

Article

Identification and Characterization of MicroRNAs and Their Targets That Respond to Powdery Mildew in Melon

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Abstract

To identify microRNAs (miRNAs) responsive to powdery mildew (PM) infection and elucidate their regulatory roles in melon PM resistance, thereby laying a foundation for deciphering the underlying molecular mechanisms, we combined high-throughput sequencing with bioinformatics analysis to screen PM-responsive miRNAs and their target genes using PM-resistant and PM-susceptible melon genotypes. In total, 113 non-redundant miRNAs were identified in both genotypes, including 70 known and 43 novel miRNAs. Subsequent differential expression analysis revealed distinct miRNA responses to PM infection between resistant and susceptible genotypes. Upon PM inoculation, 13 miRNAs showed significant differential expression in both susceptible and resistant genotypes. In addition, four miRNAs, including miR164c, miR396a, miR398a and miRn39, displayed differential expression specifically in the susceptible genotype. Conversely, five miRNAs (miR167c, miR398b, miR399g, miR530a and miRn8) showed differential expression exclusively in the resistant genotype upon PM infection. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses demonstrated that these miRNAs mediate melon's susceptibility or resistance to PM by modulating plant immune homeostasis, antioxidant metabolism, and pathogen-triggered cell death. Quantitative real-time PCR (qRT-PCR) validation confirmed a negative regulatory relationship between the expression of PM-responsive miRNAs and their predicted target genes. Collectively, our findings provide novel insights and candidate targets for further investigations into miRNA functions and regulatory mechanisms underlying melon PM resistance.

Keywords: melon; powdery mildew disease; high-throughput sequencing; bioinformatics analysis; microRNA; target gene



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1. Introduction

Melon (*Cucumis melo* L.) is an important cash crop belonging to the *Cucurbitaceae* family and is cultivated worldwide. However, melon is susceptible to powdery mildew disease (PM) at the late developmental stage, resulting in reduced fruit yield and quality, which has severely restricted the development of the melon industry. PM is a major foliar disease of melon caused primarily by the obligate biotrophic fungi *Podosphaera xanthii* and *Golovomyces cichoracearum* [1]. Severe powdery mildew infection leads to yellowing, necrosis, and premature death of leaves as the fungi siphons nutrients and impairs photosynthesis [2]. Effective control of PM remains a formidable challenge in melon production. Therefore, identification of novel, durable, and broad-spectrum resistance genes or regulatory factors

and elucidation of their functional pathways and underlying regulatory mechanisms is pivotal for developing robust breeding strategies, which will lay a solid theoretical foundation for the cultivation of powdery-mildew-resistant melon varieties.

MicroRNAs (miRNAs) are a class of small non-coding RNAs (typically 20–24 nucleotides) that function as sequence-specific regulators of gene expression, generally by guiding post-transcriptional cleavage or translational repression of target mRNAs [3,4]. Over the past few decades, with the advances in high-throughput sequencing of miRNAs and degradome analyses, thousands of miRNAs have been identified in various plant species, and their critical regulatory roles in plant growth and development have been confirmed [5–8]. Additionally, accumulating evidence has demonstrated that multiple miRNAs respond to pathogen infection and can modulate immunity. For instance, in *Arabidopsis* challenged with *Pseudomonas syringae*, miR160, miR167, miR393*, miR398, and miR773 were differentially expressed, and transgenic overexpression or suppression of some of these miRNAs led to altered resistance [9]. Moreover, some miRNAs function by generating secondary small RNAs that amplify defense signals. A notable example is miR482 in *Solanaceous* plants, which targets transcripts of Nucleotide-Binding Site Leucine-Rich Repeat (NBS-LRR) genes and triggers the production of small interfering RNAs (siRNAs) that further silence additional NBS-LRR genes [10]. NBS-LRR genes are the largest family of immune receptor genes, also known as R genes (resistance genes), which confer resistance against diverse pathogens. siRNAs are a class of small interfering RNAs with a typical length of 21–24 nucleotides in plants. They are central components of the RNA silencing pathway, a conserved gene regulatory and immune defense system in all plant species. This miRNA-mediated circuit helps modulate the expression of large R gene families and avoid autoimmunity in the absence of pathogens but can be repressed during infection to unleash R-gene-based defenses. Furthermore, in rice infected with fungal blast, members of the miR160, miR169 and miR398 families were found to be pathogen-responsive, and over-expression of miR160a and miR398b in rice plants can significantly restrict pathogen proliferation and enhance disease resistance [11,12]. These observations, along with functional assays, established a paradigm that distinct miRNAs act as fine-tuners of plant immune responses, either positively or negatively regulating defense depending on the roles of their target genes.

Recently, numerous miRNAs have been identified in melon. Nevertheless, the functional roles of these miRNAs and their target genes in melon PM resistance remain largely uncharacterized. We propose that key differentially expressed miRNAs contribute to PM defense by regulating resistance-associated target genes, and that resistant and susceptible melon genotypes possess divergent miRNA regulatory networks upon PM infection. To test this hypothesis, we investigated miRNA expression profiles and the corresponding miRNA-target regulatory modules linked to PM resistance in melon. Ultimately, our results will offer novel insights into the miRNAs and molecular mechanisms underlying PM resistance in melon.

2. Materials and Methods

2.1. Plant Materials

Melon inbred line ‘M1’, which is resistant to PM, and inbred line ‘B29’, which is susceptible to PM, were used as materials and grown in a greenhouse under a 16h light/8h dark photoperiod with day/night temperatures set at 28 °C/22 °C. M1 is a homozygous inbred line that was self-pollinated for thirteen generations from a commercial cultivar. B29 is a homozygous inbred line that was self-pollinated for nine generations from a commercial cultivar. The genetic background of these two lines has been highly stable. Melon seedlings with three true leaves were inoculated using a conidial suspension of *Podosphaera xanthii*. The pathogen strain was originally isolated from naturally diseased melon leaves collected from a local greenhouse in Jinan, Shandong province. A total of nine plants were used

for every treatment. Spores were washed from the diseased leaves with sterile distilled water and adjusted to a concentration of 1×10^6 spores/mL using a hemocytometer. Each plant was evenly sprayed with 5 mL of spore suspension until the leaf surfaces showed liquid runoff. After inoculation, the plants were kept in darkness for 12 h at 28 °C with humidity maintained above 75% [13]. Control plants of both resistant and susceptible lines were treated with sterile water (M0 and B0). All three true leaves of M1 and B29 were collected at 24 and 48 hours post-inoculation and designated as M24, M48, B24, and B48, respectively. The samples were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analyses. Each biological replicate consisted of pooled leaf tissues from three individual plants, and three biological replicates were prepared for each time point.

2.2. RNA Extraction, Library Construction and Sequencing

Total RNA was extracted from each sample using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). RNA integrity was assessed by 1.0% agarose gel electrophoresis, and the quality and concentration were measured using a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) and a Thermo NanoDrop 2100 spectrophotometer (IMPLEN, Westlake Village, CA, USA). Small RNA (sRNA) sequencing libraries were constructed using the NEBNext Ultra Small RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) and sequenced on the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA) for high-throughput sequencing.

