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Identification of self- and pathogen-targeted miRNAs from resistant and susceptible Theobroma cacao variety to black pod disease

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Abstract

Cacao (*Theobroma cacao*) is a highly valuable crop with growing demands in the global market. However, cacao farmers often face challenges posed by black pod disease caused by *Phytophthora spp.* with *P. palmivora* as the most dominant. Regulations of various gene expression influence plant resistance to pathogens. One mechanism involves targeting mRNA of virulence genes in the invading pathogens, suppressing their infection. However, resistance also could be suppressed by plant-derived miRNAs that target their own defense genes. The objective of this study is to identify differentially expressed miRNAs in black pod resistant and susceptible cacao varieties and to predict their targets in *T. cacao* and *P. palmivora* transcripts. In total, 54 known miRNAs from 40 miRNA families and 67 Novel miRNAs were identified. 17 miRNAs were differentially expressed in susceptible variety compared to resistant one, with 9 miRNAs were upregulated and 8 miRNAs downregulated. In *T. cacao* transcripts, the upregulated miRNAs were predicted to target several genes, including defense genes. The suppression of these defense genes can lead to a reduction in plant resistance against pathogen infection. While in *P. palmivora* transcripts, the upregulated miRNAs were predicted to target several genes, including *P. palmivora* effector genes. In the future, limiting expression of miRNAs that target *T. cacao*'s defense genes and applying miRNAs that target *P. palmivora* effector genes hold promise for enhancing cacao plant resistance against *P. palmivora* infection.

Introduction

The decrease in cacao (*Theobroma cacao*) productivity in Indonesia has significantly impacted plantations and associated industries. In 2015, Indonesia ranked third globally as a cacao producer, following Côte d'Ivoire and Ghana. However, over the past seven years, Indonesia's cacao production has experienced a decline, leading to a seventh position among the world's cacao producers. Nonetheless, Indonesia remains the largest cacao producer in Asia, contributing to a total of 5.7% of the Asian production[1].

Ariningsih [2] attributed the decline in cacao productivity in Indonesia to factors such as inadequate plant maintenance and the presence of pests and diseases. Among the diseases affecting cacao, the most prominent is black pod disease (BPD), caused by fungal infections from the Phytophthora genus[3]. Four species of Phytophthora have been identified as causative agents of BPD. These include *Phytophthora palmivora*, *Phytophthora megakarya*, *Phytophthora capsica*, and *Phytophthora citrophthora*. Among these species, *Phytophthora palmivora* is recognized as the most destructive, causing a global production reduction of 44%[4]. In some regions of Indonesia, losses caused by this pathogen have reached 30–50%[5].

The most common approach for BPD control in cacao is the periodic application of chemical fungicides. However, these chemicals pose environmental challenges like soil and water pollution. Additionally, fungicides can negatively impact human health and beneficial soil microorganisms[6]. Therefore, alternative strategies with limited detrimental environmental effects, such as biocontrol methods, are required. Another approach involves utilizing cacao varieties that exhibit resistance to BPD. However, resistant varieties do not always possess other desirable traits, such as high fruit yield and shorter life cycles. Consequently, breeding techniques are necessary to introduce resistance to *P. palmivora* in susceptible varieties that already display superior phenotypic characteristics.

Plant susceptibility is known to be influenced by gene expression regulation. One mechanism involves the production of miRNAs that specifically target genes associated with the defense system, thereby initiating RNA interference (RNAi) and suppressing the expression of their defense system genes[7]. According to Yang 2021 [8], a significant number of miRNAs target resistance genes, particularly NBS-LRR genes, which play a crucial role in pathogen recognition in plants due to their nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains. For instance, the heightened susceptibility of potato plants to *Verticillium dahliae* infection resulting from the overexpression of stu-miR482e can be attributed to the increased suppressive impact induced by stu-miR482e on the NBS-LRR genes responsible for disease resistance[9]. These findings highlight the potential of eliminating these miRNAs to enhance plant resistance to infection.

