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RESEARCH ARTICLE

Brassinosteroids regulated target genes and their molecular evolution and interaction in rice (*Oryza sativa* L.) response to salt stress

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Abstract

Rice growth and development are significantly affected by salt stress. Exploring salt stress-tolerant genes and their regulatory pathways is essential for sustaining productivity and ensuring food security. The brassinazole-resistant 1 (BZR1)/BRI1-EMS1 suppressor (BES1) transcription factors play pivotal roles in regulating plant development and stress responses. However, studies specifically linking BZR1 to salt tolerance in rice remain limited. In this study, 33 predicted common differentially expressed genes (cDEGs) interacting with BZR1 were identified and functionally characterized to encounter salt stress in rice. The phylogenetic relationship analysis revealed a strong evolutionary relationship between these rice genes and known Arabidopsis salt-tolerant genes. Gene ontology (GO) enrichment further confirmed that the cDEGs are significantly associated with key biological processes involved in the rice salt stress response. RNA-Seq results revealed distinct expression patterns of cDEGs between shoots and roots. In the shoots, 22 cDEGs were up-regulated, while 11 were down-regulated. In the roots, 14 cDEGs were up-regulated and 19 were down-regulated. This indicates tissue-specific regulatory responses under the experimental conditions. These findings highlight the differential regulatory roles of BZR1 across tissues under salt stress conditions. Overall, this study offers new insights into the molecular mechanisms underlying BZR1-mediated salt tolerance and identifies promising candidate genes for future experimental validation and the development of salt-tolerant rice cultivars.

Keywords: brassinosteroids; BZR1 target gene; gene expression; rice; salt stress

Introduction

Crop productivity needs to be doubled to meet the growing demand for food, as the world population is projected to reach nine billion by 2050 (1). Salinity is a major primary abiotic stress that severely impairs plant growth, development and finally yield by inhibiting key physiological processes (2, 3). Salinity primarily interferes with photosynthesis and carbon dioxide (CO_2) diffusion by reducing stomatal conductance, lowering the net photosynthetic rate and damaging photosystem II (PSII) (4). Furthermore, salinity-induced oxidative stress is exacerbated by the excessive accumulation of hydrogen peroxide (H_2O_2), which disrupts cellular homeostasis and further compromises plant health.

Rice (*Oryza sativa* L.) is highly sensitive to salinity, especially during seedling and reproductive stages. Salt stress affects rice through osmotic stress and ion toxicity. Initially, high soil salinity reduces water uptake, lowering turgor pressure and inhibiting cell expansion (5). Prolonged exposure leads to Na^+ and Cl^- accumulation, disrupting K^+ and Ca^{2+} uptake, causing ion imbalance and oxidative stress. These physiological disturbances cause membrane damage, impaired photosynthesis, stunted growth, chlorosis, poor tillering, reduced grain filling and yield loss (6).

Brassinosteroids (BRs) are naturally occurring plant hormones that play a vital role in regulating plant growth, physiological processes, development and tolerance to extreme environments throughout the life span. BRs have been widely reported to alleviate the adverse effects of salinity in various plants (7). Their effectiveness in enhancing salinity tolerance was demonstrated by the increased antioxidant enzyme activities, decreased Na^+ and Cl^- and enhanced K^+ and Ca^{2+} levels in eggplants (8). In black locust, exogenous application of BRs has been shown to reduce membrane leakage and leaf Na^+ concentration, while increasing net photosynthetic rate, stomatal conductance, transpiration rate, chlorophyll content and PSII efficiency under different levels of salinity stress (9). Former researchers reported that the BRs biosynthetic gene *BR6OX2* enhances salt stress tolerance in both apple and *Arabidopsis* (10). In *Arabidopsis*, seed germination and seedling growth of the *det2-1* and *bin2-1* mutants exhibited greater sensitivity to salt stress compared to the Columbia wild type (11). In apple, the transcription factors (TFs)

BZR1 and BZR1-2 enhance salt tolerance by modulating gibberellin biosynthesis genes *GA20ox1*, *GA20ox2* and *GA3ox1* (12).

Salinity stress is a major constraint on rice productivity. As a pioneer crop for growing in salinity affected areas, making it crucial to understand its response to salinity stress comprehensively (13, 14). BRs play a crucial role to withstand salinity stress in rice. Understanding their influence can help develop strategies for enhancing salt tolerance in rice (15). BRs regulate the expression of thousands of genes through the signalling pathway, which produces a range of biological reactions (16, 17). BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE RESISTANT 1 (BZR1) are two key TFs and the main effectors of the BRs signaling pathway. According to (18), the GSK3-like kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) phosphorylates and inactivates both TFs. Phosphorylation of BZR1 and BES1 restricts their nuclear localization and DNA-binding capacity, thereby limiting their ability to regulate BR-responsive gene expression (19, 20). BZR1 plays a pivotal role in modulating plant growth and stress responses by directly binding to the promoters of target genes involved in ion transport, reactive oxygen species (ROS) scavenging and osmotic adjustment. Recent studies have demonstrated that under salt stress, BZR1 can reprogram transcriptional responses, integrating BR signals with stress-related pathways, thereby fine-tuning gene expression for enhanced salt tolerance (21). Identifying and characterizing BZR1-regulated target genes provides valuable insights into the molecular mechanisms underlying salt stress adaptation in rice and offers potential targets for genetic improvement of stress resilience.

BZR1 is a nuclear-localized transcription factor that becomes active in response to BR signaling. When BR binds to its receptor (BRI1), a cascade is initiated that leads to the dephosphorylation and activation of BZR1, allowing it to move into the nucleus. Once activated, BZR1 binds to the promoters of BR-responsive genes, including those involved in: cell elongation and division, photosynthesis and salt stress responses (22, 23). BZR1 interacts with other hormone signaling pathways, notably abscisic acid (ABA) and ethylene, which are crucial in salt stress responses. This positions BZR1 as a central integrator of multiple stress-related signals. BZR1 activity is tightly regulated by phosphorylation (inactive state) and dephosphorylation (active state), often mediated by kinases like BIN2 and phosphatases like BSU1. Under stress, the balance of these modifications shifts, influencing stress gene expression (24). Recent studies have shown that BZR1 directly or indirectly modulates the expression of genes involved in ion homeostasis, ROS detoxification and osmotic balance, all of which are critical during salt stress (21). Overexpression of OsBZR1 (rice BZR1 homolog) has been linked to improved salt tolerance, including lower Na^+ accumulation, better K^+/Na^+ balance and improved root architecture and growth under salinity (25). Modifying BZR1 activity has been associated with maintaining biomass and grain yield under saline conditions, a crucial trait for crop resilience in salt-affected areas (21).

Researchers have been working to develop salt-tolerant plants to mitigate the adverse effects of salinity stress. However, finding new key genes conferring salt tolerance remains a challenging and time-consuming process. RNA-Seq technology has emerged as a powerful tool in this context, enabling the discovery of numerous salt-responsive genes in rice and shedding light on the complex gene networks involved in stress adaptation (26, 27). In this study, publicly available RNA-Seq data were used to compare the global transcriptional levels of two rice Jigeng-88 and introgressed lines (ILs) genotypes that differed in their salt stress tolerance. The primary goal was to find out the BZR1-regulated target genes and identify their intricate roles in rice responses to salt stress. This study provides insights into the regulatory genes and molecular pathways underlying salinity tolerance in rice.

Materials and Methods

BZR1 target genes from meta-data

According to the motif binding, we reviewed a total of 1589 genes (Supplementary Fig. 1) and selected 33 BZR1 target cDEGs (Table 1) from the RiceTFtarget (https://cbi.njau.edu.cn/RiceTFtarget/) and PlantRegMap (https://plantregmap.gao-lab.org/) databases in rice responses to salt stress. The protein-coding sequence of cDEGs was adopted from the Rice Data (https://ricedata.cn/gene/).

Basic characteristics of the target genes

To determine the basic features of the 33 target cDEGs, the chromosomal location was prepared by using the GraphPad Prism 5 (28). UniProt (https://www.uniprot.org/) and ExPasy (https://www.expasy.org/protparam/) online ProtParam tools were performed to identify the coding sequences (CDS) length (nucleotides), amino acids (aa) molecular weight, isoelectric point (pI), exon number, instability indices (ii) and gravy values (29).

Phylogenetic relationship analysis

The phylogenetic tree was prepared by the complete protein sequences of target cDEGs and Arabidopsis salt tolerant genes using MEGA-v11.0 software. The tree was reconstructed and visualized using the iTOL-v6 (https://itol.embl.de/) online tools, which were made with neighbor-joining and 1000 repeat bootstrap ways (30, 31).

