Article

Genome-Wide Expression Profiling of Small RNAs in Indian Strain of *Rhizoctonia solani* AG1-1A Reveals Differential Regulation of milRNAs during Pathogenesis and Crosstalk of Gene Regulation

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**Abstract:** *Rhizoctonia solani* AG1-1A is a necrotrophic fungus that causes sheath blight disease in rice. The reliable resistant source against this phytopathogenic fungus is not available in the gene pool of rice. Better understanding of pathogen genomics and gene regulatory networks are critical to devise alternate strategies for developing resistance against this noxious pathogen. In this study, miRNA-like RNAs (milRNAs) of an Indian strain of *R. solani* were identified by deep sequencing of small RNAs. We identified 128 known and 22 novel milRNAs from 20,963,123 sequence reads. These milRNAs showed 1725 target genes in the fungal genome which include genes associated with growth, development, pathogenesis and virulence of *R. solani*. Notably, these fungal milRNAs showed their target genes in host (rice) genome also which were later verified by qRT-PCR. The host target genes are associated with auxin metabolism, hypersensitive response, defense genes, and genes related to growth and development of rice. Osa-vacuolar-sorting receptor precursor: Rhi-miR-13, Osa-KANADI1: Rhi-miR-124, Osa-isoflavone reductase: Rhi-miR-135, Osa-nuclear transcription factor Y: Rhi-miR-13, Osa-AG1-1A Reveals Differential Regulation of milRNAs during Pathogenesis and Crosstalk of Gene Regulation. *J. Fungi* 2021, 7, 561. https://doi.org/10.3390/jof7070561

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1. Introduction

*Rhizoctonia solani* AG1-1AKühn (Teleomorph: *Thanatephorus cucumeris* (A.B. Frank Donk)) is a basidiomycete semi-saprotrophic soil-borne fungal pathogen that causes sheath blight disease in rice. In Eastern Asia, *R. solani* causes a loss of 6 million tons of rice
grains per year [1]. India suffers 10% yield loss in rice due to sheath blight disease every year [2]. The pathogen is present in the form of sclerotia or mycelium, and severely affects high yielding, semi-dwarf, and nitrogen-responsive rice cultivars. Initial symptoms are noticed on leaf sheaths near water level. Warm and humid weather, dense planting, and high nitrogen inputs are the favourable conditions for its aggravation. Until now, rice germplasm immune to \textit{R. solani} has not been known. Among several other factors, poor understanding of pathogen biology is one of the key factors that hamper the development of resistant cultivars either through breeding or transgenic approaches. Recently, efforts have been made to understand the regulation of the genome and transcriptome of \textit{R. solani} during pathogenesis [3–5].

Clear understanding of pathogen genomics and gene regulatory networks is extremely important for drawing effective strategies for resistance development against diseases. One such example is the rice–\textit{Xanthomonas oryzae pv. oryzae (Xoo)} pathosystem where deeper understanding of bacterial TALE (transcription activator-like effector) genes and their interaction with rice SWEET genes has helped developing durable and broad spectrum bacterial blight-resistant rice genotypes through marker-assisted breeding and genome editing [6–9]. Genomics has been extremely useful to facilitate host-induced gene silencing (HIGS) technology for the control of \textit{Fusarium} diseases through targeted disruption of key genes [10]. Recently, Dong and Ronald (2019) [11] presented comprehensive information on pathogen genes used for disease resistance development through genetic engineering, RNAi, and genome editing. In addition to genetic resistance, fungal genomics is crucial for fungicide development by identifying potential fungicide targets, their validation and mode of action, as demonstrated in several pathosystems [12]. For more than a decade, small RNAs have taken central stage in the area of genomics due to their versatile functions in gene regulation and metabolic pathways [13,14].

Small RNAs (sRNAs) of <200 nucleotides are classified into different categories such as miRNA, siRNA, piwiRNA, snoRNA and t-RNAs etc. Recently, more sRNAs have been discovered based on their site of synthesis, binding proteins, and secondary structure and function, for example, trans-acting siRNAs (tasiRNAs) [15], repeat associated siRNAs (rasiRNAs) [16], heterochromatic small RNAs (hcRNAs) [17], and small scan RNAs (scnR-NAs) [18] etc. MicroRNAs (miRNAs) are 21–24 nucleotide endogenous non-coding RNA molecules that work as defensive regulators in eukaryotes through post-transcriptional gene regulation. To date, numerous miRNAs have been discovered in plants and animals, but very few miRNAs have been reported in fungi [19]. Recent advancement in sequencing technologies and bioinformatics tools facilitated the identification of miRNAs in fungi [19–21]. MicroRNAs are critical regulators of host–pathogen interaction, and may help disease initiation and establishment [19,21–23].

In the model fungi \textit{Neurospora crassa}, four different types of miRNA-like RNAs (mil-RNAs) processed through a dicer-independent pathway were identified. The proteins responsible for the production of these milRNAs are different from the plants and animals to a large extent [24]. Considering the essential functions of miRNAs in gene regulation and metabolic processes, there is a necessity to study the fungal genome encoded milRNAs which would give different insights and roles of microRNAs in pathogenesis and disease development. To date, very few milRNAs have been reported in plant pathogenic fungi, which could be due to the low accumulation of milRNAs or the small number of cells at certain infection stages, and also due to lesser attention of researchers. Recently, Meng et al. (2021) [25] identified virulence-associated milRNAs from \textit{R. solani} AG1-1A infecting maize crop. With this perspective, our current study focussed on discovering the milRNAs in \textit{R. solani} causing sheath blight disease in rice. Genome-wide small RNAs isolated from a pure culture of the Indian strain of \textit{R. solani} AG1-1A were sequenced to identify the known and novel milRNAs. Later, the targets of these milRNAs were predicted in \textit{R. solani} and its host (rice) genome. Expression of 17 fungal milRNAs was analyzed in infected tissue of rice at different time points of inoculation, in different genotypes and growth stages of rice, and
also in different strains of fungi. Expression analysis of target genes present in fungal and rice genome suggested that miRNAs are critical regulators of host–pathogen interaction.

2. Material and Methods

2.1. Fungal and Plant Material

In this study, four strains of *R. solani* AG1-IA were used. These strains were Wgl-2, Chn-1, Imph-2 and Lud-1 [26]. Four rice genotypes as a fungal host were used that include susceptible (TN1 and BPT5204) and resistant (Tetep and Pankaj) cultivars.

