Journal of Integrative Agriculture 2026, 25(6): 2288–2298

Available online at ScienceDirect

Journal of Integrative Agriculture

RESEARCH ARTICLE

Multi-omics approach reveals the contribution of brassinosteroids to salt tolerance for seed germination in rice

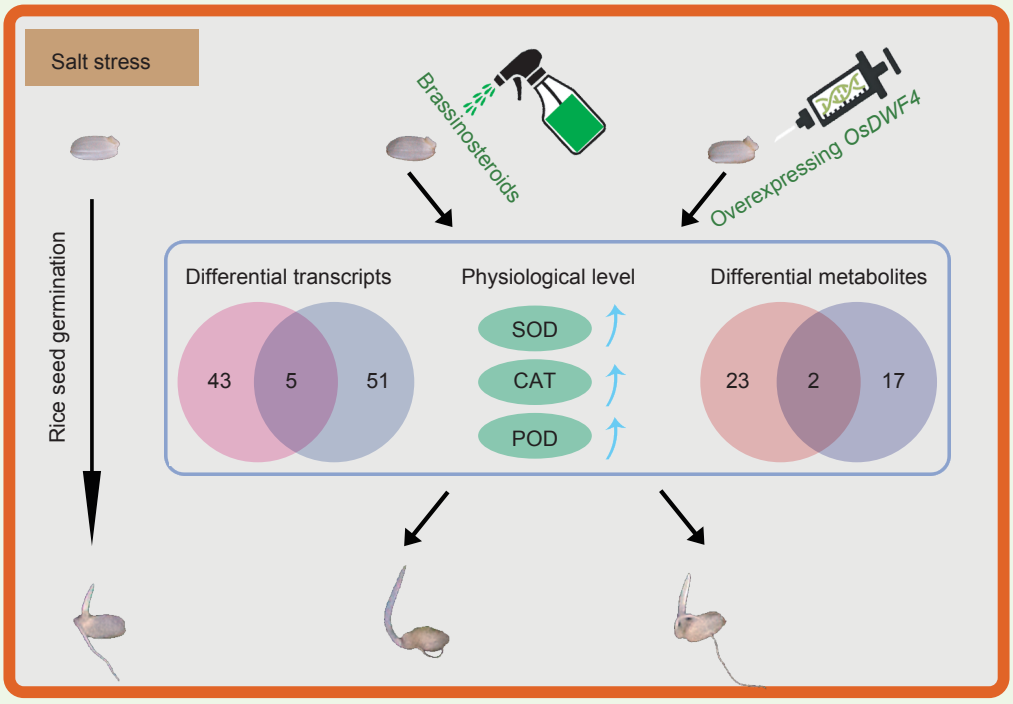
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Highlights

- Brassinosteroids (BRs) enhance the salt tolerance of rice seeds by improving their antioxidant capacity during germination.
- Five genes and two metabolites were identified as key contributors to this process.
- *OsDWF4* can promote the development and utilization of salt-tolerant rice germplasm.

Graphical abstract



SOD, superoxide dismutase; POD, peroxidase; CAT, catalase.

Received 14 April 2025; Received in revised form 16 June 2025; Accepted 24 June 2025; Available online 5 August 2025
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doi: 10.1016/j.jia.2025.08.008

Abstract

Seed germination initiates the plant life cycle, but it exhibits high sensitivity to salt stress, which is a significant environmental factor limiting rice production. Brassinosteroid (BR) is a growth-promoting phytohormone that mitigates various stresses in rice including salt, drought, and extreme temperatures. However, the mechanisms by which BR alleviates salt stress during seed germination remain inadequately characterized. This study demonstrates that seed-specific overexpression of *OsDWF4*, a rate-limiting gene in BR biosynthesis, enhances rice germination. The *DWF4-OX* lines, which have greater endogenous BR content in the seeds, showed better germination under salt stress, corroborating the results obtained through exogenous BR application. Antioxidant enzyme analyses demonstrated that BR enhances the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). Metabolomic analysis revealed that BR mitigates salt stress primarily through the biosynthesis of phenylpropanoids and secondary metabolites. Transcriptomic analysis indicated that both endogenous and exogenous BR share five co-regulated target genes and utilize a common biosynthetic pathway for stilbenoids, diarylheptanoids, and gingerols. These findings confirm the capacity of BR to enhance seed germination under salt stress and identified several BR-mediated targets for developing salt-tolerant rice varieties that are suitable for direct seeding cultivation.

Keywords: brassinosteroids, seed germination, *OsDWF4*, salt stress, multi-omics, rice

1. Introduction

Sustainable agricultural production faces multiple challenges, including escalating food demand, decreasing arable land availability, and the increasing scarcity of freshwater resources (He *et al.* 2019). Coastal and inland saline–alkali lands represent significant untapped resources with considerable potential for comprehensive utilization to potentially address the growing tensions between population needs, land availability, and food security (Wang *et al.* 2018; Zhang *et al.* 2018; Guo *et al.* 2025).

Salt stress is a major abiotic constraint limiting global agricultural sustainability, and it particularly affects rice (*Oryza sativa* L.) growth and productivity. The excessive accumulation of reactive oxygen species (ROS) under salt stress inhibits seed germination and seedling development, disrupts osmotic and ionic equilibrium, destabilizes redox homeostasis, impairs photosynthetic processes, and elevates cellular energy demands (Yang and Guo 2018; Ganie *et al.* 2021; Basu and Kumar 2024). In response to oxidative stress, rice synthesizes various ROS-detoxifying enzymes, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), which contribute to damage mitigation and cellular maintenance (Chen *et al.* 2024; Liu *et al.* 2024). Recent research has demonstrated that *SAT1/OsCYL4* performs a crucial function in enhancing saline–alkali tolerance through the ROS scavenging pathway (Li *et al.* 2025). The pseudo-histidine phosphotransfer protein OsPHP1 intensifies ion toxicity by suppressing antioxidant gene expression and osmotic regulators, consequently reducing yield (Yadav *et al.* 2025). Elucidating the regulatory mechanisms that mitigate salt stress is essential for enhancing seed germination and enabling direct rice seeding in saline soils (Mu *et al*. 2024; Dai *et al*. 2025).