Analysis of protein-protein interaction (PPI) network

The protein-protein interaction (PPI) network of cDEGs was prepared by using the STRING-v11.5 (https://string-db.org/) software with a confidence score of > 0.70 and an extreme number of interactors of 0 (32). The Cytoscape version 3.9.1 program was used to raise figuring out of the PPI networks (33). Proteins were classified as 'high' or 'low' interactive based on the number and strength of their interactions with other proteins in a PPI network. Cytoscape plugin was used in order to sort out hub genes by degree CytoHubba algorithms (34).

GO enrichment analysis

The GO analysis was prepared by using ShinyGO-v0.76 (http://bioinformatics.sdstate.edu/go76/) and visualized by the SRPLOT (https://bioinformatics.com.cn/login_en/) online tool (35). For significant data, an FDR cut-off < 0.05 was considered.

Analysis of protein co-expression network

The protein co-expression network was analyzed and built by using the whole-length protein sequences of cDEGs based on the STRING-v11.5 (https://string-db.org/) (32).

Gene expression analysis

Rice RNA-Seq data for the analysis of gene expression under salt stress were derived in roots and shoots from public database

Table 1. BRs predicted target cDEGs in rice responses to salt stress

TF	Target gene	Target gene ID	Protein type	Motif binding detail				
				Start	End	Strand	*P*-value	Motif sequence
OsBZR1	*OsAM1*	Os04g0682800	Putative, expressed	-121	-111	-	6.67e-06	CGCACGTGAGA
OsBZR1	*OsAOC*	Os03g0438100	Putative, expressed	-33	-23	-	4.34e-06	AGCACGTGGGC
OsBZR1	*OsBIP130*	Os05g0113500	Expressed	-59	-49	-	4.19e-06	CACACGTGTCC
OsBZR1	*OsBTBZ1*	Os01g0893400	Expressed	-224	-214	+	3.8e-06	CACACGTGGCG
OsBZR1	*OsbHLH148*	Os03g0741100	Expressed	-217	-207	-	5.15e-06	AGCACGTGGGA
OsBZR1	*OsbHLH38*	Os08g0432800	Putative, expressed	-1494	-1484	+	6.6e-05	GCCACGTGGCC
OsBZR1	*OsCYP2*	Os02g0121300	Putative, expressed	-72	-62	-	3.8e-06	CACACGTGGCG
OsBZR1	*OsCYP94C2b*	Os12g0150200	Putative, expressed	-189	-179	-	2.88e-06	CGCACGTGTCC
OsBZR1	*OsCBL1*	Os10g0564800	Putative, expressed	-119	-109	+	1.03e-06	CGCACGTGGGC
OsBZR1	*OsCSLD4*	Os12g0555600	Expressed	-666	-656	+	9.01e-05	CGCGCGTGGAC
OsBZR1	*OsDSG1*	Os09g0434200	Expressed	-171	-161	-	3.21e-06	CGCACGTGGCC
OsBZR1	*OsDREB1C*	Os06g0127100	Putative, expressed	-108	-98	-	2.14e-06	CGCACGTGGCG
OsBZR1	*OsGRX20*	Os08g0558200	Expressed	-40	-30	-	2.34e-06	CACACGTGGGA
OsBZR1	*OsGF14b*	Os04g0462500	Putative, expressed	-218	-208	+	3.8e-06	CACACGTGTCA
OsBZR1	*OsGPX3*	Os02g0664000	Putative, expressed	-139	-129	+	3.8e-06	CACACGTGGCG
OsBZR1	*OsGF14d*	Os11g0546900	Putative, expressed	-272	-162	-	3.8e-06	CACACGTGGCG
OsBZR1	*OsGW5L*	Os01g0190500	Expressed	-1786	-1776	+	4.25e-05	TGCACGTGCGA
OsBZR1	*OsGF14c*	Os08g0430500	Putative, expressed	-65	-55	-	3.8e-06	CACACGTGGCG
OsBZR1	*OsGF14f*	Os03g0710800	Putative, expressed	-383	-373		5.15e-06	AGCACGTGGGA
OsBZR1	*OsHAK15*	Os04g0610700	Putative, expressed	-64	-54	-	5.94e-06	AGCACGTGTGC
OsBZR1	*OsHSFC1b*	Os01g0733200	Expressed	-283	-273	+	4.56e-06	CACACGTGGCC
OsBZR1	*OsKOB1*	Os02g0817500	Putative, expressed	-53	-43	-	9.06e-06	AGCACGTGTCA
OsBZR1	*OsLEA3-2*	Os03g0322900	Putative, expressed	-81	-71	-	4.19e-06	CACACGTGTCC
OsBZR1	*OsNAC6*	Os01g0884300	Putative, expressed	-75	-65	-	3.4e-06	CACACGTGTCG
OsBZR1	*OsNHX1*	Os07g0666900	Putative, expressed	-60	-50	-	3.8e-06	CACACGTGGCG
OsBZR1	*OsNHX4*	Os06g0318500	Putative, expressed	-143	-133	-	2.14e-06	CGCACGTGGCG
OsBZR1	*OsORAP1*	Os09g0365900	Putative, expressed	-1697	-1687	+	8.08e-05	AGCTCGTGTCC
OsBZR1	*OsP5CS1*	Os05g0455500	Putative, expressed	-142	-132	-	9.25e-05	CGCGCGTGACG
OsBZR1	*OsRST1*	Os06g0685700	Putative, expressed	-457	-447	+	3.99e-06	CACACGTGGCA
OsBZR1	*OsTCP19*	Os06g0226700	Putative, expressed	-1660	-1650	+	3.79e-05	GGCACGTGTAA
OsBZR1	*OsTIP1;1*	Os03g0146100	Putative, expressed	-409	-399	-	8.67e-06	CGCACGTGTAA
OsBZR1	*OsTIFY1b*	Os03g0734900	Putative, expressed	-84	-74	+	1.79e-06	CACACGTGTGA
OsBZR1	*OsVDE*	Os04g0379700	Putative, expressed	-443	-433	-	6.3e-06	AGCACGTGGGC

Sequence Read Archive (SRA) (Accession: GSE210952; GSE167342). The expression data was analyzed and the 33 cDEGs were investigated in previous studies (36). To gain insight into the global gene expression changes related to salt stress in rice, RNA-Seq data were downloaded from the control and 60 mM NaCl for 1 week for roots and 80 mM NaCl for 14 days for shoots (26, 27). The experimental setup included root and shoot samples subjected to two treatments: control and salt stress. Each treatment was replicated as follows: control treatment with two biological replicates (n = 2) and salt treatment with three biological replicates (n = 3) for both root and shoot samples. Up - and down-regulated genes figure was drawn by using DiVenn 2.0 (https://divenn.tch.harvard.edu/) online tools (37). The heat map was prepared by using iDEP-v2.01 (http://bioinformatics.sdstate.edu/idep/) (35).

Results

Basic characteristics of the BZR1 predicted target genes

Pursuant to the genomic information of rice, chromosome localization analysis of BZR1 predicted target cDEGs (Table 1) was accomplished to clearly understand its dispersal on the chromosome (Fig. 1A-F). Besides, the length of the chromosomes was different from each other. The physical and chemical components revealed that the coding sequences (CDS) length (nucleotides) varied from 519 to 3648 bp [mean ± standard deviation (SD)=1272.91 ± 765.17], the number of amino acids (aa) of the 33 cDEGs varied from 172 to 1215 (mean ± SD = 423.30 ± 255.06), the relative molecular weight was from 1.84 to 13.22 kDa (mean ± SD = 4.61 ± 2.77), the isoelectric point (pI) covered from 4.45 to 11.11 (mean ± SD = 7.03 ± 1.88) and the instability indices (ii) were 15.28 to 71.31 (mean ± SD = 45.50 ± 12.10), respectively. Here, the gravy values of the 33 cDEGs ranged from -1.009 to 0.823 (mean ± SD = -0.23 ± 0.41) (Supplementary Table 1).

Phylogenetic relationship analysis between rice and *Arabidopsis* genes

The evolutionary tree (or phylogenetic tree) represents the evolutionary relationships among different species or other taxonomic units. Beyond simply showing lineage splits, it serves functional roles across multiple disciplines. The phylogenetic relationship between rice (*Oryza sativa*) and *Arabidopsis* (*Arabidopsis thaliana*) salt-tolerant genes sheds light on their evolutionary history and functional conservation. We analyzed the evolutionary relationships among the BZR1 predicted target cDEGs with *Arabidopsis* salt-tolerant genes and divided them into six groups (Fig. 2). The largest group was in subfamily Clade IV with 23 members and subfamily Clade VI, Clade V, have 18 and 15 members. Clade I and Clade II, have 10 and 4 members and Clade III was the smallest group containing only two genes.

