BMC Plant Biology

RESEARCH Open Access

Spermidine augments salt stress resilience in rice roots potentially by enhancing OsbZIP73's RNA binding capacity

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Abstract

Background Rice is a staple crop for over half of the global population, but soil salinization poses a significant threat to its production. As a type of polyamine, spermidine (Spd) has been shown to reduce stress-induced damage in plants, but its specific role and mechanism in protecting rice roots under salt stress require further investigation.

Results This study suggested spermidine (Spd) mitigates salt stress on rice root growth by enhancing antioxidant enzyme activity and reducing peroxide levels. Transcriptomic analysis showed that salt stress caused 333 genes to be upregulated and 1,765 to be downregulated. However, adding Spd during salt treatment significantly altered this pattern: 2,298 genes were upregulated and 844 were downregulated, which indicated Spd reverses some transcriptional changes caused by salt stress. KEGG pathway analysis suggested that Spd influenced key signaling pathways, including MAPK signaling, plant hormone signal transduction, and phenylalanine metabolism. Additionally, the bZIP transcription factor *OsbZIP73* was upregulated after Spd treatment, which is confirmed by Western blot. Further insights into the interaction between *OsbZIP73* and Spd were gained through fluorescence polarization experiments, showing that Spd enhances protein OsbZIP73's affinity for RNA. Functional enrichment analyses revealed that *OsPYL1*, *OsSPARK1*, and various SAUR family genes involved in Spd-affected pathways. The presence of G/A/C-box elements in these genes suggests they are potential targets for OsbZIP73.

Conclusions Our findings suggest a strategy of using spermidine as a chemical alleviator for salt stress and provide insights into the regulatory function of *OsbZIP73* in mitigating salt stress in rice roots.

Keywords Rice, Roots, Spermidine, Salt stress, *OsbZIP73*

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Background

Rice (*Oryza sativa*) is the major food crop grown across the globe. As a crop very sensitive to salt, soil salinization severely threatens rice's growth [1–3]. With more than 20% of the world's arable land is affected by salt stress and the affected surface is expanding [4], it is particularly important to find new strategies to mitigate salt stress effects and uncovering its underlying mechanisms.

Salt stress significantly affects crop growth and development, and plants adapt to salt stress through various physiological and molecular mechanisms [5, 6]. To mitigate the effects of high salinity, plants sense salt stress via changes in osmotic pressure and sodium ion concentration, which could trigger responsive pathways related to ion homeostasis, osmoregulation, and redox regulation. Ion homeostasis involves removing sodium ions or compartmentalizing them into vacuoles to maintain Na^+/K^+ balance [7]. Osmoregulation relies on synthesizing compatible solutes like proline, glycine betaine, and carbohydrates, aiding water retention and stabilizing cellular structures [8]. Redox regulation activates antioxidant systems to detoxify reactive oxygen species (ROS) and reduce oxidative damage, supported by phytohormones like abscisic acid (ABA), ethylene (ET), and brassinosteroids (BR), which help coordinate these pathways. Additionally, as the first organ to sense salt stress, roots exhibit more significant responses than shoots [9, 10]. In rice roots, salt signals are initiated by serine/threonine kinases. Cytosolic Ca^{2+} homeostasis maintained by binding proteins such as CaBs, DREPP, CaM, and CRT, are also crucial for root growth and development [11]. Certain proteins, like the 14-3-3 family, are notably salt-sensitive in rice roots [12]. Also, the superoxide dismutase (SOD) isozymes pathway and the catalase (CAT) enzyme pathway are vital for combating salt-induced oxidative stress in rice roots [13].

Transcription factors (TFs) have been demonstrated to play crucial roles during stress response. In *Sorghum*, salt stress causes the up regulation of transcription factors like NAC, MYB, and WRKY [14, 15]. As the largest transcription factor families in plants, basic leucine zipper (bZIP) transcription factors are also found to have diverse roles in plant response to various stresses [16, 17]. There are 89 bZIP TFs in rice and classified into 11 groups (I–XI) according to their DNA-binding specificity and amino acid sequences in basic and hinge regions. bZIP transcription factors preferentially interact with sequences termed as C-box (G*ACGTC*), G-box (C*ACGTG*) and A-box (T*ACGTA*) [17]. Among the bZIP genes, those associated with abiotic stress are the most abundant. They can regulate plant adaptation to abiotic stress through ABA signaling pathways by binding to specific target genes. In *Arabidopsis thaliana*, the transcription factor AtbZIP62 plays a critical role in regulating the Salt Overly Sensitive (SOS) signaling pathway, which maintains ion balance in the plant under salt stress conditions [18]. In *Glycine max*, overexpression of *GmbZIP1*, *GmbZIP2*, *GmbZIP44*, *GmbZIP62*, and *GmbZIP78* could enhance salt tolerance [19]. In *Helianthus annuus*, *HabZIP01*, *04*, *18*, and *48* were significantly upregulated in response to mild or severe salt stress, while *HabZIP08* was downregulated in response to salinity [20]. In rice (*Oryza sativa*), bZIP transcription factors like OsbZIP23 [21, 22], OsbZIP68 [23], OsbZIP71 [24], OsbZIP03 [25], and OsbZIP05 [26] enhance salt tolerance through the regulation of ion transporters and antioxidant enzyme activities. However, not all bZIPs positively influence salt tolerance. For instances, OsbZIP10/OsABI5 in rice are associated with reduced salt tolerance [27]. Although bZIP transcription factor family of rice plays an important role in abiotic stress, its complex regulatory mechanisms in rices' response to stress await further discovery and analysis.

Chemical interventions could assist in managing various stresses. Nitrogen-based aliphatic molecules including polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are known to promote plant tolerance [28–30]. As a precursor of other polyamines, spermidine was relatively well studied, and enhances plant tolerance to various environmental stresses [28–30]. Exogenous Spd effectively boosts both non-enzymatic and enzymatic antioxidant systems in tomato (*Solanum lycopersicum*) under high-temperature stress [31–33], enhances the activity and gene expression of antioxidant enzymes in barley seedlings to reduce oxidative damage under UV-B stress [34], and activates the expression of salt stress responsive transcription factors to alleviate ion toxicity and osmotic stress [35]. In *Leymus chinensis*, Spd mitigates osmotic damage by enhancing germination rates and growth under salt-alkali stress [36]. In sweet corn, spermidine improves seed gemination through its influence on phytohormone interactions and gene expression [37]. Additionally, application of spermidine-producing bacteria like *Bacillus subtilis* could promote plant growth [38]. Spermidine is also found to interfere with the expression of genes involved in the synthesis and signaling of different phytohormones [39, 40]. However, fewer studies have investigated spermidine's role in mitigating salt stress particularly in rice, which underscores the necessity to explore spermidine's potential to protect rice in salt-affected conditions [41–43].

Here, we subjected rice roots to salt stress and spermidine (Spd), measured physiological indices, analyzed gene expression patterns using transcriptome sequencing, and performed functional enrichment analysis of differentially expressed genes (DEGs). Furthermore, we analyzed key transcription factors affected by Spd treatment. One bZIP transcription factor, *OsbZIP73*, was

identified as the gene with higher regulatory potential by Spd. Using fluorescence polarization assay [44–47], we demonstrated that Spd could enhance the binding between recombinant protein OsbZIP73 and RNA. This study laid a foundation for understanding the molecular mechanisms of salt resistance and offers potential avenues for utilizing Spd as a salt resistance chemical in rice.