2.3. Identification of Known and Novel miRNAs

High-quality sRNA sequences were obtained after filtering the raw sequencing reads using the Fastx-toolkitv0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/, accessed on 19 July 2026), including 3'-adapter sequences, low-quality reads, and sequences that were longer than 30 nt or shorter than 18 nt. To exclude tRNA, rRNA, snRNA, snoRNA, ployA and exon sequences, all clean reads were aligned with the melon reference genome (DHL92) and Rfam 11.0 database using Bowtie2 (v2.2.8; <https://bowtie-bio.sourceforge.net/bowtie2/index.shtml>, accessed on 19 July 2026) [14]. The remaining sequences were aligned against the miRBase Release 21.0. database (<http://www.mirbase.org>, accessed on 19 July 2026) to identify known miRNAs in melon [15]. Subsequently, novel miRNAs were identified using miReap prediction software v0.2(<https://sourceforge.net/projects/mireap/>, accessed on 19 July 2026) through analyzing the precursor sequences and stem-loop structures.

2.4. Normalization and Differential Expression Analysis of miRNAs

SAMtools v1.15 (<http://samtools.sourceforge.net>, accessed on 19 July 2026) was used to normalize the read counts of each miRNA to RPM values (reads per million mapped reads) across different samples with the parameter setting following the workflow described as previously described [16]. Differential expression analysis of miRNAs was performed using DESeq2 v1.34.0 (<http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html>, accessed on 19 July 2026) with raw integer read counts as the input. The threshold was set as a fold change ≥ 2 and adjusted p -value < 0.05 [17]. Expression heatmaps were generated using TBtools v1.120 (<https://github.com/CJ-Chen/TBtools/releases>, accessed on 19 July 2026) with default core parameters as described by Chen et al. [18].

2.5. Target Gene Prediction and Functional Analysis

In plants, miRNAs regulate gene expression by imperfect base pairing with target genes, leading to mRNA cleavage or translational repression, with cleavage typically occurring between nucleotides 10 and 11 from the 5' end of the miRNA. Based on this mechanism, psRNATarget software v2 (<https://www.zhaolab.org/psRNATarget/home>, accessed on 19 July 2026) was used to predict target genes of differentially expressed

miRNAs [19]. Subsequently, GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses were performed to functionally annotate the predicted target genes. GO and KEGG enrichment analysis was performed using Goseq R package v1.64.0 (<http://bioinf.wehi.edu.au/software/goseq/>, accessed on 19 July 2026). All the annotated melon protein-coding genes were defined as the background gene universe. Fisher's exact test was adopted for enrichment statistics, and the Benjamini–Hochberg false discovery rate (FDR) was applied for multiple testing correction. Terms with adjusted $p < 0.05$ were considered significantly enriched.

2.6. qRT-PCR Analysis of miRNAs and Target Genes

Total RNA was treated with DNase I to eliminate genomic DNA contamination (TransGen, Beijing, China). A stem-loop RT primer with 6–8 nucleotides complementary to the target miRNA, which can form a hairpin structure, was designed for the reverse transcription of mature miRNAs [20]. After reverse transcription, a single-stranded cDNA of approximately 60–70 nt in length was generated, containing the reverse complement sequence of the miRNA. Subsequent PCR amplification was carried out by designing forward and reverse primers based on this cDNA. The specific reverse transcription process is as follows: 2 µg of total RNA was used, and the stem-loop primer was added to a final concentration of 2 µmol/L. The mixture was heated at 65 °C for 35 min to disrupt RNA secondary structures, then placed on ice to prevent RNA renaturation. Subsequently, 5 × Buffer, reverse transcriptase (TransGen, Beijing, China), dNTP Mix (TransGen, Beijing, China), and RNase inhibitor (TransGen, Beijing, China) were added, and the final volume was adjusted to 20 µL with ddH₂O. The reverse transcription reaction was carried out at 16 °C for 30 min, followed by 42 °C for 30 min, and finally 85 °C for 5 min. Real-time quantitative PCR was performed using TransStart Top Green qPCR SuperMix (TransGen, Beijing, China). The reaction system (10 µL) contained 5 µL of TransStart Top Green qPCR SuperMix (TransGen, Beijing, China), 1 µL each of forward and reverse primers, 1 µL of cDNA, and 2 µL of ddH₂O. The reaction conditions were as follows: 95 °C for 5 min; 95 °C for 15 s, 60 °C for 15 s, 40 cycles. All the reactions were performed with three biological replicates. *U6* and *Actin* were used as internal reference genes for miRNAs and target gene expression normalization, respectively. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [21]. The stem-loop primer sequences used for miRNA reverse transcription and the primers used for real-time PCR were synthesized by Sangon Biotech (Shanghai, China) and are listed in Supplementary Table S1 and Table S2, respectively.

3. Results

3.1. The Phenotype Analysis of M1 and B29 Against PM

Cultivated melon possesses abundant germplasm resources, which provide valuable genetic materials for PM resistance identification and mechanistic research. To investigate the role of miRNAs in PM resistance in melon, sRNA sequencing was performed using the resistant line 'M1' and the susceptible line 'B29'. As shown in Figure 1, the susceptible line 'B29' developed scattered white fungal spots one week after PM fungus infection, with lesions covering the entire leaf surface at two weeks post-infection. In contrast, the resistant line 'M1' showed no visible symptoms at either time point after inoculation, indicating a clear difference in PM resistance between the two germplasms and providing a reliable basis for further exploration of the underlying resistance mechanisms. However, previous studies on melon infected by PM fungus have demonstrated that conidial germination and epidermal penetration take place within 12 hpi, while haustorium development and stable colonization are accomplished between 24 and 48 hpi [22,23]. Thus, sampling at 24 and 48 hpi was selected to investigate early host immunity concurrent with fungal penetration and haustorium formation.

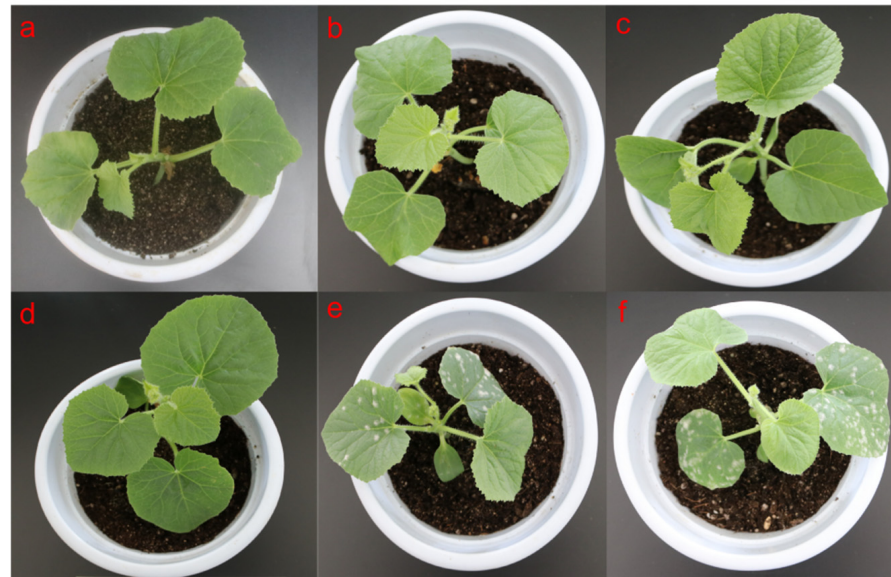


Figure 1. The different phenotypes of leaves after powdery mildew fungus infection. (a–c): The phenotype of M1 after powdery mildew fungus infection at 0, 7, 14 d; (d–f): The phenotype of B29 after powdery mildew fungus infection at 0, 7, 14 d.