2.2. RNA Isolation and Sequencing

The highly virulent Indian strain of *R. solani* AG1-IA Wgl-2 was grown in growth medium having pectin as a carbon source. The fungal sclerotium was placed in liquid medium (0.7 g K$_2$HPO$_4$, 0.5 g KH$_2$PO$_4$, 0.5 g MgSO$_4$, 0.01 g FeSO$_4$, 0.001 g ZnSO$_4$, and 10g pectin in 1 L distilled and autoclaved water) at 28 °C for 48–72 h as a still culture. The mycelium from three biological replicates was harvested and pooled to extract the RNA using TRIzol reagent (Invitrogen, Waltham, MA, USA). The quality of RNA was checked by Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA), Bioanalyzer (Agilent 2100), and agarose gel electrophoresis to check the integrity, concentration, and contamination. After quality control (QC), the sequencing library was constructed by TruSeq Small RNA Library Preparation Kit (Illumina, CA, USA). The cDNA library was prepared by sequencing adaptor ligation, reverse transcription, PCR enrichment, purification and size selection. The QC of library was analyzed by Qubit 2.0 (preliminary library concentration), Agilent 2100 (insert size), and qPCR (effective concentration of library). The QC passed libraries were fed into HiSeq 2500 sequencer for sequencing.

2.3. miRNAs Identification in *R. solani*

The sequence data were analyzed to identify the miRNAs in the *R. solani* genome. Clean reads without adaptor contamination were mapped to reference genome available in the RSIADB database [27]. Further non-coding sRNAs like rRNA, snRNA, snoRNA, and tRNAs were removed by using the Rfam database (https://rfam.xfam.org/, accessed on 1 July 2017). The mRNA sequences or coding transcripts were filtered using RSIADB database. Later, miRNA prediction was undertaken using miREAP and miRDeep2 software for identification of both known and novel miRNAs. The secondary structures of these potential novel miRNA precursors were predicted by the RNAfoldWebServer program (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi, accessed on 1 July 2017). Putatively novel miRNAs were identified based on the following criteria: (1) mature miRNA sequence should be in either of the arm of hairpin structure, (2) number of mismatches 4 or less, (3) no loop or break in miRNA sequences, (4) asymmetric bulges should be minimal in size (one or two bases) and frequency (typically one or less), especially within the miRNA/miRNA* duplex [3,20,28].

2.4. The Target Gene Prediction in *R. solani* and Rice Genome

The target genes of miRNAs in the *R. solani* genome were predicted by using Miranda software [21] and the RSIADB database. The target genes of fungal encoded miRNAs in rice genome were predicted by using the psRNA Target database [29]. The default criteria of the target prediction tool were used for identification of miRNA target genes.

2.5. Expression Analysis of Fungal miRNAs and Their Target Genes

Forty-five day old plants of rice cultivars TN1, Pankaj, BPT 5204, and Tetep were inoculated by *R. solani* AG11A Wgl-2 strain. In parallel, TN1 was inoculated by three other strains, i.e., Chn-1, Imph-2 and Lud-1. For inoculation, the fungal sclerotium was placed in the sheath tissue of rice using a transparent cello tape. The sheath tissue was harvested from different rice genotypes (TN1, Pankaj, BPT 5204, and Tetep) infected by the Wgl-2 strain. Also, infected sheath tissue was harvested from TN1 at different time
points of infection (18 h, 24 h, 48 h, 72 h, 96 h, and 5 days after inoculation) as well as from 45- (vegetative) and 80- (reproductive) day-old growth stages infected by the Wgl-2 strain. To analyze the strain specific milRNAs regulation, tissue was harvested from TN1 infected by three other strains (Chn-1, Imph-2, and Lud-1). Small RNAs and mRNAs were isolated from fungal infected rice tissue and pure culture of fungi (grown in potato dextrose agar medium) using the mirVana miRNA isolation kit (Ambion, TX, USA) according to the manufacturer’s instructions. cDNA synthesis of normalized sRNAs and mRNAs was performed using the miScript II RT kit (Qiagen, Hilden, Germany) and Improm-II reverse transcription system (Promega, Madison, WI, USA), respectively. The respective cDNA was used as template for milRNA and target gene quantification using miScript SYBR Green PCR kit (Qiagen, Germany) and SYBR Premix Ex-Taq (Takara, Shiga, Japan), respectively. Three biological replicates and two technical replicates were used for all the qRT-PCR experiments. Following the manufacturer instructions, all the kit reagents along with the milRNA and gene-specific primers (Table S1) were used for setting up the qRT-PCR reaction in a CFX96 Real-Time System (BIO-RAD, Hercules, CA, USA). The temperature profile was followed as described in previous report [30]. To ensure specificity of amplified product, melt curve analysis and agarose gel electrophoresis were used. To quantify the relative expression of milRNAs and target genes, the comparative threshold cycle (Ct) method was followed. The relative expression was calculated from $2^{-\Delta\Delta Ct}$. The standard error was calculated as reported previously [31].

3. Results
3.1. Sequence Statistics
The milRNAs encoded by the R. solani AG1 IA genome were identified through Illumina deep sequencing of small RNAs. All the sequence reads were aligned to the reference genome of R. solani available in the RSIADB database [27] and the mapped clean reads were further used to identify the known and novel milRNAs. After deep sequencing of R. solani small RNAs 20,963,123 raw reads and 18,084,903 clean reads were obtained. The clean reads were used to filter 1,231,411 reads of 18–24 nts size of which 185,760 were unique reads. 2574 reads of R. solani aligned with hairpin sequences of known milRNAs [21], while 619,582 reads were utilized for prediction of novel milRNAs.

3.2. Identification of Known and Novel milRNAs
128 known milRNAs of R. solani were identified in Indian strain of the pathogen (Table S2). Among these, Rhi-miR-16, Rhi-miR-22, Rhi-miR-35, Rhi-miR-57, Rhi-miR-91, and Rhi-miR-112 showed higher read count than the others. Twenty two novel milRNAs were predicted among which scaffold13_22000, scaffold47_37005, scaffold47_37523, scaffold47_37559, scaffold47_37341, scaffold47_37407, and scaffold47_36418 showed higher read account than others. Notably, scaffold13_22000 showed >6000 read count (Table 1). The secondary structure of a few representative novel milRNAs is shown in Figure 1.
**Table 1.** Novel miRNAs predicted from small RNA sequence data of *Rhizoctonia solani* AG1-1A Wgl-2 strain.

