Physiol Mol Biol Plants (March 2021) 27(3):633–647
https://doi.org/10.1007/s12298-021-00960-0

Deciphering the role of microRNAs during *Pi54* gene mediated *Magnaporthe oryzae* resistance response in rice

Kirti Arora[1,2] · Amit Kumar Rai[1] · B. N. Devanna[1,3] · Himanshu Dubey[1] · Alka Narula[2] · Tilak Raj Sharma[1,4]

Received: 27 January 2021 / Revised: 14 February 2021 / Accepted: 15 February 2021 / Published online: 10 March 2021

Abstract The broad-spectrum resistance gene *Pi54* confers resistance to multiple isolates of *Magnaporthe oryzae* in rice. In order to decipher the molecular mechanism underlying the *Pi54* mediated resistance in rice line Taipei309^{Pi54} (carrying *Pi54*), miRNAome study was performed at 24 h post-inoculation (hpi) with *M. oryzae*. A total of 222 known miRNAs representing 101 miRNA families were found in this study. Of these, 29 and 24 miRNAs were respectively up- and down-regulated in the resistant Taipei309^{Pi54}. Defence response (*DR*) genes, like, *NBSGO35*, and *OsWAK129b*, and genes related to transcription factors were up-regulated in Taipei309^{Pi54} line. The vast array of miRNA candidates identified here are miR159c, miR167c, miR2100, miR2118o, miR2118l, miR319a, miR393, miR395l, miR397a, miR397b, miR398, miR439g, miR531b, miR812f, and miR815c, and they manifest their role in balancing the interplay between various *DR* genes during *Pi54* mediated resistance. We also validated miRNA/target gene pairs involved in hormone signalling, and cross-talk among hormone pathways regulating the rice immunity. This study suggests that the *Pi54* gene mediated blast resistance is influenced by several microRNAs through PTI and ETI components in the rice line Taipei309^{Pi54}, leading to incompatible host–pathogen interaction.

Keywords *Magnaporthe oryzae* · MicroRNA · Rice (*Oryza sativa*) · Defence · Immunity · Blast disease

Abbreviations

hpi	Hours post inoculation
dpi	Days post inoculation
PTI	Pathogen Triggered Immunity
ETI	Effector Triggered Immunity

Introduction

The surveillance system of plants is activated largely to defend themselves by engaging in 'arms race' with the invading pathogens. Plants have evolved multiple strategies to survive and deal with the attack by diverse pests and diseases, and resistance (*R*) gene mediated host defence is one of these strategies (Abramovitch et al. 2006; Yan and Talbot 2016). The basal resistance involves passive structural barriers (Nurnberger et al. 2004), whereas the resistance response mediated by defence related (DR) proteins employs a set of transmembrane, pattern recognition receptors (PRRs) against invading pathogens. PRRs perceive and bind to the conserved pathogen associated molecular patterns (PAMPs). But PAMP triggered immunity (PTI) also does not provide complete resistance, and pathogens do evade PTI by undergoing structural and chemical modifications. The next level of plant defence, effector triggered immunity (ETI) is more specifically

✉ Tilak Raj Sharma
trsharma1965@gmail.com

[1] ICAR-National Institute for Plant Biotechnology, Pusa Campus, New Delhi 110012, India

[2] Department of Biotechnology, Jamia Hamdard, New Delhi 110062, India

[3] ICAR-National Rice Research Institute, Cuttack 753006, India

[4] Division of Crop Science, Indian Council of Agricultural Research, Krishi Bhavan, New Delhi 110 001, India

evolved and robust, triggered by pathogen effector proteins and perceived by plant R proteins (Stuckenbrock and McDonald 2009). The immunity developed through ETI largely manifests as the hypersensitive response (HR) leading to the localization of pathogen, and thereby preventing its spread (Jones and Dangl 2006).

Resistance genes are ancient and conserved across different plant species (Zhang et al. 2014). The *R* genes are very conveniently used in crop improvement programmes for disease management thus reducing the application of pesticides. Rice blast is threatening the global food security which causes a yield loss of 10–30%, and under favourable conditions for *Magnaporthe oryzae*, the losses could be up to 80% of potential yield (Skamnioti and Gurr 2009; Faivre-Rampant et al. 2011). It is of paramount importance to sustain the increasing rice production in order to meet the requirement of continually growing human population (Ray et al. 2013). Therefore, the more sustainable solution to the management of blast disease is deployment of *R* genes, which detect proteins produced by the *M. oryzae*. In rice, more than 100 blast *R* genes have been identified and mapped to its genome, and 27 of these genes have been cloned and validated for their function (Sharma et al. 2012; Ma et al. 2015; Devanna and Sharma 2018). Twenty of these *R* genes are of Nucleotide binding site- Leucine Rice Regions (NBS-LRR) category, while, *Pi54* and its orthologues (*Pi54rh* and *Pi54of*) have rudimentary NBS and small LRR domains; and *Pi-d2* and *Pi21* code for non NBS-LRR proteins (Das et al. 2012; Devanna et al. 2014). The *Pi54* gene confers resistance to many predominant strains of blast pathogen from across India, and it was cloned from Indica rice cv. Tetep (Sharma et al. 2005a; Rai et al. 2015). Further studies revealed that *Pi54* activates a multifaceted defence response in rice (Gupta et al. 2012a). The *Pi54rh* and *Pi54of* genes respectively from wild rice species *O. rhizomatis* and *O. officinalis* show variable and overlapping resistance spectrum for blast disease (Das et al. 2012; Devanna et al. 2014). Chromosomal site of integration of the *Pi54* in transgenic rice lines was also elucidated and found that no gene with significant agronomic importance was affected (Arora et al. 2015). Recently, the subcellular localization of Pi54 was analysed and, the avirulence counterpart; *Avr-Pi54* has also been cloned and characterized to establish the physical interaction between Pi54 and Avr-Pi54 proteins during ETI (Ray et al. 2016; Singh et al. 2020). During the incompatible interaction, the Pi54 protein is able to perceive AvrPi54 of *M. oryzae* and bind to it with different domains, leading to resistance response as recorded for Taipei309^{Pi54} (Rai et al., 2011). However, a mutant *AvrPi54* gene code for a modified AvrPi54 which evades scanning from Pi54 protein and leads compatible interaction, and susceptibility as observed in Taipei309 (Rai et al., 2011; Devanna et al., 2014). The broad-spectrum nature of *Pi54* has also been studied in the rice lines pyramided with *xa13* and *Xa21* genes (Singh et al. 2012). *Pi54* is being utilized along with various other disease resistance genes to develop rice lines with broad-spectrum resistance against multiple pathogens (Sharma et al. 2012). Deployment of race-specific *R*- genes is a major strategy for disease control but these genes are most prone to rapid evolution of pathogen *avr*-genes (Fukuoka et al. 2009). Therefore, an extensive understanding of the mechanism of *Pi54* mediated resistance is important for deploying this gene in rice for durable blast-resistant.

Small RNAs (siRNAs ~ 21–26 nucleotides) are ubiquitously present in plants, animals and fungi. These RNAs induce silencing of target genes through homologogy dependent interactions (Finnegan and Matzke 2003; Brant et al. 2018). They are non-coding RNAs, regulate the target gene expression by regulating pattern of chromatin methylation, mRNA degradation or translational repression (Baulcombe 2004). Characterization of host endogenous siRNAs involved in plant stress-responses is crucial for target-gene expression studies (Ruiz-Ferrer and Vionnet 2009;; Katiyar-Agarwal and Jin 2010). In plants it has also been reported that siRNAs fine-tune the PTI and ETI signalling (Padmanabhan et al. 2009; Li et al. 2017).

An indication that miRNAs regulate pathogen resistance response was first reported in the Arabidopsis plants, where flagellin flg22 from *Pseudomonas syringae pv.* tomato (*Pst*) induced an increased miR393 accumulation (Navarro et al. 2006). miR393 negatively affects the transcripts of F-box auxin receptors, thereby act in PTI signalling (Navarro et al. 2006). miRNAs also regulate plant hormone biosynthesis and signalling pathways, by regulating and fine-tuning these pathways, and thereby ETI signalling (Zhang et al. 2011). They have also been known to be part of resistance development pathways against *M. oryzae* (Li et al. 2016, 2019; Fei et al. 2016). The novel rice miRNA, miR7695 is found responsible for blast disease resistance by negatively regulating the *OsNramp6* (Natural resistance-associated macrophage protein 6) transcript. This showed the existence of regulatory network that integrates miRNA function with mRNA processing in the plant immunity (Campo et al. 2013). A set of known rice miRNAs expressed differentially upon *M. oryzae* infection were identified, and overexpression of even one of them in rice plants could enhance resistance to the *M. oryzae* (Li et al. 2014). Ectopic expression of even one of these miRNAs was sufficient to provide resistance to rice blast pathogen (Li et al. 2014). The conserved function of miRNAs and secondary siRNAs is clearly demonstrated in NB-LRR/LRR mediated ETI response in *Leguminosae* and *Solanaceae* species (Zhai et al. 2011; Shivaprasad et al. 2012). miRNAs are also reported to cleave the target *R* genes in plants (Baldrich et al. 2015), recently an apple

Md-miRLn11 was found to target NBS–LRR gene responsible for resistance against leaf spot disease in apple (Ma et al. 2014).

The miRNA database, miRBase lists over 592 precursors, and 713 mature [MSU7] miRNA sequences in rice (http://www.miRbase.org; Kozomara and Griffiths-Jones 2011). However, how do they precisely correlate with the functioning of resistance gene is not entirely understood. In this line, miRNAs associated to blast resistant in *Pi54- M. oryzae* pathosystem remains to be identified. In the present study our objectives were (1) to identify a group of known rice miRNA that were differentially expressed upon *M. oryzae* infection in resistant plants using sequencing based miRNAome analysis, (2) to identify and validate the potential miRNAs and their target genes involved in *Pi54* mediated blast resistance and (3) to establish inverse relationship of miRNA genes with their target genes in terms of expression.

Materials and methods

Plant materials, pathogen culture and experimental design

The japonica rice line (*O. sativa* ssp. Japonica) Taipei 309 (susceptible) and its near monogenic blast resistant rice line having *Pi54*; TP-*Pi54*-2 (Taipei309^{Pi54}) in T6 generation were grown and maintained in net house at a relative humidity (RH) of 70%, and 28 ± 2 °C temperature under 16/8 h light/dark regimen. *Magnaporthe oryzae* strain Moni-026 (PB-1) was used for phenotyping. Highly resistant, *O. sativa* ssp. Indica cv. Tetep and highly susceptible indica rice cv. HR12 lines were used as resistant and susceptible control, respectively for blast phenotyping.

