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RESEARCH ARTICLE

Leaf rust responsive miRNA mediated regulation of *Puccinia triticina* genes during host pathogen interaction in wheat

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Abstract

MicroRNAs are associated with immunity and are known to modulate pathogen virulence in cross-kingdom interactions in plants. In the present study, 321 known wheat miRNAs representing 120 families were predicted in response to infection with leaf rust pathogen, *Puccinia triticina*. Among total known miRNAs, 54 unique miRNAs were present in resistant and 24 in susceptible wheat genotypes. 123 targets of unique known host miRNAs were predicted in *P. triticina*, where 30 targets were predicted for S96 and 93 for R96 unique miRNAs. The functional categories of the genes in S96 were WD40-repeat-containing domain, armadillo-type fold, nucleic acid-binding and OB-fold while in R96, the functional categories of the genes were aspartic peptidase domain, glycoside hydrolase, chitinase II, calcium/calmodulin-dependent protein kinase, serine/threonine protein kinase and ribonuclease H-like domain. Thus, unique known miRNAs from susceptible and resistant wheat genotypes targeting different sets of *P. triticina* genes involved in important biological processes and in virulence suggest that miRNAs play an important role during leaf rust infection in wheat.

Keywords: Non-coding RNAs, sRNAs, target prediction, trans-kingdom RNAi, virulence, wheat miRNA

Introduction

Wheat (*Triticum aestivum* L.) is one of the most widely grown food crop, providing staple food for nearly 30% of the world population (Masarmi et al. 2023). According to a study, wheat consumption will increase by more than 30% over the next 40 years (Weigand 2011); and to fulfill this demand, the global wheat production will need to reach 858 million tons by 2050 (Alexandratos and Bruinsma 2012). Though global wheat production has been increased, its potential yield has been affected adversely by various biotic and abiotic stresses. Among the biotic stresses, fungal diseases cause significant losses to wheat production and productivity; and among the fungal diseases, rusts are the economically most important diseases (Gessese 2019). Among three rusts, leaf rust caused by *Puccinia triticina* Eriks. is most common and widespread, bringing about 10 to 20% annual yield losses (Bolton et al. 2008). The disease may cause more than 50% yield losses in susceptible genotypes (Aktar-Uz-Zaman et al. 2017). The cheap, most effective and eco-friendly option is developing resistant variety to combat leaf rust epidemics and minimize yield losses (Dubin and Brennan 2009). Search for the novel resistance gene and its transfer to broadly adapted high-yielding mega varieties will always be the priority in wheat varietal development programme. To date, more than 100 leaf rust resistance (*Lr*) genes have been documented in wheat, and ~80 have been formally designated (McIntosh et al. 2017; Prasad et al. 2020; Kumar et al. 2022). Though several *Lr* genes have been identified, mapped, cloned and utilized, due to the complexities of stage, tissue and space for the

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R-Avr interactions, the molecular mechanism underlying interaction between host and pathogen is not completely understood, which creates a problem in effective utilization of these genes.

Small RNAs (sRNAs), particularly micro RNAs (miRNAs) are endogenous non-coding RNAs of 18-22 nt in length, which regulate gene expression post-transcriptionally (Katiyar-Agrawal and Jin 2010). Various researchers reported the role of miRNAs in diverse biological process, including biotic and abiotic stress response (Wang et al. 2017; Mathur et al. 2020). Plants show two kinds of inter-connected innate immune response, namely, microbial (or pathogen)-associated molecular patterns (MAMP/PAMP)-triggered immunity (MTI/PTI) and effector-triggered immunity (ETI), in response to pathogen attack (Muthamilarasan and Prasad 2013). Small RNAs are involved in both PTI and ETI; hence play an important role in defense response against pathogens (Weiberg et al. 2013). The first stress-regulated miRNA was identified in Arabidopsis as miR393, known to be involved in antibacterial resistance against *Pseudomonas syringae*. (Navarro et al. 2006). Several other studies have also validated the role of sRNA in PTI (Navarro et al. 2006) and ETI (Shivaprasad et al. 2012).