Plant hormones play essential roles in coordinating growth and stress responses during seed germination. In rice, sodium chloride (NaCl) exposure inhibits germination by suppressing α-amylase gene expression, resulting in lower bioactive gibberellin (GA) levels (Liu *et al.* 2018). The resulting salt-induced reduction in starch mobilization directly results in delayed crop germination (Xiong *et al.* 2024). *OsHAK9* enhances seed germination under salt stress by inhibiting K^+ efflux, promoting Na^+ accumulation, and inducing transcription factors that suppress *OsGA2ox7* expression (Zeng *et al.* 2024). Abscisic acid (ABA) functions as a critical hormone in enhancing plant stress resistance (Wang *et al.* 2024). ABA-mediated salt tolerance regulation operates through two primary mechanisms: regulation of ABA synthesis and modulation of core ABA signaling (Wang W *et al.* 2016). The ABA biosynthesis genes *OsNCED3* and *OsNCED5* encode 9-*cis*-epoxycarotenoid dioxygenases that enhance salinity tolerance by increasing endogenous ABA levels (Liu C *et al.* 2021). SALT AND ABA RESPONSE ERF1 (OsSAE1) facilitates seed germination and seedling growth under salt stress by suppressing *ABSCISIC ACID-INSENSITIVE5* (*OsABI5*) expression (Li *et al.* 2022). Furthermore, Li *et al.* (2021) established that ABA-mediated salt tolerance depends on the activation of short-term brassinosteroid (BR) signaling.

BR plays a crucial role in various rice traits, including seed germination and stress resistance. OsGSK1 is a member of the GSK3/SHAGGY like protein kinase family that functions as a negative regulator in the BR signaling pathway. Knockout of *OsGSK1* enhances salt tolerance compared to the wild type (Koh *et al.* 2007). GW5-Like (GW5L), which enhances BR signaling by inhibiting GSK2 phosphorylation activity, provides salt stress resistance to rice seedlings by binding to salt-stress-responsive calmodulin OsCam1-1 (Tian *et al.* 2019). Our previous research demonstrated that the BR signaling transcription factor brassinazole-resistant 1 (OsBZR1) directly activates the transcription of *alpha-amylase isozyme 3D* (*RAmy3D*), thereby accelerating starch mobilization and promoting seed germination (Xiong *et al.* 2022b). Inhibition of the *serotonin N-acetyltransferase 2* (*SNAT2*) gene, which encodes the penultimate enzyme in melatonin biosynthesis, leads to a reduction in BR content during seed germination and increases salt stress tolerance (Hwang and Back 2019). Rice miR168a-5p responds to both BR signaling and salt stress during the germination and seedling stages through distinct downstream target

genes (Xia *et al.* 2023). However, the mechanism through which BRs enhance salt tolerance in germinating rice seeds remains poorly understood. This study demonstrates that elevating both exogenous and endogenous BR levels during germination enhances salt tolerance through brassinolide (BL) application and seed-specific overexpression of the BR biosynthesis rate-limiting enzyme OsDWF4, respectively. Our research comprehensively investigates the physiological and molecular mechanisms underlying BR-mediated germination under salt stress and proposes practical strategies, through biotechnology and exogenous BR application, to reduce salt-induced damage to rice seeds.

2. Materials and methods

2.1. Cultivation and identification of rice materials

This study used two lines of the BR synthesis-enhanced *DWF4-OX* rice and its wild-type control, the *japonica* cultivar Yandao 8 (Y8). The *DWF4-OX* lines specifically overexpress *OsDWF4* in seeds, with Y8 serving as the receptor. Additional details regarding the generation of the *pGt1::OsDWF4* transgenic lines 1 and 2 are available in our previous study (Li *et al.* 2018). All rice plants were cultivated under identical climatic and management conditions in the transgenic experimental field at Yangzhou University (Yangzhou, Jiangsu Province, China). Young leaves from the field were collected, pulverized in liquid nitrogen, and used for DNA identification. Mature seeds from high-yielding rice panicles were harvested for germination and salt tolerance evaluations.

Total DNA from rice leaves was extracted using the established CTAB method (Murray and Thompson 1980). Specific primers targeting the *hygromycin* gene were employed for PCR amplification of the desired sequence from rice genomic DNA to determine the genotype of the genetic material. The PCR products were separated using 2.0% agarose gel electrophoresis and visualized using the Bio-RAD UV 1000 Nucleic Acid Imaging System.

2.2. Seed germination analysis

Following sterilization and shelling of the rice seeds, germination experiments were conducted according to our previous procedures (Xiong *et al.* 2022b). Germination was determined by a radicle length exceeding 1 mm (Cheng *et al.* 2013). A NaCl concentration of 150 mmol L^{-1} was employed for salt treatment, while BL, the most active BR, was administered at 1 μmol L^{-1}. BL was dissolved in dimethyl sulfoxide (DMSO) and introduced 24 h after the NaCl treatment to examine its effect on seeds under salt stress. Daphylloside, obtained from Chengdu Pufeide Biotech Co., Ltd., was dissolved in ethanol.

2.3. Physiological experiments

The α-amylase activity in germinated seeds was evaluated using the starch plate assay. Seeds were sterilized with 70% (v/v) ethanol for 15 min, followed by two washes with Milli-Q water, and half-seeds without embryos were positioned on a 2% (w/v) agar plate with the cut surface contacting the agar. After 4 d of incubation in darkness at 28°C, the seeds were immersed in an iodine solution (0.1% I_2 (w/v) and 1% KI (w/v)) for 8 min. As α-amylase hydrolyzed the starch, an unstained halo formed around the seed, with halo size corresponding to α-amylase activity.

For measuring the endogenous BR content in germinated seeds, 0.5 g of sample powder was extracted, purified and analyzed *via* ultra-high-performance liquid chromatography-mass spectrometry, following the protocol described by Zheng *et al.* (2018).

The enzymatic activities of SOD, CAT, and POD in seeds were assessed using commercial kits from Suzhou Comin Biotechnology Co., Ltd., specifically the SOD-1-W Superoxide Dismutase Test Kit (WST-8 method), CAT-2-W Catalase Test Kit (Ammonium molybdate colorimetric method), and POD-1-Y Peroxidase Test Kit (Power-spectral method).

2.4. RNA sequencing (RNA-seq) and bioinformatics analysis

Library preparation used high-quality RNA (1 μg) extracted from germinated seeds, followed by high-throughput RNA sequencing on the Illumina Novaseq platform (San Diego, CA, USA). The sequencing process involved pooling various qualified libraries according to the required concentration and target data volume specifications. This generated 150 bp paired-end reads. Following quality trimming of the raw sequencing reads, the resulting data were mapped to the reference genome of *Oryza sativa japonica* (cv. Nipponbare, assembly IRGSP-1.0) from the Rice Annotation Project Database (RAP-DB; https://rapdb.dna.affrc.go.jp/) using TopHat2 alignment software. The DESeq2 R package (Love *et al.* 2014) was employed to analyze differential gene expression between the *DWF4-OX* and Y8 samples. Genes exhibiting $|\log_2(\text{fold change})| \geq 1.0$ and P-value<0.05 were classified as differentially expressed genes (DEGs) between *DWF4-OX1* and Y8.