Magnaporthe oryzae inoculation

Magnaporthe oryzae strain PB-1 was subcultured on potato dextrose agar medium (Difco-BD, NJ, USA) (39 g/l) and incubated for 15 days at 25 ± 2 °C under blue-white fluorescence light. For better sporulation fungus culture was transferred to Mathur's medium prepared in Petri plates and incubated at 25 °C under constant illumination of white fluorescent light for 7 days (55 µF/Em/s). Standard protocol for phenotyping was followed (Bonman et al. 1992). For validation of microRNAs and to identify microRNAs responsible for the blast resistance, Taipei309^{Pi54} (resistant) and Taipei309 (susceptible) rice lines were grown and maintained in triplicates along with Tetep (resistant control) and HR12 (susceptible control). A 50–60 ml of spore suspension (~ 10^6 conidia/ml) prepared in tween-20 (0.02%) and gelatin (0.1%) was spray inoculated using an atomizer on 21 days old seedlings kept in humidity chamber with 90% RH at 25 ± 2 °C. The 4-inch pots containing inoculated seedlings were kept in dark for initial 24 h and later shifted 16/8 h light/dark regimen for better infection. Leaf samples were collected in duplicate at 24 hpi; one set was used for deep transcriptome sequencing and another one kept for experimental validation. For another biological replication, disease reaction was scored and recorded at 7 days post-inoculation (DPI) (Fig. 1) using a 0–5 disease rating scale (Mackill 1992).

Small RNA library preparation and ABI SOLiD sequencing

Total RNA was isolated using purelink miRNA isolation kit (Invitrogen). The quality and quantity of RNA was analyzed on denaturing agarose gel and Nanodrop spectrophotometer. RNA integrity number was also assessed on Bioanalyzer RNA 6000 Pico chip (Agilent 2100). Enrichment of siRNA from the total RNA was carried out using Ambion flash page. Deep sequencing was performed via Applied Biosystems SOLiD (4.0) (Xcelris Genomics Pvt. Ltd., Ahmedabad, India). Four small RNA libraries were prepared using Total RNA seq kit and quality was analyzed using DNA HS chip on Bioanalyzer. Template bead preparation was carried out to generate the P2 positive beads for sequencing on SOLiD platform. Quantification of beads was done using Nanodrop spectrophotometer and workflow analysis was carried out to confirm the quality of beads. miRNA sequencing was carried out using fragment library sequencing kit of 35 bp read length. The small RNA reads (18–33 nt length) were mapped to the rice (nuclear, chloroplast, and mitochondrial); http:// rice.plantbiology.msu.edu/; version 6.0) and *M. oryzae* (http://www.broadinstitute.org) genomes using CLC genomics workbench version 7.0.

Data processing and Bioinformatics analysis

Raw reads were generated from all the four rice libraries. Reads mapped to tRNAs, rRNAs were filtered out and reads obtained after filtration were mapped against precursor miRNAs using *O. sativa* GFF file downloaded from miRBase sequence database release 16. FPKM analysis was performed for differential expression profiling of mature miRNAs (p-value ≤ 0.05). FPKM values were used to calculate the fold change as *ln* (FPKM_resistant gelatin treated sample/FPKM_resistant fungus treated sample) for resistant line, where *ln* is log2 and similarly for susceptible rice line. Based on the chi-square test derived p-values, the statistically significant (p-value ≤ 0.05) miRNAs were identified and classified as up-regulated

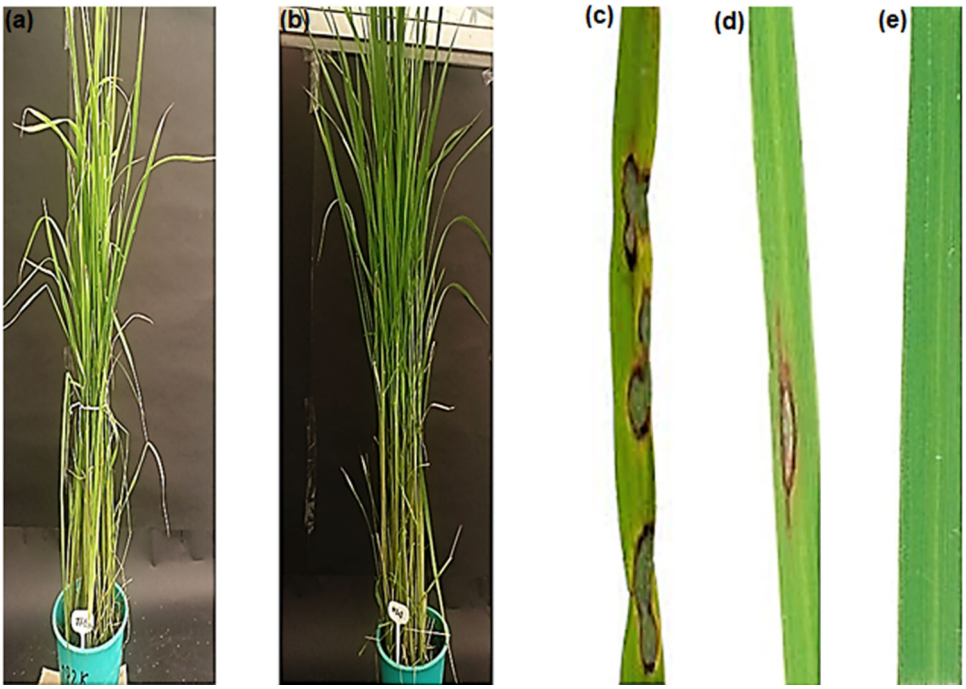

Fig. 1 Resistant and Susceptible rice plants used in this study along with their disease phenotypes. **a** Taipei309^{Pi54} rice plant (resistant). **b** Taipei309^{-} rice plant (susceptible). **c** Disease reaction on HR12 rice plant (positive control). **d** Disease reaction on Taipei309^{-} rice plant. **e** Disease reaction on Taipei309^{Pi54} rice plant

(fold change > 2.0) and down-regulated (fold change $<$ 2.0) categories. Remaining reads were mapped to rice genome using miRanalyzer (minimum read count ≥ 10) and filtered using Xcelris proprietary script and then filtered again using miRcheck script and novel microRNAs were predicted. All the miRNAs available were BLAST searched against miRBase (www.miRbase.org/) and annotated, and various analyses like GC scatter plot, Interproscan etc. were performed to reveal hypothetical and unknown genes. The set of miRNAs specifically induced in resistant line by *M. oryzae* was derived after first removing the common set of miRNAs between gelatin treated susceptible and resistant lines as well as pathogen treated susceptible and resistant lines.

Target prediction

Target prediction for selected rice miRNAs was done using psRNA Target (http://bioinfo3.noble.org/psRNATarget). The miRNA target sequences were searched with the *O. sativa* cDNA set available at the Institute for Genomic Research (TIGR, Rice Annotation Release 5.0). Default parameters were used for target gene search (score or maximum expectation at 3.0, target accessibility at 25, length of complementary scoring at 20). All the predicted target genes were evaluated by scoring as; each G:U wobble pairing 0.5 points, each indel 2.0 points and all other non-canonical Watson–Crick pairings 1.0 point. Those sequences with a total score of less than 3.0 points were considered as miRNA targets.

Validation of miRNAs and target genes by qRT-PCR

Total RNA was isolated from leaf samples, as described previously, and treated with DNAseI, reverse transcribed using oligodT primer, reverse transcriptase and deoxynucleotide triphosphatase (Qiagen, Hilden, Germany). For miRNA validation, reverse transcription of RNA to cDNA was performed using SuperScript first-strand synthesis system (Invitrogen, Carlsbad, U.S.A.). miRNAs were extended and reverse transcribed using the NCode miRNA first-strand cDNA system (Invitrogen). SYBR Green mix (Roche, Switzerland) was used in quantitative real-time PCR (Light cycler II 480) to determine the mRNA abundance. Analysis was performed using 12 independent cDNA preparations and triplicate PCR reactions. The qRT-PCR cycling conditions were: initial amplification for 1 min at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Gene expression levels was normalized by using internal control genes, U6 and 18s rRNA. Relative expression ratio was calculated using $2^{-\Delta\Delta Ct}$ with *U6* as internal control for miRNA validation and *18S rRNA* for validation of target genes. Primers used in real time qRT-PCR are listed in Table S1 and S2. All these experiments were performed in triplicate, and the results are represented in mean ± standard deviation (s.d.).

Results

The miRNAome profiling of blast resistant (Taipei309^{Pi54}) and blast susceptible (Taipei309) rice lines was studied at 24 hpi to understand the role of these miRNAs in *Pi54* mediated blast disease resistance in rice. After 7 DPI with *M. oryzae* (PB-1), blast disease resistant rice line Taipei309^{Pi54} plants showed typical hypersensitive response (HR) on the leaves while susceptible Taipei309 and HR12 plants showed typical leaf blast symptoms (Fig. 1). No disease symptoms were observed on positive control i.e. Tetep plants.

Deep sequencing of small RNAs of *M. oryzae* treated and untreated rice leaves

To identify miRNAs involved in the *Pi54* mediated rice immunity against blast pathogen, 21 days old plants were pathogen inoculated and leaf samples of Taipei309 and Taipei309^{Pi54} were harvested 24 hpi. Four small RNA libraries constructed using the small RNA isolated from leaves were subjected to deep sequencing (SoLiD 4.0). Quality check for library preparation, and size selection of cDNAs was done using Bioanalyzer and RNA integrity number (Figure S2, S3, S4). High quality sequence reads were obtained from all the samples (Table S3). Computational analysis showed that maximum ($\sim$ 33%) sequence reads from resistant samples were mapped to rice genome as compared to susceptible samples. Reads mapped to protein coding genes, rice mitochondria and chloroplast was found highest in the fungus inoculated resistant plant samples (Table S4). Reads mapped to *M. oryzae* genome in mock inoculated resistant and susceptible samples were same i.e. 0.48%, while in resistant and susceptible pathogen treated sample, 0.35% and 0.54% reads, respectively were mapped to *M. oryzae* genome (Figure S5a). Reads mapped to different non-coding RNAs like tRNA, rRNA, small nuclear and nucleolar RNAs are given in Figure S5b.

