

Full Paper

Comprehensive analysis of microRNA-Seq and target mRNAs of rice sheath blight pathogen provides new insights into pathogenic regulatory mechanisms

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Abstract

MicroRNAs (miRNAs) are ~22 nucleotide non-coding RNAs that regulate gene expression by targeting mRNAs for degradation or inhibiting protein translation. To investigate whether miRNAs regulate the pathogenesis in necrotrophic fungus *Rhizoctonia solani* AG1 IA, which causes significant yield loss in main economically important crops, and to determine the regulatory mechanism occurring during pathogenesis, we constructed hyphal small RNA libraries from six different infection periods of the rice leaf. Through sequencing and analysis, 177 miRNA-like small RNAs (miRNAs) were identified, including 15 candidate pathogenic novel miRNAs predicted by functional annotations of their target mRNAs and expression patterns of miRNAs and mRNAs during infection. Reverse transcription-quantitative polymerase chain reaction results for randomly selected miRNAs demonstrated that our novel comprehensive predictions had a high level of accuracy. In our predicted pathogenic protein-protein interaction network of *R. solani*, we added the related regulatory miRNAs of these core coding genes into the network, and could understand the relationships among these regulatory factors more clearly at the systems level. Furthermore, the putative pathogenic Rhi-miR-16, which negatively regulates target gene expression, was experimentally validated to have regulatory functions by a dual-luciferase reporter assay. Additionally, 23 candidate rice miRNAs that may involve in plant immunity against *R. solani* were discovered. This first study on novel pathogenic miRNAs of *R. solani* AG1 IA and the recognition of target genes involved in pathogenicity, as

well as rice miRNAs, participated in defence against *R. solani* could provide new insights into revealing the pathogenic mechanisms of the severe rice sheath blight disease.

Key words: rice sheath blight pathogen, microRNA, miRNA-mRNA interaction, gene expression, pathogenic mechanisms

1. Introduction

Small RNAs (sRNAs), which are derived from double-stranded RNA or hairpin-structured RNA, are non-coding RNAs of 19–30 nt in length.¹ Over the last decade, several key studies have demonstrated that sRNAs participate in various cellular processes in many organisms, including DNA damage response, the maintenance of genome integrity, and the regulation of developmental processes.^{2–4} MicroRNAs (miRNAs) are one of three major classes of sRNAs.^{4,5} And they are commonly 22 nt in length and play important roles in the post-transcriptional regulation of gene expression in plants and animals.² Mature miRNAs negatively regulate gene expression via complementary binding to the open reading frame or untranslated region (UTR) of specific target genes. The sRNA pathway has been characterized in several filamentous fungi,^{6,7} but the miRNA pathway remains poorly understood in most fungal species. Recently, it was reported that at least four types of miRNAs have been identified in *Neurospora crassa*,³ including a Dicer-dependent miRNA pathway. The production of these miRNA-like small RNAs (milRNAs) does not require quelling deficient-1 (QDE-1, a RNA-dependent RNA polymerase) or QDE-3 (a RecQ DNA helicase) but is dependent on Dicer, QDE-2 (an Argonaute protein), the exonuclease QDE-2 interacting protein, and mitochondrial ribosomal protein L3 (a RNase III domain-containing protein). These differences may indicate that fungal miRNA production evolved independently from that in plants and animals. Recently, deep sequencing technology was applied to investigate milRNA in nine filamentous fungi.^{8–16} However, neither miRNA nor milRNA has been reported in the plant pathogen *Rhizoctonia solani*, raising the question of whether miRNAs exist in *R. solani*.

Rhizoctonia solani, a basidiomycete necrotrophic fungal pathogen, is widely distributed and causes the loss of many economically important plants.¹⁷ In addition to rice, the fungus can infect the crops of approximately 50 species, including maize, barley, lettuce, sorghum, and tomato.^{18–20} As the agent of rice sheath blight disease, *R. solani* anastomosis group AG1 IA infection results in the most serious economic agricultural losses. Currently, studies of *R. solani* AG1 IA group classification,¹⁷ infection process,²¹ transcriptome, and genome²² have been reported; however, studies describing the milRNA of *R. solani* AG1 IA are lacking. Given its economic importance, we examined the regulatory role of miRNAs in host–pathogen interactions. We applied Illumina sequencing to investigate milRNA over six phases of *R. solani* infection process in rice. The main objective of our study is to explore the potential of miRNAs as pathogenetic factors and to elucidate the possible molecular basis of the host–pathogen interactions of the rice-infecting pathogen to improve the yield of food crops. In addition, *R. solani* AG1 IA milRNA was also the first miRNA reported in the *Rhizoctonia* genus, and it may serve as a model for studying pathogenic mechanisms.

2. Materials and methods

2.1. Ethics statement

The human cell lines provided by Shanghai Ying Biotechnology Co., Ltd, were purchased from the Cell Bank of the Chinese Academy of

Sciences (Shanghai, China). Protocols in this study were approved by the Cell Bank of the Chinese Academy of Sciences. All experiments were performed in accordance with the approved guidelines.

2.2. Fungal strains

The *R. solani* AG1 IA strain was selected from a heavily infected rice plant at South China Agricultural University and was designated the national standard isolate. The fungal strains were germinated and mycelia cultured on potato dextrose agar (PDA; 200 g of potato, 20 g of dextrose, and 20 g of agar) at 28 °C for 2 days. The mycelia were used for infection and RNA extraction.

2.3. RNA extraction and construction of cDNA libraries

To detect the interactions of rice and *R. solani* AG1, we collected strains at seven infection stages. The seven stages included mycelium cultured on PDA without plant; mycelium incubated on rice for 10-h; infection cushion incubated on rice for 18-h; after mycelium had invaded the host, with light necrosis appearing after 24-h; heavy necrosis after a 32-h incubation; heavy necrosis after a 48-h incubation and light grey sclerotia on infected plant tissue after a 72-h incubation. We extracted total RNA from these samples using a Fungal RNA Kit (Omega, USA). Next, cDNA libraries were constructed using a TransScript First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China). sRNA was then extracted using a mirVana™ miRNA Isolation Kit (Ambion, USA), and small cDNA libraries were constructed using a One Step PrimerScript miRNA cDNA Synthesis Kit (TransGen).