| S. No. | Novel miRNA   | miRDeep2 Score | Estimated Probability That the miRNA Candidate Is a True Positive | Total Read Count | Consensus Mature Sequence            | Precursor Coordinate          |
|-------|---------------|----------------|-----------------------------------------------------------------|------------------|-------------------------------------|-------------------------------|
| 1     | scaffold47_37407 | 193.1          | 70 +/- 24%                                                       | 379              | aaucuccuaggauuccacuug               | scaffold47:62019..62062:--   |
| 2     | scaffold47_37523 | 193            | 70 +/- 24%                                                       | 379              | aaucuccuaggauuccacuug               | scaffold47:127383..127426:--|
| 3     | scaffold47_37341 | 192.9          | 70 +/- 24%                                                       | 379              | aaucuccuaggauuccacuug               | scaffold47:26292..26335:--   |
| 4     | scaffold47_36931 | 30.3           | 70 +/- 24%                                                       | 80               | Uggacaauguucugugggguu              | scaffold47:118388..118435:+ |
| 5     | scaffold145_44745 | 17.3           | 70 +/- 24%                                                       | 37               | Gacuaaucagguugggcaauu              | scaffold145:16023..16102:+   |
| 6     | scaffold6_14191 | 6              | 66 +/- 23%                                                       | 11               | Uggaaggggauuggaccuccu              | scaffold6:504695..504742:--  |
| 7     | scaffold10_19054 | 1.3            | 35 +/- 26%                                                       | 3                | Uggcgacuguguccgcaucg              | scaffold10:751369..751434:-- |
| 8     | scaffold1_2221  | 0.9            | 16 +/- 15%                                                       | 5                | Cagcacaacggacagacgacu             | scaffold1:304739..304742:+  |
| 9     | scaffold47_37005 | 0.8            | 16 +/- 15%                                                       | 249              | Agauuuuugagccccgacuacuc           | scaffold47:124971..125020:+ |
| 10    | scaffold12_20737 | 0.8            | 16 +/- 15%                                                       | 27               | gauuuuugagcaggccccc              | scaffold12:518031..518072:+  |
| 11    | scaffold7_15416 | 0.6            | 16 +/- 15%                                                       | 2                | uguuugggugcucgugacccg             | scaffold7:443987..444035:--  |
| 12    | scaffold47_35943 | 0.4            | 16 +/- 15%                                                       | 29               | Aaggaaauuccuguggcuccu             | scaffold47:16996..17045:+   |
| 13    | scaffold47_36418 | 0.4            | 16 +/- 15%                                                       | 94               | Uuccuugggacacacgagacu             | scaffold47:65345..65392:+   |
| 14    | scaffold49_37930 | 0.4            | 16 +/- 15%                                                       | 2                | Ccucucuugggucuccggcgcuc          | scaffold49:58540..58612:+   |
| 15    | scaffold48_37687 | 0.3            | 16 +/- 15%                                                       | 14               | Ucucucaggcgcacugucucu             | scaffold48:142724..142771:+ |
| 16    | scaffold30_30807 | 0.3            | 16 +/- 15%                                                       | 30               | Augggacucgccacucgucuc             | scaffold30:89871..89933:--  |
| 17    | scaffold34_32450 | 0.2            | 16 +/- 15%                                                       | 52               | Auggcrgaugggauuccucu             | scaffold34:218833..218913:--|
| 18    | scaffold13_22000 | 0              | 16 +/- 15%                                                       | 6028             | Augggacgauuacgacucu              | scaffold13:463575..463652:--|
| 19    | scaffold130_44481 | 0             | 16 +/- 15%                                                       | 2                | ccugugagcggacgacu               | scaffold130:10887..10931:--  |
| 20    | scaffold47_37559 | 0              | 16 +/- 15%                                                       | 148              | Cguggacgggggauuccc              | scaffold47:139094..139138:--|
| 21    | scaffold22_27474 | 0              | 16 +/- 15%                                                       | 2                | Ugccccggcugaeuuccu             | scaffold22:336752..336810:+ |
| 22    | scaffold1_2114  | 0              | 16 +/- 15%                                                       | 3                | Cucugugcugcucugacuccu           | scaffold1:2908975..2909036:+ |
3.3. Identification of Target Genes in *R. solani* Genome

We identified 1725 target genes of 128 known and 22 novel miRNAs in the *R. solani* genome (Table S3). The known miRNAs targeting the genes encoding carbohydrate active enzymes, secretory proteins, and other critical proteins associated with fungal biology are listed (Tables 2 and S4). Also, the target genes of novel miRNAs were predicted (Table 3). Notably, Scaffold47_36931 showed the highest number (12) of target genes. Rhi-miR-141, Rhi-miR-120, Rhi-miR-41, and Rhi-miR-91 showed pectin esterase and pectate lyase domain-containing protein genes as their targets.
Table 2. Gene targets of *Rhizoctonia solani*-miRNAs. These target genes encode carbohydrate active enzymes and secretory proteins of fungus.

| milRNA Id     | Target Gene      | Gene Description                                      |
|---------------|------------------|--------------------------------------------------------|
|               |                  | Carbohydrate Active Enzymes                             |
| Rhi-miR-56    | AG1IA_01218      | Beta-glucosidase (EC 3.2.1.21)                          |
| Rhi-miR-120   | AG1IA_01405      | 1,3-beta-glucan synthase component GLS2                |
| Rhi-miR-122   | AG1IA_01406      | Sterol 3-beta-glycosyltransferase (EC 2.4.1.173) (Autophagy-related protein 26) |
| Rhi-miR-91    | AG1IA_02027      | Endoplasmic reticulum protein                          |
| Rhi-miR-169   | AG1IA_02441      | Beta-xyllosidase                                       |
| Rhi-miR-146   | AG1IA_02513      | Chitin deacetylase                                     |
| Rhi-miR-120   | AG1IA_02835      | Putative 1,4-alpha-glucan branching enzyme from glycoside hydrolase family GH13 |
| Rhi-miR-141   | AG1IA_02889      | Pectinesterase (EC 3.1.1.11)                          |
| Rhi-miR-150   | AG1IA_03463      | Glycosyltransferase family 2 protein                   |
| Rhi-miR-36    | AG1IA_03939      | Beta-mannosidase                                       |
| Rhi-miR-91    | AG1IA_04214      | Trehalose 6-phosphate phosphatase, glycosyltransferase family 20 protein |
| Rhi-miR-81    | AG1IA_04527      | Uridine Di Phosphate-N-acetylglucosaminyltransferase  |
| Rhi-miR-68    | AG1IA_04727      | Farnesyltransferase subunit beta                       |
| Rhi-miR-111   | AG1IA_04740      | Polysaccharide lyase family 1 protein                  |
| Rhi-miR-27    | AG1IA_04862      | 1,3-beta-glucan synthase component GLS2                |
| Rhi-miR-45    | AG1IA_05653      | Glycoside hydrolase family 51 protein                  |
| Rhi-miR-52    | AG1IA_05719      | Chitin synthase D                                      |
| Rhi-miR-119   | AG1IA_05754      | Glycogen phosphorylase                                 |
| Rhi-miR-42    | AG1IA_05803      | Alpha-galactosidase (EC 3.2.1.22) (Melibiase)          |
| Rhi-miR-139   | AG1IA_05807      | Adenylosuccinate synthetase (AMPase) (AdSS) (EC 6.3.4.4) (IMP-aspartase ligase) |
| Rhi-miR-122   | AG1IA_06014      | Glycoside hydrolase family 51 protein                  |
| Rhi-miR-41    | AG1IA_06294      | Glycoside hydrolase family 3 protein                   |
| Rhi-miR-58    | AG1IA_06593      | Alpha glucosidase II, alpha subunit, putative          |
| Rhi-miR-167   | AG1IA_07255      | Killer toxin alpha/beta                               |
| Rhi-miR-51    | AG1IA_07341      | Galactan 1,3-beta-galactosidase                        |
| Rhi-miR-120   | AG1IA_07743      | Pectinesterase (EC 3.1.1.11)                          |
| Rhi-miR-162   | AG1IA_07787      | Glycoside hydrolase family 31 protein                  |
| Rhi-miR-168   | AG1IA_07905      | Glycoside hydrolase family 95 protein                  |
| Rhi-miR-97    | AG1IA_08771      | Exo-beta 1,3-glucanase                                 |
|               |                  | Secretory Proteins                                     |
| Rhi-miR-150   | AG1IA_00157      | Polysaccharide deacetylase domain-containing protein   |
| Rhi-miR-98    | AG1IA_01858      | Uncharacterized protein                                |
| Rhi-miR-124   | AG1IA_01958      | Rad1 domain-containing protein                         |
| Rhi-miR-95    | AG1IA_02532      | Lipase domain-containing protein                       |
| Rhi-miR-144   | AG1IA_03100      | Glycosyl hydrolase family 61 domain-containing protein |
| Rhi-miR-122   | AG1IA_03118      | Uncharacterized protein                                |
| Rhi-miR-141   | AG1IA_03171      | Copper/zinc superoxide dismutase domain-containing protein |
| Rhi-miR-165   | AG1IA_05741      | Uncharacterized protein                                |
### Table 2. Cont.