Results

Spermidine promotes physiological antioxidant activity and maintains Na^+ homeostasis in rice roots

Rice variety Huanghuazhan's roots were exposed to varying concentrations of NaCl and Spd to determine the optimal treatment dosage. It was found that concentrations above 50 mM NaCl caused significant damage to rice root growth (Fig. S1a), while concentrations of Spd exceeding 0.1 mM inhibited root length (Fig. S1b). Consequently, 100 mM NaCl and 0.1 mM Spd are chosen as the optimal combination for subsequent treatment. Under this concentration, Spd significantly enhanced rice roots' length (Fig. 1a), and promotes rice seedlings' growth (Fig. 1b, Fig. S1c). As a marker for oxidative stress and lipid peroxidation, our results showed that Spd also reduced Malondialdehyde (MDA) levels (Fig. 1c). Additionally, a sodium-sensitive fluorescent probe (SBFI AM) indicated that Spd helps maintain ion homeostasis by regulating sodium ion (Na^+) concentrations (Fig. 1d). Enzymatic assays revealed that Spd enhances the activities of superoxide dismutase (SOD) and peroxidase (POD) (Fig. 1e and f). Furthermore, using fluorescent probes, we measured reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2). The results indicated a reduction in ROS and H_2O_2 fluorescent signals upon Spd treatment, contrasting with the strong signals observed under salt stress (Fig. 1g and h).

Salt stress and spermidine cause global transcriptomic changes in rice roots

To understand how spermidine (Spd) helps rice roots cope with salt stress, we conducted deep sequencing for rice roots treated with salt and Spd. Because Huanghuazhan lacks a fully sequenced genome, we did *de novo* transcriptome assembly using Trinity to evaluate the most suitable reference. This assembly yielded 216,035 transcripts and 188,284 genes by analyzing RNA sequencing data (Fig. 2a). Comparative analysis indicated that the transcriptome of Huanghuazhan rice closely matches several rice varieties, with *Oryza sativa ssp. japonica* having the most matchness (Fig. 2b). Annotation with Trembl, SwissProt, Pfam, and NR databases showed that about 65% of these genes had at least one annotation (Fig. 2c). After mapping the sequencing reads to *Oryza sativa ssp. japonica* [48], the analysis revealed that the combination treatment of NaCl and spermidine (Spd) resulted in 333 upregulated genes and 1,765 downregulated genes (Fig. 2d). In contrast, treatment with NaCl alone led to a significantly higher number of upregulated genes, totaling 2,298, and a lower number of downregulated genes, amounting to 844 (Fig. 2e). Additionally, when comparing the NaCl and Spd combination treatment to the control (CK), there were 398 upregulated and 300 downregulated genes (Fig. 2f). Hierarchical clustering of differentially expressed genes (DEGs) confirmed this pattern (Fig. S2a, S2b). Gene expression levels, measured by FPKM (Fragments Per Kilobase Million), displayed consistent patterns across all samples. The extensive summary of differentially expressed genes in Supplemental Table S1, visualizations via boxplots (Fig. S2c), gene expression density plots (Fig. S2d), and violin plots (Fig. S2e) further corroborate the reliability of our sequencing data. These results suggest that salt stress causes significant transcriptional shifts in rice roots, with spermidine providing a distinct modulation effect.

Spermidine induces transcriptional shifts under salt stress

To determine the extent to which spermidine (Spd) modulates transcriptional changes in rice roots under salt stress, several analytical techniques were used, including correlation analysis, principal component analysis (PCA), and k-means clustering. The correlation matrix revealed a significant positive relationship among control (CK), NaCl treatment, and Spd (Fig. 3a). In PCA analysis, the NaCl + Spd treatment was separated from NaCl alone, suggesting that Spd mitigates transcriptional changes caused by salt stress (Fig. 3b). k-means clustering further emphasized the difference between NaCl-only treatment and other groups, which suggested Spd counteracted NaCl-induced transcriptional shifts (Fig. 3c). Venn diagrams indicated overlapping gene expression among treatments, with a core subset of commonly regulated genes (5305 vs. 2071 genes), underscoring Spd's modulatory role (Fig. S3a, S3b). GO enrichment analysis highlighted "response to salt stress" as a key pathway influenced by Spd (Fig. S3c). KEGG enrichment analysis suggested that the MAPK signaling pathway, critical for salt stress response, might be affected by Spd (Fig. S3d). Notably, some genes that were upregulated by salt stress were often downregulated with Spd treatment (Fig. S3e, S3f). This shift in gene expression suggests that Spd can help reduce adverse effects from salt stress.

Transcription factors are involved in Spd-mediated alleviation of salt stress

To understand the molecular mechanisms and pathways behind these effects, we conducted an extensive Gene Ontology (GO) enrichment analysis to identify pathways critical for root growth and stress resilience. The GO analysis revealed significant enrichment in pathways

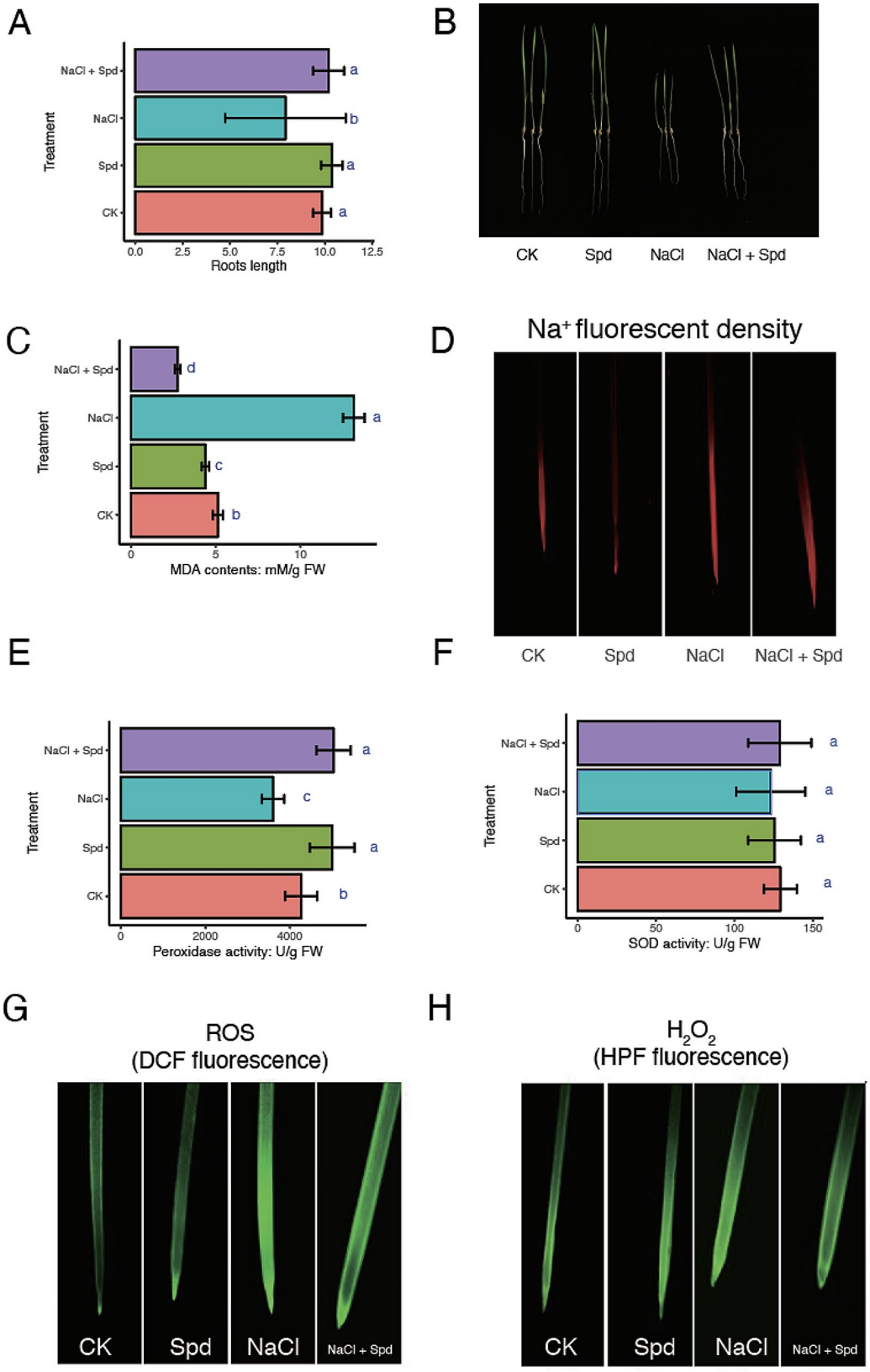
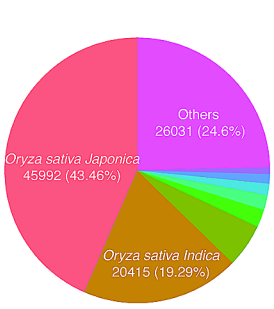