Role of miRNAs in wheat rust diseases have also been established. For instance, Feng et al. (2013) reported *tae*-miR159-mediated regulation of *ta*Myb3 expression during wheat and Chinese race *Pst* interactions. Further, several wheat genes involved in important pathways were found to be targeted by known and novel miRNAs of wheat in response to leaf rust infection (Kumar et al. 2017; Jain et al. 2020). Pathogen's miRNAs targeting host genes is also a new dimension of host-pathogen interaction. Interestingly, during plant-pathogen interaction, export of miRNAs from plants to pathogens and the silencing of important gene for virulence has recently been observed. For instance, during infection of *verticillium dahliae* on Arabidopsis and cotton, miR166 and miR159 from these plants exported to fungal hyphae of this pathogen and modulated the expression of $ca2^{+}$ dependent *CYSTEINE PROTEASE-1* and *ISOTRICHODERMIN C-15 HYDROXYLASE* respectively (Zhang et al. 2016). Similarly, genes important for virulence were found to be targeted by siRNAs from Arabidopsis in *Botrytis cinerea* (Cai et al. 2018) and *Phytophthora capsici* (Hou et al. 2019). These studies proved that the host's miRNAs can enter the pathogen and target their genes. However, little is known regarding target prediction of wheat miRNAs in *P. triticina* and role of these targeted genes in leaf rust disease development. Therefore, in this study, we have predicted the targets of known unique wheat miRNAs from resistant (HD2329+*Lr*28) and susceptible NIL (HD2329) in *P. triticina* and role of these targets in related biological processes and mechanisms were evaluated.

Materials and methods

Plant material, artificial inoculation, and sample collection

Wheat cultivar HD2329 (leaf rust susceptible at the seedling stage) and its near-isogenic line having *Lr*28 gene were used in the present study. *Lr*28 gene is present on chromosome 4AL derived from *Aegilops speltoides* (Tausch) (Naik et al. 1998) and is effective against all the pathotypes of the pathogen in India (Bipinraj et al. 2011). *P. triticina* pathotype 77-5 was used as an experimental pathogen because of its virulence and wide occurrence in the Indian subcontinent. Seeds were sown in sterile mixture of peat, sand and soil (1:1:1) and seedlings were raised to three to four leaf stage (10-15 days after sowing) in the growth chamber under ideal conditions (Temp.22°C, RH>80%, 16h light at 300 lux and 8h darkness) at National Phytotron Facility, IARI, New Delhi. Pathogen inoculum was prepared by the addition of urediniospores in Milli-Q water as suggested by Singh et al. (2012), and few drops of tween-20 were added to increase adherence of inoculum to the leaf of seedlings and applied smoothly on leaves of HD2329 and HD2329 + *Lr*28.

A total of four samples were taken as: i) HD2329 (susceptible) at 0 hour before inoculation, ii) HD2329+*Lr*28 (resistant) at 0 hour before inoculation, iii) HD2329 (susceptible) at 96 hpi (hour post-inoculation, and iv) HD2329+*Lr*28 (resistant) at 96 hpi which were designated as S0, R0, S96 and R96 respectively. The leaf samples collected as above were immediately dipped in RNA later (Qiagen) and transported to the laboratory for RNA isolation. Some part of it was kept at -80°C for further use. The disease infection was confirmed by visual examination of the leaf blades of both susceptible and resistant plants.

RNA isolation, library preparation and sequencing

Total RNA was isolated from each sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and quality of isolated RNA were checked using a nanophotometer. Total RNA was run into 15% TBE (Tris-Borate-EDTA)-Urea PAGE (Polyacrylamide Gel Electrophoresis) and sRNA bands (15–30 nt) were excised and recovered. These sRNAs were first poly(A)-tailed using poly(A) polymerase and to 5'-phosphate of the RNA, an RNA adapter was ligated. The resulting ligation products were run into 15% Urea-PAGE, purified and converted to cDNA. Single end sequencing of 75 nt long cDNAs were done using a Illumina NextSeq 500 platform (Illumina, USA).

Analysis of sRNA sequencing data and known miRNA prediction

Raw data were first processed by Illumina pipeline filter (Illumina NextSeq 500) and clean reads were obtained by removing low-quality reads and adapters. The dataset was further processed for a quality check using an in-house Perl

script. Further, adapter dimer, other RNA families (rRNA, tRNA, snRNAs, snoRNA) and repeats were removed by the UEA sRNA workbenchs (Stock et al. 2012). Subsequently, 16–35 nt long unique reads were mapped to Viridiplantae mature miRNA sequences taken from miRBase 21 (http://microrna.sanger.ac.uk) using BLAST. The unique filtered reads mapped to specific species mature miRNAs with zero mismatch and gaps in hairpin arms, were identified as known miRNAs.

Potential targets prediction of microRNAs

Potential target genes of the miRNAs in pathogen (*P. triticina*) were predicted using the psRNATarget web server. It analyses the complementary pairing between the small RNAs and their targets and utilize Smith-Waterman algorithm to calculate binding energy to evaluate target site accessibility between sRNAs and their targets. Target prediction was done by subjecting mature wheat miRNA sequences as query against *P. triticina* cDNA ASM15152v1 ensemble plant release 43 present in option "Choose cDNA library:" using psRNATarget analysis server (Dai et al. 2018. http://plantgrn.noble.org/psRNATarget/) with the default parameters and maximum expectation of 3.

