



Differential expression profiling of microRNAs and their target genes during wheat-*Bipolaris sorokiniana* pathosystem

Pradeep Sharma¹ · Om Prakash Gupta¹ · Vikas Gupta¹ · Gyanendra Singh¹ · Gyanendra Pratap Singh¹

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Abstract Wheat spot blotch, caused by *Bipolaris sorokiniana*, is a serious constraint to wheat production, reducing grain yield and consequently having severe economic impact. Several plant miRNAs have recently been discovered as regulators of gene expression involved in cellular and metabolic functions. So far reports on the roles of miRNAs in *B. sorokiniana* infection response of wheat are scanty. To further understand the defence mechanism of miRNAs-regulated cellular functions, we examined the expression patterns of 17 miRNAs and their targets involved in the interaction between wheat and *B. sorokiniana* in two contrasting wheat genotypes, Chiriya-1 and WH-147. All of the miRNAs and target genes were shown to be expressed differentially in both genotypes after *B. sorokiniana* infection. Seven and nine miRNAs were observed as up-regulated in the resistant genotype Chiriya-1 and the susceptible genotype WH147, respectively. Among the up-regulated miRNAs, ptc-miR901 (~ 10.21 times) accumulated the most in Chiriya-1 followed by ptc-miR1450 (~ 7.6 times) in WH-147. Furthermore, only two miRNAs, tae-miR156 and ptc-miR482c showed a complete inverse relation with their target genes, SPL and NBS-LRR, respectively. This research sheds light on the temporal differential regulation of miRNAs and their targets, which may play a role in wheat adaptation to *B. sorokiniana* infection.

Keywords Wheat · Disease · *Bipolaris sorokiniana* · miRNA expression analysis · Target genes

✉ Pradeep Sharma
neprads@gmail.com; Pradeep.Sharma@icar.gov.in

¹ ICAR-Indian Institute of Wheat and Barley Research, Karnal 132001, Haryana, India

Introduction

Wheat (*Triticum aestivum* L.) is one of the important cereal crops grown worldwide that serves as major source of carbohydrates and protein in economically deprived continents. Recent climate changes have aggravated the disease scenario in India which leads to increase in the incidence of spot blotch of wheat caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker. It is a seed-borne disease, in which primary infection initiated from inoculum surviving on conidia on soil, crop residues or collateral hosts. *B. sorokiniana* causing spot blotch, root-rot, leaf-spot disease, black point, seedling and head blight in cereal crops, first colonizes the older leaves at the base of wheat plant and then progresses to the upper part of the canopy (Nizam et al. 2012). Yield losses caused by *B. sorokiniana* were estimated to be 15 to 25% (Dublin and van Ginkel 1991), which can be devastating for farmers in the Eastern Gangetic Plains. On the other hand, to cope up with fungal infection, plants have evolved cascades of molecular and biochemical networks to elicit the defence response. Changes in expression patterns for genes implicated in a wide range of cellular, physiological and metabolic processes are one of the most visible aspects of fungus tolerance adaptation (Vinocur and Altman 2005). Elucidation of the molecular events underlying the biotic and abiotic stresses is important for increased survivability of the crop (McIntosh and Pretorius 2011).

The regulatory role of sRNA (small non-coding RNAs) that is 20–24 nucleotides in length has become the leading area of research. MicroRNAs (miRNAs) have been implicated to play critical roles in gene expression regulation at the post-translational level (Bartel 2004; Jones-Rhoades 2006; Singroha et al. 2021). The genes coding for miRNAs are transcribed by RNA polymerase II as pri-miRNAs

which are processed by a dicer-like enzyme (DCLs) to form Precursor miRNAs (pre-miRNAs), and further cleaved by DICER LIKE 1 (DCL1) into a miRNA/miRNA* duplex followed by the transfer of duplex from the nucleus to cytoplasm. These molecules are methylated by the HUA ENHANCER 1 (HEN1). Finally, the mature miRNA is incorporated into a RNA-INDUCED SILENCING COMPLEX (RISC) (Eldem et al. 2013) and reaches its target mRNA transcript by binding with AGRONAUTE 1 (AGO1) protein. After RISC loading, the AGO1 protein unwinds the miRNA:miRNA* duplexes, and one strand of the duplex miRNA is directed to the exosome for degradation by a small-RNA degrading nuclease, while the mature miRNA is inserted into the RISC, which contains AGO proteins (Arribas-Hernández et al. 2016). Finally, the mature miRNA directs the RISC to target mRNAs that are complementary to it.

MiRNAs with high homology to the target mRNA cause site-specific cleavage of the mRNA, whereas miRNAs with poor base pairing to the target mRNA cause translational repression and/or mRNA degradation (Sun et al. 2012). In plants, miRNAs act as negative regulators, mainly inhibiting gene expression at the level of post-transcription. The latest release of miRBase (Release 22.1, October 2018, <http://www.mirbase.org>) contains 38,589 hairpin precursors and 48,860 mature miRNAs, from 271 organisms are publicly available. Amongst them, 125 of them belong to bread wheat, while 738 and 71 miRNAs are found for rice and barley, respectively.