Protein-protein interaction (PPI) network

The protein-protein interaction (PPI) network is crucial in the field of bioinformatics. It is well known that direct and indirect connections between proteins/genes are linked by the PPI network. In this investigation, target 33 cDEGs were selected in rice response to salt stress. The highly interacted proteins are OsCBL1, OsGF14c, OsNAC6, OsGF14b, OsGF14d, OsGF14f, OsTIP1;1, OsNHX1, OsHAK15 and OsBTBZ1 (Fig. 3). The less interacted proteins are OsORAP1, OsGW5L, OsDREB1C and OsbHLH148. The red colour and highly interacted proteins are the key genes among the selected 33 cDEGs.

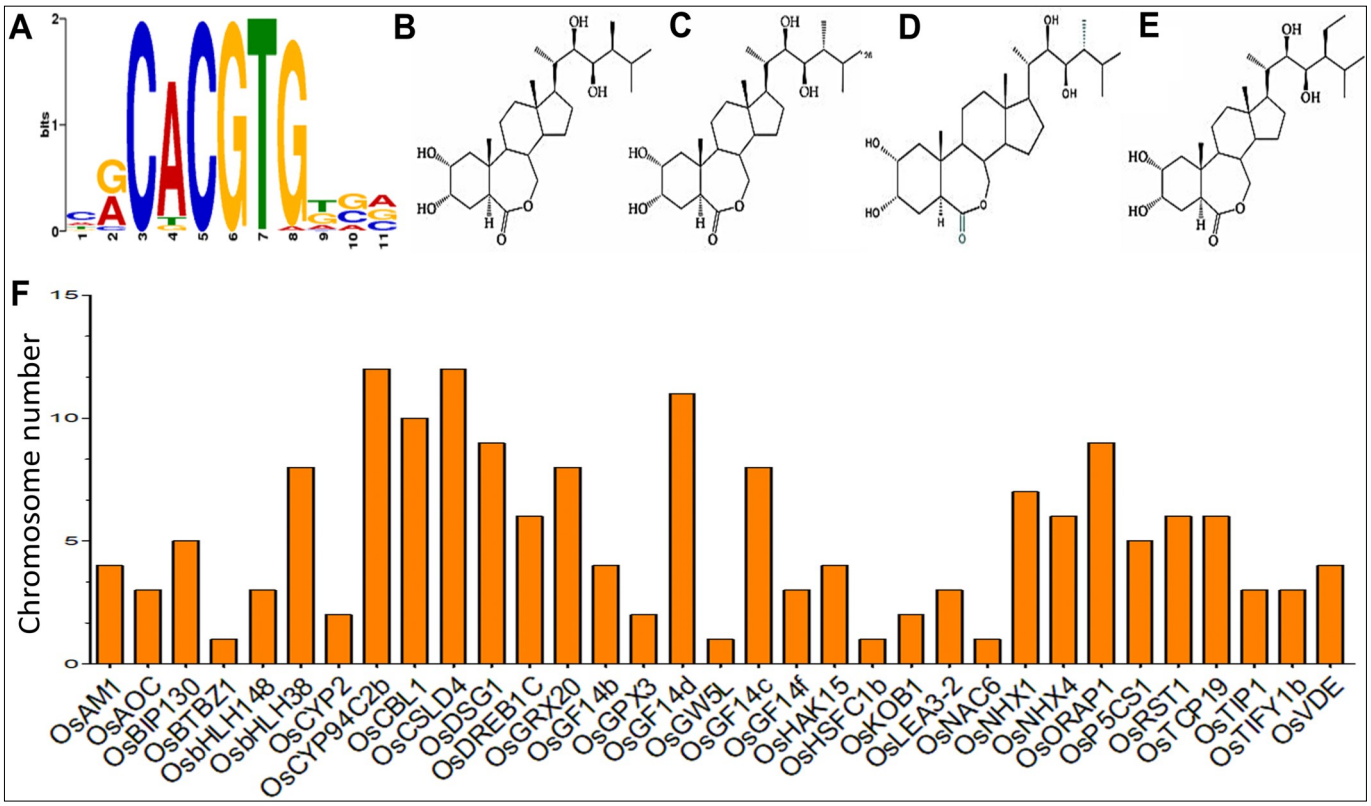

Fig. 1. BZR1 G-box, BRs structures and chromosomal locations of the BZR1 predicted target cDEGs. A. Represents the BZR1 G-box. B. Denotes the brassinolide (BL) structure. C. Indicates the 2-epibrassinolide (EBL) structure. D. Specifies the 24-epibrassinolide (EBL) structure. E. Shows the 28-homobrassinolide (HBL) structure. F. Chromosomal localizations of the 33 BZR1 predicted target cDEGs in rice responses to salt stress.

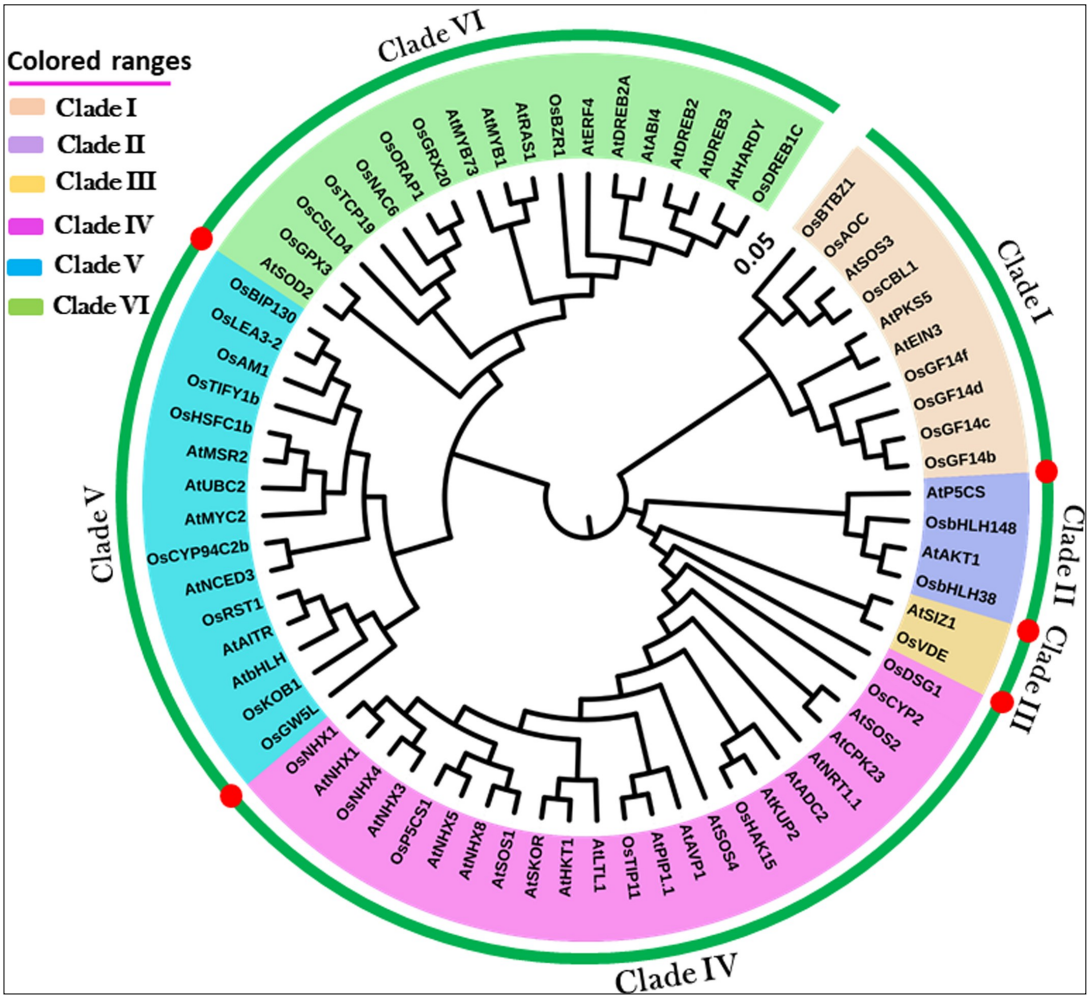

Fig. 2. Phylogenetic relationship analysis between rice cDEGs and Arabidopsis salt-tolerant genes. The neighbour-joining (NJ) tree is unrooted with 1000 repeat boot-strap methods and the sub-families of the target genes are highlighted with different coloured backgrounds. Tree description: scale length: 0.05, mode: circular, tick interval: 0, total amino acid sequences: 72 and rotation: 330 degrees.

