T.C.

ÇANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

PREDICTION OF MICRORNAS IN *MONILINIA* SPP. AND THEIR ROLES IN HOST-PATHOGEN INTERACTIONS

MASTER OF SCIENCE THESIS

KÜBRA ARSLAN

Thesis Supervisor

ASSOC. PROF. HİLAL ÖZKILINÇ

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PLAGIARISM DECLARATION PAGE

I declare that all the information and results offered in visual, audio and, written form are obtained by myself observing the academic and ethical rules. Moreover, all other results and information referred to in the thesis but not specific to this study are cited.

Kübra ARSLAN

24/06/2022

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KÜBRA ARSLAN

Çanakkale, June 2022

ÖZET

MONILINIA TÜRLERİNDE MİKRORNA'LARIN TAHMİNİ VE KONUKÇU-PATOJEN ETKİLEŞİMLERİNDEKİ ROLLERİ

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MikroRNA'lar, konukçu-patojen etkileşiminde önemli temel rollere sahiptir. Enfeksiyon sürecinde patojenlerin miRNA'ları konukçuyu, konukçu miRNA'ları ise patojeni hedefleme yeteneğine sahiptir. Bu tezde, en yaygın iki kahverengi çürüklük patojeninin (*Monilinia fructicola* ve *M. laxa*) miRNA profilleri ilk kez tanımlanmıştır. Her iki tür için seçilen izolatların in vitro'da yüksek virülenslik gösterdikleri doğrulanmıştır ve bu izolatların genomları üzerinden farklı biyoinformatik yöntemler kullanılarak miRNA tahminleri yapılmıştır. Ayrıca, *M. fructicola* türünün şeftali (*Prunus persica*) konukçusu ile etkileşimde olduğu sırada ifade ettiği milRNA dizileri keşfedilmiştir. Bu amaçla konukçu ortamı *in vitro* olarak simüle edilmiştir ve yeni nesil dizileme teknolojileri kullanılarak total küçük RNA dizilerinin okumaları alınmıştır, işlenmiştir ve biyoinformatik araçlarla analizleri gerçekleştirilmiştir. Böylece patojen tarafında konukçu ile etkileşimde hem patojenin kendisindeki hem de konukçusundaki hedef diziler belirlenmiştir. Bu dizilerin genomdaki lokasyonları da tespit edilmiştir.

Bulunan miRNA'ların hedef fonksiyon çalışmalarına göre, bu iki fungal türün *P. persica* türünü farklı yollarla enfekte ettiği ortaya çıkmıştır. Öte yandan, daha önce tanımlanmış şeftali miRNA'ları için *M. fructicola* ve *M. laxa türlerinin* transkriptomlarında hedef fonksiyon analizleri de yapılmıştır. Bulgular, *P. persica*'nın enfeksiyonlarla mücadele etmek için birden fazla mekanizma kullanıyor olabileceğini ortaya koymuştur. milRNA'ların ve hedeflerinin *M. fructicola* enfeksiyon sürecindeki rolü ilk kez araştırılmıştır. Enfeksiyon

sürecine dahil olan *M. fructicola* miRNA'larını karakterizasyonu sürecinde yeni miRNA'lar da keşfedilmiştir.

Tahribat yeteneği yüksek olan patojenler ve bunların konukçu ile etkileşimleri söz konusu olduğunda, milRNA'ların belirlenmesi ve varsayılan milRNA hedeflerinin aranması büyük bir ilerlemedir. Bu tez fungal fitopatojenlerdeki miRNA'lar ve işlevleri hakkında sınırlı bilgi bulunan bu alan için önemli bir temel bilgi sunmuştur. Ayrıca elde edilen bulgular üzerinden bu çalışmanın bir sonraki aşaması olarak bu patosistemde yeni patojen kontrol stratejileri tasarlanabilecektir.

Anahtar Kelimeler: Konukçu-patojen interaksiyonları, miRNA'lar, *Monilinia spp.*

ABSTRACT

PREDICTION OF MICRORNAS IN *MONILINIA* SPP. AND THEIR ROLES IN HOST-PATHOGEN INTERACTIONS

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MicroRNAs have important fundamental roles in host-pathogen interaction. During the infection process, miRNAs of pathogens have the ability to target the host, and host miRNAs to target the pathogen. In this thesis, miRNA profiles of the two most common brown rot pathogens (*Monilinia fructicola* and *M. laxa*) were described for the first time. It was confirmed that the selected isolates for both species showed high virulence *in vitro*, and miRNA estimations were made on the genomes of these isolates using different bioinformatics methods. In addition, milRNA sequences have been discovered that M. fructicola expresses when interacting with its peach (*Prunus persica*) host. For this purpose, the host environment was simulated in vitro and total small RNA sequences were read, processed, and analyzed using bioinformatics tools using next-generation sequencing technologies. Thus, target sequences in both the pathogen itself and the host were determined in interaction with the host on the pathogen side. The locations of these sequences in the genome have also been determined.

According to the target function studies of the miRNAs found, it was revealed that these two fungal species infect *P. persica* in different ways. On the other hand, target function analyzes were also performed in the transcriptomes of *M. fructicola* and *M. laxa* for the previously identified peach miRNAs. The findings revealed that *P. persica* may be using more than one mechanism to fight infections. The role of milRNAs and their targets in the *M. fructicola* infection process has been investigated for the first time. In the process of characterizing *M. fructicola* miRNAs involved in the infection process, new miRNAs were also discovered.

The identification of milRNAs and the search for putative milRNA targets is a major advance when it comes to highly destructive pathogens and their interactions with the host.

This thesis provided an important basis for this field, where there is limited information about miRNAs and their functions in fungal phytopathogens. In addition, in the next stage of this study, new pathogen control strategies can be designed in this pathosystem based on the findings obtained.

Keywords: Host-pathogen interactions, miRNAs, *Monilinia* spp.

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ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µl	Microliter
BLAST	Basic Local Alignment Search Tool
bp	Base-pair
cDNA	Complementary DNA
Chr	Chromosome
CUT1	Cutinase 1
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled Water
FPA	Freezed Peach Agar
g	Gram
GO	Gene ontology
kb	Kilobase
MF	*Monilinia fructicola*
MFE	Minimum Free Energy
Mg	Milli gram
milRNA	Micro RNA like small RNA
miRNA	Micro RNA
miRNA*	The complementary sequence of miRNA in pre-miRNA structure
mRNA	Messenger RNA
ng	Nanogram
NGS	Next-generation Sequencing
No	Number
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
qRT-PCR	Quantitative Real Time PCR
RNA	Ribonucleic acid
RNAseq	RNA sequencing
RPM	Revolutions per minute
rRNA	Ribosomal RNA

SAM	Sequence Alignment Map
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
sRNA	Small RNA
sRNAseq	Small RNA sequencing
tRNA	Transfer RNA
WGS	Whole Genome Sequencing

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CHAPTER 1
INTRODUCTION

1.1. *Monilinia* Species and Brown Rot Disease

Monilinia fructicola and *Monilinia laxa* are the primary pathogens responsible for of brown rot and blossom blight on stone fruits (Imre J. Holb, 2008; Hrustić et al., 2013; Ozkilinc et al., 2020). Taxonomically, they are *Ascomycetes* fungi from to the *Sclerotiniaceae* family and the *Helotiales* order. These pathogens are responsible for brown rot of stone fruits which is one of the most destructive and major diseases of those hosts. Besides these pathogens are effecting as post-harvest pathogens and could cause infections during the storage (Martini & Mari, 2014). *Monilinia fructicola* and *M. laxa* can be found in mummified fruits (either on the ground or still on the tree) twig and branch cankers from the previous year (I. J. Holb, 2004). Both sources have the potential to release spores that infect blooms and young shoots (I. J. Holb, 2004). A mummified fruit that has fallen to the ground around blossom time produces apothecia which contain spores (Figure1) . Wind or insects carry these spores (ascospores) to open or unopened blooms and young branches (I. J. Holb, 2004). The disease cycle is depicted in Figure 1.

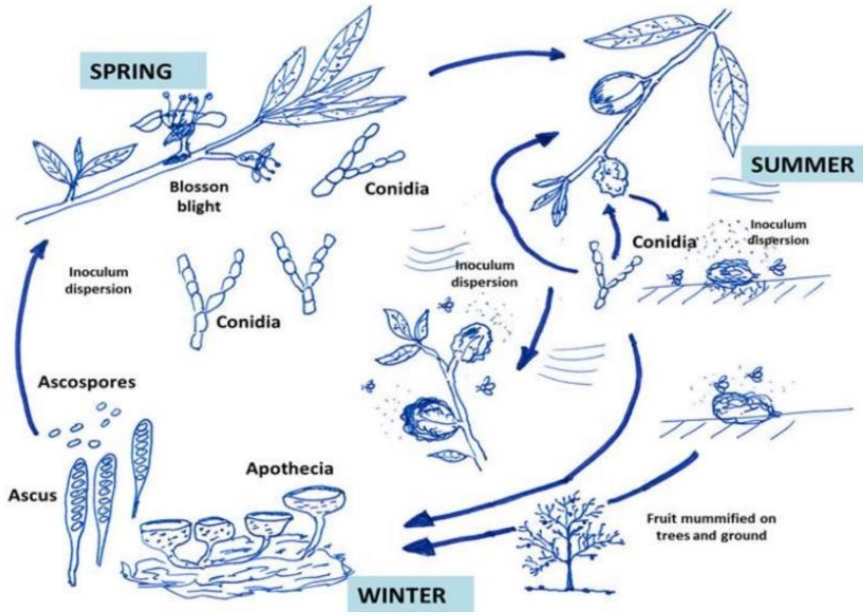


Figure 1. Brown Rot Disease Cycle (Obi et al., 2018).

1.2. Peach (*Prunus persica*): One of the Hosts of *Monilinia* Pathogens Causing Brown Rot Disease

The *P. persica* is the third most important global tree crop within the economically important Rosaceae family, and China is the greatest producer, followed by European countries (Spain and Italy) and Turkey (Food & Agriculture Organization of the United, 1997). Throughout the fruit development, the degree of resistance to *Monilinia* spp. infections varies. Sensitivity to *Monilinia spp*. is the highest during the early phases of fruit development, decreases during the green fruit or pit hardening stage, and then increases again throughout the ripening stage (Mari et al., 2003). One of the most common species in this genus is *M. fructicola*, which is more common especially during the ripening period of fruits (Mari et al., 2003; Ozkilinc et al., 2020). This species is also one of the most important quarantine pathogens on the EPPO A2 quarantine list. The estimated economic impact of peach brown rot disease is $170 million annually (https://www.rosbreed.org/home).

The main control strategies against this disease are different precautions such as the use of resistant cultivars, the cleaning of mummified fruits in the fields and lowering the post-harvest storage temperature, as well as chemical control with fungicide applications (Balsells-Llauradó et al., 2020; Martini & Mari, 2014). The emergence of this factor as a problem especially during fruit ripening and post-harvest storage processes also limits the application of chemical control methods such as fungicide. Thus, understanding host-pathogen interactions in detail may provide new insights to control the pathogen more effectively.

1.3. MicroRNAs and Their Roles

MicroRNAs (miRNAs) are small non-coding RNA group (consisting of 18-22 nucleotides) that play a key role in RNA silencing and post-transcriptional gene regulation (Bartel, 2004). A single miRNA molecule can reduce the expression of multiple genes and a gene can be targeted by several miRNA molecules(Bartel, 2009). They can suppress gene expression by base-pairing with complementary mRNA strands, leading mRNA to be

cleaved, destabilized by shortening the poly(A) tail, or translational inhibition (Bartel, 2009; Fabian et al., 2010).

Their gene regulation system over the all organisms is involved in a variety of processes, including cell division, apoptosis, carcinogenesis, and even defense responses to biotic stimuli (Croce & Calin, 2005; Hatfield et al., 2005; Lee et al., 2010). As a result, predicting an organism's miRNA profiles can help researchers better comprehend a variety of biological processes and mechanisms.