The mature miRNA sequence coordinates, present in the precursor miRNAs for all the four small RNA libraries were prepared for treated and untreated samples. Through analogy search of sequencing data using the central miRBase registry, we identified 222 known miRNAs or miRNAs* representing 101 miRNA families from the rice libraries. Highest number of miRNAs were found on chromosome number 1 followed by chromosomes number 4, 2 and 8 in resistant rice line with microRNAs number 18, 18, 15 and 15, respectively. While in susceptible line highest number of miRNAs were found on chromosome number 1, 2 and 4 with microRNAs number 20, 18 and 14, respectively (Figure S6a). Large number of miRNAs were 20 nucleotides long, followed by 23 nucleotides long in both resistant and susceptible rice lines. However, 24 nucleotides long miRNA, miR1870 was also found in the resistant line (Figure S6b).

Identification of candidate microRNAs involved in Rice-*M. oryzae* interaction

For expression profiling we used two sets, resistant (Taipei309^{Pi54}) and susceptible (Taipei309) rice lines. Of the 222 miRNAs, 53 (23.9%) and 51 (23%) miRNAs were unique to the resistant and susceptible line, respectively, and 118 miRNAs (53.2%) were common to both (Fig. 2a; Table S4). Out of 118 common miRNAs, 63 were common to resistant up-regulated and susceptible down-regulated, 26 miRNAs were common to resistant down-regulated and susceptible up-regulated (Fig. 2b). Expression profiling of

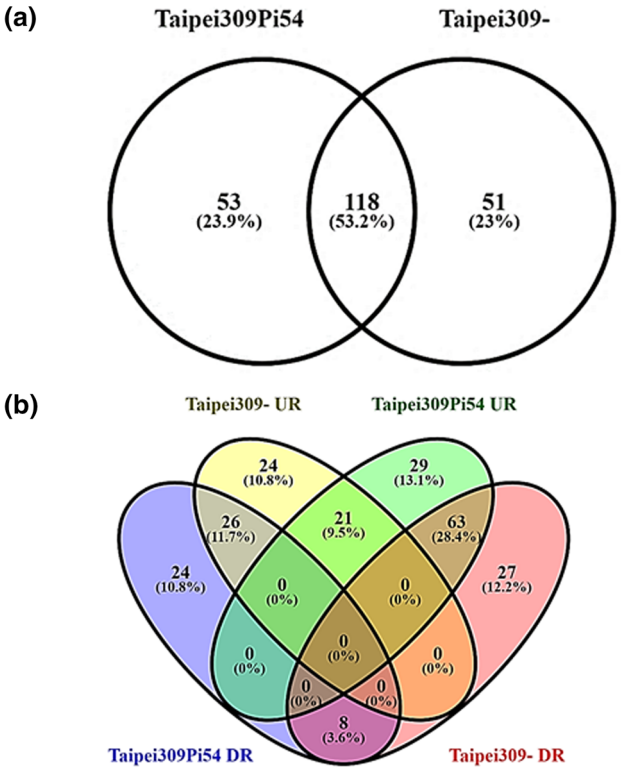

Fig. 2 Venn diagram showing overall differential regulation in Taipei309^{Pi54} (resistant) and Taipei309^{-} (susceptible) rice lines. **a** Unique miRNAs in resistant and susceptible line. **b** Common & Unique miRNAs in Taipei309^{Pi54} UR (Resistant upregulated), Taipei309^{Pi54} DR (Resistant downregulated), Taipei309^{-} UR (Susceptible upregulated), and Taipei309^{-} DR (Susceptible downregulated). 29 (13.1%) miRNAs are unique to Taipei309^{Pi54} UR, 24 (10.8%) miRNAs are unique to Taipei309^{Pi54} DR, 24 (10.8%) miRNAs are unique to Taipei309^{-} UR, 27 (12.2%) are unique to Taipei309^{-} DR. 26 (11.7%) miRNAs are common between Taipei309^{Pi54} DR & Taipei309^{-} UR. 63 (28.4%) miRNAs are common to Taipei309^{Pi54} UR & Taipei309^{-} DR, 21 (9.5%) miRNAs are common to Taipei309^{Pi54} UR & Taipei309^{-} UR. In central cluster, no miRNA is common to all the 4 categories

these common miRNAs are shown in the form of Heatmap (Fig. 3). Highest variation was observed in miR164c with 2.67 and −2.79 log_2-fold change in resistant and susceptible lines, respectively (Fig. 3a; Table S5, S6). The expression of miR1319a was -1.38 log_2-fold in the resistant and 3.13 log_2-fold in the susceptible rice lines (Fig. 3b; Table S5). We found 29 miRNAs common between resistant and susceptible up-regulated categories in which miR531b showed great shift in the expression values i.e. 0.56 and 6.36 log_2-fold expression in resistant susceptible rice lines, respectively (Figs. 3c, 4d, Table S7). Four miRNAs showed highest log2 fold change ranging from 2 to 3 while 36 miRNAs exhibited log2 fold change varying from 1 to 2, and 52 miRNAs showed log2 fold change less than 1 in the up-regulated miRNAs from resistant line (Fig. S7a; Table S7). In the present investigation, *OsWAK129b* gene (LOC_Os12g42070) targeted by miR815c showed less down-regulation in resistant rice line (− 0.08 log_2 fold) as compared to susceptible rice line (− 0.86 log_2 fold). Of all the microRNAs obtained in resistant down-regulated set, miR399h showed − 3.61 log2 fold change, miR1436 exhibited − 2.14, while 29 miRNAs fall in the category of − 2 to − 1 log2 fold change (Fig. S7b; Table S7). Further, miR531b, a common up-regulated miRNA, showed 0.56 log_2 fold change in resistant and 6.36 log_2 fold change in susceptible line. Among the common down-regulated miRNAs, three miRNAs with higher resistant log2 Fc v/s susceptible log2 Fc (R/S) was identified and these include miR1423, with R/S ratio 1.74 (− 0.46/-0.8), miR156i with R/S ratio of 2.18 (− 0.16/–0.35) and miR166e with R/S ratio of 1.94 (− 0.62/-1.2).

To examine the dynamics of miRNAs in the interaction between rice and *M. oryzae*, differentially expressed miRNAs were validated by semi qRT-PCR (Figs. S8, S9, S10, S11) and qRT-PCR. Along with the above

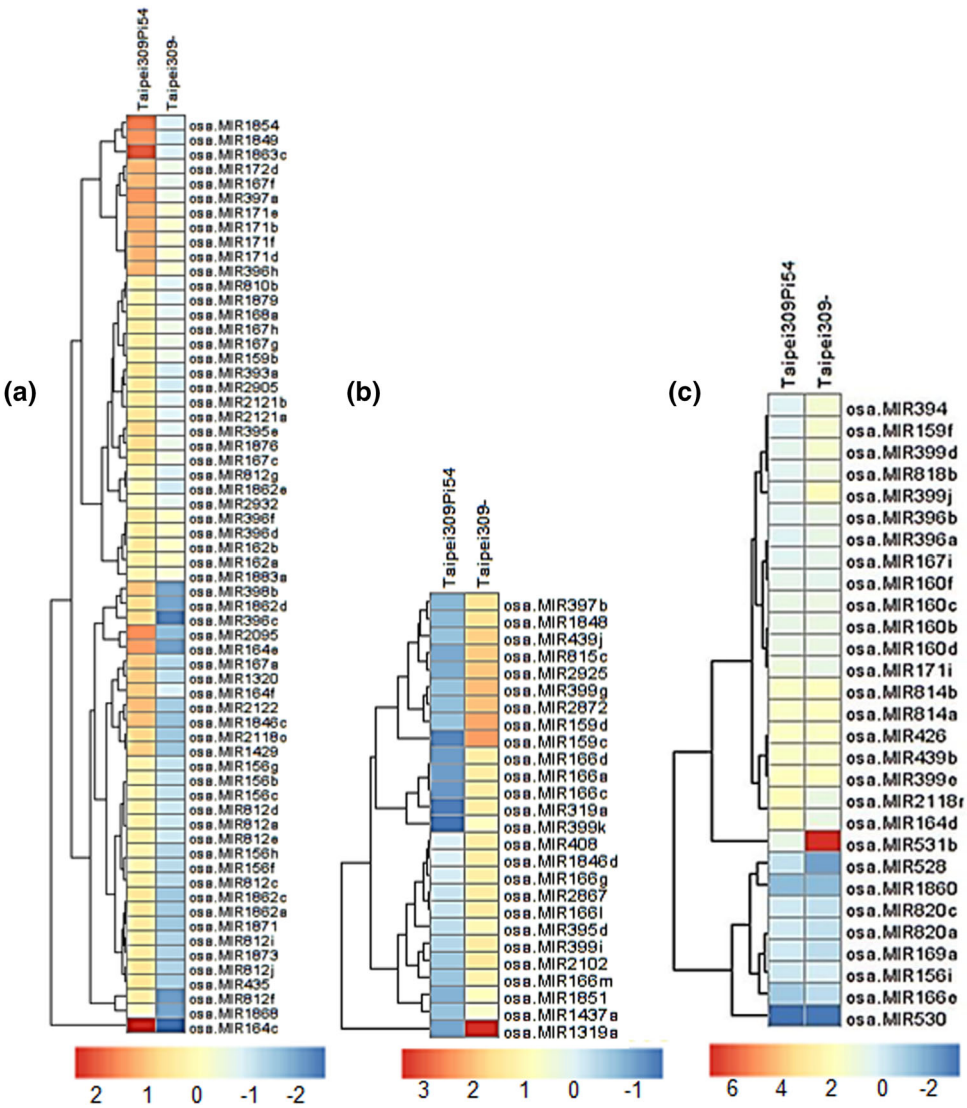

Fig. 3 Heat Map showing overall differential regulation of miRNAs in resistant rice line (Taipei309^{Pi54}). **a** Up-regulated miRNAs in resistant line. **b** Down-regulated miRNAs in resistant line. **c** Common miRNAs in resistant and susceptible line