In addition, cross kingdom RNAi is proposed as one of plant defense mechanisms against pathogen infection, which is carried out by transferring small regulatory RNAs into pathogens that attack the plant. This process leads to the silencing of virulence genes in the pathogen, thereby suppressing pathogen infection[10]. miRNAs exhibit environmentally friendly characteristics and have high specificity to their target. Koch et al. [11] demonstrate that applying CYP3-dsRNA that target *Fusarium graminearum* CYP3 gene through spraying or spray-induced gene silencing (SIGS) can effectively repress the infection of *F. graminearum* on barley. These findings highlight the potential of utilizing miRNAs as a biocontrol strategy against pathogen infection.

The objective of this study is to identify differentially expressed miRNAs in black pod disease-resistant and susceptible cacao varieties. Additionally, the study aims to identify the targets of these miRNAs within the *T. cacao* defense system gene and *P. palmivora* virulence gene. In future applications, the elimination of miRNAs that target *T. cacao* defense system genes is expected to restore the expression levels of these genes, enhancing resistance against *P. palmivora* infection and also utilizing miRNAs that target the virulence genes of *P. palmivora* as a biocontrol is also expected to suppress its infection effectively.

Results

Resistance and Susceptible varieties of T. cacao and Disease Scoring

In this research, *T. cacao* pod from resistant (healthy) and susceptible (infected) varieties (Fig. 1) were collected from the cacao plantation at the Indonesian Coffee and Cacao Research Institute. Disease scoring was performed to assess the extent of infection. Resistant cacao pod exhibited no signs of rot, whereas the susceptible cacao pod displayed one rotten zone with an area of 47.57 cm^2 (Table 1).

Table 1
Disease Scoring of samples used in miRNA extraction

Sample	Length (cm)	Circumference (cm)	Zone	Diameter 1 (cm)	Diameter 2 (cm)	Area (cm^2)
Resistant	17.7	29.8	-	-	-	-
Susceptible	19.2	26.7	1	30.8	29.8	47.57

To confirm the presence of *P. palmivora*, PCR amplification was conducted using specific primers targeting the *ITS2* and 5.8S rDNA regions of the *P. palmivora* genome. Subsequent sequencing of the amplified products indicated the presence of *P. palmivora* in the susceptible cacao pods, as shown by the aligned sequences to the *ITS2* and *5.8S rDNA* genes of *P. palmivora* in NCBI database, while the resistant cacao pods did not exhibit any traces of *P. palmivora* (Supplementary information, Table 1).

Sequencing of small RNA

miRNA extraction was performed on the samples by using a microRNA extraction kit. The extracted miRNAs were then subjected to sequencing analysis to elucidate their expression profiles. The results are displayed in (Supplementary information, Table 2). Raw reads from the sequencing results showed good quality, as indicated by the absence of low-quality reads across all four samples. The presence of reads with an N% exceeding 10% was only observed in sample S2, with a minimal percentage of 0.01%. To ensure data quality, low-quality reads were removed using Trimmomatic, resulting in a range of clean reads from 2,974,499 to 11,124,113. Subsequently, a length-based filter was applied, selecting reads with lengths between 18 and 28 base pairs. These filtered results provided the final set of clean reads for further analysis. Final clean reads obtained for samples R1, R2, S1, and S2 are 10,685,251; 2,856,473; 3,861,549; and 4,134,082, respectively.

The clean reads obtained were subsequently aligned to the cacao genome to determine their genomic positions. The mapping results revealed that in samples R1, R2, S1, and S2, a total of 3,999,744; 1,619,130; 1,218,792; and 1,192,304 reads, respectively, were successfully mapped to the cacao genome. miRNA identification revealed a range of 647 to 112,728 reads for known miRNAs and 1,703 to 46,193 reads for putative novel miRNAs.

Identification of known & novel miRNA

Known miRNA identification was conducted by aligning the reads against the miRNA cacao database derived from mirBase and pmiREN. In *T. cacao* susceptible varieties, a total of 51 known miRNAs were successfully identified, whereas in *T. cacao* resistant varieties, 33 known miRNAs were identified. The reads that were unassigned to known miRNAs were further subjected to novel miRNA prediction using MirDeep2. The prediction analysis revealed the presence of 67 novel miRNAs in *T. cacao* resistant variety and 28 novel miRNAs in *T. cacao* susceptible one (Table 2 and Supplementary information, Table 3).

