Rice

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Transcriptome Analysis Between Parents and Offspring Revealed the Early Salt Tolerance Mechanism of Rice NGY1

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Abstract

Salt stress poses a severe threat to global rice productivity, and developing salt-tolerant cultivars represents a critical strategy to address this challenge. However, the molecular mechanisms underlying salt tolerance in rice remain elusive. This study focuses on NGY1, a crossbred offspring between YF47 and SN9903, which showed superior salt tolerance compared to its parent lines during the seedling stage. RNA sequencing (RNA-seq) of seedlings harvested at distinct temporal stages of salt stress identified over 10,000 differentially expressed genes (DEGs). Functional enrichment analyses (GO and KEGG) revealed that NGY1 uniquely mobilized a broader repertoire of stress-responsive genes within shorter timeframes than its parents lines, particularly those associated with redox homeostasis, phytohormone signaling, and MAPK cascades. Meanwhile, NGY1 can rapidly upregulate genes related to salt tolerance compared to its parent during the initial stress phase. Additionally, differences in salt tolerance between NGY1 and its parents were linked to variations in alternative splicing and the high expression of certain NBS-LRR protein genes early in salt stress exposure. These findings not only provide new insights into the molecular mechanisms of salt tolerance, but also provide a theoretical basis for genetic improvement of salt tolerance in rice.

Keywords Rice, Salt-stress, Transcriptomic, Alternative splicing, Mechanism

Introduction

Rice (*Oryza sativa* L.) is a staple food for over half of the world population and plays a key role in global food security (Bandumula 2018). However, rice is a moderately salt-sensitive plant, and excessive salinity challenges rice plants, especially during the seedling and reproductive stages (Ganie et al. 2019).With soil salinization threatening more than 33% of arable land, salinity stress becomes one of major constraints for rice growth and production (Formentin et al. 2018). Several studies have shown that global crop production needs to double by 2050 to meet the projected demands from rising population, diet shifts, and increasing biofuels consumption (Ray et al. 2013). Therefore, improving salt tolerance of rice can help ease food tension. It has been proved that identification of salt-tolerant rice germplasm resources and breeding of salt-tolerant rice varieties are the most economical and effective methods to reduce the loss of rice yield caused by salinity (Ganie et al. 2021). However, the genetic resources for salt-tolerant rice breeding are still relatively scarce, because salt tolerance is a complex

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quantitative trait regulated by multiple genes, and the specific molecular mechanisms of rice from salt stress sensing and signal transduction to adaptive tolerance have not been fully clarified (Chen et al. 2021).

Rice tolerance to salinity is genotype-dependent. The different phenotypes of different varieties/local varieties under salt stress involve complex physiological mechanisms and are controlled by many genes (Pires et al. 2015; Liu et al. 2022). Considerable research efforts have been dedicated to the identification of salt-tolerant genes, leading to the discovery of hundreds of salt-stress quantitative trait loci (QTLs) across diverse rice populations to date (Nakhla et al. 2021). However, only a few QTLs showed a high significant effect (contribution of more than 30%), like *SKC1*, encoding an HKT-type transporter (*OsHKT1;5*), regulate K^+/Na^+ balance under salt stress, was finely mapped from the QTL located on chromosome 1 and successfully isolated (Ren et al. 2005). Meanwhile, the candidate genes for most QTLs were unknown due to the large localization intervals (Singh et al. 2021). In recent years, following the rapid progress of massive parallel sequencing technologies, RNA sequencing (RNA-seq) has been employed to study transcriptomic in many genotypes of salt sensitive versus tolerant rice in a comparative way (Cotsaftis et al. 2011; Razzaque et al. 2019). Using RNA-seq analysis, numerous differentially expressed genes (DEGs) have been identify across contrasting samples. For example, RNA-seq method was used to compare and analyze salt-sensitive variety Wuyunjing 30 (WYJ30) and salt-tolerant variety Zhendao 23,309 (ZD23309) seedlings under salt stress, and more than 10,000 DEGs were found, some of which play pivotal roles in strengthening salt tolerance, encompassing the response to stimulus and nucleoside binding (Liu et al. 2024). In addition, Linkage Mapping, RTM-GWAS, and RNA-seq techniques were combined to search for salt-tolerant genes in rice, which highlighted the key salt stress-related genes and possible regulatory networks (Kong et al. 2021). These transcriptomic analyses revealed the changes of key genes and pathways in rice stress response, providing a new and powerful way to analyze the mechanism of rice salt stress perception and response.

Breeding offspring with strong salt stress resistance through hybridization is a widely used breeding method at present, but environmental factors such as soil heterogeneity and climatic factors may affect physiological processes, which makes it difficult to select salt-tolerant rice varieties in the field (Qin et al. 2020). In addition, the molecular mechanisms, key genes, and regulatory networks underlying the differences in parental and progeny stress resistance are often not very clear to breeders, resulting in increased breeding time and uncertainty (Gilliham et al. 2017). Previous studies about salt-tolerant transcriptomic of rice often only focused on the gene expression differences between salt-tolerant or non-salt-tolerant varieties under salt stress or non-salt stress (Ye et al. 2022). Although a large number of salt-tolerant candidate genes have been screened in these studies, few of them can be used in actual breeding, because most of them have little effect in field or are eliminated because they affect other beneficial agronomic traits (Swarup et al. 2021). So far, several studies have summarized some advances in stress sensing and signaling, functional adaptation, and salt-tolerant breeding, understanding the complexity of salinity tolerance mechanisms in rice is still limited, and real salt-tolerant rice varieties are not yet available (Shi et al. 2023). However, there are already some relatively suitable salt-tolerant varieties in some parts of the world, and how to use them to further cultivate more effective salt-tolerant varieties without affecting other favorable traits is an urgent problem to be solved.

NanGengYan1 (NGY1) is a new middle-ripe japonica rice variety with high quality and high yield ShenNong 9903 (SN9903) as the maternal parent and high quality and salt-tolerant japonica rice YanFeng47 (YF47) as the paternity. After years of screening in saline-alkali soils, the variety NGY1 has good salt tolerance and is suitable for planting in saline-alkali soils with a salt content of less than 0.5% in regions such as Jiangsu and Shandong provinces of China. In this study, we explored the genome-wide expression differences between NGY1 and its parents YF47 and SN9903 using RNA-seq technology. The research results show that, compared with the parent variety, NGY1 has the ability to mobilize more favorable genes to combat salt stress within a short period of time. Furthermore, we found that the difference in salt tolerance between NGY1 and its parents also related to the difference in exon skipping of some genes in the early stage of salt stress and the high expression of some NBS-LRR protein genes. These findings will pave the way for further understanding the regulatory networks of salt response in crops and help to mine candidate genes for genetic improvement of salt tolerance.

Materials and Methods

Rice Varieties and Salt Stress Trials

The *japonica* rice cultivar NaGengYan1 (NGY1) and its parents YanFeng47 (YF47) and ShenNong 9903 (SN9903) of China was used in this work. Rice seeds were surface-sterilized and germinated in water solution for 4 days at 30 °C in the dark. Then, seedlings were transferred to liquid 1/2 Murashige and Skoog (MS) hydroponic culture solution and continued to grow for 10 d in a growth chamber (14-h light/10-h dark) with about 300 μmol photons m^{-2} s^{-1} light intensity at 30 °C and 70% relative humidity before treatment. Finally, the seedlings were

cultured in 1/2 MS medium with or without 100 mM NaCl for 3 days. The fresh culture medium was changed every 2 days. All the experiments were repeated three times.