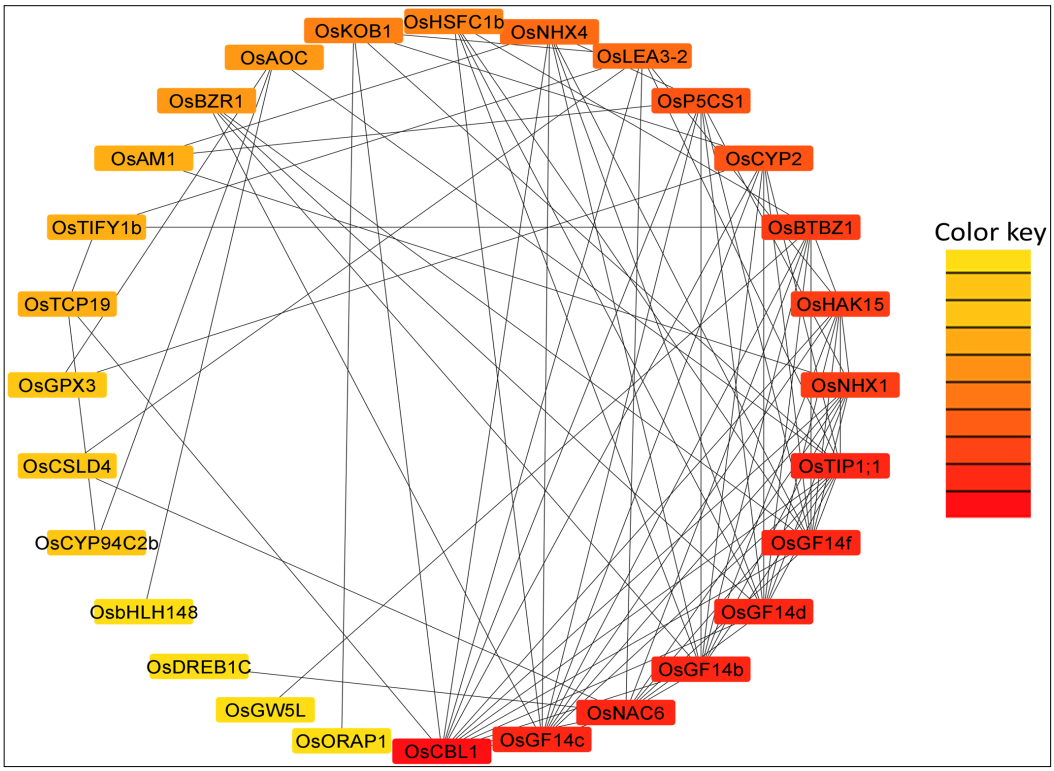

Fig. 3. PPI network for BZR1 target cDEGs in rice responses salt stress (confidence score of > 0.70 and an extreme number of interactors of 0). The Cytoscape version 3.9.1 program was used to find out the key genes in the network and the CytoHubba program was used to tier the nodes of the PPI network. The red colour indicated genes were highly interactive and the yellow colour indicated genes had low interaction with each other.

Gene ontology (GO) term enrichment analysis

According to biological process (BP) GO enrichment analysis, BZR1 predicted target OsKOB1, OsHAK15, OsAM1, OsNHX4 and OsNHX1 cDEGs are involved in potassium ion (K^+) transport (GO: 0006813) and K+ transmembrane transport (GO: 0071805). OsNAC6, OsLEA3-2, OsNHX4, NHX1 and OsCSLD4 cDEGs are response to salt stress (GO: 0009651). OsNHX4 and NHX1 cDEGs are involved in sodium ion (Na^+) transport (GO: 0006814), K^+ homeostasis (GO: 0055075), sodium (Na^+): proton (H^+) antiporter activity (GO: 0015385) and Na^+ transmembrane transport (GO: 0035725) (Table 2, Fig. 4). According to cellular component (CC) GO enrichment analysis, BZR1 predicted target OsCYP2, OsVDE1, OsAM1, OsNHX4, OsNHX1, OsGRX20 cDEGs are chloroplast (GO: 0009507) localized and OsTIP1;1, OsHAK15, OsCBL1 cDEGs are vacuole (GO: 0005773) localized (Table 2, Fig. 4). According to molecular function (MF) GO enrichment analysis, BZR1 predicted target OsGF14f, OsVDE1, OsGF14b, OsGF14c and OsGF14d cDEGs are involved in protein domain-specific binding (GO: 0019904). OsAM1, OsNHX4 and OsNHX1 genes have potassium (K^+): proton (H^+) antiporter activity (GO: 0015386) and K^+ antiporter activity (GO: 0022821). OsTIFY1b, OsDREB1C and OsTCP19 have transcription factors (GO: 0003700) that bind DNA (Table 2, Fig. 4). The detailed information about the GO BP, CC and MF are listed in Supplementary Table 2.

Protein co-expression network analysis

To interpret the co-expression network results considering OsBZR1 regulation, it's important to understand how OsBZR1 acts as a transcriptional regulator and integrates BR signalling into broader stress and developmental pathways. The interaction among OsBZR1 and OsGF14b, OsGF14c, OsGF14d and OsGF14f was experimentally determined and only OsGF14c was predicted by text mining. Proteins OsAM1, OsBTBZ1, OsCYP2, OsCYP94C2b, OsCBL1, OsCSLD4, OsGF14b, OsGPX3, OsGF14d, OsGW5L, OsGF14c, OsGF14f, OsHAK15, OsHSFC1b, OsKOB1, OsLEA3-2, OsNAC6, OsNHX1, OsNHX4, OsORAP1, OsP5CS1, OsTCP19, OsTIP1;1 and OsTIFY1b were associated with each other that was experimentally determined (Fig. 5). Proteins OsAM1 were interacted with OsP5CS1, OsNHX1 and OsNHX4 by gene neighbourhood. Also, the association of the proteins OsNAC6, OsDREB1C, OsCSLD4, OsLEA3-2, OsTIP1;1, OsCBL1, OsHAK15, OsNHX1, OsNHX4, OsGF14b, OsGF14c, OsGF14d and OsGF14f was predicted by co-expression analysis. These findings underscore the multifunctional role of OsBZR1 and highlight its central importance in coordinating BR signalling with environmental adaptation mechanisms.

Gene expression analysis

The gene expression analysis revealed a total of 33 BZR1-regulated salt-tolerant cDEGs were identified under salt stress (fold change ≥ 2, p-value ≤ 0.05). In the ILs rice variety (shoot), 22 cDEGs were up-regulated and 11 were down-regulated compared to the control, as revealed by unsupervised hierarchical clustering (Fig. 6A). In contrast, the Jigeng-88 variety (root) exhibited 14 up-regulated and 19 down-regulated cDEGs under salt stress relative to the non-saline condition (Fig. 6B). The FC values and p-values for shoot and root are presented in Supplementary Table 3. R ranges from -1 to 1. Pearson correlation analysis indicated high transcriptional consistency between control and salt stress conditions, with R^2 values of 0.9521 for ILs shoot (Fig. 6C) and 0.8817 for Jigeng-88 root (Fig. 6D). Under salt stress, ILs shoots showed strong induction of signaling and stress-related cDEGs such as OsNHX1, OsLEA3-2, OsGF14d, OsGF14b, OsGF14c, OsGF14f, OsDREB1C and OsNAC6 (Fig. 7A). In contrast, Jigeng-88 roots predominantly expressed ion transporters and stress-protective genes including OsNHX4, OsHAK15, OsTIP1;1, OsBIP130 and OsNAC6 (Fig. 7B).