2.5. qRT-PCR analysis

RNA was extracted from 25 germinated seeds of both the *DWF4-OX1* and Y8 samples using the RNAprep Pure Plant Kit (Tiangen, Beijing, China). Subsequently, 2 mg of pre-treated RNA was reverse transcribed using the SuperScript First Strand cDNA Synthesis System (Invitrogen, California, USA). Quantitative RT-PCR (qRT-PCR) was conducted on a MyiQ real-time system (Bio-Rad, California, USA) using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, Jiangsu, China). Each sample comprised three biological replicates, with *OsActin1* as the reference gene for normalization. Primer information is detailed in Appendix A.

2.6. Metabolomic profiling

The metabolomic analysis of *DWF4-OX1* and Y8 seeds was conducted with minor modifications to previously established methods (Zhou *et al.* 2022). The procedure involved metabolite extraction from samples and analysis *via* LC-MS/MS. Data processing and normalization were performed using Progenesis QI software (Nonlinear Dynamics, Newcastle, UK). Metabolite identification was based on secondary spectral information, cross-referenced with public

databases, including Lipidmaps (https://lipidmaps.org/), MassBank (http://www.massbank.jp/), HMDB (http://www.hmdb.ca/), and METLIN (https://metlin.scripps.edu/).

2.7. Data analysis

All experiments were conducted with a minimum of three replications. Statistical analysis employed analysis of variance (ANOVA) with the Duncan multiple-range test. GraphPad Prism 8 was used for graph processing and presentation.

3. Results

3.1. *OsDWF4* positively regulates rice seed germination

Previous research demonstrated that the specific overexpression of *OsDWF4* in seeds enhances rice yield and quality (Li *et al.* 2018). In this study, the *DWF4-OX* lines exhibited greater grain thickness and 1,000-grain weight, while grain length and width remained constant (Appendix B). DNA amplification verified the presence of the *hygromycin* gene in leaves of both *DWF4-OX* lines, confirming successful transformation (Appendix C). To examine the function of this gene in seed germination, subsequent studies were conducted using the *DWF4-OX* lines and their wild-type Y8. The qRT-PCR analysis revealed that *OsDWF4* expression was significantly elevated in seeds of the *DWF4-OX* lines at 2 hours after imbibition (HAI) (Appendix C). Germination assays demonstrated higher germination rates in the two *DWF4-OX* lines compared to the Y8 wild type (Fig. 1-A). Moreover, the shoot lengths of the germinated *DWF4-OX* lines exceeded those of Y8 (Fig. 1-B; Appendix D). A qualitative assessment of α-amylase activity between these rice lines, which is crucial for starch mobilization during germination, revealed larger halos around the *DWF4-OX* rice seeds in starch plate tests, indicating enhanced α-amylase production (Fig. 1-C and D). Early-stage germination studies demonstrated that BR signaling promotes starch degradation by upregulating *RAmy3D* expression (Xiong *et al.* 2022b). Accordingly, BL, a potent BR, increased *RAmy3D* expression by about twofold at 2 HAI (Fig. 1-E). Similarly, overexpression of *OsDWF4* in germinated seeds upregulated *RAmy3D* expression (Fig. 1-F). These results suggest that *OsDWF4* overexpression accelerates starch mobilization during germination, thereby enhancing seed germination.

3.2. Exogenous application and endogenous increase of BR relieve salt damage to germinated seeds

BR has been established as an enhancer of rice stress resistance, leading to an examination of its potential role in mitigating salt stress (Tian *et al.* 2019). Under NaCl treatment, the germination process of *DWF4-OX* seeds

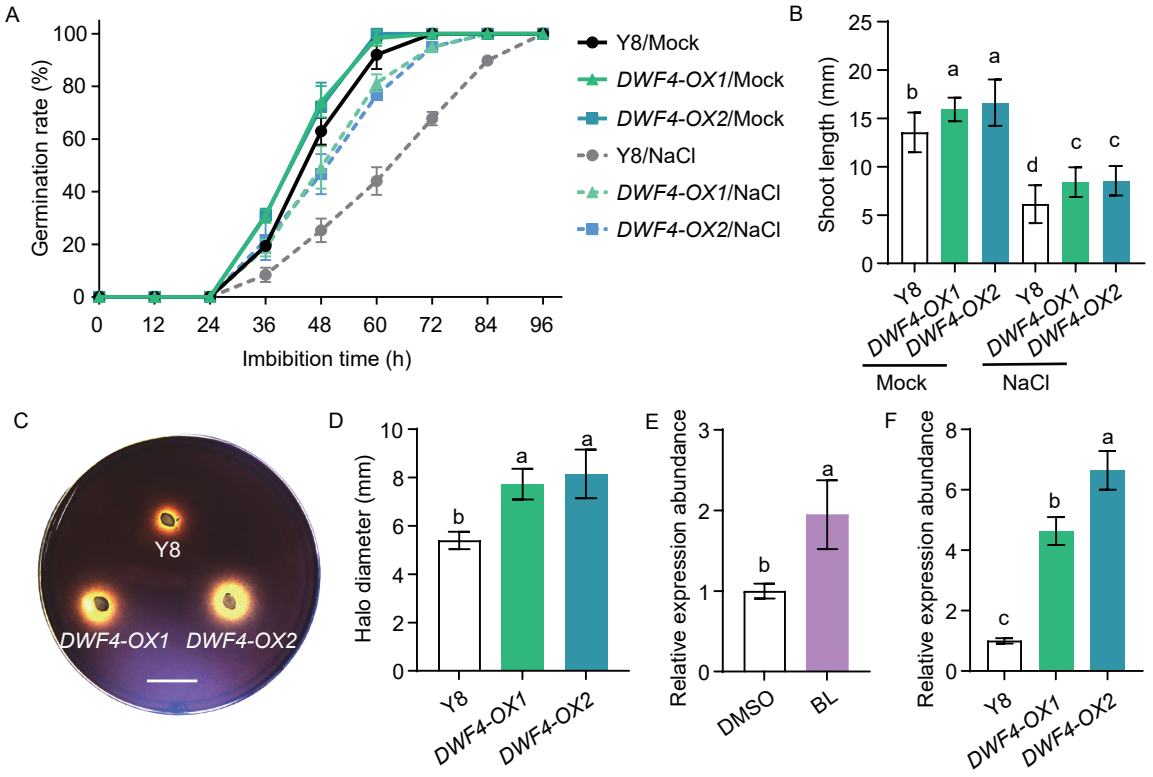


Fig. 1 *OsDWF4* promotes rice germination by regulating starch mobilization in seeds. A, germination rates from 0 to 96 hours after imbibition (HAI) of *DWF4-OX* and wild-type (WT) Yandao 8 (Y8) seeds. B, shoot lengths at 96 HAI. C, representative images comparing α-amylase activity in the seeds of the *DWF4-OX* and Y8 WT lines at 96 HAI. Bars=10 mm. D, quantitative analysis of halo diameters in starch plate tests. E and F, *RAmy3D* expression analysis at 2 HAI with BL and dimethylsulfoxide (DMSO) treatments and in seeds overexpressing *OsDWF4* and Y8 seeds. BL, the most active brassinosteroid (BR), was administered at 1 μmol L^{-1}. Error bars represent SD (n=3). Different letters indicate significant differences at P<0.05 according to Duncan's multiple range test. Each experiment was replicated at least three times.

progressed more rapidly than that of Y8 seeds (Fig. 1-A and B; Appendix D). Salt stress significantly amplified the difference in germination rates between the *OsDWF4-OX* lines and the Y8 wild type compared to the control (Fig. 1-A). At 96 HAI, overexpression of *OsDWF4* enhanced shoot length by approximately 1.20-fold under normal conditions and by 1.38-fold under salt stress (Fig. 1-B), indicating that *OsDWF4* contributes to rice salt tolerance.

