Rice

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Multi-omics-Based Construction of ncRNA-Gene-Metabolite Networks Provides New Insights Into Metabolic Regulation Under Salt Stress in Rice

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Abstract

Rice (*Oryza sativa* L.), one of the most vital staple crops globally, suffers severe yield losses due to metabolic dysregulation under salt stress. However, the systemic mechanisms by which non-coding RNAs (ncRNAs) coordinately regulate metabolic reprogramming remain elusive, and the genotype-specific regulatory networks in salt-tolerant cultivars are poorly characterized. To address this, we performed metabolomic analysis using ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) across different rice varieties under salt stress, identifying 327 metabolites, with the most notable fluctuations observed in lipids, polyamines, and phenolamides. The salt-tolerant variety Pokkali exhibited 51.96% and 31.37% fewer differentially accumulated metabolites (DAMs) in the shoots and roots respectively, compared to the salt-sensitive variety Nipponbare (NIP), which explains its superior salt-tolerant phenotype from a metabolic homeostasis perspective. Transcriptome profiling revealed 18,597 differentially expressed genes (DEGs), with 70.8% showing genotype-specific expression patterns. Pokkali-specific DEGs were markedly enriched in salt-responsive pathways, including reactive nitrogen species scavenging and ion compartmentalization. By integrating long non-coding RNA (lncRNA) and microRNA (miRNA) sequencing data, we constructed a four-tiered regulatory network comprising 6,201 DEGs, 458 miRNAs, 970 DElncRNAs, and 177 metabolites. In the regulatory network, Osa-miR408-3p was identified as a negative regulator of *Os03 g0709300* expression. Network analysis revealed that 21 polyamine and phenolamides biosynthesis-related genes were co-regulated by eight miRNAs, each forming a feedback loop with 2–11 lncRNAs. This study constructed a four-way cascade of "lncRNA-miRNA-mRNA-metabolite", and proposed a new concept of ncRNA-mediated "network regulation instead of single-gene effect".

Keywords *Oryza sativa* L., Salt stress, Multi-omics, Non-coding RNAs, Metabolic regulatory network

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Introduction

Rice (*Oryza sativa* L.) is one of the major food crops and its yield is mainly determined by traits such as panicle number, number of grains per panicle and grain weight (Dong et al. 2020). The grains are rich in substances that are beneficial to human health, including carbohydrates, fats, proteins and minerals (Abu-Ria et al. 2023). In the actual production process, rice yield is often affected by many environmental factors, such as soil moisture, ambient temperature and soil salinity (Azad et al. 2024). Under high salt concentration in soil, rice roots can produce osmotic stress, and ionic toxicity and reactive oxygen species (ROS) accumulation, thus restricting rice yield (Wang et al. 2015). Exploiting more genetically salt-tolerant resources to develop salt tolerant rice cultivars will greatly contribute to global food security. With the continuous development of sequencing technology, using transcriptome sequencing data to explore the metabolic pathways involved in plant responses to salt stress and identifying salt-tolerant candidate genes is of great significance for cultivating rice varieties with stress resistance and salt tolerance. It is of great significance for cultivating rice varieties with stress resistance and salt tolerance.

Plants are replete with various metabolites, which not only play pivotal roles participate in growth and development but also regulate adaptability to the environment. (Wang et al. 2019; Yang et al. 2022a, b; Cao et al. 2024). The metabolites have been identified in rice populations and its whole growth period, including lipids, polyamines, phenolamides, amino acids and flavonoids (Chen et al. 2016; Yang et al. 2024a, b, c). Many studies have shown that the biosynthesis of metabolites could regulate plant tolerance to salt stress. In tomato, the accumulation of polyamines and phenolamides has been shown to bolster the antioxidant system, and the by-product H_2O_2 could act as a second messenger to activate mitogen-activated protein kinase cascade and stress response genes (Sun et al. 2024). Both flavonoids and vitamin B5 had the ability to clear ROS, and their biosynthesis and accumulation could alleviate the death of plant cells under salt stress (Su et al. 2024). In wheat, an extracellular barrier could be formed by enhancing the biosynthesis of cutin and suberin, which could limit the flow of Na^+ into cells and diminish the ionic toxicity under salt stress (Wang et al. 2024a, b). In rice, regulating arbutin metabolism could promote ROS clearance induced by salt stress to enhance salt tolerance (Chen et al. 2024). Given that the salt tolerance of plants is regulated by various metabolic pathways, the analysis of metabolic changes induced by salt stress can help the mining of salt resistant metabolites.

In recent years, multi-omics integrated analysis has established a systematic research framework for elucidating rice salt stress response mechanisms by combining transcriptional-level gene expression dynamics with metabolite accumulation patterns. Recent studies revealed that *OsBCAT2* regulates the degradation of branched-chain amino acids and promotes vitamin B5 synthesis, thereby positively modulating rice salt tolerance (Sun et al. 2024). Additionally, *OsDRAP1* overexpression enhances salt tolerance by upregulating ion transport and stress-responsive genes, promoting the accumulation of metabolites such as proline and ascorbic acid, and coordinating redox homeostasis with amino acid/carbohydrate metabolic pathways. These findings provide a theoretical foundation for the precise identification of salt-tolerant genetic resources and the breeding of high-quality rice varieties (Wang et al. 2021).

It has been ascertained that ncRNA can exert an impact on the transcription process of genes. miRNA can bind to mRNA to prevent the translation process, lncRNA, when acting in cis, can modulate the expression of neighboring and downstream genes, and when operating in trans, they can interact with DNA, RNA and proteins to influence transcription (Zhao et al. 2022). They can also compete with mRNA to bind miRNA and relieve transcriptional inhibition (Meng et al. 2023). It has been widely reported that ncRNA regulates the synthesis of metabolites through gene regulation. microRNA858 represses the *SbMYB47* and regulates flavonoid biosynthesis in *Scutellaria baicalensis* (Yang et al. 2024a, b, c). The lncRNAs lnc10 and lnc11 regulate flavonoid biosynthesis in *Ginkgo biloba* (Li et al. 2024). Moreover, miRNA can jointly modulate metabolic processes in plants with lncRNA. eTM-miR858-MYB62-like module regulates anthocyanin synthesis in *Malus spectabilis*. miRNA-mRNA regulatory module plays an important role in the regulation of salt tolerance of rice, miR172-OsIDS1 and miR396b-GRF6 can regulate ROS homeostasis to enhance salt tolerance in rice (Cheng et al. 2021; Yuan et al. 2024). However, the synergistic effect of miRNA and lncRNA under salt stress is rarely reported, especially the regulation of metabolic synthesis. Therefore, it is necessary to construct a ncRNA-mediated metabolic regulatory network for in-depth exploration of the complex metabolic regulatory mechanisms under salt stress.

In this study, we compared the metabolic and transcription profiles of three rice varieties (NIP, Pokkali, and 9311) under salt stress. Firstly, a total of 327 metabolites were identified across all the samples. Notably, among these metabolites, lipids, polyamines and phenolamides were found to be readily induced by salt stress. At the same time, Pokkali had the least metabolic changes and showed a more stable metabolic process than NIP and 9311. At the transcriptional level, although Pokkali and 9311 had more stable transcription processes after salt stress, NIP also had some reported salt-tolerant genes that are specifically up-regulated, indicating that the transcriptional regulation process of rice was varietal

specific. To further analyze transcriptional differences among varieties, 458 miRNAs and 3,316 lncRNAs were identified across the whole genome. Through comparative analysis, we found that ncRNAs can affect the salt tolerance of different rice through variety-specific expression patterns, and also mediate a variety of metabolic pathways in rice to respond to salt stress. Therefore, the metabolic network regulated by ncRNA was constructed, including 6,201 DEGs, 458 miRNAs, 970 DElncRNAs, and 177 metabolites, which elucidated the complex metabolic regulatory mechanisms under salt stress. Overall, this study comprehensively analyzed the differential response of different rice varieties to salt stress from the aspects of metabolic level, transcriptional level and transcriptional regulation level, and also provided a new research method for the complex salt tolerance regulation mechanism of rice.

Materials and Methods

Plant Materials and Growth Conditions

Three rice varieties (NIP, Pokkali, and 9311) were employed in this study. The rice materials were hydroponically cultivated in the greenhouse of the Sanya Nanfan Research Institute of Hainan University. The cultivation parameters were set as follows: the light intensity was 30,000 lx, the temperature was 28 ℃, the humidity was 70%, and the light cycle was changed every 12 h.