Table 2. Top enriched GO terms by the proposed BZR1 predicted target cDEGs

GO Items	GO term ID	Enriched BZR1 predicted cDEGs
Biological process (BP)		
Potassium ion (K^+) transport	0006813	OsKOB1, OsHAK15, OsAM1, OsNHX4, OsNHX1
Salt stress response	0009651	OsNAC6, OsLEA3-2, OsNHX4, NHX1, OsCSLD4
K^+ transmembrane transport	0071805	OsHAK15, OsAM1, OsNHX4, NHX1
Osmotic stress response	0006970	OsNAC6, OsLEA3-2, OsNHX4, NHX1, OsCSLD4
Tolerance to stress	0006950	OsNAC6, OsGPX3, OsLEA3-2, OsGF14f, OsbHLH148, OsVDE, OsNHX4, OsNHX1, OsCSLD4
Sodium ion (Na^+) transmembrane transport	0035725	OsNHX4, OsNHX1
Na^+ import across plasma membrane	0098719	OsNHX4, OsNHX1
K^+ homeostasis	0055075	OsNHX4, OsNHX1
Na^+ transport	0006814	OsNHX4, OsNHX1
Positive regulation of salt stress response	1901002	OsNAC6, OsLEA3-2
Cellular component (CC)	GO term ID	Enriched BZR1 predicted cDEGs
Vacuole	0005773	OsTIP1;1, OsHAK15, OsNHX4, OsNHX1, OsCBL1
Vacuolar membrane	0005774	OsTIP1;1, OsHAK15, OsNHX4, OsNHX1
Bounding membrane of organelle	0098588	OsTIP1;1, OsHAK15, OsNHX4, OsNHX1, OsCBL1, OsCSLD4
Chloroplast	0009507	OsCYP2, OsVDE1, OsAM1, OsNHX4, OsNHX1, OsGRX20
Plastid	0009536	OsCYP2, OsVDE1, OsAM1, OsNHX4, OsNHX1, OsGRX20
Chloroplast envelope	0009941	OsAM1, OsNHX4, OsNHX1
Organelle membrane	0031090	OsTIP1;1, OsHAK15, OsNHX4, OsNHX1, OsCBL1, OsCSLD4
Plant-type vacuole	0000325	OsHAK15
Cytoplasmic vesicle	0031410	OsCSLD4
Intracellular vesicle	0097708	OsCSLD4
Molecular function (MF)	GO term ID	Enriched BZR1 predicted cDEGs
Protein domain-specific binding	0019904	OsGF14f, OsVDE1, OsGF14b, OsGF14c, OsGF14d
Potassium (K^+): proton (H^+) antiporter activity	0015386	OsAM1, OsNHX4, OsNHX1
K^+ antiporter activity	0022821	OsAM1, OsNHX4, OsNHX1
DNA-binding transcription factor activity	0003700	OsTIFY1b, OsDREB1C, OsTCP19
K^+ transmembrane transporter activity	0015079	OsHAK15, OsAM1, OsNHX4, OsNHX1
Transcription regulator activity	0140110	OsTIFY1b, OsDREB1C, OsTCP19
Transmembrane transporter activity	0022857	OsKOB1, OsTIP1;1, OsHAK15, OsAM1, OsNHX4, OsNHX1
Transporter activity	0005215	OsKOB1, OsTIP1;1, OsHAK15, OsAM1, OsNHX4, OsNHX1
Sodium (Na^+): proton (H^+) antiporter activity	0015385	OsNHX4, OsNHX1
Na^+ transmembrane transporter activity	0015081	OsNHX4, OsNHX1

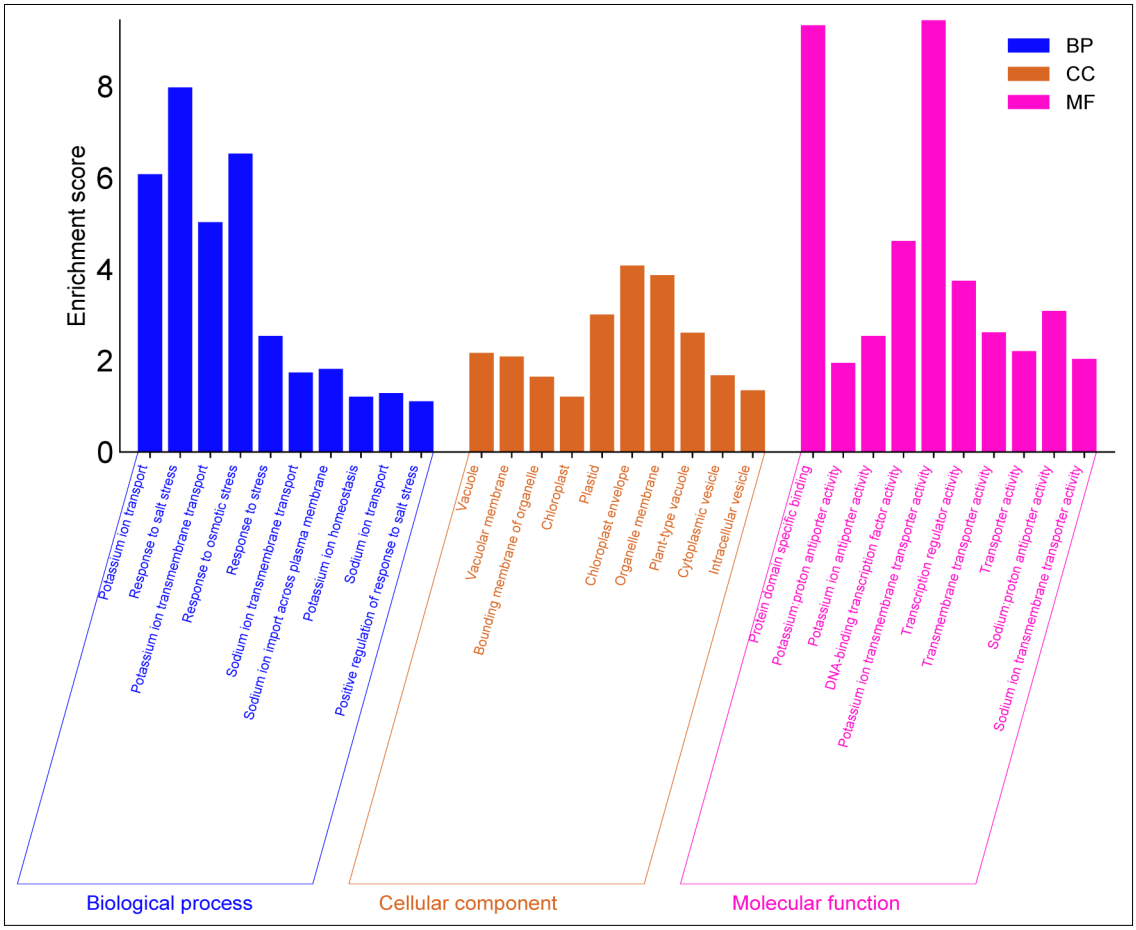

Fig. 4. Gene ontology enrichment pathway of the BZR1 predicted target cDEGs (FDR cut-off < 0.05). Predicted gene ontology, where BP represented biological process, CC represented cellular component and MF represented molecular function and the ordinate represents the number of genes enriched in GO. The GO item was represented by the abscissa.