As pathogen develops pathways to facilitate the infection process, plants encounter more pathogen attacks and develop an immune system. Pathogens are known to hijack the host machinery after successful infection and utilize it for their own growth and proliferation. Resistant plants, on the other hand, can fend off disease attacks in a variety of ways. Apart from the roles of miRNAs in organ morphogenesis, signal transduction pathways or biotic and abiotic stress in plants, it has been observed that miRNAs also play a role in complex control mechanisms during plant-fungi relationships (Yang et al., 2007). miRNAs are involved in the post-transcriptional regulation of gene expression in effector-triggered immunity (ETI) and pathogen triggered immunity (PTI) of the host (Katiyar-Agarwal & Jin, 2010). It has been revealed that transcribed miRNAs are involved in the regulation of the expression of genes involved in disease resistance proteins, serine/threonine kinases, and some transcription factors (M. Chen & Cao, 2015). Thus, a new link has emerged between miRNAs and fungal infection.

1.3.1. Evolution and Identification of MicroRNAs

miRNAs are phylogenetically significant because they are transcribed by primary RNA polymerase III and have a low rate of evolution (1 substitution per 100,000 years) (Peterson et al., 2009; Wheeler et al., 2009). There are some species with no novel miRNA family in their genome for 450,000 years, according to a molecular paleontology study (Wheeler et al., 2009). As a result, they are well preserved in plant and animal species, as evidenced by the inter-kingdom conservation of miRNAs belonging to the miR854 family (Arteaga-Vázquez et al., 2006). Therefore, despite suggestions that fungal milRNAs originated independently from plant and animal miRNAs (Lee et al., 2010), recent

investigations on their identification utilised the well-developed plant and animal miRNA prediction pipeline.

Microarray screening (Barad et al., 2004), degradome sequencing (Pantaleo et al., 2010), short RNA sequencing (Landgraf et al., 2007), and computational prediction (Mathur et al., 2020) are some of the experimental and computational methods for identifying miRNAs. Although experimental methods are more valuable and confirm the presence or function of a miRNA, wet-lab procedures can sabotage the detection of miRNAs with diverse targets or miRNAs that are only expressed under certain conditions. On the other hand, developments in next-generation sequencing technologies and computational tools have made it possible to predict miRNAs from genome data and transcriptome data could help the discovery of miRNAs, expressed at a specific time and condition (Jiang et al., 2012).

1.3.2. MicroRNAs of *Monilinia spp.*

Monilinia fructicola and *M. laxa* are the two important pathogenic species causing brown rot of *P. persica* as well as some other stone fruits worldwide (Imre J. Holb, 2008; Hrustić et al., 2013; Ozkilinc et al., 2020). Despite their destructive damages on their hosts, there are many unexplained phenomena associated with the development of the disease on fruit. Recently, a study demonstrated a comprehensive transcriptomic approach using dual-RNAseq analysis during the process by which *M. laxa*, one of the species closest to *M. fructicola*, infecting the nectarine fruit (Balsells-Llauradó et al., 2020). It was shown that *M. laxa* infection in plants altered the expressions of plant defense hormones and different plant-pathogen interaction proteins (Balsells-Llauradó et al., 2020). Thus, to better delineate the host-pathogen interactions during infection, discovery of all miRNA genes in the pathogen genomes and their targets in host genomes is essential. Then, experimentally, their expression profiles could be better interpreted.

1.4. The Aim of the Study

MicroRNAs are known to have many important roles and may shape host-pathogen interactions. Pathogen miRNAs may have been targeting both pathogen itself as well as their hosts to regulate the interaction processes. Relation of miRNAs and their pathways to the fungal infection process of şeftali (*Prunus persica*) by the pathogenic species, *M. fructicola* and *M. laxa,* have not been known and revealing this may open new avenues to understand this pathosystems. Thus, in this study, it was aimed to (i) predict the miRNA repertoires of two fungal species (*M. fructicola* and *M. laxa*) from their genomes and (ii) to find out the targets of the predicted milRNAs of the pathogens in the transcriptome of the host (*P. persica*) for exploring the target mRNAs of the host to interfere (iii) to discover the target sites of milRNAs of the *P. persica* host in the transcriptome of the pathogen species in order to get knowledge about cross-kingdom RNA interference bilaterally, and (iv) to identify milRNAs and their targets in *P. persica* during infection for *M. fructicola*. Both genomics and transcriptomics data from host and pathogen were evaluated by using diverse bioinformatics approaches.

CHAPTER 2
PREVIOUS STUDIES

The first miRNA study was conducted on *Caenorhabditis Elegans* lin-4 gene which regulates larval development by suppressing lin-14 gene (Pasquinelli et al., 2000; Reinhart et al., 2000) Afterwards miRNAs have been studied intensively on higher eukaryotes. The first fungal milRNA (micro RNA like small RNAs) study was performed *Neurospora crassa* which was about miRNAs biogenesis (Lee et al., 2010). After the discovery of miRNAs from *N. crassa*, miRNAs have been studied on many fungal organisms (R. Chen et al., 2014; J. Zhou et al., 2012).

In a study conducted on *Fusarium oxysporum*, researchers reported that 8 families of miRNA candidates were identified by using degradome sequencing, and small RNA population of *F. oxysporum* shared common features with the small RNA populations of *N. crassa* (R. Chen et al., 2014). In a medically important fungus called *Penicillium marneffei*, researchers found that differential expression of miRNAs involved in the control of thermal dimorphism (Lau et al., 2013). In a study conducted on *Trichoderma reesei*, a species that has gained great importance in the industry with its ability to degrade cellulose, was aimed to determine the miRNAs involved in cellulase production in this species (Kang et al., 2013). It has been stated that miRNAs, which play a role in cellulase induction, could be an important tool in industrial applications (Kang et al., 2013). During the *Pythium aphanidermatum* infection of, a necrotrophic fungal oomycete with high destructive ability, of turmeric, host miRNAs in the study were found to be suppressed during fungal stress (Chand et al., 2016). The decrease in miR167 expression caused an increase in the synthesis of NAC protein, which is a plant immune system element (Chand et al., 2016).

Apart from the experimental identification of fungal miRNAs which take part in the infection process, there is one recent study reporting *Fusarium oxysporum* milRNAs which target its host transcriptome. In the study, researchers predict milRNAs of *F. oxysporum* by using the miRNA data from miRbase and other fungal studies (Mathur et al., 2020).

In a study conducted on *Botrytis cineria* which is a closely related species of *Monilinia fructicola* and *M. laxa* reports the first signals about miRNAs involving a host-pathogen relationship (Jin & Wu, 2015). In a study conducted on *Sclerotinia sclerotiorum* which is a necrotrophic fungus that causes white mold disease in canola (*Brassica napus*) 53

new 227 previously known miRNAs belonging to *B. napus* were identified during *S. sclerotiorum* infection by using degradome sequencing technology, (Cao et al., 2016).

Studies on the roles of miRNAs increased between 2010 and 2016. A decline was observed until 2020 and miRNAs are now again drawing the attention in fungal systems. Inadequacy of the control strategies for fungal infections and comprehension of the importance of the host-pathogen interactions might be the cause of this regrowing interest.

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

3.1.1. Data Collection

The genome data of two isolates (one *M. fructicola* and one *M. laxa*) were chosen from our own data sources (By Ozkilinc Lab). The whole-genome sequences were preprocessed previously by our group (Arslan et al, unpublished) and those were ready to use for this study. Isolates per species from the collection were chosen as one representative by considering their virulence on apple (Ozkilinc et al., 2020) and peach hosts as explained in the following sections. The transcriptome and its annotation data of *M. fructicola* (GCA_008692225.1) and *M. laxa* (GCA_009299455.1) were retrieved from GenBank provided by Angelini et al. (2018). The transcriptome and annotation data of *P. persica* (AKXU02000000) were retrieved from GenBank and submitted by Verde et al. (2013).

3.2. Methods

3.2.1. Assessing Pathogenicity of *Monilinia* spp. Isolates on Peach Host

For the pathogenicity experiment, peaches (cultivar: Elegant Lady) with no visible lesion and similar sizes were chosen. Peach fruits were washed with tap water, 70% ethanol, and sterile distilled water and then dried with a sterile clean paper towel respectively. Seven *M. laxa* and eight *M. fructicola* isolates were selected for the pathogenicity experiment since the data for their genomes and pathogenicity levels on apple were available (Ozkilinc Lab). Mycelial plugs (1 cm) were taken from 10-day-old cultures of a representative collection of *M. fructicola* grown on Potato Dextrose Agar (PDA) and used for inoculation. Two peaches were inoculated on both sides of fruit per isolate. PDA media plugs were used as controls. After inoculations, peaches were placed in sterile clean plastic containers and incubated in the climatic chamber room at 23°C in dark.

Lesions were measured perpendicularly at day 1,2 and 3 post inoculations. The size of the first inoculum was discarded. Since there was no significant difference between replicates, average lesion sizes were considered. For genome prediction studies, one *M. fructicola* (Ti-B3-A3-2) and one *M. laxa* (Yildirim-1) isolates with high virulence were considered. Since only one species were used for miRNA sequencing, one high virulent *M. fructicola* isolate was chosen for sequencing.

3.2.2. Possible Fungal milRNA Prediction from *Monilinia spp*. Genomes

Since all possible miRNA genes were screened in the genomes of *M. fructicola* and *M.laxa*, milRNA to refer to 'microRNA like' abbreviation was preferred. The pipeline designed by Mathur et al. (2020) were used to predict milRNAs in the genomes of *M. fructicola* and *M. laxa* with small modifications. miRNA sequences of known plant, animal, viral species were retrieved from miRbase (Kozomara & Griffiths-Jones, 2014) and the miRNA sequences of fungal organisms (*Fusarium oxysporum, Verticcilium dahliae, Neurospora crassa, Penicillium marneffei, Sclerotinia sclerotiorum, Trichoderma reesei, Metarhizium anisopliae, Botrytis cinerea* (already in miRbase) were retrieved from recently published studies (R. Chen et al., 2014; Kang et al., 2013; Lau et al., 2013; J. Zhou et al., 2012; Q. Zhou et al., 2012).

The miRNA sequences were combined, and duplicate hits were removed to build a query for a BLAST search (https://ftp.ncbi.nlm.nih.gov/blast/ executables/blast+/LATEST/). The NCBI blastn search was performed by using whole miRNA data as query and *M. laxa* and *M. fructicola* genomes as subjects with strict filtering parameters (-evalue 0.1, -penalty -1, -ungapped ,-word_size 7, -max_hsps 5, -perc_identity 95).

The chromosomal positions of the acquired milRNAs were used to retrieve flanking regions of the milRNAs. The flanking regions were retrieved by using BEDtools -slop option with an optimal window size of 100 nt (Quinlan & Hall, 2010). These flanking regions were checked for the ability to form a hairpin structure by using miRNAfold webserver (Tav et al., 2016). Only the hairpins that have optimal Minimum Free Energy (MFE) and the hairpin size bigger than 70 nt were identified as possible milRNAs, and used for downstream analysis.

3.2.3. Prediction of Possible Fungal milRNA Targets in *P. persica* Transcriptome

Possible milRNAs were uploaded to psRNATarget server and *P. persica* transcriptome data were chosen as the cDNA library (Dai & Zhao, 2011). According to the default parameters only the best 5 targets of milRNAs in *P. persica* transcriptome were evaluated. The locations of these milRNAs targets were extracted from *P. persica* transcriptome by using in-house bash scripts. The target sequences were annotated, and target function analysis based on GO terms was performed by using goFEAT with default parameters (Araujo et al., 2018).