RNA Extraction and Gene Expression Analysis

Total RNA was extracted from the shoots and roots of NIP, Pokkali, and 9311 plants with or without 150 mM NaCl for 72 h. There were three biological replicates in each group, including control and treatment. Approximately 0.1 g of the sample powder was used for RNA extraction using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, cDNA synthesis was carried out using the ToloScript All-in-one RT EasyMix for qPCR kit (TOLOBIO). Real-time fluorescence quantitative PCR (qRT-PCR) analysis was conducted using the 2 × Q3 SYBR qPCR Master Mix (Universal) kit (TOLOBIO) and following the kit instructions. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Tobacco leaf total RNA extraction and reverse transcription experiments were performed using the same experimental method as for rice.

Metabolite Detection

In metabolome analysis, a combination of targeted and non-targeted metabolic methods based on ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) was used to analyze the samples (Chen et al. 2013; Luo et al. 2016). Nontargeted metabolic profiling analysis was performed using the 6560 Ion Mobility LC/Q-TOF system (Agilent, Santa Clara, CA). Data acquisition was conducted under the following conditions: ESI source in positive ion mode, drying gas (N_2) temperature of 250 °C, drying gas flow rate of 8 L/min, nebulizer gas (N_2) pressure of 35 psi, sheath gas (N_2) temperature of 375 °C, sheath gas flow rate of 11 L/min, capillary voltage of 3500 V, fragmentor voltage of 400 V, and mass scan range from 50 to 1500 Da. Data-dependent MS/MS acquisition was performed in the 'Auto MS/MS' mode.

Targeted metabolomics analysis of amino acids, polyamines, and phenolamides was conducted using dynamic multiple reaction monitoring (dMRM) mode on a 6495 triple quadrupole LC/MS system (Agilent, Santa Clara, CA). ESI source parameters were as follows: drying gas (N_2) temperature of 200 °C, drying gas flow rate of 14 L/min, nebulizer gas (N_2) pressure of 35 psi, sheath gas (N_2) temperature of 250 °C, sheath gas flow rate of 11 L/min, capillary voltage of 4000 V, high-pressure RT at 150 V, and low-pressure RF at 60 V. All data acquisitions were performed using MassHunter Workstation Data Acquisition Software.

Differentially Accumulated Metabolites Analysis

In our metabolite analysis, we conducted biological replicates, with $n = 3$ samples in each group. Taking three rice varieties (NIP, Pokkali, and 9311) as the research objects, the cumulative metabolites before and after salt treatment were compared. DAMs were screened based on the importance of variables with p-value < 0.05 and fold changes ≥ 2 or ≤ 0.5.

Construction of Strand-Specific Libraries

For lncRNA sequencing, total RNA was used as input material for the RNA sample preparations. Ribosomal RNA was removed from total RNA by using specific probes, then RNase H and DNase I were used to digest samples. After adenylation of 3' ends of DNA fragments, adaptors with hairpin loop structure were ligated to prepare for hybridization. After purification and amplification, harvested PCR products were purified. After the library construction was completed, the qualified libraries were pooled and sequenced on sequencing platforms according to effective library concentration and the required data amount.

sRNA Library Construction and Sequencing

For small RNA (sRNA) sequencing, total RNA was used as input material for the RNA sample preparations. Briefly, 3' and 5' adapters were ligated to 3' and 5' end of small RNA, respectively. Then the first strand cDNA was synthesized after hybridization with reverse transcription primer. The double-stranded cDNA library was generated through PCR enrichment. After purification and

size selection, libraries with insertions between18 to 40 bp were ready for sequencing.

Genome-Wide Identification of miRNAs

Adapters and low-quality data were removed from sRNA raw sequencing data by cutadapt (v3.1) and fastp (v0.23.4) (Breitwieser et al. 2019; Chen et al. 2018a, b). miRDeep-P2 was used to identify and quantify miRNA in the whole genome according to the latest plant miRNA identification criteria (Kuang et al. 2019). The main steps include: (1) Other types of ncRNAs were removed; (2) reads were compared to the reference genome (IRGSP-1.0); (3) The mapped reads were filtered by count to select sequences with high confidence; (4) Precursor sequence was predicted; (5) Precursor and maturation sequence information of miRNA was output. Based on two databases of known miRNA families, namely miRBase and PmiREN (Griffiths-Jones et al. 2006; Guo et al. 2022). The identified miRNAs were classified into families according to the obtained results and counts per million were used as the quantitative unit.

Bioinformatic Identification of lncRNAs

Adapter and low-quality data were removed from raw reads with fastp (v0.23.4). Then, the clean reads were compared to the rice genome by HISAT2 (v2.2.1) (Kim et al. 2015). The mapped reads were assembled by StringTie (v2.1.4) (Kovaka et al. 2019), and GffCompare (v0.12.6) was employed to pool and merge all transcriptome samples and generate a comprehensive transcriptome (Pertea and Pertea 2020). Then the lncRNA was identified based on the following screening steps: (1) Select transcripts with transcript class code "i", "x", "u", "o"; (2) Select transcripts with length ≥ 200 bp and exon number ≥ 2; (3) Utilized CPC2 to predict transcripts with coding potential, and all transcripts with a score of > 0 are discarded (Kang et al. 2017) (4) HMMER (v3.2.1) analysis was performed on the remaining transcripts, to obtain final lncRNA annotation results by excluding transcripts containing any known protein domains classified in the Pfam database (Potter et al. 2018; Finn et al. 2014). Transcripts per kilobase per million (TPM) were used as the quantitative unit.

Differentially Expressed ncRNA and mRNA Analysis

Differentially expressed lncRNAs, miRNAs, and genes were identified using the DESeq2 (v1.10.1) R package in the Bioconductor bioinformatics software package (Love et al. 2014). DElncRNAs, DEmiRNAs and DEGs were identified with criterion of fold change ≥ 2 between salt and control samples and p-value < 0.05. Then, DEGs were subjected to enrichment analysis of gene ontology (GO) functions and KEGG pathways (Ashburner et al. 2000; Kanehisa et al. 2021).

Construction of the Regulatory Network

LncRNAs regulate their upstream and downstream genes through a *cis*-acting mechanism. Generally, in this study, protein-coding genes located 100 kb upstream and downstream of lncRNAs were selected as lncRNA target genes for functional analysis. psRNATarget software was used to make prediction for DElncRNAs, miRNAs and DEGs (Dai et al. 2018). For the construction of the network, we removed DElncRNAs, miRNAs and DEGs with maximum expression levels less than 1, the pairs of lncRNA-miRNA, miRNA-mRNA, and mRNA-metabolite with a pearson correlation coefficient < −0.2 will be utilized. The regulatory network and subnetwork are constructed by Gephi.

Dual-Luciferase Reporter Assays

We amplified the CDS of the *Os03 g0709300* gene and inserted it upstream of the luciferase (LUC) sequence of the modified pGreenII 0800 vector to construct the reporter vector. Meanwhile, the precursor sequence of Osa-miR408-3p was amplified and cloned into the pEAQ-HT-DEST2 (PJF754) vector to construct the effector vector. The primers used to amplify are listed in Supplementary Table 11. A mixture of agrobacterium strain GV3101 (pSoup-p19) containing the effector and reporter vectors was injected into the leaves of 5-week-old tobacco (*Nicotiana benthamiana*). The LUC and REN activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Result

Metabolic Profiles of Different Rice Varieties Under Salt Stress

To evaluate the effects of salt stress on different rice varieties, we subjected 14-day-old NIP, Pokkali, and 9311 rice seedlings to a treatment with 150 mM NaCl for 72 h, while setting up a non-treated control group simultaneously. Compared with the control, the seedling growth of NIP and 9311 was significantly inhibited and leaf wilting occurred, whereas the wilting and yellowing of the leaves of Pokkali was less severe, which indicated that Pokkali had a certain tolerance to salinity (Fig. 1A). Then, to investigate the differences in metabolic changes of different rice varieties under salt stress, we employed a combined targeted and non-targeted metabolomics approach based on UPLC-MS/MS to detect metabolites in the shoot and root tissues of different rice varieties in both the control group and the salt-treated group. A total of 327 metabolites were identified and classified into seven classes (Fig. 1B, Table S1). Principal component analysis using these metabolite contents revealed significant metabolic changes in shoot and root tissues. Compared with the control, the metabolic changes of Pokkali shoot tissues under salt stress were less significant than those