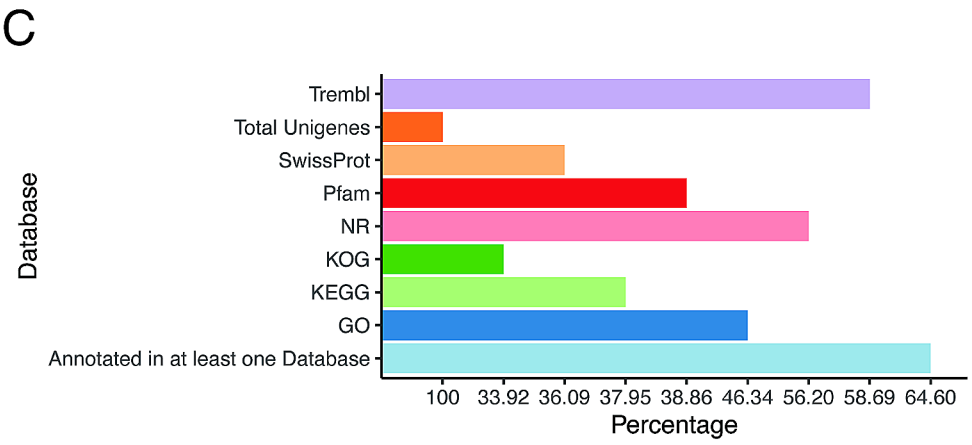
Fig. 1 The effects of spermidine on the growth and physiology of rice roots under salt treatment. (**A**)Root length. (**B**) Seedling length. (**C**) MDA acitivty. (**D**) Fluorescence of Na^+. (**E**) POD activity. (**F**) SOD activity. (**G**) DCF fluorescence. (**H**) HPF fluorescence. Control (CK), spermidine (Spd), salt (NaCl), spermidine and salt (NaCl + Spd). The different letters in the bar chart represent the significance of differences between different treatments

A

De novo assembled genes and transcripts

	Transcripts	Genes
Number	216035	188284
Mean Length	823	909
Total Bases	177740071	171127738





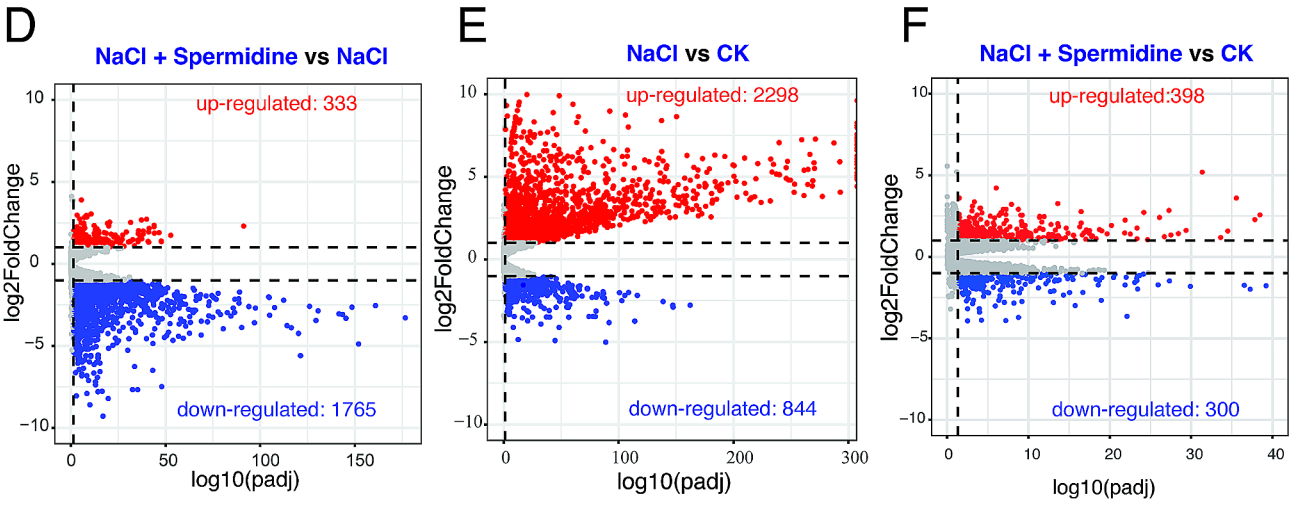


Fig. 2 Expression variation in rice after adding Spd. (**A**) Summary of assembled genes and transcripts. (**B**) Cross-species similarity analysis. (**C**) Annotation of assembled genes. (**D**) Transcriptional responses to NaCl + Spermidine treatment. (**E**) Transcriptional changes induced by NaCl stress. (**F**) Transcriptional changes compared between the NaCl + Spermidine treatment and CK

related to detoxification, cell proliferation, growth, cell viability, and reproduction (Fig. 4a). Interestingly, an enrichment in genes associated with transcriptional regulator activity suggested that Spd's mitigating effects on salt stress could be mediated through transcription factors (TFs). This finding was supported by analyses using the bioinformatics tool iTAK [49], which identified a subset of transcription factors potentially upregulated by Spd, including those in the bZIP family (Fig. 4b). Additionally, KEGG analysis indicated that Spd may also lead to broader changes in pathways such as 'Phenylalanine metabolism' (2.16%), 'Phenylpropanoid biosynthesis' (5%), and 'Plant hormone signal transduction' (7.79%) (Fig. S4). Further analysis of this pathway showed a

