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MicroRNA cross-talk between *Monilinia* fungal pathogens and peach host

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Abstract The reciprocal targeting of microRNAs (miRNA) and micro-like-RNAs (milRNA) between hosts and pathogens is critical for understanding their interactions. In this study, reciprocal miRNA targets were explored in two *Monilinia* fungal pathogens, *M. fructicola* & *M. laxa*, and their peach host (*Prunus presica*). Using in silico analysis, 355 and 266 putative miRNAs were predicted for *M. fructicola* and *M. laxa*, respectively. Number of miRNAs and their targets differed based on host and pathogen species as 209 M. *fructicola* miRNAs target 98 peach genes and 128 M. *laxa* miRNAs target 338 peach genes. On the other hand, peach miRNAs showed the species-specific responses targeting fungal pathways to struggle with its pathogens. These findings indicate distinct strategies and species-specific interactions in this pathosystem. Besides, through the in vitro experimental designs, 166 and 124 expressed miRNAs by *M. fructicola* were detected in the host-mimicked and control environments, respectively. Additionally, novel miRNAs were discovered, six of which were in the mimicked environment and the seven in the controlled environment as highlighting dynamic and specialized miRNA expression in *M. fructicola* depending on the environmental conditions. In conclusion, this study provides the first insights into miRNA-mediated interactions between *M. fructicola, M. laxa*, and peach hosts. Unrevealing the cross talk through the miRNAs in host–pathogen interactions enhances the understanding of pathogenesis and host defense mechanisms. These findings have implications for disease management strategies and contribute to the fields of basic science and evolutionary biology.

Keywords miRNA · *Monilinia* · Peach · Host–pathogen interactions

Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules that typically range in size from 18 to 22 nucleotides (Bartel, 2004; Ghildiyal & Zamore, 2009). They play critical roles in post-transcriptional gene regulation and RNA silencing (Bartel, 2004; Ha & Kim, 2014). MiRNAs can inhibit the expression of multiple genes with a single molecule, or target a single gene with multiple molecules, by either cleaving or shortening the poly(A) tail on mRNA or

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by binding to complementary sequences on the target mRNA, which triggers mRNA degradation (Bartel, 2009; Fabian et al., 2010). As such, they appear to be important key points for regulating interactions within and between organisms. MicroRNAs are known to be highly conserved across species even at higher taxonomic levels that can be extended even between kingdoms (Cuperus et al., 2011; Moran et al., 2017). For example, the miR854 family is conserved in both plant and animal species (Arteaga-Vázquez et al., 2006). The very low evolution rate of RNA polymerase III (1 substitution per 100,000 years), which is responsible for miRNA transcription, may also contribute to this conservation of miRNAs (Geiduschek & Tocchini-Valentini, 1988; Peterson et al., 2009; Wheeler et al., 2009). According to a molecular paleontology study, some species have not had a novel miRNA family in their genome for 450,000 years (Wheeler et al., 2009). Although miRNAs have been known to be anciently conserved, class- or species-specific ones are also known (Gómez-Martín et al., 2023; Jagadeeswaran et al., 2009; Zhang et al., 2008). While some miRNA families are conserved in fungi, their conservation is more variable compared to other kingdoms (Chen et al., 2014; Johnson et al., 2022; Lee et al., 2010a). Fungal miRNAs have distinct characteristics and may have evolved separately from plant and animal miRNAs (Jones-Rhoades et al., 2006; Lee et al., 2010a).

Some of the experimental and computational techniques for identifying miRNAs include microarray screening (Barad et al., 2004), degradome sequencing (Pantaleo et al., 2010), short RNA sequencing (Landgraf et al., 2007), and more recently computational prediction (Mathur et al., 2020). MiRNAs expressed under certain conditions can be detected through experimental designs (Jiang et al., 2012). The small-RNA-seq technology and bioinformatic tools for the annotation of miRNAs have remarkably increased the high-quality data for the identification of miRNAs encoded in genomes and their possible targets (Johnson et al., 2022; Morozova & Marra, 2008).

After the discovery of the first miRNA to control larval development by silencing the Lin-14 gene of *Caenorhabditis elegans* (Pasquinelli et al., 2000; Reinhart et al., 2000), the extensive research for miRNAs in different eukaryotic groups has increased. The first fungus-related study on milRNAs (microRNA like RNAs) was directed at *Neurospora crassa* (Lee et al., 2010a). Subsequently, miRNA-based studies have been directed at many fungal species with many interesting questions. (Chen et al., 2014; Lau et al., 2013; Zhou et al., 2012a). Different functions of miRNAs discovered in fungal groups have also been described. For example, the differential expression of certain miRNAs has been elucidated in the regulation of thermal dimorphism in the therapeutically important fungus *Penicillium marneffei* (Lau et al., 2013). As another example, miRNAs that play an important role in the cellulose degradation pathway of *Trichoderma reesei*, a fungus that has an important role in the industry with its function of degrading cellulose, were discovered (Kang et al., 2013). On the other hand, many fungal species are pathogens that can cause serious damage to their host plants, and the role of miRNAs in these host–pathogen relationships is increasing research interest.

Pathogen-to-host and host-to-pathogen miRNA molecules also play important regulatory roles in this reciprocal battle between host and pathogen (Yang et al., 2007). For example, the regulatory roles of miRNAs are known in both effector-induced immunity (ETI) and pathogen-induced immune (PTI) responses (Baldrich & San Segundo, 2016; Chen & Cao, 2015; Katiyar-Agarwal & Jin, 2010). During infection by *Sclerotinia sclerotiorum*, a necrotrophic fungus that causes white mold in canola (*Brassica napus*), 53 novel and previously known 227 miRNAs were identified in the host (Cao et al., 2016). Besides, it has been discovered that *Pythium aphanidermatum*, a necrotrophic oomycete of turmeric plant, interfere many genes in the host via its miRNAs during the infection (Chand et al., 2016). As another example, different expression profiles of some miRNAs were detected in tomato leaves against infection by the fungal pathogen *Botrytis cinerea* and three of these miRNAs were found to be strongly associated with the response to this pathogen infection (Jin & Wu, 2015). It was computationally predicted in transcriptome datasets for 13 fungi that miRNAs from fungal pathogens target resistance genes in plant hosts, whereas miRNAs in hosts target pathogen virulence genes (Mathur et al., 2020). Moreover, a recent study has been published on the role of miRNAs in regulating the expression of effectors that play an important role in facilitating the infection of plant pathogenic fungi (Xu et al., 2023). It proved that the effector gene related to the expression of the VmSP1 protein, which

is known to suppress plant immunity and is associated with virulence of the pathogen, in the apple tree Valsa canker agent *Valsa mali* is regulated by a fungal milRNA at the post-transcriptional level (Xu et al., 2023). These findings contribute to the growing body of knowledge on the discovery, identification, and functions of miRNAs in the interaction between fungal pathogens and their hosts. Such knowledge is crucial not only for advancing our understanding of the underlying mechanisms but also for developing alternative strategies to control these pathogens.