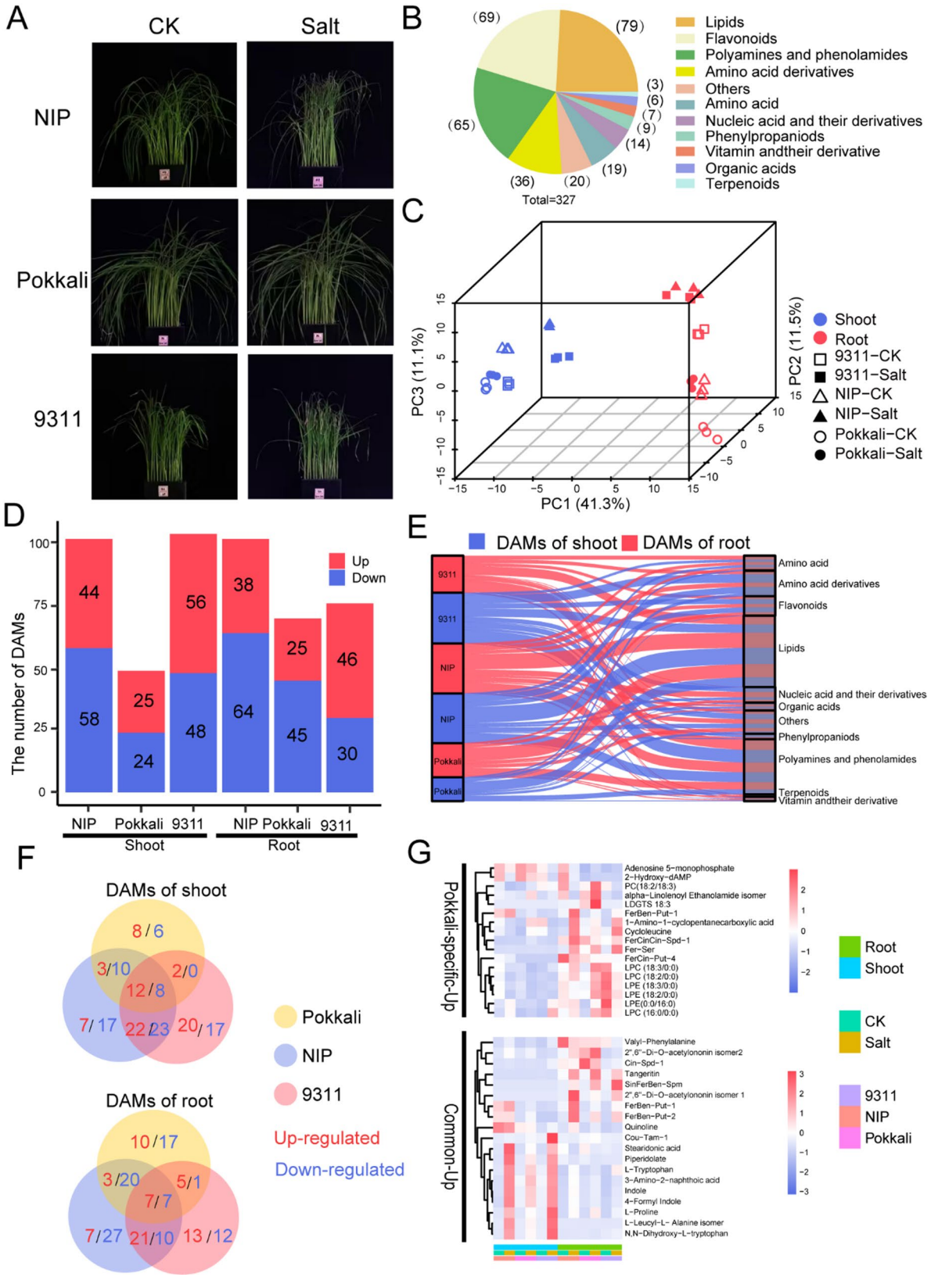

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Fig. 1 Metabolite profiles of rice from different varieties and tissues under salt stress. **A** Phenotypic characteristics of the NIP, Pokkali, and 9311 under salt stress. **B** Composition and proportion of different metabolites in rice. **C** PCA of the metabolites in different tissues and varieties under salt stress in rice. **D** The number of DAMs in different samples under salt stress. **E** Sankey diagram of identified DAMs in six comparative groups. **F** Venn diagram analysis of identified DAMs in the shoots and roots of different rice varieties after salt treatment. **G** Heatmap of DAMs that are specifically up-regulated in Pokkali and common

of other varieties (Fig. 1C). A total of 102, 49, and 104 DAMs were identified in the shoots, and 102, 70, and 76 in the roots of NIP, Pokkali, and 9311 salt-treated plants, respectively, compared with the controls (Fig. 1D, Table S2). Pokkali showed that its DAMs were 51.96% less in the shoots and 31.37% less in the roots than that of the NIP. This indicates that the metabolic processes of Pokkali were more stable under salt stress (Fig. 1D). This is consistent with the fact that salt-tolerant rice varieties always maintain a relatively stable physiological state to sustain their normal growth under salt stress. We established a three-dimensional metabolic classification system based on the compositional profiles of different varieties under control (CK) conditions, categorizing metabolites into dominant, suppressed, and balance groups according to their accumulation levels. Dynamic analysis under salt stress revealed that balance metabolites accounted for 59.21% and 53.33% in shoot and root tissues, respectively (Fig. S1). These indicate that salt stress-induced metabolic reprogramming is a key factor driving the differential response among varieties. In addition, lipids, polyamines and phenolamides accounted for the largest proportion of DAMs (Fig. 1E). According to the species-specific analysis of DAMs among the three varieties, 8 DAMs were found to be specifically upregulated in the Pokkali shoot, mainly comprising metabolites of polyamines and phenolamides and lipids, while 10 specifically upregulated DAMs were detected in the Pokkali roots, with lipids being the predominant type (Fig. 1F). Among the common upregulated DAMs, polyamines and phenolamides, which accounted for the largest proportion, three of them were significantly more upregulated in Pokkali and 9311 than in NIP (Fig. 1G). These results indicate that the metabolic pathways of lipids, polyamines, and phenolamides in rice are more susceptible to induction by salt stress, while the salt-tolerant variety Pokkali is less prone to salt-induced changes. This provides key metabolic pathways and signaling pathways for deciphering the adaptation mechanism of rice to salt stress.

Transcriptional Differences Among Rice Varieties Under Salt Stress

In order to investigate the effects of salt stress on transcript levels in shoot and root of different rice varieties, we conducted an analysis of salt-responsive genes (Fig. S2). NIP, Pokkali, and 9311 were identified with 9,420, 4,611 and 4,929 DEGs in the shoots and 7,624, 5,111 and 4,767 DEGs in the roots, respectively (Fig. 2A, Table S3). It was evident that the number of DEGs was significantly higher in NIP compared to Pokkali and 9311, indicating that Pokkali exhibited a less perturbed transcriptional profile under salt stress. Gene expression analysis of three rice varieties under CK conditions revealed that balanced genes accounted for 35.37% and 48.65% of DEGs in shoot and root tissues, respectively (Fig. S3). These findings indicate that most of the changes in gene expression are specifically triggered by salt stress rather than intrinsic breed differences. Transcriptome analysis revealed that a total of 18,597 unique DEGs were identified under salt stress. Among them, 13,040 and 12,022 DEGs were identified in the shoots and roots. It's worth noting that 49.49% and 73.61% of these exhibited genotype-specific expression patterns, accounting for 70.8% of the total DEGs (Fig. 2B). This indicates that different varieties possess their own unique genetic mechanisms to adapt to the salt-stressed environment. Then, we clustered all DEGs according to their expression levels, clustering them into 10 classes both in shoots and roots (shoot: S1-S10; root: R1-R10) (Fig. 2C and Fig. S4). Genes of clusters R6, R9, and R10 were highly expressed under salt stress in 9311, NIP, and Pokkali, respectively. In addition, we also found some reported salt-tolerant genes existed in these clusters (Fig. 2D, E, Fig. S5). Further GO enrichment showed that R6 was significantly enriched in "photosynthesis", "hydrogen peroxide catabolic process". Salt stress can increase the expression and activity of *OsAPx8* in the roots of rice, which belongs to the R6 gene cluster, thereby enhancing the ability to scavenge reactive oxygen species and thus enabling the plant to resist salt stress (Fig. 2E) (Jiang et al. 2016). R9 was significantly enriched in "regulation of response to salt stress", "regulation of response to osmotic stress". The OsbHLH035 belonging to R9 can be induced for expression and it regulates the expression of genes related to salt stress response, activates the downstream salt resistance signaling pathway, and enhances the tolerance of rice to salt stress (Fig. 2F) (Chen et al. 2018a, b). R10 was significantly enriched in "cell wall organization or biogenesis" and "response to iron ion" (Fig. 2D). Transgenic plants of *OsMORE1a* induce multiple genes related to the cell wall (Kim et al. 2022). *OsYSL15* is involved in the transportation of iron ions (Fig. 2G) (Inoue et al. 2009). As the outer barrier of the cell, the cell wall can resist the adverse effects of a high-salt environment. The transportation of iron ions can maintain the intracellular ion balance, thereby