The plant length, root length, and fresh weight of NGY1, YF47 and SN9903 seedlings were manually determined in triplicate for each treatment, and each replicate contained at least 5 randomly selected seedlings. Total chlorophyll content was determined with reference to the previous method (Lu et al. 2022).

RNA-seq and Bioinformatic Analysis

For obtaining rice materials for RNA-seq, the above 10 days old seedlings were treated with 1/2 MS nutrient solution containing 200 mM NaCl, seedlings were sampled at 0, 1, and 12 h. Total RNA was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cDNA library was sequenced using the Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China). DESeq2 was used to identify differentially expressed genes (DEGs) with these thresholds: false discovery rate (FDR) < 0.05 and fold change ≥ 2. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis referred to previous methods (Li et al. 2021). Bioinformatic analysis was performed using Omicsmart, a real-time interactive online platform for data analysis (http://www.omicsmart.com). The sequencing data has all been archived in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA1260548.

Quantitative Real-Time PCR (RT–qPCR)

Three biological replicates, each comprising three individual plants, were used for RT–qPCR. Total RNA was extracted from plant tissue using the plant RNA Extract Kit (TIANGEN Biotech, Beijing, China). The cDNA was synthesized with the HiScript II Q RT SuperMix (+gDNA wiper) for qPCR (Vazyme Biotech, Nanjing, China). The RT–qPCR was performed using a LightCycler 480 II Real time PCR (Roche, Basel, Switzerland) with AceQ® qPCR SYBR Green Master Mix (Vazyme Biotech, Nanjing, China). The relative level of expression was calculated using the formula $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$. The primers used for RT-qPCR analyses are listed in Table S1.

H_2O_2 Content Analysis

The rice seeds were allowed to germinate for 4 days, followed by 10 days of normal cultivation. Subsequently, the seedlings were subjected to hydroponic treatments with either 200 mM NaCl solution or control solution without NaCl for 12 h. Fresh seedling tissues (0.1 g) were collected and immediately snap-frozen in liquid nitrogen for H_2O_2 quantification using the Hydrogen Peroxide (H_2O_2) Content Assay Kit (Solarbio, Beijing, China). The absorbance of the final solution was detected at 415 nm. The experiment was repeated three times.

Statistical Analyses

The two-tailed Student's *t*-test ($P < 0.05$) were used to identify the statistical significance of the difference. The Microsoft Excel 2016 for Windows V7 was used for all statistical analyses.

Results

Phenotypic Variability of NGY1, SN9903 and YF47 Under Salt Stress

To investigate the phenotypic variation of NGY1, SN9903 and YF47 under salt stress, 10-day-old rice seedlings were treated with nutrient solution containing 100 mM NaCl. It is worth noting that the three rice varieties showed visible differences in plant height after 10 days of normal cultivation (Fig. S1). After 72 h of salt treatment, all seedlings showed symptoms of salt damage. Visual damages were observed as leaf rolling and whitish, reduction in root growth and seedling height in SN9903 (Fig. 1a). Similar salt damage was found in YF47, but not in NGY1 (Fig. 1a). Further data analysis showed that NGY1 was significantly superior to its parents in plant height, root length, fresh weight and total chlorophyll content at seedling stage under salt stress, indicating that NGY1 was more resistant to salt (Fig. 1b, c, d and e). In addition, NGY1 had better seedling height, root length and fresh weight than its parent under normal conditions, which may be related to its stronger salt tolerance (Fig. 1b, c and d). The order of salt tolerance of the three rice varieties was as follows: NGY1 > YF47 > SN9903.

RNA-seq Analysis and Differentially Expressed Genes Results

To further explore the molecular mechanisms accounting for the disparities in salt tolerance among the three rice genotypes, a high-throughput sequencing system was employed for comparative transcriptome analysis. In general, differences in responses at the during the early growth stage reflect the overall tolerance of rice to salt throughout its life cycle, so 10-day-old rice seedlings were subjected to 0 h, 1 h and 12 h salt stress and samples were collected for transcriptomic analysis. A total of 27 cDNA libraries were constructed, representing three rice cultivars and three salt treatment times, each with three biological replicates. These libraries were sequenced using the Illumina deep sequencing platform. Each sequencing data exceeded 6G readings, with an average high quality reads of ≥ 99.13% and mapped genome percentage of ≥ 93.29% (Table S2).

The principal component analysis (PCA) of the RNA-seq data revealed principal components (PCs) 1 and 2 accounted for 87.5% and 4.5% of the total variability,

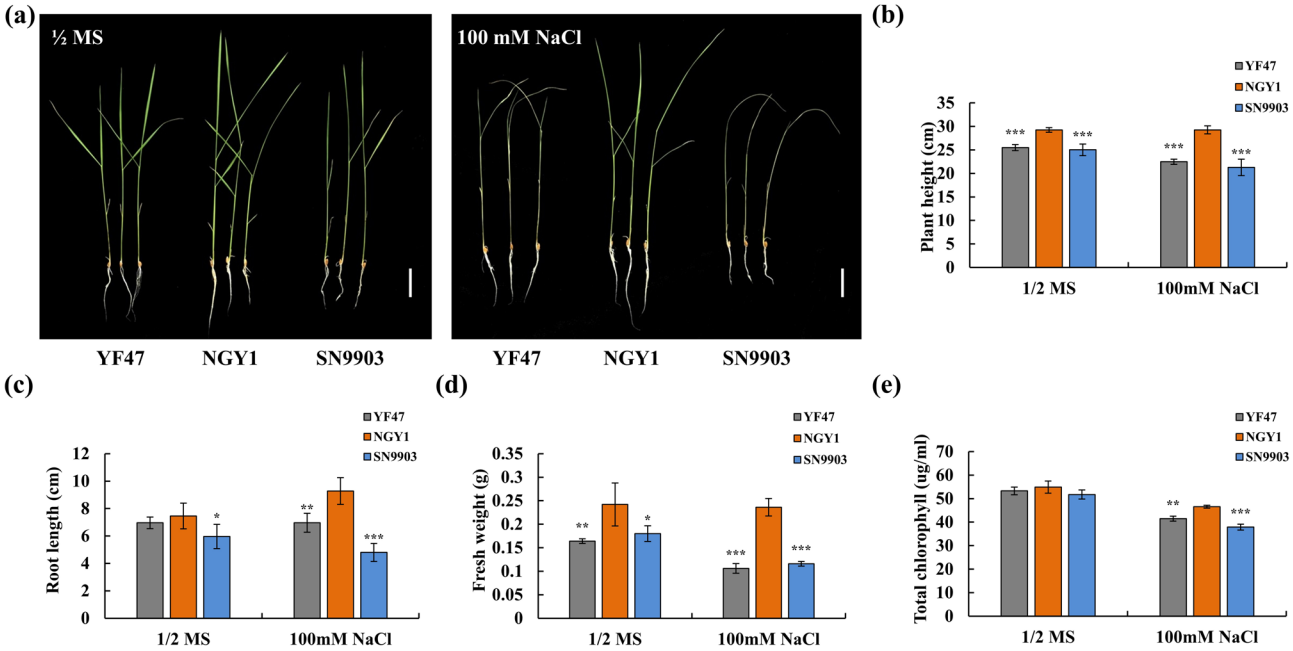

Fig. 1 Morpho-physiological analyses of NGY1, YF47 and SN9903 rice plants in control and salt stress conditions. **a**–**e** The seedlings were cultured normally for 10 days, and then switched to 1/2 MS nutrient solution with or without 100 mM NaCl for 72 h. The plant height (**b**), root length (**c**), fresh weight (**d**) and total chlorophyll content (**e**) of seedlings of NGY1, YF47 and SN9903 under 1/2 MS or salt treatment. Data are expressed as an average of five biological replicates ± SD; Student's *t*-test analysis indicates a significant difference (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001). White scale bar = 3 cm

