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Identification and analysis of miRNA - mRNA regulatory modules associated with resistance to bacterial leaf streak in rice

Baowei Wu[1], Xiaoyu Zhang[2], Jialiang Zhao[1], Bohong Zeng[1] and Zhibin Cao[1*]

Abstract

Background B acterial leaf streak (BLS) is a bacterial disease that severely affects rice leaves, leading to significant yield reductions. microRNAs (miRNAs) are short non-coding RNAs extensively involved in the growth, development, and stress responses of plants and animals. However, miRNAs that regulate the response of rice to bacterial leaf streak are still relatively scarce.

Results The indica rice variety Dular exhibits resistance to BLS, whereas the variety 9311 is highly susceptible to the disease. By conducting miRNA sequencing and transcriptome sequencing on both Dular and 9311 before and after BLS inoculation, we identified 19 miRNAs that were significantly downregulated at both 12 and 24 h post-inoculation in Dular, and 9 miRNAs that were significantly upregulated at the same time points in 9311. Additionally, through degradome sequencing, we identified 23 miRNA- mRNA regulatory modules that likely play crucial roles in rice resistance to BLS, and 4 miRNA- mRNA regulatory modules that may be important in rice susceptibility to the disease.

Discussion Current studies on rice disease resistance miRNAs primarily focus on those involved in resistance to rice blast and bacterial blight, with the miRNA-target mRNA regulatory mechanisms for BLS remaining unclear. This study has identified miRNA-mRNA modules that may play significant roles in rice responses to BLS, contributing to the understanding of the miRNA regulatory network involved in rice defense against BLS infection.

Keywords MiRNA, Bacterial leaf streak, miRNA-seq, RNA-seq, miRNA-mRNA regulatory network

Introduction

Bacterial leaf streak (BLS) is a significant bacterial disease affecting rice leaves, caused by the pathogenic variant *Xanthomonas oryzae* pv. *oryzicola* (Xoc), which infects the plant through wounds or stomata. This disease leads to a reduction in rice yield, with a 10% decrease in mild cases and over 40% in severe cases, posing a significant threat to global rice production. Currently, the main strategy for controlling this disease focuses on identifying disease-resistant genes and using them to develop resistant varieties [1]. microRNAs (miRNAs) are short non-coding RNAs, typically 20 to 24 nucleotides in length, widely present in plants [2, 3]. The processing of miRNAs

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in plants is now well understood: first, RNA polymerase II transcribes the initial miRNA precursor, pri-miRNA (primary miRNA). Then, pri-miRNA is processed into pre-miRNA by the action of a Dicer-like (DCL) protein complex. The pre-miRNA is subsequently transported through the nuclear pore into the cytoplasm, where it is further cleaved by Dicer protein to form the mature short miRNA. Most miRNAs are highly conserved through evolution. Mature miRNAs associate with Argonaute (AGO) proteins to form the RNA-induced silencing complex (RISC) [4, 5]. RISC functions through two primary mechanisms: directly pairing with target mRNAs to induce cleavage and degradation, or binding to the 3' UTR of target genes to inhibit their translation, thereby regulating gene expression [6–8].

Currently, the miRNA database miRBase (Release 22.1) includes 38,589 precursor miRNA sequences and 48,885 mature miRNAs across 271 species. Rice has the highest number of miRNAs recorded, with 604 pre-miRNA sequences and 738 mature miRNAs [9]. Despite this extensive cataloging, the target genes and biological functions of many rice miRNAs remain incompletely understood.

Research has demonstrated that miRNAs play crucial regulatory roles in plant responses to both biotic and abiotic stresses by modulating the expression of target genes, thereby affecting disease resistance and adaptability [10–14]. For instance, Maize miR167 is the first miRNA identified to be associated with resistance to maize chlorotic mottle virus (MCMV). Studies have shown that miR167 positively regulates maize resistance to MCMV. Further research revealed that the auxin response factors *ZmARF3* and *ZmARF30* are target genes of miR167, and miR167 negatively regulates *ZmARF3* and *ZmARF30*, thus influencing maize resistance to MCMV. This mechanism explains how miR167 regulates maize resistance to MCMV by targeting *ZmARF3/30* [15]. Wheat leaf rust is a fungal disease that severely affects the wheat leaves. Studies have shown that the candidate resistance gene Lr46-*RLK3*, associated with leaf rust resistance, is regulated by miR164, while Lr46-*Glu2* and Lr46-*RLK2* are regulated by miR9780, and the expression of Lr46-*Glu2* is further influenced by miR5384. These findings suggest that these miRNAs may play a crucial role in wheat's response to leaf rust [16]. rice miR160a targets *OsARF8* (Auxin Response Factor 8), and mutants of *ARF8* exhibit resistance to rice blast, highlighting miR160a's role in regulating rice blast resistance [17]. Additionally, rice miR168 targets *OsAGO1* (Argonaute proteins 1), a key component of the RISC complex essential for miRNA function. Overexpression of miR168 in rice reduces resistance to rice blast and delays the flowering period, whereas downregulation of miR168 shortens the flowering period, increases yield, and enhances blast resistance. Furthermore, miR535 regulates tillering, miR164 controls the flowering period, and miR1320 modulates disease resistance, underscoring the miR168-*OsAGO1* module's role in balancing yield and disease resistance in rice [18]. Another example is rice miR171, which targets *SCL6* (SCARECROW-like 6). Overexpression of miR171b increases resistance to rice blast and delays flowering, while suppression of miR171 expression reduces resistance and accelerates flowering. *SCL6* mutants display phenotypes similar to miR171b overexpression lines, indicating miR171b' s role in coordinating resistance and flowering time [19]. Additionally, rice miR1871 targets *OsMFAP1* (Microfibrillar-Associated Protein 1); downregulation of miR1871 elevates *OsMFAP1* expression, enhancing resistance to rice blast and increasing individual plant yield. This suggests that the miR1871-*OsMFAP1* module is pivotal in regulating rice blast resistance and yield [20]. Although several rice miRNAs have been identified in the regulation of rice blast response, research specifically focused on miRNAs involved in BLS remains limited. What remains intriguing is which miRNAs are involved in regulating the rice response to bacterial leaf streak disease?

In this study, we selected a resistant variety, Dular, and a susceptible variety, 9311, employing miRNA sequencing, degradome sequencing, and transcriptome sequencing. The objective is to identify miRNA-mRNA modules that play significant roles in rice's response to BLS infection. This research aims to provide critical insights into the miRNA - mRNA regulatory mechanisms underlying rice's defense against BLS, facilitating the identification and functional analysis of key regulatory elements involved in this process.

Materials and methods

Rice cultivation and bacterial leaf streak inoculation

Seeds of the indica rice varieties Dular and 9311 were soaked overnight. The water-imbibed seeds were then transferred to petri dishes lined with filter paper and placed in an incubator to promote germination. The incubator was set to a temperature regime of 30 °C with 13 h of light and 26 °C with 11 h of darkness, The light intensity was set to high, and the humidity was set to 60%. After one week, uniformly growing seedlings were selected and transplanted into soil, continuing cultivation within the incubator.

After approximately six weeks of growth, when the rice reached the tillering stage, uniformly growing plants were selected for bacterial leaf streak (BLS) inoculation. First, the frozen stock of the highly virulent RS105 strain of the rice BLS pathogen was activated, and the bacterial suspension was adjusted to an OD_{600} of 0.5 using PBS buffer. The suspension was then inoculated onto rice leaves using the needle puncture method. Each rice

plant was inoculated on two fully expanded leaves, with more than 30 plants per variety. Following inoculation, the incubator temperature was adjusted to 30 °C for 14 h and 26 °C for 10 h, The light intensity was set to high, and the humidity was set to 60%, and the growth containers were covered with cling film to maintain humidity for 24 h. After the humidification period, the cling film was removed, and cultivation was continued.

Statistics of lesion length after inoculation

Fourteen days after inoculation with the RS105 physiological race of bacterial leaf streak (BLS), the phenotypic responses of the plants were evaluated. Lesion lengths on the inoculated leaves were measured using a ruler, with over 50 lesions recorded for each rice variety.

Statistical analysis and significance testing

All data are presented as the mean ± standard error of biological replicates. Statistical analysis was performed using t-tests. Asterisks indicate significant differences at the following levels: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Sample collection and RNA sequencing

Rice leaves from the Dular variety (labeled as R_mock, R_12h, and R_24h) and the 9311 variety (labeled as S_mock, S_12h, and S_24h) were collected before inoculation and at 12 and 24 hours post-inoculation with bacterial leaf streak (BLS). At each time point, three biological replicates were obtained, each sample consisted of at least 1 g of tissue. 18 samples were immediately snap-frozen in liquid nitrogen and sent to Lianchuan BioTech (Hangzhou, China) for miRNA sequencing, transcriptome sequencing using the Illumina NovaSeq 6000, miRNA sequencing libraries were prepared using the TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, USA). After sequencing, 3' adapters and unwanted sequences were removed to obtain clean data. Small RNAs ranging from 18 to 25 nucleotides were retained, and the sequences were aligned to mRNA, RFam, and Repbase databases (excluding miRNAs) for filtering. The filtered reads, after length and RFam database filtering, were aligned to precursor sequences and the genome for miRNA identification. miRNAs with a p-value < 0.05 were selected as differentially expressed.

RNA sequencing of these 18 samples was performed by Lianchuan BioTech using the Illumina NovaSeq 6000. After sequencing, adapter sequences and low-quality reads were removed, yielding clean reads. Gene expression levels were quantified using FPKM (Fragments per kilobase million). Differentially expressed genes (DEGs) were identified based on a fold change ≥ 2 (| log2FC| ≥1) and a q-value < 0.05. Gene ontology (GO) analysis was performed to classify gene functions, while KEGG pathway analysis was used to identify enriched metabolic and signaling pathways. P-values were corrected for FDR, and GO terms and pathways with an FDR ≤ 0.05 were considered significantly enriched. GSEA analysis was performed using GO and KEGG datasets to identify gene sets with high Enrichment Scores (ES). Significant gene sets were selected based on the criteria of p-value < 0.05 and FDR ≤ 0.05.