3.2. Overview of sRNA Profiles in Melon

To preliminarily investigate the regulatory role of miRNAs in PM resistance in melon, 18 independent sRNA libraries generated from leaf samples of melon resistant line ‘M1’ and susceptible line ‘B29’ collected at three time points after PM inoculation with three biological replicates were constructed and sequenced, yielding approximately 12,088,574 to 15,879,104 total reads per library. An alignment analysis revealed that 80.12% to 82.92% of the reads could be mapped to the melon reference genome (Supplementary Table S3). Pearson correlation analysis based on normalized RPM values showed high correlation ($r > 0.8$) among all the biological replicate groups, indicating excellent reproducibility of our sequencing data (Supplementary Figure S1). As shown in Figure 2, sRNA lengths across all the libraries were predominantly distributed between 20 and 24 nucleotides (nt), with the 22-nt sRNAs being the most abundant (>20%), followed by 21-nt and 24-nt sRNAs (both ~10%). Notably, no significant differences of the proportion of sRNAs were observed among the three stages, suggesting that PM infection does not significantly alter the global sRNA length distribution in melon.

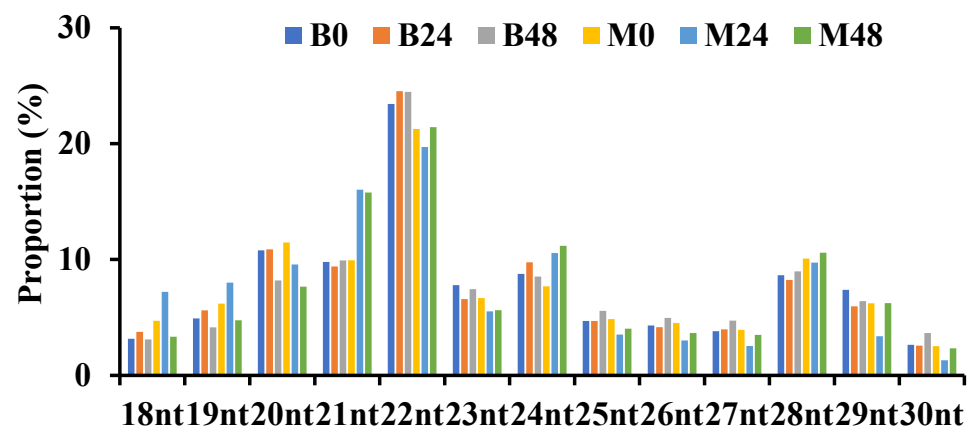


Figure 2. Length distribution of sRNA in melon. B0: Control of susceptible lines; B24 and B48: Samples collected at 24 and 48 hours post-inoculation of susceptible lines; M0: Control of resistant lines; M24 and M48: Samples collected at 24 and 48 hours post-inoculation of resistant lines.

3.3. Identification of Known and Novel miRNAs in Melon

To identify known miRNAs in melon, unannotated reads with perfect matches to the melon reference genome were aligned against all the plant mature miRNA sequences deposited in miRBase Release 21.0. In total, we identified 70 known miRNAs assigned to 29 miRNA families (Table 1). Of these families, 25 are evolutionarily conserved, with homologs detected in at least two distinct plant species. In addition, the remaining four families were less conserved and species-specific, including miR1863, miR2111, miR7129, miR7130. The miR169 and miR399 families have nine and six members, respectively. Both of the miR156 and miR172 families have five members. In addition, 13 miRNA families have only one member, 7 miRNA families have two members, 2 miRNA families have three members, and 3 miRNA families have four members (Figure 3). After identification of the known miRNAs, the remaining reads were used to identify novel miRNAs by predicting the hairpin structures of their precursor sequences. A total of 43 novel miRNAs were identified in this study and named miRn1 to miRn43. We further evaluated the miRNA distribution among the libraries and found that 70 known miRNAs and 43 novel miRNAs were commonly present in the control and PM-treated samples of both the sensitive and tolerant genotypes.

Table 1. Sequences of known and novel miRNAs identified in melon.

Known miRNAs	miRNA Sequences	Length (nt)	Conserved in Other Plants
miR156a	UGACAGAAGAGAGUGAGCAC	20	Arabidopsis, Rice, Maize
miR156b	UUGACAGAAGAUAGAGAGCAC	21	Arabidopsis, Rice, Maize
miR156e	UUGACAGAAGAUAGAGGGCAC	21	Arabidopsis, Rice, Maize
miR156f	UGACAGAAGAUAGAGAGCAC	20	Arabidopsis, Rice, Maize
miR156h	UGACAGAAGAGAGGGAGCAC	20	Arabidopsis, Rice, Maize
miR159a	UUUGGAUUGAAGGGAGCUCUA	21	Arabidopsis, Rice, Maize
miR159b	AUUGGAUUGAAGGGAGCUCCU	21	Arabidopsis, Rice, Maize
miR160a	UGCCUGGCUCCCUGUAUGCCA	21	Arabidopsis, Rice, Maize
miR160d	UGCCUGGCUCCCUGAAUGCCA	21	Arabidopsis, Rice, Maize
miR162	UCGAUAAACCUCUGCAUCCAG	21	Arabidopsis, Rice, Maize
miR164a	UGGAGAAGCAGGGCAGGUGCU	21	Arabidopsis, Rice, Maize
miR164b	UGGAGAGGCAGGGCACAUGCU	21	Arabidopsis, Rice, Maize
miR164c	UGGAGAAGCAGGGCAGGUGCA	21	Arabidopsis, Rice, Maize
miR166c	UCGGACCAGGCUUCAUCCCC	21	Arabidopsis, Rice, Maize
miR166e	UCGGACCAGGCUUCAUCCUC	21	Arabidopsis, Rice, Maize
miR166g	UCGGACCAGGCUUCAUCCC	20	Arabidopsis, Rice, Maize
miR166i	UCGGACCAGGCUUCAUUCUC	20	Arabidopsis, Rice, Maize
miR167a	UGAAGCUGCCAGCAUGAUCUA	21	Arabidopsis, Rice, Maize
miR167c	UGAAGCUGCCAGCAUGAUCUU	21	Arabidopsis, Rice, Maize
miR167d	UGAAGCUGCCAGCAUGAUCUG	21	Arabidopsis, Rice, Maize
miR167e	UCAAGCUGCCAGCAUGAUCUA	21	Arabidopsis, Rice, Maize
miR168	UCGCUUGGUGCAGGUCGGGA	20	Arabidopsis, Rice, Maize
miR169b	UAGCCAAAAAUGACUUGCCUG	21	Arabidopsis, Rice, Maize
miR169g	AAGCCAAGGAUGAAUUGCCGG	21	Arabidopsis, Rice, Maize
miR169h	CAGCCAAGGAUGACUUGCCGG	21	Arabidopsis, Rice, Maize
miR169k	UGAGCCAAGGAUGACUUGCCU	21	Arabidopsis, Rice, Maize
miR169m	UAGCCAAAAAUGACUUGCCUGC	22	Arabidopsis, Rice, Maize
miR169n	UAGCCAAGGAUGACUUGCCUG	21	Arabidopsis, Rice, Maize
miR169o	UAGCCAAAGAUGACUUGCCUG	21	Arabidopsis, Rice, Maize
miR169q	UGAGCCAAAGAUGACUUGCCU	21	Arabidopsis, Rice, Maize
miR169r	GAGCCAAGAAUGACUUGCCGG	21	Arabidopsis, Rice, Maize
miR171c	UGAUUGAGCCGUGCCAAUAUC	21	Arabidopsis, Rice, Maize
miR171d	UUGAGCCGUGCCAAUAUCACG	21	Arabidopsis, Rice, Maize
miR171e	UGAUUGAGCCGCGCCAAUAUC	21	Arabidopsis, Rice, Maize

Table 1. Cont.