respectively, and the data for three biological replicates were clustered closely and were separated by the time point and genotypes (Fig. 2a). Using the DESeq2 comparison of all groups, we identified differential expression genes (DEGs) (|Log2FoldChange| > 1, $P < 0.05$) between the NGY1, SN9903 and YF47 at each salt treatment time point. A large number of DEGs in NGY1 were identified, especially at 12 h (3951 upregulated and 4493 downregulated) (Fig. 2b). In addition, salt treatment induced more dramatic transcriptional changes in NGY1 than in SN9903 and YF47 at 1 h (Fig. 2b). In total, over 10,000 DEGs were identified in all salt conditions. Among these DEGs, 888 and 5070 genes were commonly regulated in all cultivars at 1 h and 12 h, respectively, suggesting that more genes changed significantly the transcription levels with the extension of the salt stress time (Fig. 2c, d). It is worth noting that after 1 h of salt stress, the number of up-regulated genes were higher than down-regulated genes, but after 12 h, the number of down-regulated genes were higher than up-regulated genes in all rice varieties tested (Fig. 2b). Together, the data indicated that the salt condition induced dynamic transcriptional regulation in rice and there are differences in parental and progeny responses.

Functional Classification and Annotation of DEGs

GO analyses of DEGs in each variety were carried out to identify the specific gene with main biological functions. We compared the top 10 GO terms of NGY1, SN9903 and YF47 under salt stress (0 h to 1 h or 12 h). After 1 h of salt stress, three varieties all showed significant enrichment in GO terms related to transcriptional initiation response, such as transcription, DNA-templated (GO:0006351), nucleic acid-templated transcription (GO:0097659), and RNA biosynthetic process (GO:0032774) (Fig. 3a, b, c). However, NGY1 enrich more the amount of DEGs in above GO terms than SN9903 and YF47, suggesting that NGY1 had a stronger early response to salt than its parents (Fig. 3a, b and c). After 12 h of salt stress, NGY1 shows more differences than its parents in the top 10 GO terms. First of all, compared with salt stress for 1 h, the gap between the amount of DEGs enriched by NGY1 and its parents was further increased (Fig. 3d, e, f). Secondly, the DEGs enrichment significance of NGY1 is lower than that of the parents (Fig. 3d, e, f). Finally, in the top 10 GO terms, the biological processes of NGY1 are different from YF47 and SN9903 (Fig. 3d, e, f). For example, DEGs of YF47 and SN9903 are more enriched in hydrogen peroxide metabolic process (GO:0042743) and reactive oxygen species metabolic process (GO:0072593) under 12 h salt stress, while NGY1 is not (Fig. 3d, e, f). Although some DEGs of NGY1 was found to be enriched in oxidation-reduction process in the top 20 GO terms, the significance of enrichment was lower than that of its parents (Fig. S2). This suggests that sustained salt stress leads to more oxidative stress in YF47 and SN9903 than in NGY1, as it strives to balance the intracellular REDOX condition in parents. Further quantitative analysis of H_2O_2 levels at

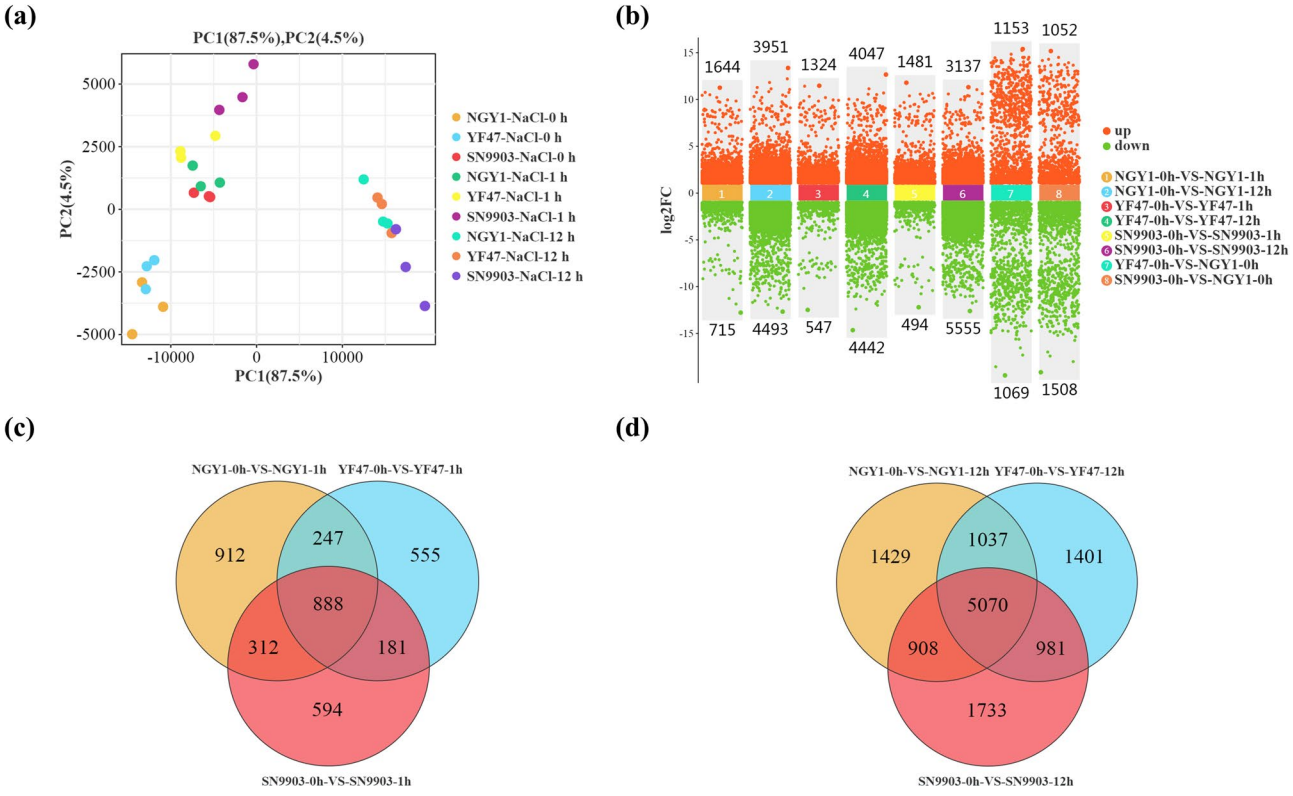

Fig. 2 Sequencing data distribution and differentially expressed genes (DEGs). **a** Principal component analysis (PCA) based on the expressed genes. **b** Total number of DEGs in NGY1 vs. YF47 or SN9903 (0 h, salt stress), NGY1 vs. YF47 or SN9903 (1 h, salt stress) and NGY1 vs. YF47 or SN9903 (12 h, salt stress) comparisons. **c** Venn diagram of DEGs in the 0 h vs. 1 h comparisons (NGY1, YF47, and SN9903). **d** Venn diagram of DEGs in the 0 h vs. 12 h comparisons (NGY1, YF47, and SN9903)