Gene ontology analysis of predicted target genes in pathogen

Gene ontology (GO) analysis of pathogen gene predicted through psRNATarget was done using Blast2GO tool. CDS (FASTA) sequences of predicted target genes were downloaded from Ensembl Fungi database (https://fungi.ensembl.org/info/data/ftp/index.html) and were BLAST search against nonredundant (nr) protein database of NCBI. BLAST results were analysed and mapped to GO terms using Blast2GO software. GO IDs obtained against each gene were used for making gene ontology graph using wego 2.0 tool (https://wego.genomics.cn/) (Ye et al. 2018).

Gene enrichment analysis

Gene enrichment analysis of the predicted target genes of the pathogen was carried out to know the functional category of these genes using ShinyGOv0.61 web tool (Ge et al. 2020) in which enrichment analysis based on hypergeometric distribution and FDR correction were selected. The predicted target gene ID obtained as a result from the psRNATarget were used as an input for gene enrichment analysis in this web tool; *P. triticina* STRINGdb was chosen as best matching species and cut off was taken as P-value 0.05(FDR).

Construction of miRNA-gene network

The miRNA-mediated regulatory network of known miRNAs of R96 and S96 and their predicted targets was constructed by Cytoscape version 3.4.0 (Kohl et al. 2011). miRNAs, their targets and respective unpaired energy (UPE) was taken as input for the construction of miRNA-gene network. Cytoscape is an open-source software package that provides a visualization that helps to connect miRNAs to their respective target genes.

Results

Quality control, pre-processing and features of processed data

A total of 270061615, 195841332, 261000268 and 154139043 raw sequence reads were obtained for S0, S96, R0 and R96, respectively. After removing adaptors, low-quality sequences and sizes ranged from 16 to 35nt, a total of 8240950, 4291834, 9095651 and 4515879 clean unique reads remained in S0, S96, R0 and R96 libraries, respectively (Table 1.). R0 had maximum percentages of total filtered reads (58.39%) and unique filtered reads (31.81%) among all four samples. In all the samples 24 nt long filtered reads accounted maximum percentage of unique reads, except S96 where 16 nt long unique reads had the maximum percentage. In S0, R0 and R96 the percentage of unique reads of 24 nt length were 16, 12 and 20% while in S96 the percentage of 16 nt long unique reads was 10%.

Prediction of known miRNAs

321 known miRNAs belonging to 120 families were predicted in all four samples (Supplementary_data_1). The known miRNAs predicted in samples S0, S96, R0 and R96 were 270, 206, 240 and 236, respectively. Among these samples, common known miRNAs were 173; miRNAs exclusively present in S0 were 40, S96 were 2, R0 were 18 and R96 were 15.

Among 120 miRNA families, miR169 had maximum number of miRNAs (25) followed by miR156 (22), miR166 (15), miR159 (14), miR396 (13), miR167 (12), miR164 (10) and miR395 (10), were the few families in descending order with context to number of miRNA per family (Fig. 1.). Among 106 families predicted in S0 sample, family miR156 had maximum number of miRNAs (20) while among 85 families predicted in S96 sample, family miR156 had maximum number of miRNAs (17). Further, 95 families were predicted in

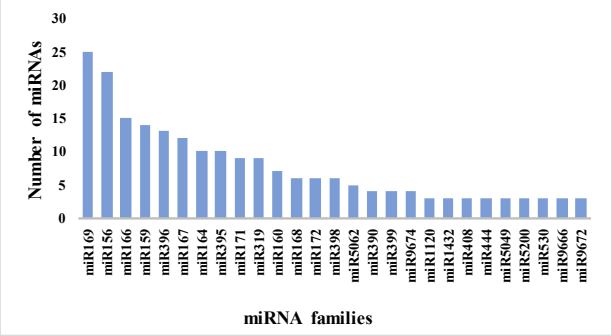

Fig. 1. Number of known miRNAs per family

Table 1. Statistics of raw and processed reads

Samples	Total number of sequences in input file		Sequences remaining after 3′ adaptor removal		Sequences remaining after length range filtering (16-35nt)		Percentage of reads after length filtration (16-35 nt)	
	Total reads	Unique reads	Total reads	Unique reads	Total reads	Unique reads	Total reads (%)	Unique reads (%)
S0	270061615	28216468	240913810	12593004	108546503	8240950	40	29
S96	195841332	16710625	187965454	7013090	58263915	4291834	30	26
R0	261000268	28585043	251178240	13125595	152410436	9095651	58	32
R96	154139043	19159223	144973611	6893693	48807637	4515879	32	24

R0 sample where family miR156 had a maximum number of miRNAs (20), while 101 families were predicted in R96 sample where family miR156 had maximum number of miRNAs (17).