Degradome sequencing analysis

The R_mock and S_mock samples were sent to Lianchuan BioTech for degradome sequencing using the Illumina HiSeq 2500. NRPM (normalized reads per million) was used to align the data with the database, removing redundant sequences. The remaining alignable sequences were mapped to the cDNA database of the sequenced species to generate the degradome density file. miRNA target genes were identified by integrating the cleavage site prediction data. A regulatory network of the selected miRNAs and their targets was visualized using Cytoscape.

RNA extraction

Following sample collection, rice leaves were immediately flash-frozen in liquid nitrogen and ground into a fine powder using a mechanical grinder. Total RNA was extracted with TRIzol reagent RNAiso Plus (Takara, China) according to the manufacturer's instructions. RNA quantity and quality were assessed using a NanoDrop (Thermo Fisher, USA). High-quality RNA samples were stored at − 80 °C for subsequent analyses.

qPCR analysis of MiRNA and mRNA

For miRNA quantification, the stem-loop method was employed using the miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme, China). Briefly, total RNA was reverse-transcribed using miRNA-specific stem-loop primers together with U6-specific downstream primers, and the resulting cDNA was subsequently diluted 20-fold. *U6* (GenBank: XR_003239727.1) served as the internal reference gene. qPCR was performed with Takara TB Green® Premix Ex Taq™ II kit (Takara, China), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

For mRNA quantification, total RNA was first treated with HiScript IV RT SuperMix for qPCR (+ gDNA wiper) (Vazyme, China) to remove genomic DNA and then reverse-transcribed. Subsequent qPCR was conducted using the Takara TB Green® Premix Ex Taq™ II kit, with *Actin* (LOC_Os03g50885.1) serving as the internal reference gene. The $2^{-\Delta\Delta Ct}$ method was also used to analyze these data. All primer sequences were designed using Primer Premier 5.0 and are provided in Supplementary Table S1.

Results

Phenotypic response to bacterial leaf streak inoculation

In this study, we inoculated two indica rice varieties, Dular and 9311, with the highly virulent *Xanthomonas oryzae* pv. *oryzicola* strain RS105. Nine days post-inoculation, we observed the phenotypic responses of both varieties. The results showed that the Dular variety exhibited a clear resistant phenotype, while 9311 displayed a susceptible phenotype (Fig. 1A). Statistical analysis further revealed that the lesion length in Dular was significantly shorter than that in 9311 at 9 days post-inoculation (Fig. 1B). These findings indicate that Dular has strong resistance to bacterial leaf streak, whereas 9311 is more susceptible to the disease.

Identification and profiling of MiRNAs involved in bacterial leaf streak response

To identify and screen miRNAs involved in the regulation of rice responses to bacterial leaf streak (BLS) infection, we collected leaf samples at the five-leaf stage from the BLS-resistant variety Dular (R) and the susceptible variety 9311 (S), as well as from plants inoculated with the RS105 strain at 12 and 24 h post-inoculation, for miRNA sequencing. Correlation analysis showed high reproducibility within the same sample group (Fig. 2A). Principal component analysis (PCA) revealed that samples from the resistant variety R clustered together, while samples from the susceptible variety S formed a separate cluster (Fig. 2B), indicating good biological reproducibility within each group.

Statistical analysis of the miRNA mature sequence lengths showed that most rice miRNAs ranged from 21 to 24 nucleotides, with 24-nt miRNAs representing the largest proportion (Fig. 2C), accounting for 49.6% of all unique sRNAs sequenced. Given the high conservation of miRNAs across species, we performed a conservation analysis of the sequenced miRNAs. The results indicated that the majority of the miRNA precursor sequences aligned with the rice genome, followed by alignments with genomes of species such as apple, soybean, and grape (Fig. 2D).

To identify differentially expressed miRNAs (DEMs), we applied a threshold of $p < 0.05$. In the susceptible variety, 31 miRNAs were upregulated and 50 downregulated 12 h post-inoculation, while 37 miRNAs were upregulated and 28 downregulated at 24 h post-inoculation, compared to the mock treatment. In the resistant variety, 13 miRNAs were upregulated and 35 downregulated at 12 h post-inoculation, while 8 miRNAs were upregulated and 31 downregulated at 24 h post-inoculation (Fig. 2E). These results indicate that downregulated miRNAs predominantly respond to BLS infection in rice. Venn diagram analysis of the DEMs across the four sample groups revealed no shared miRNAs between the resistant variety R and the susceptible variety S before or after BLS inoculation (Fig. 2F).

Differential MiRNA expression in resistant and susceptible rice varieties

Given the significant differences between the resistant variety R and the susceptible variety S, and the Venn diagram analysis showing no common DEMs between the two varieties, we separately analyzed the DEMs in each variety. The results revealed that, compared to the mock-treated resistant variety, 21 miRNAs were commonly differentially expressed at both 12 and 24 h post-inoculation

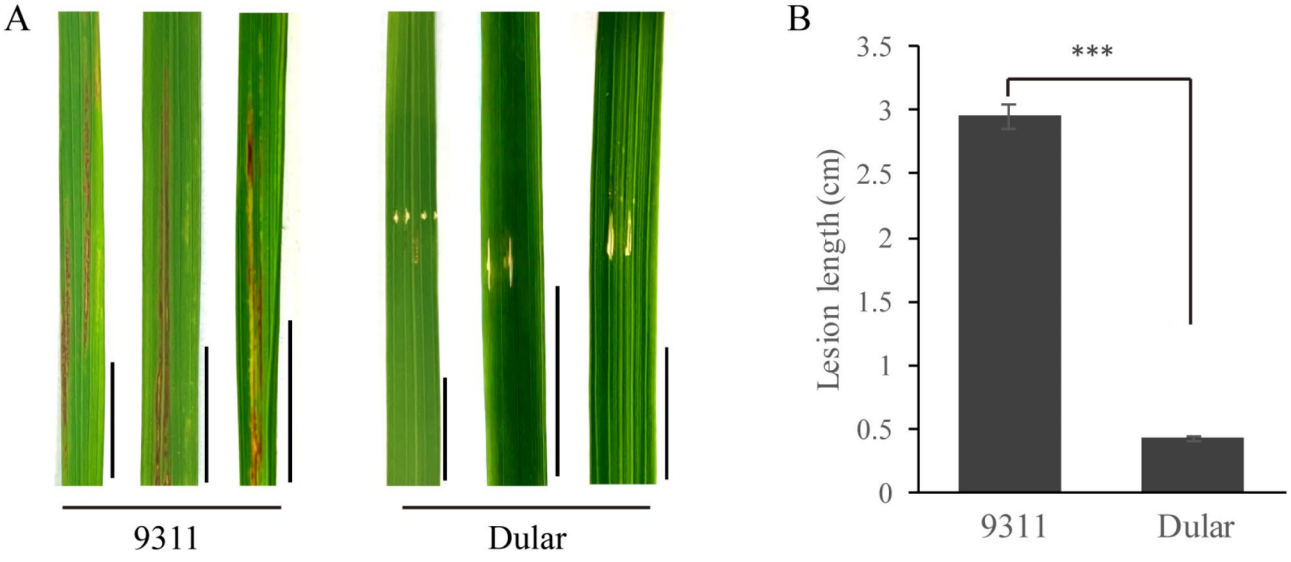

Fig. 1 Phenotype and lesion length of rice inoculated with bacterial stripe disease. **A**, Leaf phenotypes of 9311 and Dualr varieties after 9 days of inoculation with RS105. Bar, 1 cm; **B**, Statistics of lesion length of 9311 and Dualr varieties after 9 days of inoculation with RS105. The number of measured lesions exceeded 30, and significance analysis was performed using t-test. Asterisks on the bar chart indicate statistical significance, *** representing $p < 0.001$