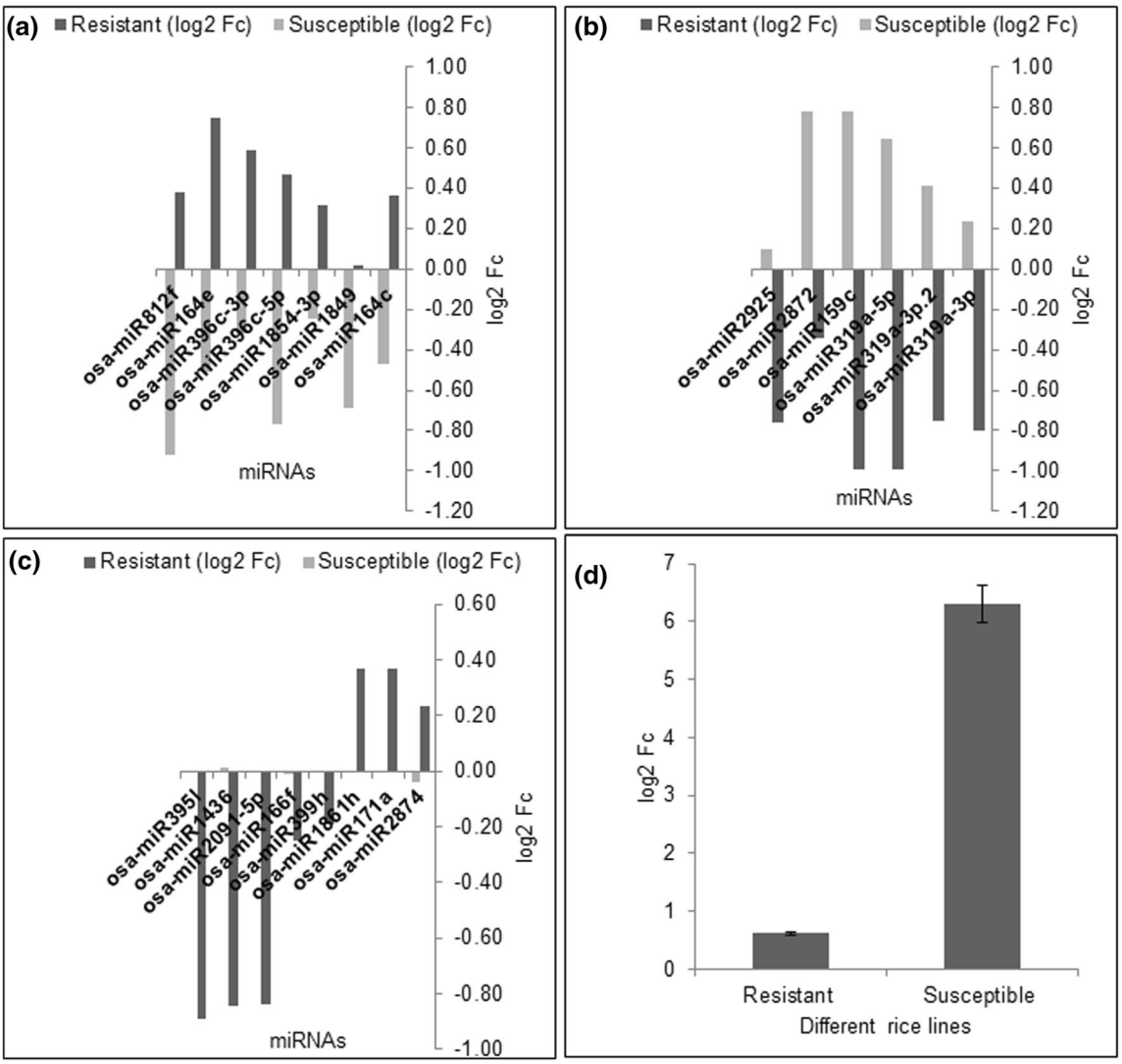

Fig. 4 Expression profiling of various blast responsive positive and negative regulators with respect to their profiling in resistant as well as susceptible rice lines. **a** Positive regulators of rice blast, **b** Negative regulators of blast resistance, **c** Positive and negative regulators present exclusively in resistant rice line, **d** Expression profiling of osa-miR531b in resistance and susceptible rice lines

classification of miRNAs in different categories, we further classified miRNAs into three groups based on their expression pattern upon *M. oryzae* inoculation: (1) up-regulated in Taipei309^{Pi54}, resistant line but down-regulated in Taipei309, (2) down-regulated in Taipei309^{Pi54} but up-regulated in Taipei309 rice line, (3) exclusive to the rice line Taipei309^{Pi54}. In a manner first class should play positive role, second class should play negative role, but third class should play either positive or negative roles in providing resistance to rice against rice blast pathogen. Validations of deep sequencing results by qRT-PCR further emphasized miRNAs as positive and negative regulators in rice immunity. Positive regulators of rice immunity were miR164c, miR1849, miR1854-3p, miR396c-5p, miR396c-3p, miR164e, miR812f which showed increase in their expression in resistant but decrease in susceptible rice line as shown in Fig. 4a, d (Table S5, S6). Other category of positive regulators miR2874, miR171a, and miR1861h was specific to resistant line (Fig. 4a). Negative regulators were miR319a, miR159c, miR2872, miR2925 which showed decrease in the resistant but increase in the susceptible lines (Fig. 4a–c). Also, some other negative regulators miR399h, miR166f, miR2091-5p, miR1436, and miR395l were specific to the resistant line (Fig. 4c).

Expression pattern and validation of miRNA target genes

To understand whether defence related genes are constitutively activated in resistant line, expression of pathogenesis related (PR) target genes was analyzed. Target prediction was done for each miRNA, for 92 up-regulated

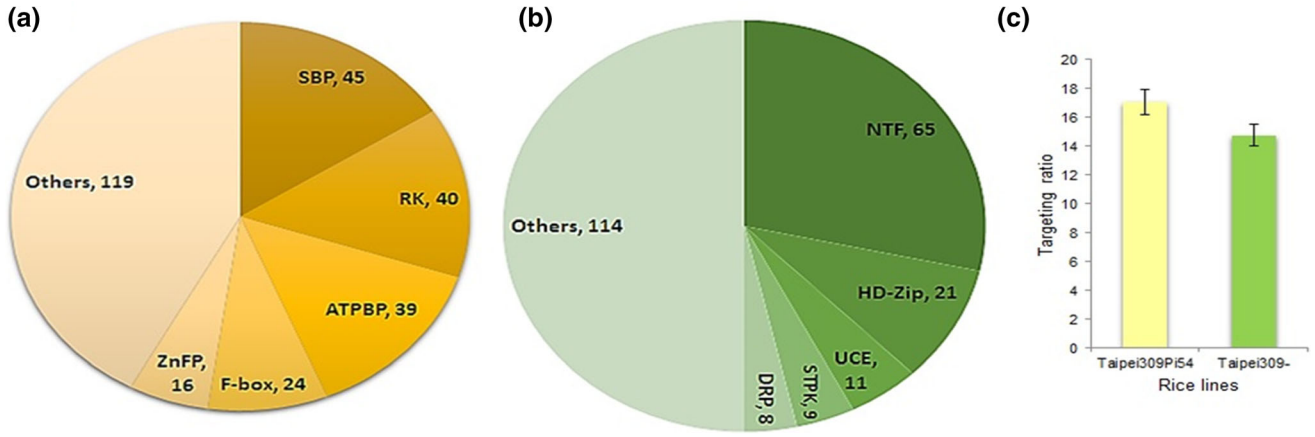

Fig. 5 Analysis of Target genes. Functional annotation & Target Ratio of predicted targets of differentially expressing microRNAs in Resistant rice line (Taipei309^{Pi54}). **a** Upregulated microRNA targets **b** Down regulated microRNA targets. **c** Targeting ratio analysis of resistant and susceptible rice lines (Abbreviations: SBP- Squamosa promoter-binding domain, ZnFP- Zinc finger proteins, ATPBP- ATP binding protein, DRP- Disease resistance proteins like RPP13, F-box- F-box domain containing protein, HD-Zip- Class III HD-Zip protein 4, NTF- Nuclear transcription factor Y, RK- Receptor kinases, STPK's- Serine/threonine-protein kinase, UCE- Ubiquitin conjugating enzyme

resistant miRNA a total of 1722 targets were retrieved, and for 50 down-regulated resistant miRNAs we predicted 702 targets. In susceptible line, 620 and 1479 targets were identified for 50 up-regulated and 90 down-regulated miRNAs, respectively (Table S8). Functional annotations of target genes were depicted for both up-regulated and down-regulated miRNAs of resistant line. Highest number of targets predicted belonged to categories of squamosa binding domain (SBP), receptor kinases (RKs), and ATP binding domain while lowest categories were of Jasmonate-o-methyltransferase (JOM), map kinases (MAPK), WRKY and ZnF for up-regulated miRNAs in resistant line (Fig. 5a). Highest number of targets predicted were for the categories of nuclear transcription factors (NTF), Class-III HD-Zip domain (HD-Zip), and Ubiquitin conjugating enzyme (UCE) while lowest categories of WRKY domain (OsWRKY), RNA recognition motif family protein (RRMF), and squamosa binding domain (SBP) for down-regulated miRNAs in resistant line (Fig. 5b). In total 142 miRNAs in resistant line showed a total of 2424 targets giving targeting ratio of 17.07 and 140 miRNAs in susceptible line showed 2099 targets with a targeting ratio of 14.99 (Fig. 5c).

Target genes with E- value ranging from 2.5 to 3.0 and genes responsible for disease resistance were selected for validation by semi qRT-PCR (Figure S12, S13, S14, S15) and qRT-PCR. Majority of the target genes for up-regulated miRNAs showed down-regulation, demonstrating their inverse relation with miRNAs in terms of expression (Fig. 6a). Highest down-regulation was observed in NBSDS target gene with log2 fold change of -1.59. Lowest down-regulation was seen in Zinc finger gene (ZnF) of log2 fold change value of − 0.33. Out of 12 genes targeted by down-regulated miRNAs from resistant plants, 4 target genes showed down-regulation, with log2 fold change values ranging from − 0.08 to − 0.61. Highest up-regulation was observed for Transcription factors (TCP) gene which showed 0.50-fold change (Fig. 6a). The *OsWAK129b* gene (LOC_Os12g42070) targeted by miR815c showed less down-regulation in resistant rice line − 0.08 $\log_2$ fold) as compared to susceptible rice line (− 0.86 $\log_2$ fold). Majority of the genes targeted by up-regulated susceptible miRNAs showed down-regulation except two genes NBSGO and Aspartic Proteinase Nepenthesin (APN) which showed up-regulation of 0.58-fold and 0.55-fold, respectively. Highest down-regulation was observed in case of Zinc finger gene (ZnF) with log2 fold change value of -2.05 followed by *Oryza sativa* wall associated kinases (*OsWAK*) gene with log2 fold change value of − 0.86 (Fig. 6b). Out of 8 target genes, targeted by down-regulated miRNAs from susceptible plants, 7 genes showed desired up-regulation trend except Transpose gene with log2 fold change of −0.39. Highest up-regulation was observed in NAC domain with 0.58-fold change (Fig. 6b). For correlation analysis of a particular miRNA and its target gene, expression values were calculated. Out of 13 miRNA-target pairs chosen, 10 pairs showed inverse relation with each other while 3 pairs showed direct relation (Fig. S16).