With the availability of the wheat genome sequence, several groups have discovered wheat miRNAs which are conserved across plant species (Achakzai et al. 2018). However, owing to the complexity of the wheat genome and the spatial/temporal nature of miRNA expression, a large number of miRNAs still remain to be discovered. Recent findings support that miRNAs are involved in plant responses to a wide array of biotic stresses such as powdery mildews (Wu et al. 2014) and rusts in wheat (Feng et al. 2015; Gupta et al. 2012; Kumar et al. 2017). Seeing the huge genome size of the bread wheat, more number of miRNAs is expected to be identified (Brenchely et al. 2012). Recently, identification of miRNAs using high-throughput sequencing techniques has become low cost and easily available. Meng et al. (2013) identified 605 miRNAs representing 540 families at grain filling stage in wheat using deep sequencing approach. Though, computational predictions using new bioinformatics tools can also be used for miRNA and its target prediction (Pandey et al. 2013; Jaiswal et al. 2019). Transcriptional regulation of many miRNAs and genes in response to biotic stress has been widely reported in various crops (Gupta et al. 2012; Kumar et al. 2017; Inal et al. 2014; Feng et al. 2015). However, the role of miRNAs in wheat infected by *B. sorokiniana* is very

poorly known. In this work, we investigated the expression profiling of miRNAs associated in cellular and biochemical functions related to host defence response in two contrasting wheat genotypes, Chiriya-1 and WH-147 which has been infected with *B. sorokiniana*. In addition, we also used qPCR to validate target transcripts of responsive miRNAs.

Material and methods

Plant material

The plant material included 64 wheat genotypes representing registered genetic stocks for different traits, advanced lines, known resistant sources of spot blotch and few released varieties (Supplementary Table 1). Planting was conducted in the first week of December so that the post anthesis stage coincided with the warm and humid conditions required for proper disease development. The genotypes were planted in the poly-house in a single row plot of 1 m length keeping row to row and plant to plant distances of 25 cm and 5 cm, respectively at ICAR-Indian Institute Wheat Barley Research, Karnal, India. To enhance disease development and spread, a mixture of susceptible genotypes was planted as spreader rows at borders and every 10 rows. To grow a good crop, recommended agronomic practices were used.

Creation of epiphytotic conditions and phenotyping

Spot blotch disease was created artificially in the poly-house to minimise environmental effects. A mixture of *B. sorokiniana* isolates collected from Faizabad, Pantnagar, Coochbehar and Kalyani, all of which have been identified as hot spots for spot blotch incidence in India. The pure cultures of individual *B. sorokiniana* isolates were maintained on potato dextrose agar media (Chand et al. 2003; Pandey et al. 2008) and sorghum grains were used for large-scale multiplication of *B. sorokiniana* spores (Chand et al. 2013) was multiplied on autoclaved sorghum grains. The inoculated sorghum grains were incubated for about 6 weeks at room temperature with constant shaking to promote the fungal development. The infected sorghum grains were subsequently sprinkled in between the planted rows as well as in the spreader rows. Apart from that the spores were extracted from the infected sorghum grains in water. The spore suspension was adjusted to about 104 spores/ml of water which was evenly sprayed at three different Zadoks growth stages viz., tillering (GS-20), flag leaf emergence (GS-37) and anthesis (GS-65) in the evening hours. To maintain sufficient humidity, misting as well as irrigation was given to the plants after inoculations.

Disease assessment was done for each plot visually at early dough stage (GS-83) as per Zadoks scale (Zadoks et al. 1974). Recording of spot blotch incidence was done following a double-digit scale (00–99) as a modification of Saari and Prescott's severity scale (Saari and Prescott, 1975). The upper most leaves, the flag leaf (digit D1) and the penultimate leaf (digit D2) were considered for scoring the disease. The disease severity (DS) was calculated as given below:

$$\text{Disease severity (\%)} = (D1/9) \times (D2/9) \times 100$$

Genotypes were classified into six groups based on the reactions to spot blotch as highly resistant (0–10), resistant (11–20), moderately resistant (21–40), moderately susceptible (41–60), susceptible (61–80) and highly susceptible (81–100) based on disease severity.

Selection of miRNAs and target genes, Primer designing

Based on a review of the literature, we identified 17 miRNAs and five target genes involved in defence of different fungal pathogens (Table 2). The mature miRNA sequences were retrieved and downloaded from miRNA Registry database (<http://miRNA.sanger.ac.uk>). MiRNA-specific forward primers and a universal reverse primer along with internal control were designed using BioEdit software version 5.09.04 (Supplementary Table 2). Similarly, primers for target genes of miRNAs were designed by using PrimerBlast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) (Supplementary Table 3). β -Actin (Forward Primer: 5'-CAA ATC ATG TTT GAG ACC TTC AAT G-3' and Reverse primer 5'-ACC AGA ATC CAA CAC GAT ACC TG-3') was used as a reference gene for data normalization of target genes as described earlier (Kaur et al. 2017). These primers were then used for analysis of hairpin structure, homodimer and heterodimer formation by using OligoAnalyser (<http://eu.idtdna.com/calc/analyser>), and primers were made final if they were having ΔG less than -9 kcal/mol. All the primer sequences were validated by using gel electrophoresis of PCR amplicons and the presence of just one peak on the thermal dissociation (T_m) curve generated by the thermal denaturation protocol, which was performed after each real-time PCR run.

Isolation of miRNAs and total RNAs

For the miRNA investigations, two contrasting wheat genotypes viz., Chiriya-1 (resistant) and WH-147 (sensitive) for *B. sorokiniana* were used. After 48 h, samples were collected from the leaves of plants that had been

sprayed with sterile water (mock) and infected with disease and kept at -80 °C until use.