enabling the plant to resist stress. These results indicate that different rice varieties have specific transcriptional regulatory mechanisms in response to salinity changes. It reveals the complex and diverse adaptive strategies evolved in rice and provides crucial clues for an in-depth interpretation of the molecular basis of rice responses to salt stress.

Widespread Identification of ncRNAs Under Salt Stress in Multiple Rice Varieties and Tissues

To investigate the response of ncRNAs to salt stress in rice, we first conducted a genome-wide identification of miRNAs and lncRNAs in the shoots and roots of three rice varieties under salt stress. There were 641 pre-miRNAs regions present in the rice reference genome, generating 458 uniquely mature miRNAs, including 125 known miRNAs and 333 unknown miRNAs (Fig. 3A and Table S6). The length of identified miRNAs was about 21nt, which was the same as previously reported (Fig. 3B and Fig. S7 A) (Fan et al. 2015). A total of 3,316 lncRNAs were identified, including 2,020 intergenic lncRNAs, 698 sense lncRNAs, 452 antisense lncRNAs, and 146 intronic lncRNAs (Fig. 3C and Table S7). A total of 2,040 lncRNAs exhibited highly conserved characteristics in the three rice varieties, accounting for 61.52% of the lncRNAs identified in the three varieties (Fig. 3D). Through an analysis of structural features, compared to the mRNA of the whole transcriptome, lncRNAs exhibited fewer exons, with most of them consisting of 2 to 4 exons, particularly two-exon lncRNAs, which accounted for 68.88% of the total number of them, and most of the lncRNAs (87.85%) were 200–3000 nt in length (Fig. 3E, F). Through the distribution of ncRNAs and mRNAs on chromosomes across the genome, we found that lncRNAs were evenly distributed across the chromosomes, indicating that they have no clear preference for specific genomic locations, while mRNA and miRNA were distributed on both ends of the chromosomes (Fig. 3G). Performing identifications across diverse varieties and tissues can expand the detection range and enhance the likelihood of uncovering novel ncRNA molecules and their isoforms. Furthermore, a holistic comprehension of their functions in multiple aspects, including rice growth and development, as well as responses to stress, can be facilitated.

NcRNAs in Different Rice Varieties Respond to Salt Stress Through Specific Regulation Mechanisms

To clarify the mechanism of action by which ncRNAs function in rice salt stress, we first compared the salt-induced DEncRNAs in three rice varieties. Specifically, 18, 29 and 49 DEmiRNAs were identified in the shoots of NIP, Pokkali, and 9311 respectively, while 33, 30 and 19 DEmiRNAs were identified in the roots (Table S8). At the same time, we identified 550, 261 and 347 DElncRNAs in shoots of NIP, Pokkali, and 9311 and 418, 340 and 406 DElncRNAs in roots, respectively (Table S9). We found that 66.1% of DEmiRNAs and 80.38% of DElncRNAs exhibited genotype-specific expression patterns in roots. This indicates that different rice varieties possess specific ncRNAs to respond to salt stress, leading to the formation of distinct evolutionary adaptation directions. (Fig. 4B, C, Fig. S8 and Table S9). By clustering the known DEmiRNAs in shoots and roots, a small branch contained Osa-miRseq2 (Osa-miRN221a), Osa-miRseq19 (Osa-miR171b), and Osa-miRseq314 (Osa-miR528-5p) in the root expression profile (Fig. 4D). The three miRNAs showed similar expression patterns when induced by salinity, with increased expression levels in Pokkali and decreased expression levels in 9311 and NIP (Fig. 4D). The overexpression of Osa-miR528-5p increases the accumulation of abscisic acid and ascorbic acid, and decreases the accumulation of ROS to increase the salt tolerance of rice (Wang et al. 2021). We detected the expression levels of Osa-miR171b in the roots of three rice varieties through qRT-PCR analysis, and the results showed that they were consistent with the transcriptome sequencing data (Fig. S10D-F). Meanwhile, we detected the expression level of Osa-miRseq89 (Osa-miR408-3p), which was in the same gene cluster as the reported salt tolerance regulatory factor Osa-miRseq406 (Osa-miR398b). The qRT-PCR verification result was also in line with the expression trend of sequencing data in the three rice varieties (Fig. S10 A-C). Two evolutionary branches containing four miRNAs were found in the shoot expression profile. Among them, knockout of Osa-miRseq303 (Osa-miR396e) can increase rice yield. Overexpression of Osa-miRseq171 (Osa-miR393a) can reduce the tolerance of rice to saline-alkali stress (Fig. S9) (Zhang et al. 2020; Gao et al. 2010a, b). Under salt stress, the expression level of these miRNAs remained basically unchanged in Pokkali, while the expression levels in 9311 and NIP increased significantly. We conducted qRT-PCR verification on Osa-miRseq294 (Osa-miR164d) of the same cluster of Osa-miR396e, and the results were consistent with the sequencing data (Fig. S10 G-I). These results indicate the accuracy of our sequencing data. Variety-specific ncRNAs reflect the genetic divergence that has taken place among different rice varieties during evolution. In contrast, the shared ncRNAs perform universal functions throughout the growth and development of rice. Collectively, these ncRNAs are indispensable for the normal growth and survival of rice.

To investigate the role of specifically expressed ncRNAs in different varieties in regulating genes transcriptional levels under salt stress, we predicted the target genes of these DEncRNAs. We found some overlapping genes between the genes potentially regulated by DEncRNAs and DEGs, and conducted GO and KEGG enrichment