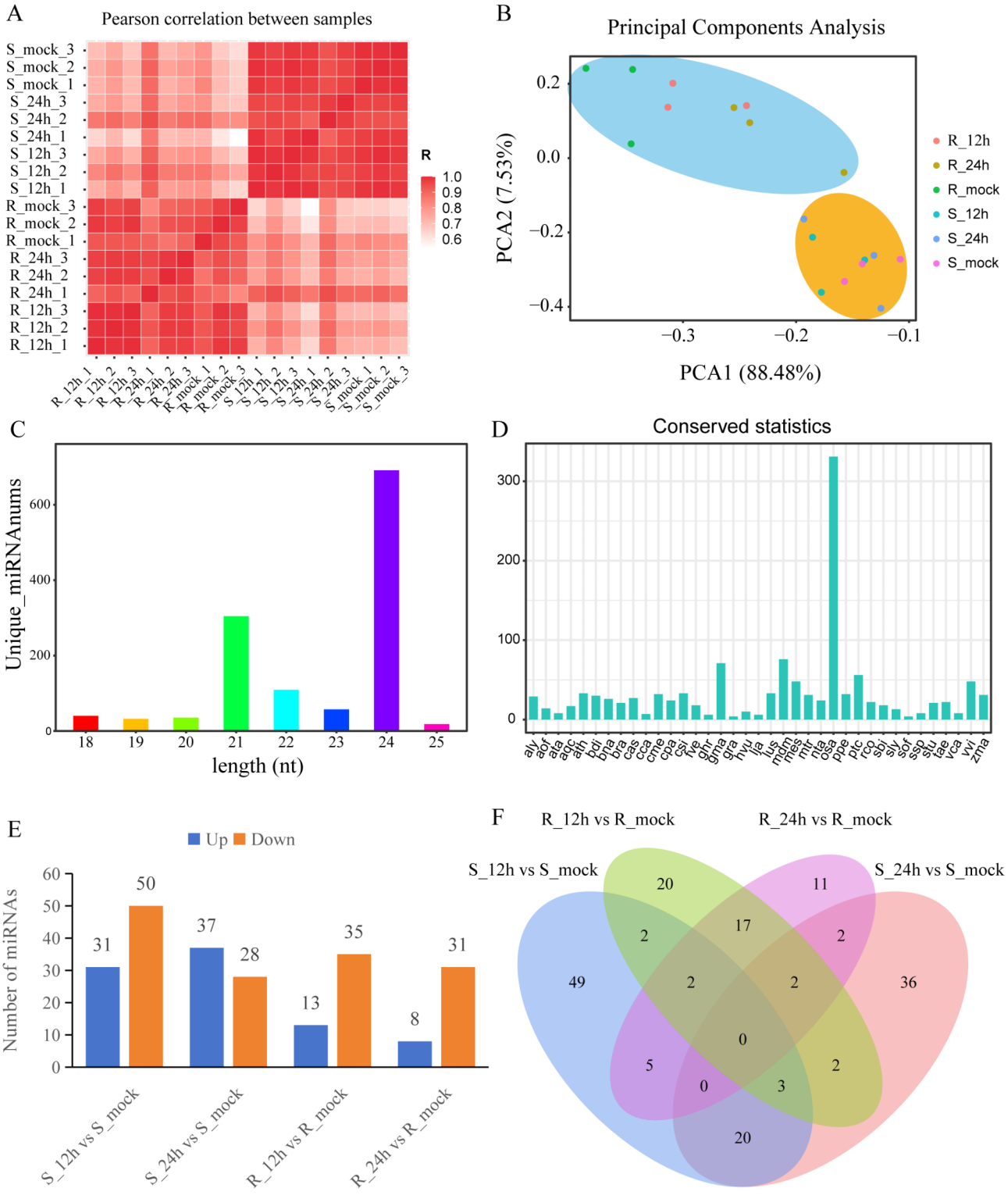

Fig. 2 miRNA sequencing. **A**, Sample correlation analysis in miRNA sequencing. **B**, Principal component analysis of miRNA sequencing data. **C**, miRNA length statistics in miRNA sequencing. **D**, Conservation analysis of miRNAs obtained by sequencing in different species. **E**, Statistics of the number of DEMs. **F**, Venn diagram of DEMs

with BLS (Fig. 3A). In the susceptible variety, 23 miRNAs were commonly differentially expressed at both 12 and 24 h post-inoculation, compared to the mock-treated samples (Fig. 3B).

Further analysis of the expression levels of these commonly DEMs showed that, in the resistant variety, 2 miRNAs were upregulated and 19 were downregulated at both 12 and 24 h post-inoculation (Fig. 3C). In contrast, in the susceptible variety, 9 miRNAs were upregulated and 14 were downregulated at both time points (Fig. 3D). Notably, five miRNAs that were upregulated at 12 h post-inoculation in the resistant variety were downregulated at the same time point in the susceptible variety: osa-miR531a_L-1R-2, osa-MIR159f-p5, osa-MIR5813-p5, osa-miR166d-5p, and osa-MIR162b-p5. Additionally, four miRNAs were upregulated at 12 h in the resistant variety and downregulated at 24 h in the susceptible variety: osa-miR531a_L-1R-2, osa-MIR444f-p3, osa-miR166h-5p, and osa-miR166k-5p (Supplementary Figure S1).

These results suggest that, following infection with bacterial leaf streak, the expression of miRNAs in rice primarily exhibits a downregulation trend. The 19 miRNAs that were consistently downregulated at both 12 and 24 h post-inoculation in the resistant variety are likely to play a crucial role in rice's resistance to bacterial leaf streak.

MiRNA targets identified in resistant and susceptible rice varieties

miRNAs play a critical role in plant stress responses by negatively regulating the expression of target genes. To identify the target genes of miRNAs, we performed degradome sequencing on the resistant variety Dular (R) and the susceptible variety 9311 (S). A total of 9,505 target genes corresponding to 505 miRNAs were identified. Using a threshold of $p < 0.05$, we further filtered these results and identified 330 target genes for 130 miRNAs (Supplementary Table S2).

Next, we focused on the 19 miRNAs that were downregulated in the resistant variety R after infection. Degradome sequencing revealed their respective target genes. Consistent with previous reports, we identified the following targets: miR159a targets *OsGAMYBL* (Os06g0605600, Os01g0812000), miR162a and miR162b target *OsDCL1* (Os03g0121800), miR166c targets *OsHOX9* (Os10g0480200) and *OsHOX10* (Os03g0109400), miR166k also targets *OsHOX9* (Os10g0480200), miR408 targets *OsUCL5* (Os02g0731400) and Os04g0518200, miR528 targets *D3* (Os06g0154200) and *OsUCL23* (Os08g0137400), and miR812n targets Os02g0553500 (Fig. 4).

GO and KEGG enrichment analysis of MiRNA target genes in resistant and susceptible rice varieties

Based on degradome data, we conducted GO and KEGG enrichment analyses of the target genes of DEMs in the resistant variety Dular and the susceptible variety 9311. In the resistant variety Dular, the target genes of miRNAs differentially expressed at different time points before and after infection were mainly enriched in biological processes such as cold response, fungal response, photosynthetic electron transport, and MAPK kinase activity (Fig. 5A). KEGG pathway analysis revealed that these target genes were primarily enriched in pathways related to photosynthesis and nucleocytoplasmic transport (Fig. 5B). In the susceptible variety 9311, the target genes of DEMs across different time points before and after infection were enriched in GO terms related to hormone response, cold response, auxin-activated signaling, protein phosphorylation, and DNA binding (Fig. 5C). The KEGG analysis revealed that these target genes are primarily enriched in pathways related to plant-pathogen interactions and porphyrin metabolism (Fig. 5D). Due to the low number of DEMs in the 9311 (S), the number of target genes identified for these miRNAs in the degradome sequencing was also low. As a result, fewer GO terms and KEGG pathways with p-values less than 0.05 were observed in Fig. 5B and D. Additionally, we conducted an association analysis between the identified target genes and their corresponding miRNAs using a Sankey diagram (Supplementary Figure S2). The miR166 family (e.g., miR166c, miR166h, and miR166k) was found to target numerous mRNAs, primarily involved in functions such as DNA binding, protein binding, and oxidoreductase activity, indicating the significant regulatory role of the miR166 family in rice biological processes. In contrast, the target mRNAs of miR528 and miR408a were predominantly associated with stress-related functions, including defense response, electron transfer activity, and oxidative stress response, suggesting their critical roles in rice adaptation to environmental stresses. These findings highlight the pivotal role of the miRNA–mRNA regulatory network in rice growth, development, and stress response mechanisms.

These results suggest that the mRNA targets of miRNAs involved in rice response to bacterial leaf streak infection are associated with key biological processes, including signal transduction, hormone regulation, and kinase activity. These findings provide new insights into the role of miRNAs in rice's response to bacterial leaf streak and highlight key genes and metabolic pathways that may regulate these processes.