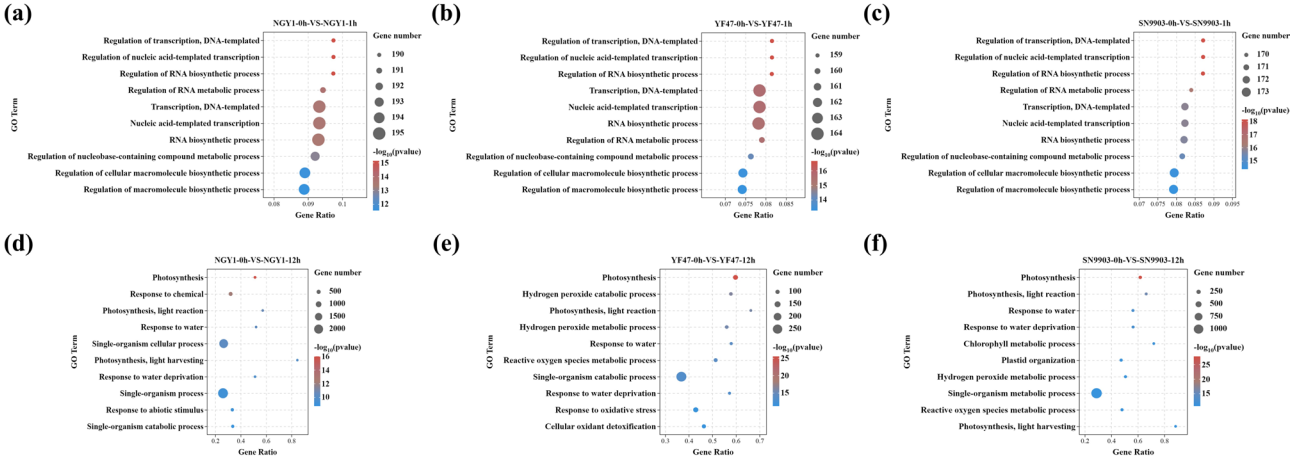

Fig. 3 The diversion in the top 10 enriched GO pathways between the three rice varieties under different salt stress time. **a**–**c** The top 10 GO pathways significantly enriched with DEGs in NGY1 (**a**), YF47 (**b**), and SN9903 (**c**) after 1 h of salt stress. **d**–**f** The top 10 GO pathways significantly enriched with DEGs in the NGY1 (**a**), YF47 (**b**), and SN9903 (**c**) after 12 h of salt stress

12 h post-salt stress revealed ngy1 exhibited significantly lower ROS accumulation compared to its parental cultivars under salt stress condition (Fig. S3).

In addition, after 1 h of salt stress, KEGG analysis showed that three rice varieties were significantly enriched in plant hormone signal transduction, MAPK signaling pathway and biosynthesis of secondary metabolites (top 3), and the number of the enriched genes of NGY1 were higher than those of YF47 and SN9903, with NGY1 > YF47 > SN9903 (Fig. 4a, b, c). Due to the important role of hormone and MAPK pathway in plant abiotic stress (Singh and Jwa 2013), it can be judged that YF47

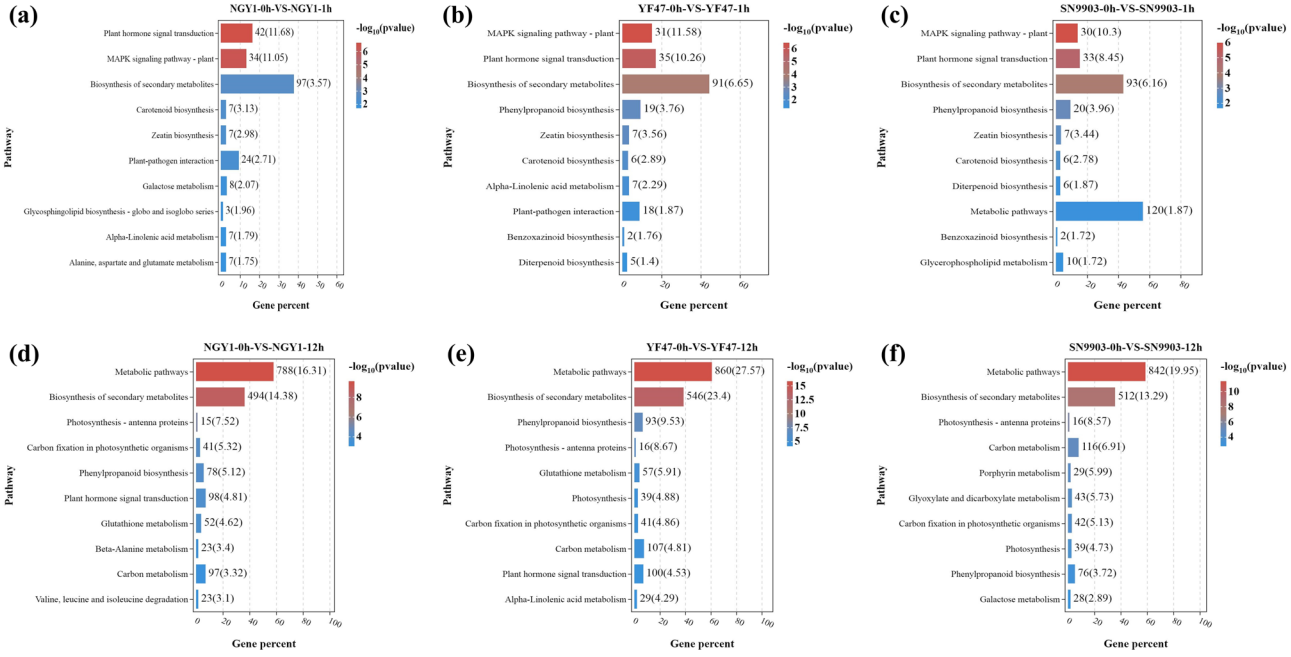

Fig. 4 The top 10 KEGG enrichment pathways among three rice varieties under different salt stress times. (a-c) The top 10 KEGG pathways significantly enriched with DEGs in NGY1 (**a**), YF47 (**b**), and SN9903 (**c**) after 1 h of salt stress. **d**–**f** The top 10 KEGG pathways significantly enriched with DEGs in the NGY1 (**d**), YF47 (**e**), and SN9903 (**f**) after 12 h of salt stress

can mobilize more favorable genes to combat salt stress in a short period of time than its parents. After 12 h of salt stress, the DEGs of the NGY1 were significantly enriched in metabolic pathways, biosynthesis of secondary metabolites and carbon metabolism, and the number and intensity of the enriched genes of NGY1 were lower than those of YF47 and SN9903 (Fig. 4b, c, d). These results indicated that salt stress had threatened the normal metabolism of rice with the prolongation of treatment time, and the adaptability of NGY1 to salt stress was better than that of its parent.

Analyzing the Regulatory Change of Known Salt-Tolerance Related Genes

To further explore the molecular reasons for the differences in salt tolerance between NGY1 and its parents, transcriptional changes in the salt tolerance genes present in the three breeds were analyzed. A total of 104 salt-tolerance related genes were found in DEGs, which were not concentrated in a single rice variety (Additional Data 1). After 1 h of salt stress, more salt-tolerant genes were identified in NGY1 than in its parents, and most of the salt-tolerant genes were significantly up-regulated, while only a few salt-tolerant genes were down-regulated (Fig. 5a). After 1 h of salt stress, among the co-upregulated salt-tolerance related genes, *LEA17* had a high relative expression abundance in all three varieties, and *SKC1* was the only co-downregulated salt-tolerant gene among the three rice varieties, which in NGY1 was more down-regulated than YF47 and SN9903 (Fig. 5a). Furthermore, after 1 h of salt stress, *OsNF-YC13* and *HS18.8* were significantly down-regulated in NGY1, but had no significant changes in the parents, and *STRK1* was up-regulated in the parents, but had no significant changes in NGY1 (Fig. 5a). Among the 15 salt-tolerance genes whose transcriptional changes occurred only in NGY1, the up-regulated expression of TSPO was the highest, and the down-regulated expression of OsNF-YC13 was the lowest (Fig. 5a). These data indicate that NGY1 can mobilize more up-regulated expression of salt-stress-related genes in a short period of time after salt stress compared to its parents.