2.4. Cloning and sequencing of *R. solani* AG1 sRNAs

Fractions of sRNA between 15 and 30 nt were extracted from the seven-staged samples on a 15% denaturing polyacrylamide gel. Next, a 5' adaptor and a 3' adaptor were ligated sequentially to 15 µg of sRNA. Then, the short RNAs were converted to DNA by reverse transcription-polymerase chain reaction (RT-PCR) and sequenced on an Illumina GA II machine (BGI, Shenzhen, China). The sequenced short read data were deposited in Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) under the accession number GSE68236.

2.5. Identification of miRNAs

After removing adaptor contaminants and low-quality tags from the raw reads, the remaining sRNA sequences were utilized in further analysis. We compared sRNAs against known non-coding RNAs (i.e. rRNA, tRNA, snRNA, and snoRNA) deposited in the Rfam database²³ (version: 10.1) to identify other ncRNAs using BLAST (version 2.2.26). sRNAs belonging to the 'rRNA, etc.' class were removed. The prediction of *R. solani* AG1 IA milRNAs was performed using MIREAP (version 0.2; <http://sourceforge.net/projects/mireap>) and miRDeep2 software. Both methods identified known miRNAs and novel candidates with a canonical hairpin structure. Some changes in the published workflow were adopted to predict

miRNAs. Tags that appeared in at least four libraries were selected. The abundance of each tag in each library was normalized to transcripts per million (TPM), and tags with an abundance >2.5 TPM were used to identify candidate miRNAs. Based on the normalized data, we calculated the Pearson correlation coefficient between miRNAs and performed hierarchical cluster analysis using the hclust function in R based on the average agglomeration method, which was used to analyse the expression pattern of the miRNAs. These candidate miRNAs were also aligned to the *R. solani* AG1 IA genome sequence (accession AFRT0000000.1).²⁴

2.6. Target gene prediction

Miranda v3.3a²⁵ software was used to predict targets of candidate miRNAs with the following parameters: gap open penalty (−8), a gap extend penalty (−2), score threshold (90), energy threshold (−23) kcal/mol, and scaling parameter (2).

2.7. Identification of rice miRNA and their target genes

During the miRNA prediction as shown above, we had obtained the clean reads without other ncRNAs. These reads were aligned to the hairpin sequences of rice miRNAs in the miRBase database²⁶ using BLASTN. Then, we analysed the reads that were perfectly matched to rice miRNA sequences. And reads in at least three libraries (10-h, 18-h, 24-h, 32-h, 48-h, and 72-h) at more than 2.5 TPM were considered as rice miRNA sequence. We further confirmed that all these reads were not matched to *R. solani* AG1 IA genome sequences by performing BLASTN alignment. As several miRNA sequences in miRBase were actually the same to each other (such as the same sequences found for osa-miR159a.1 and osa-miR159b), they were considered as one miRNA here. Finally, 23 rice miRNAs were discovered. The target genes of these miRNAs had been reported in the miRBase database and one study by Zhu et al.,²⁷ and these resources were used for analysis in our study.

2.8. Transcriptome

The *R. solani* AG1 IA transcriptome was detailed in our previous study.^{22,24} Six transcriptome libraries for plant infection at 10-, 18-, 24-, 32-, 48-, and 72-h were prepared and sequenced. The expected fragments per kilobase of transcript per million fragments (FPKM) values were calculated. The FPKM values of miRNA targets were used to calculate the Pearson correlation coefficient and to perform hierarchical cluster analysis (the hclust function in R).

2.9. Construction of the protein-protein interaction network

The protein-protein interactions (PPIs) were obtained in our previous study²⁸ that identify interactions based on the interolog approach and domain-domain interactions and assess the interactions by gene expression profile, gene ontology (GO) annotation, and the results of yeast two-hybrid assay.

2.10. Analysis of miRNA expression using RT-qPCR

Experimental validation of miRNA-like RNAs was performed using the miRNA Purification Kit (Cwbio, CW0627), miRNA cDNA Kit (Cwbio, CW2141), and miRNA Real-Time PCR Assay Kit (Cwbio, CW2142). Briefly, a poly-A tail was added to the 3' end of total RNA. Then, the RNA was reverse-transcribed with an oligo-dT adaptor. Quantitative PCR (qPCR) was then performed using Synergy Brands green detection with a forward primer for mature

miRNA sequences and a universal adaptor reverse primer.²⁹ To calculate the expression levels of the target gene, the $2^{-\Delta\Delta CT}$ relative quantification method was employed.³⁰ All reactions were repeated three times with three biological samples and were detected by 0.8% (w/v) agarose gel electrophoresis.

2.11. Dual-luciferase reporter assay

This assay system was provided by Shanghai Ying Biotechnology Co., Ltd. We used the psiCHECK-2 dual-luciferase reporter vector, which contained both the synthetic Renilla luciferase gene and the synthetic firefly luciferase gene, each with its own promoter and poly (A)-addition sites.³¹ The target gene of Rhi-miR-16 was synthesized using XhoI and NotI sites at 5' and 3' ends, respectively, and was inserted between the XhoI and NotI restriction sites in the multiple cloning regions of the Renilla Luciferase 3'-UTR. As a negative control, we designed the following mutated Rhi-miR-16 targeting oligonucleotide: 5'-CGCTAGTTCAGAGTGGACGGTCT-3'. For luciferase assays, HEK293T cells were co-transfected with 100 ng of the reporter plasmid (psiCHECK2-AG1IA_02173) and 100 nm of the mimic. Cells were grown at 37°C in 5% CO₂ for 48 h. Renilla and firefly luciferase activities were measured using the Dual-GloTM Luciferase Assay System Kit (Promega).

3. Results

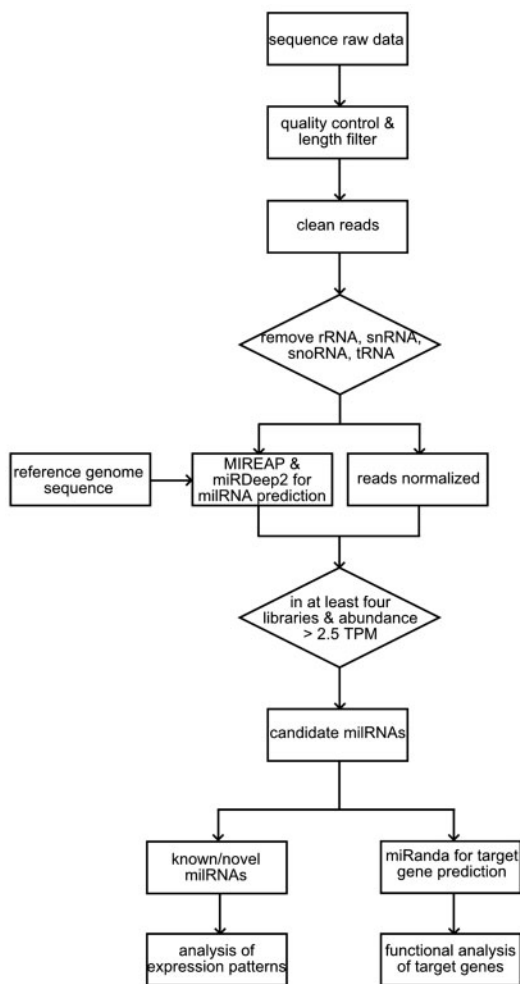
3.1. Identification of miRNAs in infection stages