Peach production is important both economically and agriculturally, with its widespread cultivation occurring on multiple continents, adapting to various climates and becoming a significant component of agriculture in these regions (https://www.fao.org). However, pre-harvest and post-harvest diseases in peach fruit cause serious economic and quality losses, and the primary actors of these problems globally are *Monilinia fructicola* and *Monilinia laxa* (Abate et al., 2018; Holb, 2008; Hrustić et al., 2013; Ozkilinc et al., 2020). Elucidating the host–pathogen relationships in the Peach-*Monilinia* pathosystem is crucial for both fundamental science and the development of more practical applications for the control of these pathogens. Big datasets were obtained for these pathogens at the genomic and transcriptomic level (Angelini et al., 2018; Durak et al., 2021) and their virulence and host–pathogen relationships were studied (Ozkilinc et al., 2020; Balsells-Llauradó et al., 2020; Fidanoğlu et al., 2023). However, until now, there is no known data on miRNAs that play important key roles in the regulation of host–pathogen relationships and infection processes during in the battle between the kingdoms. In addition, scientific research conducted in this direction will also constitute an important resource for RNA-based control strategies, the importance and necessity of which are increasingly emphasized as an alternative to traditional fungicides.

Thus, the objectives of this study were to (i) predict the miRNA repertoires of two fungi (*M. fructicola* and *M. laxa*) from their genomes, (ii) identify the targets of the predicted miRNAs of the pathogens in the transcriptome of the peach host (*P. persica*), and (iii) identify pathogen-targeting host miRNAs. To achieve these goals, we used genome data of both pathogen species produced in our previous studies (Durak & Ozkilinc, 2023) and host transcriptome data which is publicly available. Furthermore, miRNAs expressed in *M. fructicola* in host-mimicking in vitro conditions were identified by using next-generation sequencing data. Thus, for the first time, miRNAs that play a role in host–pathogen relationships in this pathosystem were discovered.

Methods

Fungal samples and data collection

One isolate from two species (*M. fructicola* and *M. laxa*) was selected. The selected isolate codes were Ti-B3-A3-2 for *M. fructicola* and Yildirim-1 for *M. laxa*. Criteria taken into account in the isolate selection: (1) They are highly virulent, according to the results obtained from our previous study, in which the virulence of a large collection of both pathogenic species was evaluated (Ozkilinc et al., 2020); (2) We have genome data (SRA accession number for the chosen samples: SRS13297834, SRS13297846 under the BioProject PRJNA846280), so that it can be used in miRNA annotation. In addition, the pathogenicity of the fifteen candidate isolates was also confirmed on the peach host (cultivar name: Elegant Lady) following the protocol by Ozkilinc et al. (2020). Mycelial plaques of 1 cm in diameter were obtained from 10-day-old cultures grown on Potato Dextrose Agar (PDA). These plugs were placed in the holes drilled in the same size on both sides of each fruit. Two fruits were used per species. PDA media plugs were inserted into the control fruits. After inoculation, the peaches were placed in clean plastic boxes and taken into the incubator at 23 °C in the dark. After inoculation, daily controls were made, and lesion sizes were measured perpendicularly and the size of the first inoculum was subtracted. Since there was no statistically significant difference among the replicates, mean lesion sizes were used for each isolate.

The transcriptome and its annotation data for these two species were retrieved from GenBank (*M. fructicola*: GCA_008692225.1 and *M. laxa*: GCA_009299455.1) provided by Angelini et al. (2018). The transcriptome and annotation data of peach (*Prunus persica*) were retrieved from GenBank (Accession number: AKXU02000000) which was submitted by Verde et al. (2013).

Genome-wide predictions of milRNAs (microRNA-like RNAs) in *Monilinia spp.*

Since it is aimed to identify all potential miRNA genes at the genome level, the use of the term "microRNA-like (milRNA)" is considered more appropriate. The assembled whole genome data of *M. fructicola* and *M. laxa* were used as mentioned above. With minor changes, the pipeline developed by Mathur et al. (2020) was used to predict milRNAs in the genomes. The miRNA sequences of fungal organisms (*Fusarium oxysporum, Verticillium dahliae, Neurospora crassa, Sclerotinia sclerotiorum, Trichoderma reesei, Metarhizium anisopliae, and Botrytis cinerea*) were retrieved from recently published studies and known plant, animal and viral miRNAs were retrieved from miRbase (Chen et al., 2014; Kang et al., 2013; Kozomara & Griffiths-Jones, 2014; Lau et al., 2013; Zhou et al., 2012a, b; https://mirbase.org/).

The BLAST database was locally created using the "makeblastdb" command on the genomes of *M. laxa* and *M. fructicola*. The miRNA sequences from various fungal species and miRBase were compiled as query subjects, with redundant hits removed (https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/). For the NCBI blastn search, stringent filtering options (-evalue 0.1, -penalty -1, -ungapped, max hsps 5, word size 7, and perc identity 95) were used.

The flanking areas of the acquired milRNAs were retrieved using the genomic locations of the milRNAs. The flanking regions were retrieved by using BEDtools -slop option with an optimal window size of 100 nt (Quinlan & Hall, 2010). Using the miRNAfold website, these flanking regions were tested for their capacity to form a hairpin shape (Tav et al., 2016). Only the hairpins that have optimal Minimum Free Energy (MFE) and a hairpin size bigger than 70 nt were identified as possible milRNAs and used for downstream analysis (Supplementary Information).

In silico identification of target genes of fungal miRNAs in peach (*Prunus persica*) host

Potential milRNAs predicted from both species as explained above were submitted to the psRNATarget website (Dai & Zhao, 2011). The transcriptome data of *P. persica* version 298_v2.1 were chosen as a cDNA library in the psRNATarget server (Dai & Zhao, 2011). Only the top five milRNA targets in the *P. persica* transcriptome were assessed using the default settings and scoring Schema V2. Using custom bash scripts, the sites of these milRNA targets were retrieved from the *P. persica* transcriptome. Target function analysis based on GO terms was carried out by using goFEAT with default parameters after the target sequences were annotated (Araujo et al., 2018).

In silico identification of target genes of peach (*P. persica*) miRNAs in *Monilinia* spp. pathogens

Targets of miRNAs from *P. persica* were gathered from miRBase and searched in the transcriptome data of *M. fructicola* and *M. laxa* by using the psRNATarget server (www.mirbase.org; (Dai & Zhao, 2011)). The reference genome annotations of *M. fructicola* and *M. laxa* were used to manually annotate the sequences that *P. persica* miRNAs target. Target mapping of miRNAs identified in both the pathogens' own genomes and in peach hosts was visualized using Shinycircos (Yu et al., 2018).