Discussion

The present study aimed at understanding the crosstalk between host miRNAs and blast resistance gene *Pi45* during infection with *M. oryzae*. The deciphering of the

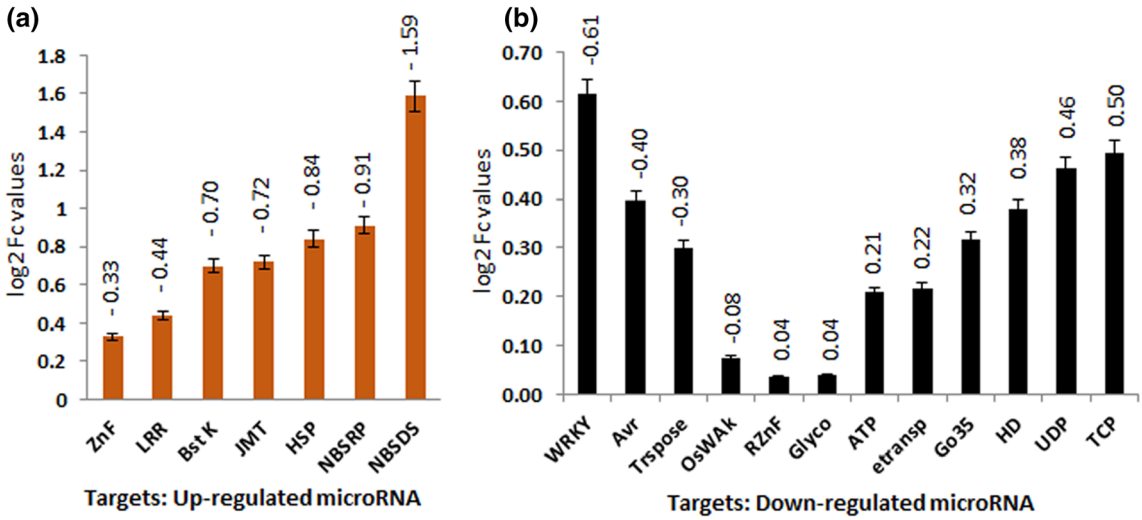

Fig. 6 Real time expressions of target genes in transgenic lines. **a** Targets of Upregulated miRNAs, **b** Targets of Downregulated miRNAs (Abbreviations: ATP-ATP binding domain 1 family, BstK-Brassinosteroid serine/threonine protein kinase, etransp- electron transporter, Go35/NBSGO- NBSGO (go35) NBS-LRR domain protein, Glyco- secondary cell wall-related glycosyltransferase family 47, HD- class III HD-Zip protein 4, HSP- Heat shock protein hsp20, JMT- Jasmonate o-methytransferase, LRR- Leucine rich repeat receptor protein, NBSDS- NBS-LRR gene, NBSRP- NBS-LRR type disease resistance protein Rps1-k-2, OsWAk- *Oryza sativa* wall associated kinases, RZnF- Ring Zinc finger protein-like domain, TCP-TCP family transcription factor containing protein, Trspose- Membrane transporter D1, UDP-UDP-glucoronosyl and UDP-glucosyl transferase family protein, WRKY- WRKY domain containing proteins, ZnF- ZOS1-04—C2H2 Zinc finger domain)

components contributing to rice immunity downstream of *Pi54-AvrPi54* (*R-Avr*) interaction showed the presence of a fine regulatory mechanism mediated by miRNAs. Moreover, availability of cloned *R-Avr* gene pairs have facilitated the dissection of the molecular events responsible for recognition and signalling at the early stages of pathogen infection. The rice PTI and ETI signalling pathways converge at the downstream immune responses, like ROS production, cell wall reinforcement, and defence gene activation; which mostly are regulated by MAPK cascades, TFs, epigenetic modifiers, plant hormones, and small RNAs (Liu et al. 2014). Small RNAs regulate expression of genes at post transcription level, and characterization of these RNAs in plant under stress responses is an active area of research (Sunkar et al. 2008). Also, studies have supported the miRNA's role as post-transcriptional gene regulators in plant immunity, specifically in Rice- *M. oryzae* interaction (Campo et al. 2013; Li et al. 2016). Present study was designed to understand the crosstalk between host miRNAs and fungal pathogen underlying the resistance mechanism, and to select miRNA candidates having role in *Pi54* mediated resistance. The transgenic event TP-*Pi54*-2 has consistently showed durable resistance to *M. oryzae* (Arora et al. 2015). Therefore, we used TP-*Pi54*-2 (Taipei309^{Pi54}) in the subsequent study of understanding the role of miRNAs in immune response of rice against *M. oryzae*. miRNAome profiling of resistant and susceptible rice leaves at 24 hpi would contribute for better understanding the molecular mechanisms involved in *Pi54* mediated rice blast resistance.

Magnaporthe oryzae responsive differentially regulated rice miRNAs

In the present investigation, miR2874, miR171a, and miR1861h were exclusively upregulated in Taipei309^{Pi54}. Role of these miRNAs is well established under abiotic stress tolerance, but no report in biotic stresses. Hence. these miRNAs are the novel candidates for understanding rice- *M. oryzae* interaction. miR2874 is unique for its strong siRNA coding potential (Chen et al. 2011), while miR171a is a highly convincing miRNA candidate which has perfect antisense match with the coding region, and directed a precise cleavage and degradation of mRNA pathway that resembles RNAi guided by siRNAs (Liu et al. 2008). The miR171a targets various transcription factors (TF) important in plant defence including Squamosa promoter binding (SPB), MYB domain, bZIP domain and Zinc finger domain (ZnF). Transcriptional activation of these genes further regulates gene expression and signal transduction that probably play role in stress responses. Other members of miR1861 family were reported as basal regulator in rice-*M. oryzae* interaction (Li et al. 2014). Another class of positive regulators which showed respective decreased and increased expression in susceptible and resistant rice line were miR164c, miR164e, miR1849,

miR1854-3p, miR2925, miR396c-5p, miR396c-3p and miR812f. Some members of miR164 family negatively regulate TF's like NAC domain, CUC and NAM domains, which behave as crucial components for transcriptional activation of plant immune system against pathogens (Sieber et al. 2007; Huang et al. 2012). It was reported that NAM negatively regulates wheat resistance to stripe rust, and also drought resistance in rice (Fang et al. 2014). The miR1849 is unique to rice as its expression is present in rice but not in other crops (Campo et al. 2013). miR1854 was responsive to drought and salinity stress in cotton (Xie et al. 2015). miR396c behaved as negative regulator in Rice-*M. oryzae* interaction (Campo et al. 2013; Li et al. 2014), but in our study it behaved as positive regulator. miR812f was predicted to cleave a putative 1-aminocyclopropane-1-carboxylate oxidase, involved in the regulation of ethylene production (Vriezen et al. 1999); low expression in various abiotic stresses (Kansal et al. 2015). Earlier, it was reported that in response to *M. oryzae*, Taipei309^{Pi54} plants showed up-regulation of genes coding for ethylene biosynthesis (Gupta et al. 2012a).

Unique miRNAs which behave as negative regulators of rice immunity found in this study include miR399h, miR166f, miR2091-5p, miR1436 and miR395l. Role of these miRNAs were established under different biotic stresses, but this report is novel for rice-*M. oryzae* interaction. Different studies define their role; miR399h is one of the most effective miRNAs against Potato Virus Y (Iqbal et al. 2016), artificial miRNA of miR166f (amiRNA-166f) conferred resistance against *Grapevine virus A* in *Vitis vinifera* (Roumi et al. 2012). Similar to our study, a member of miR166 family, miR166k/l is reported to be a negative regulator in Rice-*M. oryzae* interaction (Li et al. 2014). miRNAs; miR2091 and miR1436 were responsive to southern rice black-streaked dwarf virus at 20 dpi (Xu et al. 2014). miR1436 also regulates genes involved in starch synthesis pathways in rice which may play a significant role in transducing signal in the host by regulating sugar transport in response to pathogen attack (Zheng et al. 2012). Another class of negative regulators which showed increased expression in susceptible, but decrease in resistant lines included miR319a, miR159c, miR2872, and miR2925. miR319a-5p and miR159c showed highest down-regulation meaning up-regulation of their target genes coding for Zinc ion binding proteins and MYB domain proteins having central role in providing immunity to fungal pathogen in rice (Chen et al. 2011). Interestingly the functional validation of miR319b, a member of miR319 family has confirmed that *M. oryzae* through miRNA319b suppresses jasmonic acid (JA) synthetic and downstream signalling components leading to susceptibility (Zhang et al. 2018). miR2872 has the ability to form long hairpin structures with strong siRNA-coding potential (Chen et al. 2011), and its role in any of the biotic or abiotic stress is not yet reported. Therefore, miR2872 may be a novel candidate miRNA involved in rice- *M. oryzae* interaction and could be a candidate for developing blast resistance rice lines.

In the present investigation, miR439g showed higher up-regulation in susceptible rice line, leads to down-regulation of its target gene dirigent-like-protein which has been reported to have function in lignification of vascular plants and callose deposition (Davin and Lewis 2000; Jin-long et al. 2012). Pathogen attack on susceptible plants does not lead to lignification while in resistant plants lignification at the infection sites lead to hypersensitive reaction (Flor 1971; Stukenbrock and McDonald 2009). Callose deposition and lignification was already reported in the case of *Pi54- M. oryzae* interaction in rice (Rai et al. 2011). miR439g is also reported as a negative regulator of rice resistance for *M. oryzae* (Li et al. 2014), therefore supporting the findings of the present study. Hence, systemic resistance was enhanced in response to pathogen in resistant plants owing to lignification and callose deposition.