To identify miRNAs in *R. solani* AG1 IA, we analysed sequencing data collected pre-infection and at six different stages of infection, including 10 h (10-h), 18-h, 24-h, 32-h, 48-h, and 72-h after infection. A total of 77,029,030 clean reads were sequenced. For each library, greater than 1 million unique reads were obtained, at least 67% of which were singletons (Table 1). The lengths of all sequenced sRNAs range from 18 to 30 nt, with the majority distribution on 19–25 nt (Supplementary Fig. S1), which is similar to the standard distribution of sRNAs in plants and animals.² The composition of the deep sequenced sRNA dataset is very complex and includes a large number of other non-coding RNA species, such as rRNA, tRNA, snRNA, and snoRNA (Supplementary Table S1). Given this complexity and the disproportionate number of singletons in our data that possibly originate from sequencing error, we utilized a method (Fig. 1) originally developed for fungal miRNA prediction with some alterations to the rice miRNA detection workflow.³² In our analysis, reads matched to other non-coding RNA types were removed, and the *R. solani* AG1 IA genome sequence²² was used as the reference for miRNA detection. Previous research on fungal miRNA detection has indicated that few miRNAs are conserved compared with other eukaryotes.^{8–15} Two popular and powerful tools, MIREAP and miRDeep2,^{33,34} can identify fungal miRNAs; however, fewer than 45 miRNAs were found for each of four reported fungi,^{8,9,13,15} which may be due to the limitation of analytical workflows. However, each method also has its limits of prediction in fungi. Here, we used both MIREAP and miRDeep2 to comprehensively analyse our sequencing data, avoiding their shortcomings, and identified a total of 177 candidate miRNAs, each identified in at least four libraries at more than 2.5 TPM (Supplementary Table S2). Each of the 177 candidate miRNAs, of which 17 miRNAs were overlapped predicted by MIREAP and miRDeep2, was supported by at least 11 reads in one of the libraries (Supplementary Table S2). Three miRNAs were chosen as examples and are presented in Supplementary Figs S2 and S3.

Table 1. Summary of *R. solani* AG1 IA microRNA-like RNA sequencing

	IA	1-1	1-2	1-3	1-4	1-5	1-6
Clean data	11,141,234	11,888,887	11,146,287	11,196,662	10,174,721	10,566,997	10,914,242
Unique reads	2,273,938	2,020,357	1,852,268	1,825,295	1,083,067	1,768,179	2,229,097
Singletons	1,540,073	1,478,502	1,333,911	1,307,798	796,228	1,240,766	1,606,571
Reads length (nt)	18~30	18~30	18~30	18~30	18~30	18~30	18~30
Mapped reads	1,715,684	1,542,499	1,413,949	1,383,724	651,796	1,057,442	1,303,246
Mapped per (%)	75.45	76.35	76.34	75.81	60.18	59.80	58.47

Mapped reads: a count of the unique reads that matched to *R. solani* AG1 IA genome sequence. IA: library without plant infection; 1-1, 1-2, 1-3, 1-4, 1-5, and 1-6: libraries after infection at 10-h, 18-h, 24-h, 32-h, 48-h, and 72-h, respectively.

**Figure 1.** Workflow for the prediction of candidate miRNAs.

3.2. Conserved and novel miRNAs

A comparison of sequences between candidate miRNAs in *R. solani* AG1 IA and published miRNAs in miRBase,²⁶ detailing miRNAs in other fungi,^{8–15} revealed that 109 miRNAs exhibited partial sequence conservation (sequence coverage > 50%, identity \geq 0.88) (Supplementary Table S3). Fourteen miRNAs in *R. solani* AG1 IA matched 14 miRNA mature sequences belonging to 14 families in miRBase with alignment coverage greater than 70% (greater for Rhi-miR-94; Supplementary Tables S3 and S4). Among the remaining 95 semi-conserved miRNAs, 24 (25.26%) had expression greater

than 10 TPM during the infection stages. In addition, 68 novel miRNAs were not conserved among fungi and were exclusively found in *R. solani* AG1 IA. At least 15 novel miRNAs exhibited expression levels greater than 20 TPM during the infection stages, suggesting their involvement in the pathogenic mechanism of *Rhizoctonia*. Further study of these miRNAs is needed to improve the understanding of their involvement in the pathogenesis of filamentous fungi.

3.3. Target prediction of miRNAs

Based on the genome sequence of *R. solani* AG1 IA,²² the 3'-UTRs of 653 genes were predicted to be target regions for 157 miRNAs using Miranda²⁵ (Supplementary Table S5). Targets were not identified for 20 miRNAs, which could be explained by several reasons: they may lack a target, their target may not be annotated in the genome, there may be mismatches between genome and miRNA³⁵ or their targets may be outside of the 3'-UTR.³⁶

Pleiotropic miRNA may target several mRNAs in a given signaling pathway or different mRNAs in converging pathways to exert a large effect on a cell.³⁷ Many of the miRNAs examined in this work regulated multiple mRNA targets. In fact, only 30 (19.11%) miRNAs regulated a single target (Supplementary Table S5). The largest number of targets that we identified for one miRNA (Rhi-miR-135) is 50. Fifteen miRNAs had more than 10 targets, and 103 genes were predicted to be regulated by at least two miRNAs (Supplementary Table S5). Of the 653 targeted genes, 342 (52.37%) have been annotated,²² including transcription factors (TFs), glycoside hydrolases (GHs), haloacid dehalogenase-like hydrolase, ABC transporters, cytochrome P450, E3 ubiquitin-protein ligase, heterotrimeric G proteins, and mitotic checkpoint proteins (Supplementary Table S6). Among them, some of the predicted target factors could be involved in pathogenicity, including cell wall-degrading enzymes and virulence-associated factors in the transduction signal pathway in the Pathogen–Host Interaction (PHI) database.³⁸ Target genes with possible functions in pathogenesis were discovered using GO terms and the EuKaryotic Orthologous Groups, which included pathogenic-related GO classifications (such as hydrolase activity annotated in GH10), large numbers of signal transduction genes, and genes involved in secretion (Supplementary Tables S7 and S8; Supplementary Fig. S4). During infection stages, secreted proteins are typically important factors, which may indicate their involvement in pathogenesis. For example, the expression of AG1IA_00621 (a putative secreted protein) was up-regulated during the infection stages from 10-h to 32-h, while the expression of its miRNA regulator Rhi-miR-91 decreased during these stages (Supplementary Tables S2 and S6).