3.2.4. Prediction of Possible P. persica milRNA Targets in the Fungal Transcriptomes

Only the best target per one *P. persica* miRNA sequence was evaluated by using psRNAtarget on both *M. fructicola* and *M. laxa* transcriptome. Annotation of the sequences targeted by *P. persica* miRNAs was done manually according to the reference genome annotations of *M. fructicola* and *M. laxa*. For visualization of all possible targets of pathogen miRNAs considering both the host and the pathogen Shinycircos was used (Yu et al., 2018).

3.2.5. Mimicking Pathogenicity on Freezed Peach Agar (FPA)

Since RNA isolation was not successful in detached fruit inoculation experiments, another mimicking the host environment approach byconsidering the study by (Maximiano et al., 2021) followed. For validation and quantification of FPA induce pathogenic activities in *M. fructicola*, the *CUT1* gene (which is an important effector gene of *M. fructicola*) (Wang et al., 2002) expression was measured for Ti-B3-A3-2 isolate. CUT1 gene is an important effector gene which is secreted by the pathogen to degrade the outer surface of the fruit hence up-regulation of CUT1 gene expression can show that media induces pathogenicity. Primers for M. fructicola *CUT1* primers was previously designed by our lab group (Fidanoglu and Ozkilinc, unpublished data). For this, mycelial plugs were taken from 10-day-old PDA

cultures of isolates and placed on fresh PDA and FPA (20% Freezed dried peach and 10% g Agar). Approximately 100 mg samples grown on FPA, and PDA were first scraped from the filter paper and ground in liquid nitrogen using a mortar and pestle. Frozen tissues were immediately homogenized in TRizol reagent and centrifuged at 14000 RPM for 1 min to get rid of contamination of cell debris. Phase separation was obtained by using chloroform. After precipitation in isopropanol at 80°C for 24 hours, samples were washed with pure ethanol and diluted in RNAse-free water. RNA quantification was performed using the Fluorimeter method Qubit® RNA Assay Kit (InvitrogenTM).

cDNA was synthesized from 15 µg of total RNA by using the GoScript Reverse Transcription System (Promega, USA) by following the manufacturer's instructions. The qRT-PCR experiments were performed using the GoTaq qPCR Master Mix (Promega, USA) according to the manufacturer's instructions. The experiment was performed using three biological replicas and three technical replicates in each qRT-PCR run. Relative *CUT1* gene expressions were calculated using the comparative CT method ($2^{-\Delta\Delta CT}$).

3.2.6. sRNA, cDNA Library Construction and Next-Generation Sequencing

Total RNA isolation from the Ti-B3-A3-A2 isolate grown on FPA and PDA (two biological replicates) was performed using the same protocol in the Section 3.2.5. The quality and quantity of total RNAs were analyzed by using Qubit and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). For the library construction TruSeq Small RNA Library Prep Kit was used according to the manufacturer's protocol. Libraries were then submitted to Illumına HiSeq 2500 small RNA sequencing (Macrogen Inc., Next-Generation Sequencing Service, Geumcheon-gu, Seoul, South Korea).

3.2.7. sRNA Data Analysis

Raw reads obtained from sRNAseq were first subjected to quality check, with the removal of low-quality reads and adapter sequences by using Trimmomatic and Fastqc (Bolger et al., 2014). The reads with poly-A tail were removed and reads longer than 34nt and shorter than 18nt were discarded by using Cutadapt (Martin, 2011). The clean reads obtained from each media were combined by using FASTX-Toolkit (Assaf & Hannon,

2010). The reads were aligned with rRNA, tRNA, snRNA and snoRNA sequences in the Rfam database (Martin, 2011), and repeat sequences in RepBase (Jurka et al., 2005) by using Bowtie (Langmead & Salzberg, 2012) and aligned reads were discarded from downstream analyses.

3.2.8. Identification of milRNAs in *M. fructicola*

The filtered reads were aligned against known mature miRNA sequences from the miRBase using Bowtie (maximum 2 mismatches allowed) to identify known miRNA sequences (Langmead & Salzberg, 2012). The unique reads that do not align against the miRBase are used for novel miRNA prediction. For novel miRNA prediction miRDeep2 package was used with default parameters (Friedländer et al., 2012). The precursor sequences of potentially novel miRNAs were predicted by using the RNAfold utility of the ViennaRNA package (Lorenz et al., 2011).

3.2.9. Target Prediction of milRNAs and Statistical Analysis

To identify FPA induced milRNAs, known milRNAs and novel milRNAs were combined and counts data for four sample were obtained by aligning milRNA sequences to genome by using Bowtie v2 and idxstats function in Samtools (Li et al., 2009). Raw miRNA counts from each sample were used to normalize expressions of milRNAs. The fold changes of milRNAs levels in samples grown on FPA were calculated relative to that in PDA. The miRNAs with fold-change in expression $\log2(FPA/PDA) \geq 1$ and adjusted *p*-value < 0.05 were considered as differentially expressed miRNAs, DEseq2 tool was used to identify the differentially expressed miRNAs (Love et al., 2014).

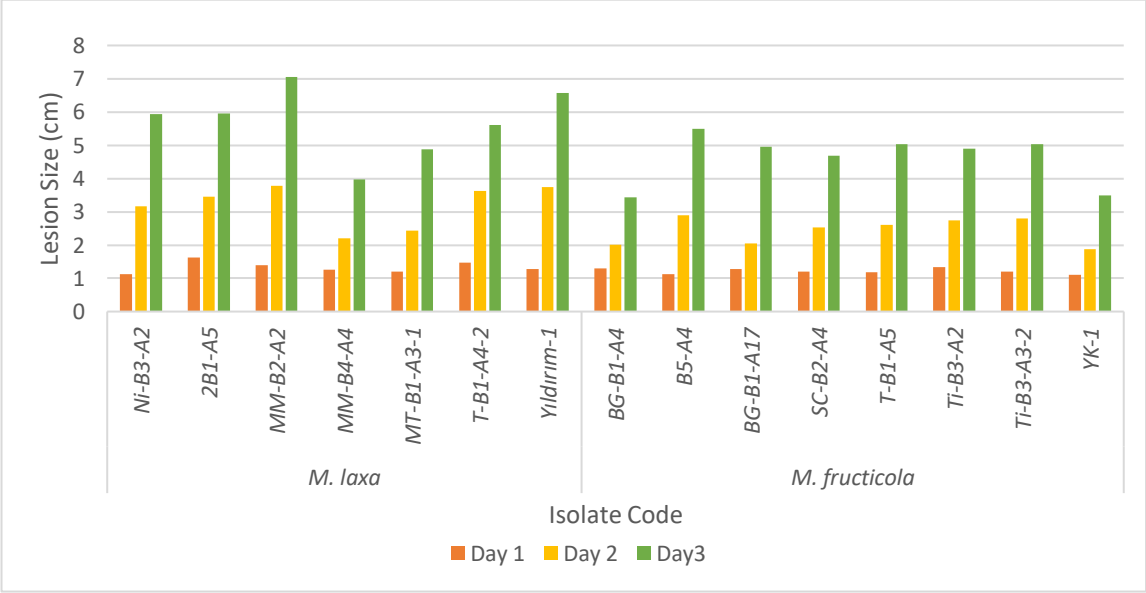
For target prediction, differentially expressed milRNAs and transcriptome sequences were uploaded to the psRNATarget server (Dai & Zhao, 2011). The targets of MF-FPA1 and MF-FPA2 miRNAs were also checked with *P. persica* transcriptome which is already present in the server.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Pathogenicity of *Monilinia* Isolates on *P. persica*

Typical brown rot symptoms developed throughout the duration of the *in vitro* pathogenicity experiment. No lesions were observed on control fruits. The experiment was ended on the 4th day since the lesions covered the whole surface in some fruits. There was no significant difference between lesion sizes of the replicates, hence the mean of the total lesions was evaluated per isolate. Seven *M. laxa* isolates showed variable pathogenicity levels in the range of 3.975-6.56 cm. Pathogenicity levels of eight *M. fructicola* isolates ranged between 3.425-5.025 (Figure 2). For downstream analysis, one highly virulent isolate was chosen from each species (Ti-B3-A3-2 from *M. fructicola*, Yıldırım-1 of *M. laxa*)

Figure 2. The measurements of pathogenicity experiment. Average lesion sizes for each isolate were shown as for each measurement day Day 1: orange, Day 2: yellow, Day 3, green).



4.2. Fungal milRNA Prediction

For milRNA prediction from the genomes of highly virulent *M.fructicola* and *M. laxa* isolates, a homology-based approach was used with strict filtering parameters. The retrieved hits were filtered to ensure that the query and subject sequences were either identical or had only one mismatch. There were 1319 blast hits from miRBase miRNAs and 103 hits from fungal milRNAs for *M. fructicola*, 1280 hits from miRBase miRNAs and 142 hits from fungal milRNAs for *M. laxa* (Figure 3). Apart from miRBase, mostly published miRNAs of *Verticillium dahliae* and *Sclerotinia sclerotiorum* were found in the genomes of *M. fructicola* and *M. laxa* The distribution of blast hits through the scaffolds of the genomes of *M. fructicola* and *M. laxa* was also evaluated and represented (Figure 4-5).

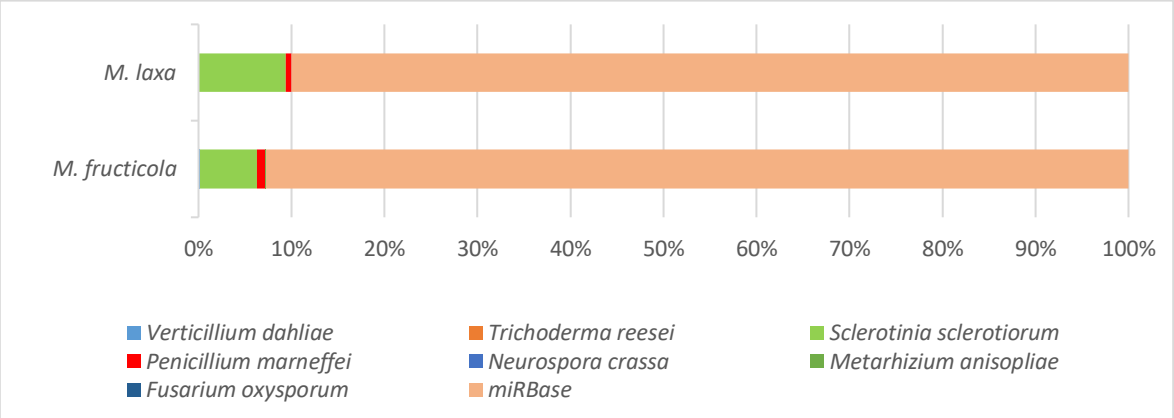


Figure 3. The percentage of fungal and miRBase miRNAs that is able to map to genomes of *M. fructicola* and *M. laxa*.

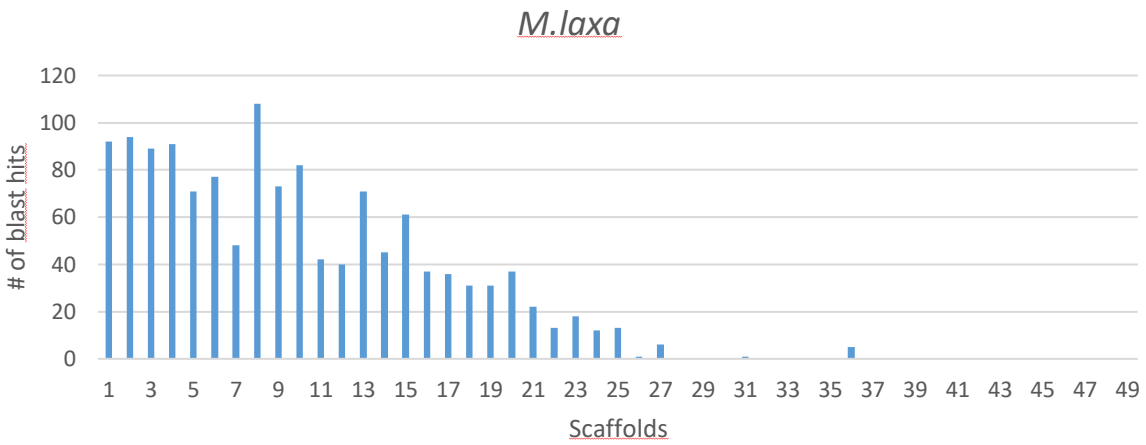


Figure 4. The distribution of milRNAs through the scaffolds of the genome of *M. laxa*.