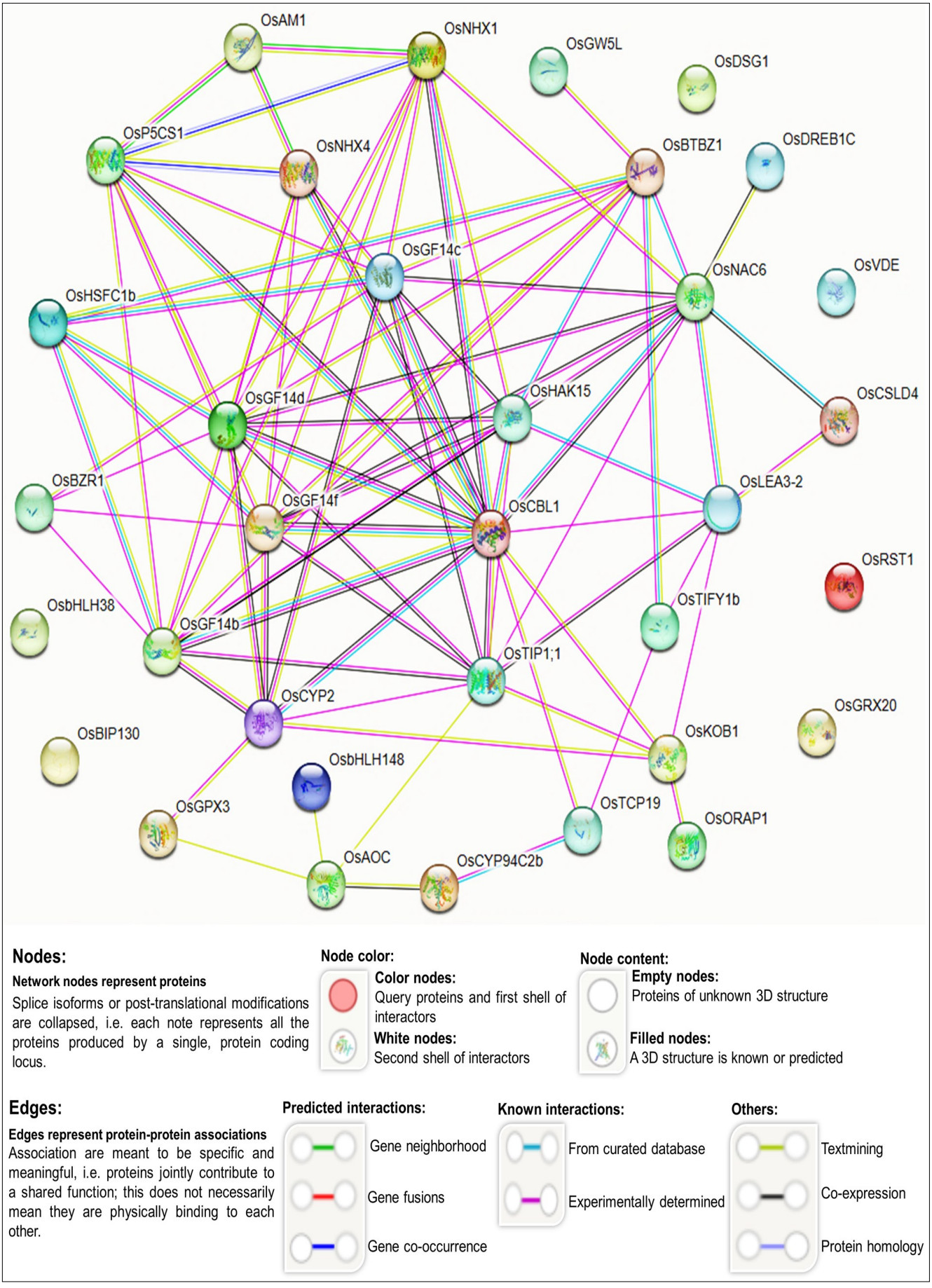

Fig. 5. Protein co-expression analysis of 33 BZR1 predicted desired cDEGs. Nodes: 34; edges: 87; mean node degree: 5.12; mean local clustering coefficient: 0.363; predicted number of edges: 62; enrichment p-value: 0.00153. The various coloured connective lines represent the various approaches used to predict interactions.

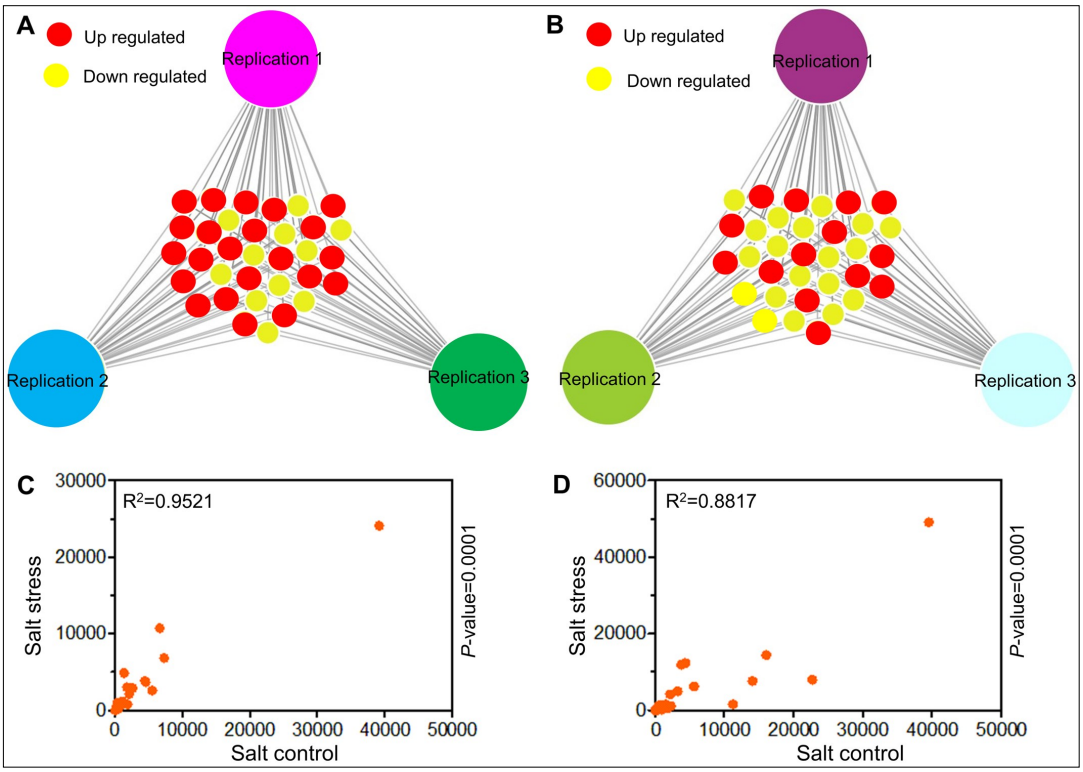

Fig. 6. Up and down-regulated cDEGs. A. Up-regulated cDEGs to compare between the control and salt stress conditions in the ILs rice variety for shoot. B. Down-regulated cDEGs to compare between the control and salt stress in the Jigeng-88 rice variety for root. Red color represented the up-regulated cDEGs and yellow color represented the down-regulated cDEGs. C. Pearson correlation analysis of cDEGs for shoots. D. Pearson correlation analysis of cDEGs for roots.

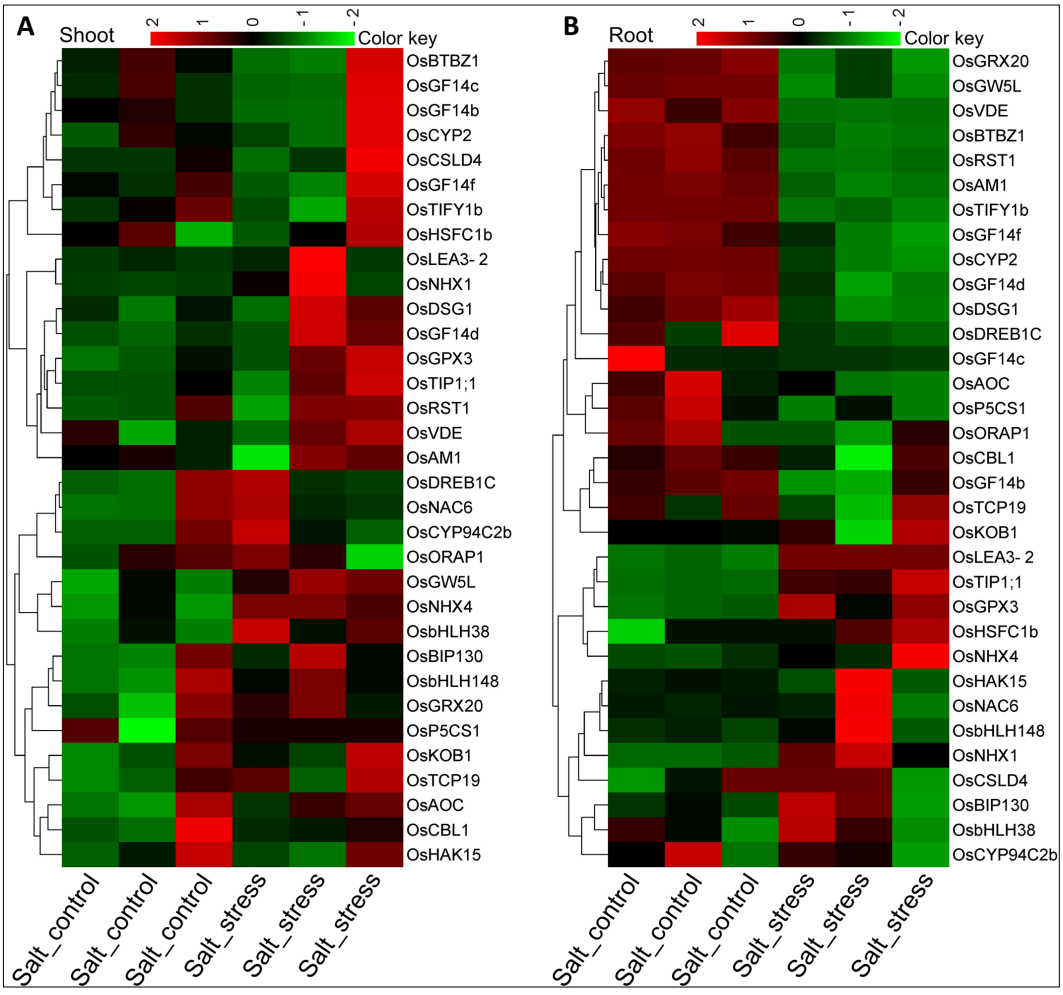

Fig. 7. Heatmap for the control and salt stress conditions between two rice varieties. A. Heat map for the comparison to the control and salt stress in ILs and Jigeng-88 rice variety for shoots. B. Heat map for the comparison between the salt stress and the control in ILs and Jigeng-88 rice variety for roots. Rice RNA-seq data for the expression analysis were derived from NCBI gene expression SRA data (Accession: GSE210952; GSE167342). The average linkage clustering algorithm and the Pearson correlation distance metric were applied.