Known miRNAs	miRNA Sequences	Length (nt)	Conserved in Other Plants
miR171h	UGAUUGAGCCGCGUCAUAUC	21	Arabidopsis, Rice, Maize
miR172a	GGAAUCUUGAUGAUGCUGCAG	21	Arabidopsis, Rice, Maize
miR172b	AGAAUCUUGAUGAUGCUGCAU	21	Arabidopsis, Rice, Maize
miR172d	GGAAUCUUGAUGAUGCUGCAU	21	Arabidopsis, Rice, Maize
miR172e	AGAAUCUUGAUGAUGCUGCAG	21	Arabidopsis, Rice, Maize
miR172f	UGAAUCUUGAUGAUGCCGCAC	21	Arabidopsis, Rice, Maize
miR1863	AGCUCUGAUACCAUGUUAGAUAUUG	24	Rice, Melon
miR2111a	UAAUCUGCAUCCUGAGGUUA	21	Arabidopsis, Soybean
miR319b	UUGGACUGAAGGGAGCUC	20	Arabidopsis, Rice, Maize
miR319c	UUGGACUGAAGGGAGCUCU	20	Arabidopsis, Rice, Maize
miR390b	AAGCUCAGGAGGGAUAGCGCC	21	Arabidopsis, Rice, Maize
miR393a	UCCAAAGGGAUCGCAUUGAUC	21	Arabidopsis, Rice, Maize
miR394b	UUGGCAUUCUGUCCACCUC	20	Arabidopsis, Rice, Maize.
miR395a	CUGAAGUGUUUGGGGGAACUC	21	Arabidopsis, Rice, Maize
miR395b	UUGAAGUGUUUGGGGGAACUC	21	Arabidopsis, Rice, Maize
miR396a	UUCCACAGCUUUCUUGAACUU	21	Arabidopsis, Rice, Maize
miR396b	UUCCACAGCUUUCUUGAACUG	21	Arabidopsis, Rice, Maize
miR396e	UUCCACGGCUUUCUUGAACUG	21	Arabidopsis, Rice, Maize
miR397	UCAUUGAGUGCAGCGUUGAUG	21	Arabidopsis, Rice, Maize
miR398a	UGUGUUCUCAGGUCGCCCCUG	21	Arabidopsis, Rice, Maize
miR398b	UGUGUUCUCAGGUCACCCU	21	Arabidopsis, Rice, Maize
miR399a	UGCCAAAGGAGAUUUGCCCCG	21	Arabidopsis, Rice, Maize
miR399b	UGCCAAAGGAGAGUUGCCCUA	21	Arabidopsis, Rice, Maize
miR399c	UGCCAAAGGAGAUUUGCCCCG	21	Arabidopsis, Rice, Maize
miR399d	UGCCAAAGGAGAGUUGCCCU	21	Arabidopsis, Rice, Maize
miR399f	UGCCAAAGGAGAAUUGCAC	19	Arabidopsis, Rice, Maize
miR399g	AGGGCUUCUCUCCAUUGGCAGG	22	Arabidopsis, Rice, Maize
miR408	AUGCACUGCCUCUCCCCUGGC	21	Soybean, Maize
miR477a	ACCUCUCCUCAAAGGCUUCCAA	21	Tomato, Grape
miR477b	CUCUCCUCAAAGGCUUCUG	20	Tomato, Grape
miR530a	UGCAUUUGCACCUGCACCUU	20	Rice, Soybean
miR530b	UGCAUUUGCACCUACACCUU	20	Rice, Soybean
miR7129	AGUCAAAUCUAAACGAUCGUGUAU	24	Melon
miR7130	GUUUGGAAUGUGCGAGAUGUGUGC	24	Melon
miR854	GAUGAGGAUAGUGAGGAGGAG	21	Arabidopsis, Poplar, Tea plant
miR858	UCUCGUUGUCUGUUCGACCUU	21	Arabidopsis, Tomato, Grape
Novel miRNAs	miRNA sequences	Length (nt)	
miRn1	AGUCAAUUCGAACAUAGCUCAGUG	24	
miRn2	AAAAGGUCAGAGGUUCGAAUCUCC	24	
miRn3	UUGGACUGAAGGGAGCUC	20	
miRn4	CAUGUUAUUGAGGAAGCUCUUG	22	
miRn5	AUUUACGAUCUGUAUAACAAAGUC	24	
miRn6	UUGGACUGAAGGGAGCUCU	20	
miRn7	UCCACAGCUUUCUUGAACUU	21	
miRn8	UUAACGAUCUAGGUUAACAUGAU	24	
miRn9	UGCCAAAAGAGACUUGCCCUG	21	
miRn10	UUAUAUCUUGGCGGUCUGUAAAGC	24	
miRn11	UCAUGCCAUCUCUUGGAUU	20	
miRn12	AUAGACGCUGGUAGAUAGAUAUC	24	
miRn13	UGAAUAGAUUGUGAGUGGAAC	21	
miRn14	UGGACCACCAUUCUGAUACC	21	
miRn15	UGGAGAAGCAGGGCACGUGCA	21	
miRn16	AAGCUCAGGAGGGAUAGCGCC	21	
miRn17	UUCUCUCCCUCAAAGGGCUUCGA	22	

Table 1. Cont.

Novel miRNAs	miRNA sequences	Length (nt)
miRn18	AAUGAUCUGUUCUAAUAUCUAAGC	24
miRn19	AUUUAUAGAUCUGUCAGUGAUAAC	24
miRn20	UUGUGAGCUCGAAAUUGAAUACG	24
miRn21	AUUGACGAACUAAUUUAUACUCCAU	24
miRn22	AAAGUCUAUCACUGAUAGACCAUG	24
miRn23	UUAGUAUCUAUCGAUGUUUAUCAU	24
miRn24	UCCAUCUCUUGCACACUGG	20
miRn25	UUUCGAUCGAGUUUUUAUAUGUAAC	24
miRn26	UCAGCGCUUCAUUCAAUCACA	21
miRn27	AUAGUCUGUGUUUGAAGUGCAU	22
miRn28	AUCGAUCUAUCGACCUUUACUCAU	24
miRn29	UUACUAUUGAAGGGAGGAAGU	21
miRn30	UCUUCGAUCUUGUCAUUUUCUGCU	24
miRn31	UUUCGGAUUGUACCUUAGUUCUCC	24
miRn32	AGUGUGCUACUUUUGAAAUGUAAA	24
miRn33	UUCUUGAUCUUGUCAUUUUCUGCU	24
miRn34	UUCCACAGCUUUCUUGAACUU	21
miRn35	UCAUUGACAUUGUGGUUGACU	21
miRn36	UUCUAUUGUCUUCAUUUGUGA	21
miRn37	ACCGAUGGUCGUAUGUCCGAAU	22
miRn38	AACGUUCUGUUUGUGUGAAACUAC	24
miRn39	UUGACAGAAGAGAGUGAGCAC	21
miRn40	AUUUAAUUGUCAUUGUAGGCCAUU	24
miRn41	ACACGACUGGGUACAU AUGACUCC	24
miRn42	UUCGAUCUCUAGAAUUUUAUCCUU	24
miRn43	AUAUAUCCGAUCUACGACUAAGGU	24