The *DWF4-OX* lines demonstrated substantially higher BR contents compared to Y8, confirming that *DWF4-OX* germinated seeds provide a suitable model for greater endogenous BR synthesis (Fig. 2-A). A more pronounced increase in the BR content was observed in *OsDWF4-OX* seeds under salt stress compared to normal conditions (Fig. 2-A). Germination assays under salt stress revealed that *DWF4-OX* seeds achieved 100% germination about 12 h earlier than Y8 (Fig. 2-B). At 120 HAI, the shoot lengths of germinated *DWF4-OX* seeds exceeded those of Y8 (Fig. 2-C and D). To determine whether exogenous BL application affects germination under salt stress, BL was applied to NaCl-treated seeds for 24 h. The germination characteristics of BL-treated seeds paralleled those of *DWF4-OX* seeds, with both exhibiting enhanced germination rates and increased shoot lengths under salt stress (Fig. 2-B–D). Moreover, BL treatment suppressed *OsDWF4* expression during seed germination (Appendix E), consistent with the regulatory mechanism of

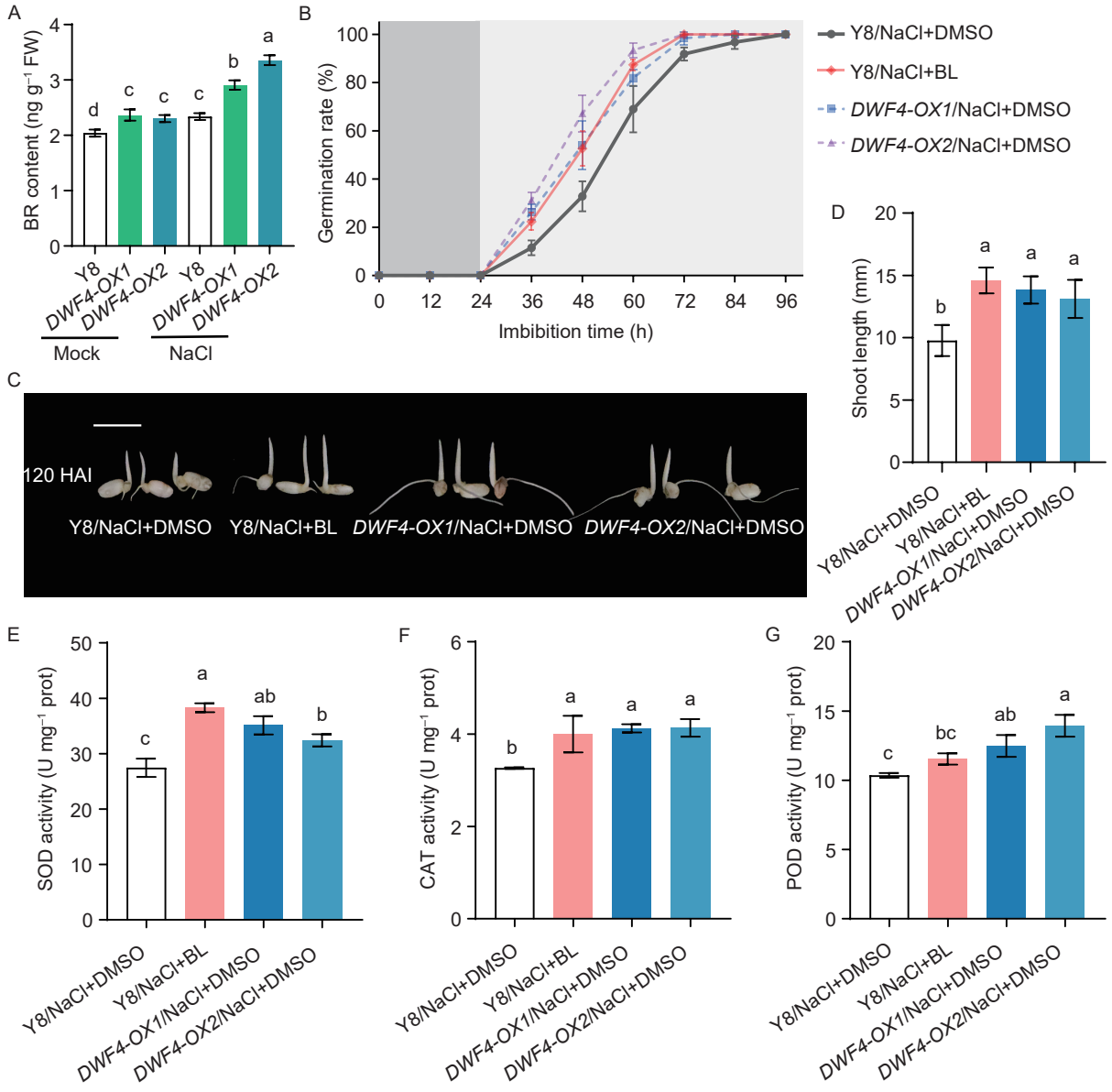


Fig. 2 Physiological and biochemical analysis of brassinosteroid (BR)-mediated seed germination under salt stress. A, BR contents in *DWF4-OX1*, *DWF4-OX2* and Yandao 8 (Y8) seeds after 36 h of treatment with either NaCl (150 mmol L^{-1}) or a Mock solution. Three experimental conditions were applied: Y8 treated with NaCl; *DWF4-OX* rice treated with NaCl; and Y8 exposed to BL (1 μmol L^{-1}) after 24 h of NaCl treatment. B–D, germination rates (B), diagram of germinated seeds at 120 hours after imbibition (HAI) (C), and shoot lengths at 120 HAI (D) for all treatments. E–G, analysis of antioxidant enzyme activities, including superoxide dismutase (SOD) (E), catalase (CAT) (F), and peroxidase (POD) (G). DMSO, dimethylsulfoxide. Error bars represent SD (n=3). Different letters indicate significant differences at P<0.05 according to Duncan's multiple range test. Each experiment was repeated at least three times.

feedback inhibition of BR biosynthesis. These findings suggest that elevated levels of endogenous or exogenous BR enhance rice salt tolerance during seed germination.