Unique miRNAs present in resistant (R96) and susceptible (S96) NILs

Unique known miRNAs present in the resistant (R96) and susceptible plant samples (S96) were identified to predict their target in the pathogen. Among total 206 and 236 known miRNAs present in S96 in R96 samples, 24 and 54 unique known miRNAs were found to present in S96 and R96 sample, respectively (Fig. 2.). Among total known miRNAs, common miRNAs were excluded and only unique miRNAs present in resistant and susceptible plants after the inoculation were taken for downstream analysis to know their specific role in compatible and incompatible reaction during host pathogen interaction.

Target prediction of host miRNAs in pathogen and gene enrichment analysis of the target genes

A total of 123 targets of unique known host miRNAs (miRNAs from wheat) in the pathogen (*P. triticina)* were predicted using psRNATarget tool, where 30 targets were predicted for S96 and 93 for R96 unique miRNAs. Further, it was observed that unique miRNAs of R96 target a greater number of pathogen genes as compared to S96 (Table 2.). Gene enrichment analysis using ShinyGOv0.61 of predicted pathogen target genes resulted in total 24 functional categories of the gene, where 7 categories were of S96 and 17 were of R96 samples. Interestingly, target prediction and gene enrichment analysis showed that the unique host miRNAs targeted different functional categories of the genes in the pathogen. In case of S96, functional categories of the genes, WD40-repeat-containing domain, armadillo-type fold, nucleic acid-binding, OB-fold etc. were noticed (Table 2). In case of R96, the functional categories of the genes were aspartic peptidase domain, glycoside hydrolase, chitinase II, calcium/calmodulin-dependent/calcium-dependent protein kinase, serine/threonine/dual specificity protein kinase, ribonuclease H-like domain, etc. (Table 2). The genes targeted in R96 samples were mostly involved in pathogenicity, virulence or acting as effector molecules.

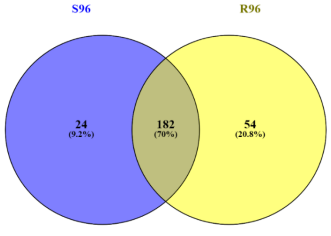

Fig. 2. Unique miRNAs in R96 and S96

Gene ontology analysis of predicted target genes in pathogen

GO terms of both S96 and R96 samples were compared using WEGO 2.0 (Fig. 3a). Comparative gene ontology analysis resulted in only two categories of gene ontology as cellular component and biological process. In cellular component category, the percentage of target gene accounted for R96 and S96, respectively in these sub-categories were as follows: cell (38, 70.8), cell part (38, 70.8), organelle (30.4, 58.3), intracellular organelle (30.4, 58.3), membrane-bounded organelle (29.1, 54.2), organelle part (20.3, 45.8), intracellular organelle part (20.3, 45.8), intracellular part (35.4, 70.8), intracellular (35.4, 70.8) and protein containing complex (15.2, 45.8). Here, among total 10 sub-categories, the top three abundant were cell, cell part and organelle. In the biological process category only one sub-category was found as biological regulation and the percentage of gene accounted for it in R96 and S96 were 15.2 and 41.7, respectively. A bar graph showing GO terms with significant gene number differences were presented in (Fig. 3b), where top three GO categories were protein-containing complex (GO:0032991), intracellular (GO:0005622) and intracellular part (GO:0044424).

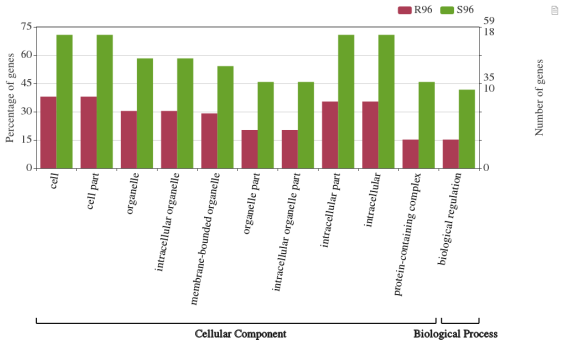

Fig. 3a. Comparison of GO terms of S96 and R96 samples

Table 2. Functional categories of pathogen genes targeted by wheat unique miRNAs (S96) and R96