| milRNA Id   | Target Gene      | Gene Description                                           |
|-------------|------------------|------------------------------------------------------------|
| Rhi-miR-141 | AG1IA_06494      | Uncharacterized protein                                    |
| Rhi-miR-122 | AG1IA_07117      | Uncharacterized protein                                    |
| Rhi-miR-52  | AG1IA_07216      | Uncharacterized protein                                    |
| Rhi-miR-19  | AG1IA_07698      | Transcription initiation factor TFIID complex 60 kDa subunit|
| Rhi-miR-54  | AG1IA_08056      | Tyrosinase domain-containing protein                       |
| Rhi-miR-144 | AG1IA_08227      | Uncharacterized protein                                    |
| Rhi-miR-90  | AG1IA_08293      | Cytochrome P450 domain-containing protein                  |
| Rhi-miR-146 | AG1IA_08653      | Uncharacterized protein                                    |
| Rhi-miR-122 | AG1IA_08771      | Uncharacterized protein                                    |
| Rhi-miR-144 | AG1IA_09802      | Protein tyrosine kinase domain-containing protein           |
| Rhi-miR-130 | AG1IA_10060      | Cytochrome P450 domain-containing protein                  |

### Table 3. Target genes of *Rhizoctonia solani* novel miRNAs.

| S. No. | Novel milRNA | Target Gene | Gene Description                                           |
|--------|--------------|-------------|------------------------------------------------------------|
| 1      | scaffold47_37407 | AG1IA_04067 | Ubiquitin conjugating enzyme family protein               |
| 2      | scaffold47_37407 | AG1IA_02707 | MFS transporter, putative                                  |
| 3      | scaffold47_37523 | AG1IA_04067 | Ubiquitin conjugating enzyme family protein               |
| 4      | scaffold47_37523 | AG1IA_02707 | MFS transporter, putative                                  |
| 5      | scaffold47_37431 | AG1IA_04067 | Ubiquitin conjugating enzyme family protein               |
| 6      | scaffold47_37341 | AG1IA_02707 | MFS transporter, putative                                  |
| 7      | scaffold47_36931 | AG1IA_08169 | Phosphatidylinositol 3-kinase tor2                         |
| 8      | scaffold47_36931 | AG1IA_05744 | DNA-directed RNA polymerase subunit (EC 2.7.7.6)          |
| 9      | scaffold47_36931 | AG1IA_06882 | EOS1 domain-containing protein                             |
| 10     | scaffold47_36931 | AG1IA_06165 | ABC transporter                                           |
| 11     | scaffold47_36931 | AG1IA_07629 | Uncharacterized protein                                    |
| 12     | scaffold47_36931 | AG1IA_07663 | ATP-dependent rRNA helicase RRP3                         |
| 13     | scaffold47_36931 | AG1IA_04769 | Fungal zn(2)-Cys(6) binuclear cluster domain-containing protein |
| 14     | scaffold47_36931 | AG1IA_08613 | BMRI protein                                              |
| 15     | scaffold47_36931 | AG1IA_00053 | Uncharacterized protein                                    |
| 16     | scaffold47_36931 | AG1IA_06822 | Uncharacterized protein                                    |
| 17     | scaffold47_36931 | AG1IA_07843 | Uncharacterized protein                                    |
| 18     | scaffold47_36931 | AG1IA_08211 | Molybdenum cofactor biosynthesis protein                  |
| 19     | scaffold145_44745| AG1IA_06624 | Uncharacterized protein                                    |
| 20     | scaffold145_44745| AG1IA_10187 | RNase H domain-containing protein                          |
| 21     | scaffold145_44745| AG1IA_10310 | TFIIB domain-containing protein                            |
| 22     | scaffold145_44745| AG1IA_02688 | GPI transamidase component PIG-S                          |
| 23     | scaffold145_44745| AG1IA_05493 | Uncharacterized protein                                    |
| 24     | scaffold145_44745| AG1IA_04447 | HLH domain-containing protein                              |

Abbreviations: MFS—Major Facilitator Superfamily, TFIIB—Transcription Factor IIA, GPI—Glycosylphosphatidylinositol, PIG-S—Phosphatidylinositol glycan biosynthesis class S protein, HLH—Helix Loop Helix.
3.4. Identification of R. solani-miRNAs Target Genes in Oryza Sativa Genome

In addition to identifying the gene targets of fungal encoded miRNAs in fungal genome, target genes of these miRNAs were also searched in its host genome. Interestingly, 22 known and 2 novel miRNAs showed their target genes in the rice genome. Some of these notable target genes:miRNA regulatory pairs are Osa-KANADI1:Rhi-miR-124, Osa-vacuolar-sorting receptor precursor:Rhi-miR-13, Osa-nuclear transcription factor Y:Rhi-miR-131, Osa-isoflavone reductase: Rhi-miR-135, Osa-OsFBX438: Rhi-miR-142, Osa-NB-ARC domain containing protein: Rhi-miR-18, Osa-plastocyanin-like domain containing protein:Rhi-miR-22, Osa-cytochrome P450 81E1: Rhi-miR-26, Osa-response regulator receiver domain containing protein: Rhi-miR-43, Osa-serine/threonine-protein kinase receptor precursor: Rhi-miR-61, Osa-OsMADS58: Rhi-miR-66, and Osa-extra-large G-protein-related: Rhi-miR-81. Among the novel miRNAs, Scaffold130_44481 miRNA targets the Osa-plastocyanin-like domain containing protein while scaffold47_37559 targets transferase family protein (Table 4).