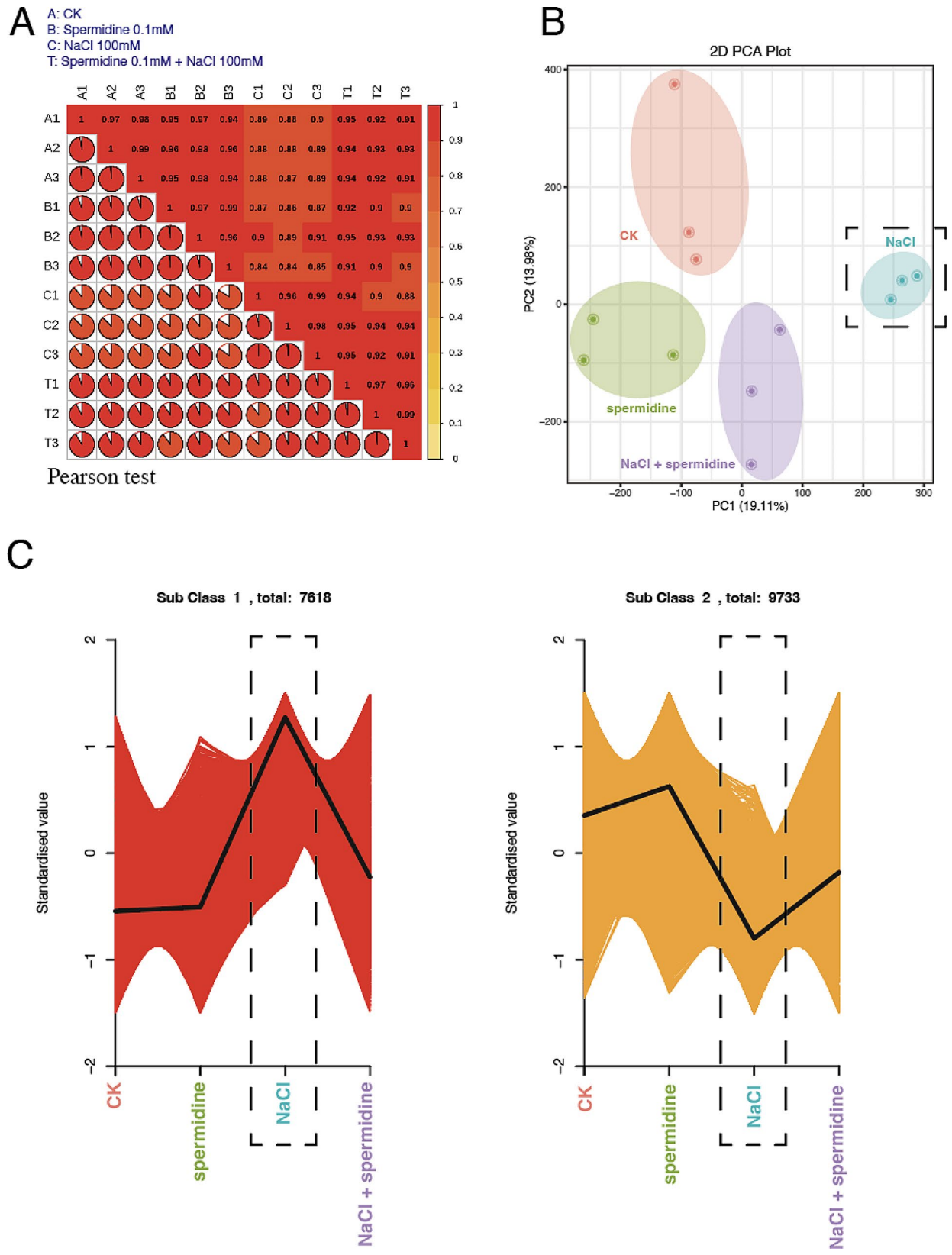


Fig. 3 Spermidine-mediated restoration of rice from salt stress. (**A**) Correlation analysis of treatment effects. (**B**) Principal Component Analysis (PCA) of rice samples with three replicates. (**C**) k-means clustering reveals two subclasses among different treatment groups. In the figure, labels A, B, C, and T correspond to CK (Control), Spermidine, NaCl (Salt), and NaCl + Spermidine treatments, respectively. The numbers 1 to 3 indicate the three replicates for each treatment

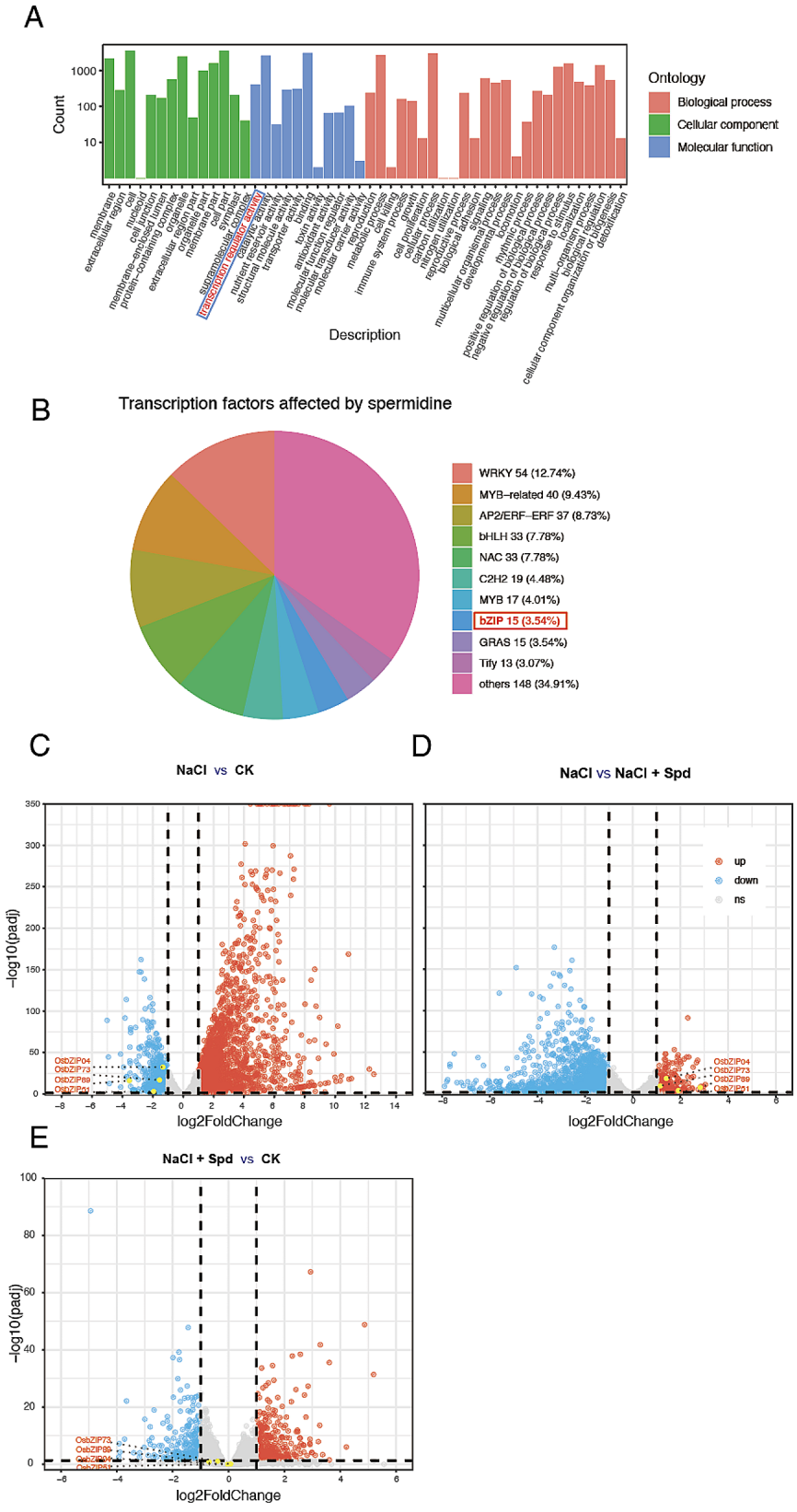


Fig. 4 Gene ontology annotation analysis. (**A**) Gene ontology annotation of total genes affected by spermidine. (**B**) Annotation of spermidineaffected transcription factors. Comparative volcano plots highlighting transcription factor responds to (**C**) salt stress and (**D**) spermidine treatment. (**E**) Comparion between spermidine + salt stress treatment and CK

strong impact on genes from the auxin-responsive SAUR family and ethylene-responsive transcription factors (Supplemental Table S2). In addition, 43 transcription factors were identified downregulated under salt stress but upregulated in response to Spd treatment (Supplemental Table S3, Fig. 4c). Among these, four (*OsbZIP04*, *OsbZIP51*, *OsbZIP73*, and *OsbZIP89*) of them belong to bZIP transcription factors family (Fig. 4d). Additionally, when comparing the NaCl+Spd treatment to the control (CK), these transcription factors showed significant differential expression, indicating a compromised effect where the gene expression levels do not fully revert to control conditions but show a mitigated response (Fig. 4e). Because *OsbZIP73* is most well-known for its association with abiotic stress responses among the four bZIP transcription factor family genes [50, 51], we chose to focus on *OsbZIP73* for downstream validation.