To analyse miRNA expression behaviour, total miRNAs were isolated from 100 mg of leaf tissue using mirVana™ isolation Kit (Ambion) according to the manufacturer's instructions. Total RNA was extracted for target transcripts using TRIzol® Reagent (Ambion, USA) following the manufacturer's protocol. The quality and quantity of miRNAs were determined using a Nano-Drop spectrophotometer, ND-1000 (NanoDrop Technologies, USA).

srcDNA library construction

For the miRNAs expression profiles, a small RNA (srcDNA) library was generated as described previously (Seungil et al. 2006). miRNAs were poly A tailed at 37 °C for 45 min in 50 μ l reaction volume comprising 0.3 μ g RNA and 5U *E. Coli* poly(A) polymerase (NEB). The poly (A) tailed small RNA was isolated from sample using a purification cartridge included in mirVana Probe and Marker Kit (Ambion) and following the manufacturer's instructions and also reported by Gupta et al. (2012).

Expression analysis of miRNA targeted transcripts

To quantify the expression profile of selected miRNAs target transcripts, we synthesized the cDNA from total RNA using Novagen® first-strand cDNA synthesis kit (Merck, Germany) according to the user's manual for the expression analysis of various target genes of selected miRNAs and as previously described (Kaur et al. 2017). The concentration and purity of srcDNA and cDNA were quantified by using Nano-Drop spectrophotometer, ND-1000 (NanoDrop Technologies, USA).

Validation of miRNAs and target genes using qPCR

Before conducting the expression study, we tested the primer efficiency by PCR-amplifying all of the miRNAs and their corresponding target genes. To validate, a srcDNA library was used to amplify 17 miRNAs using a specific forward primer and an RTQ universal reverse primer (Supplementary Table 2). About ~ 93 bp amplicon was recovered using 3% agarose gel. After PCR confirmation as described (Gupta et al. 2012), quantitative real-time PCR was performed on CFX real time system (BioRad, USA) as follow: the total volume of the PCR reaction was 10 μ l of 29 Light CyclerR 480 SYBR Green 1 master mix (Roche), 0.8 μ l RTQ-UNIR primer (10 μ M), 0.8 μ l forward primer (10 μ M) and 2 μ l (10 ng/ μ l) of srcDNA to a final reaction volume of 20 μ l. The threshold cycle (Ct) value of the technical triplicates and standard deviation was calculated. The relative expression level of

all the miRNAs was calculated by using the comparative $2^{-\Delta\Delta Ct}$ method. For internal control in qRT-PCR and data normalization, β -actin and U6 snRNA were used.

Results

Screening of wheat genotype for *B. sorokiniana* infection under field conditions

Spot blotch disease of wheat, caused by *B. sorokiniana*, is a serious disease of wheat in the Warm and humid environment of South East Asia. In poly-house conditions, the genotypes were tested against mixture of *B. sorokiniana* isolates. Disease score was recorded and genotypes were classified into six groups viz., highly resistant (0–10), resistant (11–20), moderately resistant (21–40), moderately susceptible (41–60), susceptible (61–80) and highly susceptible (81–100) based on disease severity (Fig. 1). Out of 64 lines, 11 genotypes were recorded as highly resistant, 7 resistant, 5 moderately resistant, 17 moderately susceptible, 14 susceptible and 10 highly susceptible (Table 1). The genotypes Chirya-1, GS/2019–20/5048, GS/2019–20/5048, GS/2019–20/9003 and GS/2019–20/9003 were recorded highly resistant to spot blotch whereas genotypes DBW 46, FLW 13, FLW 31, PAU 16,058, Sonalika, WH 147, GS/2019–20/4036, GS/2019–20/4045, GS/2019–20/4046 and GS/2019–20/4057 were recorded highly susceptible.

MicroRNA profiling in resistant cultivar Chiriy-1 during *B. sorokiniana* infection

Based on the previous research describing the function of miRNAs in crop-fungus interactions, 17 miRNAs were chosen to explore their crucial role during *B. sorokiniana* infection in two contrasting wheat genotypes, Chiriy-1 (resistant) and WH-147 (sensitive). These 17 miRNAs derived from different plant species, includes *Triticum*

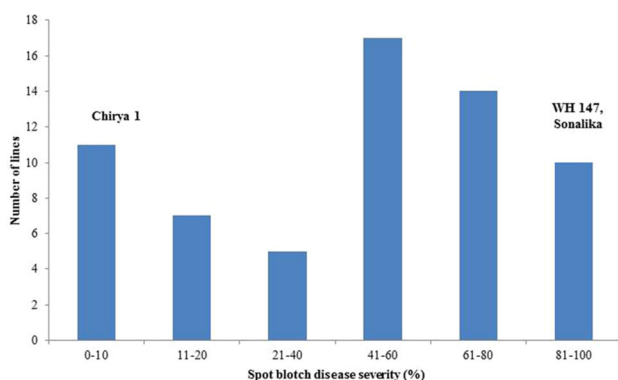


Fig. 1 Histogram of spot blotch disease severity in selected advance wheat lines and released wheat genotypes

Table 1 Reaction of genotypes against a mixture of *Bipolaris sorokiniana* isolates under polyhouse conditions