After 12 h of salt stress, compared with 1 h of salt stress, the number of salt-tolerant genes with transcriptional changes in all varieties was significantly increased, and the number of up-regulated genes was still higher than that of down-regulated genes (Fig. 5 and Additional Data 1). Surprisingly, after 12 h of salt stress, the number of salt-tolerance related genes identified in NGY1's DEGs was lower than that of its parents, and the number of down-regulated genes in the parents was much higher than that in the NGY1 (Fig. 5b and Additional Data 1). Among the co-upregulated genes, *LOC4324157* and *LEA17*, *TSPO*, *SNAC1* and *OsHSP18.0-CIII* had the highest expression difference after 12 h of salt stress, and *LEA17* maintained a high expression difference among all rice varieties after 1 h and 12 h of salt stress (Fig. 5a, b). Under 1 h of salt stress, the up-regulation degree of *LOC4324157* and *SNAC1* in NGY1 was higher than that of parents, but the transcriptional differences were

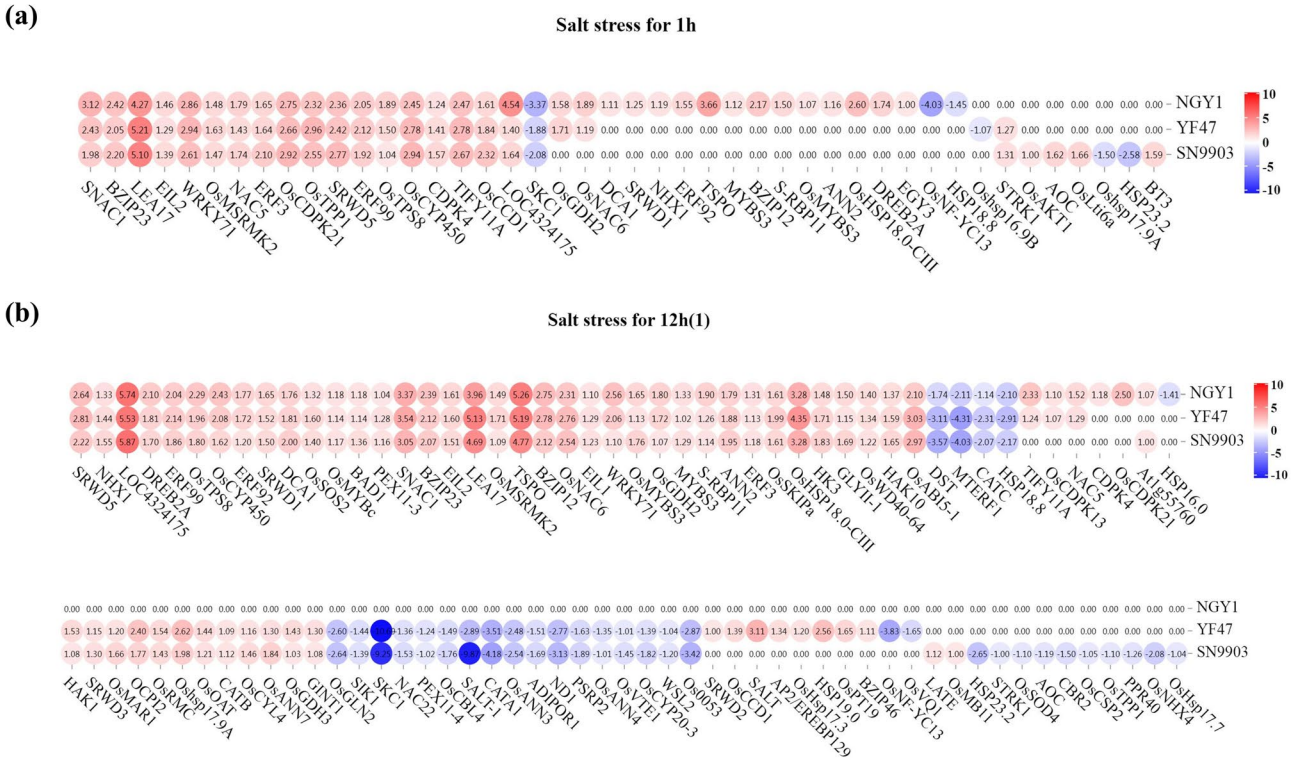

Fig. 5 Expression changes of the salt tolerance related genes. **a** Salt-tolerance related genes of DEGs at the transcriptional level in the three rice varieties after 1 h of salt stress. **b** Salt-tolerance related genes of DEGs at the transcriptional level in the three rice varieties after 12 h of salt stress

similar after 12 h (Fig. 5a, b). *TSPO* and *OsHSP18.0-CIII* were not differentially expressed in parents after 1 h of salt stress, but were differentially expressed in parents and offspring after 12 h of salt stress, and the degree of difference was similar among varieties (Fig. 5a, b). After 12 h of salt stress, four of the five down-regulated genes in NGY1 were less down-regulated than their parents (*DST*, *MTERF1*, *CATC* and *HSP18.8*), except for *HSP16.0*, which was differentially expressed only in NGY1 (Fig. 5b). After 12 h of salt stress, only among the differentially expressed salt-stress-related genes in the parents, the number of down-regulated genes in SN9903 was more than that in YF47 (Fig. 5b). It was worth noting that *SKC1*, which was significantly down-regulated in all three varieties after 1 h salt stress, was only differentially expressed in the parents after 12 h salt stress, and the down-regulated degree was greater (Fig. 5a, b). Among the other two genes down-regulated at 1 h of salt stress in NGY1, *HSP18.8* was significantly down-regulated in all three varieties with prolonged treatment time, while *OSNF-YC13* was down-regulated only in YF47 after 12 h of salt stress (Fig. 5a, b). These data indicate that with the persistence of salt stress, NGY1 is more adaptive to salt stress than it parents, suggesting that there are other genes or ways in its body to regulate its salt tolerance.