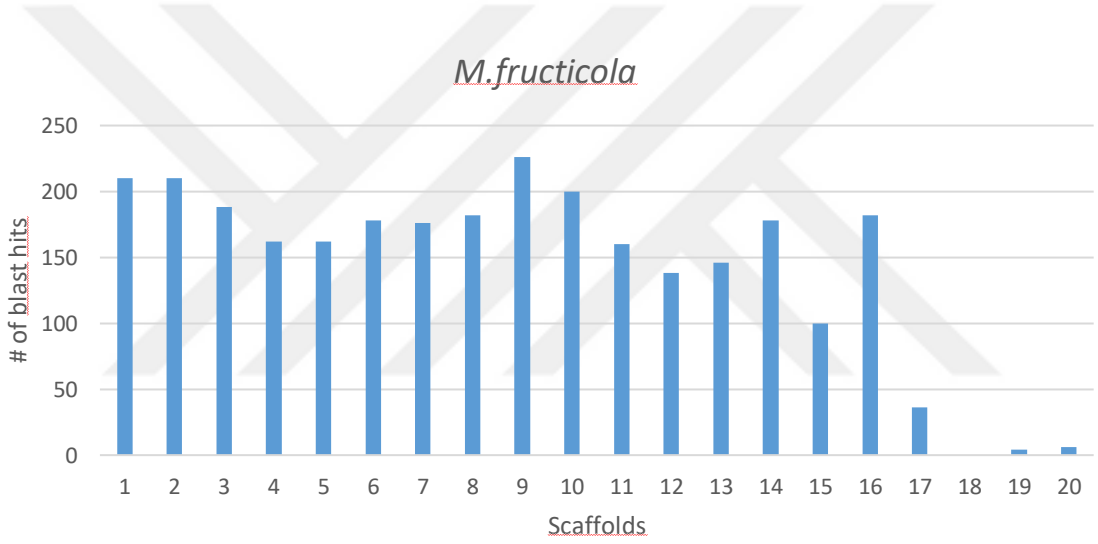


Figure 5. The distribution of unfiltered milRNAs through the scaffolds of the genome of *M. fructicola*.

Furthermore, each window flanking the genomic location's sequence was examined to see if it formed a hairpin-like structure. About 80% of the putative hairpin structures were eliminated after failing to meet additional criteria such as minimum hairpin length, MFE, and miRNA/miRNA* complementarity. For *M. fructicola* only 20.2% milRNAs were capable to form 355 hairpins, and for *M. laxa* only 16% milRNAs were able to form 266 hairpins.

4.3. Possible Fungal milRNA Targets in the Transcriptomes of the Pathogen and the Host

According to results from psRNAtarget, 209 milRNAs of *M. fructicola* targets only 98 genes from *P. persica* transcriptome, and 128 milRNAs of *M. laxa* target 338 genes from *P. persica* transcriptome (Figure 6). Gene Ontology analysis of the genes targeted by fungal milRNAs showed milRNAs target various regions in *P. persica* transcriptome including genes that play important role in cell cycle, defense response and hormonal pathways (Figure 7-8).

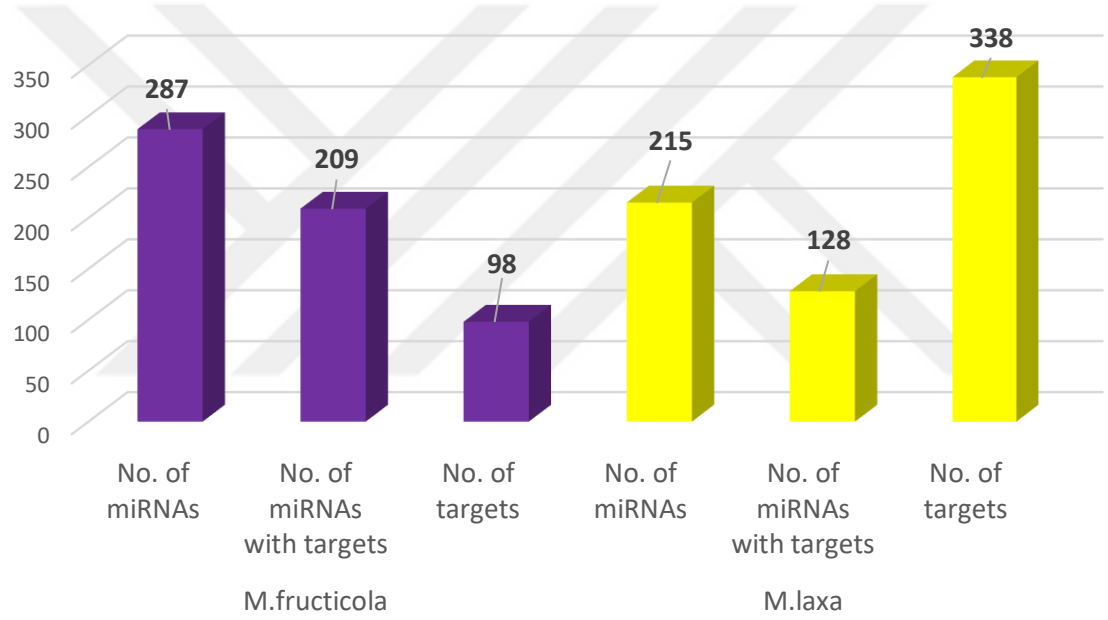


Figure 6. Number of miRNAs from both species with their targets in host transcriptomes. No. of miRNAs: Number of milRNAs that can form hairpin, No. of miRNAs with the targets: Number of milRNAs that targets *P. persica* genes. No. of targets: Number of *P. persica* unique target genes

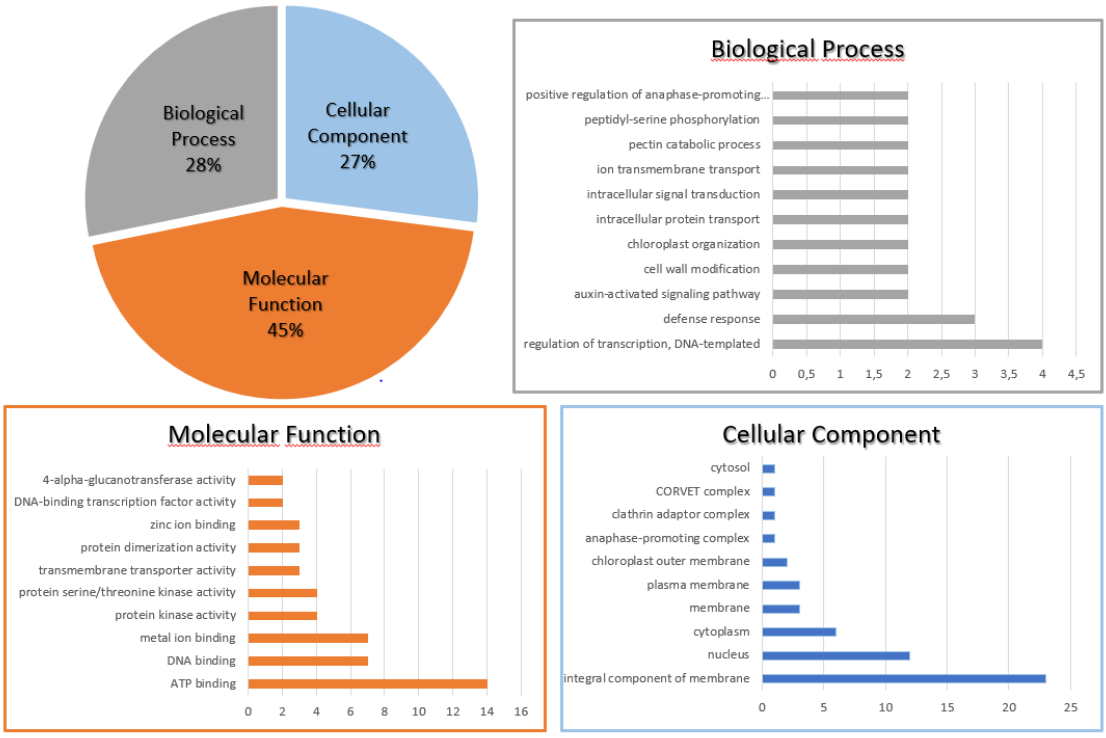


Figure 7. Target function analysis of *P. persica* genes that were targeted by *M. fructicola* milRNAs based on GO terms.

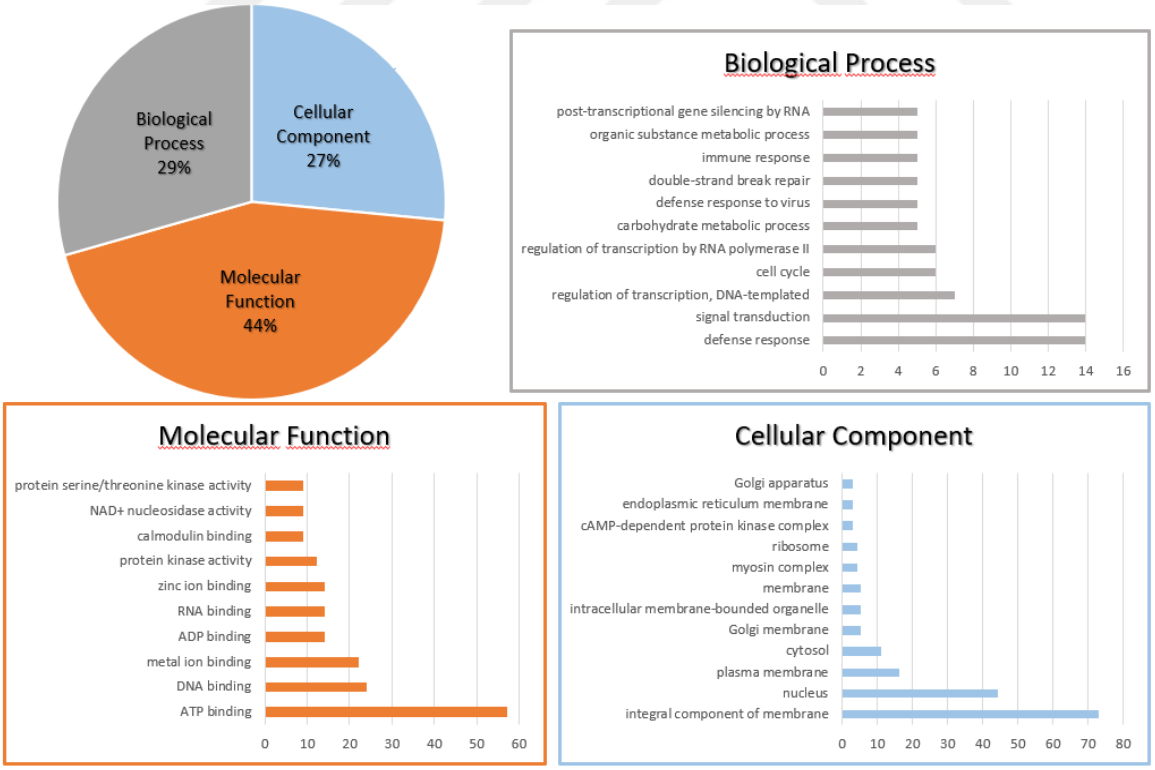


Figure 8. Target function analysis of *P. persica* genes that were targeted by *M. fructicola* milRNAs based on GO terms.