Table 4. Target genes of Rhizoctonia solani miRNAs in rice genome; 24 miRNAs showed their target as rice genes encoding proteins associated with defense and other metabolic processes.

| miRNAs   | Target Gene Accession | Expect | Gene Description                                      | Inhibition      |
|----------|-----------------------|--------|-------------------------------------------------------|-----------------|
| Rhi-miR-1 | LOC_Os01g47740.2      | 3      | cDNA | zinc finger, C3HC4 type domain containing protein, expressed | Translation     |
| Rhi-miR-111 | LOC_Os07g48720.3 | 2.5    | cDNA | MAG2, putative, expressed                               | Cleavage        |
| Rhi-miR-124 | LOC_Os09g23200.1     | 2.5    | cDNA | KANADI1, putative, expressed                            | Cleavage        |
| Rhi-miR-130 | LOC_Os10g20630.1     | 1      | cDNA | vacuolar-sorting receptor precursor, putative, expressed | Cleavage        |
| Rhi-miR-131 | LOC_Os11g02464.1     | 2.5    | cDNA | vacuolar-sorting receptor precursor, putative, expressed | Cleavage        |
| Rhi-miR-135 | LOC_Os12g42400.4     | 2.5    | cDNA | nuclear transcription factor Y subunit, putative, expressed | Cleavage        |
| Rhi-miR-131 | LOC_Os12g42400.1     | 2.5    | cDNA | nuclear transcription factor Y subunit, putative, expressed | Cleavage        |
| Rhi-miR-142 | LOC_Os02g58790.5     | 3      | cDNA | cell division inhibitor, putative, expressed           | Cleavage        |
| Rhi-miR-159 | LOC_Os02g58790.2     | 3      | cDNA | cell division inhibitor, putative, expressed           | Cleavage        |
| Rhi-miR-160 | LOC_Os02g58790.1     | 3      | cDNA | cell division inhibitor, putative, expressed           | Cleavage        |
| Rhi-miR-181 | LOC_Os02g58790.4     | 3      | cDNA | cell division inhibitor, putative, expressed           | Cleavage        |
| Rhi-miR-182 | LOC_Os02g58790.3     | 3      | cDNA | cell division inhibitor, putative, expressed           | Cleavage        |
| Rhi-miR-183 | LOC_Os02g58790.1     | 3      | cDNA | cell division inhibitor, putative, expressed           | Cleavage        |
| Rhi-miR-184 | LOC_Os01g55200.1     | 3      | cDNA | potassium channel KAT1, putative, expressed            | Cleavage        |
| Rhi-miR-185 | LOC_Os12g16290.1     | 2.5    | cDNA | isoflavone reductase, putative, expressed              | Cleavage        |
| Rhi-miR-186 | LOC_Os05g45180.1     | 3      | cDNA | anthocyanidin 5,3-O-glucosyltransferase, putative, expressed | Cleavage        |
| Rhi-miR-187 | LOC_Os12g03740.1     | 2.5    | cDNA | OsFBX438-F-box domain containing protein, expressed   | Cleavage        |
| Rhi-miR-188 | LOC_Os12g43720.1     | 3      | cDNA | early-responsive to dehydration protein-related, putative, expressed | Cleavage        |
| Rhi-miR-189 | LOC_Os03g24410.1     | 1.5    | cDNA | expressed protein                                      | Cleavage        |
| Rhi-miR-190 | LOC_Os08g30660.1     | 2.5    | cDNA | NB-ARC domain containing protein, expressed           | Cleavage        |
| Rhi-miR-191 | LOC_Os12g02570.2     | 2.5    | cDNA | expressed protein                                      | Cleavage        |
| Rhi-miR-192 | LOC_Os12g02570.1     | 2.5    | cDNA | expressed protein                                      | Cleavage        |
### Table 4. Cont.

| milRNAs     | Target Gene Accession | Expect | Gene Description                                      | Inhibition  |
|-------------|-----------------------|--------|-------------------------------------------------------|-------------|
| Rhi-miR-20  | LOC_Os01g25430.1      | 2      | cDNA | expressed protein                                       | Cleavage    |
|             | LOC_Os07g48200.2      | 2.5    | cDNA | B3 DNA binding domain containing protein, putative, expressed | Cleavage    |
|             | LOC_Os07g48200.1      | 2.5    | cDNA | B3 DNA binding domain containing protein, putative, expressed | Cleavage    |
| Rhi-miR-22  | LOC_Os02g49350.1      | 2.5    | cDNA | plastocyanin-like domain containing protein, putative, expressed | Cleavage    |
| Rhi-miR-26  | LOC_Os03g55250.1      | 2.5    | cDNA | cytochrome P450 81E1, putative, expressed               | Cleavage    |
| Rhi-miR-43  | LOC_Os04g28160.1      | 3      | cDNA | response regulator receiver domain containing protein, expressed | Cleavage    |
| Rhi-miR-51  | LOC_Os04g34390.1      | 3      | cDNA | serine/threonine-protein kinase receptor precursor, putative, expressed | Cleavage    |
|             | LOC_Os10g26660.2      | 2.5    | cDNA | expressed protein                                       | Translation |
| Rhi-miR-61  | LOC_Os08g40620.1      | 2.5    | cDNA | rabGAP/TBC domain-containing protein, putative, expressed | Cleavage    |
|             | LOC_Os08g40620.2      | 2.5    | cDNA | rabGAP/TBC domain-containing protein, putative, expressed | Cleavage    |
| Rhi-miR-66  | LOC_Os05g11414.1      | 2.5    | cDNA | OsMADS58-MADS-box family gene with MIKCc type-box, expressed | Cleavage    |
| Rhi-miR-81  | LOC_Os05g50910.2      | 3      | cDNA | extra-large G-protein-related, putative, expressed      | Cleavage    |
|             | LOC_Os05g50910.1      | 3      | cDNA | extra-large G-protein-related, putative, expressed      | Cleavage    |
| Rhi-miR-89  | LOC_Os06g33020.1      | 2.5    | cDNA | retrotransposon protein, putative, Ty3-gypsy subclass, expressed | Cleavage    |
|             | LOC_Os06g32890.1      | 2.5    | cDNA | retrotransposon protein, putative, Ty3-gypsy subclass, expressed | Cleavage    |
| Rhi-miR-9   | LOC_Os02g35820.2      | 2.5    | cDNA | retrotransposon protein, putative, unclassified, expressed | Cleavage    |
|             | LOC_Os02g35820.1      | 2.5    | cDNA | retrotransposon protein, putative, unclassified, expressed | Cleavage    |
|             | LOC_Os07g04230.1      | 2.5    | cDNA | retrotransposon protein, putative, unclassified, expressed | Cleavage    |
| Rhi-miR-97  | LOC_Os07g47950.1      | 2.5    | cDNA | expressed protein                                       | Cleavage    |
| scaffold130_44481 | LOC_Os09g29390.1  | 2.5    | cDNA | plastocyanin-like domain containing protein, putative, expressed | Cleavage    |
| scaffold47_37559 | LOC_Os04g52164.1  | 2      | cDNA | transferase family protein, putative, expressed         | Cleavage    |
|             | LOC_Os04g52164.2      | 2      | cDNA | transferase family protein, putative, expressed         | Cleavage    |