Identification of the Significant Differential Alternative Splicing in Rice Genotypes

There has been evidence that alternative splicing (AS) is closely related to plant salt tolerance(Cui et al. 2014; Yu et al. 2021). In order to further explore the reason why salt tolerance of NGY1 was stronger than that of its parent, we analyzed the changes of AS in three varieties, including retained intron (RI), skipped exon (SE), alternative 3′ splice site (A3SS), alternative 5′ splice site (A5SS) and mutually exclusive exons (MXE). A total of nearly 25,000 AS events were detected in all samples tested, most of which were SE (over 1/2) and RI events (Fig. S4). After 1 h of salt stress, the number of AS events increased significantly in each variety, except for the RI events of NGY1, which decreased slightly (Fig. 6a). With the continuation of salt stress, the number of SE events in NGY1 continued to increase, while the number of SE events in its parents decreased (Fig. 6a). Notably, regardless of salt stress, the five types of AS events in NGY1 occurred in higher numbers than in their parents (Fig. 6a). In addition, the comparative analysis of differences showed that under normal conditions, the proportion of various types of AS events between NGY1 and the two parents was similar (Fig. 6b, e). After 1 h of salt stress, the proportion of different types of AS events between NGY1 and the two parents showed great difference, and the difference gradually weakened with the continuation of salt stress

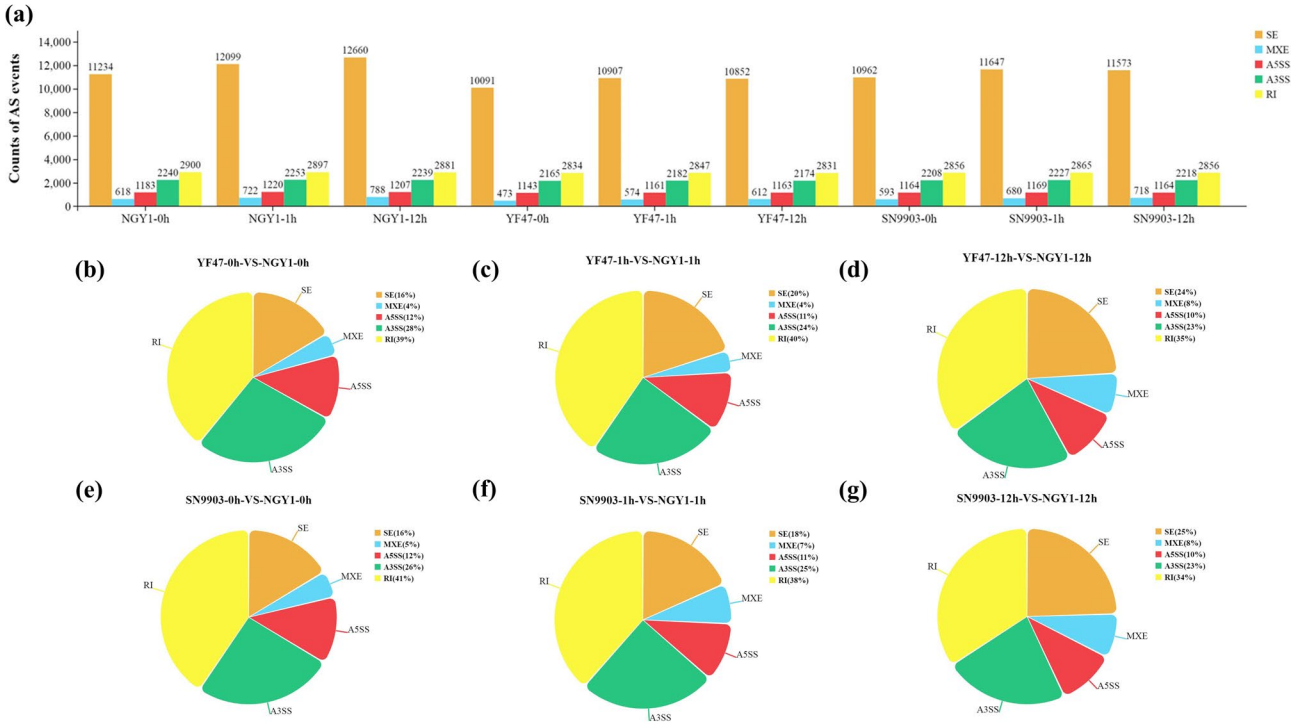

Fig. 6 Global alternative splicing analysis of NGY1, YF47, and SN9903 plants under salt stress. **a** Number statistics of different kinds of alternative splicing (AS) events in NGY1, YF47, and SN9903 plants under different salt stress time. **b**–**d** The difference of AS event proportion between NGY1 and YF47 under 0 h (**b**), 1 h (**c**) or 12 h (**d**) of salt stress. **e**–**g** The difference of AS event proportion between NGY1 and SN9903 under 0 h (**e**), 1 h (**f**) or 12 h (g) of salt stress

(Fig. 6c, d, f, g). Furthermore, as salt stress persisted, the proportion of differed SE events between NGY1 and the parents increased significantly (Fig. 6b, c, d, e, f, g). The GO analysis was performed on the genes with significant SE differences between NGY1 and its parents after 1 h salt stress, and it was found that most of them were concentrated in cellular process, metabolic process and biological regulation (Fig. S5). Meanwhile, two salt-tolerance related genes *NFXL2* and *OTS1* were identified in the YF47-1 h-VS-NGY1-1 h comparison group, and four salt-tolerance related genes *NFXL2*, *OsANN9*, *OsCBSX4* and *OTS1* were identified in the SN9903-1 h-VS-NGY1-1 h comparison group (Additional Data 2). This may be one reason why NGY1 is closer to YF47 in salt tolerance than SN9903. Together, these results suggest that differences in SE events between NGY1 and parents may be one of the reasons for differences in salt tolerance and which play a key role in the early stages of salt stress.

Identification of Other Highly Induced DEGs Identified in NGY1 Under Salinity Stress

In order to further investigate whether there are unknown salt-tolerance related genes that play a key role in NGY1's good salt tolerance, we conducted further analysis of the transcriptome data. Since the above analysis has proved that 1 h salt treatment is the key node for the difference in salt tolerance between NGY1 and its parents, the DEGs between NGY1 and its parents after 1 h salt stress were compared, and the DEGs were analyzed by GO. After 1 h of salt stress, the concentration of DEGs between NGY1 and its parents was mainly related to the defense response (GO:0006952), response to stress (GO:0006950) and response to stimulus (GO:0050896) (Fig. 7a, b). Because the expression of genes enriched in GO:0006952 term had the largest difference, DEGs that was common differentially expressed in NGY1 compare with YF47 or SN9903 was further selected in this GO term for heat mapping (Fig. 7c). Cluster analysis further showed that the expression of these genes was similar in the parents after 1 h of salt stress, but different in the NGY1 (Fig. 7c). In addition, in these DEGs, the number of genes in NGY1 with higher expression than that of the parents was less than the genes with lower expression than that of the parents (Fig. 7c). Further statistical analysis of the genes whose expression levels in Fig. 7c were higher than those of parents showed that most of them belonged to the NBS-LRR protein family (Table 1). Some NBS-LRR protein gene in Table 1 were randomly selected for RT-qPCR, and their expression similar to the RNA-seq data, which verified the accuracy of RNA-seq (Fig. 7d). It is worth noting that the expression levels of these genes in NGY1 were higher than those of their parents regardless of salt stress, and the differences gradually increased with the persistence of salt stress (Fig. 7d).