4.4. Fungal Target Genes of *P. persica* miRNAs

The targets of known miRNAs of *P. persica* on *M. fructicola* and *M. laxa* genomes were evaluated. Due to a large number of target genes analyses were made default values in psRNAtarget webserver with some modifications (Maximum number of expectation=1, No. of best targets= 5). The number of *P. persica* miRNAs targeting *M. fructicola* and *M. laxa* genomes was 102 and 104, respectively. Annotation of target sequences shows different genes that are involved in cellular mechanisms (Figure 9-10). Furthermore, the distribution of fungal milRNAs and their targets in the *P. persica* genome and *P. persica* miRNAs and their targets in fungal genomes were displayed (Figure 11-12)

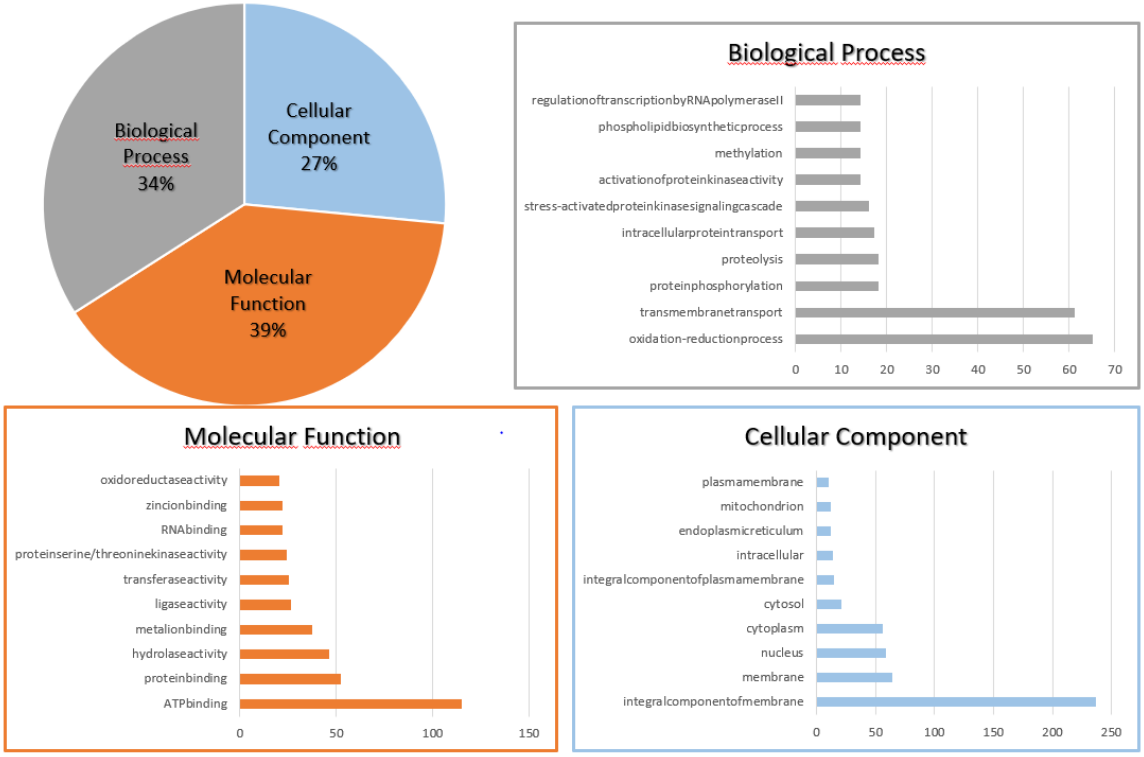


Figure 9. Target function analysis of *M. fructicola* genes that were targeted by *P. persica* miRNAs based on GO terms.

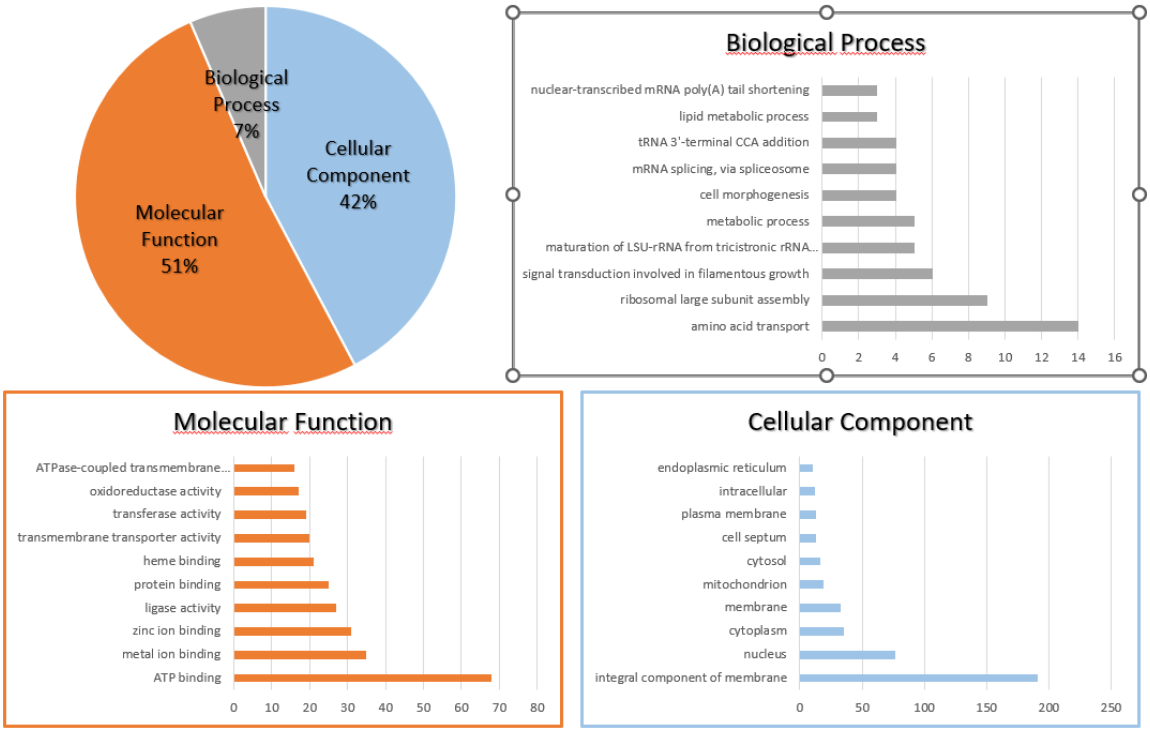


Figure 10. Target function analysis of *P. persica* genes that were targeted by *M. fructicola* based on GO terms.

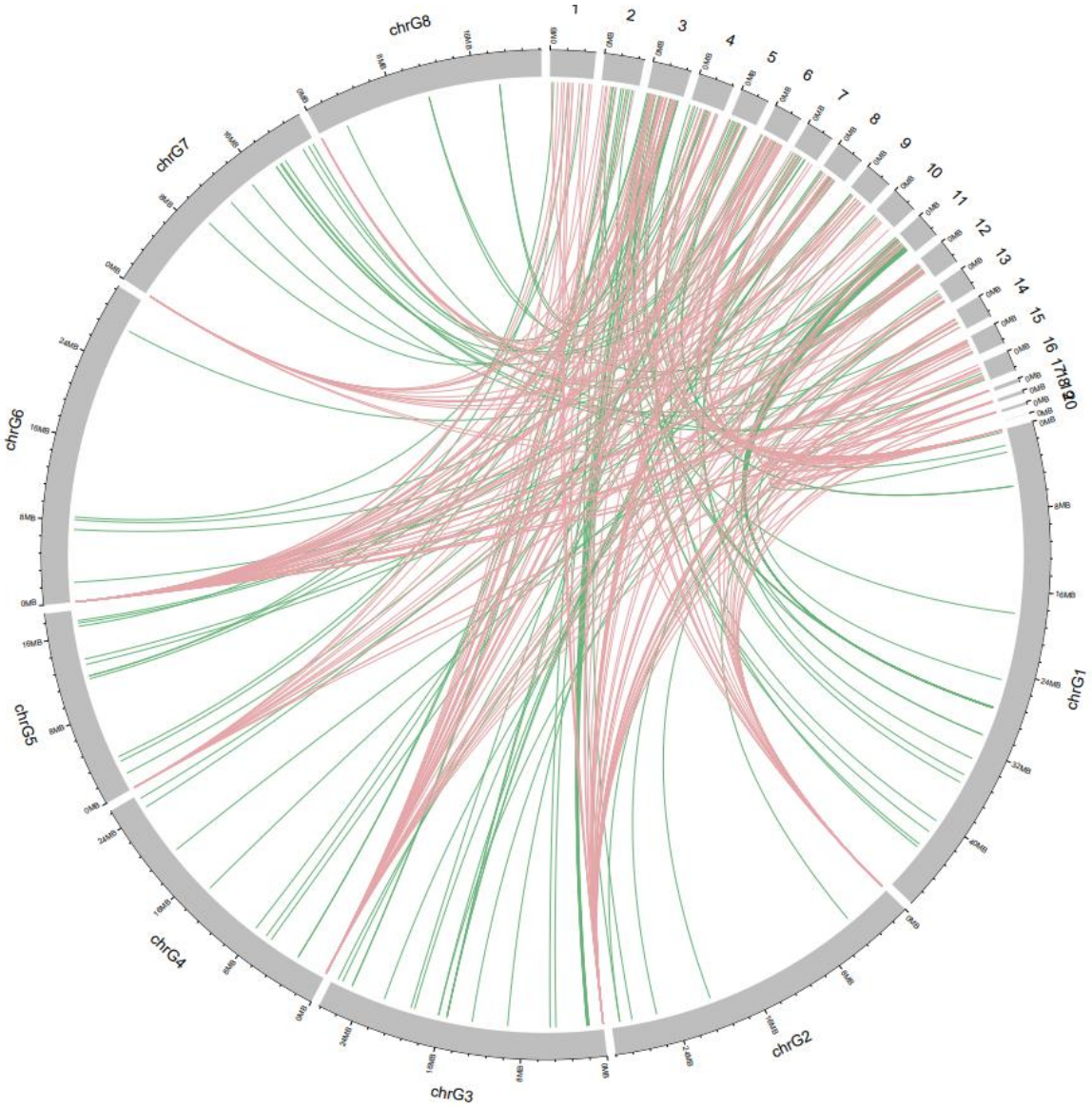


Figure 11. The distribution of *M. fructicola* milRNAs and their targets in the *P. persica* genome (green lines) and *P. persica* miRNAs and their targets in fungal genomes (red lines) are displayed. chrG1-chrG8 are the chromosomes of *P. persica*, the range of 1-20 are the *M. fructicola* scaffolds.