Mimicking in vitro host environment and testing of success of mimicry by an effector gene expression

The strategy of mimicking the host environment was used considering the study by Maximiano et al. (2021). Freeze-dried peach fruit was used to provide a similar host media and the media name called "Freeze-dried Peach Agar" (FPA). To prepare FPA, 20% Freeze-dried peach and 10% g agar per 1 lt of distilled water were shaken vigorously before autoclaving. To determine whether host mimicry was successful, it was planned to measure of the expression levels of an effector gene in FPA and standard PDA medium for the isolate Ti-B3-A3-2. *Cutinase 1* (*CUT1*), one of the effector genes of fungal pathogens, was selected. In a previous study of our group, this gene was in silico extracted from the whole genome of *M. fructicola,* and the primers were designed and used successfully in Real-Time PCR studies (Fidanoglu and Ozkilinc, unpublished data).

PDA and FPA media were prepared and after pouring them into the Petri dishes, a nitrocellulose membrane was placed on each media. Mycelial plugs from 10-day-old PDA cultures were taken and placed on freshly prepared PDA and FPA media. Three PDA and three FPA were used for each replicate of

the isolate. Then cultures were incubated at 23 °C for 6–7 days depending on the size of the lesion. Then, total RNA extractions were performed. The mycelium grown on filter papers was scraped off. 100 mg of mycelium was weighed and crushed using a mortar and pestle in liquid nitrogen. TRizol reagent was added immediately and centrifuged at 14000 RPM for 1 min. The RNA, obtained after the chloroform step and washing with ethanol, was precipitated by isopropanol treatment for 24 h at -80 °C. RNA was dissolved with RNAase-free distilled water. RNA concentrations were measured by using a fluorimeter (Qubit®, InvitrogenTM), and the samples were kept at -80 °C.

The GoScript Reverse Transcription System (Promega, USA) was used to obtain cDNA from the RNA samples by using 15 µg of total RNA per sample and following the manufacturer's instructions. The GoTaq qPCR Master Mix (Promega, USA) was used for the qRT-PCR studies in accordance with the manufacturer's guidelines. Three biological replicas and three technical replicas were used in the experiment for each qRT-PCR run. The comparative CT technique (2-ΔΔCT) was used to determine the relative *CUT1* gene expressions where Mf*Actin* used as a reference gene (Schnabel & Dai, 2004).

microRNA sequencing and identification of *M. fructicola* milRNAs

The total RNA of an isolate of *M. fructicola* (Ti-B3-A3-A2) grown on PDA and FDA as described above was used for small-RNA sequencing. RNA samples from two biological replicates from each media were sent for next-generation sequencing. According to the manufacturer's instructions, the TruSeq Small RNA Library Prep Kit was used by the company. Library construction and small RNA sequencing using an Illumina HiSeq 2500 was performed by Macrogen (Macrogen Inc., Next-Generation Sequencing Service, Geumcheon-gu, Seoul, South Korea).

Adapter sequences and poor-quality reads were removed from raw reads using Trimmomatic and the quality control was done using FastQC (Andrews, 2010; Bolger et al., 2014). Using Cutadapt, the reads with poly-A tails were eliminated, and reads longer than 34 nt and shorter than 18 nt were discarded (Martin, 2011). After the cleaning steps, reads from biological replicates were pooled using the FASTX-Toolkit (Gordon A, Hannon GJ: Fastx-toolkit. FastQ/A short-reads pre-processing tools, unpublished). Using the software Bowtie (Langmead & Salzberg, 2012), the matched reads with repeat sequences in RepBase (Jurka et al., 2005) and rRNA, tRNA, snRNA, and snoRNA sequences in the Rfam database were discarded.

Filtered reads were matched to mature miRNA sequences from miRBase using Bowtie to identify known miRNA sequences (maximum of 2 mismatches allowed) (Langmead & Salzberg, 2012). MiRNA sequences that did not match those in the miRBase database were evaluated as novel microRNAs. MiRDeep2 software was used for novel miRNA prediction with default settings (Friedländer et al., 2012) (Supplementary Information). The ViennaRNA package's RNAfold utility was used to predict the hairpin formation ability of the precursor sequences of potentially novel miRNAs (Lorenz et al., 2011).

Results

Pathogenicity of *Monilinia* isolates on peach host

In vitro pathogenicity test on peach fruits was terminated on the 4th day because the surface of all fruits was covered with lesions. The typical brown rot symptoms were developed on inoculated fruits and the controls did not have lesions. Lesion measurements of the tested isolates taken during the three days were recorded. The lesion sizes of seven *M. laxa* isolates ranged from 3.975 to 6.56 cm. The eight isolates of *M. fructicola* had lesion sizes ranging from 3.425 to 5.025. (Supplementary Figure 1). Thus, all isolates were detected as pathogenic on peach fruit. In this part of the study, the level of virulence among the isolates was not taken into account. After confirmation of pathogenicity of the isolates in peach fruit, downstream analysis were continued with two isolates selected (Ti-B3-A3-2 from *M. fructicola*, Yıldırım-1 of *M. laxa*) by taking into account the criteria specified in the method section.

In silico fungal milRNA prediction

The genomes of highly virulent *M. fructicola* and *M. laxa* isolates selected were used to predict milRNA using a homology-based approach with strict filtering

criteria. The retrieved hits were filtered to ensure that the subject and query sequences were either identical or had just one mismatch. For *M. fructicola*, there were 1319 blast hits from fungal milRNAs and 103 hits from miRBase miRNAs, while for *M. laxa*, there were 1280 hits from fungal milRNAs and 142 hits. The percentage of miRBase miRNAs was found higher than the fungal miRNAs which was able to map genomes *of M. fructicola* and *M. laxa* (Fig. 1a, b). Apart from miRBase, the genomes of *M. fructicola* and *M. laxa* included many known miRNAs from *Verticillium dahliae* and *Sclerotinia sclerotiorum*. Blast hit scores were evaluated throughout the scaffolds of the genomes (Fig. 2a-b). miRNA sequence hits were quite changed among the scaffolds of *M. laxa* and many of the scaffolds did not include any hit (Fig. 2a). Except for scaffold 18, all the scaffolds of *M. fructicola* had the miRNA hits. (Fig. 2b).

Each milRNA sequence, including flanking region, was checked for hairpin-like structure formation ability. Almost 80% of the putative hairpin constructs were discarded as they did not meet requirements such as minimum hairpin length, MFE, and complementarity between miRNA and miRNA targets. Only 20.2% of milRNAs from *M. fructicola* and 16% of milRNAs from *M. laxa* were able to generate 355 and 266 hairpins, respectively.