Spermidine increases binding affinity between OsbZIP73 and RNAs

Based on earlier results indicating that *OsbZIP73* was downregulated under salt stress but upregulated after spermidine's (Spd) treatments from transcriptome analysis, we firstly confirmed its expression profile using Western blotting (Fig. 5a). Next, to check if OsbZIP73 protein contains any special domains related to its role in salt stress, bioinformatics tools including SEG, CAST, fLPS, and GBSC [52] were used to predict its binding properties. Interestingly, these tools all predicted that OsbZIP73 contains low-complexity domains (indicated by squares). Low-complexity domains are often associated with phase separation, and they drive the formation of protective condensates, which are basically consisted of proteins and RNAs (Fig. 5c). This discovery led us to test whether Spd drives OsbZIP73 to form protein-RNA condensates. To do that, we purified recombinant OsbZIP73 protein (Fig. 5b) and used fluorescence polarization assays to test its binding to RNA. The data indicated that Spd significantly increased the binding affinity between OsbZIP73 and RNA under the treatment of NaCl (Fig. 5d and e). To check if these domains are specific to some rice varieties, we compared the sequences of Huanghuazhan and *Oryza sativa ssp. japonica* to check if there are any potential genetic variations. The analysis indicated that the CDS sequence of *OsbZIP73* in huanghuazhan was completely consistent with that of Nipponbare, which implied that Spd might induce common mechanisms via OsbZIP73 in different rice varieties (Fig. 5f). We further investigated potential targets of the transcription factor OsbZIP73 under Spd treatment. Given previous findings indicating Spd's influence on pathways like 'Phenylalanine metabolism', 'MAPK signaling pathways', and 'Plant hormone signal transduction' (Fig. S4), coupled with OsbZIP73's known recognition of C-box/G-box/A-box promoter sequences in target genes, we performed a cis-element analysis (Fig. S5). This cis-element and G/C/A-box analysis identified *OsPYL1* (MAPK), *OsSPARK1* (Receptor protein kinase), *OsPAL3* (Phenylalanine), *OsPAL4* (Phenylalanine), and *OCBL6* (MAPK) as potential target genes for *OsbZIP73* (Supplemental Table S4). The presence of motifs recognized by OsbZIP73 in the promoter regions of these genes suggests they may be direct targets of this transcription factor.

Discussion

In our investigation, we identified that exogenous spermidine (Spd) has a significant effect on enhancing salt stress resilience in rice roots, and *OsbZIP73* plays an important role in this process (Fig. 6).

Spermidine has been acknowledged for its role in various stress responses in plants [21–23], to explore the impact of spermidine (Spd) on rice growth under salt stress, we designed precise preliminary experiments to determine the level of salt concentration that markedly hindered rice growth, and the Spd concentration capable of mitigating salt-induced damage. Subsequently, four treatments (CK, 0.1 mM Spd, 100 mM NaCl, 0.1 mM Spd+100 mM NaCl) were devised to assess the impacts of various treatments on rice growth. The results indicated that Spd presents the ability to enhance rice root growth to a certain degree, by elevating the activity of antioxidant enzymes and reducing peroxide levels in rice. The physiological indices improved by spermidine (Spd) through mitigating abiotic stress have been extensively examined across various plant species [35, 53, 54]. Our findings align closely with these outcomes, as they primarily show enhanced antioxidant enzyme activity, diminishing peroxide levels within the plant and maintaining essential ion equilibrium. However, the molecular mechanisms involved have not yet been elucidated.

To uncover the molecular mechanism by which spermidine (Spd) alleviates salt stress impacts on rice growth, transcriptome sequencing was carried out on root tissues subjected to four distinct treatments. An analysis of gene expression patterns revealed that, with Spd treatment, most differentially expressed genes affected by salt stress experienced a shift towards restoration. Genes upregulated by salt stress showed downregulation upon Spd treatment, while those with the opposite expression pattern exhibited similar alterations. This suggests Spd alters the expression patterns of most genes, potentially due to transcription factors. We then screened transcription factors with differential expression among treatments and found that 15 bZIP transcription factors exhibited varying expression levels under salt stress and Spd treatment. Four of these (*OsbZIP04*, *OsbZIP51*, *OsbZIP73*, and *OsbZIP89*) were downregulated by salt stress and significantly upregulated by Spd, which suggested they