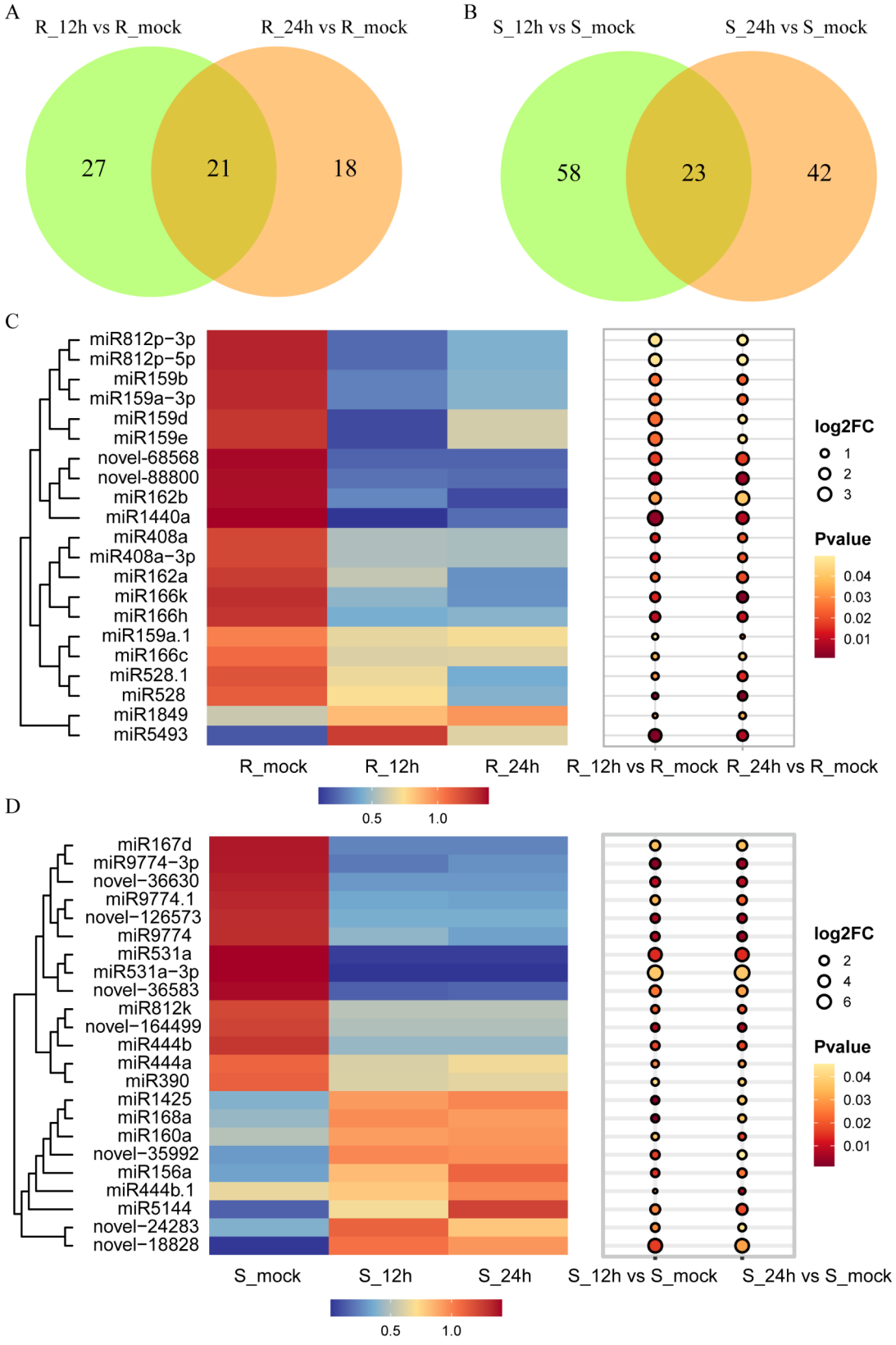

Fig. 3 Differentially expressed (DE) miRNA heatmap and bubble plot. **A**, Statistics of DE miRNAs in resistant rice materials. **B**, Statistics of DE miRNAs in susceptible rice materials. **C**, Expression calorimetric maps of DE miRNAs in resistant materials at 12 h and 24 h after inoculation with RS105. **D**, Calorimetric maps of DE miRNAs in susceptible materials at 12 h and 24 h after RS105 inoculation

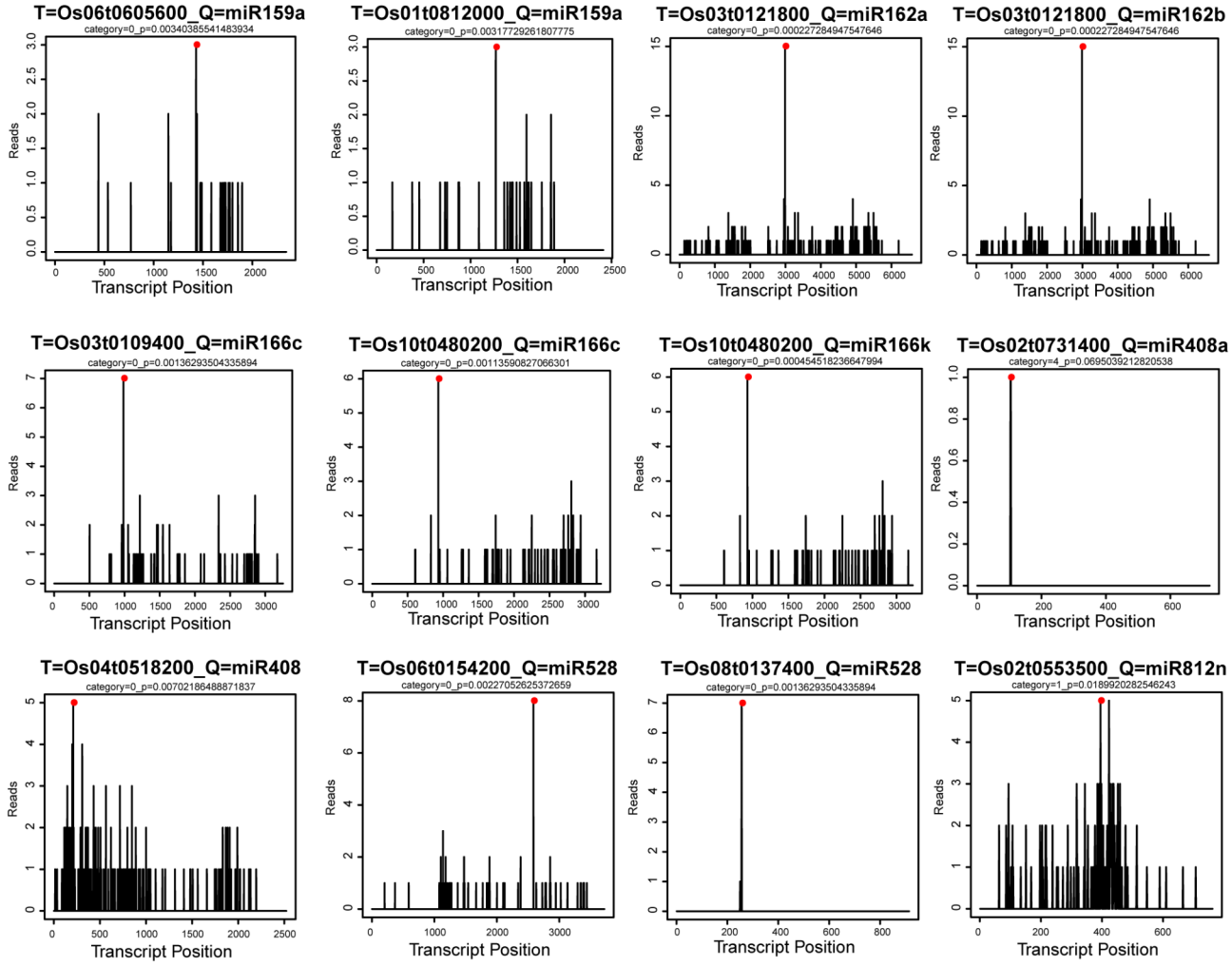

Fig. 4 The T-plot of miRNA-regulated target genes detected by degradomome sequencing

Transcriptome sequencing of resistant and susceptible rice varieties under bacterial leaf streak infection

To further identify key genes involved in the response to bacterial blight infection, transcriptome sequencing was performed on samples from the resistant variety R and the susceptible variety S, both before and at 12 and 24 h post-infection. Principal component analysis (PCA) revealed that the non-infected samples of both the resistant (R) and susceptible (S) varieties clustered together, while the infected samples formed a distinct group (Fig. 6A). Correlation analysis of the samples confirmed good biological replicability within each group (Fig. 6B). We set a threshold of fold change ($|\log2FC| \geq 1$) and q-value < 0.05 for screening differentially expressed genes (DEGs). The results showed that in the resistant variety, compared to the mock-inoculated R_mock, 4,869 genes were upregulated and 5,550 downregulated at 12 h post-infection (R_12 h), and 2,655 genes were upregulated and 2,417 downregulated at 24 h post-infection (R_24 h). In the susceptible variety, compared to the mock-inoculated S_mock, 4,674 genes were upregulated and 4,892 downregulated at 12 h post-infection, and 4,466 genes were upregulated and 4,791 downregulated at 24 h (Fig. 6C).

Further analysis of the DEGs in both the resistant and susceptible varieties revealed 2,305 commonly differentially expressed genes, including 1,217 upregulated and 1,059 downregulated genes (Fig. 6D). GO and KEGG enrichment analysis showed that the 1,217 upregulated genes were primarily involved in processes such as oxidative detoxification, response to oxidative stress, defense against bacterial infections, and hydrogen peroxide decomposition, while the 1,059 downregulated genes were enriched in processes related to transmembrane transport, auxin-activated signaling pathways, and brassinosteroid biosynthesis (Fig. 6E). These results suggest that bacterial blight infection triggers multiple stress response signaling pathways in rice, activating defense mechanisms.

Additionally, we focused on 17 genes enriched in bacterial defense responses, which were significantly