Discussion

BRs play crucial role in regulating gene expression associated with salt stress tolerance in rice. Their molecular evolution and interaction with other signalling pathways offer promising avenues for developing salt-tolerant rice varieties. The current study comprehensively analyzed the characteristics, phylogenetic relationships, protein-protein interactions and functional annotations of BZR1-predicted target cDEGs in rice under salt stress conditions, providing valuable insights into the genetic and molecular basis of salinity tolerance.

The BZR1 target cDEGs were distributed across multiple rice chromosomes, with variations in their sequence length, molecular weight, isoelectric points and instability indices. This variability underscores the functional diversity of these genes in rice response to salt stress. For instance, the CDS ranged from 519 to 3648 bp and the number of amino acids varied from 172 to 1215, reflecting their roles in diverse cellular processes (Fig. 1F). For chromosomal distributions, diverse existing studies are in line with our findings which were reported in sunflower (38), rice (39), radish (40) and peanut (41) plants.

Phylogenetic relationship analysis between BZR1 cDEGs in rice and Arabidopsis salt-tolerant genes highlighted the evolutionary conservation and divergence of salt-tolerant genes between rice and Arabidopsis which is crucial for discovering common genetic mechanisms, enabling gene transfer, improving crop resilience and deepening our understanding of plant adaptation to environmental stress (42-44). Clustering the cDEGs into six distinct clades suggests functional redundancy and diversification in their roles under salt stress. The largest clade (Clade IV) with 23 members indicated a potentially central role in salt tolerance, whereas smaller clades may represent specialized or niche functions. It suggested that predicted cDEGs had been highly conserved in plant evolution. Same-group proteins have performed similar structures and functions (45). In group I, there are OsGF14b, OsGF14c, OsGF14d and OsGF14f cDEGs and these cDEGs have different consensus sequences (Fig. S2). Under salt stress, OsGF14b (46), OsGF14c (47), OsGF14d (47) and OsGF14f (47) cDEGs are highly expressed. The phylogenetic analysis was conducted using salt tolerant genes and classified using a similar methodology, 15 groups in danshen (48), 15 groups in tomato (49), 12 groups in maize (50) and 3 groups in rice (51).

The PPI network analysis identified highly interactive hub proteins, such as OsCBL1, OsGF14c, OsNAC6, OsGF14b, OsGF14d, OsGF14f, OsTIP1;1, OsNHX1, OsHAK15 and OsBTBZ1, which are critical in salt stress response (Fig. 3). The high interaction scores of these proteins suggest their central role in mediating salt stress adaptation pathways. Conversely, proteins with fewer interactions (e.g., OsORAP1, OsGW5L, OsDREB1C and OsbHLH148) might represent specialized functions or mechanisms that are less studied. The PPI network illustrated the connections between related genes and proteins (34). Previous studies demonstrated that PPI refer to the connections between two or more proteins, with biochemical, hydrophobic and electrostatic factors playing a role in these interactions (52) . In rice, salt tolerance is a complex trait involving various molecular mechanisms and cellular processes such as ion transport, oxidative stress response and cell membrane stability. These findings suggest that key hub proteins such as OsCBL1, OsGF14 family members, OsNAC6 and OsNHX1 may play central regulatory roles in the rice response to salt stress, highlighting their potential as critical targets for genetic improvement and stress-resilient crop development.

GO enrichment analysis revealed complex traits like salt tolerance in plants such as rice and provided insights into rice adaptation to saline environments (53). GO analysis revealed that BZR1 target genes were involved in essential biological processes, i.e. ion transport, osmotic stress response and salt stress tolerance. Key genes like *OsKOB1*, *OsHAK15*, *OsAM1*, *OsNHX4* and *OsNHX1* play pivotal roles in sodium and potassium ion transport, maintaining ionic homeostasis and mitigating salt-induced damage. Localization of certain genes (e.g., *OsCYP2*, *OsVDE1*) in chloroplasts and others (e.g., *OsTIP1;1*, *OsHAK15*) in vacuoles indicates compartmentalized stress responses. Molecular functions like potassium-proton antiporter activity and transcription factor binding further demonstrate the multifunctionality of these cDEGs in salt tolerance. According to BP GO enrichment analysis, BZR1 predicted target *OsKOB1* (54, 55), *OsHAK15* (56), *OsAM1* (57), *OsNHX4* (58-60) and *OsNHX1* (58, 61) cDEGs are involved in potassium ion (K^+) potassium ion (K^+) transport and K^+ transmembrane transport. *OsNAC6* (62, 63), *OsLEA3-2* (64), *OsNHX4*, *NHX1* and OsCSLD4 (65, 66) cDEGs are response to salt stress. *OsNHX4* and *NHX1* cDEGs are involved in sodium ion (Na^+) transport, K^+ homeostasis, sodium (Na^+): proton (H^+) antiporter activity and Na^+ transmembrane transport (Table 2, Fig. 4). These processes coordinate the uptake and compartmentalization of sodium ions, manage oxidative stress through reactive oxygen species (ROS) detoxification and regulate stress-responsive gene expression.

GO-CC terms enriched under salt stress reflect the spatial organization of stress-responsive activities within the rice cell. Key components include the plasma membrane, vacuole, mitochondrion and chloroplast (67). Localization of certain cDEGs (e.g., OsCYP2, OsVDE1) in chloroplasts and others (e.g., OsTIP1;1, OsHAK15) in vacuoles indicates compartmentalized stress responses. According to cellular component (CC) GO enrichment analysis, BZR1 predicted target *OsCYP2* (68), *OsVDE1* (69), *OsAM1*, *OsNHX4*, *OsNHX1*, *OsGRX20* (70) cDEGs are chloroplast localized and *OsTIP1;1* (71), *OsHAK15, OsCBL1* (72) cDEGs are vacuole localized (Table 2, Fig. 4). Vacuoles serve as storage compartments to sequester excess Na^+, thereby protecting the cytosol. Mitochondria and chloroplasts, as energy and ROS-generating organelles, are vital for stress signalling and adaptation and their associated proteins are often differentially expressed during salt stress to optimize energy usage and minimize oxidative damage (73). GO-MF terms like potassium-proton antiporter activity and transcription factor binding further demonstrate the multifunctionality of these cDEGs in salt tolerance. At the molecular level, GO-MF annotations highlight functions such as ion channel activity, transporter activity, transcription factor binding and oxidoreductase activity. These molecular functions are directly involved in executing stress responses-facilitating ion fluxes, activating stress-responsive genes and modulating redox status (74). According to GO-MF enrichment analysis, BZR1 predicted target *OsGF14f*, *OsVDE1*, *OsGF14b*, *OsGF14c* and *OsGF14d* cDEGs are involved in protein domain-specific binding. *OsAM1*, *OsNHX4* and *OsNHX1* cDEGs have potassium (K^+): proton (H^+) antiporter activity and K^+ antiporter activity. O*sTIFY1b* (75), *OsDREB1C* (76, 77) and *OsTCP19* (78) cDEGs have TFs that bind DNA (Table 2, Fig. 4). By using the GO enrichment pathway, the similar type conclusions were consistent with existing studies by previous researchers (79-87).