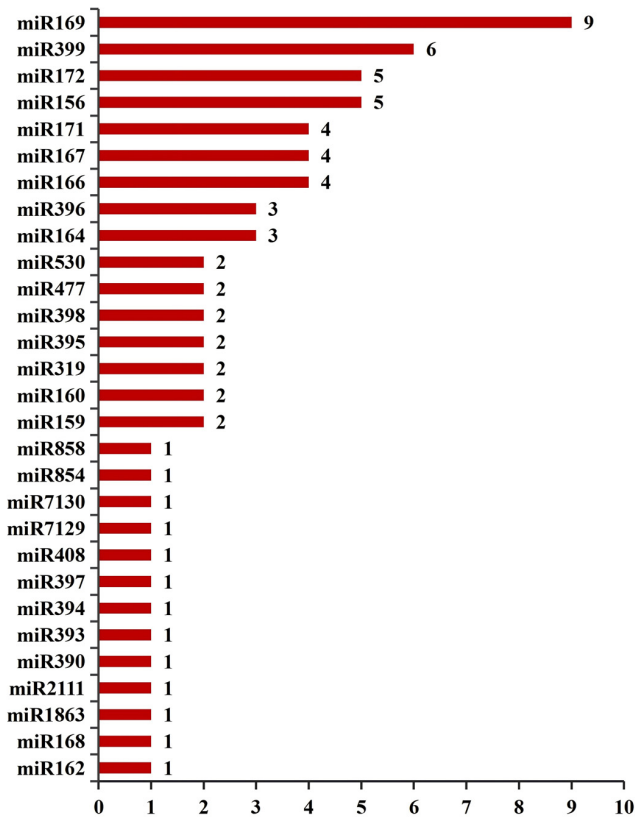


Figure 3. Numbers of members of known miRNA families in melon.

3.4. Differential Expression of miRNAs in Response to PM Infection and qRT-PCR Validation in Melon

To identify PM-responsive miRNAs, differential expression analyses were performed between PM infected samples and control samples for both PM-resistant and PM-susceptible genotypes. A total of 13 known miRNAs and eight novel miRNAs were differentially expressed between the mock and PM-infected samples. Among them, 12 miRNAs were upregulated and 6 miRNAs were downregulated in the susceptible genotype, and 11 miRNAs were upregulated and 7 miRNAs were downregulated in the resistant genotype (Figure 4). Notably, miR398b, miR399g, miR530a and miRn8 were specifically upregulated in the resistant genotype, while miR167c was specifically downregulated in the resistant genotype. These five miRNAs showed no significant expression changes in the susceptible genotype (Figure 5). Additionally, miR164a, miR397, and miR477 exhibited higher basal expression in resistant lines compared to susceptible lines prior to infection, suggesting that they may pre-activate defense signaling pathways to enable faster and stronger resistance responses upon PM infection. The differential expression levels of eight miRNAs were experimentally validated using qRT-PCR, and the results were highly consistent with the high throughput sequencing results (Figure 6).

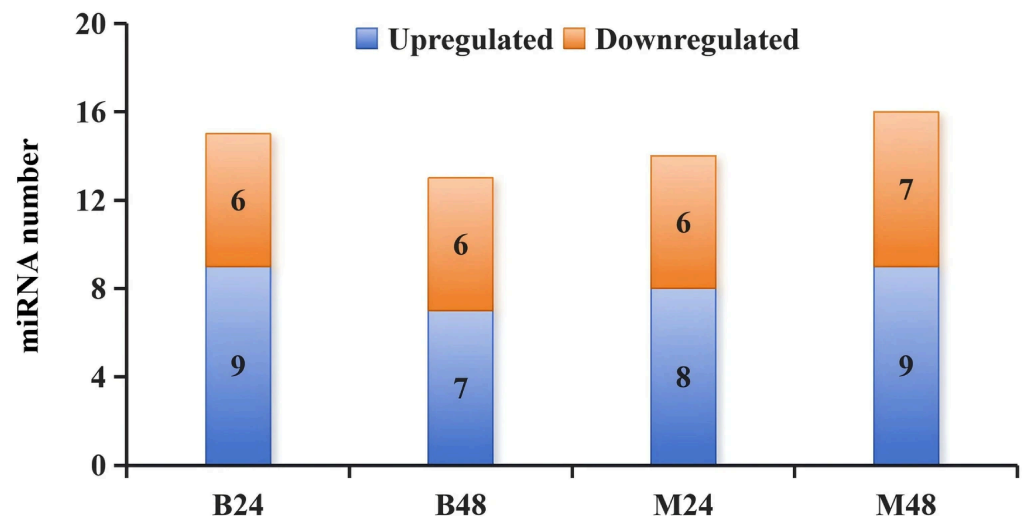


Figure 4. Statistical analysis of differentially expressed miRNA in resistant and susceptible lines in response to PM infection. B24: Comparison between samples collected at 24 hours post-inoculation with control treated with sterile water in susceptible lines; B48: Comparison between samples collected at 48 hours post-inoculation with control treated with sterile water in susceptible lines; M24: Comparison between samples collected at 24 hours post-inoculation with control treated with sterile water in resistant lines; M48: Comparison between samples collected at 48 hours post-inoculation with control treated with sterile water in resistant lines.

3.5. Target Gene Prediction and Functional Characterization of Differentially Expressed miRNAs

MiRNAs primarily regulate gene expression at the post-transcriptional level by binding to target mRNAs through reverse complementarity, leading to mRNA cleavage or translational repression. Therefore, identification and analysis of target genes are fundamental to understanding the regulatory role of miRNAs. To further elucidate the mechanism of miRNA regulation in melon resistance to PM, bioinformatic approaches were employed to predict target genes of the 21 differentially expressed miRNAs (Supplementary Table S4). In total, 187 target genes were predicted. miR408 targeted the largest number of genes (23), whereas miRn11 targeted the fewest (only 2). GO functional annotation classified these target genes into three categories: biological process, cellular component, and molecular function. Most target genes were enriched in biological processes, indicating that miRNAs

may regulate diverse genes to participate in multiple biological processes during defense against PM infection (Figure 7). KEGG pathway enrichment analysis revealed significant enrichment in the plant–pathogen interactions and MAPK signaling pathways, which contained 35 and 27 target genes, respectively (Figure 8). This suggests that miRNAs may confer resistance by regulating genes involved in plant immune responses and protein kinase signaling pathways. These findings provide a theoretical basis for understanding the role of miRNAs in regulating melon defense against PM infection.