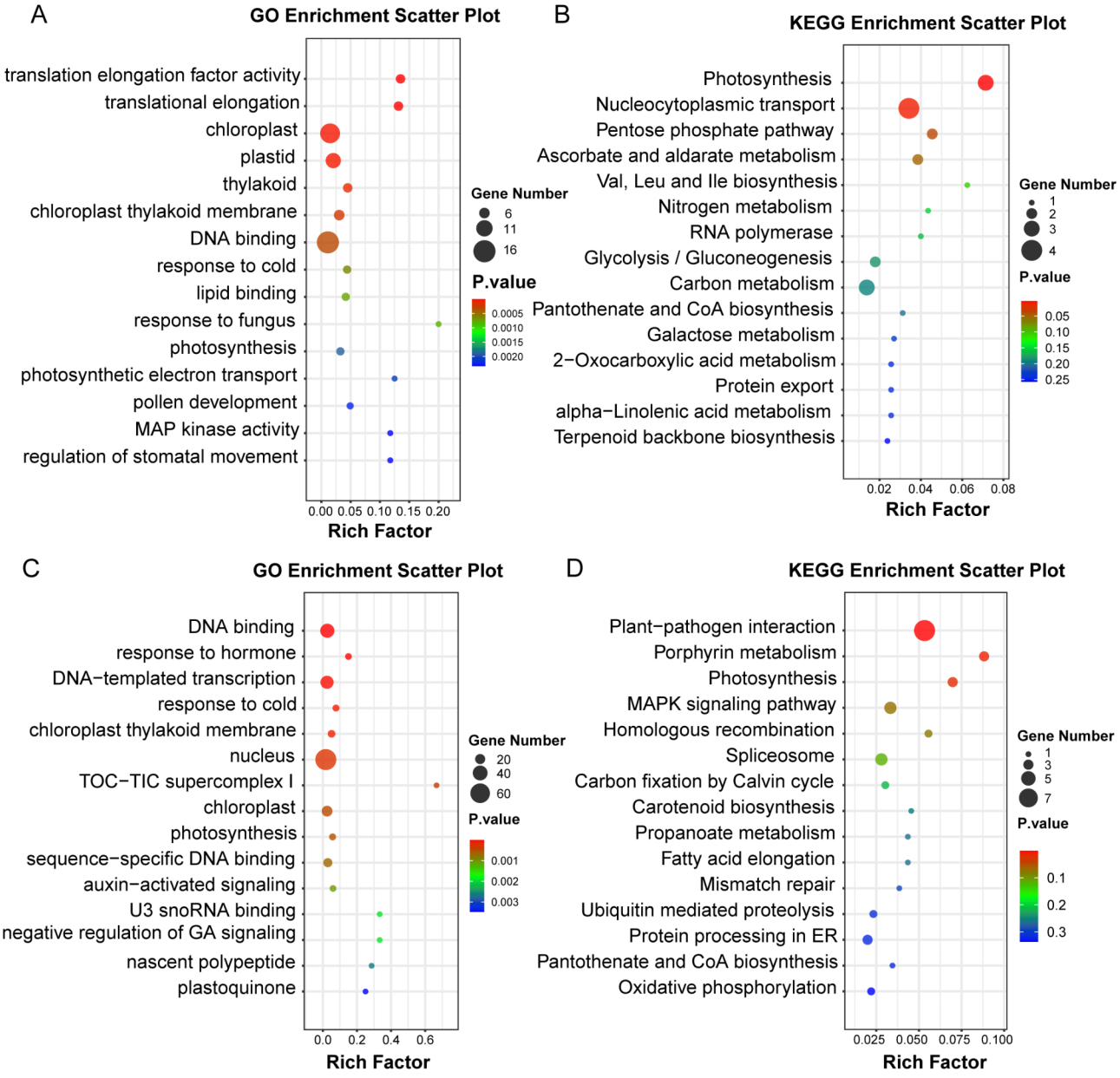

Fig. 5 Enrichment analysis of miRNA target genes. **A**, GO enrichment analysis of the target genes of DEMs in the resistant variety before and after infection. **B**, KEGG enrichment analysis of the target genes of DEMs in the resistant variety before and after infection. **C**, GO enrichment analysis of the target genes of DEMs in the susceptible variety before and after infection. **D**, KEGG enrichment analysis of the target genes of DEMs in the susceptible variety before and after infection

upregulated at both 12 and 24 h post-infection in both resistant and susceptible varieties (Fig. 6F). Notably, Xa21 binding protein Xb3, associated with the major bacterial blight resistance gene *Xa21*, was identified, along with two rice pathogenesis-related proteins, *OsPR4b* and *OsPR4c*, which have been previously implicated in positive regulation of resistance to rice blast and sheath blight. These findings suggest that several of the identified genes likely play crucial roles in the rice response to bacterial blight infection.

GSEA analysis reveals key biological pathways in response to bacterial blight infection

To complement the GO and KEGG enrichment analyses and identify potentially overlooked functional genes, we performed Gene Set Enrichment Analysis (GSEA) on the transcriptome data. The results revealed significant enrichment of gene sets related to several key biological processes in the resistant variety samples before and after bacterial blight infection. Specifically, GO terms such as cellular oxidative detoxification, chitinase activity, and systemic acquired resistance, as well as KEGG pathways

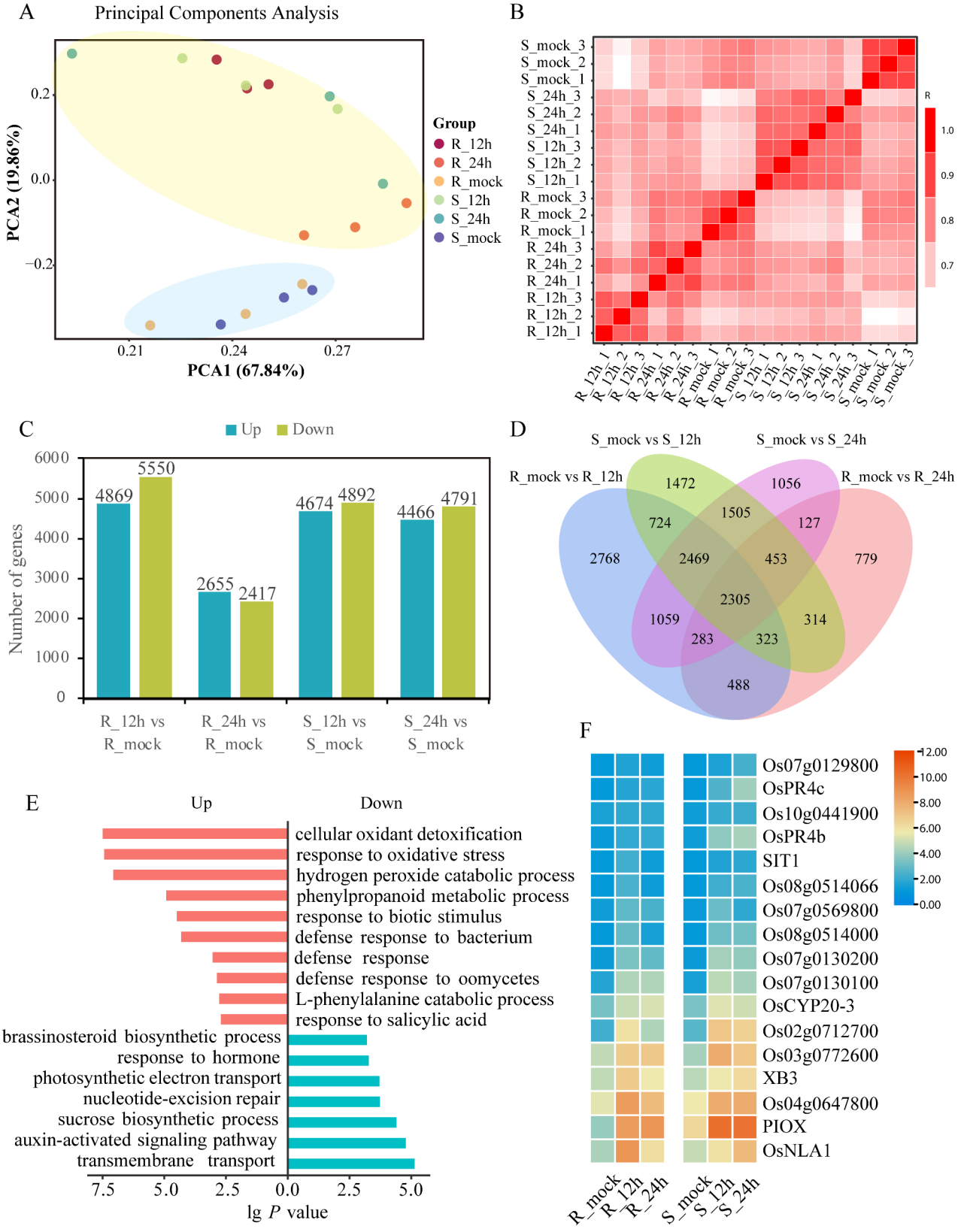

Fig. 6 Transcriptome analysis of rice response to bacterial streak disease. **A**, Principal component analysis of transcriptome sequencing. **B**, Repeatability analysis of transcriptome sequencing. **C**, Statistics of the number of differentially expressed genes before and after bacterial streak disease inoculation. **D**, Differential representation of gene Wayne diagram. **E**, Differential expression gene GO enrichment analysis. **F**, GO enriched into gene expression patterns in response to bacterial defense

related to phenylpropanoid and flavonoid biosynthesis, were upregulated (Fig. 7A and B).

In the susceptible variety, GSEA similarly identified enrichment patterns comparable to those in the resistant variety. GO terms related to chitinase activity, peroxidase activity, and oxidative stress response, as well as KEGG pathways involved in MAPK signaling, phenylpropanoid biosynthesis, and plant-pathogen interactions, were significantly enriched, with upregulation observed across these gene sets (Fig. 7C and D). These findings provide a broader perspective, indicating that a variety of defense-related pathways, including systemic acquired resistance, cell wall degradation (via chitinase), oxidative stress response, and key secondary metabolite biosynthetic pathways, are activated in rice following BLS infection.

Validation of differentially expressed genes

To verify the accuracy of the high-throughput sequencing data, we randomly selected two DEMs, miR162a and miR166k, from the miRNA sequencing data for qPCR validation using the stem-loop method. The miRNA sequencing data indicated that the abundance of miR162b and miR166k in the resistant variety Dular significantly decreased at 12 h and 24 h after inoculation with the RS105 strain of *Xanthomonas oryzae* (Fig. 8A). qPCR using the stem-loop method confirmed that the expression levels of miR162b and miR166k were significantly downregulated following inoculation with RS105 (Fig. 8B).

Additionally, we selected 10 differentially expressed genes from the RNA-seq data for qPCR validation of their expression changes before and after inoculation with RS105 in both the resistant variety Dular and the susceptible variety 9311. The qPCR results showed that five genes (LOC_Os12g25090, LOC_Os04g43200, LOC_Os07g35560, LOC_Os07g46060, LOC_Os03g56160) were significantly upregulated at 12 h (Fig. 8C) and 24 h (Fig. 8D) post-inoculation in Dular. Similarly, these