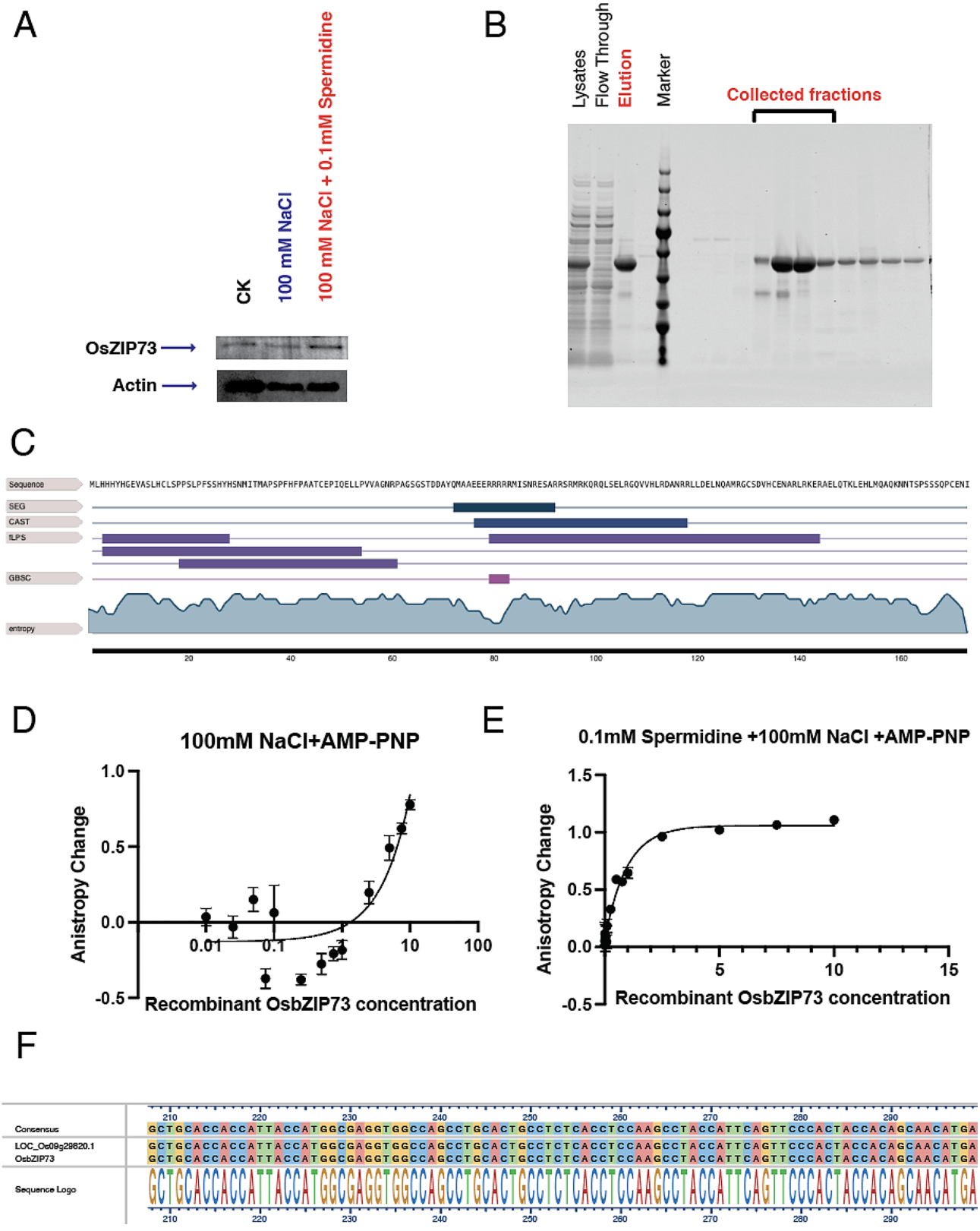


Fig. 5 Characterization of *OsbZIP73*. (**A**) Western Blot analysis of OsZIP73 in CK, NaCl, and NaCl + Spermidine-treated samples. (**B**) Coomassie blue staining of recombinant OsbZIP73. (**C**) Low complex domain prediction with bioinformatics tools SEG, CAST, fLPS and GBSC. Fluorescence polarization assay of recombinant OsZIP73 protein and FAM-labeled $(AGCU)_5$ RNA without (**D**) or with (**E**) the treatment of 0.1mM Spermidine. (**F**) Sequence alignment for *OsbZIP73* of Huanghuazhan and *Oryza sativa ssp. Japonica*

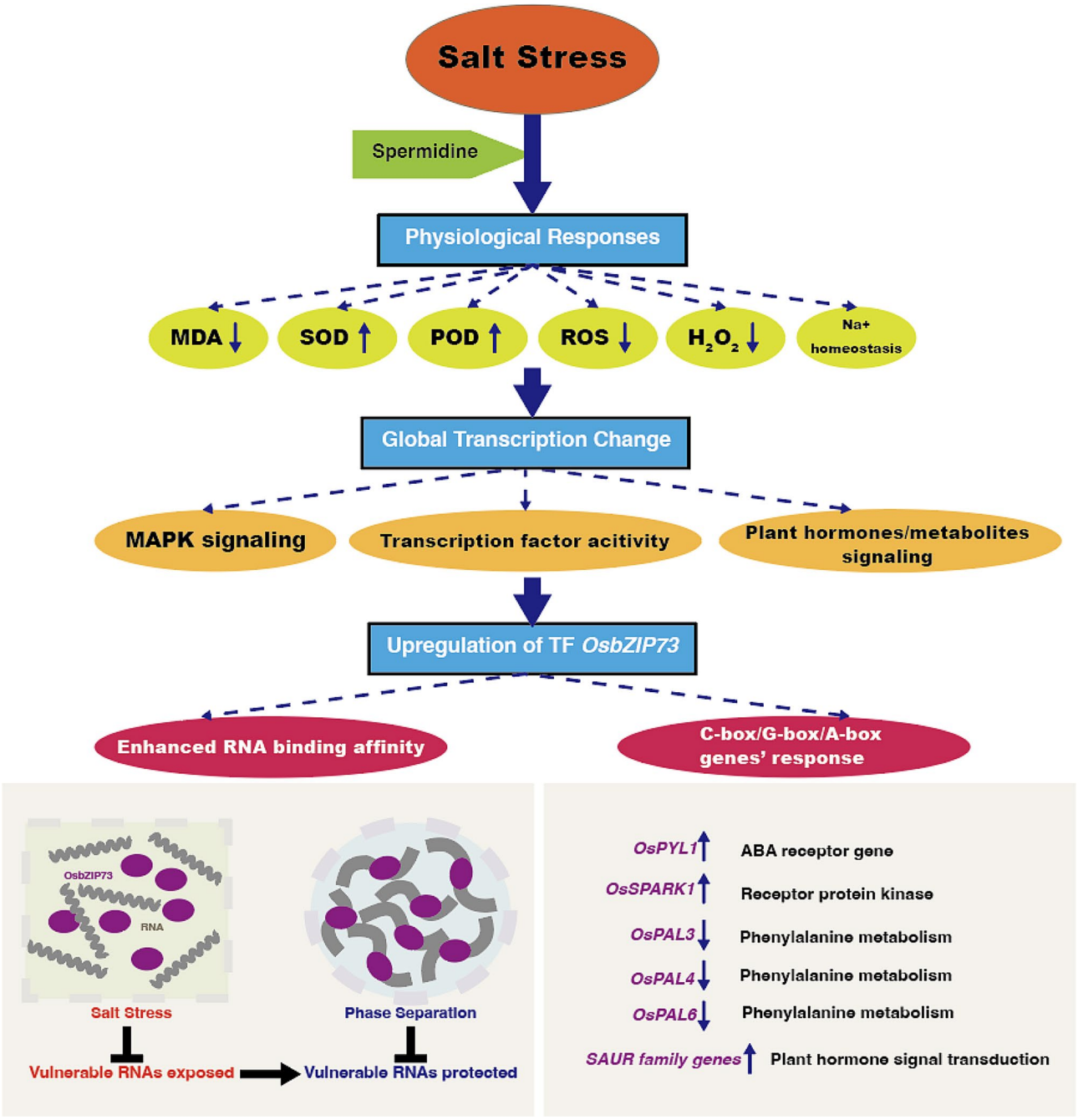


Fig. 6 Mechanisms of spermidine-mediated salt stress alleviation

may alter downstream gene regulation and thus allowing rice to adapt to salt stress environments. Among these, only *OsbZIP73* had been reported to be associated with abiotic stress. Previous studies showed that *OsbZIP73* improves rice's cold tolerance by regulating endogenous ABA levels and reducing ROS content [55, 56], which are pathways critical for resistance to abiotic stress. Consequently, *OsbZIP73* was selected as a candidate gene to verify its role in enhancing salt tolerance in rice when Spd was added. Western blot experiments confirmed that *OsbZIP73* expression levels in different treatments closely matched transcriptome sequencing results.

Phase separation is a cutting-edge research area in biology, offering new insights into biological processes [57–59]. During growth and development, plants face environmental changes such as variations in light, temperature, water, and salt levels. Plant cells must quickly regulate gene expression to adapt. Membrane-free organelles formed through the phase separation of biomolecules compartmentalize and concentrate biomolecules.

This process plays a crucial role in plant growth, development, and stress responses [47, 48]. Prior research has shown that transcription factors can quickly change conformation and form complexes when exposed to salt stress, therefore enhancing their resistance to osmotic stress [60, 61]. Our study found that adding Spd significantly increased the ability of protein OsbZIP73 to bind to RNA, suggesting that OsbZIP73 might aid in salt tolerance through phase separation by forming biomolecular condensates. These condensates likely compartmentalize RNAs and proteins, protecting them from stress-induced damage.

Furthermore, we performed KEGG functional enrichment analyses of differentially expressed genes identified through transcriptome sequencing. This was done to understand the potential pathways through which OsbZIP73 enhances salt tolerance in rice with Spd. The analysis showed that the differentially expressed genes under salt stress with or without exogenous Spd, were significantly enriched in pathways related to abiotic stress including the MAPK signaling pathway, plant hormone signal transduction, phenylpropanoid biosynthesis, and phenylalanine metabolism. In addition, previous studies have shown that OsbZIP73 can regulate downstream genes containing the AGCT core sequence in their promoter [55, 56]. Therefore, we examined the promoter sequences (2000 bp upstream of the transcription start site) of genes enriched in these pathways. We identified two specific motifs recognized by the transcription factor OsbZIP73 in the promoter regions of several rice genes (Fig. S5a). These motifs were found in the promoters of *OsPYL1*, *OsSPARK1*, *OsPAL3*, *OsPAL4*, *OsPAL6*, and *OsSAUR29*. The presence of these motifs is indicated near the transcription start sites (TSS) (Fig. S5b). The proximity of these motifs to the TSS suggests that they may play a crucial role in regulating the expression of these genes in response to salt stress. Given that these motifs are known to be binding sites for OsbZIP73, it is reasonable to propose that *OsPYL1*, *OsSPARK1*, *OsPAL3*, *OsPAL4*, *OsPAL6*, and *OsSAUR29* are potential targets of OsbZIP73. This regulatory interaction could be important for understanding how these genes contribute to salt stress resilience in rice.