Potential fungal milRNA targets in the transcriptomes of the pathogen and the host

Results from psRNAtarget showed that 128 milRNAs of *M. laxa* targeted 338 genes from the *P. persica* transcriptome, while 209 milRNAs of *M. fructicola* only targeted 98 genes (Fig. 3). Fungal milRNAs target a variety of locations in the *P. persica* transcriptome, including genes crucial for the cell cycle, immune response, and hormone pathways, according to the Gene Ontology study of the genes they targeted (Fig. 4).

miRNAs targeting each other between the host and the pathogens

Transcriptome data for both the host and two pathogen species were rigorously evaluated for miRNAs targeted to host-to-pathogen genes and pathogen-to-host genes. After analysis with strict filtering options, the target genes of miRNAs identified in the host transcriptome data were numerically determined as 102 for *M. fructicola* and 104 for *M. laxa*. Functional annotation analyses for these targeted genes were identified in relation to many biological processes. Notably, the most enriched biological processes in *M. fructicola* involved oxidation–reduction processes, protein phosphorylation, and transmembrane transport. Meanwhile, in *M. laxa*, the predominant processes included amino acid transport and ribosomal large subunit assembly (Fig. 5). The distribution of genes targeted in the pathogen species by the host miRNAs along the scaffolds of the fungal genomes and the location of target genes in the host targeted by pathogenic species along host chromosomes are presented visually (Fig. 6a-b).

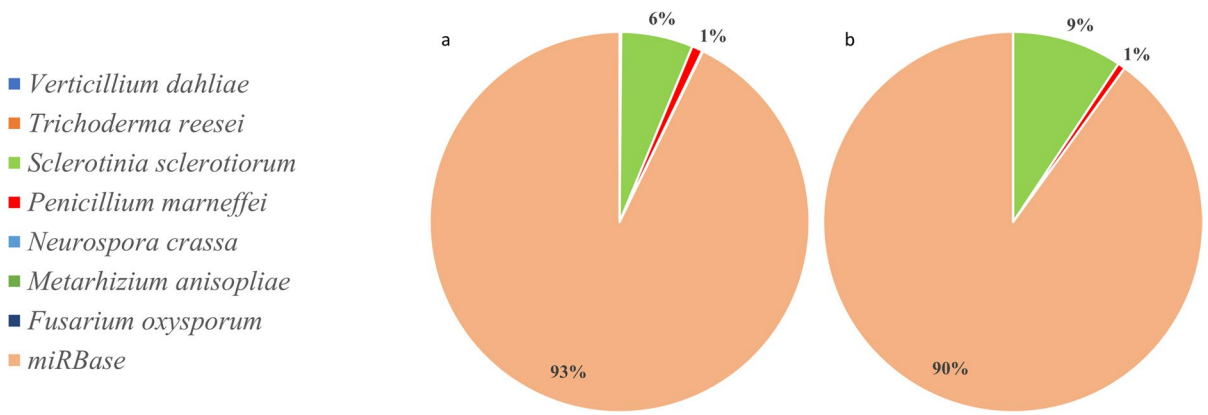


Fig. 1 The percentage of fungal and miRBase miRNAs that is able to map to genomes of *M. fructicola* (**a**) and *M. laxa* (**b**)

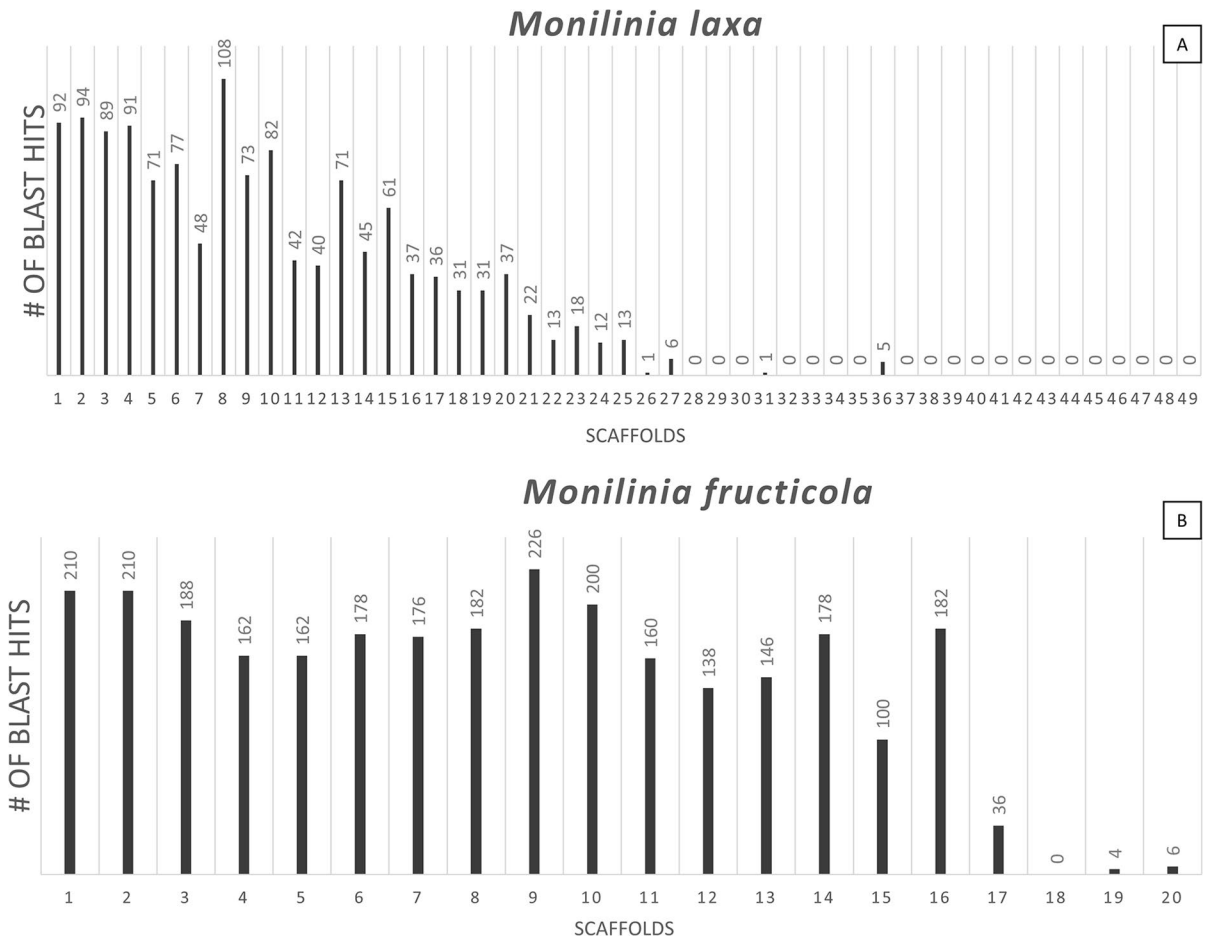


Fig. 2 Distributions of milRNAs in the genomes of *M. laxa* (**A**) and *M. fructicola* (**B**)