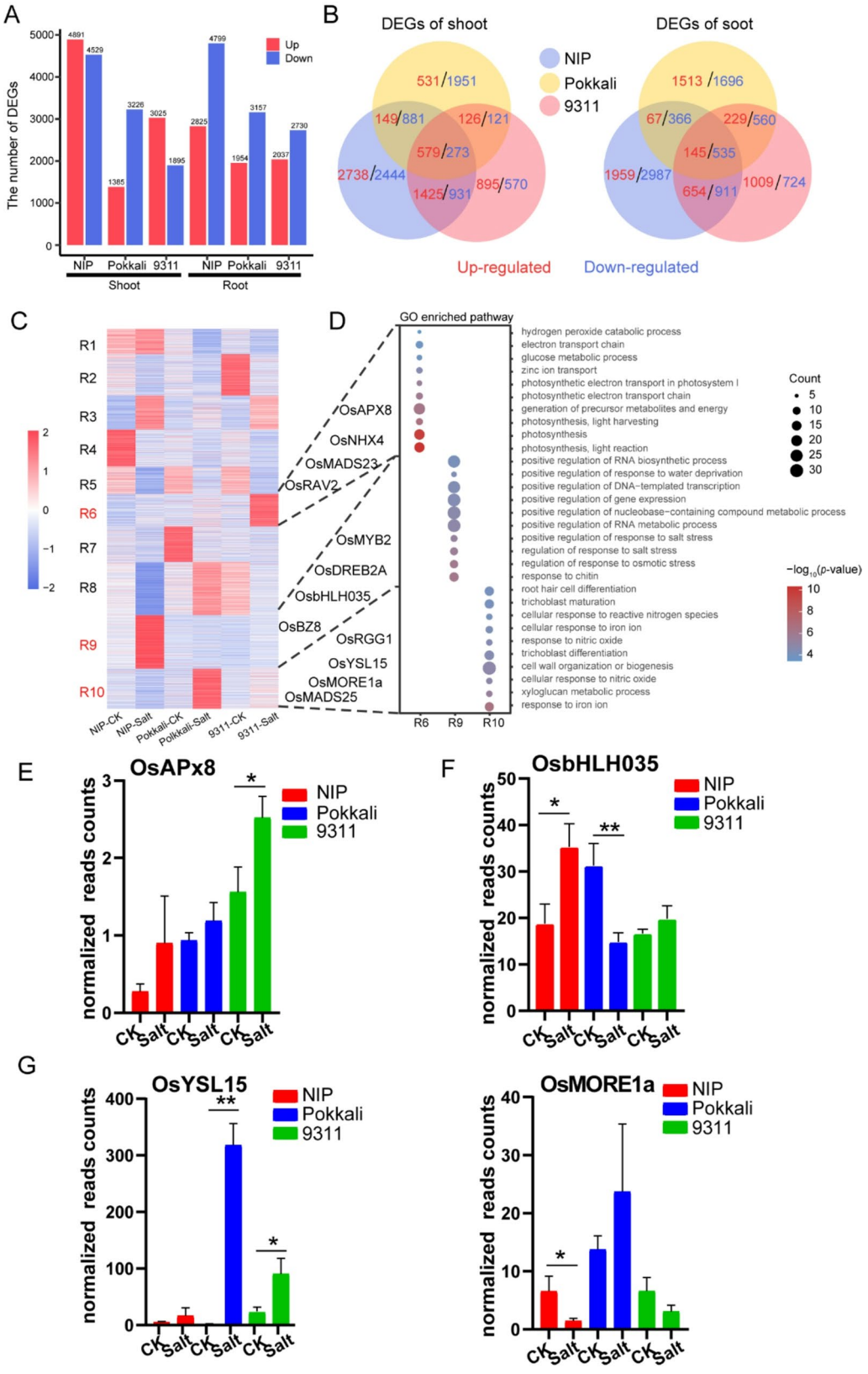

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Fig. 2 Transcriptional profiles of rice from different varieties in shoot and root under salt stress. **A** Histogram of DEGs identified in different rice varieties and tissue groups under salt stress. **B** Venn diagram analysis of DEGs identified in different rice varieties in shoot and roots. **C** Heatmap shows the DEGs expression pattern in each cluster in the roots (from top to bottom represents cluster 1 to cluster 10). **D** For clusters in R6, R9, R10, the top ten GO terms with the most significant *p*-values are listed. **E**–**G** Expression profiles of representative genes in R6, R9 and R10 in root under salt stress. The x-axis represents different varieties before and after salt treatment. The y-axis represents the gene expression level, measured in TPM

analysis of these overlapping genes (Fig. 4E-G, Fig. S11 and Fig. S12). The results showed that the target genes of common DEncRNAs were significantly enriched in "response to water" and "biosynthesis of various plant secondary metabolites" (Fig. 4F, G). The target genes of Pokkali-specific DEncRNAs were significantly enriched in "secondary metabolic process", "phenylpropanoid biosynthetic process" (Fig. 4F, G). The target genes of NIP-specific DEncRNAs were primarily involved in "amino acid transport", "phenylalanine, tyrosine and tryptophan biosynthesis" (Fig. 4F, G). ncRNAs respond to salt stress by regulating various metabolic pathways in rice. It indicates that different varieties initiate distinct physiological and biochemical reactions along with metabolic pathways through the specific expression of miRNAs and lncRNAs. It provides novel insights and research directions for a more profound comprehension of the molecular mechanisms underlying rice salt tolerance.

Construct Multi-dimensional Regulatory Network to Reveal the Complex Mechanisms of Rice Under Salt Stress

In view of the regulatory role of ncRNAs in metabolic biosynthesis and the competitive binding of lncRNAs and mRNA to miRNA, we constructed a four-tiered regulatory network mediated by ncRNAs to reveal the complex metabolic regulatory mechanisms under salt stress. The network contained 6,201 DEGs, 458 miRNAs, 970 DElncRNAs, and 177 metabolites (CV > 60%) (Fig. 5A and Table S10). In this regulatory network, we found several regulatory modules that had been reported to be involved in salt tolerance in rice, such as Osa-miR393a-Os05 g0489100, Osa-miR398b-OsCSD2, Osa-miR168-OsAGO1a and Osa-miR396c-OsGRF10 (Gao et al. 2010a, b; Lu et al. 2010; Xia et al. 2023). Overexpression of Osa-miR528-5p can enhance salt tolerance, and it is downregulated in both NIP and 9311. Consistent with its expression trend, Osa-miR408 has been reported to enhance the adaptation of *Zea mays* to salt stress (Qin et al. 2023). miR408 is one of the most evolutionarily conserved miRNAs in terrestrial plants, which predominantly targets genes encoding the plantacyanin and laccases in *Arabidopsis thaliana* (Yang et al. 2024a, b, c). Our regulatory network has discovered a new Osa-miR408-Os03 g0709300 regulatory module under salt stress in rice. *Os03 g0709300*, encoding a plastocyanin-like domain-containing protein, represents a novel potential regulator of salt tolerance in rice. We identified a subnetwork centered around the *Os03 g0709300* gene, which involved 16 mRNA-metabolite pairs and 10 mRNA-miRNA pairs (Fig. 5B). We found that the genes in this regulatory module are related to polyamines and phenolamides, flavonoid and amino acids and their derivatives (Fig. 5B). To explore the regulatory mechanism between Osa-miR408-3p and the target gene *Os03 g0709300*, through the prediction of the psRNATarget website, we determined that the binding site of Osa-mir408-3p and *Os03 g0709300* is located in the coding region of *Os03 g0709300* (Fig. 5F). To verify the relationship between them, we constructed the effector vector and the reporter vector (Fig. 5F). Using qRT-PCR, we found that transcriptional expression of *Os03 g0709300* was significantly suppressed when both Pre-miR408-3p and *Os03 g0709300* were expressed in tobacco compared to *Os03 g0709300* alone (Fig. 5C-D). The expression of LUC was also significantly down-regulated when both Pre-miR408-3p and *Os03 g0709300* were expressed in tobacco compared to *Os03 g0709300* alone (Fig. 5E). These results indicate that Osa-miR408-3p negatively regulates the expression of *Os03 g0709300*. Moreover, this complex and elaborate regulatory network can uncover the metabolic transduction pathways in rice under salt stress. It contributes to a comprehensive understanding of the molecular regulatory mechanisms underlying rice salt tolerance and provides potential molecular targets for the genetic improvement of rice varieties.

Polyamines and phenolamides are a class of metabolites that undergo significant differential changes induced by salt stress, and polyamines can improve plant salt resistance. Therefore, the analysis of the regulatory mechanism of the synthesis of these substances can promote the analysis of the mechanism of salt tolerance in rice. We found that there were 21 DEGs in the synthesis pathway of this metabolite, of which 6 DEGs were regulated by 8 miRNAs, while each miRNA had 2–11 potential target lncRNAs (Fig. 5G, Table S10). The regulation of rice salt tolerance by the miR396 family has been widely reported (Gao et al. 2010a, b; Yuan et al. 2024), and we found that in the regulatory network Osa-miRseq296 (Osa-miR396a-5p) might regulate spermine biosynthesis through the target gene *OsSPMS1* (Fig. 5G). The increase of spermine content is conducive to the improvement of salt tolerance of rice, and miR396-SPMS may be a new regulatory module of salt tolerance mechanism of rice. In addition, several key genes in the pathway were also identified by several newly identified miRNA targets. For