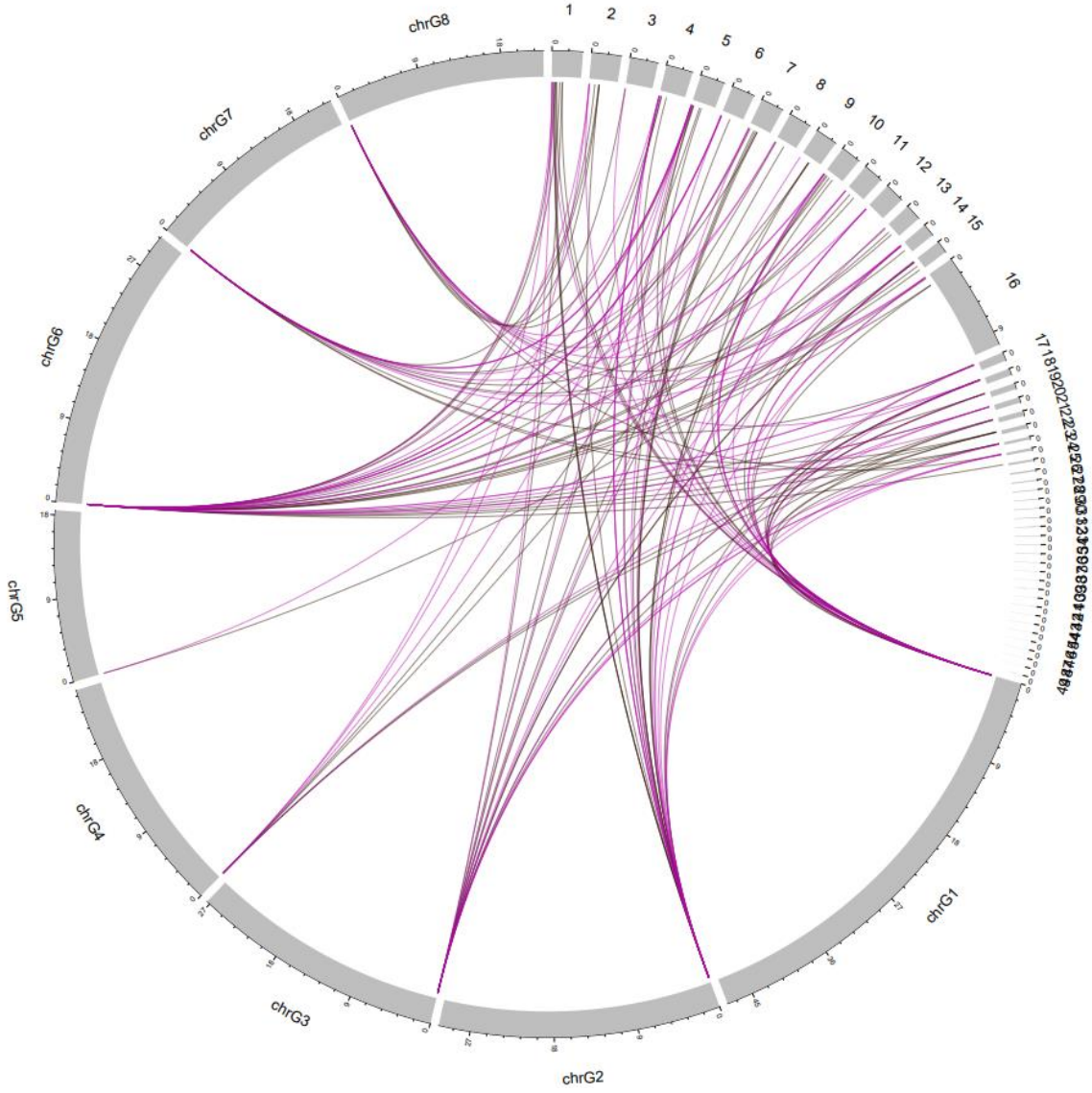


Figure 12. The distribution of *M. laxa* milRNAs and their targets in the *P. persica* genome (black lines) and *P. persica* miRNAs and their targets in fungal genomes (pink lines) are displayed. chrG1-chrG8 are the chromosomes of *P. persica*, and the range of 1-49 are the *M. laxa* scaffolds.

4.5. Mimicking Host Environment To Induce Pathogen Virulence

The expression profile of the *CUT1* gene was evaluated by qRT-PCR. To normalize the qRT-PCR results, Actin gene was used as an internal control. *CUT1* gene expression in the isolate grown on FPA was presented with *CUT1* gene expression 3.88 times more than the same isolate grown on PDA (Figure 13), which confirms the induction of fungal virulence on FPA cultures.

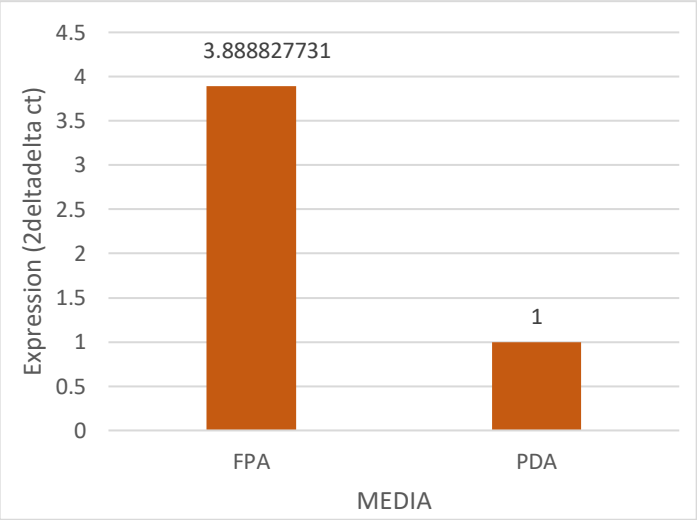


Figure 13. mRNA expression levels of *CUT1* genes in Ti-B3-A3-2 isolate. FPA: Freezed peach agar media, PDA: Control

4.6. Sequencing Statistics and sRNA Data Analysis

In total four small RNA NGS libraries were constructed, representing one isolate (Ti-B3-A3-2) grown on two different media with two biological replicates. For each of the four sequenced samples (MF-FPA-1, MF-FPA-3, MF-PDA1, MF-PDA2) approximately 28 million reads were obtained via high-throughput sequencing (Table 1). The adaptor-trimmed clean sequence reads were subjected to QC analysis, revealing that the length of clean reads ranged from 10-1500 with peaks at 15-30 nt (Figure 13). The alignment of clean reads against the Rfam database showed that approximately 15% of sequences aligned to known rRNA, tRNA, snRNA, and snoRNA and were removed from the milRNA prediction analysis.

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

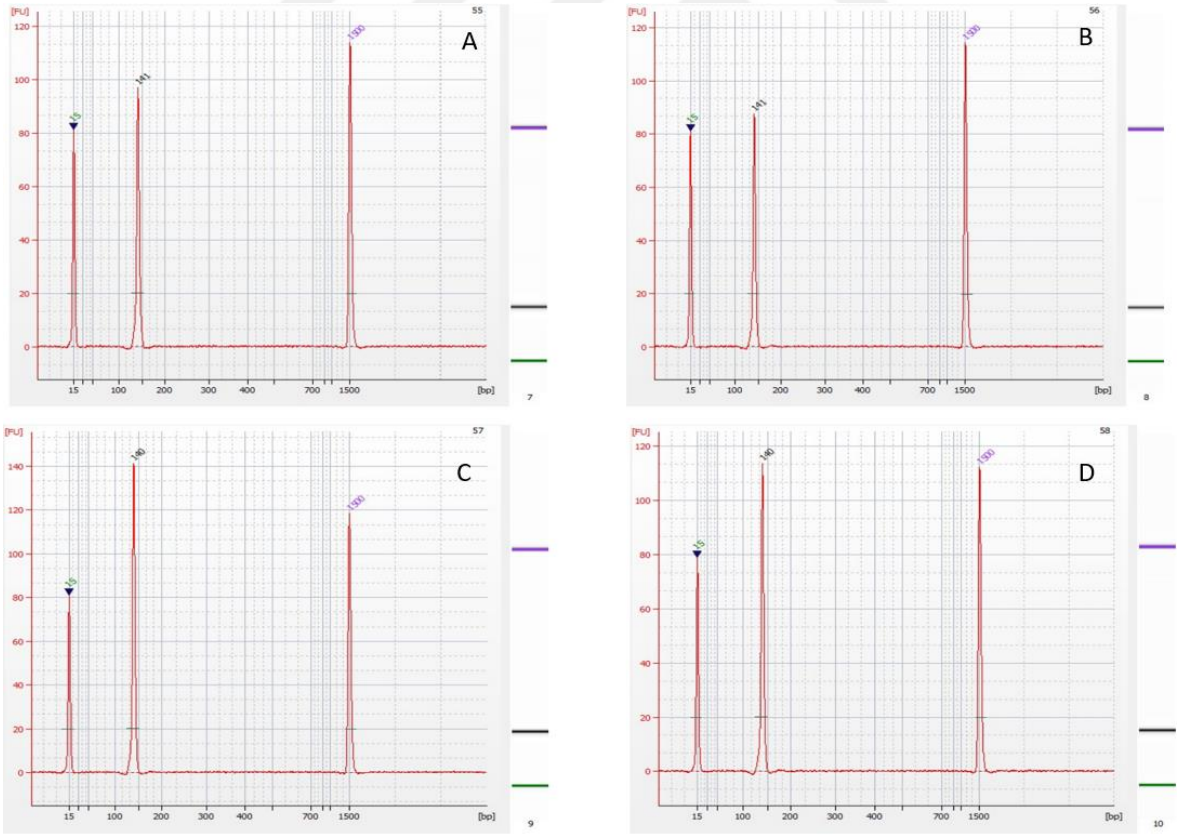


Figure 14. Frequency of library reads according to their sizes. A: MF-FPA-1, B: MF-FPA-3, C: MF-PDA-1, D: MF-PDA-2.

4.7. Identification of Potential *Monilinia fructicola* milRNAs from sRNA data

The filtered reads are aligned against the known miRNAs from the miRBase database using Bowtie to identify conserved or known miRNAs from alignments. 166, 124 known milRNAs were found in MF-FPA and MF-PDA reads, respectively. Known milRNA reads constitutes only 0.01% of the total reads.

The unique reads that do not align against the miRBase database are used for novel miRNA prediction. The reads not aligning to known plant miRNAs are first mapped to the reference genome using Bowtie with a maximum of 2 mismatches for novel miRNA prediction. miRDeep2 package was used to predict novel milRNA sequences, and RNAfold was used to predict the hairpin formation ability of novel milRNAs. Among samples 6 novel milRNAs were predicted from MF-FPA data sets and 7 novel milRNAs were predicted from MF-PDA data sets. These novel milRNAs were the only milRNAs that can form suitable hairpin structures (Table 2-3).

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.