3.4. Pathogenic protein-protein network regulation

The PPI network of *R. solani* AG1 IA²⁸ contains the interacting partners of 120 miRNA targets, including 14 PHI genes and 6 secreted proteins (Supplementary Table S9). Among them, some of the genes involved in the cell cycle are contained in one subnetwork (Supplementary Fig. S5), including AG1IA_05392 (a mitotic checkpoint protein), AG1IA_06586 (a g2/mitotic-specific cyclin), AG1IA_03893 (a cell division regulator), AG1IA_00552 (a cyclin-dependent kinase regulatory subunit), and AG1IA_08213 (an exocyst complex protein). AG1IA_08213 regulated by Rhi-miR-48 is involved in the exocyst complex, which plays roles in exocytosis, cell migration, and growth.³⁹ The network interaction between AG1IA_08213 and AG1IA_05392 is regulated by Rhi-miR-115, supporting that AG1IA_08213 is involved in the cell cycle. Two PHI genes—AG1IA_03893 (cell division control) that showed a peak in transcript level at 10-h stage and AG1IA_06586 (a g2/mitotic specific cyclin) that was abundantly expressed during infection process, regulated by Rhi-miR-95 and Rhi-miR-85, respectively—interact with one another, possibly indicating an important role for miRNAs in pathogenesis. In one subnetwork, an ABC-A transporter (AG1IA_05406) interacts with AG1IA_03552 (peptidase), which is subsequently regulated by Rhi-miR-75. Because the AG1IA_05406 homologue (MGG_00937) in *Magnaporthe oryzae* is essential for formation of the appressoria, a specialized cell of fungal pathogens that is used to infect plants,⁴⁰ it is possible that AG1IA_05406 plays a similar role in *R. solani* AG1 IA. This notion is also supported by its expression pattern, which peaks 24-h after infection.²² However, to confirm the function of this gene, further experimental approaches are needed.

AG1IA_05961 is a candidate G protein beta subunit ($G\beta$), which is an important basic unit of the heterotrimeric G protein-mediated signalling pathway. Heterotrimeric G protein-mediated signalling plays a central role in the virulence, mycelial growth, and development of filamentous fungi.⁴¹ In a yeast two-hybrid assay, we explored the protein interactions of a hub (i.e. connected proteins with central roles in network architecture) containing AG1IA_05961 and its nine partners.²⁸ We identified three key miRNA regulators of this hub (Rhi-miR-44, Rhi-miR-120, and Rhi-miR-77) in the transduction signal pathway (Supplementary Fig. S6).

In the ubiquitin ligase (AG1IA_01341) hub, 13 partners, including transporters, actin, ubiquitin, and uncharacterized proteins, were predicted as PPIs. This ubiquitin ligase was predicted to be a potential effector of *R. solani* AG1 IA during infection stages,²² and Rhi-miR-83 might regulate a key factor in this subnetwork, supporting by more than 2-fold up-regulation of Rhi-miR-83 during 0-h and 18-h stages and the decreased transcription of AG1IA_01341 during infection. In total, functional annotation of miRNA target mRNAs reveals that miRNAs have complex interactions in PPI networks involved in pathogenesis, transcription, signal transduction, and cell cycle regulation.

3.5. miRNA expression patterns during rice infection

Based on the normalized expression of miRNAs during infection stages, hierarchical clustering (see Materials and methods section) identifies eight major clusters (Fig. 2A). In clade A, expression peaks appear without fungal infection (0-h), and expression declines during the 10-h and 18-h stages. In clades B and C, miRNAs exhibit increased expression at the 10-h and 18-h stages. For clades D, miRNAs display exaggerated expression peaks at 24-h. Different expression patterns are found for miRNAs in clade H, which exhibits

increased expression from 10-h to 24-h. miRNAs were found to target 631 genes expressed during infection stages in a previous study²² (Fig. 2B; Supplementary Fig. S7). Different expression patterns for target genes are noted for most targets in clades M, O, and P, which exhibit the highest expression at 32-h, 10-h, and 24-h, respectively (Fig. 2B). Five TFs were clustered into clades J and O, including two fungal-specific TFs (AG1IA_05790 and AG1IA_03810) that display expression peaks at the 18-h stage and one bZIP TF (AG1IA_03425) that displays an expression peak at the 10-h stage. AG1IA_05790 is predicted to be regulated by Rhi-miR-54, Rhi-miR-145, and Rhi-miR-163. In contrast, AG1IA_03810 is regulated by Rhi-miR-16. The peak expression of these genes suggests their involvement in regulating other infection-related genes. One secreted protein (AG1IA_00123, GH9) in clade J is regulated by Rhi-miR-165 and is up-regulated at least 2-fold during 10-h and 18-h stages. The expression peak at 18-h for this GH9 enzyme might help to identify its potential role of cellulose digestion. The fungal ABC transporter G family members are connected to pleiotropic drug resistance phenomena and are related to translocation of phospholipid molecules.^{42–44} During the infection by *R. solani*, the rice antimicrobials (such as secondary metabolites and small molecules) can be activated and accumulated in response to the pathogen.⁴⁵ To facilitate the rice infection, *R. solani* ABC-G transporters may be contributed to drug resistance. One ABC-G transporter (AG1IA_02225; in clade O) shows the abundance of expression, especially for the peak occurred at 10-h (Supplementary Fig. S7), indicating its important role in pathogenesis, which may also be supported by the down-regulated expression of its putative regulator Rhi-miR-57 during 0-h and 10-h stages. In clade M, the expression of GH6 (AG1IA_08328), which is regulated by Rhi-miR-144, is at its highest during the 32-h stage. Previous research reported that biotrophic fungi lack the cellulose of GH6 and hemibiotrophic and necrotrophic fungi employ it to degrade plant cell wall.^{46–49} AG1IA_08328 was found to use plant cell walls as a substrate²², suggesting its involvement in the infection process and an important role for Rhi-miR-144. AG1IA_05961 is regulated by Rhi-miR-120 and Rhi-miR-145, and an interacting protein (AG1IA_04857, phosphoglucomutase) is regulated by Rhi-miR-77 (Supplementary Fig. S6A), which may facilitate a greater understanding of the heterotrimeric G protein-mediated signalling pathway in *R. solani*. miRNA may regulate multiple targets that exhibit different expression patterns, whereas miRNAs with similar expression patterns may regulate different expressed targets. Rhi-miR-16 is predicted to specifically regulate six mRNAs that are regulated by Rhi-miR-16 alone (Fig. 2 and Supplementary Table S5). Among these targets, AG1IA_01440 (hypothetical protein) is not expressed during infection, whereas the others differentiate into five different clusters containing AG1IA_02173 (dynactin), AG1IA_03810 (fungal-specific TF), AG1IA_04392 (dehydrogenase E1 and transketolase), AG1IA_05100 (CE10), and AG1IA_07163 (hypothetical protein) in clades P, J, N, K, and O, respectively. The transcriptome expression patterns of these genes in the five clades are distinct. The different expression patterns of the targets of the three miRNAs (Rhi-miR-92, Rhi-miR-94, and Rhi-miR-151) selected for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) confirmation also yielded similar results (Figs 2 and 3A). To investigate the expression patterns of target genes for the miRNAs forming one cluster, targets regulated by four miRNA clusters were selected for analysis and the diversity of expression patterns were represented (Supplementary Fig. S8), supporting that miRNA-dependent regulatory network is very complex.⁵⁰ However, considering the peak and valley points of expression for target mRNAs, the miRNA