Wheat miRNAs	Targeted pathogen genes	Functional category of target genes
S96		
miR2118c	PTTG_00821T0, PTTG_04981T0	Translation initiation factor 2, alpha subunit, C-terminal
miR2118c, miR319c	PTTG_01510T0 PTTG_04981T0	Nucleic acid-binding, OB-fold
miR159f, miR399a, miR166m, miR166a-5p	PTTG_02823T0, PTTG_06107T0 PTTG_08765T0, PTTG_09105T0	P-loop containing nucleoside triphosphate hydrolase
miR156f, miR169c, miR169v	PTTG_06676T0, PTTG_09266T0	WD40 repeat
miR156f, miR169c, miR169v	PTTG_06676T0, PTTG_09266T0	WD40/YVTN repeat-like-containing domain
miR156f, miR5062b-5p	PTTG_03426T0, PTTG_05449T0	Armadillo-type fold
miR156f, miR169c, miR169v	PTTG_06676T0, PTTG_09266T0	WD40-repeat-containing domain
R96		
miR827-5p	PTTG_00730T0, PTTG_03260T0 PTTG_10061T0, PTTG_10249T0 PTTG_10301T0, PTTG_10332T0 PTTG_10544T0, PTTG_10753T0 PTTG_10929T0, PTTG_11342T0	Aspartic peptidase domain
miR395p-3p	PTTG_02506T0, PTTG_06669T0	Glycoside hydrolase, chitinase active site
miR395p-3p, miR171b, miR168b	PTTG_02506T0, PTTG_04012T0 PTTG_05391T0, PTTG_06669T0	Glycoside hydrolase, catalytic domain
miR395p-3p, miR171b, miR168b	PTTG_02506T0, PTTG_04012T0 PTTG_05391T0, PTTG_06669T0	Glycoside hydrolase superfamily
miR395p-3p	PTTG_02506T0, PTTG_06669T0	Chitinase insertion domain
miR395p-3p	PTTG_02506T0, PTTG_06669T0	Glycoside hydrolase family 18, catalytic domain
miR395p-3p	PTTG_02506T0, PTTG_06669T0	Chitinase II
miR395p-3p, miR395h	PTTG_03746T0, PTTG_04619T0	Calcium/calmodulin-dependent/calcium-dependent protein kinase
miR395p-3p	PTTG_02506T0, PTTG_06669T0	Glycosyl hydrolases family 18
miR395p-3p, miR395h, miR826a	PTTG_00230T0, PTTG_03746T0 PTTG_04619T0	Serine/threonine/dual specificity protein kinase, catalytic domain
miR827-5p, miR319h, miR9666a-3p, miR166p	PTTG_00730T0, PTTG_01510T0 PTTG_04859T0, PTTG_04969T0 PTTG_10301T0	Ribonuclease H-like domain
miR168b, miR780.1	PTTG_05728T0, PTTG_08336T0	G-protein beta WD-40 repeat
miR395p-3p, miR395h, miR826a	PTTG_00230T0, PTTG_03746T0 PTTG_04619T0	Protein kinase domain
miR395p-3p, miR395h, miR826a	PTTG_00230T0, PTTG_03746T0 PTTG_04619T0	Protein kinase domain
miR827-5p, miR166p	PTTG_00730T0, PTTG_04969T0 PTTG_10301T0	Integrase core domain
miR827-5p, miR166p	PTTG_00730T0, PTTG_04969T0 PTTG_10301T0	Integrase, catalytic core
miR395p-3p, miR395h, miR826a	PTTG_00230T0, PTTG_03746T0 PTTG_04619T0	Protein kinase-like domain

miRNA -mediated regulatory network of known miRNAs and their targets

The miRNA-mediated regulatory network of known miRNAs of R96 and S96 and their targets was constructed by cytoscape (version 3.4.0) (Fig. 4a. and 4b.). Network analysis of wheat miRNA and their target genes showed that miR395p-3p, miR395h, miR827-5p etc., were targeting several genes of the pathogen like glycoside hydrolase,