Infection type	Scale	Number of lines
Highly resistant	0–10	11
Resistant	11–20	7
Moderately resistant	21–40	5
Moderately susceptible	41–60	17
Susceptible	61–80	14
Highly susceptible	81–100	10

aestivum (6), *Phycotrella trichocarp* (6), *Oryza sativa* (2), *Physcomitrella patens* (1), *Chlamydomonas reinhardtii* (1), and *Solanum lycopersicum* (1). These miRNAs have a role in various cellular and molecular responses, including disease resistance (Table 2). qPCR study of these miRNAs revealed varying degree of accumulation in Chiriy-1 genotypes infected with *B. sorokiniana* (Fig. 2). We observed that 7 miRNAs were involved in up-regulation, including ptc-miR169-3p, ptc-miR166a, ptc-miR1450, osa-miR2118a, osa-miR1869, ppt-miR901 and cre-miR1169-3p while 10 were down-regulated, including ptc-miR482c, ptc-miR1447, ptc-miR1448, tae-miR156, tae-miR160, tae-miR167, tae-miR396-5p, tae-miR444a, tae-miR1138, sly-miR1917 in resistant wheat genotype Chiriy-1 (Figs. 2, 3 and 4). Furthermore, ppt-miR901 was observed as significantly elevated (~ 10.21 times), followed by cre-miR1169-3p (~ 8.2 times), and ptc-miR169-3p (~ eight times). Similarly, amongst down regulated miRNAs, tae-miR156 (~ 0.12 times) and tae-miR396-5p (~ 0.12 times) were substantially down-regulated (Figs. 3 and 4).

MicroRNA profiling in susceptible cultivar WH-147 during *B. sorokiniana* infection

The comparative expression study of these 17 miRNAs selected from different plant species in susceptible wheat genotype WH-147 revealed the same trend of differential expression behaviour as Chiriy-1. We observed that, as compared to Chiriy-1, 9 miRNAs were up-regulated: ptc-miR169-3p, ptc-miR482c, ptc-miR1448, ptc-miR1450, tae-miR156, tae-miR167, osa-miR2118a, ppt-miR901, cre-miR1169-3p while 8 miRNAs viz. ptc-miR166a, ptc-miR1447, tae-miR160, tae-miR396-5p, tae-miR444a, tae-miR1138, osa-miR1869 and sly-miR1917 were down-regulated in wheat genotype WH-147 genotypes (Figs. 2, 3 and 4). Among the 9 up-regulated miRNAs, five were found to exhibit a consistent pattern of expression in both genotypes: ptc-miR169-3p, ptc-miR1450, osa-miR2118a, ppt-miR901 and cre-miR1169-3p (Figs. 2 and 4). Nonetheless, six miRNAs, namely ptc-miR1447, tae-

Table 2 Role and regulation of different miRNAs used in the present study

miRNA	Plants	Fungus	Regulation	Target	Function	References	Regulation in present study	
							Chiriya-1	WH147
miR169	<i>P. beijingensis</i> <i>T. aestivum</i>	<i>D. gregaria</i> <i>B. sorokiniana</i>	↑ ↑	CCAAT-binding (TF), chloroplast photosystem ii subunit x	Flowering timing, photosynthesis	Chen et al. (2012); Sun, (2012); Inal et al. (2014)	↑	↑
miR482	Cotton <i>P. beijingensis</i> <i>T. aestivum</i>	<i>V. dahliae</i> <i>D. gregaria</i> <i>B. sorokiniana</i>	↓ ↑ ↑	Nucleotide-binding site leucine-rich repeat (NBS-LRR)	Biotic stress	Yin et al. (2012); Lu et al. (2008); Chen et al. (2012)	↓	↑
miR166	<i>P. trichocarpa</i> Cotton	<i>B. dothidea</i> <i>V. dahliae</i>	↑ ↑	HD-ZIPIII (TF), Ca^{2+} -dependent cysteine protease (<i>Clp-1</i>)	Leaf and vascular development, disease resistance	Zhao et al. (2012); Sun, (2012); Zhang et al. (2016)	↑	↓
miR1447	<i>P. beijingensis</i>	<i>D. gregaria</i>	↑	Disease resistance protein	Biotic stress	Yin et al. (2012); Chen et al. (2012)	↓	↓
miR1448	Cotton <i>P. beijingensis</i> <i>P. trichocarpa</i>	<i>V. dahliae</i> <i>D. gregaria</i> <i>B. dothidea</i>	↓ ↑ ↑	Disease resistance protein, glutathione S-conjugate, ABC transporter, ATP-binding cassette transport protein	Biotic stress	Yin et al. (2012); Chen et al. (2012); Zhao et al. (2012); Lu et al. (2008)	↓	↑
miR1450	<i>P. trichocarpa</i>	<i>B. dothidea</i>	↑	Leucine-rich repeat transmembrane protein kinase	Disease resistance	Zhao et al. (2012)	↑	↑
miR156	<i>T. aestivum</i> <i>P. beijingensis</i> <i>P. trichocarpa</i> <i>P. taeda</i> <i>T. aestivum</i>	<i>E. graminis f. sp. tritici</i> <i>D. gregaria</i> <i>B. dothidea</i> <i>C. quercuum f. sp. fusiforme</i> <i>B. sorokiniana</i>	↓ ↑ ↑ ↓ ↓	Squamosa-promoter binding protein-like	Plant development	Lu et al. (2007); Xin et al. (2010); Zhao et al. (2012); Chen et al. (2012)	↓	↑
miR160	<i>P. trichocarpa</i> <i>P. taeda</i>	<i>B. dothidea</i> <i>C. quercuum f. sp. fusiforme</i>	↑ ↓	Auxin response factor, Aux/IAA	Signaling pathway, flower development, biotic stress	Lu et al. (2007); Zhao et al. (2012); Sun, (2012)	↓	↓
miR167	<i>T. aestivum</i>	<i>P. graminis f. sp. tritici</i>	↑	ARF (TF)	Signaling pathway, flower development	Gupta et al. (2012)	↓	↑
miR396	<i>P. beijingensis</i>	<i>D. gregaria</i>	↑	50S ribosomal protein L20, growth regulating factor	Translation, Leaf development, response to red light	Chen et al. (2012); Sun, (2012)	↓	↓
miR444	<i>T. aestivum</i>	<i>P. graminis f. sp. tritici</i>	↑	C3HC4 type family protein, MADS-box (TF)	Growth, development, and stress response, flowering time regulation	Gupta et al. (2012); Sun, (2012)	↓	↓