Table 2
The 10 most abundant known & novel miRNAs identified from *T. Cacao* libraries.

miRNA ID	Sequence (5'-3')	Chromosome	Start position	End position	Strand	R1	R2	S1	S2
Tca-MIR159d	TTGGACTGAAGGGAGCTCCT	NC_023616.1	25272801	25272821	+	48938	22272	357	528
Tca-MIR3954a	TTGGACAGAGTAATCACGGTCG	NC_023622.1	30293905	30293926	-	2992	182	111	311
Tca-MIR482a	TCTTCCCAACACCTCCCATACC	NC_023624.1	22810014	22810035	+	20440	950	8	20
Tca-MIRN9	TTCACTTCTCTGATTTGGGCGA	NC_023619.1	19178360	19178381	-	9948	341	2	19
Tca-MIR472a	TCTTCCCTACTCCACCCATCCC	NC_023621.1	40294561	40294582	-	6525	490	3	21
Tca-MIRN7068a	ACTCTCTTTCGAAGGCTTCAAG	NC_023625.1	35431242	35431263	-	5592	445	2	24
tcc-miR162	TCGATAAACCTCTGCATCCAG	NC_023623.1	22612635	22612655	-	1260	754	23	18
tcc-miR166a	TCGGACCAGGCTTCATTCCCC	NC_023623.1	28905616	28905636	-	2302	183	20	30
Tca-MIR159b	TTGGACTGAAGGGAGCTCCC	NC_023623.1	32655000	32655019	-	687	111	21	61
tcc-miR403a	TTAGATTCACGCACAAACTCG	NC_023622.1	32849355	32849375	-	3260	212	3	16
Novel-miR59	CAGTTTAATATCTGATACATG	NC_023625.1	4587917	4587943	-	659	26	1183	1418
Novel-miR1	TTGCCGAGTCCGCCCATGCCTA	NC_023624.1	22796221	22796242	+	31310	2312	84	128
Novel-miR6	GATAAATCATTGCAATTGTTG	NC_023619.1	21211154	21211177	+	520	202	305	322
Novel-miR42	GATAACATCATAGGATTA	NC_023622.1	29085332	29085349	+	788	100	49	237
Novel-miR18	GCGGCGATGATGATTGAAAA	NC_023618.1	883902	883920	+	55	10	34	50
Novel-miR5	CTTTTAACCGACTTTTGCACTCCC	NC_023617.1	4348336	4348356	-	1914	285	9	10
Novel-miR2	TTGCCGGCTCCGCCCATTCCAA	NC_023624.1	22809818	22809839	+	3627	117	0	3
Novel-miR4	ACAGGGAACAGGCTGAGCATG	NC_023625.1	34189717	34189737	-	1180	107	13	8
Novel-miR34	AATGTAAATCTGAACAATTC	NC_023619.1	572382	572402	+	27	8	5	20
Novel-miR39	AGCTCCTGAGATATGCAAGC	NC_023623.1	4147363	4147383	-	816	70	0	0

We found that the length of the identified miRNAs ranged from 18 to 27 nucleotides, with the majority (60 miRNAs) having a length of 21 nucleotides. Following this, miRNAs with a length of 24 nucleotides ranked second with the highest count of 26 miRNAs, while miRNAs with a length of 22 nucleotides accounted for 18 miRNAs. miRNAs with lengths beyond the categories were present in fewer numbers, with less than 5 miRNAs each (Fig. 2a). The identified known and novel miRNAs are also distributed across 10 chromosomes within the cacao genome. Chromosomes NC_023621.1, NC_023633.1, and NC_023625.1 have the highest abundance of miRNAs, exceeding 15 in number, while the remaining 7 chromosomes, NC_023616.1, NC_023617.1, NC_023618.1, NC_023619.1, NC_023620.1, NC_023622.1, and NC_023624.1, contained fewer than 15 miRNAs (Fig. 2b).