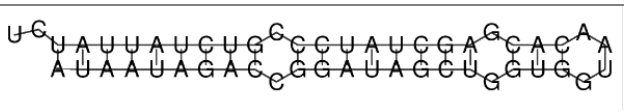
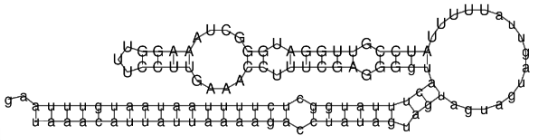
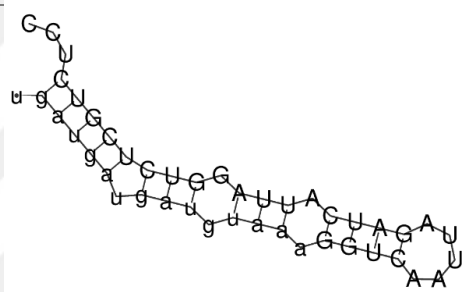
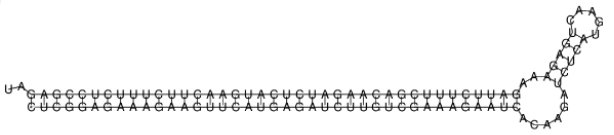
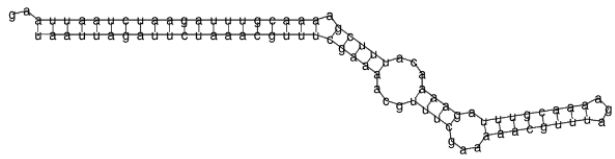
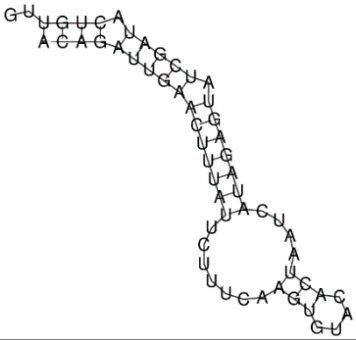
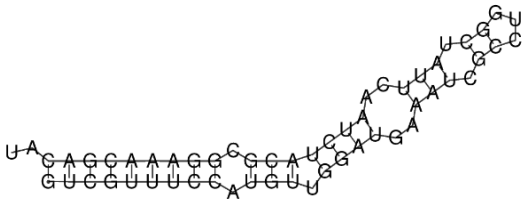
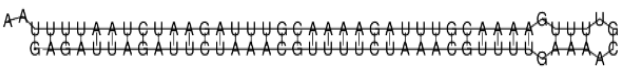
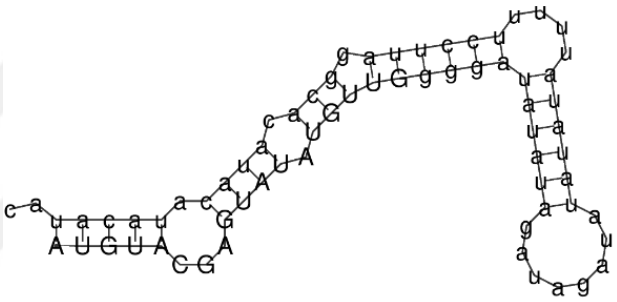
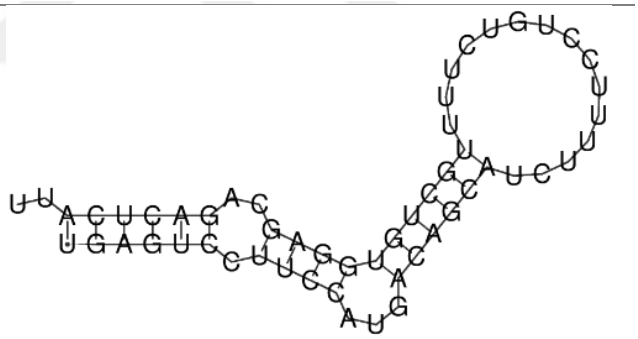
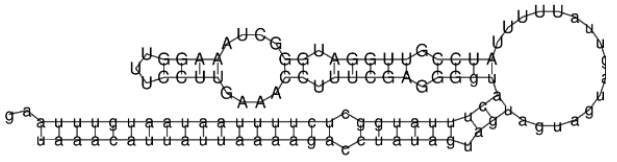
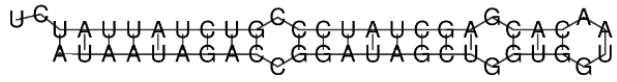
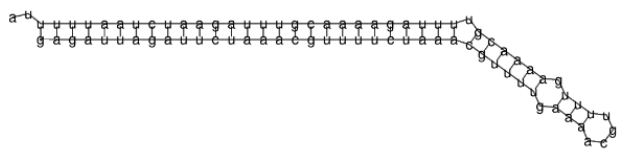
milRNA IDs	Library ID	MFE	Mature milRNA sequence	2D structure
VICG01000001.1_25886	MF-PDA	-67.60	ATAATAGACCGGATAGCTGGTG	
VICG01000016.1_4478	MF-PDA	-81.40	TAAACATTATTAAAAGACCT	
VICG01000002.1_12298	MF-PDA	-66.40	ATCATTAGGTCTCGTCTCC	
VICG01000002.1_2912	MF-PDA	-89.30	CATGAACTTCTTTCTCCGAGAT	
VICG01000004.1_4550	MF-PDA	-60.50	TAATTAGATTCTAAACGTTTC	
VICG01000005.1_4608	MF-PDA	-51.70	CATAGAGTATCGATACTGTTG	
VICG01000004.1_6348	MF-PDA	-74.80	GTCGTTTCCATGTTGGATGAA	

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
VICG01000004.1_6702	MF-FPA	-70.90	GAGATTAGATTCTAAACGTTT	
VICG01000011.1_941	MF-FPA	-68.40	ATGTACGAGTATATGTTGGG	
VICG01000001.1_1581	MF-FPA	-65.20	GCTGTGGAGCAGACTCATT	
VICG01000016.1_1516	MF-FPA	-81.30	TAAACATTATTAAAAGACCT	
VICG01000001.1_9611	MF-FPA	-67.60	ATAATAGACCGGATAGCTGGTG	
VICG01000002.1_3732	MF-FPA	-63.40	GAGATTAGATTCTAAACGTTT	

4.8. Differentially Expressed milRNAs and Target Prediction

Target prediction analyses were performed on whole milRNA data of MF-FPA and MF-PDA libraries. MF-FPA milRNAs were also checked if they have the ability to target *P. persica* genes. MF-FPA and MF-PDA milRNAs targeted 90 and 62 genes of *M. fructicola*, respectively. Even though the same strict parameters were applied to target prediction analysis in P. persica, MF-FPA milRNAs targeted 123 genes (Figure 16).

Differentially expressed miRNAs were determined using DESeq2. On the heatmap, the best differentially expressed 20 milRNAs presented as two main clusters.7 of the MF-PDA milRNAs and 13 MF-FPA milRNAs were upregulated (Figure 15). Only the best targets of differentially expressed milRNAs were represented in Appendix Table 1.

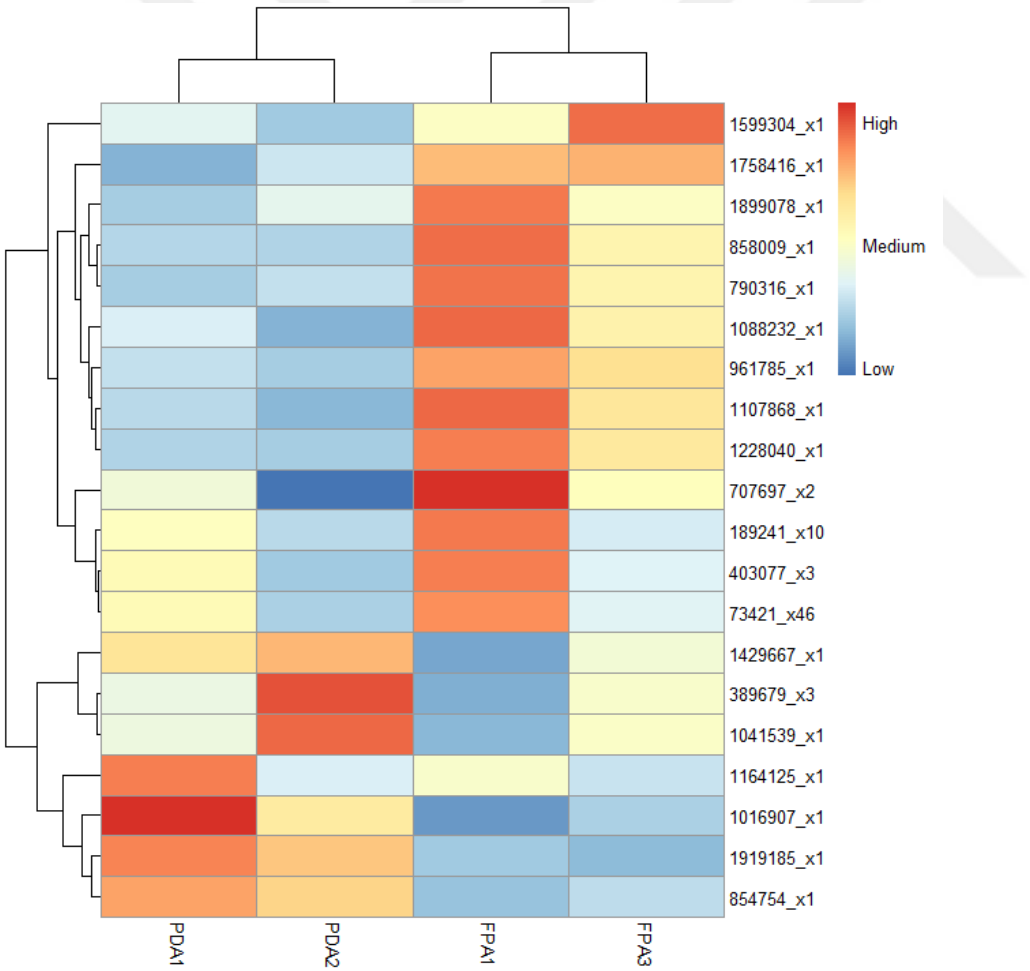


Figure 15. Heatmap of the differentially expressed miRNAs. The color scale indicates the relative expression level of differentially expressed M. fructicola milRNAs.

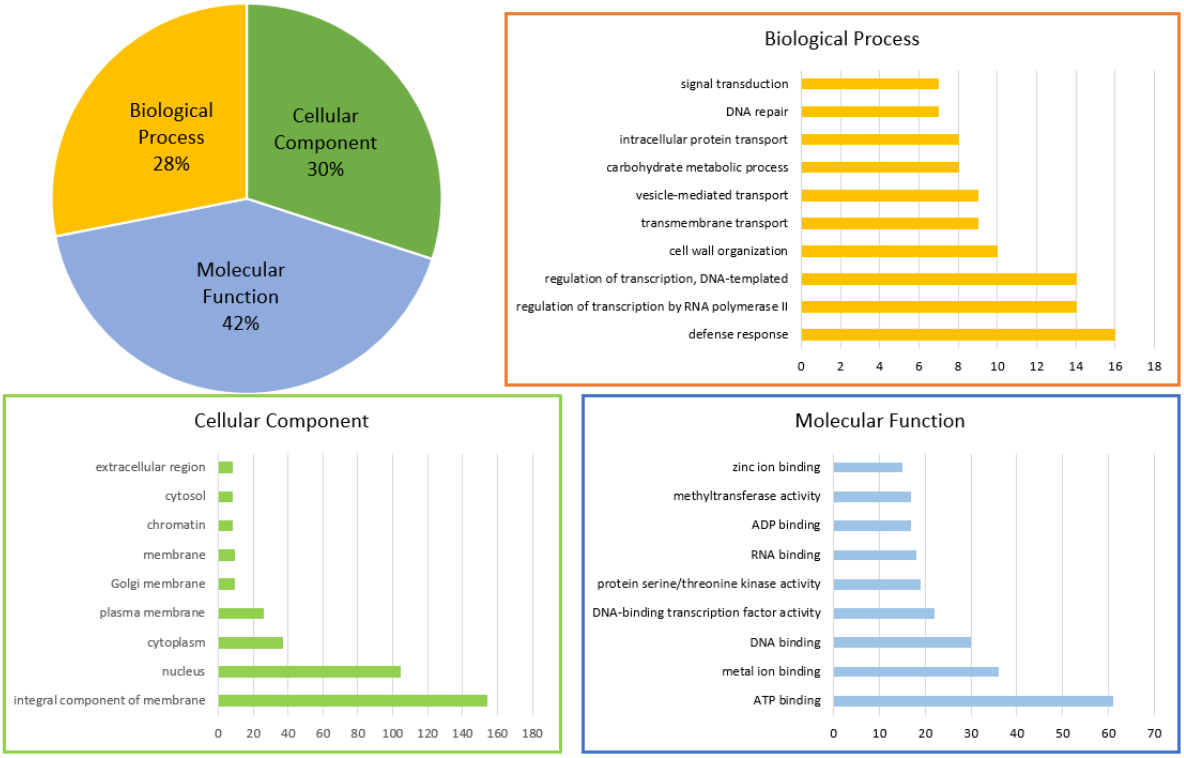


Figure 16. Target function analysis of *P. persica* genes that were targeted by *M. fructicola* (MF-FPA) based on GO terms.

4.9. Discussion

In this thesis, we presented miRNA profiles of the two most prominentspecies of brown rot of peach fruit for the first time. Besides, *M. fructicola* miRNA sequences expressed in the host mimicking environment were discovered and the novel expressed miRNA was explored. The study presented both a workflow to analyze fungal miRNAs and much new information about miRNAs of *Monilinia* pathogens, especially for those involved in host-pathogen interactions. Considering scarce information on miRNAs of fungal plant pathogens and their roles, this study contributed important fundamental knowledge to the field. These findings could have the potential to be used to devise new pathogen control strategiesin a further future studies.