#### 3.5. qRT-PCR Expression Analysis of milRNAs

Seventeen milRNAs (known and novel) were selected based on read count, target genes in *R. solani*, and also based on the target genes in rice. The relative expression of fungal encoded milRNAs during host (rice) infection was analyzed at different time points of infection, different age and genetic makeup of host tissue, and also in different strains of fungi. The number of expressed milRNAs varied in different experiments, therefore, only those milRNA were included which showed expression in at least one sample.
3.5.1. At Different Time Points of Inoculation

TN1, the susceptible rice cultivar, was inoculated with \textit{R. solani} to study the expression levels of fungal-encoded miRNAs at different time points of infection. While comparing with the expression of miRNAs in \textit{R. solani} grown in PDA medium, all the miRNAs except Rhi-miR-160 showed down-regulation during the rice infection at all time points. Furthermore, the miRNAs were differentially regulated at different time point of infection. Novel miRNAs scaffold130_44481 and scaffold47_37005, and known miRNAs Rhi-miR-35 and Rhi-miR-169 expressed at all time points, while scaffold7_15416 and Rhi-miR-160 expressed at all the time points except at 18 h. Among all the miRNAs analyzed, only Rhi-miR-160 showed up-regulation (at 48 h and 72 h time points). Scaffold1_2221 expressed at 48 h, 72 h, 96 h and 5d while scaffold47_37559 showed expression at 18 h, 24 h, and 5 days. Expression of Rhi-miR-22, Rhi-miR-43, Rhi-miR-120 and Rhi-miR-146 was observed exclusively at a specific time point of infection, i.e., 72 h, 5 days, 48 h and 5 days respectively. Notably, the Rhi-miR-43 showed down-regulation at 5 days. While analyzing the expression of miRNAs at different time points, most of the miRNAs showed expression at 5 days (Figure 2).

![Figure 2](image-url)
3.5.2. In Different Growth Stages of Host Plant

The expression of fungal encoded miRNAs was influenced by the growth stage of its host. All the miRNAs except Rhi-miR-16 showed down-regulation at both the growth stages during infection when compared with the expression level of miRNAs in fungi grown in PDA medium. Among the two growth stages, scaffold7_15416, Rhi-miR-160, and Rhi-miR-169 showed more expression at reproductive stage (80 days old) of host than vegetative (45 days old). Rhi-miR-43 showed down-regulation in a 45-day-old host and the expression levels further declined in an 80-day-old host. Interestingly, Rhi-miR-16 did not show expression in a 45-day-old host but was up-regulated in an 80-day-old host; whereas Rhi-miR-146 did not show expression in 80 days old host but was down-regulated in a 45-day-old host. Scaffold1_2221 and Rhi-miR-135 also showed stage specific expression, i.e., down-regulation in 45 and 80-day-old hosts, respectively. Scaffold7_15416, scaffold130_44481, scaffold47_37559, scaffold47_37005, Rhi-miR-43, Rhi-miR-35, Rhi-miR-160, and Rhi-miR-169 showed down-regulation in both 45-day-old and 80-day-old hosts (Figure 3).

**Figure 3.** qRT-PCR expression analysis of miRNAs in sheath tissue of 45 days and 80 days old TN1 plants after *R. solani* inoculation. X-axis: samples; Y-axis: Expression level of the individual miRNA relative to its expression in *R. solani* grown in PDA. Error bars indicate the mean ± standard error (SE) of three biological replicates.
3.5.3. In Different Rice Genotypes

Expression of fungal-encoded miRNAs was analysed during the infection of four different genotypes of rice, i.e., Tetep, BPT5204, Pankaj, and TN1. Forty-five day-old plants of these genotypes were inoculated with Wgl-2 strain of *R. solani* and samples were collected after 5 days of inoculation. All the miRNAs except Rhi-miR-160 showed down-regulation during infection of four rice genotypes when compared with the expression level of miRNAs in fungi grown in PDA medium. Seven miRNAs: scaffold7_15416, scaffold130_44481, scaffold47_37559, scaffold47_37005, Rhi-miR-35, Rhi-miR-160, and Rhi-miR-169 showed expression in all the genotypes. These miRNAs were down-regulated during infection of rice genotypes. Among these, expression of scaffold130_44481 was more in tolerant (Tetep and Pankaj) than the susceptible (TN1 and BPT5204) rice genotypes. Expression of scaffold47_37559 was greater in Pankaj than the other three genotypes while, Rhi-miR-169 expression was lesser in Pankaj than the other three genotypes. Rhi-miR-160 showed up-regulation in Tetep and BPT5204 but down-regulation in Pankaj and TN1. Rhi-miR-120 and Rhi-miR-135 showed exclusive expression in Pankaj while Rhi-miR-146 showed exclusive expression in TN1. Scaffold1_2221 expressed in all genotypes except BPT5204 (Figure 4).

![Figure 4. qRT-PCR expression analysis of miRNAs in different rice genotypes after *R. solani* inoculation. X-axis: samples (*R. solani* inoculated sheath tissue of four rice genotypes); Y-axis: Expression level of the individual miRNA relative to its expression in *R. solani* grown in PDA. Error bars indicate the mean ± S.E. of three biological replicates.](image-url)
3.5.4. In Different Strains of Fungus

Expression of miRNAs of four different fungal strains was analysed during the infection of the TN1 rice genotype. All the miRNAs except scaffold1_2221 showed down-regulation during infection in all fungal strains when compared with the expression level of respective miRNAs in fungi grown in PDA medium. Novel miRNAs scaffold7_15416, scaffold1_2221, scaffold130_44481, scaffold47_3559, scaffold47_37005 and known miRNAs Rhi-miR-35, Rhi-miR-160 and Rhi-miR-169 showed expression in all the four strains of fungi while infecting its host. Among these, scaffold7_15416, scaffold1_2221, scaffold47_35559, and Rhi-miR-160 showed more expression in Imph-2 strain than three other fungal strains. Furthermore, scaffold47_35943, scaffold47_36931, Rhi-miR-43, Rhi-miR-57, and Rhi-miR-135 showed expression only in Imph-2 strain while infecting TN1. Notably, scaffold1_2221 was up-regulated in Imph-2 strain but down-regulated in the other three strains. Rhi-miR-16 and Rhi-miR-146 showed exclusive expression in Chn-1 and Wgl-2 strains, respectively. Rhi-miR-120 did not show expression in the Wgl-2 strain (Figure 5).