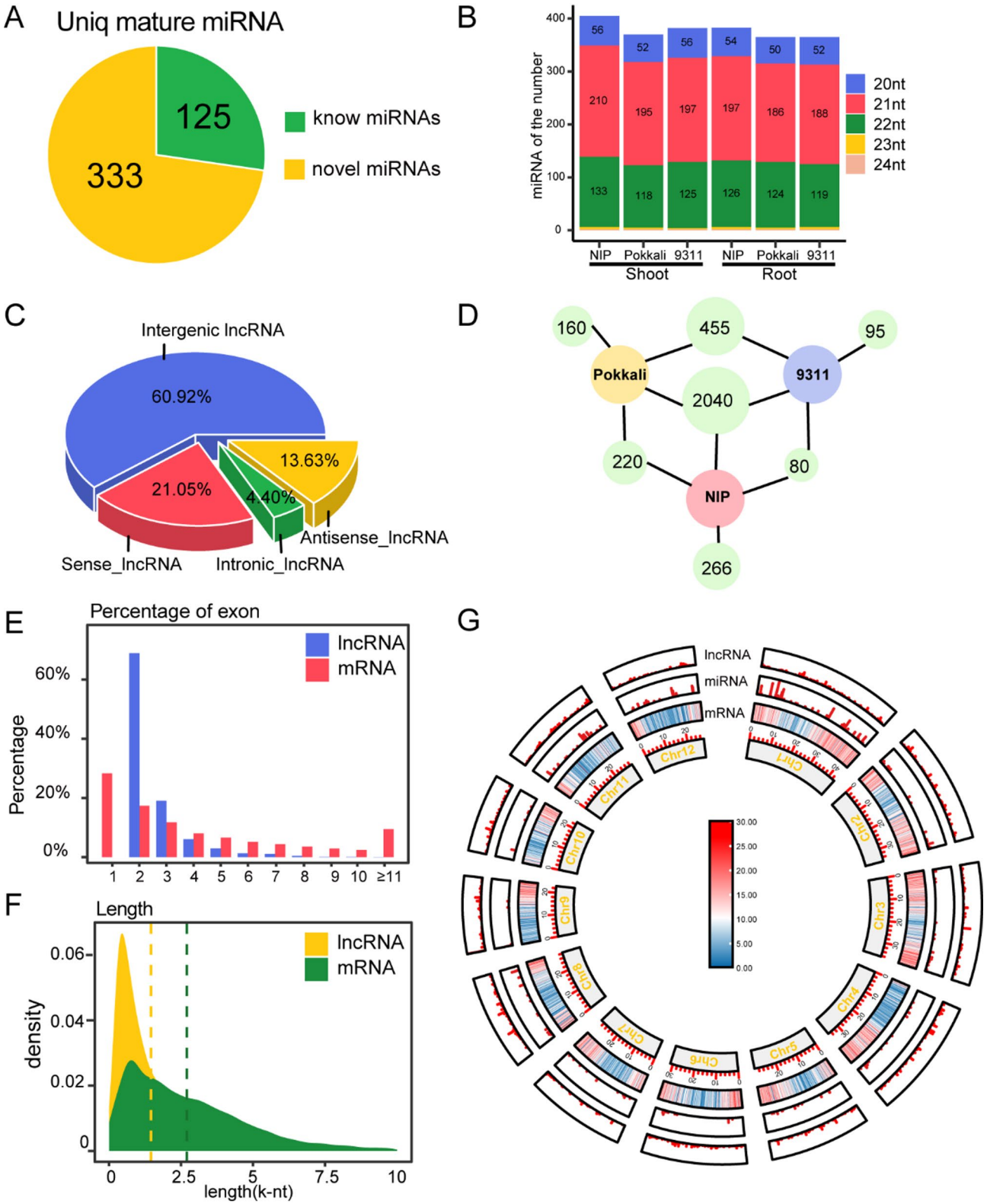

Fig. 3 Genome-wide identification and characterization of ncRNAs in rice under salt stress. **A** The number of known and novel mature miRNAs identified in all samples. **B** The length of identified miRNAs. **C** Percentage of classified lncRNAs types. **D** The number of lncRNAs identified in different rice varieties. **E**, **F** Comparison of length and exon number of lncRNA and mRNA. **G** Distribution of lncRNAs, miRNAs and mRNAs on the chromosome. The innermost two tracks represent the 12 chromosomes (Chr1 - Chr12) and gene density of the rice genome. The other tracks (the lncRNA and miRNA tracks) represent the respective densities of identified lncRNAs and miRNAs in the genome

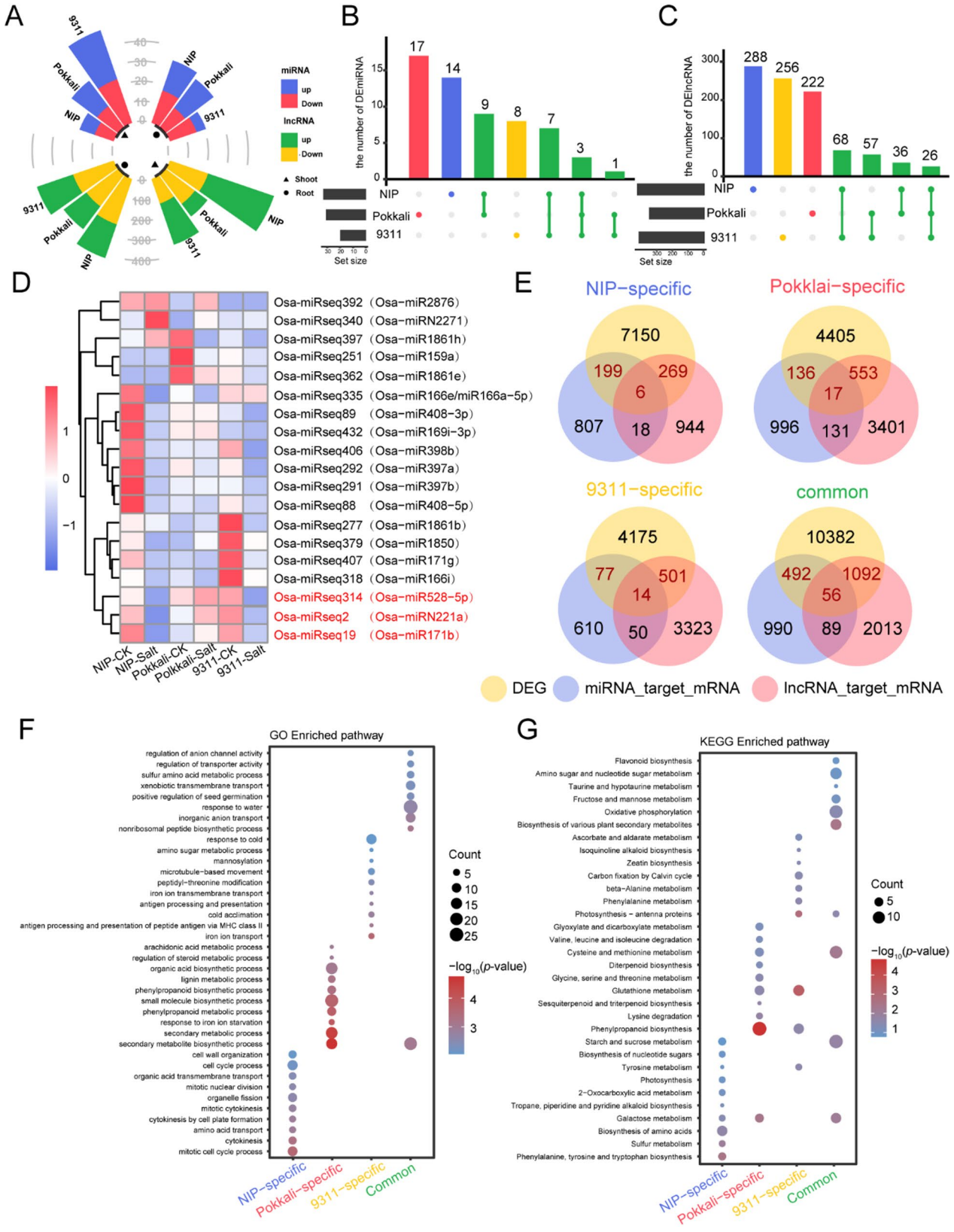

Fig. 4 (See legend on next page.)