Analysis of Differentially Expressed miRNA

The PCA plot visualization (Fig. 3a) shows the grouping of miRNAs into distinctive clusters. miRNAs from resistant variety of TSH858, R1 and R2, clustered together in the red-colored plot, while miRNAs from susceptible variety of Sulawesi 1, S1 and S2, clustered in the blue-colored plot. This indicates a distinct separation between the resistant and susceptible samples based on their miRNA quantification. The PCA plot provides insights into the differences in principal component values within the two sample groups under investigation.

As much as 17 miRNAs with *p*adj value < 0.005 (Fig. 3b) were identified as differentially expressed out of a total of 117 miRNA normalization variables. The visualization of the volcano plot indicates these miRNAs with red plots. Out of them, nine miRNAs were upregulated while the other eight were downregulated. The most upregulated miRNA was Novel-miR59 with a $\log_2$FoldChange value of 7, indicating a significant increase in expression. On the other hand, tcc-miR399a showed the most pronounced decrease in expression with a $\log_2$FoldChange value of -6, as shown in Supplementary information, Table 4 and Table 5. The expression pattern of miRNAs, as depicted in Fig. 3c, exhibited a high degree of consistency in both the resistant and susceptible replicates.

Eight of the 17 differentially expressed miRNAs are putative novel miRNAs: Novel-miR2, Novel-miR6, Novel-miR9, Novel-miR18, Novel-miR24, Novel-miR39, Novel-miR42, and Novel-miR59. The remaining miRNAs are known and can be retrieved from both the miRbase and pmiREN databases.

Target Prediction on T. cacao and P. palmivora Genome

Known miRNAs and novel miRNAs were subjected to target gene prediction in the transcripts of *T. cacao* and *P. palmivora*. psRNATarget analysis revealed that 54 known miRNAs and 67 novel miRNAs were predicted to regulate up to 14,716 genes in the susceptible *T. cacao* genotype. Among these, 12,398 genes were regulated through cleavage and 2,318 genes were regulated through translational repression. In the 9 upregulated miRNAs, several miRNAs exhibit a broad range of target genes. For instance, miR156b displaying the highest number of target genes with 188 target genes. The following miR156b are Novel-miR34, miR167a, Novel-miR18, Novel-miR6, Novel-miR59, miR3954a, miR3627a, and Novel-miR42 which target 158, 157, 156, 154, 147, 135, 116, and 4 genes, respectively.

Among the targets of upregulated miRNAs in *T. cacao* transcripts, 17 disease-resistance genes are regulated by miR156b, miR167a, Novel-miR6, Novel-miR34, and Novel-miR59. Furthermore, miR3627a, Novel-miR6, miR3954a, and Novel-miR34 were predicted to target 7 L-type lectin-domain containing receptor kinase genes involved in defense response against oomycetes. Additionally, Novel-miR34, Novel-miR59, and miR156b were predicted to target cellulose synthase-like protein genes and expansin genes, which involved in cell wall synthesis and extension, an important stage in plant defense mechanisms against pathogen infection. Moreover, upregulated miRNAs, such as MIR3627a, target processes related to photosynthesis, including the activation of the RuBisCO protein. MiR167a and Novel-miR6 target genes associated with protein ubiquitination, while Novel-miR18 targets processes involved in DNA damage checkpoint signaling and DNA repair.

In the case of downregulated miRNAs, a total of 1085 transcripts in *T. cacao* were predicted to be targeted. Among these, 207 disease-resistant genes were identified as targets for Novel-miR2, Novel-miR39, Novel-miR9, Tca-MIR472a, Tca-MIR482a, and Tca-MIRN9. Furthermore, several miRNAs were found to target 60 receptor protein kinase genes, which play a vital role in plant defense by recognizing pathogens. These miRNAs include Novel-miR2, Novel-miR39, Novel-miR9, Tca-MIR472a, Tca-MIRN9, and tcc-miR396c. Notably, Novel-miR9 was predicted to target receptor protein kinases containing L-type lectin-domains. In addition to targeting disease resistance genes, Novel-miR2 and Novel-miR9 were also predicted to target genes involved in cell wall structural components. According to the prediction, one particular miRNA, tcc-miR399a, is expected to target proteins that undergo the process of ubiquitination.