Expression of pathogen regulated rice miRNAs correlates inversely with their target gene expression

It has been reported that miRNAs possess nucleotide complementarity with their target genes leading to down-regulation of their expression. In the present study, the target genes were chosen with expression value between 2.5 and 3.0 $\log_2$ fold higher and also whose role has been reported in disease resistance (Cao et al. 2014; Pandey et al. 2014). Except a recent report, functional role of target genes has not been reported for Rice-*M. oryzae* interactions; hence the present study reports a set of target genes having their role in pathways leading to rice immunity for *M. oryzae*. Recently, the functional role miR319 target gene; OsTCP21 was validated and found that OsTCP21 is a TF and is a positive regulator of blast resistance through jasmonic acid (JA) signalling (Zhang et al. 2018). Common target genes between resistant and susceptible rice lines also followed same regulation trend as their miRNA genes. The up-regulated miRNAs have target genes in blast resistant line as compared to the susceptible line, whereas target genes of down-regulated miRNAs were more in susceptible line compared to the resistant line. As miRNA genes follow inverse relation in terms of expression with their target genes (Lakhotia et al. 2014), same trend was observed in the present investigation. Validation of negative correlation between miRNAs and their target genes has been done in various biotic and abiotic stress responsive miRNAs in wheat, rice, tomato and potato (Pandey et al. 2014; Luan et al. 2014; Lakhotia et al. 2014; Zhang et al.

2018; Chandran et al. 2019). In the present study, thirteen pairs of miRNAs and their target genes were validated, and majority of them showed inverse relation with their target genes. Different categories of target genes selected in this study were NBSDS, NBSGO, NBSGO35, NBSRP, LRR, NBS-LRR. Majority of which were related to plant immunity to biotic stresses, especially related to blast resistance and were the most special category for all the *R* genes. The R proteins have a series of LRRs, NBS and a putative amino terminal signalling domain. LRR domains of plant R proteins are the determinants of response specificity with effector proteins and their action may lead to HR (McHale et al. 2006). Other selected targets in the present study were ZnF and RZnF (Ring zinc finger protein), which are most common DNA binding motifs found in eukaryotic TFs and perform specialised functions like transcription, translation and zinc sensing etc. (Laity et al. 2001). Also, ZnF play a vital role in providing immunity to rice against blast pathogen (Gupta et al. 2012b). Therefore, functional role of all the target genes observed in our study indicates their crucial role in multiple pathways by which *Pi54* mediates disease resistance. Similarly, Dong et al. (2018) reported negative correlation between miRNAs with their targets, and a total of 50 novel miRNAs were identified though the comparative study involving susceptible rice line ZhongerRuanzhan and its resistant mutant H4 after challenging *M. oryzae*. Also, Baldrich et al. (2015) using deep sequencing of small RNA have identified a set of miRNAs/target gene in rice during a *M. oryzae* elicitor induced defence response.

Targeting ratio of miRNA is the average number of target genes per microRNA gene (Coruh et al. 2015). More the number of genes targeted by a miRNA, greater is the role it plays in regulation of different signalling pathways. We obtained targeting ratio 17.07 for blast resistant rice lines and 14.99 for susceptible rice lines. Targeting ratio was found to be more in resistant line than susceptible one. Difference in targeting ratio also highlighted functional role of *Pi54* in blast resistance as both resistant and susceptible lines differ for only one gene; *Pi54*, suggesting its role in providing immunity to blast pathogen. In the present investigation, *OsWAK129b* (LOC_Os12g42070) a target for miR815c showed less down-regulation in resistant rice line as compared to susceptible rice line. *OsWAK* of rice are wall associated kinases from receptor like kinases (RLKs) subfamily in plants which plays important roles in cell expansion, heavy metal stress tolerance, pathogen resistance, and plant development (Zhang et al. 2005). Also, different miRNAs like miR395l, miR167c that targeted *NBSGO35* (RPP13) gene (LOC_Os11g44580) showed comparatively more up-regulation in resistant line. The target *NBSGO35* is a type of NBS-LRR proteins. Also, miR2100 and miR159c that targeted RZnF (LOC_Os03g31320) showed little up-regulation in resistant line, and higher down-regulation in its susceptible counterpart. The RING-finger is a specialised type of Zn-finger and is probably mediating the protein–protein interactions. Stable zinc finger motifs or scaffolds perform specialized functions of mRNA trafficking, transcription, translation, protein folding, cytoskeleton organisation, cell adhesion, and chromatin remodelling (Nakamura 2011). Both miR397a and miR397b may be involved in the network of *Pi54* mediated resistance. Their target gene, LOC_Os03g16610.1, which code for Laccases is responsible for lignification of vascular tissue, and provides defence against pathogen invasion (Mayer and Staples 2002). Laccases were found to be one of the differentially regulated genes in Taipei309^{Pi54} rice line, validating its role and importance in the present study (Gupta et al. 2012a). Therefore, miR815c, miR395l, miR167c, miR2100, miR159c, miR397a, and miR397b may manifest their role by balancing the interplay between various defense response genes during *Pi54* mediated blast disease resistance in rice.

Pi54 mediated resistance development with different miRNA candidates and target genes

The *Pi54* mediated resistance is manifested through an array of various miRNA candidates, which directly or indirectly impact the resistance mechanism. Among these miRNAs, miR531b, a common up-regulated miRNA, showed much lower up-regulation in resistant line than the susceptible counterpart. miR531b targets LOC_Os12g19530.1, an ATP binding protein (Zhu et al. 2008). Therefore, the lower expression of this miRNA in the resistant line indicates higher up-regulation of its target gene, hence more ATP binding which leads to activation of NBS domain in the signalling pathway as compared to susceptible thereby fine tuning of *Pi54* mediated resistance. Three common down-regulated miRNAs with higher resistant log2 Fc v/s susceptible log2 Fc (R/S) ratio were identified. Among them, miR1423 with target protein kinase domain gene is novel for biotic stress studies (Sunkar et al. 2008). miR156i with target genes teosinte glume architecture and squamosa promoter-binding-like protein may play role in defense mechanism. This was down-regulated in response to rice strip virus (Du et al. 2011), and also controls plant development and hormone signalling (Campo et al. 2013). Third miRNA, miR166e was reported to be down regulated in response to rice strip virus and its first target gene is a HD-Zip TF that leads to transcriptional activation of various defensive genes responsible for plant immunity (Guo et al. 2012). miR166e has another target; SGT1, this protein is an essential regulator of cell cycle, and a crucial component of *R* gene-

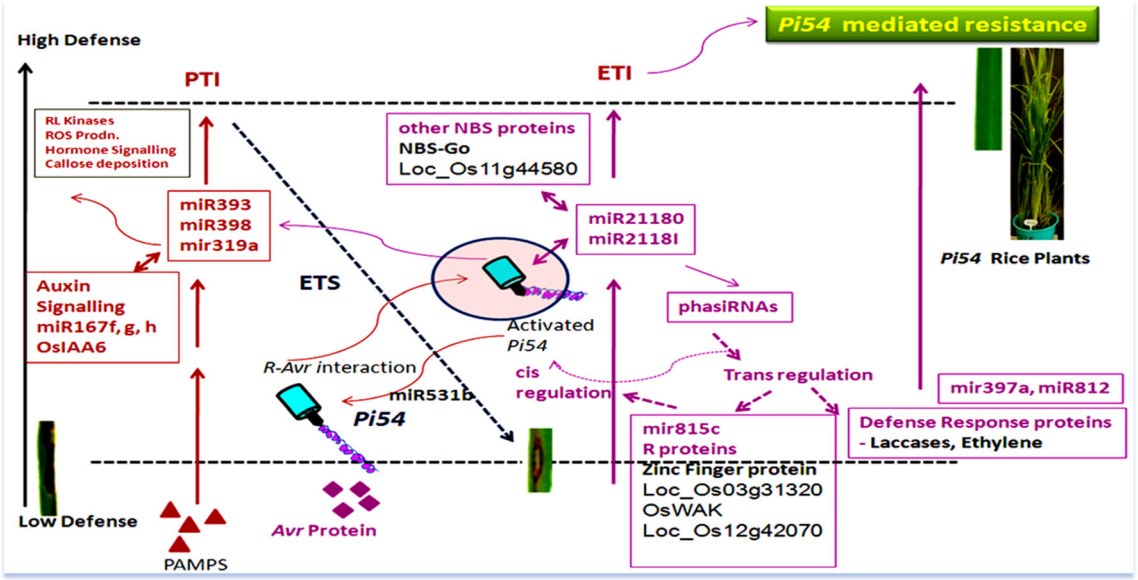

Fig. 7 miRNA and *Pi54* mediated reprogramming of Zig-Zag-Zig model for disease resistance. Black broken lines show low defense and high defense levels. Black dotted line with arrow represents suppression of immunity by Avr proteins. Black arrow shows the progress from low defense to high defense levels. Red arrow and text show components of PTI while pink arrow and text represent ETI components and its progress

mediated disease resistance in plants (Azevedo et al. 2002; Kumar et al. 2014). miR166e was also reported to be down-regulated in rice-*M. oryzae* interaction and its target is START domain (Campo et al. 2013). These miRNAs and their target genes manifested networking pathways of different miRNAs leading to *Pi54* mediated blast resistance. These findings therefore indicate that rice line Tetep is capable of activation of basal resistance against rice blast by activating the *Pi54* gene. A better understanding of the mechanisms of miRNAs in disease progression would shed light on management strategies for rice diseases.