Gene co-expression network analysis allows for the identification of hub genes, which may serve as targets for improving salt resistance in rice through genetic manipulation or breeding (42). In the present study, the co-expression network revealed robust interactions among genes such as *OsBZR1* and *OsGF14b*, *OsGF14c*, *OsGF14d*, *OsGF14f* and *OsGF14c*. The co-expression network significantly contributed to rice salt tolerance. Additionally, a yeast two-hybrid screen revealed 14-3-3 (*OsGF14b*, *OsGF14c*, *OsGF14d* and *OsGF14f*) proteins as OsBZR1-interacting proteins (88). Experimentally validated associations provide strong evidence of their collaborative roles in salt tolerance pathways. Predicted interactions through text mining and neighborhood analysis highlight potential regulatory mechanisms that warrant further investigation. From previous studies, 42 leaf-rolling genes by using a similar type of network analysis in rice (89). The six candidate proteins, i.e., *OsDSG1*, *OsVDE*, *OsRST1*, *OsGRX20*, *OsbHLH38* and *OsBIP130* have no PPI network, according to network analysis. It could reflect an independent or specialized function, a condition where interactions are unnecessary, or it might indicate misfolding or a particular cellular context (43). Despite not being part of the central PPI network, these genes may still play important roles in the salt stress response. *OsDSG1* is involved in early seed development and stress signaling. It may affect seed viability and germination under stress (90). *OsVDE* functions in the xanthophyll cycle for photoprotection. It helps manage oxidative stress caused by salt-induced ROS (69). *OsRST1* is likely involved in post-transcriptional gene silencing, influencing gene regulation during salt stress adaptation (91). *OsGRX20* contributes to redox homeostasis and detoxification of ROS, which are commonly induced by salt stress (72). *OsbHLH38* regulates gene expression related to abiotic stress responses (92). *OsBIP130* likely acts as a chaperone in protein folding and the endoplasmic reticulum stress response, which can be triggered by salinity (3). These proteins may contribute to salt stress tolerance through parallel or complementary pathways, independent of direct PPIs.

Differential expression analysis demonstrated that a significant portion of BZR1-regulated cDEGs were upregulated under salt stress, confirming their involvement in stress adaptation. This dynamic regulation underscores the importance of these genes in enhancing rice tolerance to high salinity conditions. The high R^2 values reflect an overall strong correlation between control and stress conditions, indicating that the global transcriptome remains largely stable, with only specific subsets of genes responding to salt stress. Based on fold change (FC) and p-value thresholds and considering the clustering patterns, the observed expression changes are statistically significant. In the ILs, the expression of genes such as OsNHX1 (ion homeostasis), OsLEA3-2 (late embryogenesis protein), the OsGF14 family (14-3-3 signaling), OsDREB1C and OsNAC6 (TFs) suggests a coordinated transcriptional regulatory response in shoots, likely orchestrated by BZR1. In contrast, Jigeng-88 shows upregulation of OsNHX4, OsHAK15 (potassium transporter), OsTIP1;1 (aquaporin), OsBIP130 and OsNAC6 in roots, reflecting a physiological emphasis on maintaining ionic and osmotic homeostasis under salt stress. The differences in gene expression patterns between IL shoots and Jigeng-88 roots under salt stress are both statistically significant and biologically meaningful, supporting the notion of tissue-specific transcriptional regulation by BZR1 in salt tolerance. All target salt-tolerant cDEGs were highly expressed for both roots and shoots under salt stress conditions compared to the control conditions (Fig. 7A and B). Among the 33 cDEGs, *OsNHX4*, *OsNHX4*, *OsP5CS1* (93) and *OsGW5L* (94) were positively regulated under salt stress in rice which is consistent with our results. Taken together, these findings provide important insights into the spatial regulation and functional divergence of BZR1-mediated gene networks under salt stress. The differential expression patterns between shoot and root tissues across the two rice genotypes suggest that enhancing salt tolerance may require tissue-specific gene modulation. Overall, these findings implied that different salt-tolerant cDEGs might play various functions in rice under salt stress.

The integration of genomic, phylogenetic and functional analyses provides a comprehensive understanding of the BZR1-regulated salt-tolerant cDEGs in rice. Key genes and pathways identified in this study present promising targets for genetic engineering and breeding programs aimed at improving rice resilience to salt stress. Future studies should aim to validate the functional roles of hub genes and investigate their possible uses in enhancing crop development.

Challenges and unexplored aspects

1. While 33 cDEGs interacting with BZR1 were identified and characterized bioinformatically, their direct functional roles in conferring salt tolerance have not been experimentally validated (e.g., through knockout or overexpression studies). This remains a crucial gap in confirming their biological significance.
2. The observed differential expression between shoots and roots under salt stress suggests complex, tissue-specific regulation. However, the exact upstream signals and downstream pathways involved in this differential regulation remain largely unexplored.
3. Although interaction with BZR1 was predicted, direct binding of BZR1 to the promoter regions of these cDEGs (e.g., through ChIP-Seq or EMSA) has not been demonstrated. This limits the mechanistic understanding of how BZR1 modulates these genes.
4. While phylogenetic analysis indicated similarity between rice and Arabidopsis genes, it remains unclear whether these conserved genes perform analogous functions in salt stress tolerance across species. Functional conservation across species needs to be validated.

Future directions

To determine whether the BZR1 transcription factor (TF) interacts with other genes under salt stress, several experimental approaches and computational tools can be used:

1. Yeast one-hybrid (Y1H) assay is used to study DNA-protein interactions. It can identify transcription factors, such as BZR1, that bind to specific DNA elements within gene promoters. As BZR1 regulates gene expression by binding to promoter regions, Y1H is a reliable method for validating predicted BR-responsive target gene interactions.
2. Yeast two-hybrid (Y2H) screening using BZR1 as bait can identify protein-protein interactions, including co-factors or other transcription factors that associate with BZR1 under salt stress conditions.
3. Chromatin immunoprecipitation followed by sequencing (ChIP-Seq) using a BZR1-specific antibody can reveal genomic regions bound by BZR1 *in vivo* during salt stress. This technique helps pinpoint direct BZR1 targets by identifying promoter or enhancer regions to which BZR1 binds.

4. Bioinformatic promoter analysis of genes upregulated under salt stress can uncover putative BZR1 binding motifs, such as E-boxes or brassinosteroid-responsive elements. Tools like MEME or JASPAR can be used to predict conserved binding sites within these promoters.
5. Reporter gene assays, such as GUS (β-glucuronidase) or luciferase constructs driven by salt-responsive gene promoters, can be used to experimentally validate BZR1 binding. Changes in reporter activity in response to salt stress would indicate regulatory effects mediated by BZR1.
6. Identification and manipulation of key BZR1-regulated genes involved in salt stress responses offer a promising strategy for engineering crops with enhanced salinity tolerance, an increasingly critical trait for agriculture in arid and coastal regions.
7. BZR1 target genes present precise candidates for CRISPR/Cas9-mediated genome editing; for example, upregulating BZR1-activated genes or silencing associated negative regulators could bolster plant defense mechanisms under salt stress conditions.
8. BZR1-regulated genes may also function as molecular markers for screening salt-tolerant genotypes in breeding programs, thereby expediting the development of resilient cultivars through marker-assisted selection without relying on transgenic approaches.

Conclusion

This study provides compelling evidence that BZR1 plays a regulatory role in rice salt stress response through its interaction with a distinct set of cDEGs. The identification of highly tolerant cDEGs such as *OsNHX1*, *OsNAC6* and *OsGF14* in shoots, along with *OsNHX4*, *OsTIP1;1* and *OsHAK15* in roots, underscores their critical roles in the salt stress response. These genes represent strong candidates for functional validation and potential use in breeding programs aimed at enhancing salt tolerance in rice. The GO enrichment and phylogenetic analyses further support the involvement of these genes in salt tolerance, with strong homology to known salt-responsive genes in Arabidopsis. These findings offer new insights into the molecular framework of BZR1 -mediated salt stress adaptation in rice and highlight valuable candidate genes for genetic improvement. Ultimately, this research contributes foundational knowledge toward the development of salt-tolerant rice varieties, aiding in the pursuit of stable rice productivity under salinity stress conditions. A deeper understanding of homeostatic regulation and its crosstalk with phytohormones, particularly BRs, will be essential for future advances. Continued investigation into BR signaling pathways and the role of BZR1 transcription factors in salt stress responses will further inform the identification of key regulatory genes and promote the development of resilient rice varieties.

Authors' contributions

MAM conceptualized the research work, conducted bioinformatics analysis, developed methodology and drafted the manuscript. SAJ developed the methodology, investigated the literature and conducted formal analysis. MNM supervised the research work and edited the manuscript. MH developed the methodology, drafted the manuscript and reviewed and edited the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The Authors do not have any conflicts of interest to declare.

Ethical issues: None

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