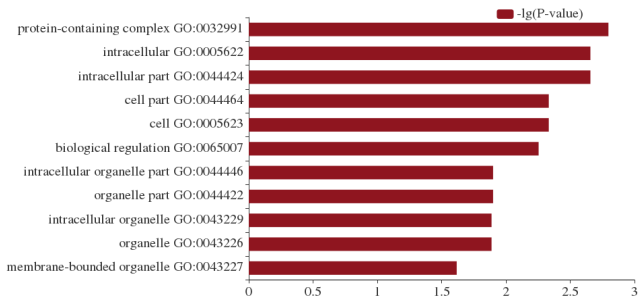

Fig. 3b. GO terms with significant gene number differences between R96 and S96

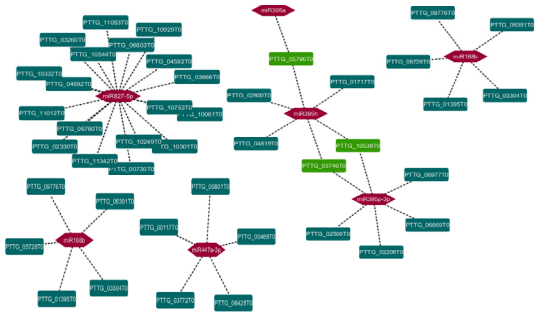

Fig. 4a. Cytoscape networks of known miRNAs of R96 and their targets

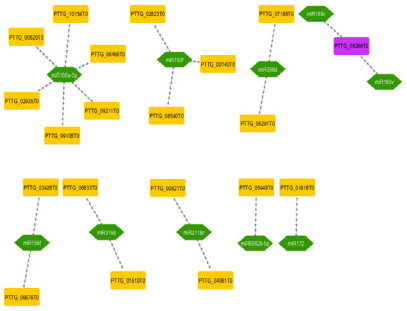

Fig. 4b. Cytoscape networks of known miRNAs of S96 and their targets

chitinase II, protein kinase domain etc. and miR395p-3p, miR171b, miR168b targeted glycoside hydrolase superfamily functional category genes. Further, we observed that unique wheat miRNAs, miR395p-3p, miR395h, miR826a targeted specificity protein kinase, catalytic domain functional category genes of the pathogen *P. triticina*. Interestingly, wheat miRNAs miR395p-3p and miR395h from a resistant plant i.e., R96 targeted almost every functional category of the gene of the pathogen. Regulatory network analysis helped us to identify the complex interaction between wheat miRNAs and their targets in pathogen, implicating their multilayer role in host pathogen interaction.

In R96 known miRNAs are shown as source node in crimson colour in hexagon shape while target genes are shown as target node in steel blue colour in round rectangle shape. The green round rectangle boxes represent the genes predicted to be targeted by more than one miRNA. miRNA targeting genes were indicated by separate arrow the length of which correspond to unpaired energy (UPE).

In S96 known miRNAs are shown as source node in green colour in hexagon shape while target genes are shown as target node in yellow colour in round rectangle shape. The purple round rectangle box represents the genes predicted to be targeted by more than one miRNA. miRNA targeting genes were indicated by separate arrow, the length of which correspond to unpaired energy (UPE)

Discussion

Host-pathogen interaction and their evolution is a continuous phenomenon. During this interaction, host sRNAs may regulate the expression of important genes of host (Kumar et al. 2017; Jain et al. 2020) and pathogen (Hou et al. 2019; Mathur et al. 2020). Similarly, pathogen originated sRNAs may regulate the expression of important genes of host (Mueth et al. 2015; Wang et al. 2017) as well as pathogen *i.e.,* its own gene (Mueth et al. 2015). The present study identified the important target genes in *P. triticina*, targeted by unique miRNAs of wheat NILs in compatible and incompatible reactions.

As reported earlier that the export of sRNAs from plant to pathogen silencing important genes involved in virulence, provided a starting point for this study. In the present study, a greater number of the pathogen's genes were targeted by unique known miRNAs of R96 than S96 and were mostly involved in virulence. Contrary to R96, the targets of unique known miRNAs of S96 were mainly involved in different biological processes. We found that the miRNA induced during resistance (R96) in wheat predominantly targeted the aspartic peptidase and glycoside hydrolase genes in *P. triticina* (Table 2). Aspartic peptidases are known to affect spore formation and germination in fungus (Yuan et al. 1989) and secreted peptidases may act as virulence factors (Krishnan et al. 2018). They degrade the proteins present in the cell walls of plants during infection and release the amino acids, which constitute the principal source of nitrogen and sulphur. Aspartic peptidases have also been reported to be involved in pathogenesis of several economically important phytopathogenic fungi (Krishnan et al. 2018). Mathur et al. (2020) found several target genes in fungi having cysteine-type peptidase activity in interaction between rice and *Fusarium verticilliodes*, barley and *Ustilago maydis* and maize and *Sporisorium reilianum*. Glycoside hydrolase (GH) family represents an ancient chitinase family (Funkhouser and Aronson 2007), where chitinases are known to play an important role in nutrition, morphogenesis and development processes in fungi (Langner and Gohre 2016). In this study, several target genes were annotated as chitinase and similar to this, Mathur et al. (2020) has reported that hvu-miR397 from barley, tae-miR2032 from wheat, zma-miR414 from maize and ghr-miR414d from cotton targeted family 9 glycosyl hydrolase in *Ustilago maydis,* Class 5 chitinase chi100 in *Fusarium graminearum,* Chitin