Zig-zag model of immunity is the fundamental theme that describes the different stages of resistance in plants in response to recognition of either of the conserved PAMP molecules or highly variable effector (Avr) molecules originating from pathogen (Jones and Dangl 2006). Disease resistance (R) proteins containing NBS-LRR domains which play a crucial role in this model have been shown to be further regulated by small RNAs, particularly microRNAs (Zhang et al. 2016). Higher expression of a dominant *Pi54* resistance gene in response to *M. oryzae* incursion is known to activate a cascade of genes encoding defence response enzymes (Gupta et al. 2012a, 2012b). However, in silico protein modelling followed by interaction analysis between Pi54 and four candidate effector protein models discovered that it interacted best at the LRR domain of Pi54 protein (Ray et al. 2016), which is the same hypothesis as predicted by miR531b regarding ATP binding and activation of LRR domain in the present study. On the basis of results obtained in the present investigation and recent knowledge on the involvement of microRNA in regulation of disease resistance, we have proposed a functional pathway (Fig. 7) which integrates steps of *Pi54* mediated modulation of microRNA levels leading to reprogramming of plant response to stress (Campo et al. 2013; Li et al. 2014, 2016; Baldrich and Segundo 2016). Such microRNAs previously known to be involved in all different stages of PTI and ETI, were also found to be differentially expressing in rice plants having functional *Pi54* mediated resistance system. Reactive oxygen species (ROS) production and callose deposition on cell walls by RLKs and hormone signalling have been previously shown to be associated with *Pi54* resistance pathway. Differential expression of miR393, miR398 and miR319a in *Pi54* plants indicate its involvement in PAMP triggered immunity which operates through predicted microRNA targets; RLK and hormone signalling genes. Several members of miR167 family which target Auxin signalling genes like *OSIAA6* were also found to be showing differential expression in these plants. Upon the detection of Avr molecules by LRR domain of Pi54, this LRR domains get activated after internal re-orientation to expose the NBS domain. This study finds several microRNAs such as miR21180 and miR2118l which target NBS domain containing proteins were differentially expressed in resistant and susceptible lines. NBSGO protein coding gene LOC_Os11g44580 was confirmed to be up-regulated in resistant lines which are a target of miR21180. The miR-*Pi54* interactions lead to the production of phased secondary RNAs (phasiRNAs) which are known to propagate

regulatory effects on additional target genes through trans- and cis- regulation mechanisms. Differential expression of miR815c which targets Zinc finger protein coding gene LOC_Os3g31320 and OsWAK gene showed its involvement in trans regulation of defence pathway genes. In a similar study, miRNA target genes ZnF, LOC_Os06g47850.1 and rice WRKY DNA-binding LOC_Os02g08440 were found differentially expressed during rice-*M. oryzae* interaction (Dong et al. 2018). Similarly, target genes of differentially expressing microRNAs, miR397a and miR812f such as laccases and ethylene hormone signalling might be involved in *Pi54* mediated resistance pathway. The miR531b which is known to be involved in cis regulation of R genes could also be facilitating *Pi54* co repression and deactivation after pathogen resistance is successfully achieved.

The present study identified the list of microRNAs having a role in PTI and ETI components of Pi54 gene mediated blast resistance in rice, and these identified miRNAs are potential candidates for functional analysis and subsequent incorporation into rice blast resistance development programmes.

Supplementary Information The online version contains supplementary material available at(https://doi.org/10.1007/s12298-021-00960-0) .

Author contributions Planned and designed the research: TR Sharma; performed experiments: Kirti Arora; conducted fieldwork: K Arora, AK Rai, BN Devanna; analysed data: K Arora, AK Rai, H Dubey; wrote and edited the manuscript: K Arora, BN Devanna, AK Rai, Alka Narula, TR Sharma.

Funding Department of Biotechnology, Government of India, and Indian Council of Agricultural Research.

Declaration

Conflict of interest On behalf of all the authors, corresponding author states that there is no conflict of interest.

References

Abramovitch RB, Anderson JC, Martin GB (2006) Bacterial elicitation and evasion of plant innate immunity. Nat Rev Mol Cell Biol 7:601–611

Arora K, Rai AK, Gupta SK, Singh K, Narula A, Sharma TR (2015) Phenotypic expression of blast resistance gene *Pi54* is not affected by its chromosomal position. Plant Cell Rep 34:63–70

Azevedo C, Sadanandom A, Kitagawa K, Freialdenhoven A, Shirasu K, Schulze-Lefert P (2002) The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. Science 295:2073–2076

Baldrich P, Campo S, Wu MT, Liu TT, Hsing YI, San Segundo B (2015) MicroRNA-mediated regulation of gene expression in the response of rice plants to fungal elicitors. RNA Biol 12(8):847–863

Baldrich P, San Segundo B (2016) MicroRNAs in rice innate immunity. Rice. https://doi.org/10.1186/s12284-016-0078-5

Baulcombe D (2004) RNA silencing in plants. Nature 431:356–363

Bonman JM, Khush GS, Nelson RJ (1992) Breeding rice for resistance to pests. Annu Rev Phytopathol 30:507–528

Brant EJ, Budak H (2018) Plant small non-coding RNAs and their roles in biotic stresses. Front Plant Sci. https://doi.org/10.3389/fpls.2018.01038

Campo S, Peris-Peris C, Siré C, Moreno AB, Donaire L, Zytnicki M, Notredame C, Llave C, San Segundo B (2013) Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the Nramp6 (Natural resistance-associated macrophage protein 6) gene involved in pathogen resistance. New Phytol 199:212–227

Cao X, Wu Z, Jiang F, Zhou R, Yang Z (2014) Identification of chilling stress-responsive tomato microRNAs and their target genes by high-throughput sequencing and degradome analysis. BMC Genomics. https://doi.org/10.1186/1471-2164-15-113

Chandran V, Wang H, Gao F, Cao X-L, Chen Y-P, Li G-B, Zhu Y, Yang X-M, Zhang L-L, Zhao Z-X, Zhao J-H, Wang Y-G, Li S, Fan J, Li Y, Zhao J-Q, Li S-Q, Wang W-M (2019) miR396-OsGRFs module balances growth and rice blast disease-resistance. Front Plant Sci. https://doi.org/10.3389/fpls.2018.01999

Chen D, Meng Y, Yuan C, Bai L, Huang D, Lv S, Wu P, Chen LL, Chen M (2011) Plant siRNAs from introns mediate DNA methylation of host genes. RNA 17:1012–1024

Coruh C, Cho SH, Shahid S, Liu Q, Wierzbicki A, Axtell MJ (2015) Comprehensive annotation of *Physcomitrella patens* small RNA loci reveals that the heterochromatic short interfering RNA pathway is largely conserved in land plants. Plant Cell 27:2148–2162

Cui Y, Zhao Y, Lu Y, Su X, Chen Y, Shen Y, Lin J, Li X (2021) In vivo single-particle tracking of the aquaporin AtPIP2; 1 in stomata reveals cell type-specific dynamics. Plant Physiol. https://doi.org/10.1093/plphys/kiab007

Das A, Soubam D, Singh PK, Thakur S, Singh NK, Sharma TR (2012) A novel blast resistance gene, *Pi54rh* cloned from wild species of rice, *Oryza rhizomatis* confers broad spectrum resistance to *Magnaporthe oryzae*. Funct Integr Genomics 12:215–228

Davin LB, Lewis NG (2000) Dirigent Proteins and Dirigent Sites Explain the Mystery of Specificity of Radical Precursor Coupling in Lignin and Lignin Biosynthesis. Plant Physiol 123:453–462

Devanna BN, Sharma TR (2018) Wheat blast disease management: cues from the advancements in molecular biology of rice-*Magnaporthe* pathosystem. J Plant Biochem Biotechnol 27:249–259

Devanna NB, Vijayan J, Sharma TR (2014) The blast resistance gene *Pi54of* cloned from *Oryza officinalis* interacts with *Avr-Pi54* through its novel non-LRR domains. PLoS ONE. https://doi.org/10.1371/journal.pone.0104840

Dong S, Zhang J, Sun D et al (2018) Identification of *Magnaporthe oryzae*-elicited rice novel miRNAs and their targets by miRNA and degradome sequencing. Eur J Plant Pathol. https://doi.org/10.1007/s10658-017-1399-y

Du Z, Xiao D, Wu J, Jia D, Yuan Z, Liu Y, Hu L, Han Z, Wei T, Lin Q, Wu Z (2011) p2 of Rice stripe virus (RSV) interacts with OsSGS3 and is a silencing suppressor. Molecular plant Pathol 12:808–814

Faivre-Rampant O, Bruschi G, Abbruscato P, Cavigiolo S, Picco AM, Borgo L, Lupotto E, Piffanelli P (2011) Assessment of genetic diversity in Italian rice germplasm related to agronomic traits and blast resistance (*Magnaporthe oryzae*). Mol breed 27:233–246

Fang Y, Xie K, Xiong L (2014) Conserved miR164-targeted NAC genes negatively regulate drought resistance in rice. J Exp Bot 65:2119–2135

Fei Q, Zhang Y, Xia R, Meyers BC (2016) Small RNAs add zing to the zig-zag-zig model of plant defenses. Mol Plant-Microbe Interact 29:165–169

Finnegan EJ, Matzke MA (2003) The small RNA world. J cell Sci 116:4689–4693

Flor H (1971) Current status of the gene-for-gene concept. Annu Rev Phytopathol 9:275–296

Fukuoka S, Saka N, Koga H, Ono K, Shimizu T, Ebana K, Hayashi N, Takahashi A, Hirochika H, Okuno K, Yano M (2009) Loss of function of a proline-containing protein confers durable disease resistance in rice. Science 325:998–1001

Guo W, Wu G, Yan F, Lu Y, Zheng H, Lin L, Chen H, Chen J (2012) Identification of novel *Oryza sativa* miRNAs in deep sequencing-based small RNA libraries of rice infected with rice stripe virus. PLoS ONE. https://doi.org/10.1371/journal.pone.0046443

Gupta SK, Rai AK, Kanwar SS, Chand D, Singh NK, Sharma TR (2012a) The single functional blast resistance gene *Pi54* activates a complex defence mechanism in rice. J Exp Bot 63:757–772

Gupta SK, Rai AK, Kanwar SS, Sharma TR (2012b) Comparative analysis of zinc finger proteins involved in plant disease resistance. PLoS ONE. https://doi.org/10.1371/journal.pone.0042578

Huang T, López-Giráldez F, Townsend JP, Irish VF (2012) RBE controls microRNA164 expression to effect floral organogenesis. Development 139:2161–2169

Iqbal MS, Hafeez MN, Ali A, Rashid B, Tabassum B, Nasir IA (2016) Prediction of host-derived miRNAs with the potential to target PVY in potato plants. Front Genet. https://doi.org/10.3389/fgene.2016.00159

Jin-long G, Li-ping X, Jing-ping F, Ya-chun S, Hua-ying F, You-xiong Q, Jing-sheng X (2012) A novel dirigent protein gene with highly stem-specific expression from sugarcane, response to drought, salt and oxidative stresses. Plant cell Rep 31:1801–1812