Up to 8,917 *P. palmivora* genes were predicted to be controlled by 54 known miRNAs and 67 novel miRNAs, with 7,245 genes were regulated through cleavage and 1,672 genes through translational inhibition. Among the 9 upregulated miRNAs, several miRNAs exhibit multiple target genes in *P. palmivora* transcripts, where miR167a and Novel-miR18 are miRNAs with the highest number of target genes, up to 132 and 130 target genes, respectively. Then followed by Novel-miR6, miR3627a, Novel-miR59, miR156b, miR3954a, and Novel-miR34 with the number of target genes 97, 80, 76, 64, 63, and 52 respectively. However, Novel-miR42 was found to have no target genes.

The 9 upregulated miRNAs were predicted to regulate up to 694 genes in *P. palmivora*. Among these, 12 genes were RxLR effector genes, responsible for *P. palmivora* virulence factors that elicit plant hypersensitive response. Novel-miR59, miR3627a, Novel-miR6, miR156b, and miR167a regulated these 12 genes. Moreover, Novel-miR6 and Novel-miR-18 were also predicted to target Crinkler (CRN) family protein effector genes, which play a role in *P. palmivora* virulence factors by inducing necrosis in host plants. Other upregulated miRNAs were also predicted to target important genes in *P. palmivora*, including miR167a and Novel-miR18, which target cytochrome oxidase subunit genes involved in cellular respiration, miR3627a targeting vesicle coat complex genes involved in intracellular protein transport, Novel-miR34, miR167a, miR3954a, Novel-miR18, Novel-miR59, Novel-miR6 and miR156b targeting protein kinases involved in protein phosphorylation, and miR3954a targeting transcription factor genes.

Meanwhile, the 8 downregulated miRNAs were predicted to target up to 621 genes. Among these genes, 8 were identified as RxLR effector genes, regulated by Novel-miR2, miR482a, miRN9, miR396c and miR399a, while 4 were identified as Crinkler family (CRN) effector genes, regulated by miR396c and Novel-miR-39. Other downregulated miRNAs were also predicted to target important genes in *P. palmivora*. For instance, miR482a which target cytochrome oxidase subunit genes, miR396c targeting pre-mRNA-splicing factor SYF1-like protein gene involved in pre-mRNA splicing, miR472a targeting transmembrane protein gene, and Novel-miR39, miR396c, miR399a and novel-miR9 targeting TKL/DRK protein kinase gene.

Discussion

Cacao, belonging to the genus Theobroma, is a crop of significant economic importance[12]. Understanding the molecular defense mechanisms of cacao plants and their ability to suppress pathogen development is crucial in tackling black pod disease caused by *P. palmivora*. Nonetheless, no previous analysis has compared the occurrence of miRNAs in regulating genes between resistant and susceptible species affected by BPD caused by *P. palmivora.* In this study, the identification of miRNAs that potentially target important mRNAs involved in plant defense and pathogen suppression was successfully conducted.

The present study identified a total of 54 known miRNAs, consisting of 40 distinct miRNA families. Among these families, miR160, miR396, miR399, and miR159 families exhibited a higher representation, each consisting of 3 miRNA species. The remaining families consisted of either 1 or 2 miRNAs. In another study, Cheng et al. [13] successfully identified 31 miRNA families in banana roots infected with *Fusarium oxysporum* f. sp. *Cubense.* Meanwhile, Rabuma et al. [7] also succeeded in identifying 24 miRNA families in chili leaves infected with *Phytophthora capsica.* Xin et al. [14] also conducted a study on miRNA identification of wheat infected with *Erysiphe graminis*, pathogen that causes powdery mildew disease in wheat, where the result showed that there were 132 miRNA families identified.