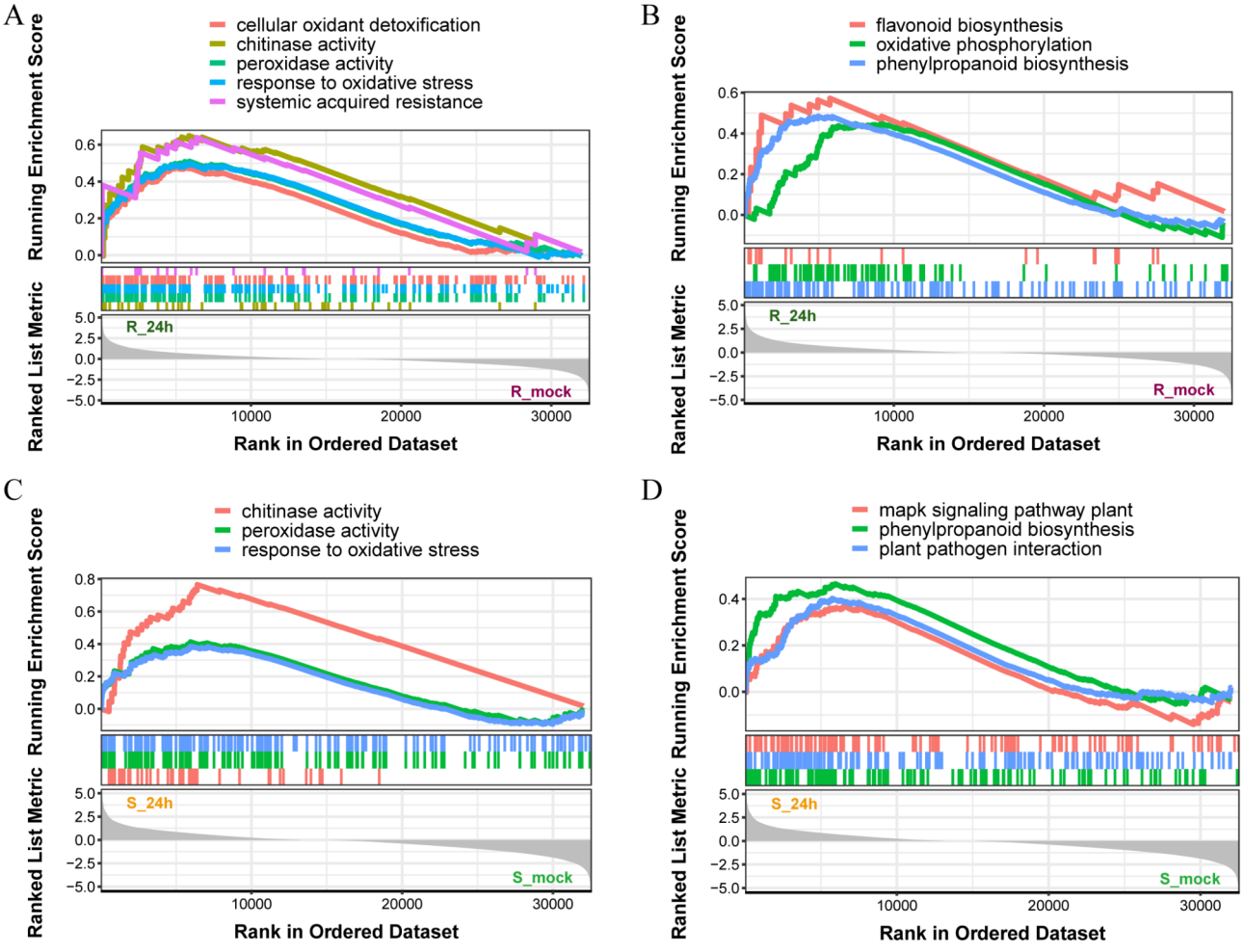

Fig. 7 GSEA Enrichment analysis. **A**, The gene set enrichment analysis of GO strips before and after inoculation of disease-resistant materials. **B**, The gene set enrichment analysis of KEGG before and after inoculation of disease-resistant materials. **C**, The enrichment analysis of GO stripe gene set of susceptible materials before and after inoculation. **D**, The gene set enrichment analysis of KEGG of susceptible materials before and after inoculation

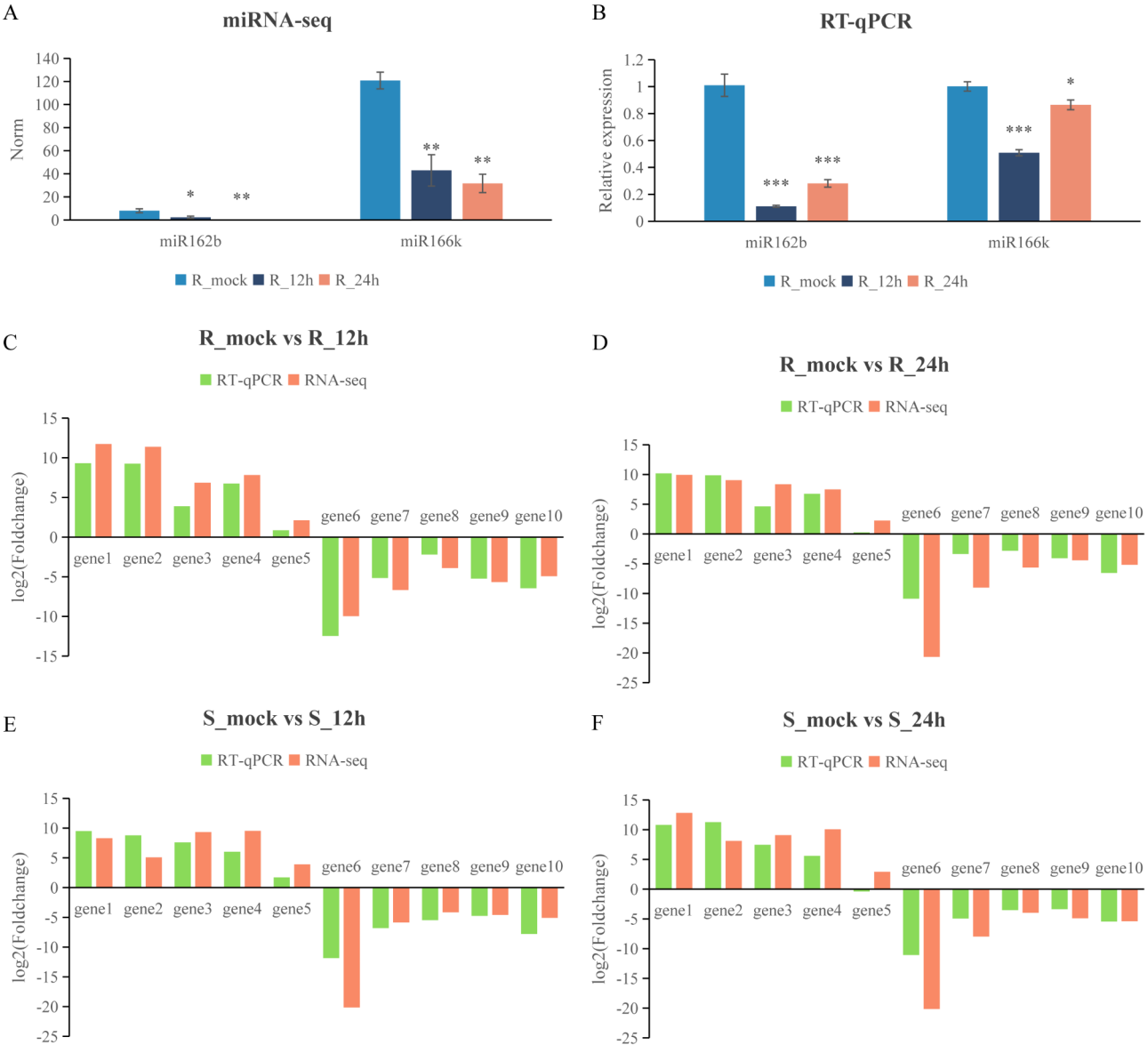

Fig. 8 Validation of RNA-seq data by RT-qPCR. **A**, Abundance of miR162b and miR166k determined from miRNA sequencing data. **B**, Changes in the abundance of miR162b and miR166k in the resistant variety Dular after inoculation with RS105, as measured by stem-loop RT-qPCR. **C**, Expression changes of 10 randomly selected genes in the resistant variety Dular at 12 h post-inoculation with RS105. **D**, Expression changes of 10 randomly selected genes in the resistant variety Dular at 24 h post-inoculation with RS105. **E**, Expression changes of 10 randomly selected genes in the susceptible variety 9311 at 12 h post-inoculation with RS105. **F**. Expression changes of 10 randomly selected genes in the susceptible variety 9311 at 24 h post-inoculation with RS105. In RT-qPCR analysis, gene expression levels were calculated using the $\log_2$FC values, the expression levels of miR166k were determined using $\log_2$(FC + 1)

genes were upregulated in the susceptible variety 9311 at both 12 h and 24 h post-inoculation (Fig. 8E and F). Conversely, five other genes (LOC_Os05g12320, LOC_Os03g22634, LOC_Os10g34790, LOC_Os03g43010, LOC_Os02g51900) were significantly downregulated at both time points in both Dular and 9311 (Fig. 8C-F). The qPCR results aligned with the RNA-seq data, supporting the reliability of the RNA-seq results.

miRNA-mRNA eegulatory networks responding to bacterial leaf streak infection

miRNAs regulate gene expression through two primary mechanisms: one involves binding to the CDS of target mRNAs, promoting their degradation, while the other binds to the 3' UTR of target mRNAs, inhibiting their translation. The former leads to a decrease in target mRNA levels, while the latter results in reduced protein accumulation, without affecting mRNA abundance. In plants, most miRNAs regulate target genes through the degradation mechanism. To identify key miRNAs and

mRNAs involved in the response to bacterial leaf blight infection, we conducted miRNA sequencing, transcriptome sequencing, and degradome sequencing.