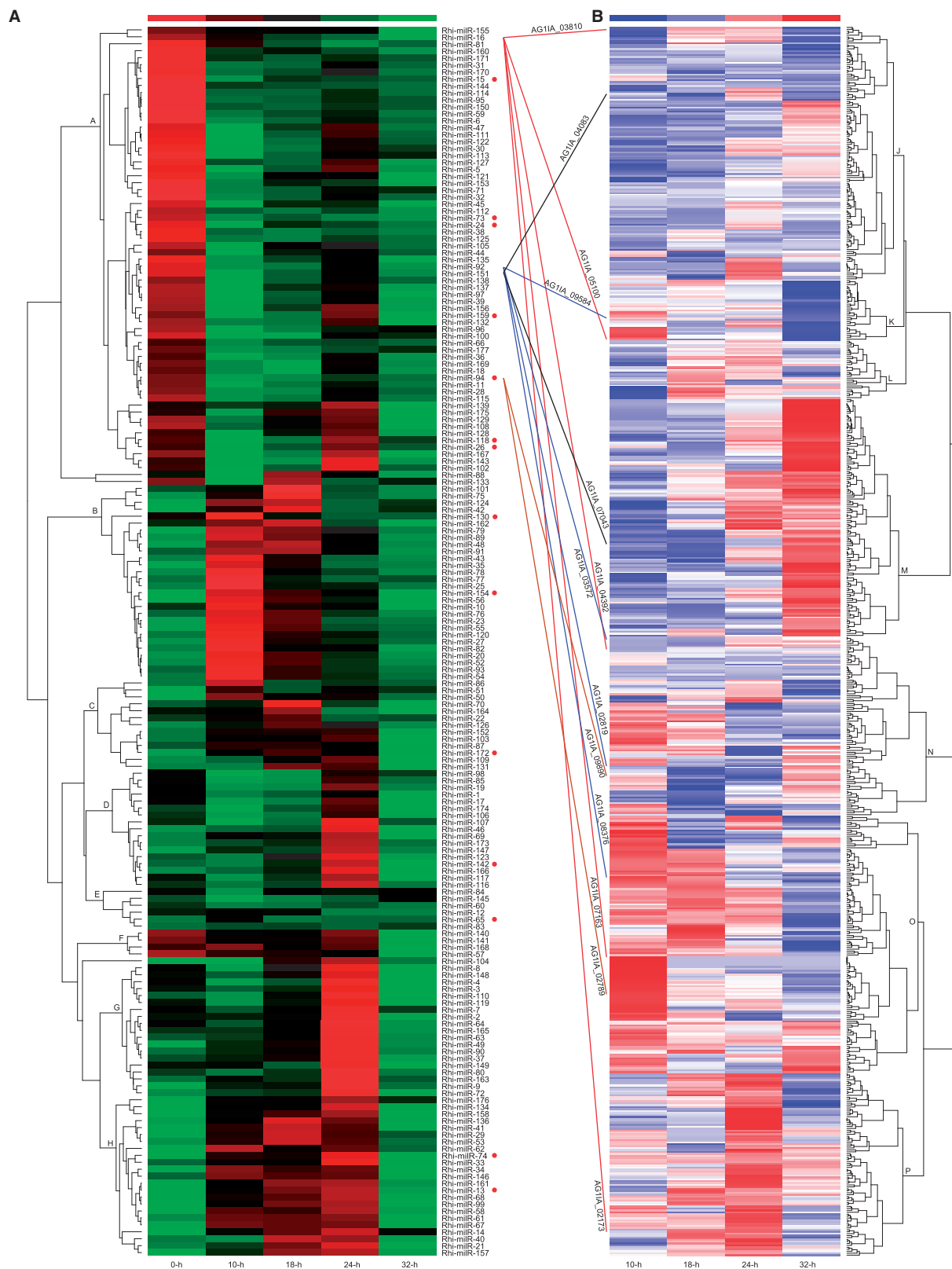


Figure 2. Expression patterns of miRNAs and transcriptome expression of target mRNAs. (A) Eight major clusters (A-H) are presented for expression of the miRNAs. Fourteen conserved miRNAs are denoted with red circles. (B) The transcriptome expression of 631 target mRNAs known to be regulated by miRNAs is presented, revealing seven major clades (J-P). The following examples of miRNA-mRNA interactions are shown: five mRNAs (AG11A_02173, AG11A_03810, AG11A_07163, AG11A_05100, and AG11A_04392) regulated by Rhi-miR-16 (red lines), four mRNAs (AG11A_02819, AG11A_03572, AG11A_08376, and AG11A_09584) regulated by Rhi-miR-92 (blue lines), two mRNAs (AG11A_02789 and AG11A_09890) regulated by Rhi-miR-94 (brown lines), and two mRNAs (AG11A_04083 and AG11A_07043) regulated by Rhi-miR-151 (black lines).