Several SAUR family genes are enriched in the plant hormone signal transduction pathway. Among these, four genes (*OsSAUR7*, *OsSAUR10*, *OsSAUR27*, and *OsSAUR29*) were significantly upregulated in the treatment with Spd under salt stress and significantly downregulated in the Salt-only treatment. Research on the promoter sequences of these four SAUR family genes revealed the presence of A-box sequences in the promoter of *OsSAUR10* and *OsSAUR29*, which implied that they may be regulated by *OsbZIP73* (Supplemental Table S4). Meanwhile, we also detected ABA responsive elements in the promoters of these two genes, and the ABA related pathways have been shown to be closely related to salt stress (Fig. 6) [62, 63]. Prior studies have shown that auxin synthesis and transport are closely linked to plant salt tolerance [64]. Additionally, SAUR proteins can interact with calmodulin *in vitro* [65, 66], and regulate H^+-ATPases and K^+ channels [67]. These findings suggest that the *OsSAUR* genes may enhance salt tolerance in rice by regulating ion transport through interactions with calmodulin. However, more evidence is required to confirm the mechanisms. KEGG enrichment analysis also revealed that significantly different genes are enriched in the phenylpropanoid biosynthesis and phenylalanine metabolism pathways. This is consistent with research findings in tomatoes [68] and mulberries [69], suggesting that phenylpropanoid biosynthesis may play an important role in rice salt tolerance. Further research in our study identified three genes related to phenylalanine metabolism: *OsPAL3* (LOC_Os02g41670), *OsPAL4* (LOC_Os02g41680), and *OsPAL6* (LOC_Os04g43800). The promoter sequences of these genes contain both the ACGT core sequence (Supplemental Table S4) and bZIP-responsive elements (Supplemental Table S5), which is necessary for OsbZIP73 binding. Thus, we speculate that expression changes in *OsbZIP73* after adding Spd may have caused changes in the expression levels of these two genes, affecting rice's response to salt stress through phenylpropanoid biosynthesis and phenylalanine metabolism pathways.

To sum up, the ability of OsbZIP73 to influence these pathways indicates a broader role in salt stress resilience. The observed protein-RNA binding suggests that Spd might enhance the formation of protective biomolecular condensates, providing a novel mechanism for salt stress response in rice roots. Despite these insights, many questions remain. Further research such as overexpression and knockout studies is required to understand how OsbZIP73 interacts with RNAs and downstream genes. Exploring its broader implications for stress tolerance could reveal its potential as a target for further research and application. Future studies should explore the molecular pathways and cofactors involved in these interactions, as well as the potential applications of Spd in enhancing salt stress resilience in other crops.

Conclusions

Our study initially observed that exogenous spermidine (Spd) enhances rice's resilience to salt stress at both physiological and transcriptomic levels. Further Functional analyses show that Spd improves the plant's tolerance by influencing genes in key pathways, including MAPK signaling, hormone signal transduction, and phenylpropanoid biosynthesis. Additionally, the affinity assay reveals that Spd enhances OsbZIP73's protein-RNA

binding, suggesting the formation of protective biomolecular condensates which compartmentalize and shield biomolecules from stress-induced damage. Cis-element and promoter analyses identify *OsPYL1*, *OsPYL3*, *OsPYL4*, *OsPYL6*, *OsSPARK1*, and various *SAUR* family genes contain OsbZIP73-responsive sequences, highlighting their roles in maintaining rice resilience. These findings suggest exogenous spermidine can alleviate salt stress and may enhance salt stress resilience in rice and other crops potentially by modulating OsbZIP73 activity.

Methods

Chemicals and materials

The rice cultivar used in this study was Huanghuazhan rice, which was provided by the Rice Research Institute of Guangdong Academy of Agricultural Sciences (China). Spermidine was purchased from Sigma-Aldrich (Prodct No. S2626), 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) were provided by Beijing Solebold Technology Co., Ltd. Hydroxyphenyl-fluorescein (HPF) and SBFI AM (Na^+ indicator) were purchased from Shanghai Maokang Biotechnology Co., Ltd.

Preparation and treatment of plant materials

Rice seeds with full grains or similar sizes were surface sterilized with 5% hydrogen peroxide for 15 min, rinsed with deionized water 3 to 5 times, and placed into a constant temperature incubator (28 °C) for 1 day to promote germination. Seedlings with consistent appearance were then cultured in a hydroponic box containing 1/2 strength Hoagland nutrient solution. Samples were divided into three groups for different treatments. To evaluate the effects of NaCl on growth of rice roots, seeds were placed onto a raft on the surface of 1/2 strength Hoagland nutrient solution containing no added NaCl or 25, 50, 100, 150, or 200 mM NaCl. After 72 h of treatment, the length of rice roots was measured and calculated by Image J software. To assess the effect of Spd on the rice root system under NaCl stress, rice seedlings were treated with the appropriate NaCl concentration and 0.1, 0.2, 0.3, 0.4, or 0.5 mM Spd was added at the same time. As a control, rice seedlings were treated with either nutrient solution or Spd. Once the optimal concentrations of Spd and NaCl were defined, all root samples were treated with the optimized concentrations and used for physiological analysis and histochemical testing. Simultaneously, we performed transcriptome sequencing on root tissues of four samples treated with optimized concentrations (control, NaCl treated only, Spd treated only and NaCl and Spd treated simultaneously) to investigate the impact of NaCl on growth of rice roots, and unravel the molecular mechanisms that drive the alterations in root response to salt treatment following the introduction of spermidine. Three replicates for each treatment resulting in a total of 12 transcriptome datasets.

Library preparation and RNA sequencing

A total of 1 μg total RNA for each sample extracted by TRIzol was used for downstream RNA sequencing. Samples were indexed and treated using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB) following the manufacturer's instructions. Finally, enriched samples were pre-treated with USER Enzyme (NEB, USA) at 37 °C for 15 min and denatured at 95 °C. Pre-treated samples were PCR-amplified using Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index Primers from Illunima. Indexed samples were clustered using a cBot Cluster Generation System with a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) and then sequenced on an Illumina Hiseq platform to generate a length of 120–150 bp paired-end reads. The sequencing data were deposited in the GEO (Gene Expression Omnibus, accession number: **GSE189665**).

Raw RNA sequencing data processing

The fastp tool [70] was used to remove adapters and low-quality reads from the raw data. In detail, sequenced reads with N content exceeding 10% and a quality score Q<=20 over more than 50% of the total length of the read were removed. To assemble the transcriptome of Huanghuazhan rice, the transcriptome assembly tool Trinity was applied to the sequencing reads [71]. In addition, Corset [72] was used to group the relevant transcripts into gene clusters. To predict coding sequences (CDSs) from the transcriptome assembled *de novo* by Trinity [73], the TransDecoder (https://github.com/TransDecoder/TransDecoder/releases) tool was employed to identify candidate coding regions. Gene functions were then annotated and evaluated using DIAMOND [74] or HMMER [75] from the following databases: Nr (NCBI non-redundant protein sequences); Swiss-Prot (a manually annotated and reviewed protein sequence database); Trembl (a variety of new documentation files and TrEMBL, a computer-annotated supplement to SWISS-PROT); KEGG (Kyoto Encyclopedia of Genes and Genomes); GO (Gene Ontology); KOG/COG (COG: Clusters of Orthologous Groups of proteins; KOG: euKaryotic Ortholog Groups); and Pfam (Protein family).