Table 2 continued

miRNA	Plants	Fungus	Regulation	Target	Function	References	Regulation in present study	
							Chiriyal	WH147
miR1138	<i>T. aestivum</i>	<i>P. graminis f. sp. tritici</i>	↑	eIF-4b	Protein biosynthesis	Gupta et al. (2012)	↓	↓
miR2118	Cotton	<i>V. dahliae</i>	↓	TIR-NBS-LRR	Disease resistance	Yin et al. (2012)	↑	↑
miR1869	<i>T. aestivum</i>	<i>F. culmorum</i>	↓	Copine family proteins	Biotic stress	Inal et al. (2014)	↑	↓
miR901	<i>T. aestivum</i>	<i>F. culmorum</i>	↓	–	Biotic stress	Inal et al. (2014)	↑	↑
miR1169	<i>T. aestivum</i>	<i>B. sorokiniana</i>	↓	Leucine-rich repeat receptor	Disease resistance	Inal et al. (2014)	↑	↑
miR1917	Cotton	<i>V. dahliae</i>	↓	Triple response 4	Ethylene responses	Yin et al. (2012)	↓	↓

*↓ -denotes downregulation; ↑- denotes upregulation

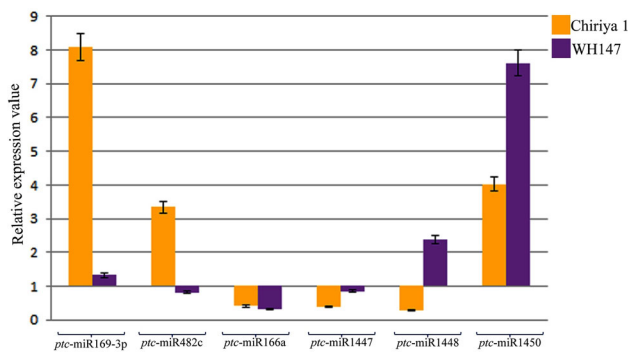


Fig. 2 qRT-PCR validation of selected miRNAs from *Populus trichocarpa*. The histograms display the relative values for the quantified expressions of the miRNAs. The analyses were performed as triplicates, and the error bars were indicated on each column

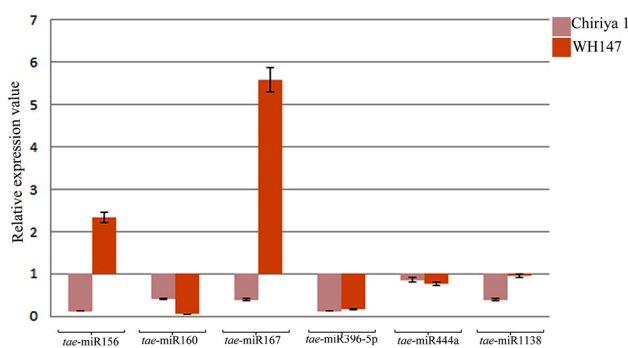


Fig. 3 qRT-PCR validation of selected miRNAs from *Triticum aestivum*. The histograms indicate the relative values for the quantified expressions of the miRNAs. The analyses were performed as triplicates, and the error bars were indicated on each column

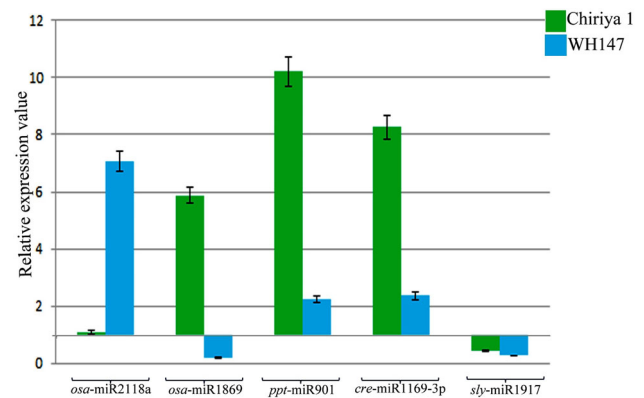


Fig. 4 qRT-PCR validation of selected miRNAs from *Oryza*, *Physcomitrella patens*, *Chlamydomonas reinhardtii* and *Solanum lycopersicum*. The histograms show the relative values for the quantified expressions of the miRNAs. The analyses were performed as triplicates, and the error bars were indicated on each column

miR160, tae-miR396-5p, tae-miR444a, tae-miR1138, and sly-miR1917 showed common trend of accumulation in both the genotypes (Figs. 2, 3 and 4). Amongst up-regulated miRNAs, ptc-miR1450 (~ 7.6 times), osa-miR2118a (~ 7.06 times) and tae-miR167 (~ 5.5 times) was observed to be the most strongly up-regulated (Figs. 2, 3 and 4). Similarly, tae-miR160 (~ 0.04 times), tae-miR444a (~ 0.16 times), and osa-miR1869 (~ 0.19 times) were shown to be down-regulated miRNAs (Figs. 3 and 4).