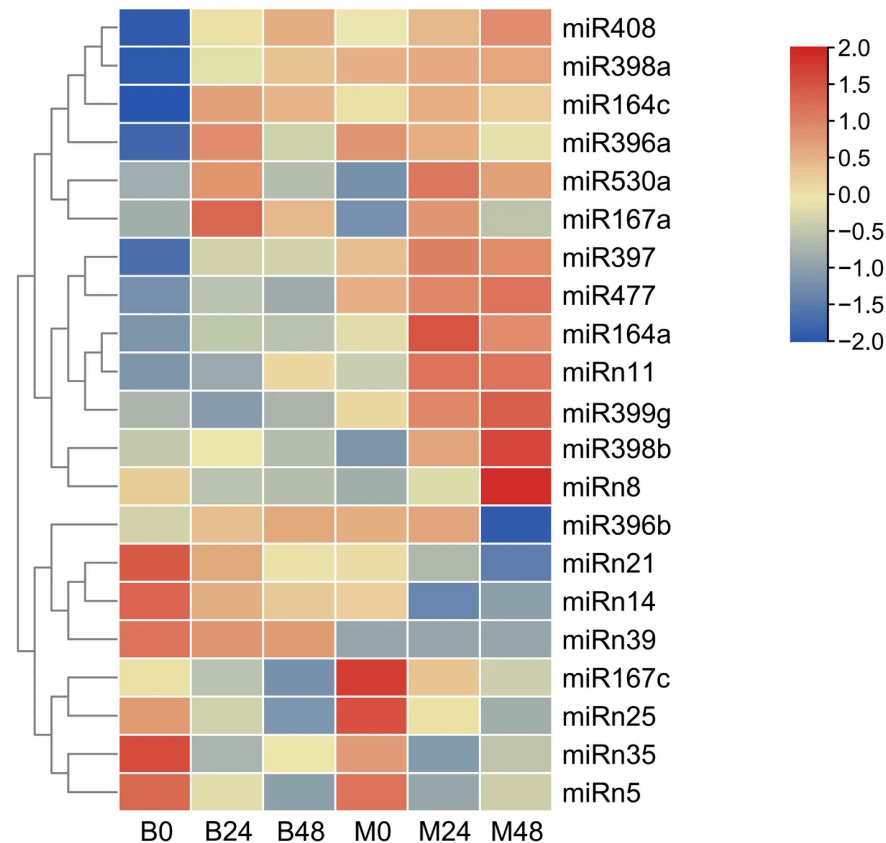


Figure 5. Heat map analysis of differentially expressed miRNA in resistant and susceptible lines in response to PM infection. B0: Control of susceptible lines; B24 and B48: Samples collected at 24 and 48 hours post-inoculation of susceptible lines; M0: Control of resistant lines; M24 and M48: Samples collected at 24 and 48 hours post-inoculation of resistant lines.

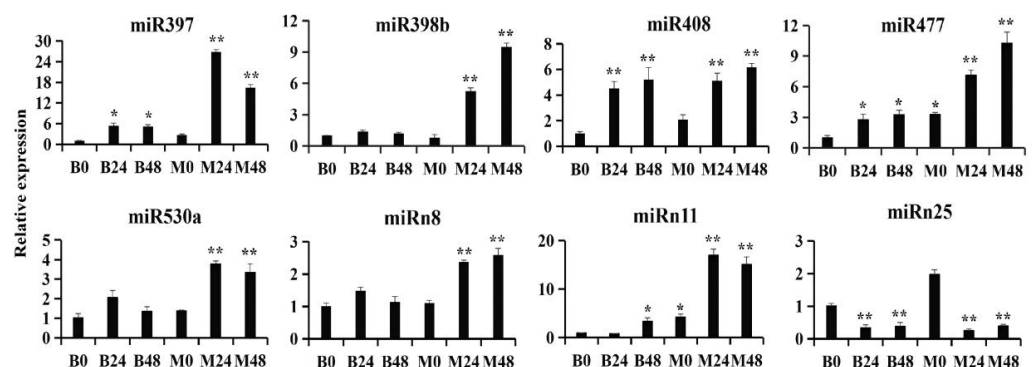


Figure 6. qRT-PCR validation of differentially expressed miRNA. Error bars indicate $\pm$ SD of three biological replicates. Asterisks indicate a significant change (* $p < 0.05$; ** $p < 0.01$). B0: Control of susceptible lines; B24 and B48: Samples collected at 24 and 48 hours post-inoculation of susceptible lines; M0: Control of resistant lines; M24 and M48: Samples collected at 24 and 48 hours post-inoculation of resistant lines.

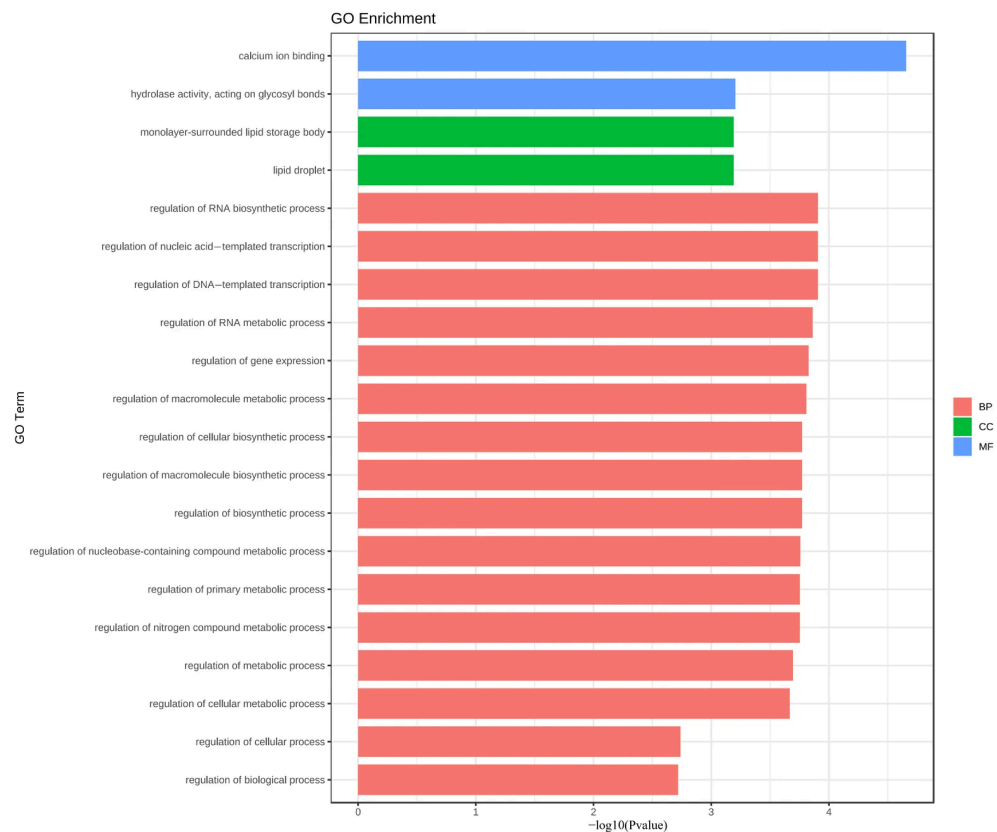


Figure 7. GO analysis of target genes of differentially expressed miRNAs in resistant and susceptible melon seedlings in response to PM infection.