3.3. BR improves the antioxidant capacity in germinated seeds under salt stress

Salt stress triggers the accumulation of reactive oxygen species (ROS) in rice seeds, resulting in oxidative damage (Chen *et al.* 2024). In response, rice activates an antioxidant defense system that uses enzymes such as SOD, CAT, and POD to minimize damage (Liu H *et al.* 2021; Liu *et al.* 2024). The activities of these antioxidant enzymes were evaluated at 36 HAI with salt treatment. BL application enhanced the activities of SOD, CAT, and POD under salt stress (Fig. 2-E–G). Correspondingly, an elevated endogenous BR content through genetic modification enhanced the activities of these antioxidant enzymes (Fig. 2-E–G).

3.4. Metabolite profiling of salt-treated seeds rescued by BR

To examine the effects of BR on seed metabolites under salt stress, a metabolomic analysis was performed on rice seeds at 36 HAI. The comparison of seeds treated with or without BL identified 25 differentially abundant metabolites (DAMs) in salt-treated seeds (Fig. 3-A). A comparison between *DWF4-OX1* and Y8 revealed 19 DAMs in salt-treated seeds (Fig. 3-A). A Venn diagram analysis demonstrated that the expression patterns of two DAMs following the increases in both exogenous and endogenous BR were consistent: daphylloside exhibited downregulation, while azithromycin (INN) showed upregulation (Fig. 3-A–C; Appendix F). Although treatment with daphylloside showed no effect on germination under normal conditions, it inhibited germination under salt stress by reducing the germination rate and shoot length of seeds (Appendix G).

A Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was conducted to investigate the potential functions of the DAMs. This analysis indicated that phenylpropanoid biosynthesis, comprising 33 metabolites, represented the most significantly enriched pathway (Fig. 3-D; Appendix H). Furthermore, pathways including cysteine and methionine metabolism, containing 47 metabolites, and

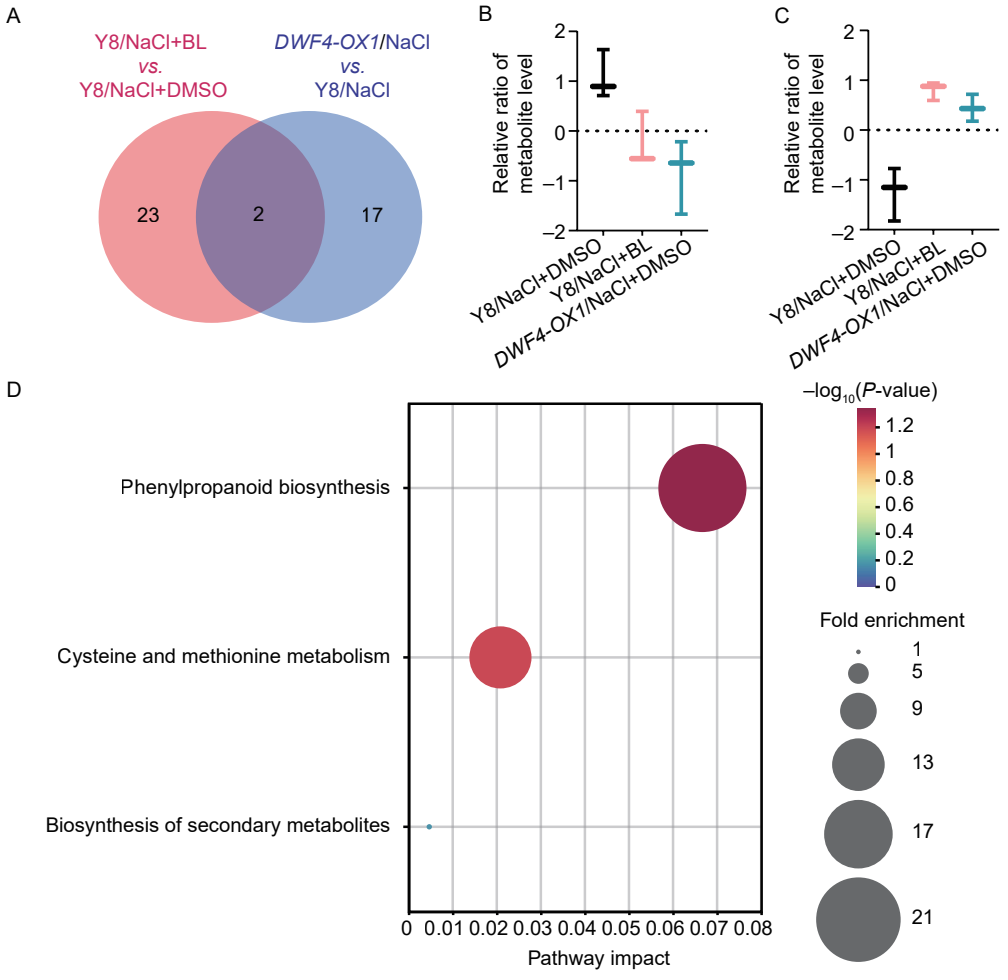


Fig. 3 Metabolomic analysis of brassinosteroid (BR)-mediated germination under salt stress in seeds treated for 36 h. A, Venn diagram of overlapping and differentially abundant metabolites (DAMs) from comparisons between Yandao 8 (Y8)/NaCl, Y8/NaCl+BL (the most active BR, was administered at 1 μmol L^{-1}), and *DWF4-OX1*/NaCl. B and C, abundances of the two DAMs daphylloside and azithromycin (INN). DMSO, dimethylsulfoxide. D, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of significantly enriched metabolic pathways.

biosynthesis of secondary metabolites, encompassing 623 metabolites, emerged as the crucial metabolic processes through which BR mitigates salt stress damage (Fig. 3-D; Appendix H).

3.5. BR-mediated transcriptional regulation of salt-responsive genes in rice seeds

The transcriptomic analysis identified genes regulated by BR in salt-stressed seeds. Under salt treatment, 48 DEGs were identified in response to BL treatment, comprising 28 downregulated and 20 upregulated genes (Fig. 4-A; Appendix I). In *DWF4-OX1* seeds, 56 genes showed significant expression changes compared to Y8 seeds, with 15 downregulated and 41 upregulated (Fig. 4-B; Appendix J). Among these, *OsXIP*, which encodes a xylanase inhibitor protein, demonstrated greater expression under NaCl treatment (Tokunaga and Esaka 2007). The qRT-PCR analysis confirmed elevated *OsXIP* expression in *DWF4-OX* rice compared to wild-type Y8 rice under salt stress, corroborating the RNA-seq data (Appendices J and K). A Venn diagram analysis identified five genes with similar expression patterns in both groups, with two downregulated and three upregulated (Fig. 4-C). Specifically, *Os07g0526400* (*polyketidesynthase15*, *OsPKS15*) and *Os07g0638600* (*classIIIperoxidase105*, *prx105*) showed reduced expression, while *Os02g0288100* (*pectinmethylesterase8*, *OsPME8*), *Os04g0602500*, and *Os01g0341750* exhibited increased expression (Fig. 4-D). The qRT-PCR validation of these five DEGs in germinated seeds confirmed expression patterns consistent with the RNA-seq data, validating the reliability of the RNA-seq results (Fig. 4-E).