Validation of target genes of selected miRNAs using qPCR and regulatory network analysis

In order to see the effect of different miRNAs on the expression behaviour of their corresponding genes in resistant and susceptible wheat genotypes, we performed the expression analysis of five target genes namely SPL, MADS box, ARF, HDZ and NBS-LRR targeted by tae-miR156, tae-miR444a, tae-miR160, ptc-miR166a, and ptc-miR482c, respectively (Fig. 5). We observed a difference in the expression patterns of all the target genes when compared to the associated miRNAs (Fig. 5). Only two miRNAs, tae-miR156 and ptc-miR482c, shows a perfect inverse relationship with their target genes (Fig. 5). Furthermore, the target gene (NBS-LRR) of ptc-miR482c exhibited the greatest up-regulation (3.05 times), whereas HDZ protein expression (0.07 times) was the least (ptc-miR166a) (Fig. 5).

To explore the possible regulatory association between miRNA and their targets, the putative miRNA-target relationship was investigated by searching against the analysed miRNAs using the psRNATarget tool (<https://www.zhao lab.org/psRNATarget/>). The results showed that 17 miRNAs target a total of 179 wheat transcripts (Supplementary Table 4) and a possible regulatory network as shown in Fig. 6 was constructed using cytoscape (<http://cytoscape.org/>), among which osa-miR2118a target TRIAECS424BL1044950.1 (Mitogen-activated protein kinase 6) while tae-miR167 targets TRIAECS422BL0383990.1, TRIAECS420286040.2, TRIAECS422DL0501640.1 and TRIAECS423B0732040.2 (Auxin responsive factor 8) further validate the role of miRNA imparting role in disease resistance.

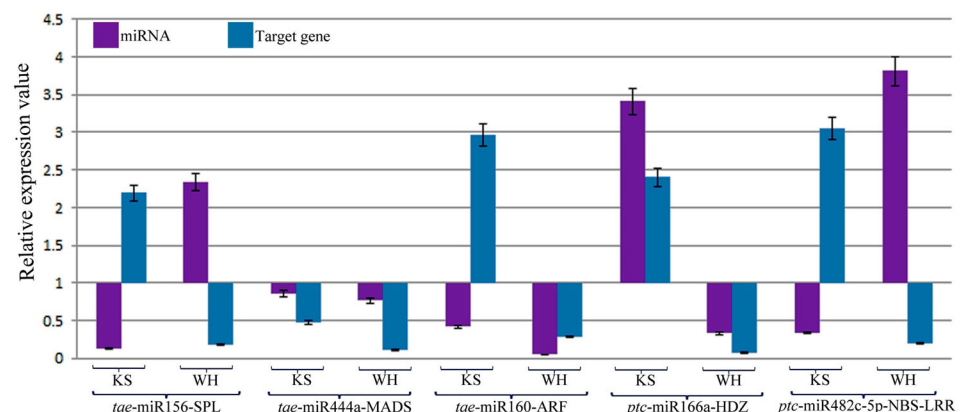
Discussion

MicroRNAs control gene expression in response to abiotic and biotic stress. The onset of the disease triggers early host defence responses. These defensive responses comprise of hormone signalling pathway regulated by lipoxygenase activity, a cell to cell signalling mechanism involving lignin biosynthesis and a translational initiation factor-regulated protein synthesis.

So far, a large number of miRNAs from model plant species have been identified but only a few of these miRNAs has been functionally characterized. In the current investigation some of the chosen miRNAs had not previously been shown to be stress responsive, whereas others were recognised to be implicated in fungal stress (Zhao et al. 2012). miR156 has been shown to have a role in the response of fungal stress in pine, poplar, and wheat (Xin et al. 2010).

MiR156 has been shown to have a role in the response to fungal stress in pine, poplar, and wheat (Xin et al. 2010). miR169 expression was shown to be down-regulated in soybean in response to pathogen stress (Subramanian et al. 2008), but up-regulated in poplar in response to fungal attack (Gupta et al. 2014). miR169 has the ability to target a number of RNAs, including Pto kinase 1, a well-known resistance gene (Inal et al. 2014). As a result, its expression level in infected samples is predicted to be greater compared to control. Our result of increased accumulation of ptc-miR169-3p in resistant cultivar Chiriya-1 upon *B. sorokiniana* infection suggests its active role in defence mechanism in wheat compared to WH147. It has been reported that a nucleotide-binding site leucine-rich repeat (NBS-LRR) protein, targeted by miR482, was suppressed in tomato when inoculated with *Pseudomonas syringe* (DC3000) (Gupta et al. 2014). Since a resistant cultivar needs to synthesize more defence proteins than a susceptible cultivar does, higher expression levels of such target