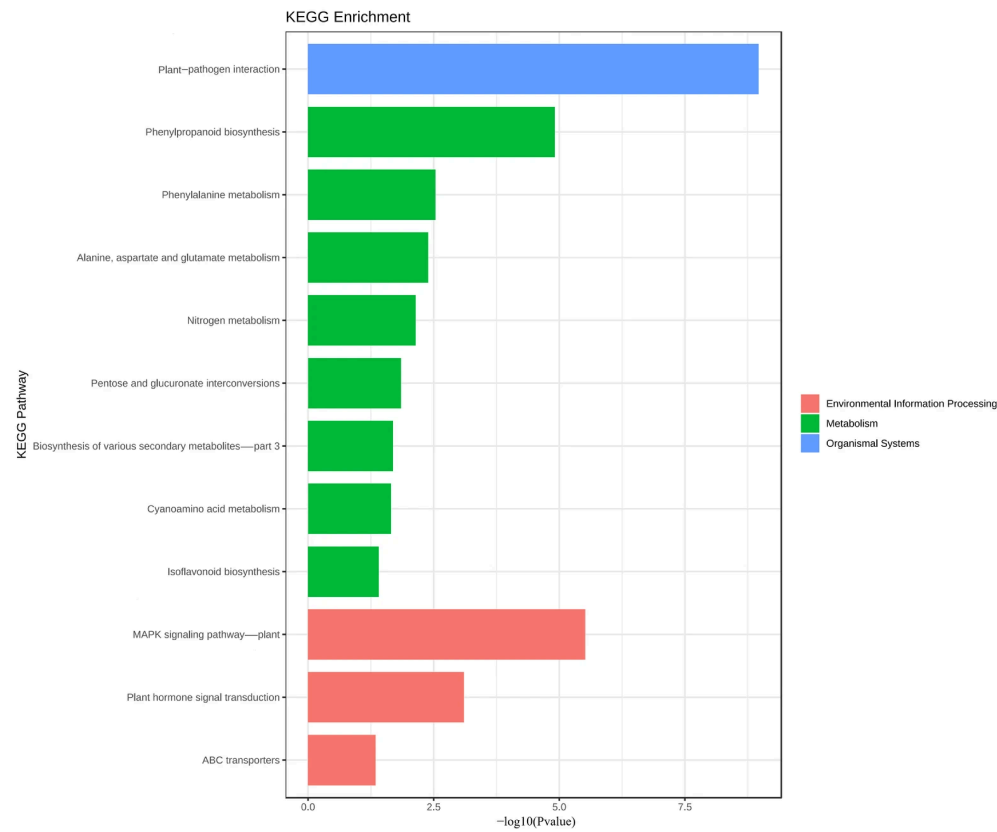


Figure 8. KEGG analysis of target genes of differentially expressed miRNAs in resistant and susceptible melon seedlings in response to PM infection.

3.6. Correlation Analysis Between miRNAs and Target Gene Expression

To explore the regulatory relationships between miRNAs and their target genes, qRT-PCR was used to detect the expression of eight target genes of miR167c, miR398b, miR399g and miRn8 before and after PM infection. As shown in Figure 9, *MELO3C026955* and *MELO3C008957* are target genes of miR398b, encoding superoxide dismutase and an LRR family receptor-like kinase, respectively. After PM infection, the expression of these two genes showed no significant changes in the susceptible genotype but was markedly down-regulated in the resistant genotype. Their expression patterns were negatively correlated with miR398b abundance, which is consistent with the canonical miRNA-target regulatory mode. Similarly, miR399g and its targets *MELO3C017200* and *MELO3C021852*, as well as miRn8 and its targets *MELO3C003188* and *MELO3C023120*, also exhibited significant negative expression correlations. In addition, *MELO3C025737* and *MELO3C002443*, the target genes of miR167c, encode a Dof transcription factor and an F-box protein, respectively. Following PM inoculation, these two genes were significantly upregulated in the resistant genotype but remained unaltered in the susceptible genotype, indicating their positive regulatory roles in melon defense against PM infection.

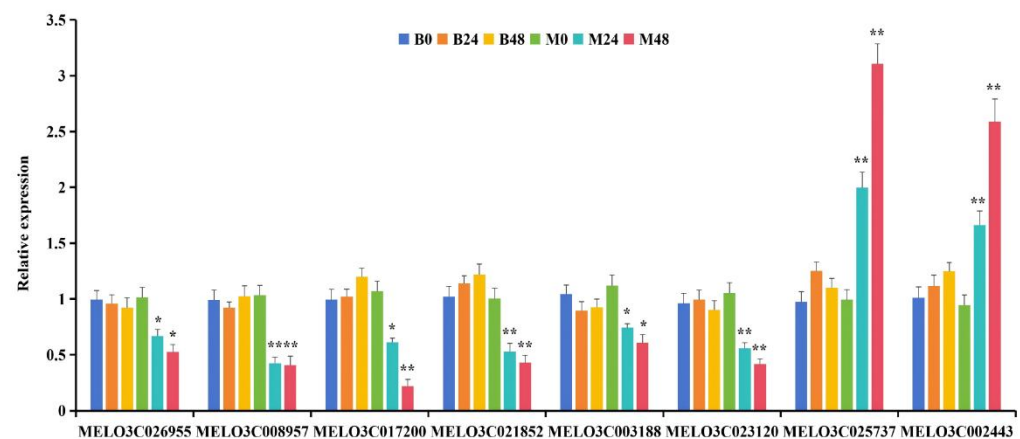


Figure 9. Expression analysis of target genes after PM infection. Error bars indicate $\pm$ SD of three biological replicates. Asterisks indicate a significant change (* $p < 0.05$; ** $p < 0.01$). B0: Control of susceptible lines; B24 and B48: Samples collected at 24 and 48 hours post-inoculation of susceptible lines; M0: Control of resistant lines; M24 and M48: Samples collected at 24 and 48 hours post-inoculation of resistant lines.

4. Discussion

Numerous previous studies have deciphered the core regulatory mechanisms of powdery mildew resistance in cucurbit crops, which can be categorized into two major forms: race-specific resistance mediated by NBS-LRR genes and broad-spectrum durable resistance derived from loss-of-function *mlo* mutations [24,25]. As important regulators of gene expression, miRNAs play crucial roles in various biological processes, such as plant growth, development, and responses to abiotic and biotic stresses. However, studies on the involvement of miRNAs in powdery mildew resistance in melon remain unreported. Our research team has developed a broad-spectrum, highly PM-resistant melon inbred line ‘M1’, which exhibits stable resistance under both natural conditions and artificial inoculation in a greenhouse, providing valuable material resources for comprehensively dissecting the molecular mechanisms underlying resistance. Notably, M1 and B29 were selected from different commercial cultivars with distinct genetic backgrounds. Therefore, the detected molecular variations between the two lines are not completely resistance-specific and may be partially influenced by intrinsic genetic differences. Further validation using

near-isogenic lines will exclude background noise and improve the accuracy of future mechanistic studies.

To explore the regulatory functions of miRNAs in melon PM resistance, high-throughput sRNA sequencing was performed using leaf tissues from PM-resistant and PM-susceptible melons after PM inoculation. Distinct from rice, tomato, and soybean [26–28], where the accumulation of 24 nt sRNAs is significantly higher than that of 21 nt and 22 nt sRNAs, melon has the highest proportion of 22 nt sRNAs, followed by 21 nt and 24 nt sRNAs. This length distribution pattern is consistent with the findings in cucumber, wheat, and grape [29–31]. Previous research has indicated that 21 and 22 nt sRNAs are mainly miRNAs and trans-acting siRNAs (ta-siRNAs), which regulate gene expression through post-transcriptional silencing. In contrast, 24 nt sRNAs are mainly phased siRNAs (pha-siRNAs) enriched in repetitive sequences and transposon regions, playing a core role in heterochromatin modification and RNA-directed DNA methylation. The high abundance of 22 nt sRNAs in melon suggests that the fine regulatory network dominated by miRNAs/ta-siRNAs plays a predominant regulatory role in melon.