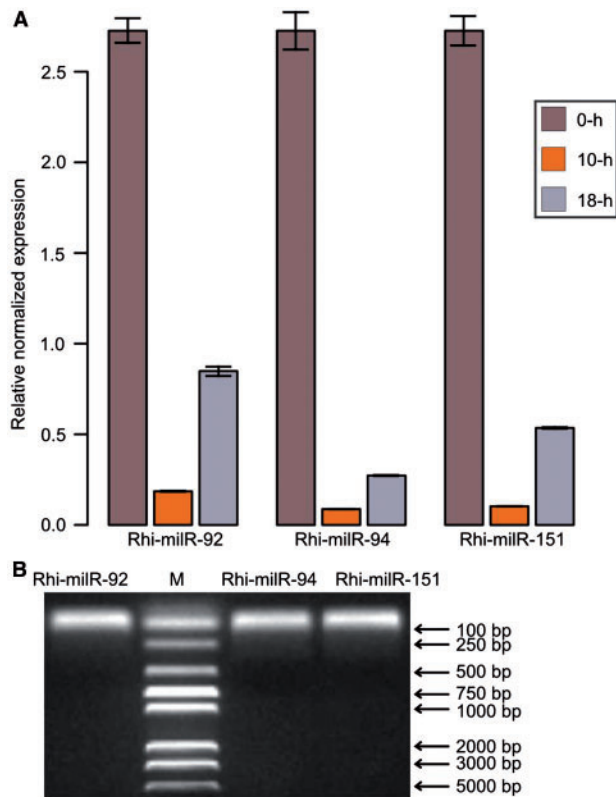


Figure 3. Validation of differentially expressed miRNAs obtained in high-throughput sequencing by RT-qPCR/RT-PCR. (A) The expression of selected miRNAs (Rhi-miR-92, Rhi-miR-94, and Rhi-miR-151) was assayed in the first three stages and normalized to the 18S control gene. All analyses were performed with three biological replicates. Each bar represents the mean \pm standard deviation (S.D.). (B) RT-PCR for three novel miRNAs (Rhi-miR-92, Rhi-miR-94, and Rhi-miR-151) providing experimental proof that these miRNAs exist in fungi.

expressions seemed to be correlated with the transcript levels of their possible target genes during early infection stages (Supplementary Fig. S8E), such as: occurrence of 0-h peaks for miRNAs in cluster A and 10-h peaks for targets; 10-h and 18-h peaks for miRNAs in cluster B, and 24-h peaks and 10-h valleys for targets; 10-h and 18-h peaks for miRNA in cluster C, and 24-h peaks and 18-h valleys for targets.

3.6. Confirmation of miRNA differential expression during infection

The purpose of this study was to explore the role of miRNAs involved in host–pathogen interactions. During the process of rice infection, the 10-h and 18-h stages are crucial for successful infection by *R. solani* AG1 IA. Mycelium rapidly develops on the rice surface at 10-h, and infection cushions form at 18-h after infection. We analysed miRNA expression in the initial three samples (0-h, 10-h, and 18-h) by RT-qPCR and found that the expression of randomly selected miRNAs (Rhi-miR-92, Rhi-miR-94, and Rhi-miR-151) declined at 0–10 and 0–18 h stages, suggesting up-regulated of their targets during the key infection stages (Fig. 3A). The Rhi-miR-92 target gene AG1IA_09584 (hydrolase domain-containing protein) was particularly abundant at 10-h and 18-h. After the pathogen infection, special antimicrobials will be produced by rice in response to

the pathogen.⁴⁵ The expression peak at 10-h for AG1IA_09584 that encodes a member of haloacid dehalogenase-like hydrolase superfamily may indicate its involvement in pathogenic progress by degrading the antimicrobial compounds from rice. The trend in miRNA expression level in the initial three samples was similar to that of the normalized tags (Fig. 3A, Supplementary Table S2). RT-PCR results confirm that these three miRNAs exist in *R. solani* AG1 IA (Fig. 3B).

3.7. Validation of Rhi-miR-16 targets

The interactions between a miRNA and its targeted mRNA site(s) are important for understanding miRNA function. The predicted target of Rhi-miR-16 is AG1IA_02173 (Fig. 4A and B). To test the Rhi-miR-16 mimic and its target gene interactions, an exogenous dual-luciferase reporter assay was utilized. HEK293T cells co-transfected with a reporter plasmid (psiCHECK2-AG1IA_02173) and the Rhi-miR-16 mimic decreased the luciferase expression ratio relative to cells transfected with only the psiCHECK-2_AG1IA_02173 reporter. However, the negative control, which contained a mutated Rhi-miR-16 targeting site reporter (psiCHECK2-AG1IA_02173-mut) co-transfected with Rhi-miR-16 mimic (Supplementary Table S10), exhibited the same luciferase expression ratio relative to cells transfected exclusively with the psiCHECK-2_AG1IA_02173-mut reporter (Supplementary Table S11). These results indicate that the Rhi-miR-16 mimic for the psiAG1IA_02173 reporter was inhibitory ($P < 0.05$) (Fig. 4C, Supplementary Table S12), but the Rhi-miR-16 mimic for the psiAG1IA_02173-mut reporter was not ($P > 0.05$) (Supplementary Table S12). Therefore, Rhi-miR-16 appears to regulate the predicted target gene AG1IA_02173 (dynactin). Dynactin, an additional multisubunit complex, is required for efficient dynein-mediated transport of vesicles *in vitro*;⁵¹ plays an important role in early endosome motility and apical recycling, which is involved in hyphal tip growth and pathogenicity;⁵² and results in abnormal hyphal growth and defective in nuclear distribution when mutated.⁵³ This finding suggests that miRNA is associated with the exocytosis, infection, and growth of *R. solani* AG1 IA.