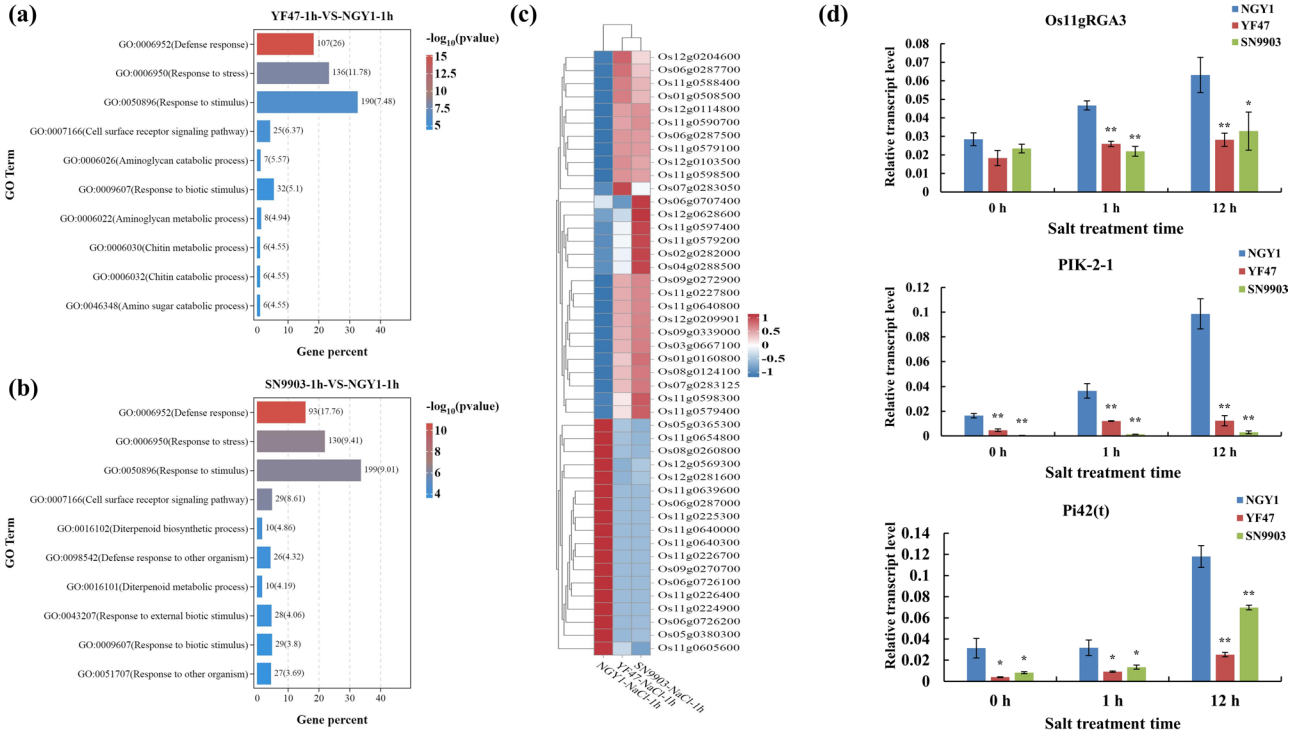

Fig. 7 Other highly regulated DEGs identified in salinity stressed NGY1 plants. **a**, **b** The top 10 significantly enriched GO pathways of DEGs in NGY1 and YF47 (a) or SN9903 (b) after 1 h of salt stress. **c** Heatmap of the differentially expressed genes that are significantly enriched in GO:0006952 pathway in NGY1, YF47 or SN9903 after 1 h of salt stress. **d** RT-qPCR analyses of *Os11gRGA3*, *PIK-2-1* and *Pi42(t)* in NGY1, YF47, and SN9903 different salt stress times. Expression was normalized to that of Actin. Values were mean ± SD ($n = 3$). Student's *t*-test analysis indicates a significant difference (*$P < 0.05$ and **$P < 0.01$)

Table 1 DEG statistics of GO:0006952 term with higher expression in NGY1 than YF47 and SN9903 after 1 h salt stress

ID	NGY1 (FPKM)	YF47 (FPKM)	SN9903 (FPKM)	Symbol	Description
Os06g0287000	0.737	0.001	0.001	RPP13L3	Similar to NBS-LRR type R protein, Nbs4-Pi
Os11g0639600	8.585	0.001	0.001	RPP13L3-1	NB-ARC domain containing protein (Contains the LRR domain)
Os11g0224900	3.114	0.067	0.054	Os11gRGA3	Similar to NBS-LRR protein (Fragment)
Os06g0726100	92.858	4.192	4.349	Cht3*	Similar to Seed chitinase-c
Os11g0225300	4.839	0.001	0.001	Os11gRGA5	Nucleotide binding site-leucine rich repeats (NBS-LRRs) protein, Resistance protein, Resistance to the blast fungus, (Nippponbare: susceptible to the blast fungus carrying the AVR-Pia
Os11g0226400	3.742	0.001	0.008	RGA5	Similar to Nitrate-induced NOI protein, expressed
Os11g0640300	2.369	0.001	0.001	RPM1	NB-ARC domain containing protein (Contains the LRR domain)
Os05g0365300	5.476	1.086	0.654	OsBBR1	Nucleotide-binding site leucine-rich repeat (NBS-LRR) protein, Bacterial blight resistanc
Os06g0726200	320.190	86.023	88.983	Cht1*(Chi1)	Similar to Chitinase 1
Os11g0226700	0.832	0.001	0.001	RGA4	Hypothetical conserved gene
Os12g0569300	115.333	31.035	40.082	--	Thaumatin, pathogenesis-related family protein
Os09g0270700	0.677	0.001	0.001	RPM1-1	Disease resistance protein domain containing protein
Os11g0654800	2.663	1.103	1.019	PIK-2	Conserved hypothetical protein
Os12g0281600	5.267	1.969	2.231	Pi42(t)	NB-ARC domain containing protein (Contains the LRR domain)
Os11g0640000	0.381	0.001	0.001	--	NB-ARC domain containing protein (Contains the LRR domain)
Os05g0380300	1.573	0.599	0.629	A1.1-4	Similar to NBS-LRR protein (Fragment)
Os08g0260800	0.264	0.016	0.007	RPM1-2	Similar to NB-ARC domain containing protein (Contains the LRR domain)
Os11g0605600	2.106	0.520	0.025	PIK-2-1	Similar to NB-ARC domain containing protein, expressed (Contains the LRR domain)

These evidences suggest that the differential expression of some NBS-LRR protein genes may be one of the reasons why salt tolerance of NGY1 is better than that of its parents.

Discussion

Due to global climate change and man-made causes, more and more irrigable land is affected by salinization, threatening food security (Horie et al. 2012). As the primary food crop for the effective utilization of saline-alkali land, it is an important task to understand the response of rice to salt stress from the genetic level and to select salt-tolerant varieties (Bhatt et al. 2020). Altering gene expression has been shown to improve plant tolerance to abiotic stress(Orellana et al. 2010; Hirayama and Shinozaki 2010). In this study, the gene expression changes of salt-tolerant rice variety NGY1 and its parents (YF47 and SN9903) were analyzed by RNA-seq technology under salt stress, and the mechanism of salt tolerance in rice was investigated.

As a moderately salt-sensitive crop, rice is especially sensitive to salt stress at seedling stage (Chang et al. 2019). Phenotypic and physiological analysis showed that NGY1 was more tolerant to salt than its parents at seedling stage (Fig. 1). Transcriptome analysis showed that more genes in NGY1 were changed after 1 h of salt stress than their parents, and this phenomenon disappeared after 12 h of salt stress (Fig. 2b). It can be concluded that salt stress 1 h may be the key node for NGY1 to be better than its parents in salt tolerance. Further GO analysis showed that after 1 h of salt stress, the DEGs identified in all varieties were significantly enriched in the transcriptional initiation response, but the number of DEGs enriched in NGY1 was more than that in its parents (Fig. 3a, b, c). In addition, after 1 h of salt stress, the number of DEGs enriched in plant hormone signaling, MAPK signaling and secondary metabolite biosynthesis pathway in NGY1 was more than that of parents (Fig. 4a, b, c). Plant hormones, MAPK pathways and secondary signals have been shown to be significantly related to salt stress (Yu et al. 2020; Yang and Guo 2018; Jan et al. 2021). These data support our view that 1 h after salt stress, NGY1 has the ability to mobilize more favorable genes for salt stress resistance than its parents. The importance of early response to salt stress has also been highlighted by a previous study in which salt-tolerant rice *Pokkali* was able to induce more transcripts than salt-sensitive rice IR29 at 1 h after salt stress (Kawasaki et al. 2001). The difference is that while many transcripts in *Pokkali* are down-regulated in response to the initial salt shock, NGY1 is more up-regulated. However, both up-regulated and down-regulated DEGs in NGY1 were higher than those in parents at 1 h after salt stress (Fig. 2b). Other studies have shown that a large number of genes are upregulated to achieve salt tolerance in different genetic backgrounds (Ismail and Horie 2017; Akbar et al. 2022). Therefore, the mobilization of more favorable gene upregulation in a short period of time may be critical for NGY1 to resist salt stress. Notably, after 12 h of salt stress, SN9903, which was the least salt-tolerant, identified more differentially expressed genes than NGY1 and YF47, but most of them were down-regulated (Figs. 1 and 2b and d).