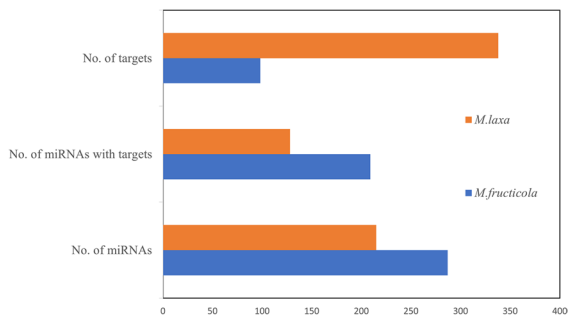


Fig. 3 No. of miRNAs: Number of milRNAs that can form hairpins, No. of miRNAs with the targets: Number of milRNAs that target P. persica genes. No. of targets: Number of P. persica unique target genes

Confirmation of success of the mimicked host environment

CUT1 (Cutinase 1) gene expression was found to be 3.88 times higher in the isolate grown on FPA than in the isolate cultured on PDA. This showed that a significant increase in the expression level of the effector gene *Cut1* was induced in FPA cultures prepared with the peach host material ($P < 0.001$). This significant increase in *CUT1,* one of the effector genes associated with pathogen virulence in the fungal pathogens (e.g. Lee et al., 2010b), detected in FPA medium containing tissues of the peach fruit provided evidence that the host environment was successfully represented.

Data quality statistics of sRNA sequences from in vitro experiments

High-throughput sequencing was used to collect about 28 million reads for each of the four sequenced samples (MF-FPA-1, MF-FPA-3, MF-PDA1, MF-PDA2) (Table 1). QC assessment of the adaptor-trimmed clean sequence reads showed that

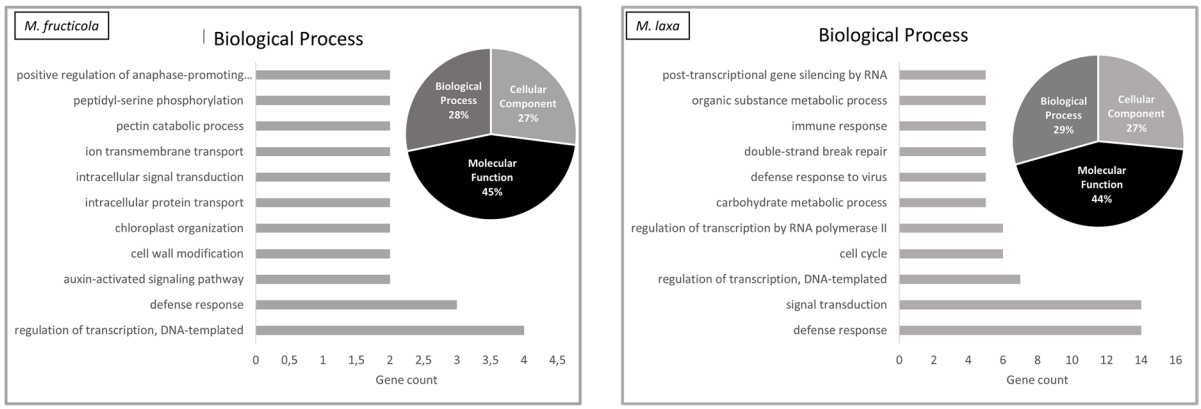


Fig. 4 Target function analysis of peach genes targeted by fungal milRNAs based on GO terms (*M. fructicola* and *M. laxa*)

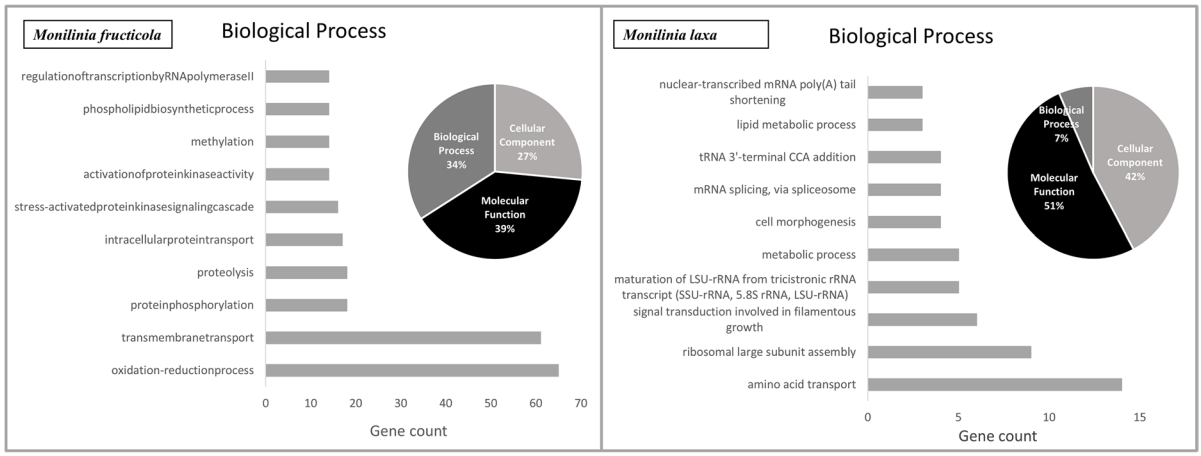


Fig. 5 Target function analysis of fungal genes targeted by peach miRNAs based on GO terms (*M. fructicola* and *M. laxa*)

the length of the clean reads ranged from 10–1500 with peaks at 15–30 nt (Supplementary Figure 2). About 15% of the sequences that linked to known rRNA, tRNA, snRNA, and snoRNA were excluded from the milRNA prediction study after clean read alignment against the Rfam database.

milRNAs experimentally expressed during *Monilinia fructicola* and peach host interaction

The miRNAs determined in the small RNA reads of the *M. fructicola* isolate cultured on the host-mimicked FPA and standard PDA media were 166 and 124, respectively. Among those, only 0.01% of the total readings were known milRNA reads found in the miRBase database. Among the rest, six novel milRNAs were predicted expressed in the FPA media and seven novel milRNAs were expressed in the PDA media. These novel miRNAs were named, and their hairpin structures were obtained (Tables 2 and 3).

The target of miRNAs expressed in two different media was predicted. The host targets of expressed and identified miRNAs of *M. fructicola* in FDA and PDA media were 90 and 62 even though the same stringent criteria were used for target prediction analysis in *P. persica* (Fig. 7).