We performed a computational pipeline with strict filtering parameters in order to identify the milRNA profile of *M. fructicola* and *M. laxa*. An in-house database of miRNAs which contains seven fungal species and mature miRNA sequences retrieved from miRBase,

was used as the subject of blastn search against *M. fructicola* and *M. laxa* genome. We employed a homology-based approach to predict potential miRNAs in *M. fructicola* and *M. laxa* genomes, based on the homologies reported between milRNAs from other fungi and miRNAs of plant and animal origin (Gao et al., 2013). The homology-based blastn hits of milRNAs from other fungal species were lower than the milRNAs in miRBase. In a study conducted for *Fusarium oxysporum*, milRNAs were identified by the same main pipeline but with different parameters (Mathur et al., 2020). The number of blastn reads was much higher than our study (328 hits to the miRBase and 8860 hits to the other fungal miRNAs). However, the parameters used in this study were much more strict to obtain true possible milRNAs. These results werethe first outlook of *Monilinia* spp. milRNAs and might be an indication of a difference in the diversity of *M. fructicola* miRNAs considering other selected fungal organisms.

Known miRNA genes can be coded within protein-coding genes and intergenic regions in the genome. The location of miRNA genes in a genome can determine their expression and function. For example, human miRNA genes were discovered on all autosomes and X chromosomes, however, a few miRNA genes were predicted on the Y chromosome but not confirmed thoroughly (Ghorai & Ghosh, 2014). The conservation of miRNA genes largely depends on their locationsand, a miRNA might have a function well in order to be evolutionarily selected. As shown in Figure 2, there were no possible predicted miRNA genes in 28-30, 32-35, 37-49th scaffolds of the *M. laxa* genome, and results were similar to finding in the *M. fructicola* genome. This might indicate that the lack of conserved regions led to the absence of miRNAs. In addition, these results might indicate that last scaffolds of both species do not contain important or vital genes that need to be expressed, so that miRNAs are not conserved in these regions. From another perspective, there is a hypothesis in consideration for the biogenesis of novel miRNAs impliying that miRNAs are synthesized from the antisense transcript region of their target genes (De Felippes et al., 2008). By taking the consideration this hypothesis, the results support that absence of genes in the last scaffolds led to the absence of miRNAs. The annotation and conservation of these last scaffolds needed to be inspected.

Hairpin formation ability of miRNA genes is a unique identifier and distinguishing feature of miRNAs. To filter out significant milRNA sequences, the sequence of each window flanking region of filtered milRNA sequences was checked if it can form a hairpin

structure. The majority of milRNAs were discarded from the downstream analysis since they could not form a hairpin structure or pass additional filtering parameters such as minimum hairpin length, MFE, and miRNA/miRNA* complementarity.

Interactions between hosts and pathogens are among the most complicated and interesting questions in the area of ecology and evolutionary biology. It is also important to develop better management strategies against pathogens. Pathogens use a variety of virulence elements to obtain and acquire the supplies accessible within their hosts, while hosts defend themselves with multilevel defenses against to pathogens.

According to the target prediction results, *M. laxa* had the ability to target more *P. persica* genes than *M. fructicola*. Even though *M. laxa* has less milRNA genes than *M fructicola*, milRNAs of *M. laxa* have shown better focusing on *P. persica* genes. From the results in Section 4.3 and a study published by Ozkılınc et al. (2020), we can confirm the results that *M. laxa* is more aggressive than *M. fructicola* under certain experimental conditions. The annotation of targeted *P. persica* genes presented various essential mechanisms.

In a study conducted on *M. laxa*, it has been proven that there must be an interference mechanism among the the genes related to hormone mechanisms in nectarine. In this case, particularly auxin is a crucial plant hormone that regulates growth and development (Balsells-Llauradó et al., 2020). The results shown in Figure 6 presented that especially *M. fructicola* targets auxin hormone-related pathways, which indicates showing that this pathogen could be interfering with important developmental stages in plants. Moreover one of the *P. persica* target genes by *M. laxa* is responsible for plant immune response. This might indicate that when infected by *M. laxa*, plant immune response would be suppressed, hence plant might become more sensitive to *M. laxa* and other diseases. Both fungal species not only target different crucial cell processes of *P. persica* but also weakens the immune defense response of *P. persica*..

Prunus persica has much larger genome content compared to the genomes of its fungal pathogens. The results of the target prediction of *P. persica* miRNAs forced us to use more strict parameters to get the best targets since *P. persica* miRNAs target a large number genes in *M. fructicola* and *M. laxa*. Annotation of targeted genes presented a high number of genes in the complex and important cellular fungal mechanisms. *P. persica* milRNAs

target mostly oxidation-reduction processes, transport systems, and protein phosphorylation mechanisms of *M. fructicola*. These are vital in cells and should not be disrupted. For example; protein phosphorylation is one of the most common and important post-transcriptional regulators via changing the structural conformation of the protein. Thus,down-regulation of this protein leds to dysfunctional post-translational regulation. There are target genes not only important metabolic pathways but also fungal defense pathways. Stress responses are necessary for pathogenic fungi to survive in the host's hostile environment, and it is certain' clear that stress-activated protein kinase (SAPK) pathways play a key role in mediating such responses and virulence in many fungal infections (Brown et al., 2017). On the other hand, *P. persica* miRNAs targeted mostly amino acid transportation, signal transduction in filamentous growth, and another essential metabolic process rather than defense response in *M. laxa*. These resultsimplicate that although these species are closely related *P. persica* use different strategies to combat with battles *M. fructicola* and *M. laxa* in different ways.

The quantity of research on one topic would be influenced by the previous experiences of a country.. For example; if brown rot disease on *P. persica* was not detected in a country,then researchers would ignore the disease because they are not able to supply materials for the experiments. Likewise the number of studies on *Monilinia spp*. is higher in the countries with high *P. persica* production(https://www.webofscience.com). Considering our facilities we had the only chance to perform our pathogenicity experiments as detached fruit *in vitro* conditions. However, we faced low of RNA yield possibly due toRNAses and cellular degradation after detaching the fruit from the tree. To overcome this problem, we explored a new way to mimic the host environment. For this, a study by Maximiano et al (2020) was a good example. In our studies, we also confirmed that mimicking model environment is successful because the expression of *CUT1,* one of the fungal effector genes, was induced up to four-folds in FPA media in comparison to classical PDA media. Thus, this novel method could be easily used in othersome studies to study host-pathogen interactions. Although FPA does not represent the plant environment, the presence of plant components in the medium induce *CUT1* expression 4 times more than PDA and mimicking in planta conditions. The use of FPA is easier than in planta growth, avoiding contamination or the seasonal availability of fresh peach. The simulation of plant condition *in vitro* provided host-pathogens interactions on phytopathogens in a time and space independent manner.

Novel miRNA prediction is based on unique readings that do not align with the miRBase database. For novel miRNA prediction, reads that do not align to known plant miRNAs are first mapped to the reference genome using Bowtie with a maximum of two mismatches (Langmead & Salzberg, 2012). Novel milRNA sequences were predicted using the miRDeep2 program, and hairpin forming capacity was predicted using RNAfold (Friedländer et al., 2012; Lorenz et al., 2011) . Six new milRNAs were predicted from MF-FPA data sets and seven new milRNAs were predicted from MF-PDA data sets among the samples. The only milRNAs that can form acceptable hairpin structures were identified as these novel milRNAs. The fact that miRBase alignments were too low would be caused by a lack of a lot of fungal miRNAs in miRBase.

milRNAs differentially expressed between MF-FPA and MF-PDA were computed and the targets of milRNAs that were differentially expressed were predicted only in *M. fructicola* transcriptomes. Additionally, the targets of MF-FPA milRNAs were searched in the *P.persica* transcriptome. To be able to employ the DEseq2 algorithm, researchers must include at least 3 biological replicates in order to enhance statistical power. Other differential expression pipelines contain different complex statistical methods. To be able to detect differentially expressed milRNAs, other pipelines will be performed.

Regardless of the differential expression, targets of all MF-FPA milRNAs have identified both *M. fructicola* and *P. persica* transcriptomes as well. Not all the results were identical to the results of milRNAs from the genomic prediction but also indicate that *M. fructicola* express milRNAs that can target the immune system of *P. persica*. Additionally *M. fructicola* targets crucial cell processes.

CHAPTER 5
CONCLUSION

Identifing milRNAs and search for the possible milRNA targets is an important breakthough when it comes to relationships of destructive pathogenswith their hosts. This study is the first attempt to understand molecular mechanism of the battle between peach and *Monilinia* spp. In this thesis, whole possible milRNA repertoire of high virulent *Monilinia* spp. isolates predicted by using different computational approaches. In addition to mimicking in planta environment in vitro, milRNAs that possibly involve the infection process and their possible targets were identified.

A homology-based computational pipeline was performed in order to investigate milRNAs of *Monilinia spp*. for the first time. Outcomes of milRNA diversity showed differences in *Monilinia spp*. milRNAs even though these species are closely related. The distribution of milRNAs was also determined for the first time and demonstrated that different genomic locations contain possible milRNAs, there were large genomic locations which did not preserve milRNAs at all. Target function analysis indicated that these two fungal species might be using different pathways to infect *P. persica*.

On the other side of the battle, target function analysis were performed for previously known peach miRNAs in the transcriptomes of *M. fructicola* and *M. laxa*. The results indicated that *P. persica* might be using different pathways to overcome these infections.

milRNAs and their targets in the infection process was evaluated for the first time for *M. fructicola*. Lack of publicly available fungal miRNA data led to a low number of miRNAs. A comprehensive novel miRNA prediction pipeline were used to characterize *M. fructicola* miRNAs involved in infection process.

This thesis is the first attemp to understand molecular mechanisms of host-pathogen interactions of *Monilinia spp*. Due to the limited information available on miRNAs in fungal phytopathogens and their roles, this work added to the field's core knowledge. As part of a future investigation, these results could be used to develop novel pathogen control strategies.

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APPENDIX

Tablo 1

The annotations of both *P. persica* genes and *M. fructicola* genes which are targeted by differentially expressed milRNA

milRNA ID	miRBase ID	DEseq Result	Annotation of M.fructicola targets	Annotation of P.persica target genes
1599304	oni-miR-10614	Upregulated	class VII chitin synthase regulator of G protein sequence	Prupe.4G247900.1 Endocytosis, vesicle-mediated transport, cytoplasmic vesicle
1758416	oni-miR-10614	Upregulated	class VII chitin synthase regulator of G protein sequence	-
1899078	oan-miR-7414-3p	Upregulated	zinc knuckle domain-containing protein	-
858009	oan-miR-146b-3p	Upregulated	zinc knuckle domain-containing protein	Prupe.5G226600.2
790316	rno-miR-487b-5p	Upregulated	WD40 repeat-like protein	Prupe.3G226900.1 Zinc ion binding
1088232	oan-miR-146b-3p	Upregulated	glycoside hydrolase family 13 protein	
961785	stu-miR8004	Upregulated	IQ calmodulin-binding domain-containing protein AAA-domain-containing protein putative Fork head protein like protein AAA-domain-containing protein putative Fork head protein like protein	Prupe.2G162700.1 K efflux antiporter, chloroplast, potassium:proton antiporter activity, membrane, integral component of membrane
1107868	oan-miR-146b-3p	Upregulated	glycoside hydrolase family 13 protein	Prupe.4G115300.2 nitrogen compound metabolic process, urease activator complex
1228040	oan-miR-146b-3p	Upregulated	glycoside hydrolase family 13 protein	Prupe.1G241400.1 Phloem development
707697	bta-miR-12064	Upregulated	trafficking protein-like protein particle complex subunit 3	Prupe.1G468200.1 oxidoreductase activity, metal ion binding, dioxygenase activity
189241	pab-miR482i	Upregulated	putative cysteine peptidase protein	-
403077	pab-miR482i	Upregulated	putative cysteine peptidase protein	-
73421	pab-miR482i	Upregulated	putative cysteine peptidase protein	Prupe.6G046900.1 DNA-binding transcription factor activity, sequence-specific DNA binding