3.8. Rice miRNAs and their target genes

During the infection, the complex plant defence responses to the broad host-range necrotrophs will be initiated, which may contrast and share mechanisms with biotrophs and is significant to dissect the host–pathogen interactions.⁵⁴ In rice, several miRNAs are discovered to involve in plant immunity against the hemibiotroph *M. oryzae*.⁵⁵ However, no rice miRNA is reported to induce responses to the necrotroph *R. solani* AG1 IA. We further investigated the sequencing sRNA data and identified 23 rice miRNAs in at least three libraries at more than 2.5 TPM (Supplementary Table S13; Supplementary Fig. S9). Among these miRNAs, two miRNAs (osa-miR164a/b/f and osa-miR166k/l-3p) previously indicated to involve in rice immunity against *M. oryzae*⁵⁵ were also identified, and four miRNAs (osa-miR164e, osa-miR396f-5p, osa-miR530-5p, and osa-miR6253) without expression in rice response to *M. oryzae* (Supplementary Data in study by Li, et al.⁵⁵) were found in this study, suggesting the multifaceted host resistance mechanisms in rice. For the 59 target genes of these miRNAs, at least of 43 (72.88%) were TFs (such as MYB TFs; Supplementary Table S14) that may regulate responses to necrotrophs. Members of MYB TFs had been reported to mediate responses to necrotrophic fungi with a diversity of virulence strategies through different mechanisms.⁵⁴ Moreover, other resistance-related genes (such as Os01g02360, a receptor-like kinase) that may contribute to rice defence against *R. solani* were identified as well. Our

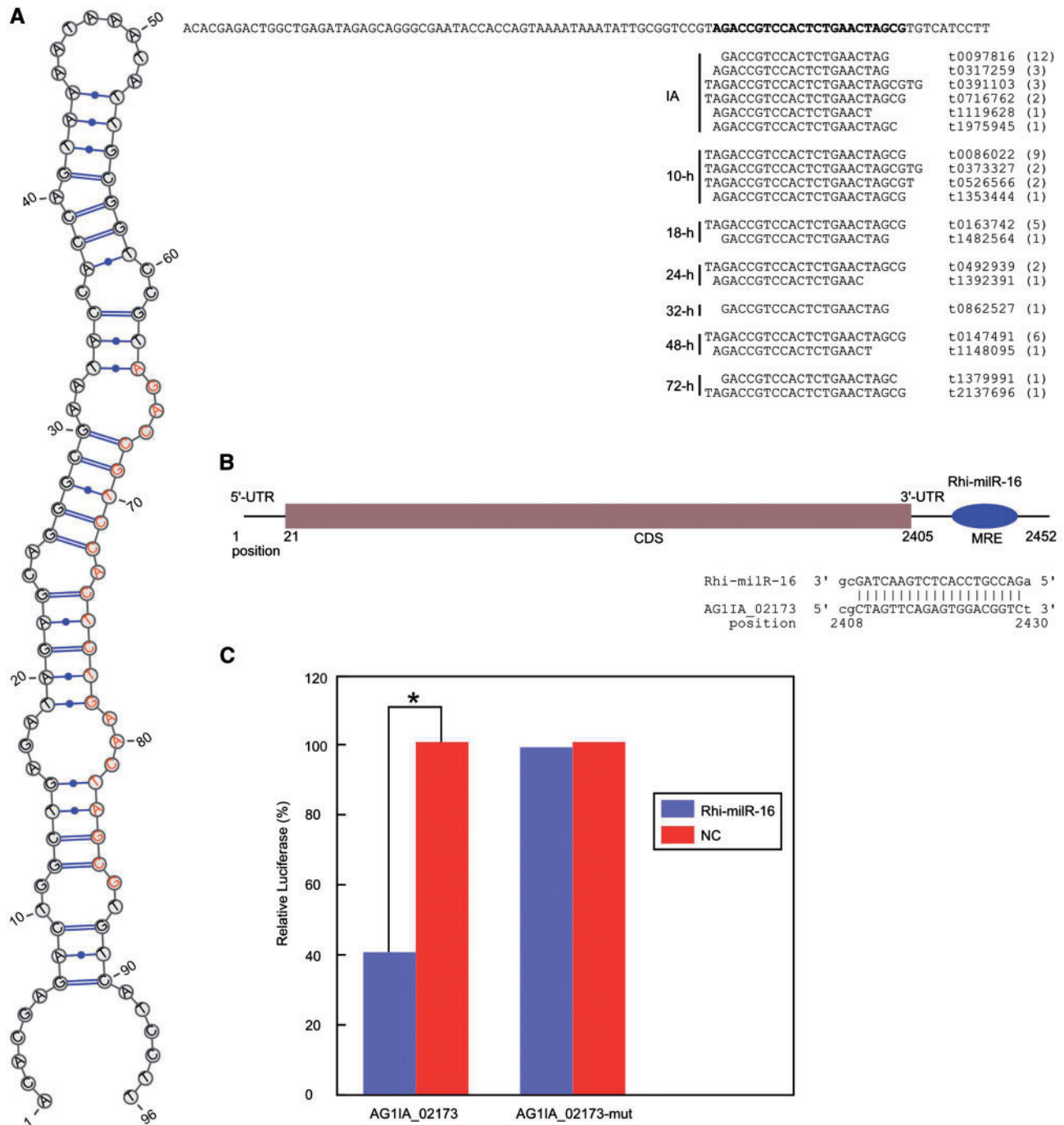


Figure 4. Validation of the predicted Rhi-miR-16 target. (A) Rhi-miR-16 secondary structure. (B) The Rhi-miR-16 targeting sequence in the 3'-UTR of AG11A_02173 mRNA. (C) Rhi-miR-16 targets AG11A_02173 through a targeting sequence located at its 3'-UTR. Dual-luciferase reporter assays were performed to test the interaction of Rhi-miR-16 and its targeting sequence in the 3'-UTR using constructs containing the predicted targeting sequence and a mutated targeting sequence cloned into the 3'-UTR of the reporter gene. The data represent three independent experiments with three measurements. * indicates $P < 0.05$.

analyses provided clues to investigate interactions between rice and *R. solani*, albeit future research are required to confirm the host resistance mechanisms.