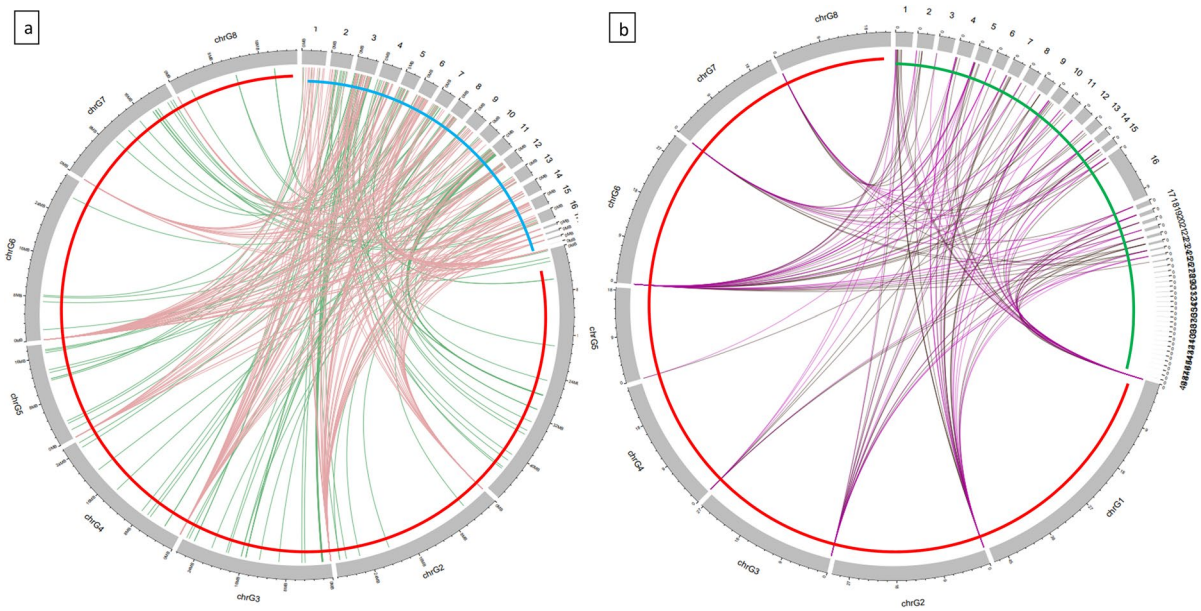


Fig. 6 **A** The distribution of *M. fructicola* milRNAs and their targets in the *P. persica* genome (green lines) and *P. persica* miRNAs and their targets in fungal genomes (red lines) are displayed. chrG1-chrG8 are the chromosomes of *P. persica* (inner red line), and the range of 1–20 are the *M. fructicola* scaffolds (inner blue lines). **B** The distribution of *M. laxa* milRNAs and their targets in the *P. persica* genome (black lines) and *P. persica* miRNAs and their targets in fungal genomes (pink lines) are displayed. chrG1-chrG8 are the chromosomes of *P. persica* (inner red line), and the range of 1–49 are the *M. laxa* scaffolds (inner green line)

Table 1 The raw data statistics and the average values of the total read bases, the number of reads, GC/AT contents (%), and the ratio of bases with Phred quality scores over 20 and 30 for each library

Sample ID	Total read bases (bp)	Total reads	GC(%)	AT(%)	Q20(%)	Q30(%)
MF-FPA-1*	1,522,744,689	29,857,739	52.1	47.9	97.98	95.78
MF-FPA-3*	1,435,524,438	28,147,538	52.02	47.98	97.91	95.66
MF-PDA-1**	1,306,863,678	25,624,778	52.19	47.81	97.99	95.74
MF-PDA-2**	1,576,196,259	30,905,809	51.92	48.08	98.08	95.99

**M. fructicola* sRNA-seq library from the samples grown on Freezed Peach Agar

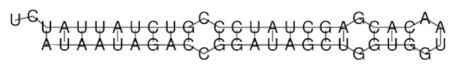
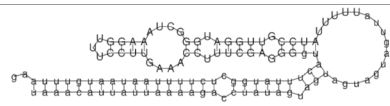
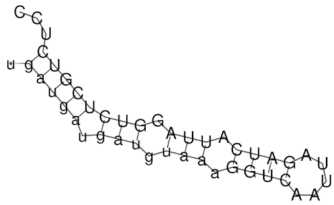
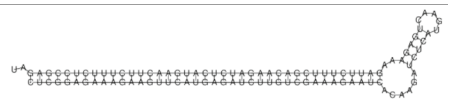
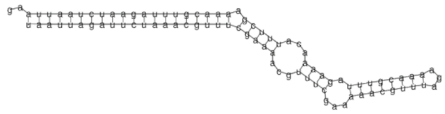
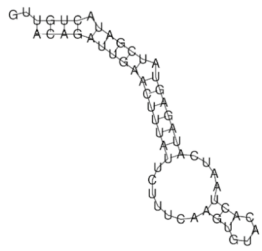
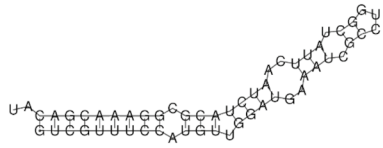
***M. fructicola* sRNA-seq library from the samples grown on Potato Dextrose Agar

Discussion

For the first time, the miRNA profiles and their targets in the peach host genome for the two most common species of brown rot pathogens were explored. In addition, differentially expressed miRNAs of *M. fructicola* were discovered in the host mimicry environment, which was designed and validated on *M. fructicola* for the first time in this study. This first study through prediction and in vitro host mimicry approaches discovered miRNAs, including all milRNA-based interactions, between two devastating brown rot pathogens and their peach fruit host.

Despite using very strict filtering in computational pipelines, a great number of miRNAs were detected in the genomes of both fungal species. Among these miRNAs detected in the pathogen genomes, those targeting the peach host were more numerous in *M. laxa* than in *M. fructicola*. This may indicate that *M. laxa* could use a more comprehensive and multifaceted approach in its pathogenicity strategy for its peach host. Or it may indicate a more aggressive strategy on