Gene enrichment analysis

Gene expression levels were estimated using RSEM [76], and then the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value for each gene was calculated based on gene length. DESeq2 [77] was next used to determine the enriched genes from pairwise comparisons, each sample being represented by three replicates.

Corrected *P*-value (*FDR*) < 0.05 and $|Log_2(\text{fold-change})| \geq 1$ were used as the thresholds for significant differential expression. For GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) annotation, enrichment analysis was performed based on the hypergeometric test. The plant transcription factor prediction tool iTAK [49] was used to predict related TFs.

Physiological measurements

The fluorescent probes DCFH-DA, DHE, and HPF were used to detect the levels of ROS, O_2^-, and H_2O_2, and the sodium ion indicator probe SBFI AM was used to determine the Na^+ content of rice root tips. Treated rice roots samples were immediately flash-frozen with liquid nitrogen after sample collection and stored at −20℃ until use. The activity of antioxidant enzymes was determined by measuring the photochemical reduction of nitro blue tetrazolium (NBT), and the activity of H_2O_2 (Shu 2016) and MDA (Parida 2004) was determined as described previously. Image Pro-plus (Media Cybernetics) was used to quantitatively analyze fluorescence, and SPSS (Sigma) was used to perform ANOVA analysis.

Plasmids construction

To create the His-tagged recombinant protein, we initiated by synthesizing the cDNA of Huanghuazhan rice using FastKing RT kit (KR116). Following this, PCR amplification was performed using specific forward 5'-ATGCTGCACCACCATTACCA-3' and reverse primer 5'-TCAAATATTCTCGCATGGCTGT-3'. Subsequently, the PCR product was integrated into a vector and its sequence was verified through Sanger sequencing. For further refinement, the inserted sequence, along with an mCherry tag, was introduced into the pColdI- vector using the Infusion (Takara). The resulting construct, pColdI-*OsbZIP73*, served as the plasmid for subsequent purification of the recombinant protein.

Recombinant protein purification

E. coli BL21 Star (DE3) cells, transformed with pColdI-*OsbZIP73* plasmids, were initially grown in 1 L of LB medium supplemented with ampicillin at 37 °C until reaching an OD_{600} of 0.6. Subsequently, the cells were cooled at 4 °C for 30 min and then incubated overnight at 15 °C in the presence of 1 mM IPTG. Afterwards, the cells were harvested by centrifugation at 8,000 g for 2 min, rapidly frozen in liquid nitrogen, and preserved at -80 °C. To extract the protein, the cell pellets were resuspended in bacterial lysis buffer, which consisted of 20 mM HEPES, 500 mM NaCl, 10 mM imidazole, 0.5% NP-40, and 10 mM β-mercaptoethanol, adjusted to a pH of 7.5 with NaOH. This suspension was then sonicated on ice. Following sonication, the lysate was clarified by centrifugation at 10,000 g for 20 min at 4 °C. The supernatant was incubated with 3 ml of pre-equilibrated Ni-NTA Superflow agarose beads (QIAGEN) for 1 h at 4 °C in a sealed gravity column (Bio-Rad). The beads were subsequently washed: first with 50 ml of high-salt wash buffer (20 mM HEPES, 1 M NaCl, 20 mM imidazole, and 10 mM β-mercaptoethanol, adjusted to pH 7.5 by NaOH), and then with 50 mL of low-salt wash buffer (20 mM HEPES-NaOH pH 7.5, 10 mM NaCl, 20 mM imidazole, and 10 mM β-mercaptoethanol, adjusted to pH 7.5 by NaOH).

The His-tagged protein was eluted using 8 ml of elution buffer (20 mM HEPES, 10 mM NaCl, 250 mM imidazole, 10% glycerol, and 10 mM β-mercaptoethanol, adjusted to pH 7.5 by NaOH). The eluted protein underwent further purification through an NGC chromatography system (Bio-Rad). Specifically, it was loaded onto a HiTrap 1 ml Heparin HP column (Cytiva) and separated using a gradient of increasing salt concentration, employing a mixture of buffer A (20 mM HEPES-NaOH pH 7.5, 10 mM NaCl, 10% glycerol, and 1 mM DTT) and buffer B (20 mM HEPES-NaOH pH 7.5, 1 M NaCl, 10% glycerol, and 1 mM DTT). The fractions containing the desired protein were collected and subjected to buffer exchange using a PD-10 column (Cytiva) into a storage buffer (20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 10% glycerol, and 1 mM DTT). The protein was concentrated using an Amicon Ultra-4 10 kDa MWCO (Millipore) according to the manufacturer's guidelines. Finally, the recombinant protein was rapidly frozen with liquid nitrogen and stored at -80 °C.

Immunoblotting

Rice roots are lysed using SDS-lysis buffer (R0070, Solarbio) and then loaded onto SDS-PAGE gel. Proteins are transferred onto a PVDF membrane for subsequent antibody detection. The membrane was blocked, incubated with a primary OsbZIP73-specific antibody (A2065, ABclonal), washed, and then exposed to a secondary antibody conjugated to HRP (AS014, ABclonal). OsbZIP73 expression was visualized and quantified using ECL reagents. For internal control, anti-actin monoclonal antibody for Plant-HRP (K800001M-HRP, Solarbio) was used. Blots were cut prior to hybridization with antibodies during blotting.

Fluorescence polarization assay

Proteins (0–10 μM) were incubated in a reaction mixture containing 14.4 mM HEPES-NaOH, 100 mM NaCl, 1 mM $MgCl_2$, 0.36 mM TCEP, 14.4% glycerol, 0.1% DMSO, and 10 nM 5′ FAM-labelled RNA. This incubation was performed both with and without the presence of 0.1mM Spermidine in a 10 μl reaction for 30 min at 25 °C. Different conditions were employed for specific experiments: 1 mM AMP-PNP was used for AMP-PNP

assays. Fluorescence polarization measurements were conducted using an Infinite F-200 PRO (TECAN) instrument and plotted by Prism 10 software.

Promoter and cis-acting element analysis

The promoter sequence (a sequence of 2000 bp upstream of the transcription start site) of the selected genes were extracted by TBtools-II [78]. An online database called PlantPAN 4.0 [79] (http://plantpan.itps.ncku.edu.tw/plantpan4/index.html) was employed to identify the cis-acting regulatory elements within each gene's promoter region. Additionally, the JASPAR 2024 [80] and RSAT [81] were utilized to confirm the presence of the cis-acting elements in the selected genes.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05492-9.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Acknowledgements
Not applicable

Author contributions
Conceptualization, S.X. and M.C.;Methodology, S.X., M.C., and D.S.;Formal analysis, M.C., and D.S.;Investigation, S.X., M.C., and D.S.;Resources, S.X. and H.Y; Writing – Original Draft, M.C.;Writing – Review & Editing, S.X., M.C., and D.S.;Visualization, D.S. and M.C.;Supervision, S.X., and M.C.;Funding Acquisition, S.X., M.C., D.S., and H.Y.

Funding
This work was financially supported by Research Startup Funding of Guangdong Ocean University (060302052103, 060302052307), the National Innovation and Entrepreneurship Training Program (CXXL2020013) and the Special Project of Seed Industry Vitalization under Rural Revitalization Strategy in Guangdong Province (2022NPY00014).

Data availability
The datasets generated and/or analysed during the current study are available in the GEO repository (GSE189665, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189665).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 16 October 2023 / Accepted: 6 August 2024
Published online: 20 August 2024

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