Figure 5. qRT-PCR expression analysis of miRNAs in sheath tissue of TN1 after inoculation with different strains of *R. solani*. X-axis: samples (sheath tissue of TN1 inoculated with four different strains of *R. solani*); Y-axis: Expression level of the individual miRNA relative to its expression in *R. solani* grown in PDA. Error bars indicate the mean ± S.E.
3.5.5. Expression Analysis of Fungal and Plant Target Genes

Expression of seven plant target genes and two fungal target genes was analysed in *R. solani*-infected rice (TN1) tissue. Along with the target genes, the expression of the respective regulatory miRNA was also analysed. Among the plant target genes, the regulons Osa-KANADI1:Rhi-miR-124, Osa-vacuolar-sorting receptor precursor:Rhi-miR-13, Osa-plastocyanin-like domain containing protein:Rhi-miR-22, and Osa-serine/threonine-protein kinase receptor precursor: Rhi-miR-51 showed anticipated reciprocal trend of expression. In these cases, the fungal encoded miRNAs and their respective plant target genes showed opposite trend of expression during the disease development (Figure 6a). Similarly, fungal genes AGA1IA_02889 and AGA1IA_07743 and their regulatory miRNAs Rhi-miR-141 and Rhi-miR-120 showed a contrasting expression pattern in infected tissue of rice (Figure 6b).

4. Discussion

MicroRNAs are regulatory genes that play critical roles in cellular processes of almost all eukaryotes [14]. It was believed that fungi do not possess microRNAs until the discovery of miRNA-like small RNAs (miRNAs) produced through at least four different biogenesis pathways in model fungi *N. crassa* [6,32]. Compared to plants and animals, there are very limited reports on miRNAs of phytopathogenic fungi which are primary agents causing diseases and huge loss to agriculture crops. In the absence of resistance in the rice germplasm against *R. solani*, it is extremely important to decipher the gene regulatory networks and biology of pathogens to devise effective strategies for controlling the menace of sheath blight disease in rice [31,33–35]. We analyzed the miRNAs of sheath blight disease causing pathogen *R. solani* AG1 IA by RNAseq of small RNAs. A total of 128 known and 22 novel miRNAs were identified. Most of the miRNAs showed low abundance which is accordance with earlier study of fungal miRNAs [36]. Notably, known miRNAs-Rhi-miR-16, Rhi-miR-22, Rhi-miR-35, Rhi-miR-57, Rhi-miR-91, and Rhi-miR-112 and novel miRNAs-scaffold13_22000, scaffold47_37005, scaffold47_37523, scaffold47_37559, scaffold47_37341, scaffold47_37407, and scaffold47_36418 showed more abundance than other microRNAs suggesting their significant role in fungal gene regulation. The novel miRNA scaffold13_22000 showed >6000 read count which emphasizes that several novel players identified in this study might regulate fungal genome. Earlier studies
suggested that such novel miRNAs may have crucial roles in pathogenesis and virulence of *R. solani* [21,25]. 1725 target genes of 128 known and 22 novel miRNAs were identified that are involved in various metabolic and developmental processes of fungi indicating that miRNAs are major regulators of the *R. solani* genome. The role of miRNAs in fungal growth and development has been recently described in *Sclerotinia sclerotiorum* [37]. A number of miRNAs were identified in the soil-borne fungal pathogen *Verticillium dahlia* causing wilt diseases in several agriculture crops. One of the miRNAs (VdmilRNA1) was demonstrated to mediate epigenetic repression of a virulence gene (VdHy1) in pathogenic fungi [38]. Furthermore, the role of miRNAs (CmmiR4 and CmliR16) in fruiting body development was demonstrated in *Cordyceps militaris* [39]. The miRNAs-Rhi-miR-141, Rhi-miR-120, Rhi-miR-41, and Rhi-miR-91 target pectinesterase and pectate lyase domain-containing protein genes signifying their crucial role in plant cell wall degradation and pathogenesis. miRNAs of *R. solani* targeting carbohydrate active enzymes and other fungal genes including those involved in virulence and pathogen–host interaction were reported recently [25]. The genes encoding plant cell wall degrading enzymes of *R. solani* play significant role in disease initiation and establishment [4]. Vm-miR16 of *Valsamali* regulates the expression of pectinase genes during *V. Mali*–host interaction [40].

Interestingly, *R. solani* miRNAs showed their targets in the host (rice) genome also. A similar observation was made on *R. solani* targeting maize genes [25]. Recently, cross kingdom regulation of genes by miRNAs during host–pathogen interaction has been demonstrated in several pathosystems [41]. Bidirectional cross-kingdom transport of microRNAs or small RNAs through naked form, combined with RNA-binding proteins or enclosed by vesicles between fungal pathogens and its host plants has been reported [42]. In an interesting observation, Cui et al. (2019) [43] demonstrated that bba-miR1 encoded by pathogenic fungus *Beauveria bassiana* hijacks the RNA-interference machinery of a host (mosquito) to attenuate host immunity and facilitate infection. The fungus *Sclerotinia sclerotiorum* was reported to produce at least 374 distinct highly abundant sRNAs during infection of *Arabidopsis thaliana* and *Phaseolus vulgaris*. Targets of these small RNAs were significantly down-regulated during infection in *A. thaliana* [44].

Rhi-miR-124 targets Osa-KANADI1. In *Arabidopsis*, KANADI1 acts as a transcriptional repressor of genes involved in auxin biosynthesis, auxin transport, and auxin response [45]. Fungal pathogens control the auxin levels and auxin signalling pathways that significantly influence the defense network in plants [46]. In the wheat–*Zymoseptoria tritici* pathosystem, Ma et al. [47] showed that as an immune response, the fungal infection induced wheat small RNAs that regulate auxin-related genes. Rhi-miR-13 regulates Osa-vacuolar-sorting receptor (VSR) of rice which is responsible for targeting defense-related soluble proteins to the vacuole. Plant vacuoles play an important role in defense by releasing hydrolytic enzymes and antimicrobial compounds upon pathogen infection that leads to programmed cell death due to hypersensitive response [48]. Suppression of rice VSR by *R. solani* miRNA might suppress the plant defense by inhibiting the hypersensitive response. Notably, the target probability in the case of Osa-vacuolar-sorting receptor precursorRhi-miR-13 is very high with expected value of 1.0. Therefore, further probing of this miRNA-target gene regulation network can add significant information on host-pathogen molecular cross-talk. Rhi-miR-131 targets nuclear transcription factor Y (NFY) subunit of rice which is also regulated by the host miRNA, i.e., Osa-miR169. The over-expression of Osa-miR169 in rice led to repression of NFY resulting in hypersusceptibility of transgenic rice against blast fungus *Magnaporthe oryzae* [49]. Repression of NFY by Rhi-miR-131 may weaken the defense response of rice against *R. solani*. Other host target genes of fungal miRNAs included isoflavone reductase, OsFBX438-F-box domain containing protein, NB-ARC domain containing protein, serine/threonine-protein kinase receptor, and rabGAP/TBC domain-containing protein which are involved in plant defense against pathogens [50–54]. *R. solani*-encoded miRNAs target maize defense genes during infection and negatively regulate resistance of maize [25]. Notably, the regulons such as Osa-KANADII:Rhi-miR-124, Osa-vacuolar-sorting receptor precursor:Rhi-miR-13,
Osa-plastocyanin-like domain containing protein: Rhi-miR-22, and Osa-serine/threonine-protein kinase receptor precursor: Rhi-miR-51 were verified by analysing their expression through qRT-PCR during host–fungal interaction and disease development. Recent reports demonstrate a role of cross-kingdom RNAi in regulation of genes associated with pathogen virulence and host resistance. For example, small RNAs of phytopathogenic fungi *Botrytis cinerea* trigger the silencing of immunity-related genes of its hosts *A. Thaliana* and *Solanium lycopersicum* [55]. A similar observation has also recently been reported in the case of *Puccinia triticina* and *P. striiformis* [56,57]. In addition to plant defense genes, fungal miRNAs showed their target as genes associated with growth and the development of the host.