Fig. 5 qRT-PCR validation of selected miRNAs and their target genes. Histogram show the relative values for the quantified expressions of the miRNAs. The analyses were performed as triplicates, and the error bars were indicated on each column



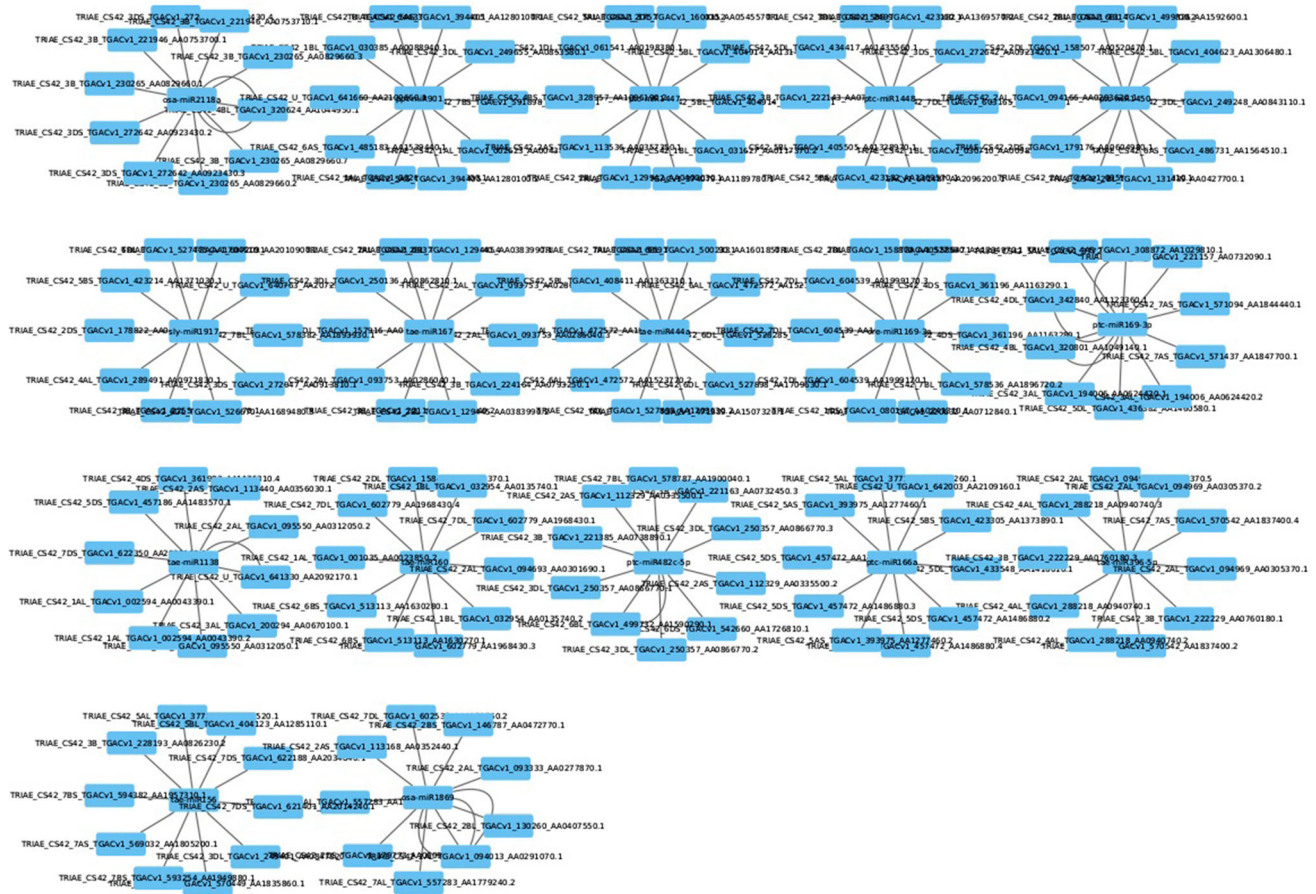


Fig. 6 A schematic representation of the regulatory network relationships between the putative miRNAs and their targets in wheat. The 17 miRNAs that regulate target genes of wheat are shown as circles

genes in resistant cultivar was expected. Our results showed that compared to susceptible genotype WH-147, down regulation of *ptc-miR482c-5p* in resistant genotype Chiriya-1 might be crucial to accumulation more defence related target gene in Chiriya-1. Similarly, miR166 has been reported to target a fungal virulent gene, *Ca²⁺-dependent cysteine protease (Clp-1)*, which decreases disease resistance against fungal pathogen in cotton and the expression of miR166 was observed to be higher in resistant cotton cultivar upon fungal infection suggesting decreased virulence compared to susceptible cultivars (Zhang et al. 2016). Our result of increased expression of *ptc-miR166a* in Chiriya-1 might suggest same defence mechanism operating in cotton. miR1447, miR1448 which were reported to target disease resistance proteins (Lu et al. 2005, 2008) were observed to be differentially regulated upon *D. gregaria* infection with miR1447 being increased and miR1448 is being decreased. In contrast, we observed reverse relation of expression of miR1447 and miR1448 in both the resistant and susceptible genotype (Fig. 3 and 4) suggesting a different mechanism of response in wheat. Zhao et al. (2012) showed up-regulation of

miRNA1450 targeting LRR-transmembrane protein kinase gene involved in defence mechanism in *Populus trichocarpa* against *Botryosphaeria dothidea* (Zhao et al. 2012). We also observed increased expression of this miRNA in both the wheat cultivars suggesting similar defence mechanism.

