F.P.D. performed the experiments; J.W., Y.B.L., X.Q.Z., and Y.Z. analyzed the data; J.L.X. and Z.K.L. assisted with manuscript revision; T.Y.Z. revised the manuscript; B.Y.F. and W.S.W. designed the experiments and drafted the manuscript. All authors read and approved this manuscript.

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SUPPORTING INFORMATION

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