synthase 3 in *Sporisorium reilianum and* class v chitinase in *Verticillium dahliae* respectively. Wheat miRNA, miR395p-3p and miR395h were found target calcium/calmodulin-dependent/calcium-dependent protein kinase genes in this study. Similar results were also reported by Ramachandran et al. (2020) and Mathur et al. (2020) where, miRNAs from wheat and other crop species were found to target different genes of fungal pathogen having calcium/calmodulin-dependent protein kinase activity.

The present study found that miR827-5p, miR319h, miR9666a-3p and miR166p targeted ribonuclease H-like domain in *P. triticina* while, ribonuclease-like effector protein in wheat is known to enhance susceptibility to *B. graminis* f.sp. *tritici*. Wheat miRNA tae-miR40, tae-miR38 and MIR395b were found to target the gene encoding ribonuclease h protein at1g65750 and PSTG_04009T0 gene having 5′-3′ exoribonuclease activity respectively in *Puccinia striiformis* f. sp. *tritici* (Ramachandran et al. 2020). WD40 domain-containing proteins, also known as WD40-repeat proteins, are one of the largest protein families in eukaryotes and depicts essential roles in fundamental cellular and biological processes (He et al. 2018; Hu et al. 2017). These proteins play pivotal roles in cell division, cytoskeleton assembly, DNA replication, RNA processing, apoptosis, and signal transduction (He et al. 2018). We observed that miR168b and miR780.1 from R96 targeted G-protein beta WD-40 repeat in fungus and similar to our observation, Mathur et al. (2020) reported that zma-miR414 from maize targeted WD repeat-containing protein JIP5 of *Sporisorium reilianum and* ghr-miR482d from cotton targeted WD repeat-containing protein of *Verticillium dahliae*. Similarly, many wheat miRNAs were reported to target *Puccinia striiformis* f. sp. *tritici* genes encoding WD-40 repeat containing protein (Ramachandran et al. 2020). Hou et al. (2019) found that secondary siRNAs produced from Arabidopsis during natural infection of oomycete pathogen *Phytophthora capsici,* targeted important genes of the pathogen, resulted in developmental deficiency in *phytophthora* and subsequently abolishment of virulence. Interestingly, sRNAs from wheat stripe rust fungus (*Puccinia striiformis* f.sp. *tritici*) targeted many wheat genes like NBS-LRR proteins, serine/threonine kinases etc., involved in rust resistance in cereals (Mueth et al. 2015). They also identified targets of these sRNAs into *P. striiformis*. Among the target genes predicted in *P. striiformis,* 13% were assigned to "kinase activity". Other targets were, genes related to vesicle-mediated intracellular transport, protein turnover, protein phosphorylation, signal transduction etc. In addition to this, *Pst*-milR1 (*Puccinia striiformis* f. sp. *tritici* microRNA-like RNA 1) was found to impair wheat resistance to *Pst* by suppressing the wheat pathogenesis-related 2 gene (Wang et al. 2017). Together these findings suggested the possible role of host miRNAs in regulating pathogen's genes through cross-kingdom RNAi.