The six miRNAs namely *tae-miR156*, *tae-miR160*, *tae-miR167*, *tae-miR396-5p*, *tae-miR444a*, *tae-miR1138* have been validated for their different role in different plants exposed to various fungal pathogen (Gupta et al. 2014). miR156 has been reported to be down-regulated in *T. aestivum* and *P. taeda* exposed to *Erysiphe graminis* f. sp. *tritici* and *Cronartium quercuum*, respectively while up-regulated in *Populus beijingensis* and *P. trichocarpa* exposed to *Dothiorella gregaria* and *B. dothidea* respectively. Similarly, miR160 showed up-regulation in *Populus trichocarpa* while down-regulation in *P. taeda* upon exposure with *B. dothidea* and *C. quercuum* respectively (Lu et al. 2007; Xin et al. 2010; Chen et al. 2012, 2018; Zhao et al. 2012). Our result showed down regulation of these two miRNA in resistant cultivar Chiriya-1 while inverse relation in WH-147 upon *B. sorokiniana* infection

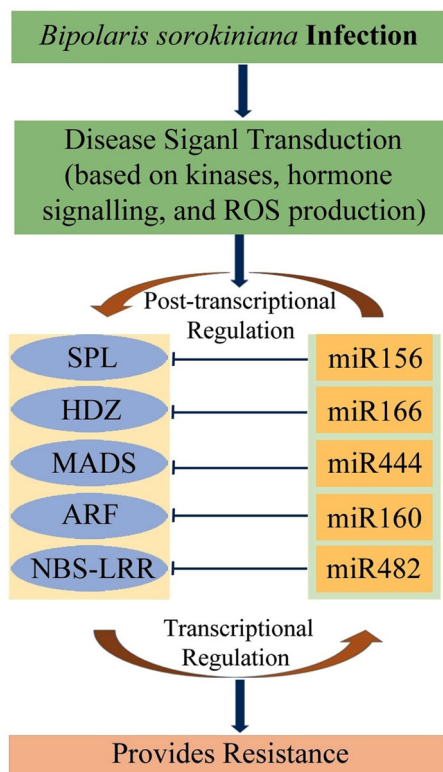


Fig. 7 Possible disease resistance regulatory mechanism during *Bipolaris sorokiniana* infection in wheat. ROS: reactive oxygen species

suggesting the different function of same miRNA to different function in different species under different set of pathogens. Likewise, expression of miR164, miR444 and miR1138 have been shown to be up-regulated by targeting their corresponding target genes ARF, MADS box and eIF^{-4b} under *Puccinia graminis* f. sp. *tritici* infection (Gupta et al. 2012) while miR396 targeting 50S ribosomal protein L20 was reported to be up-regulated in *P. beijingensis* under *D. gregaria* fungal infection respectively (Chen et al. 2012). Our result of differential expression of these four miRNAs in resistant and susceptible cultivars signifies their role during hormonal imbalances and translation machinery during *B. sorokiniana* infection. Upon infection with *B. sorokiniana*, wheat plants activates miRNA mediated defense pathway involving disease signal transduction based on different kinases, hormone signaling, and production of reactive oxygen species (ROS). These pathways are significantly modulated by interaction of miRNAs and target genes, usually transcription factors (TFs) in response to *B. sorokiniana* infection. These regulatory interaction of miRNAs and TFs could reorganize the molecular machinery to impart resistance. Thus, we propose the possible regulatory interaction model of miRNA-target pairs during *B. sorokiniana* infection in wheat (Figs. 6 and 7).

MiR2118 and miR1917 by targeting TIR-NBS-LRR and triple response 4 protein respectively, have been characterized for their role during disease resistance in cotton under *V. dahlia* fungal invasion where they were observed as down regulated (Yin et al. 2012). Up-regulation of miR2118 and down-regulation of miR1917 in both the cultivars, Chiriy-1 and WH-147 suggesting the interference in accumulation of their corresponding target genes modulating the disease response under *B. sorokiniana* infection. Leucine-rich repeat receptor, and copine family proteins which are targeted by miR1169-3p, and miR1869 are induced upon pathogen attack (Jambunathan et al. 2001; Kulcheski et al. 2011; Eldem et al. 2013). Inal et al. (2014) showed that the expression of miR901 is down-regulated in wheat upon fungal-inoculation with *F. culmorum*, *B. sorokiniana* in the both resistant and susceptible cultivars. The contrast result of inverse expression in our study in both the cultivars might be linked to different genetic background leading to differential response. Recognizing mRNAs regulated by miRNAs will help us better understand the biological functions of miRNAs (Tan et al. 2012). We constructed interaction networks of the differentially expressed miRNA-targets. osa-miR2118a target was predicted to regulate cognate target gene (MAP kinases) while taе-miR167 predicted to regulate three genes coding for Auxin responsive factor 8 regulators. The overall analyses indicated that fungal-responsive wheat miRNAs were differentially regulated in a pathogen and cultivar specific manner. This suggested that such genetic variation among cultivars could be amenable to selection for disease resistance in terms of miRNA expression upon fungal stresses.

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Author contribution PS conceptualized and performed the experiments. VG did phenotyping and generated data, PS and OPG wrote the first draft, PS, GS, and GPS edited the final draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

Consent to participate NA.

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