In this study, 113 miRNAs were identified in leaf tissues of PM-resistant melon ‘M1’ and susceptible melon ‘B29’ through high-throughput sequencing. By comparison with the miRBase database, 70 known miRNAs belonging to 29 families were identified, among which 25 families were highly conserved and 4 families were less conserved, suggesting that most miRNAs in melon are widely conserved across plants and may regulate fundamental biological processes, while lowly conserved miRNAs may contribute to species-specific resistance mechanisms. Differential expression analysis revealed 11 up-regulated and 6 downregulated miRNAs in the susceptible genotype, and 11 upregulated and 7 downregulated miRNAs in the resistant genotype. Notably, miR398b, miR399g, miR530a and miRn8 were specifically upregulated in the resistant genotype, while miR167c was specifically downregulated, with no significant expression changes observed in the susceptible genotype. These findings suggest that these miRNAs may play critical roles in PM defense by specifically regulating disease-resistance-related genes. For example, in rice, miR398b has been shown to enhance resistance to *Magnaporthe oryzae*. Transgenic rice overexpressing miR398b exhibits delayed infection progression, reduced lesion expansion, and stronger disease resistance. Further research showed that miR398b could alter total SOD activity and promote H₂O₂ accumulation by targeting multiple superoxide dismutase genes (*CSD1*, *CSD2*, *CCSD*, *SODX*) and suppressing their expression, thereby activating defense-related genes and hypersensitive responses to strengthen immunity [32]. Moreover, miR398 has been identified as the only shared regulatory factor in the three-tiered immune network of rice, including PAMP-triggered immunity (PTI), effector-triggered susceptibility (ETS), and effector-triggered immunity (ETI), exhibiting a broad-spectrum disease resistance profile characterized by ‘promoting PTI and ETI while suppressing ETS’ [33]. Additionally, miR399 has been implicated in phosphate metabolism regulation, indirectly enhancing resistance by reinforcing cell walls and other defense processes [34,35]. The attenuated response of miRNA in ‘B29’ may weaken defense signal transduction, making it more susceptible to PM infection. These results align with known miRNA-mediated pathogen defense mechanisms in plants and provide molecular clues for understanding melon-specific resistance against PM.

In addition, four miRNAs, miR164c, miR396a, miR398a and miRn39, exhibited distinct expression patterns exclusively in susceptible genotypes upon PM infection, implying their critical roles in mediating plant susceptibility rather than disease resistance. As key immunity-related regulatory microRNAs, these candidates participate in modulating plant immune homeostasis, antioxidant metabolism, and cell defense responses, thereby determining the differential disease phenotypes between susceptible and resistant genotypes.

For instance, miR164c belongs to a conserved plant miRNA family and exerts regulatory functions primarily by post-transcriptional repression of NAC transcription factors. Numerous studies have validated that miR164 acts as a negative regulator of disease resistance in multiple plant–fungal pathogen interaction systems [36,37]. The miR164–NAC module participates in the regulation of phytohormone signaling, reactive oxygen species homeostasis, pathogen-triggered cell death, and the expression of defense-related genes [38]. In this study, miR164c exhibited specific differential expression only in susceptible melon materials upon PM infection, suggesting that upregulated miR164c may promote plant susceptibility. Further genetic functional validation of miR164c and its target NAC genes is required to dissect the molecular pathway through which miR164c modulates melon susceptibility to PM. Furthermore, miR396a acts as a conserved negative regulator of plant fungal disease resistance and a key driver of plant susceptibility [39]. It primarily targets and represses GRF family transcription factors, which are core positive regulators of plant immune activation and defense reprogramming. Functional studies have confirmed that suppressed GRF expression impairs multiple critical defense responses, including pathogen-induced ROS burst, cell wall callose deposition, and the transcription of SA-dependent defense genes and PR disease-resistant genes [40,41]. In susceptible plant materials, PM infection specifically induces the upregulation of miR396a, which continuously inhibits the host's endogenous immune cascades. This persistent suppression of defense signaling eliminates the host's inhibitory effect on fungal proliferation, ultimately facilitating PM colonization and spread and the development of typical susceptible symptoms.

GO functional annotation revealed that the target genes of differentially expressed miRNAs are primarily categorized into three classes: biological process, cellular component, and molecular function, with most enriched in biological processes, indicating that miRNAs likely modulate multiple biological processes during melon defense responses. KEGG enrichment further showed significant enrichment of target genes in the plant–pathogen interaction pathway and the MAPK signaling pathway, suggesting that miRNAs may enhance melon resistance to PM by regulating key genes in immune response and kinase signaling cascades. For instance, miR408 may enhance disease resistance by regulating critical genes in the plant–pathogen interaction pathway, while miRn11 may participate in immune regulation via the MAPK pathway. The plant–pathogen interaction pathway involves pattern recognition receptors (PRRs) and effector recognition, triggering downstream defenses such as callose deposition and reactive oxygen species (ROS) burst [42]. The MAPK cascade mediates intracellular signal transduction to activate defense-related gene expression [43]. Enrichment of these pathways in the resistant line 'M1' implies miRNAs may enhance pathogen recognition and response by suppressing negative regulators or activating positive ones. For example, miRNAs might target genes that inhibit kinase activity, thereby relieving the suppression of immune signaling. This regulatory pattern is consistent with miRNA-mediated immunity in model plants such as *Arabidopsis* and rice [44,45]. qRT-PCR analysis showed that the target genes of miR167c, miR398b, miR399g, and miRn8 exhibited no significant expression changes in the susceptible line 'B29' but displayed inverse expression patterns in the resistant line 'M1', indicating their expression is closely linked to resistance, suggesting that the resistant line 'M1' may possess a specialized signaling network that senses PM infection and activates effective defense through coordinated regulation of these miRNAs and their targets. In contrast, the susceptible line lacks such efficient sensing or regulatory capacity, leading to defective defense responses and increased PM susceptibility.

5. Conclusions

Previous studies have demonstrated that pathogen infection triggers differential accumulation of miRNAs in diverse plant species. These pathogen-responsive miRNAs further regulate downstream target genes implicated in plant–pathogen interactions. This study identified PM-responsive miRNAs and their target genes involved in the plant–pathogen interaction pathway and the MAPK signaling pathways in melon and systematically revealed the regulatory mechanisms of miRNAs underlying defense responses. These findings provide a theoretical basis for understanding the molecular mechanisms of melon’s susceptibility and resistance to PM from the miRNA perspective. Moreover, this study offers potential molecular markers and target genes for molecular breeding of PM-resistant melon varieties, highlighting significant theoretical and application values.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12091141/s1>, Figure S1: The correlation coefficients among all the samples; Table S1: Stem-loop primer sequences used for miRNA reverse transcription; Table S2: Oligonucleotide primer sequences used for qRT-PCR; Table S3: Summary statistics of sRNA-seq data and mapping results; Table S4: Function annotation of target genes of differentially expressed miRNAs.

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Data Availability Statement: The raw sequence data reported in this paper have been deposited in the NCBI sequence read archive database (accession number: SRR9129105-SRR9129122).

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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