Several target genes directly or indirectly involved in translation, cellular and biological processes in the pathogen were targeted by host miRNA. In S96 miR2118c targeted the genes encoding translation initiation factor 2, alpha subunit, C-terminal. Eukaryotic IF2 (eIF2) is the major initiator tRNA carrier responsible for loading eukaryotic Met-tRNAi^{Met} onto the 40S ribosomal subunit (Kim et al. 2018), and α subunit of eIF2 regulates the activity of eIF2 (Naveau et al. 2013). Ramachandran et al. (2020) found that, tae-miR66, tae-miR67, tae-miR68 and tae-miR69 from wheat targeted *Puccinia striiformis* f. sp. *tritici* gene encoding eIF2 beta subunit. Further, we found that miR2118c and miR319c targeted nucleic acid-binding, OB-fold genes in fungi (Table 2). The OB-fold is a small structural motif originally named for its oligonucleotide/oligosaccharide binding properties. Genes with OB-fold motif have been identified important for many biological processes, including transcription, translation, DNA replication, repair, recombination, telomere maintenance and cold shock response (Theobald et al. 2003). In our study, miR156f andmiR5062b-5p were found to target *P. triticina* gene encoding Armadillo-type fold. Similarly, Ramachandran et al. (2020) reported that tae-miR32 targeted *Puccinia striiformis* f. sp. *tritici* gene encoding armadillo beta-catenin repeat-like protein. We found that miR156f, miR169c and miR169v were targeting WD40/YVTN repeat-like-containing domain genes in *P. triticina* as like zma-miR414 and ghr-miR482d targeted WD repeat-containing protein of *Sporisorium reilianum* and *Verticillium dahlia,* respectively. Similarly, Ramachandran et al. (2020) found that many miRNAs like tae-miR150, tae-miR66, etc. targeted *Puccinia striiformis* f. sp. *tritici* genes encoding WD-40 repeat containing protein. In plant pathogenic fungus *Fusarium*, WD-40 proteins were reported to have many important functions as well as required for virulence, though the WD repeat containing protein Rack1 have not been characterized (Shim et al. 2006). WD-40 repeat containing proteins have been targeted by unique miRNAs of R96 and S96 (Table 2), which might implicate that these miRNAs are important for basal defense of the plants. Further, concluding remark can only be given after studying the expression of these miRNAs and their targets or by functional validation of these miRNAs.

In S96, miR159f, miR399a, miR166m and miR166a-5p targeted P-loop containing nucleoside triphosphate hydrolase in fungus. Typically, P-loop NTPases hydrolyze the β–γ phosphate bond of a bound nucleoside triphosphate. Effector candidates from *Rhizophagus clarus* showed homology to the *Trametes versicolor* P-loop protein containing a nucleoside triphosphate hydrolase domain (P-loop NTPase) and its cellular role was to involve in cell envelope (Toro and Brachmann 2016). However, the NB-ARC (a P-loop containing nucleoside triphosphate hydrolase) in wheat was found to involve protein conformational changes

and signaling (Andersen et al. 2020). It was found that several miRNAs from R96 targeted various fungal virulence genes *i.e.*, aspartic peptidases, protein kinases etc. were similar to our observation *i.e.,* miR166 and miR159 from *Arabidopsis* and cotton modulated the expression of ca2+-dependent *CYSTEINE PROTEASE-1* (*Clp-1*) and *ISOTRICHODERMIN C-15 HYDROXYLASE* (*HiC-15*), respectively (Zhang et al. 2016a). Further, siRNAs derived from *Verticillium dahliae hygrophobin 1(VdH1)* gene in transgenic cotton, found to provide resistance to verticillium wilt (Zhang et al. 2016b). Several such observation of sRNA-mediated silencing of fungal genes involved in virulence (Nowara et al. 2010; Cai et al. 2018) further supports the present findings.

The higher number of unique wheat miRNAs and their targets in R96 than S96 might indicate that a greater number of unique miRNAs have been produced in resistant plants in response to pathogen attack. These miRNAs have targeted a greater number of important pathogen genes. Further, exclusive miRNAs present in the resistant stage *i.e.,* R96 might be providing resistance by virtue of targeting significant number of genes involved in the virulence or pathogenicity of the pathogen and the absence of these miRNAs in susceptible plant *i.e.,* S96 might be one of the reasons of susceptibility. Small RNAs and dsRNAs were found to induce gene silencing in pathogens even when applied externally (Koch et al. 2016; Rosa et al. 2018), further supporting the hypothesis that miRNAs may travel from plants to pathogens in response to pathogen attack. Therefore, exclusive miRNAs present in the resistant plants (R96) targeting virulent genes involved in the pathogenicity of the pathogen supports previous results that host may send miRNAs to the pathogen during host-pathogen interaction to counteract the pathogen establishment and/or its dispersal. The present study provides evidence for role of miRNAs in host-pathogen interaction and their continuous evolution. Although the exact role of miRNA in wheat leaf rust resistance is unknown, the results of present study provide the foundation for understanding miRNA-mediated resistance response to leaf rust. The identified correlations between miRNA and its targets in pathogens will lay the groundwork to unravel the complex miRNA-mediated regulatory networks involving the secrets of host-pathogen interactions and will help in refining biotechnological approaches for improving biotic stress resistance in wheat.

Author's contribution

Conceptualization of research (NJ, PKS, KVP); Designing of the experiments (NJ, PKS, KVP); Contribution of experimental materials (HK, PKS); Execution of field/lab experiments and data collection (AS, NS); Analysis of data and interpretation (AS, SS, PP); Preparation of the manuscript (AS, NJ).

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