To elucidate the functions of the identified DEGs, Gene Ontology (GO) and KEGG enrichment analyses were performed for both the exogenous BR application and endogenous BR increase samples. GO analysis of the comparison Y8/NaCl+BL *vs*. Y8/NaCl+DMSO identified the five most enriched terms as cell wall, external encapsulating structure, oxygen binding, phytoene dehydrogenase activity, and root hair elongation (Fig. 5-A). In the *DWF4-OX1*/NaCl *vs*. Y8/NaCl comparison, the top five enriched terms were pectinesterase activity, carboxylic ester hydrolase activity, plant-type vacuole membrane, pectinesterase inhibitor activity, and cellular response to salt stress (Fig. 5-B). The KEGG enrichment analysis revealed a significant pathway common to both Y8/NaCl+BL *vs*. Y8/NaCl+DMSO and *DWF4-OX1*/NaCl *vs*. Y8/NaCl: the stilbenoid, diarylheptanoid, and gingerol biosynthesis pathway (Fig. 5-C and D; Appendices

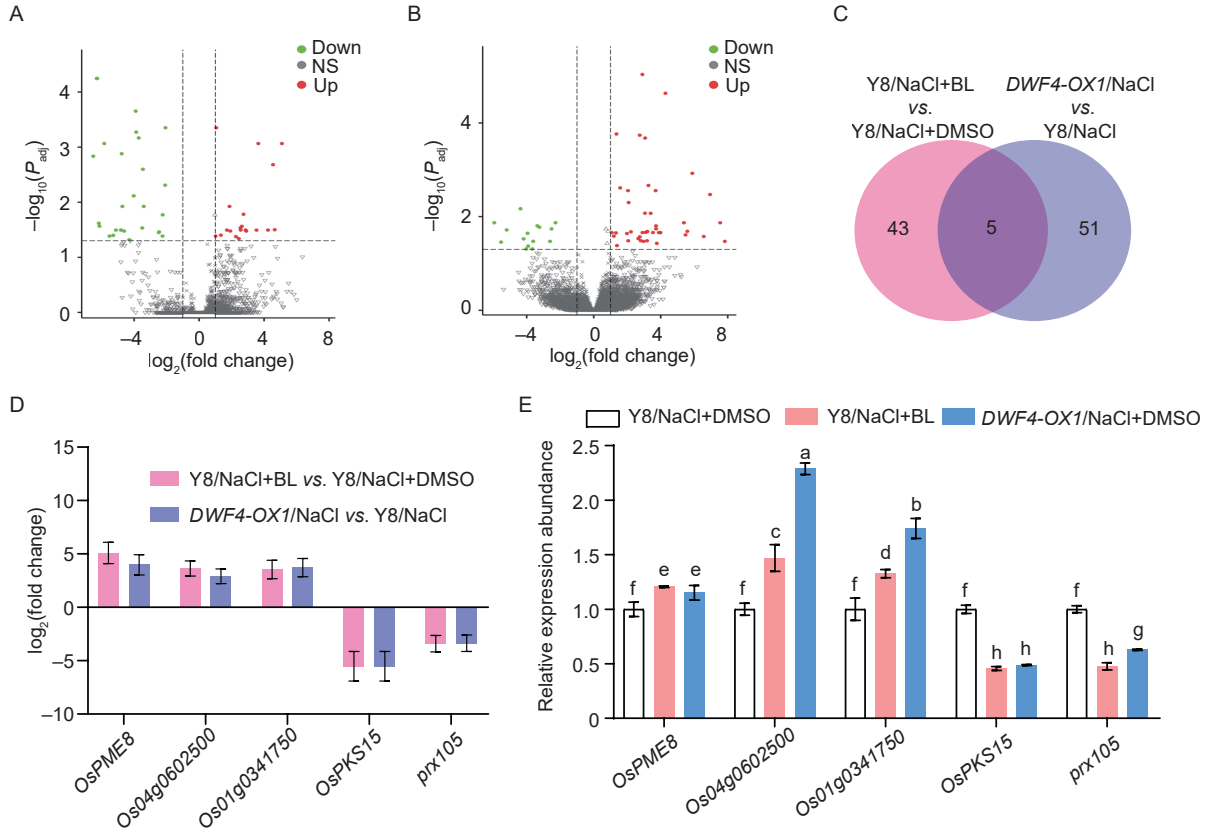


Fig. 4 Transcriptomic identification of brassinosteroid (BR)-responsive genes under salt stress using seeds treated for 36 h. A and B, Volcano plots of differentially expressed genes (DEGs) in Yandao 8 (Y8)/NaCl+BL *vs*. Y8/NaCl+dimethylsulfoxide (DMSO) (A) and *DWF4-OX1*/NaCl *vs*. Y8/NaCl (B). Solid circles represent the P_{adj}<0.05 and |$\log_2$(fold change)|≥1.0 criteria. Down, down-regulated genes; NS, not significant; Up, up-regulated genes. C, Venn diagram showing overlapping DEGs from comparisons of Y8/NaCl, Y8/NaCl+BL (the most active BR, was administered at 1 μmol L^{-1}) and *DWF4-OX1*/NaCl. D, relative expression abundances of five DEGs in the RNA-seq analysis. E, validation of RNA-seq data for the five DEGs by qRT-PCR. *Actin1* was used as an internal control for normalization. Error bars represent SD (*n*=3). Different letters indicate significant differences at *P*<0.05 according to Duncan's multiple range test. Each experiment was replicated three or more times.

L and M). Furthermore, alpha-linolenic acid metabolism, linoleic acid metabolism, and phenylpropanoid biosynthesis were enriched in the exogenous BR treatment, while amino sugar and nucleotide sugar metabolism and thiamine metabolism showed enrichment in the case of increased endogenous BR (Fig. 5-C and D).

3.6. Correlation analysis of transcriptomic, metabolomic, and physiological data

An analysis of Pearson correlation coefficients revealed the relationships between representative differential genes, metabolites, and physiological indicators in salt-stressed seeds (Fig. 6). The heat map illustrates correlations among the five DEGs, two DAMs, germination rate at 48 HAI, and antioxidant enzymes (SOD, CAT, and POD). This analysis demonstrated significant positive correlations between these factors and germination rate, as well as the antioxidant enzymes SOD, CAT, and POD. Daphylloside demonstrated a strong positive correlation with *prx105* expression, but significant negative correlations with SOD and *Os01g0341750*. INN and *OsPKS15* showed no significant correlations with the other analyzed parameters. The three BR-induced upregulated genes under salt stress conditions exhibited positive correlations with each other and positive effects on antioxidant enzyme activities. Conversely, the downregulated *prx105* gene exhibited negative correlations with both antioxidant enzymes and the upregulated genes.