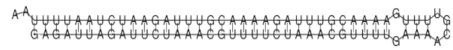
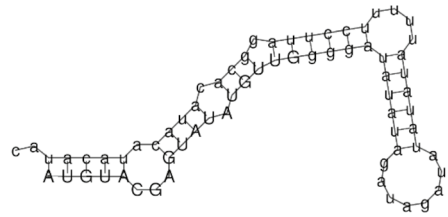
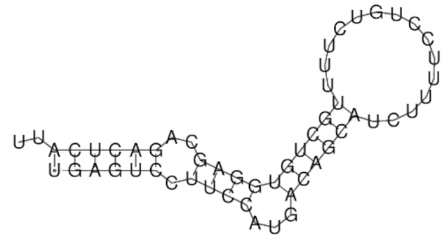
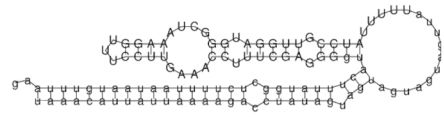
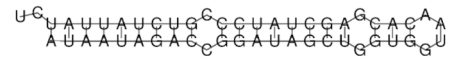
Table 2 The basic information about novel miRNAs found in MF-PDA libraries, MFE: Minimum Folding Energy (kcal/mol), mature milRNA sequences of novel milRNAs, and 2D structures predicted from premilRNAs

milRNA IDs	Library ID	MFE	Mature milRNA sequence	2D structure
VICG010000 01.1_25886	MF-PDA	-67.60	ATAATAGACCGGATAGCTGGTG	
VICG010000 16.1_4478	MF-PDA	-81.40	TAAACATTATTAAAAGACCT	
VICG010000 02.1_12298	MF-PDA	-66.40	ATCATTAGGTCTCGTCTCC	
VICG010000 02.1_2912	MF-PDA	-89.30	CATGAACTTCTTTCTCCGAGAT	
VICG010000 04.1_4550	MF-PDA	-60.50	TAATTAGATTCTAAACGTTTC	
VICG010000 05.1_4608	MF-PDA	-51.70	CATAGAGTATCGATACTGTTG	
VICG010000 04.1_6348	MF-PDA	-74.80	GTCGTTTCCATGTTGGATGAA	

the peach, as observed in the in vitro pathogenicity experiments in this study.

Protein-coding genes and intergenic regions within a genome can encompass miRNA genes (Olena & Patton, 2010). The expression and function of miRNA genes can be influenced by their genomic locations. For instance, while human miRNA genes have been discovered on all autosomes and the X chromosomes, the confirmation of a few identified miRNA genes on the Y chromosome remains incomplete (Ghorai & Ghosh, 2014). The conservation of miRNA genes largely depends on their specific locations, with selective pressure favoring miRNAs that offer advantageous functions during evolution (Chen & Rajewsky, 2007). In our study, we observed that some of the scaffolds from both pathogen species did not contain any potential predicted miRNA genes. One plausible explanation for the absence of miRNAs in these locations could be the presence of non-conserved or variable sequences. However, it is important to note that the annotations of these scaffolds require further investigation for confirmation, and our research group is actively working on the genome annotations. Another perspective being

Table 3 The basic informations about novel miRNAs found in MF-FPA libraries, MFE: Minimum Folding Energy (kcal/mol), mature milRNA sequences of novel milRNAs, and 2D structures predicted from premilRNAs

milRNA IDs	Library ID	MFE	Mature milRNA sequence	2D structure
VICG010000 04.1_6702	MF-FPA	-70.90	GAGATTAGATTCTAAACGTTTT	
VICG010000 11.1_941	MF-FPA	-68.40	ATGTACGAGTATATGTTGGG	
VICG010000 01.1_1581	MF-FPA	-65.20	GCTGTGGAGCAGACTCATT	
VICG010000 16.1_1516	MF-FPA	-81.30	TAAACATTATTAAAGACCT	
VICG010000 01.1_9611	MF-FPA	-67.60	ATAATAGACCGGATAGCTGGTG	
VICG010000 02.1_3732	MF-FPA	-63.40	GAGATTAGATTCTAAACGTTT	

discussed is the theory proposing the synthesis of new miRNAs from the antisense transcript regions of target genes (De Felippes et al., 2008) The identification of these novel miRNAs in chromosomal regions provides further support for this theory. The absence of miRNAs in certain areas might be attributed to the lack of conserved elements. Furthermore, these findings imply that last numbered scaffolds of both *M. laxa* and *M. fructicola* genomes might not encompass crucial or significant genes, thus explaining the lack of conservation of miRNAs in these regions. Overall, our study sheds light on the intricate relationship between miRNA genes and their genomic locations, emphasizing the need for further research to elucidate the functional significance and evolutionary dynamics of miRNAs in diverse fungal species.

While miRNAs in pathogenic fungal organisms target vital biological pathways in their hosts, there are miRNAs in the hosts that play a role in the control against their pathogens, as well. In a recent study, gene expression changes were investigated at the transcriptome level in the interaction between *M. laxa* and nectarine host, and it was reported that host hormone controls were also affected during this interaction (Balsells-Llauradó et al., 2020). This finding is supported with our finding that pathogen miRNAs target hormonal control systems, for example *M. fructicola* miRNAs target the auxin hormone-related pathway,

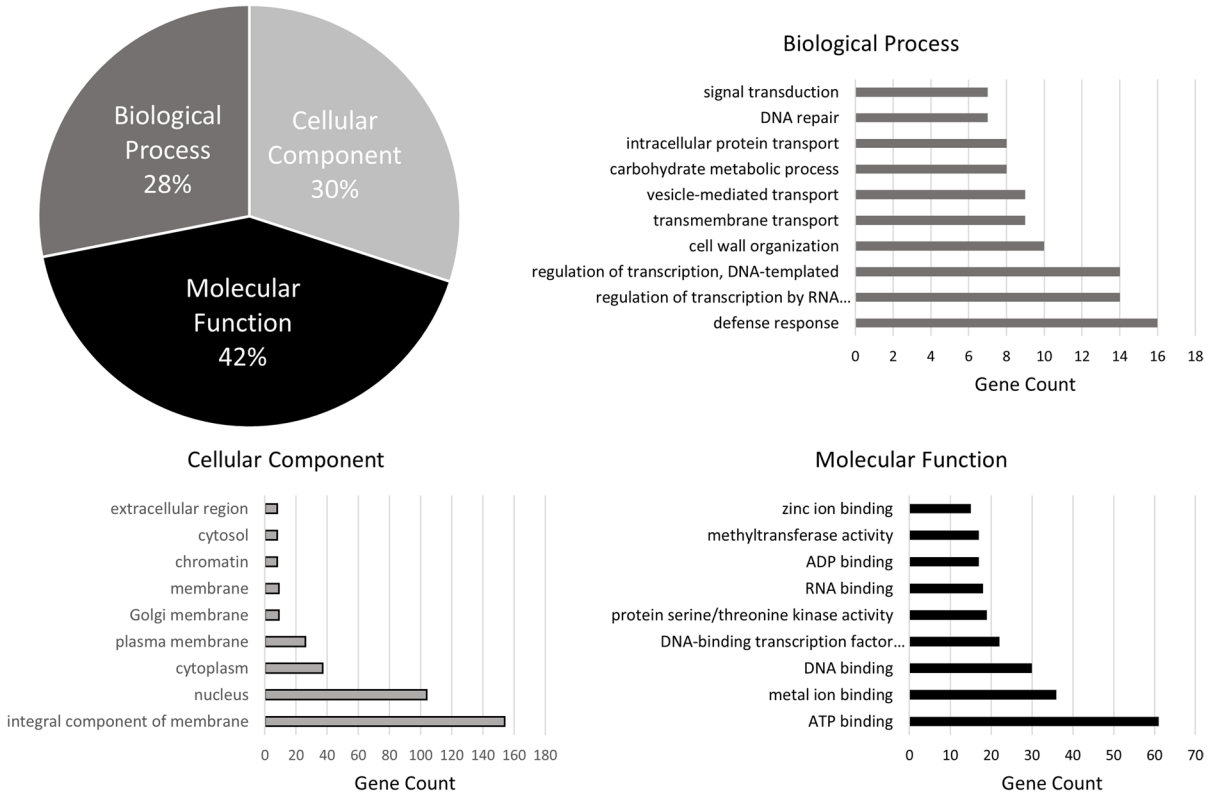


Fig. 7 Target function analysis of *P. persica* genes that were targeted by *M. fructicola* (MF-FPA) based on GO terms