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Fig. 4 Differential expression of lncRNAs and miRNAs under salt stress in rice. **A** The number of DEmiRNAs and DElncRNAs in shoots and roots. **B**, **C** Upset plot for DEmiRNAs and DElncRNAs in different varieties in the roots. **D** Heatmap of known DEmiRNAs in roots. **E** Venn diagram showing the common and unique parts between the screened DEGs, cis-regulated target genes of DElncRNAs, and target genes of DEmiRNAs. **F**, **G** Enriched GO and KEGG pathways of DEGs that are targets of DElncRNAs and miRNAs in NIP-specific, Pokkali-specific, 9311-specific, and Common. High to low (indicated by the *p*-value). The size of bubbles indicates the number of DEGs the larger the number the larger the symbol

example, *OsCPA3* was associated with three new miRNAs, and *OsADC1*, *OsADC2*, and *OsPAO3* were each associated with a new miRNA (Fig. 5G). This network breaks through the research on a single regulatory level. It constructs a metabolic regulation framework from four different levels, covering ncRNA regulatory mechanisms across multiple dimensions, including the transcriptional level, translational level, and metabolic level. This provides a more comprehensive and systematic view of metabolic regulation, enabling a deeper understanding of the complex regulatory processes in rice under salt stress.

Discussion

In recent years, abiotic stresses, including salt stress, have severely affected the growth and yield of rice (Lin et al. 2025; Zhang et al. 2024; Wang et al. 2024a, b; Nie et al. 2024). Previous studies have shown that metabolites play a positive regulatory role in rice response to abiotic stress (Jiang et al. 2019; Yang et al. 2022a, b; Guo et al. 2024). In our study, metabolite analyses showed that differences in metabolites between tissues were more significant than differences in metabolites between species. Lipids, polyamines and phenolamides are the main metabolites that respond to changes in salt stress. The transcriptome results showed that the salt response of NIP was higher than that of Pokkali and 9311. Under salt stress conditions, not only do different ncRNAs exist in different rice varieties, but these ncRNAs are specific and conserved in different rice varieties. Finally, we constructed a metabolic regulatory network of rice ncRNAs under salt stress, which provides a unique resource for the discovery of new regulators and regulatory mechanisms of salt tolerance in rice.

Polyamines, phenolamides, and lipid metabolites have emerged as critical players in plant adaptation to salinity stress, acting through dynamic regulation of redox homeostasis, membrane stability, and stress signaling (Alcázar et al. 2020). In this study, a total of 327 metabolites were identified using targeted and non-targeted metabolite assays, mainly including polyamine, phenolicamide, flavonoid and lipid. By comparing the differential metabolites accumulated under salt stress in three varieties, it was found that the four polyamines and phenolamines metabolites specifically upregulated in Pokkali might constitute a conserved salt tolerance mechanism in plant species. Previous studies have shown that there is a gene cluster in tomatoes that regulates the accumulation of phenolic amides and thereby enhances the salt tolerance of tomatoes (Yang et al. 2024a, b, c). In rice, overexpression of the *OsPAO3* gene can increase the polyamine content in the coleoptile of seeds and enhance the salt tolerance of rice (Liu et al. 2022). These research results correspond to the upward trend of polyamine and phenolic amine metabolites in Pokkali in this study, further confirming the important role of the accumulation of polyamine and phenolic amine in enhancing the salt tolerance of plants. Furthermore, in this study, glycerophospholipids metabolites accounted for a relatively large proportion among the specifically upregulated metabolites of Pokkali. Such as phosphatidylcholine (PC), lyso-phosphatidylcholine (LPC) and lyso-phosphatidylethanolamine (LPE). Existing studies have shown that exogenous addition of PC can significantly enhance the tolerance of peach trees to salt stress and reduce the damage caused by salt stress to peach trees (Sun et al. 2022, 2023). However, the role of PC in the salt stress response of rice has not been fully characterized. More importantly, the relationship between LPC and LPE and the plant salt stress response has not been reported before. In this study, the contents of these metabolites in the salt-tolerant variety Pokkali increased significantly, suggesting that they may play a potential positive regulatory role in the salt tolerance process of rice. This discovery provides a brand-new direction for in-depth exploration of the role of lipid metabolism in the salt tolerance mechanism of rice.

A large number of studies have demonstrated that genes affect salt tolerance of rice through transcriptional regulation. For example, *OsMYB2* mediates the gene expression of the amino acid transporter-encoding gene *OsANT1*, which positively regulates the growth and salt tolerance of rice (Nie et al. 2024). This study, by integrating the mRNA, ncRNA and metabolite data of three rice varieties, not only verified the cross-variety conserved salt stress response modules, such as the synergistic upregulation of the ion channel gene *OsGORK* and the abscisic acid (ABA) signaling core factor *OsRab16A* (Ganguly et al. 2012). ncRNAs can regulate gene expression, miRNAs can degrade genes by complementary pairing with gene bases, and lncRNAs can affect genes by competitively binding to miRNAs. In recent years, an increasing number of ncRNAs have been found to be involved in plant growth and development regulation and abiotic stress response, including salt stress. In our study, we identified three specific miRNAs (Osa-miR528-5p, Osa-miRN221 A and Osa-miR171b) in the

salt-tolerant variety Pokkali, revealing the refined regulation of the core network by the species-specific epigenetic regulatory layer. Among them, Osa-miR528-5p has been functionally confirmed to enhance salt tolerance by promoting the synthesis of ABA by inhibiting the expression of the l-ascorbate oxidase (AO) (Wang et al. 2021). In addition, our data further reveal its co-expression with the glycosyltransferase gene *OsGAUT4* and the accumulation of amino acid derivatives. However, the two novel miRNAs (Osa-miRN221a and Osa-miR171b) exhibit unique targeting patterns. By negatively regulating the histone deacetylase gene *OsHDT1* and the cytokinin glycosylase gene *cZOGT1*, the two may synergically enhance chromatin openness and maintain the level of active cytokinin, thereby alleviating growth inhibition caused by salt stress (Zhao et al. 2014; Kudo et al. 2012). These findings provide a comprehensive and valuable resource for studying the molecular mechanism of rice response to salt stress. However, there are limitations to our study of ncRNAs. Compared with the rice database, the number of known miRNAs identified is relatively small, and there are limitations to further studies. The regulatory mechanism of the identified lncRNAs under salt stress in rice remains unclear. Furthermore, circular RNA (circRNA), as an important vector of competitive endogenous RNA (ceRNA), has not been involved in this study, and the key regulatory layer may have been omitted.

Although omics techniques have systematically identified the core response genes and metabolic pathways of salt stress in rice, current research still has significant limitations (Zhu et al. 2024). The dynamic modification effect of epigenetic regulatory layers, such as ncRNA, on the metabolic network has not been revealed. The traditional ceRNA theory is limited to the competitive binding between RNA molecules and lacks a systematic analysis of the participation of metabolites in the regulatory circuit. However, previous studies have mostly focused on the mRNA and metabolomics levels, making it difficult to analyze the metabolic synergistic regulatory mechanism at the ncRNA molecular level. In order to further clarify the metabolic regulatory role in the process of rice resistance to salt stress, combined with the analysis results of mRNA, ncRNAs and metabolomics, the classical ceRNA theory was extended to the multi-dimensional interaction framework of "lncRNA-miRNA-mRNA-metabolites", jointly revealing a new mechanism by which ncRNAs regulate metabolic homeostasis through epigenetic reprogramming.

In our study, we constructed a metabolic regulatory network involving ncRNAs based on the multi-omics data of shoot and root tissues of three rice varieties under salt stress, and elucidated their involvement in rice salt stress defense. The prominent advantage of this network is reflected in that through the joint analysis of multiple varieties and multiple tissues, it can precisely explore the key regulatory factors with variety specificity. The study revealed that Osa-miR528, Osa-miR171b, and Osa-miR221a were specifically upregulated in Pokkali roots under salt stress, with Osa-miR528 overexpression significantly enhancing salt tolerance (Wang et al. 2021). These findings demonstrate that this regulatory network can effectively identify potential salt-tolerant regulatory targets and provide candidate genes for molecular design of salt-tolerant varieties. Existing studies predominantly focus on unidimensional metabolite-gene regulatory relationships. For instance, overexpression of the *OsNCED3* gene has been demonstrated to enhance rice salt tolerance by promoting trehalose biosynthesis (Ye et al. 2023). In contrast, our study achieved a more comprehensive understanding of the regulatory mechanisms through the integration of metabolomic data, establishing connections between ncRNAs and metabolic pathways to further elucidate the salt tolerance mechanisms in rice. However, the regulatory network constructed in this study has certain limitations. While the integration of multi-cultivar and multi-tissue data enhanced the comprehensiveness of our analysis, it simultaneously increased the complexity of the network, potentially elevating false-positive results. Future studies could employ degradome sequencing to experimentally validate the regulatory relationships between candidate miRNAs and their target genes (Zhuo et al. 2022). Although we validated several miRNA-target interactions including Osa-miR408 and its target gene *Os03 g0709300* through qRT-PCR and LUC, the regulatory relationships between lncRNAs and miRNAs, as well as those involving genes and metabolites, require further experimental confirmation. In practical applications, previous studies have identified *OsWRKY53* as a key regulator of salt tolerance in rice through genome-wide association studies (GWAS) (Yu et al. 2023). The regulatory network constructed in this study can be applied to GWAS for identifying salt tolerance-associated single nucleotide polymorphisms in ncRNA regions, thereby accelerating molecular marker development and population genetic improvement. In addition, the conserved network modules discovered in rice can guide the stress resistance engineering of other crops by prioritizing homologous genes and pathways.