With the continuous salt stress, there was an explosive increase of DEGs in all rice varieties, both up-regulated and down-regulated DEGs (Fig. 2b). The GO enrichment of DEGs in NGY1 and the parents showed greater differences after 12 h salt stress than after 1 h salt stress. One of the differences was the lower significance and higher number of DEGs enriched REDOX process in NGY1 than its parents (Fig. 3d, e, f and Fig. S2). Further physiological experiments showed that YF47 and SN9903 accumulated more H_2O_2 after 12 h of salts stress than NGY1 (Fig. S3). Many studies have shown that oxidative stress caused by salt stress is one of the causes of plant damage, and improving REDOX capacity is beneficial to salt tolerance of rice (Vaidyanathan et al. 2003; Hazman et al. 2015; Zhao et al. 2022). Therefore, the reason for less damage to NGY1 under continuous salt stress may be that NGY1 is able to mobilize more redox-related genes in response to salt stress than its parents. Furthermore, KEGG analysis showed that the number and significance of DEGs enriched in the metabolic pathways and biosynthesis of secondary metabolites pathways of NGY1 were weaker than those of the parents after 12 h salt stress. Existing studies have shown that salt stress can induce the expression of metabolite and secondary metabolite-related genes in rice (Rajkumari et al. 2023; Chandran et al. 2019). Therefore, it can be judged that continuous salt stress is less harmful to NGY1 than its parents, which is consistent with our phenotypic data (Figs. 1 and 4d, e and f).

There have been many reports on the transcriptional changes in rice under salt stress, however, few reports have investigated the transcriptional differences between parents and offspring under salt stress (Ren et al. 2022, 2023). In theory, the salt tolerance genes of the offspring are all derived from the parents, so the key reason for the better salt tolerance of the offspring than that of the parents may be the difference in the transcription level of salt tolerance genes. In previous studies, *TPSO*, *OsNF-YC13* and *SKC1* have been shown to be key transporters regulating the salt tolerance response in rice, which is associated with REDOX and Na^+/K^+ homeostasis in rice plants under salt stress (Pandey et al. 2021; Manimaran et al. 2017; Alnayef et al. 2020). In this study, we found that more genes related to salt tolerance were up-regulated in NGY1 compared to the parents under short-term salt stress (Fig. 5a). Among the salt tolerance-related DEGs

identified only in NGY1, *TPSO* (tryptophan-rich sensory protein/translocator protein) had the highest transcription level after 1 h salt stress (Fig. 5a). After 1 h of salt stress, the number of down-regulated salt tolerance-related DEGs identified in all rice varieties was fewer, with the largest down-regulation of *OsNF-YC13*, which were identified only in NGY1 (Fig. 5a). *SKC1* was the only DEGs with down-regulated expression related to salt tolerance that was identified in all rice varieties, but the degree of down-regulation was greater in NGY1 than in the parents (Fig. 5a). Notably, as salt stress continued, *TPSO* was identified to be up-regulated in all rice varieties, and *SKC1* returned to its pre-stress level in NGY1, while down-regulation of *SKC1* expression occurred in the parents (Fig. 5b). These results suggest that the early response of the related salt tolerance genes to salt stress is the key to determine that NGY1 is more tolerant to salt than its parents.

In addition, the AS events changes in NGY1 were also different from those in the parents during salt stress. Previous studies have shown that salt stress can lead to a significant increase in AS events, indicating that AS events play an important role in plant response to salt stress (Ding et al. 2014). In this study, the number of all types of AS events in NGY1 was higher than that in the parents, regardless of salt stress (Fig. 6a). Among all detected AS events, the number of SE events increased in NGY1 as salt stress continued but decreased in parents, and the proportion of different SE events between NGY1 and the parents increased significantly (Fig. 6). Since RI is the most prevalent AS form in rice, most previous studies have focused only on RI's role under salt stress, with little attention paid to SE (Mirdar Mansuri et al. 2019). *NFXL2* and *OTS1* were identified as two genes with differential SE events in NGY1 and its parents after 1 h of salt stress, which were found to be related to the changes in ROS and root development under salt stress (Additional Data 2)(Srivastava et al. 2016; Schmidt et al. 2013). This may be one of the reasons why NGY1 has a more developed root system than its parent under salt stress (Fig. 1c). A report on RNA-seq analysis of three rice leaves with different salinity tolerance showed that variety-specific mRNA AS during the early stages of salt stress may be significantly associated with salt tolerance in rice (Jian et al. 2022). Our study suggests that SE events at the early stage of salt stress may play a major role in salt stress tolerance in rice. However, since most SE events were newly identified, its important role in salt stress in rice requires further investigation. (Fig. S4).

At the early stage of salt stress, the DEGs between NGY1 and its parents were mainly enriched in the response to defense response, stress and stimulus (Fig. 7a, b). Interestingly, some NBS-LRR protein family genes were found to be associated with salt stress in this report, which was often associated with rice blast in previous studies (Yuan et al. 2011; Okuyama et al. 2011; Guo et al. 2016) (Fig. 7; Table 1). A heterologous transient transformation experiment showed that overexpression of *ZmNBS25* (a maize NBS-LRR gene) caused increased electrolyte leakage and ROS accumulation in tobacco leaves (Xu et al. 2018). A recent report indicated that *OsBRW1*, coded a NBS-LRR protein, is closely related to the accumulation of intracellular reactive oxygen species (Ma et al. 2025). Given that ROS and electrolyte leakage are also important indicators of salt tolerance in plants, it can be speculated that the NBS-LRR protein genes may regulate the response of rice to salt stress by regulating related pathways. In particular, the transcription levels of some NBS-LRR protein family genes (*Os11gRGA3*, *PIK-2-1* and *Pi42(t)*) in NGY1 were higher than those in its parents regardless of salt stress, which might be one of the reasons why NGY1 was better than its parents in salt tolerance (Fig. 7d).

Conclusion

In summary, our results suggest that differences in transcription and AS (SE) of related genes under short-term salt stress between the NGY1, YF47 and SN9903 may be a key reason for the difference in salinity tolerance between the offspring and the parent. At the same time, our analysis revealed some genes that may play key roles in salinity tolerance in rice (like NBS-LRR protein family genes), and they can be used as valuable resources to provide theoretical basis for salt tolerance breeding in rice.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author Contributions

C.L. and Z.Y.D. planned the research. C.L., K.L, W.H.L, T.C., S.Y., L.H., X.D.W., L.Z., L.H.Z, C.F.Z., Q.Y.Z. and Z.Z performed the experiments. C.L. analyzed the data and wrote the manuscript. Y.D.Z. revised the manuscript. C.L.W. reviewed the manuscript. All authors approved the final paper.

Data Availability

The sequencing data has all been archived in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA1260548.

Declarations

Competing Interests
The authors declare no competing interests.

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