thereby attempting to interfere the control of growth and development of the host as shown in this study. Besides, it has been determined that both fungal species have miRNAs that can weaken the immunological defense mechanism of their hosts, *P. persica*, and attack several vital cell functions. Specifically, mtr-miR2673a detected in *M. laxa* was found to target a gene ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase plays important in the plant immune system in *P. persica*. This suggests that when *M. laxa* infects this host, it can directly weaken the host immune system, thus rendering the plant weak against other pathogens. It has been suggested that milRNAs in the rice sheath blight pathogen *Rhizoctonia solani* mediate pathogenic mechanisms by regulating target genes related many different pathways such translocation and degradation of antimicrobial substances from the host; signal transduction; transcription factors that control pathogenic factors during the infection process; and cell wall-degrading enzymes (Lin et al., 2016). In the same study by Lin et al. (2016), it was reported that the expression of four miRNAs detected in the rice host were expressed during *R. solani* infection, but did not play a role in host immunity against *Magnaporthe oryzae*, which was previously reported in another study (Li et al., 2014). Virulence strategies of pathogens can be shaped according to different defense responses in their hosts, and host responses can provide different responses against different pathogens (Mengiste, 2012). Thus, the amount and diversity of target mRNAs of milRNAs can be expected to have significant effects on shaping pathogen aggressiveness, host immunity, and ultimately host–pathogen relationships.

Target estimates of the pathogens of miRNAs in *P. persica*, which has a much larger genome than the fungal species, were also obtained considering very strict parameters to reach the most reliable data. As expected, crucial cellular pathways in fungal processes were targeted by host miRNAs such as transport systems, oxidation–reduction pathways, and protein phosphorylation mechanisms (Fig. 5). Among those, protein phosphorylation seems a significant

post-transcriptional regulator, which modifies the structural conformation of the protein, thus, properly post-translational regulation and the down-regulation of this protein. Target genes are also be expected as associated with fungal defense and/or processes such as stress-activated protein kinase (SAPK) pathways are crucial in regulating stress responses and virulence in many fungal infections (Brown et al., 2017). On the other hand, *P. persica* miRNAs appeared to target primarily the transport of amino acids, key growth-related metabolic pathways such as filamentous growth signaling in *M. laxa*, and mainly the genes that are responsible for oxidation–reduction process, protein biosynthesis, and transportation for *M. fructicola*. These findings suggest that despite the close kinship of these species, *P. persica* uses different tactics to defeat or control *M. fructicola* and *M. laxa*.

To understand host–pathogen interactions, of course, in vivo studies allow for more real scenarios to be explored. However, due to time dependency such as fruit ripening process, physical possibilities to design in vivo studies, and RNA degradation because of RNAase released from the host or some other reasons, requires an alternative to in vivo studies. Such an good example for in vitro host mimic was recently shown for *Sclerotinia sclerotiorum* (Maximiano et al., 2021). In this study, peach host mimicry for *M. fructicola* was designed, tested and approved for the first time. It appears that the pathogen's attack movements against the host are triggered even in the in vitro environment containing peach fruit tissue particles. It is known that cutinase enzymes play a role in breaking the cutin layer of the host during the infection process of fungal pathogens and that gene expressions related to this enzyme are associated with pathogen virulence (e.g. Rogers et al., 1994; Lee et al., 2010b; Auyong et al., 2015; Villafana & Rampersad, 2020). The expression of *CUT1*, one of the fungal effector genes (Lee et al., 2010b), was up to four times more upregulated in FPA media than in traditional PDA conditions. By these results, there has also been a supportive to investigate the mimicry environments of different hosts in this pathosystem, and our team continues to work in this direction.

Novel miRNA prediction in fungal species offers new discoveries and targets regarding regulatory networks and gene expression patterns in these organisms. In this study, the six novel miRNAs expressed in FPA and the seven expressed in PDA were discovered for *M. fruticola* isolate. The computationally predicted novel miRNAs in these species require experimental validation to determine their conservation and biological roles. Mutant studies showed that some of the genes are required for certain miRNA biogenesis in *Penicillium marneffei* by specifically targeting novel miRNAs using genetic manipulation techniques, researchers have been able to investigate the impact of their loss on fungal growth, development, and pathogenicity (Lau et al., 2013). Knocking-out of a gene encoding Dicer-like protein in *Fusairum verticilloides* showed that this gene is related regulation of milRNAs and in turn affect the pathogenicity of the fungus in the interaction of maize kernels (Qu et al., 2023). Similarly, knockout or mutant studies can be planned to reveal the roles of some miRNA genes. Further research should focus on functional analysis through knock-out experiments and target validation assays.

MiRNAs involved in host–pathogen interactions are also very important molecular targets for disease control strategies. These reciprocal miRNA targets in the *Monilinia*-peach pathosystem were elucidated by using both in silico and in vitro approaches. This study presented the first important data in terms of miRNAs specific to this pathosystem and became a model study with some of the approaches used. Although fungal pathogen-host interactions are a versatile biological complex, important data have been obtained regarding the role of miRNAs in this pathosystem. In addition, various target miRNAs that can be evaluated in disease management have been obtained. Furthermore, in order to continue the research in this direction in more detail, it is important and necessary to identify differentially expressed miRNAs by using a larger number of samples and replicates and to confirm the associated candidate miRNA expressions with real-time PCR. These additional analyses will enable us to confirm the specific roles played by individual miRNAs in the intricate dynamics of the *Monilinia*-peach interaction.

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Author contributions KA performed both dry- and wet-lab works and data analyses under supervision of HO. HO directed the research, recommended data analyses, and contributed to all parts. Authors wrote the article together and approved the manuscript.

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Data availability The data generated and used in this study are available in NCBI GenBank under the BioProject with the accession number PRJNA1014700. Besides, predicted miRNA sequences of both *M. laxa* and *M. fructicola* and miRNA sequences of *M.fructicola* from sRNA sequencing data are provided in Supplementary Information file.

Declarations

Competing interests The authors declare no competing interests.

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