To further investigate the miRNA-mRNA regulatory modules in rice's response to bacterial leaf blight, we focused on miRNA-mRNA modules that exhibit a negative correlation in expression levels after infection. Our analysis identified 27 miRNA-mRNA pairs with such negative correlations (Fig. 9). Among these, 23 modules showed downregulation of miRNA expression and upregulation of their target gene expression following infection, involving 8 miRNAs (miR159a、miR159b、miR159f、miR162b、miR166g、miR166h、miR166k and miR528) and 23 target genes. Notable miRNA families include miR159, miR162, miR166, miR408, and miR528. Previous studies have shown that miR159a plays a positive regulatory role in rice immunity against rice blast fungus. Transgenic rice overexpressing miR159a exhibits enhanced resistance to rice blast, while miR159a suppression results in higher susceptibility [21]. miR162a targets *OsDCL1*, regulating rice immunity against rice blast. Overexpressing miR162a in transgenic rice results

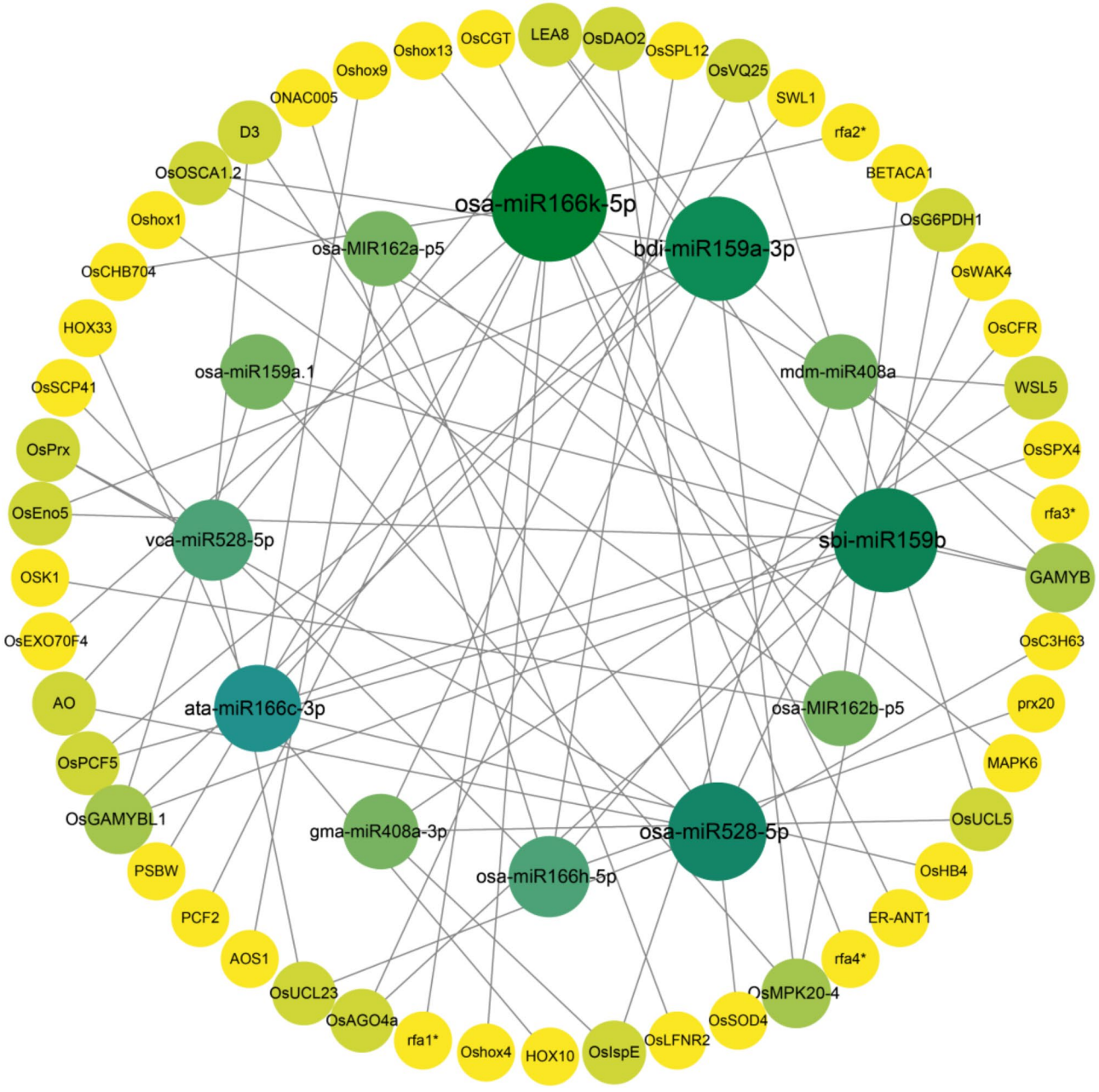

Fig. 9 Key miRNA-mRNA regulatory network after bacterial leaf streak infection in rice

in enhanced resistance, albeit with a slight reduction in yield, indicating that miR162a plays a critical role in balancing disease resistance and yield in rice [22]. miR166k-166 h targets the *EIN2* gene and positively regulates rice resistance to both rice blast and rice sheath blight [23]. In maize, overexpression of miR408b weakens resistance to *Fusarium verticillioides* (Corn ear rot), suggesting that miR408b negatively regulates maize's disease resistance [24]. miR528 targets *OsAO* and regulates the accumulation of reactive oxygen species (ROS), affecting rice resistance to both rice blast and rice stripe virus [25, 26].

In these 23 miRNA-mRNA modules, miRNAs were suppressed while their target genes were induced during bacterial leaf blight infection, suggesting their potential key roles in rice's defense against this disease. Additionally, four other miRNA-mRNA modules showed upregulation of miRNA expression and downregulation of target gene expression, All four of these identified miRNA–mRNA pairs involve miR169a, suggesting that miR169a and its four regulated target genes may play pivotal roles in rice susceptibility to bacterial leaf streak (The information on the 27 pairs of miRNA-mRNA regulatory modules is provided in Supplementary Table 3).

Key miRNA-mRNA modules in rice response to bacterial leaf blight

We focused on five miRNA-mRNA modules: miR159a-*OsGAMYBL1*, miR159f-*LYL1*, miR166h-*prx20*, miR166k-*rfa1*/*rfa2*/*rfa3*/*rfa4*, and miR528-Os12g0561200. In these modules, the miRNAs were upregulated during bacterial leaf blight infection, while their corresponding target genes were downregulated. This expression pattern strongly suggests that these five miRNA-mRNA modules likely play critical roles in rice's response to bacterial leaf blight. Additionally, we observed significant changes in the miR169a-*COPT4* module. miR169a was induced in response to bacterial leaf blight infection, while its target gene, *COPT4*, was significantly downregulated during infection (Fig. 10). This indicates that the miR169a-*COPT4* module may be involved in the susceptibility of rice to bacterial leaf blight.

Discussion

MiRNAs involved in rice response to bacterial leaf blight

miRNAs, as short non-coding RNAs, play crucial roles in various biological processes such as plant growth, development, and stress responses [27–29]. Despite the significant biological functions of miRNAs, research on their role in regulating rice's response to bacterial leaf blight remains limited. miR167d targets the *ARF12* gene, and studies have shown that, 24 h after inoculation with rice blast fungus, miR167d expression is significantly downregulated in resistant varieties, while it is upregulated in susceptible varieties. Further studies revealed that overexpression of miR167d in rice reduces its resistance to rice blast, while the use of a target mimic (MIM167d) enhances resistance. Knocking out the target gene *ARF12* in rice also decreases its resistance to rice blast, suggesting that miR167d regulates resistance to rice blast by modulating the expression of *ARF12* [30]. miR395b targets and cleaves two sulfate transporter genes, *OsSULTR2;1* and *OsSULTR2;2*, preventing the transport of sulfate ions from old leaves to new ones, leading to accumulation of sulfate ions in the xylem vessels. miR395 also targets *OsAPS1*, a gene encoding ATP sulfurylase, inhibiting the assimilation of sulfate ions into adenosine-5'-phosphosulfate. This accumulation of sulfate ions in the leaves and xylem suppresses the growth of both bacterial leaf blight and white leaf blight pathogens, thereby enhancing rice resistance to these diseases [31]. In contrast to bacterial leaf blight, much more is known about miRNAs regulating rice's response to rice blast and white leaf blight. miR535 targets the SPL gene *OsSPL4*, and overexpression of *OsSPL4* in transgenic rice enhances resistance to rice blast, increasing the expression of defense-related genes and ROS accumulation. In contrast, the *spl4* knockout mutant is more susceptible, highlighting that miR535, by targeting *OsSPL4*, negatively regulates rice resistance to rice blast [32]. miR812w, a novel member of the miR812 family, enhances rice resistance to rice blast when overexpressed, while CRISPR/Cas9-mediated knockout of miR812w decreases resistance to bacterial leaf blight, suggesting its role in modulating rice resistance to rice blast [33]. miR827 is induced by rice blast infection and targets the genes SPX-*MFS1* and SPX-*MFS2*. Overexpression of miR827 significantly downregulates the expression of defense-related genes such as *OsPR1a* and *OsPR1b*. While overexpression of miR827 reduces rice resistance to rice blast, knocking out miR827 enhances its resistance, suggesting a new mechanism by which miR827 regulates rice resistance to rice blast [34]. miR1873, a rice-specific miRNA, targets LOC_Os05g01790. Overexpression of miR1873 weakens the rice defense response, while overexpressing its target mimic or LOC_Os05g01790 enhances the defense response, leading to increased expression of defense-related genes, higher H_2O_2 accumulation, slower progression of rice blast infection, and improved resistance to rice blast [35]. In this study, we performed GO and KEGG enrichment analyses of the target genes of DEMs and found several biological pathways that were significantly enriched between the different treatment groups. For instance, the GO analysis enriched terms related to hormone response, photosynthetic electron transport, cold response, auxin-activated signaling pathways, protein phosphorylation, kinase activity, and ATP binding. The KEGG analysis primarily enriched pathways related