4. Discussion

4.1. BR assists rice seeds against salt stress during germination

Salt stress significantly impairs crop productivity, and enhancing crop salt tolerance through plant hormone pathways presents a viable strategy. As a hormone associated with stress resistance, ABA has been extensively investigated regarding salt tolerance (Wang *et al.* 2024; Liu *et al.* 2025). Knockout of *ABA8′-Hydroxylase* (*OsABA8ox1*), which degrades ABA, delays germination but increases seed survival after exposure to 150 mmol L^{-1} NaCl (Fu *et al.* 2022). OsABI5, a critical regulator in the ABA signaling pathway, adversely affects seed germination, seedling growth, and salt tolerance in rice (Li *et al.* 2022). qSE3 enhances salt tolerance by promoting ABA accumulation and activating ABA signaling pathway genes in salt-stressed rice seeds, thereby reducing ROS accumulation (He *et al.* 2019). Furthermore, research indicates that the OsGAPC1-OsSGL module negatively influences salt tolerance by regulating ABA biosynthesis and stress-related gene expression in rice (Jiang *et al.* 2024).

This investigation examined BR, a plant hormone that interacts with ABA, and proposes enhancing salt stress resistance in rice seeds through exogenous BR application and endogenous BR enhancement. Germination analyses demonstrated that BL treatment enhanced the germination rate and shoot length of rice seeds under salt stress (Fig. 2-B–D). Comparable results were observed in *DWF4-OX* seeds, where an elevated endogenous BR content, resulting from greater *OsDWF4* expression, improved seed germination (Fig. 2-A–D). The increased activities of SOD, CAT, and POD in BR-treated and BR synthesis-enhanced seeds contributed to these improvements (Fig. 2-E–G). Moreover, BL treatment suppressed *OsDWF4* expression in germinating seeds (Appendix E), aligning with previous findings in rice seedlings (Sharma *et al.* 2013).

4.2. BR-regulated metabolites and genes associated with salt tolerance were identified by multi-omics analysis

Metabolomics is an advanced multi-omics technique that reveals crucial insights into stress responses and their

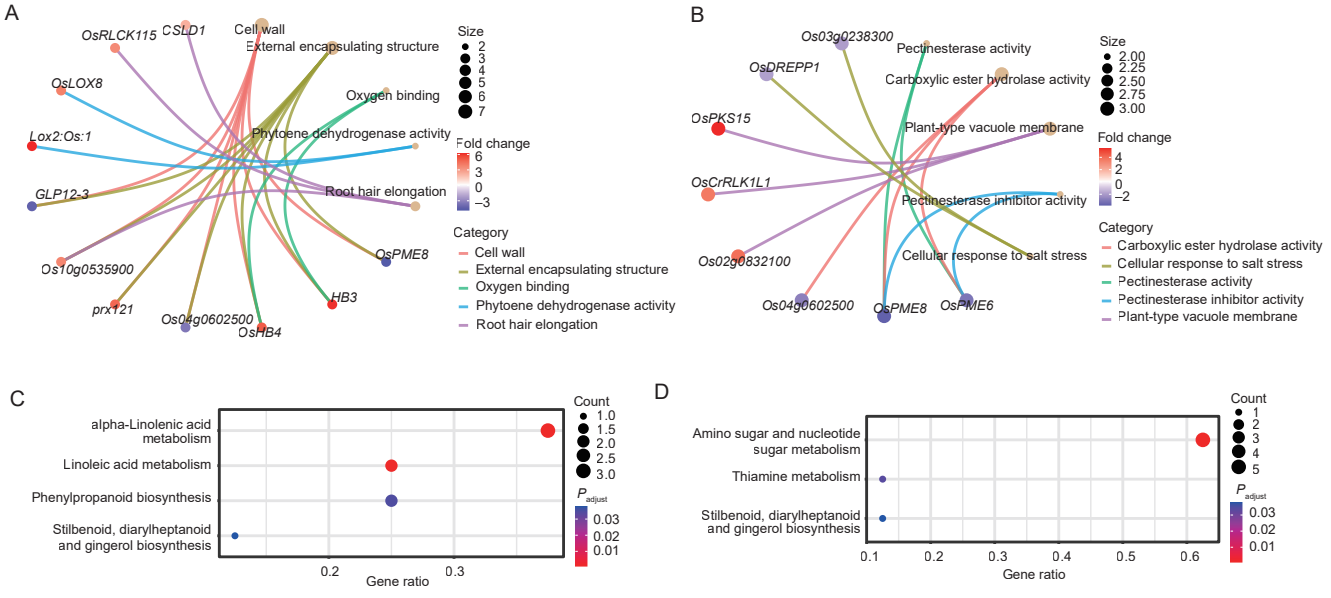


Fig. 5 Functional annotations of differentially expressed genes (DEGs) in the transcriptomic analysis. A and B, Gene Ontology (GO) classification of DEGs in Yandao 8 (Y8)/NaCl+BL *vs*. Y8/NaCl+dimethylsulfoxide (DMSO) (A) and *DWF4-OX1*/NaCl *vs*. Y8/NaCl (B). C and D, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment for DEGs in Y8/NaCl+BL *vs*. Y8/NaCl+DMSO (C) and *DWF4-OX1*/NaCl *vs*. Y8/NaCl (D).

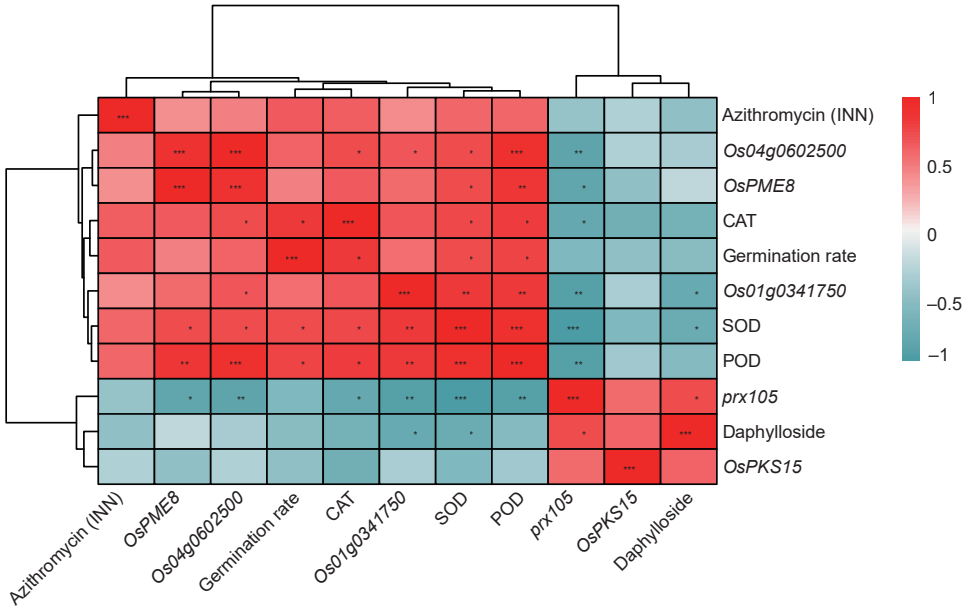


Fig. 6 Correlation analysis integrating transcriptomic, metabolomic, and physiological data. Spearman's rank or Pearson's correlation was used to analyze the correlations between the relative abundance of biochemical parameters and physiological data. Red indicates a positive correlation, and green indicates a negative correlation. SOD, superoxide dismutase; POD, peroxidase; CAT, catalase. The color intensity represents the strength of each correlation. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