Conclusion

In conclusion, the lipid, polyamine, and phenolamide metabolic pathways in rice are more susceptible to induction under salt stress. Compared with NIP and 9311, when subjected to salt stress, the salt-tolerant variety Pokkali exhibits more stable metabolic and transcriptional levels under salt stress. Moreover, these three rice varieties respond to salt stress through the specific expression of ncRNAs. By extensively identifying

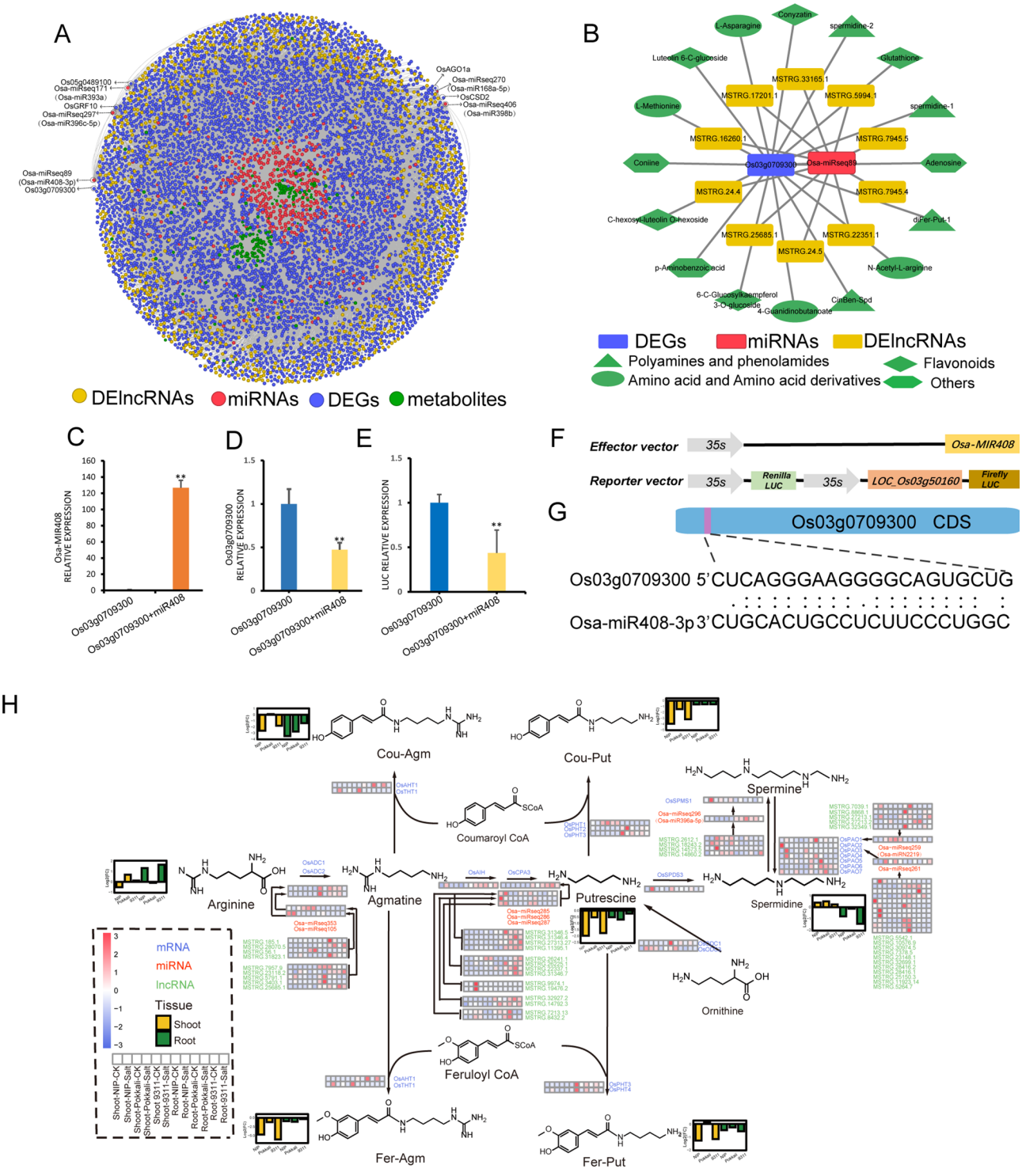

Fig. 5 Construction of the metabolic regulatory network of ncRNA under salt stress. **A** Construction of the ncRNAs mediated metabolic regulatory network. The yellow dots represent DElncRNAs; Red dots represent miRNAs; Blue dots represent DEGs; Green dots represent metabolites. **B**Subnetwork centered on the core Osa-miRseq89 (Osa-miR408-3p). The yellow round rectangle represent DElncRNAs; Red round rectangle represent miRNAs; Blue round rectangle represent DEGs; Different green shapes represent metabolites. Triangle represent polyamines and phenolamides; Ellipse represent amino acid and amino acid derivatives; Diamond represent flavonoids; exagon represent others. **C** Expression level of pre-miR408-3p in tobacco leaves. **D-E** Expression of *Os03 g0709300* and LUC after the co-expression of Osa-miR1408-3p and *Os03 g0709300* in tobacco leaves. **F** Sche-matic diagram of the effector and reporter vectors. **G** Binding site of Osa-miR408-3p on the target gene *Os03 g0709300*. **H** The synthetic pathways of polyamines and phenolamides in rice. The heatmap represents the expression levels of DEGs, miRNAs, and DElncRNAs in shoots and roots of different rice varieties under salt stress. Color scale placed horizontally at the bottom of the diagram.Accumulation fold changes of DAMs from different varieties in the shoots and roots are indicated by the bar chart after salt treatment.The yellow bars represent the shoots, while the green bars represent the roots.

non-coding RNAs in different tissues of different rice varieties under salt stress, we finally constructed a four-tiered regulatory network (lncRNA-miRNA-mRNA-metabolite) mediated by ncRNAs under salt stress. Osa-miR408 in the network shows differential expression in rice roots after salt stress, and its negative regulation of the expression of the target gene *Os03 g0709300* has been verified. This network offers elaborate regulatory relationships for the molecular mechanisms of rice salt stress at the transcriptional, translational, and metabolic levels, laying a foundation for promoting the breeding of salt-tolerant rice varieties.

Abbreviations

NIP	Nipponbare
DAM	Differentially accumulated metabolite
DEG	Differentially expressed gene
ncRNA	Non-coding RNA
miRNA	MicroRNA
lncRNA	Long non-coding RNA
circRNA	Circular RNA
sRNA	Small RNA
LUC	Dual-luciferase reporter assays
ceRNA	Competing endogenous RNA
ROS	Reactive oxygen species
PC	Phosphatidylcholine
LPC	Lyso-phosphatidylcholine
LPE	Lyso-phosphatidylethanolamine
ABA	Abscisic acid
GWAS	Genome-wide association studies

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-025-00811-6.

Supplementary material 1

Supplementary material 2

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Not applicable for this study.

Author Contributions
J.Y. conceived the project and supervised the study. H.T. and X.O. conducted the data analysis and curation. C.Y., S.L. and J.Z. performed the metabolite profiling. X.H., E.L., G.X. and Q.S. anticipated in the material preparation. H.T., C.W. and X.H. wrote the first draft. J.Y. reviewed, edited, and finalized the manuscript. All authors discussed the results and commented on the manuscript.

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Data Availability
All data generated or analysed during this study are included in this published article (and its supplementary information files).

Declarations

Competing Interests
The authors declare no competing interests.

Ethical Approval
Not applicable.

Consent To Participate
Not applicable.

Consent for Publication
Not applicable.

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