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329

Kansal S, Devi RM, Balyan SC, Arora MK, Singh AK, Mathur S, Raghuvanshi S (2015) Unique miRNome during anthesis in drought-tolerant indica rice var. Nagina 22. Planta 241:1543–1559

Katiyar-Agarwal S, Jin H (2010) Role of small RNAs in host-microbe interactions. Ann Rev Phytopathol 48:225–246

Kozomara A, Griffiths-Jones S (2011) MiRBase: Integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. 39:D152–D157. https://doi.org/10.1093/nar/gkq1027

Kumar D, Kirti PB (2014) Pathogen-induced SGT 1 of Arachis diogoi induces cell death and enhanced disease resistance in tobacco and peanut. Plant Biotech J 13:73–84

Laity JH, Lee BM, Wright PE (2001) Zinc finger proteins: new insights into structural and functional diversity. Membranes 11:39–46

Lakhotia N, Joshi G, Bhardwaj AR, Katiyar-Agarwal S, Agarwal M, Jagannath A, Goel S, Kumar A (2014) Identification and characterization of miRNAome in root, stem, leaf and tuber developmental stages of potato (*Solanum tuberosum L.*) by high-throughput sequencing. BMC Plant Biol. https://doi.org/10.1186/1471-2229-14-6

Li S, Castillo-González C, Yu B, Zhang X (2017) The functions of plant small RNAs in development and in stress responses. Plant J 90:654–670

Li Y, Lu YG, Shi Y, Wu L, Xu YJ, Huang F, Guo XY, Zhang Y, Fan J, Zhao JQ, Zhang HY (2014) Multiple rice microRNAs are involved in immunity against the blast fungus *Magnaporthe oryzae*. Plant Physiol 164:1077–1092

Li ZY, Xia J, Chen Z, Yu Y, Li QF, Zhang YC, Zhang JP, Wang CY, Zhu XY, Zhang W, Chen YQ (2016) Large-scale rewiring of innate immunity circuitry and microRNA regulation during initial rice blast infection. Sci Rep. https://doi.org/10.1038/srep25493

Li Y, Martin J, Jeyakumar J, Feng Q, Zhao Z-X, Fan J, Khaskheli MI, Wang W-M (2019) The roles of rice microRNAs in rice-*Magnaporthe oryzae* interaction. Phytopathol Res. https://doi.org/10.1186/s42483-019-0040-8

Liu HH, TianLiWuZheng XYJCACC (2008) Microarray-based analysis of stress regulated microRNAs in *Arabidopsis thaliana*. RNA 14:836–884

Liu W, Liu J, Triplet L, Leach JE, Wang G-L (2014) Novel insights into rice innate immunity against bacterial and fungal pathogens. Annu Rev Phytopathol 52:213–241

Luan Y, Wang W, Liu P (2014) Identification and functional analysis of novel and conserved microRNAs in tomato. Mol Biol Rep 41:5385–5394

Ma C, Lu Y, Bai S, Zhang W, Duan X, Meng D, Wang Z, Wang A, Zhou Z, Li T (2014) Cloning and characterization of miRNAs and their targets, including a novel miRNA-targeted NBS-LRR protein class gene in apple (Golden Delicious). Mol Plant 7:218–230

Ma J, Lei C, Xu X, Hao K, Wang J, Cheng Z, Ma X, Ma J, Zhou K, Zhang X, Guo X (2015) *Pi64*, encoding a novel CC-NBS-LRR protein, confers resistance to leaf and neck blast in rice. Mol Plant Microbe Interact 28:558–568

Mackill DJ (1992) Inheritance of blast resistance in near-isogenic lines of rice. Phytopathol 82:746–749

Mayer AM, Staples RC (2002) Laccase: new functions for an old enzyme. Phytochemistry 60:551–565

McHale L, Tan X, Koehl P, Michelmore RW (2006) Plant NBS-LRR proteins: adaptable guards. Genome Biol. https://doi.org/10.1186/gb-2006-7-4-212

Nakamura N (2011) The role of the transmembrane RING finger proteins in cellular and organelle function. Membranes 1:354–393

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JD (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312:436–443

Nurnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev 198:249–266

Padmanabhan C, Zhang X, Jin H (2009) Host small RNAs are big contributors to plant innate immunity. Curr Opin Plant Biol 12:465–472

Pandey R, Joshi G, Bhardwaj AR, Agarwal M, Katiyar-Agarwal S (2014) A comprehensive genome-wide study on tissue-specific and abiotic stress-specific miRNAs in *Triticum aestivum*. PLoS ONE. https://doi.org/10.1371/journal.pone.0095800

Rai AK, Kumar SP, Gupta SK, Gautam N, Singh NK, Sharma TR (2011) Functional complementation of rice blast resistance gene *Pi-kh* (*Pi54*) conferring resistance to diverse strains of *Magnaporthe oryzae*. J Plant Biochem Biotechnol 20:55–65

Ray DK, Mueller ND, West PC, Foley JA (2013) Yield trends are insufficient to double global crop production by 2050. PLoS ONE. https://doi.org/10.1371/journal.pone.0066428

Ray S, Singh PK, Gupta DK, Mahato AK, Sarkar C, Rathour R, Singh NK, Sharma TR (2016) Analysis of *Magnaporthe oryzae* genome reveals a fungal effector, which is able to induce resistance response in transgenic rice line containing resistance gene, *Pi54*. Front Plant Sci. https://doi.org/10.3389/fpls.2016.01140

Roumi V, Afsharifar A, Saldarelli P, Niazi A, Martelli GP, Izadpanah K (2012) Transient expression of artificial microRNAs confers resistance to Grapevine virus A in *Nicotiana benthamiana*. J Plant Pathol 94:643–649

Ruiz-Ferrer V, Voinnet O (2009) Roles of plant small RNAs in biotic stress responses. Annu Rev Plant Biol 60:485–510

Sharma TR, Rai AK, Gupta SK, Vijayan J, Devanna BN, Ray S (2012) Rice blast management through host-plant resistance: retrospect and prospects. Agric Res 1:37–52

Sharma TR, Shanker P, Singh BK, Jana TK, Madhav MS, Gaikwad K, Singh NK, Plaha P, Rathour R (2005) Molecular mapping of rice blast resistance gene *Pi-kh* in the rice variety Tetep. J Plant Biochem Biotechnol 14:127–133

Shivaprasad PV, Chen HM, Patel K, Bond DM, Santos BA, Baulcombe DC (2012) A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. Plant Cell 24:859–874

Sieber P, Wellmer F, Gheyselinck J, Riechmann JL, Meyerowitz EM (2007) Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. Development 134:1051–1060

Singh A, Singh VK, Singh SP, Pandian RT, Ellur RK, Singh D, Bhowmick PK, Krishnan SG, Nagarajan M, Vinod KK, Singh UD (2012) Molecular breeding for the development of multiple disease resistance in Basmati rice. AoB Plants. https://doi.org/10.1093/aobpla/pls029

Singh J, Gupta SK, Devanna BN, Singh S, Upadhyay A, Sharma TR (2020) Blast resistance gene Pi54 over-expressed in rice to understand its cellular and sub-cellular localization and response to different pathogens. Sci Rep. https://doi.org/10.1038/s41598-020-59027-x

Skamnioti P, Gurr SJ (2009) Against the grain: safeguarding rice from rice blast disease. Trends Biotechnol 27:141–150

Stukenbrock EH, McDonald BA (2009) Population genetics of fungal and oomycete effectors involved in gene-for-gene interactions. Mol Plant Microbe Interact 22:371–380

Sunkar R, Zhou X, Zheng Y, Zhang W, Zhu JK (2008) Identification of novel and candidate miRNAs in rice by high throughput sequencing. BMC Plant Biol. https://doi.org/10.1186/1471-2229-8-25

Vriezen WH, Hulzink R, Mariani C, Voesenek LA (1999) 1-Aminocyclopropane-1-carboxylate oxidase activity limits ethylene biosynthesis in *Rumex palustris* during submergence. Plant Physiol 121:189–196

Xie F, Wang Q, Sun R, Zhang B (2015) Deep sequencing reveals important roles of microRNAs in response to drought and salinity stress in cotton. J Exp Bot 66:789–804

Xu D, Mou G, Wang K, Zhou G (2014) MicroRNAs responding to southern rice black-streaked dwarf virus infection and their target genes associated with symptom development in rice. Virus Res 190:60–68

Yan X, Talbot NJ (2016) Investigating the cell biology of plant infection by the rice blast fungus *Magnaporthe oryzae*. Curr Opin Microbiol 34:147–153

Zhai C, Lin F, Dong Z, He X, Yuan B, Zeng X, Wan L, Pan Q (2011) The isolation and characterization of *Pik*, a rice blast resistance gene which emerged after rice domestication. New Phytol 189:321–334

Zhang H, Wu Z, Wang C, Li Y, Xu J-R (2014) Germination and infectivity of microconidia in the Rice blast fungus *Magnaporthe oryzae*. Nat Commun. https://doi.org/10.1038/ncomms5518

Zhang S, Chen C, Li L, Meng L, Singh J, Jiang N, Deng X-W, He Z-H, Lemaux PG (2005) Evolutionary expansion, gene structure, and expression of the rice wall-associated kinase gene family6. Plant Physiol 139:1107–1112

Zhang X, Bao Y, Shan D, Wang Z, Song X, Wang Z, Wang J, He L, Wu L, Zhang Z, Niu D, Jin H, Zhao H (2018) Magnaporthe oryzae Induces the expression of a MicroRNA to suppress the immune response in rice. Plant Physiol 177(1):352–368

Zhang X, Zou Z, Gong P, Zhang J, Ziaf K, Li H, Xiao F, Ye Z (2011) Over-expression of microRNA169 confers enhanced drought tolerance to tomato. Biotechnol Lett 33:403–409

Zhang Y, Xia R, Kuang H, Meyers BC (2016) The diversification of plant NBS-LRR defense genes directs the evolution of MicroRNAs that target them. Mo Biol Evol 33(10):2692–2705

Zheng Y, Li YF, Sunkar R, Zhang W (2012) SeqTar: an effective method for identifying microRNA guided cleavage sites from degradome of polyadenylated transcripts in plants. Nucleic acids Res. https://doi.org/10.1093/nar/gkr1092

Zhu QH, Spriggs A, Matthew L, Fan L, Kennedy G, Gubler F, Helliwell C (2008) A diverse set of microRNAs and microRNA-like small RNAs in developing rice grains. Genome Res 18:1456–1465