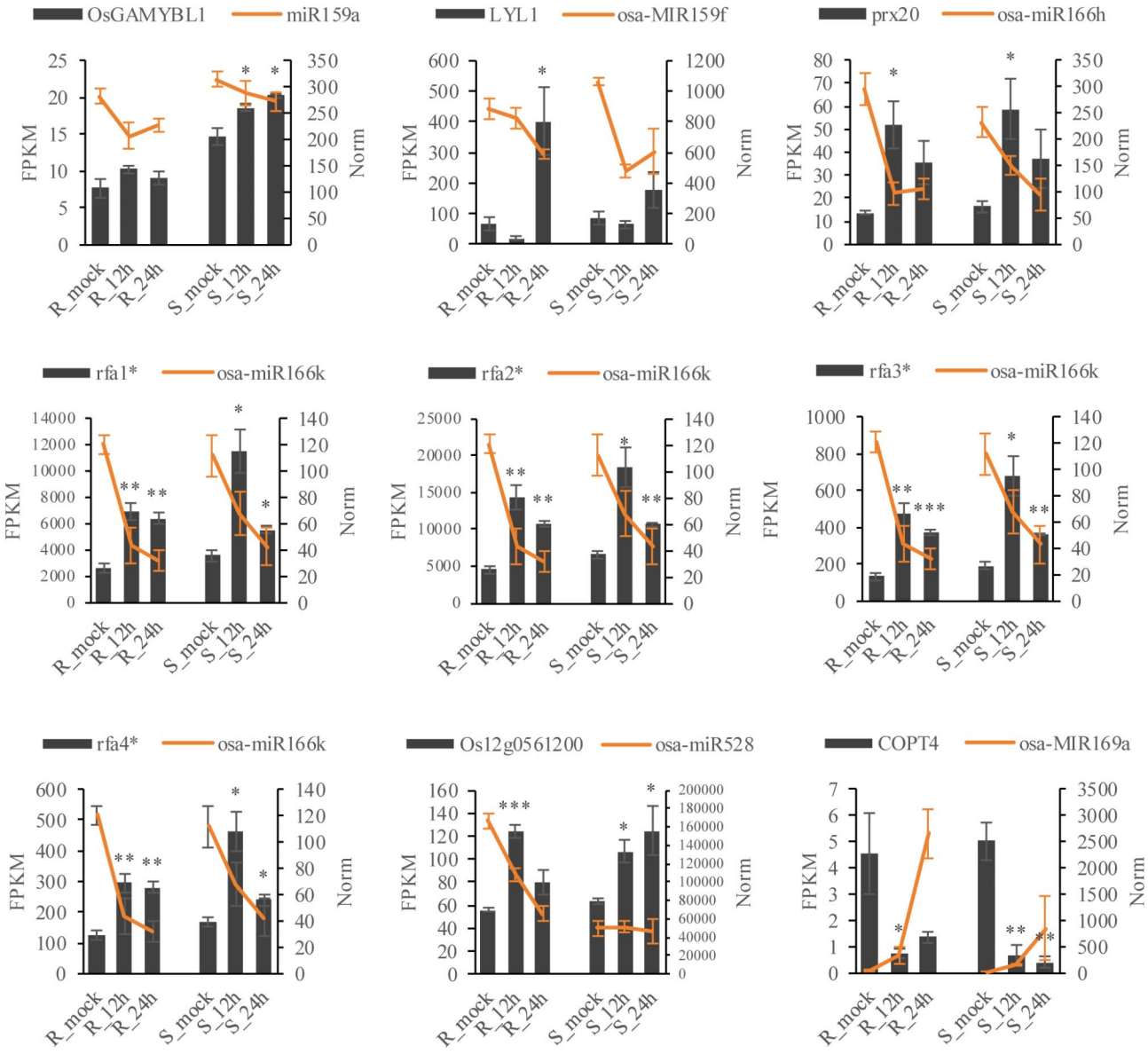

Fig. 10 miRNA-mRNA involved in the regulation of rice response to bacterial leaf streak

to plant-pathogen interactions, which aligns with our understanding of plant responses to pathogen infection. Similar to previous studies, other research has also highlighted the key roles of these pathways in plant disease resistance. For example, researchers performed transcriptome sequencing on rice leaves infected with bacterial blight, and their GO and KEGG analyses similarly enriched terms related to hormone response and MAPK signaling pathways [36].

These findings indicate that research on miRNAs regulating disease resistance in rice has primarily focused on rice blast, with relatively fewer studies on bacterial leaf blight. In this study, we performed miRNA sequencing on both resistant and susceptible rice varieties under bacterial leaf blight infection and identified 19 miRNAs that were downregulated in the resistant variety, and 9 miRNAs upregulated in the susceptible variety. Additionally, we identified 2 miRNAs upregulated in the resistant variety and 14 downregulated in the susceptible variety.

QTLs and genes regulating resistance to bacterial leaf blight in rice

Compared to the study of miRNAs regulating rice's response to bacterial leaf blight, research on quantitative trait loci (QTLs) and mRNAs involved in resistance to this disease is more extensive. Based on rice genome-wide association studies (GWAS) and transcriptomic analysis, a QTL locus, qBLS6.2, was identified on chromosome 6. Further gene editing experiments confirmed that *OsBLS6.2* is a resistance gene. Knockout of this gene

significantly enhanced the resistance of rice to bacterial leaf blight [37]. During bacterial leaf blight infection, the expression of small heat shock protein *OsHSP18.0* is notably upregulated. Overexpression of *OsHSP18.0* in rice significantly improves its resistance to bacterial leaf blight, while RNAi-mediated suppression of *OsHSP18.0* reduces resistance, suggesting that *OsHSP18.0* positively regulates bacterial leaf blight resistance in rice [38]. Multiple rice glucosidase genes are induced during bacterial leaf blight infection, but not by white leaf blight. Mutant lines of the glucosidase genes *OsBGLU20* and *OsBGLU23* exhibit longer lesion lengths and increased bacterial growth after infection, compared to wild-type plants. Furthermore, the expression levels of defense-related genes such as *OsPR1a*, *OsPR5*, and *OsWRKY72* are significantly downregulated when *OsBGLU20* and *OsBGLU23* are suppressed. These findings suggest that *OsBGLU20* and *OsBGLU23* play a positive role in regulating rice resistance to bacterial leaf blight [39]. The receptor-like cytoplasmic kinase *NRRB* is induced by bacterial leaf blight and is highly expressed in both leaves and leaf sheaths. RNAi-mediated silencing of *NRRB* enhances rice's resistance to bacterial leaf blight, as well as activating the expression of pathogenesis-related genes such as *PR1a* and *WRKY13* [40]. The rice sulfated peptide receptor gene *OsPSKR1*, which possesses kinase activity, is significantly upregulated after inoculation with the RS105 strain of bacterial leaf blight. Transgenic rice overexpressing *OsPSKR1* shows enhanced resistance to RS105, with increased expression of disease-related genes in the salicylic acid (SA) pathway. These results indicate that *OsPSKR1* functions as a PSK receptor that regulates rice resistance to bacterial leaf blight by activating disease-related genes in the SA pathway [41]. Moreover, polygalacturonase-inhibiting protein *OsPGIP1* is strongly induced during bacterial leaf blight infection. Overexpression of *OsPGIP1* in rice enhances its resistance, while RNAi lines show significantly reduced resistance, highlighting that *OsPGIP1* positively regulates bacterial leaf blight resistance [42].

Researchers have also fine-mapped numerous resistance genes for bacterial leaf blight using QTL analysis. A BSA analysis of F_2 populations derived from resistant variety X455 and susceptible variety JG30 identified the *Xo2* resistance gene, which was fine-mapped to a 110 kb region on chromosome 2 [43]. Another recessive resistance gene, *bls1*, was fine-mapped to a 21 kb region on chromosome 6, and genetic analysis revealed *OsMAPK6* as a candidate gene for this QTL. The potential target of *OsMAPK6* is the *RGA4* gene, which is known to mediate bacterial leaf blight resistance [44]. In addition to resistance genes, increasing numbers of susceptibility genes have also been identified. For example, editing the promoter region of the rice hexokinase gene *OsHXK5* using CRISPR/Cas9 enhances rice's resistance to bacterial leaf blight, while overexpression of *OsHXK5* increases susceptibility to the disease [45]. The bacterial leaf blight pathogen RS105 secretes the effector *Tal5d*, which binds to the promoter of the susceptibility gene *OsSULRT3;6*, activating its expression. *OsSULRT3;6* encodes a sulfate transporter. Editing the effector-binding elements (EBEs) in the *OsSULRT3;6* promoter using CRISPR/Cas9 significantly enhances rice resistance to bacterial leaf blight [46].

In conclusion, while much has been done to identify resistance genes for rice blast, white leaf blight, and sheath blight, relatively few resistance genes for bacterial leaf blight have been identified. Further exploration and identification of these resistance genes are crucial for advancing our understanding of rice disease resistance mechanisms and for the development of rice varieties with enhanced resistance to bacterial leaf blight.

Conclusion

In this study, we performed miRNA sequencing, degradome sequencing, and transcriptome sequencing on both the resistant variety Dular and the susceptible variety 9311 after inoculation with the RS105 strain of *Xanthomonas oryzae* to identify key regulatory modules. A total of 19 miRNAs were significantly downregulated at various time points in the resistant variety, while 9 miRNAs were significantly upregulated in the susceptible variety. These 28 DEMs are likely to play crucial roles in rice's resistance to bacterial leaf blight.

Further analysis integrating degradome and transcriptome data led to the identification of 27 miRNA-mRNA regulatory modules with negative correlations in expression during bacterial leaf blight infection. Among these, 23 miRNA-mRNA modules (involving miR159a, miR159b, miR159f, miR162b, miR166g, miR166h, miR166k, and miR528) are likely involved in enhancing resistance to the disease, while the remaining 4 modules, associated with miR169a, may play a role in susceptibility. Based on these findings, we constructed a miRNA-mRNA regulatory network that elucidates the mechanisms underlying rice's response to bacterial leaf blight infection.

This study provides new insights into the functional roles of miRNA-mRNA modules in regulating rice's defense against bacterial leaf blight and offers valuable genetic resources for disease-resistant rice breeding.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11404-4.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Not applicable.

Author contributions
WBW and CZB conceptualized and designed the experiment. WBW carried out the experiments and prepared the manuscript. ZXY, ZJL and ZBH contributed to the analysis of the sequencing data.

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Data availability
The miRNA sequencing data in this study are available in Sequence Read Archive (SRA) under Bioproject accession number PRJNA1198404. The transcriptome sequencing data from 18 samples have been deposited in SRA under accession number PRJNA1198665.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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