4. Discussion

Over 15,000 miRNAs have been identified in plants, animals, viruses, and some unicellular organisms.^{2,5,26,56,57} Many studies have

reported that fungi encode Dicer-like and Argonaute proteins and that gene expression can be specifically knocked down using RNAi-based methods.⁵⁸⁻⁶¹ To date, few miRNAs have been reported in plant pathogenic fungi, which could be due to the low accumulation of miRNA or the small number of cells at certain infection stages. Traditional sequencing of sRNAs preferentially identifies abundant miRNAs.⁶² As a result, less abundant or tissue-specific miRNAs may remain undiscovered in many organisms. Recently, sequencing

Table 2. List of 15 candidate miRNAs involved in pathogenesis

miRNAs	Clade	Peak	Target Genes	Peak	Description	PHI ID	PHI Description
Rhi-miR-95	A	0-h	AG1IA_03893	10-h	Cell division control	PHI:346	Reduced virulence
Rhi-miR-92	A	0-h	AG1IA_09584	10-h	Hydrolase domain-containing protein		
Rhi-miR-16	A	0-h	AG1IA_03810	18-h	Fungal-specific TF		
			AG1IA_02173	24-h	dynactin		
Rhi-miR-44	A	0-h	AG1IA_00962	32-h	G-gamma domain-containing protein		
Rhi-miR-144	A	0-h	AG1IA_08328	32-h	GH6		
Rhi-miR-120	B	10-h	AG1IA_05961	10-h	G-protein beta subunit	PHI:300	Reduced virulence
Rhi-miR-56	B	10-h	AG1IA_03425	10-h	bZIP TF		
Rhi-miR-54	B	10-h	AG1IA_05790	18-h	Fungal-specific TF		
Rhi-miR-77	B	10-h	AG1IA_04857	32-h	Phosphoglucomutase		
Rhi-miR-85	D	24-h	AG1IA_06586	24-h	g2/mitotic-specific cyclin	PHI:338	Reduced virulence
Rhi-miR-145	E	32-h	AG1IA_05961	10-h	G-protein beta subunit	PHI:300	Reduced virulence
			AG1IA_05790	18-h	Fungal-specific TF		
Rhi-miR-83	E	32-h	AG1IA_01341	10-h	Ubiquitin ligase		
Rhi-miR-163	G	24-h	AG1IA_05790	18-h	Fungal-specific TF		
Rhi-miR-165	G	24-h	AG1IA_00123	18-h	GH9		
Rhi-miR-57	F	0-h	AG1IA_02225	10-h	ABC transporter G family	PHI:391	Unaffected pathogenicity

The expression peaks of miRNAs and target mRNAs and PHI annotations are shown. Among these targets, AG1IA_00123 is a putative secreted protein.

technologies have allowed the generation of large libraries of sRNAs, which facilitates the identification of less abundant miRNAs.²⁶ Here, a large number of sRNAs from different infection stages of *R. solani* AG1 were sequenced using high-throughput Illumina sequencing. Our results verified that the plant pathogen *R. solani* produces miRNAs and identified novel miRNAs in basidiomycetes. Future studies involving the construction of transformants overexpressing or knocking out particular miRNAs in *R. solani* AG1 will help to confirm their functions. Although the miRNA pathway remains poorly understood, our study provides important evidence for this pathway and broadens our knowledge of the pathogenic mechanism and biology of *R. solani* at the non-coding RNA level.

The successful identification of miRNAs shows that MIREAP and miRDeep2 are powerful tools^{33,34} to identify candidate miRNAs based on high-throughput sequencing reads and reference genome sequences. For fungal miRNA discovery,^{8,9,13–15} fewer than 45 miRNAs were identified in each species from four sRNA sequencing libraries, with the exception of *Aspergillus flavus*.¹⁴ These studies of fungal miRNA^{8,9,13–15} suggest that the generation of more sequencing data from multiple libraries and the use of suitable prediction workflows should help identify miRNAs. For plant pathogens, the study of miRNAs expressed during infection is lacking. In our work, a method was proposed for fungal miRNA prediction in which the rice miRNA detection workflow was altered, which identified highly credible miRNAs supporting by at least 11 reads in one of the libraries. Sequence comparison indicates that 109 (61.58%) candidate miRNAs share partially conserved sequences with reported miRNAs, suggesting the high accuracy of miRNA prediction by our method as well. Moreover, confirmation of our results by RT-qPCR/RT-PCR supports the accuracy of miRNA identification. The dual-luciferase reporter assay has been used widely to validate human miRNA targets.⁶³ Due to the lack of effective transformation systems and reporter genes for *R. solani* AG1 IA, HEK293T cells were transfected with a dual-luciferase reporter vector to validate predicted miRNA targets. In future work, we will explore methods to validate the predicted miRNA targets in rice sheath blight pathogen. Moreover, based on expression data obtained during the infection stages and on the pathogenic PPI subnetwork, the expression pattern

and character of predicted *R. solani* miRNAs were revealed. This information could help us to understand pathogenic factors during key infection stages at the systems level. From our analysis, 15 candidate pathogenic miRNAs are identified (Table 2), which is supported by the expression of miRNAs and their target genes, and functions of targets, as well as PHI annotations. Although gene expression was affected by comprehensive factors, such as TFs and alternative splicing, infection-related miRNAs are important pathogenic factors. The results may suggest that the pathogenic mechanisms of miRNAs in the rice sheath blight pathogen are mediated by regulating target ABC-G transporter and hydrolase involved in drug resistance and translocation of various molecules and degrading the antimicrobial compounds from host after penetration respectively, by regulating virulence-associated factors associated with transduction signal pathway, by regulating TFs that may regulate pathogenic factors during infection, and by regulating cell wall-degrading enzymes involving in fungal pathogenicity (Table 2), albeit further research are required to confirm. Our future work will clarify the miRNA pathways and identify critical pathogenic factors. In addition, the diversity of virulence strategies in pathogens corresponds to different host defence response mechanisms,⁵⁴ which is also supported by the identification of four rice miRNAs that seemed do not involve in rice immunity against *M. oryzae*⁵⁵ but expressed during *R. solani* infection. The target genes of these four miRNAs included TFs that may represent major impacts on rice immunity against the rice sheath blight pathogen. As the mechanisms of interactions between *R. solani* and rice still remain poor understand, the discovery of rice miRNAs involved in host defence responses to *R. solani* will facilitate research on pathogen virulence and host resistance.

Authors' contribution

R.L. and L.H. contributed equally to this work. A.Z. and P.L. managed the project. P.Q. prepared the RNA samples. A.Z. and R.L. designed the analysis. R.L. and A.Z. performed the bioinformatics analysis. Y.W., Z.Y., Q.D., S.L., S.W., W.W., and J.H. participated in miRNA expression and regulation analyses. L.H. performed the experiments. R.L., L.H., A.Z., and P.L. prepared the figures and tables. W.W. and H.L.

assisted with preparing the supplementary figures and supplementary tables. R.L. and A.Z. submitted the data to the NCBI. R.L., L.H., and A.Z. wrote the paper. All authors reviewed the manuscript.

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Accession numbers

All data have been deposited at NCBI GEO under the accession code GSE68236.

Conflict of interest

None declared.

Supplementary data

Supplementary data are available at www.dnaresearch.oxfordjournals.org.

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