This study provides a first comprehensive analysis of miRNAs expression during pathogen infection as the expression of 17 miRNAs was analysed at different time points of infection, at different growth stages of the host, in different genotypes (with different genetic make-up) of host, and also in different strains of fungi. The down-regulation of all miRNAs except Rhi-miR-160 was noticed at all the time points of infection suggesting the induction of their target genes during the disease initiation and establishment. The differential regulation of miRNAs at different time points of infection suggests that fungal genes are regulated by miRNAs during disease initiation and progression. Scaffold130_44481, scaffold47_37005, Rhi-miR-35, and Rhi-miR-169 showed expression throughout all time points of infection indicating their central role in infection. Expression of Rhi-miR-22, Rhi-miR-43, Rhi-miR-120, and Rhi-miR-146 was observed specifically at 72 h, 5d, 48 h, and 5d respectively, suggesting that fungi recruit specific miRNAs as the infection progresses. During the infection and disease progression, *R. solani* requires mycotoxin production and penetration of mycelium inside the host cells. Studies in *Aspergillus flavus* and *Fusarium oxysporum* f. sp. *Niveum* highlighted the significant roles of miRNAs in the mycelium growth and mycotoxin biosynthesis [22,58]. Lin et al. (2016) [21] analysed the differential expression pattern of *R. solani* miRNAs during infection and suggested their roles in disease initiation and establishment [58]. Differential regulation of miRNAs during sclerotial development in *Sclerotinia sclerotiorum* was observed by analyzing small RNAs at different time points [59].

At the time of infection, expression of scaffold7_15416, Rhi-miR-160, and Rhi-miR-169 was more at the reproductive stage than vegetative stage of rice while Rhi-miR-16 did not show expression in vegetative stage but was up-regulated in the reproductive stage. By contrast, Rhi-miR-146 was down-regulated in the vegetative stage but no expression was observed in the reproductive stage. These results suggest that growth stage of host plant influences the miRNAs expression of fungi. Scaffold1_2221 was down-regulated in the vegetative stage while Rhi-miR-135 was down-regulated in reproductive stage, indicating their specific role during pathogenesis of different-aged hosts. Scaffold7_15416, scaffold130_44481, scaffold47_37559, scaffold47_37005, Rhi-miR-43, Rhi-miR-35, Rhi-miR-160, and Rhi-miR-169 were down-regulated at both the growth stages signifying their essential roles during infection.

Expression of seven-miRNAs scaffold7_15416, scaffold130_44481, scaffold47_37559, scaffold47_37005, Rhi-miR-35, Rhi-miR-160, and Rhi-miR-169 was down-regulated while infecting the four rice genotypes suggesting their key roles in regulating the genes during infection. Expression of scaffold130_44481 and scaffold47_37559 was greater in tolerant genotypes than susceptible rice genotypes while Rhi-miR-120 and Rhi-miR-135 showed exclusive expression in Pankaj indicating that regulation by miRNAs is significantly influenced by host genetic architecture. Expression of other miRNAs also varied in different genotypes of its host.

Expression of scaffold7_15416, scaffold1_2221, scaffold130_44481, scaffold47_3559, scaffold47_37005, Rhi-miR-35, Rhi-miR-160, and Rhi-miR-169 was observed in all the four strains of fungi while infecting its host suggesting their primary role in infection of the host. Notably, expression of scaffold7_15416, scaffold1_2221, scaffold47_37559, and Rhi-miR-160 was greater in Imph-2 strain while scaffold47_35943, scaffold47_36931,
Rhi-miR-43, Rhi-miR-57, and Rhi-miR-135 were exclusively expressed in Imph-2 strain indicating that different strains of a fungus have evolved differential miRNA mediated gene regulation. Imph-2 and Chn-1 are mild strains of *R. solani* [26]. Rh-miR-16 showed exclusive expression in Chn-1 while Rhi-miR-146 showed exclusive expression in the Wgl-2 strain. Expression pattern of miRNAs in this study indicate that miRNAs may play critical role in determining the virulence of fungal strains. *Vm*-miR16 of the phytopathogenic fungus *V. mali* regulates the expression of virulence genes [40].

Fungal genome-encoded microRNA-like RNAs are relatively less explored genetic elements that play a major role in regulation and expression of genes associated with growth, development, pathogenesis, and virulence. We made a comprehensive effort by combining RNAseq and qRT-PCR assays to decipher the miRNAs of an Indian strain of *R. solani* AG1-1A causing sheath blight disease of rice. Several miRNA candidates targeting key genes of *R. solani* were identified which need to be further characterized to understand their specific biological roles. The novel miRNAs are significant additions to understanding of the complexity of gene regulation of *R. solani*. The novel miRNA scaffold13_22000 showing very high expression needs to be characterized for its role in pathogen–host interaction and fungal metabolism. Further probing the regulation of rice genes by fungal miRNAs will be a milestone in understanding the molecular basis of host–pathogen interaction. Some of the gene regulatory networks such as Osa-KANADI1:Rhi-miR-124, Osa-vacuolar-sorting receptor precursor:Rhi-miR-13, and Osa-nuclear transcription factor Y:Rhi-miR-131 can be studied on priority. Also, the better understanding regulation of rice defense genes by *R. solani* miRNAs may help in executing more effective strategies against this deadly plant pathogen. Detailed expression analysis of miRNAs at different time points of infection, at different growth stages of hosts, in different genotypes of hosts, and in different strains of fungi provide ample information to plan future studies for understanding the gene regulation mechanism of *R. solani*. Our results indicate that miRNAs may contribute to almost all the metabolic processes of the *R. solani* pathogen.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/jof7070561/s1, Table S1: List of primers used in this study for qRT-PCR assays. Table S2: List of known miRNAs identified in this study. Table S3: List of target genes of known and novel miRNAs. Table S4: Gene ontology analysis of selected target genes.

**Author Contributions:** Conceived and designed the experiments: S.K.M. Performed the experiments: N.B.P. and S.K.M. Analysis of data: S.K.M., N.B.P., S.K.S., S.N. Wrote the article: S.K.M., N.B.P., S.M.B., C.V.D.R., R.M.S. Provided the resources: V.P., Y.C.M. and R.M.S. All authors have read and agreed to the published version of the manuscript.

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