underlying physiological mechanisms through metabolite profile analysis. The metabolomic analysis identified two overlapping DAMs in both BR-treated seeds and BR synthesis-enhanced seeds (Fig. 3-A). Under the influence of BR, daphylloside levels decreased while INN levels increased in salt-stressed seeds (Fig. 3-B and C). Daphylloside treatment further inhibited seed germination under salt stress (Appendix G). The KEGG enrichment analysis of metabolomic data identified the phenylpropanoid biosynthesis pathway as crucial in BR-mediated salt tolerance (Fig. 3-D; Appendix D). Phenylpropanoid-derived compounds, including flavonoids, stilbenes, lignin, and various phenolic acids, enhance salt tolerance in plants (Yu *et al.* 2023).

Notably, this pathway was also enriched in our transcriptomic analysis. The phenylpropanoid biosynthesis pathway exhibited significant alterations in response to exogenous BR treatment, while no such changes were observed with endogenous BR enhancement (Fig. 5-C and D). Transcriptomic data from Gao Y *et al.* (2024) demonstrated that the AP2/ERF transcription factor *OsRAV1*, which positively regulates salt tolerance, controls seed vigor during rice germination through the phenylpropanoid biosynthesis pathway. The GO and KEGG analyses revealed that exogenous and endogenous BR enhanced salt tolerance in germinating seeds through distinct pathways, except for the stilbenoid, diarylheptanoid, and gingerol biosynthesis pathway (Fig. 5; Appendices L and M). Previous studies have identified this pathway as being associated with drought and salt stress responses in plants (Wang X *et al.* 2016; Qian *et al.* 2021). Significantly, five credible DEGs were identified from the two comparison groups, so they potentially serve as downstream targets for BR-mediated mitigation of salt stress in germinating seeds (Fig. 4-C and D; Appendices I and J). For instance, *prx105* is a Class III peroxidase family member that demonstrates potential in ROS scavenging activity. A functionally similar gene, *OsPrx30*, has been shown to mediate ROS accumulation induced by bacterial blight in rice (Liu H *et al.* 2021). Our correlation analysis further confirmed a negative correlation between *prx105* expression and antioxidant enzyme activities (Fig. 6).

4.3. *OsDWF4* can be used for salt-tolerant germplasm development and utilization

BRs play crucial roles in regulating various traits in rice, including leaf angle, seed germination, and stress resistance, presenting opportunities for their use in crop breeding and improvement (Xiong *et al.* 2022a; Zhang *et al.* 2023, 2024; Gao X *et al.* 2024; Yang *et al.* 2024). Recently, Yin *et al.* (2025) emphasized the potential of utilizing the crosstalk between BRs and other plant hormones to develop high-yielding and stress-resistant crop varieties. Under salt stress conditions, 24-epibrassinolide, an active by-product of brassinolide biosynthesis, contributes to reduced ROS production and the maintenance of ionic homeostasis (Tanveer *et al.* 2018). BR-defective barley demonstrates increased susceptibility to stress under harsh field conditions (Dockter and Hansson 2015). The BR biosynthesis regulator *OsGSR1* moderately activates BR signaling to enhance ABA-mediated salt tolerance during the rice seedling stage (Li *et al.* 2021). However, excessive BR enhancement may be undesirable, as its pleiotropic effects can result in issues such as excessively wide leaf angles that would hinder dense planting. Therefore, fine-tuning BR content and signaling through the manipulation of BR synthetic pathway branches or downstream genes represents an effective strategy for modern crop breeding (Tong and Chu 2018; Zhao *et al.* 2018; Tian *et al.* 2021; Lu *et al.* 2025).

To address the dose-dependent effects of BR, complementary BR synthesis pathways regulated by *OsDWF4* were examined to investigate salt tolerance during rice germination. Previous research has shown that rice overexpressing *OsDWF4* demonstrates higher yields, and the mutant of *OsDWF4* is better suited for high-yield conditions at various plant densities due to its erect leaves (Sakamoto *et al.* 2006; Li *et al.* 2018). Our findings indicate that *OsDWF4* regulates seed germination by promoting starch mobilization (Fig. 1). Like with BL treatment, *DWF4-OX* seeds exhibited enhanced salt tolerance (Fig. 2-A–C). Notably, overexpressing *OsDWF4* promoted seed germination more effectively under salt stress than under normal conditions, compared with the Y8 wild type (Fig. 1-A and B). This enhancement may be partially attributed to the higher BR content induced by salt (Fig. 2-A). These findings support the potential of *OsDWF4* as a target for improving both yield and salt tolerance during germination while highlighting the importance of careful BR synthesis management. Future research should address salt stress challenges in direct-seeded rice by identifying beneficial alleles or using tissue-specific promoters to precisely regulate *OsDWF4* expression. This approach aligns with recent strategies to optimize plant height through beneficial haplotypes, enabling the simultaneous improvement of rice yield and salt tolerance (Quan *et al.* 2024).

5. Conclusion

Whether applied externally or enhanced endogenously through overexpression of *OsDWF4*, BRs can promote rice seed germination under salt stress conditions. Our analysis using a multi-omics approach revealed substantial alterations in the biosynthetic pathways of stilbenoid, diarylheptanoid, gingerol and phenylpropanoid. Furthermore, five downstream BR-regulated targets were identified as essential components contributing to enhanced salt tolerance in germinated seeds.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (32301853), the Natural Science Foundation of Jiangsu Province, China (BK20230715), the Basic Science (Natural Science) Research Project of Higher Education Institutions of Jiangsu Province, China (23KJB210013), the Yellow Sea Wetland Project, China (HHSDKT202303), the Programs from the Government of Jiangsu Province, China (JBGS[2021]001 and PAPD), and the School-level Research Projects of the Yancheng Institute of Technology, China (xjr2022042). We would like to thank EditChecks (https://editchecks.com.cn/) for providing linguistic assistance in the preparation of this manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Appendices associated with this paper are available at https://doi.org/10.1016/j.jia.2025.08.008

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