Subsequently, we discovered a set of 67 miRNAs classified as novel, predicted by using miRDeep2. The accuracy of miRDeep2 in identifying miRNAs ranged from 98.6–99.9%, and it successfully reported hundreds of novel miRNAs. The prediction process involved utilizing high throughput sequencing data, mapped reads, and regenerating miRNA precursors using the miRDeep2 program. To differentiate novel miRNAs from known ones, the generated miRNAs were compared to the known miRNA database[15]. In a similar study by Cheng et al. [13] that employed the same program, 28 novel miRNAs responsive to *Fusarium oxysporum* f. sp. cubense tropical race 4 (Foc TR4) were successfully identified in banana roots. However, only 5 novel miRNAs were found to have putative target genes. Another study by Liu et al. [16] analyzed putative novel miRNAs and successfully identified 310 novel miRNAs associated with the ripening process of hot pepper (*Capsicum annum* L.).

In our study, the identified miRNAs exhibited a length range of 18 to 27 nucleotides, with the majority (60 miRNAs) having a length of 21 nucleotides. This finding agrees with Zhang et al. [17] research on Peony flowers with identified miRNAs lengths ranging from 18 to 24 nucleotides. Guo et al. [18] also identified soybean miRNAs with lengths varying from 18 to 25 nucleotides. Another study that aligns with these findings is Liu et al. [16], which observed that miRNAs vary in length from 18 to 28 nucleotides during the stage of chili fruit ripening. Most miRNAs fall within the range of 20 to 24 nucleotides, with 24 nucleotides being the most abundant, additionally, 23 nucleotides are the typical length observed for miRNAs. Through numerous comparisons of miRNA lengths derived from numerous studies, it becomes evident that the length of miRNAs varies depending on the organism type and the specific role of miRNA in regulating processes within certain plants or growth phases.

We also predicted the pre-miRNAs structure of some known and novel pre-miRNA with the highest abundance (Fig. 4). pre-miRNAs must form a loop structure to be processed into mature miRNA. The stability of the pre-miRNA structure is determined by its MFE (minimum free energy) value, where a lower MFE indicates a more stable structure. The MFE of some miRNA with the highest abundance ranging from − 39.70 to -25.60 kcal/mol for known miRNA and − 42.60 to -14.30 kcal/mol for novel miRNA. In another study, Tiwari et al. [19] showed that the MFE of known and novel pre-miRNA of potato plant with low nitrogen stress were ranging between − 114.9 to -19.9 kcal/mol for known miRNAs and − 166.4 to -15.4 kcal/mol for novel miRNAs. Hence, the stability of pre-miRNA secondary structure will also determine the abundance of miRNAs in response to pathogen.

Upon pathogen infection, plants activate their initial line of defense by initiating pattern-triggered immunity (PTI) as a first defense barrier and effector-triggered immunity (ETI) as a second barrier[20,21]. Varied responses to pathogen attacks are observed in resistant and susceptible plant varieties, influenced by miRNA expression towards target genes[22]. miRNAs regulate gene expression during plant-pathogen interactions[13]. For instance, in susceptible chili pepper plants, the excessive expression of miRNA398, which targets receptor protein kinase, enhances susceptibility to rot disease[22]. This indicates that plant susceptibility arises from the regulation of gene expression, could be mediated by miRNAs suppressing their disease-resistance genes.

The PsRNA analysis of the *T. cacao* genome identified 9 upregulated miRNAs predicted to target 1,112 genes. miR165b has been identified as a potential regulator of the putative *Theobroma cacao* gene that encodes the disease resistance RPP13-like protein through mRNA cleavage. Similarly, Novel-miR34 is predicted to target the same gene by inhibiting its translation at the mRNA level. Lewis et al. [23] postulated in their research that the ZAR1 protein, which shares homology with RPP13, serves as a recognition factor for type III bacterial pathogen effectors (T3Es) like *Pseudomonas syringae*. ZAR1 belongs to the coiled-coil (CC) nucleotide-binding site and leucine-rich repeat (NBS-LRR) protein class, which includes the R protein.

Furthermore, miR167a, Novel-miR34, and Novel-miR6 have been predicted to target disease-resistance genes, specifically RGA, At4g19060, and At4g27220, through mRNA cleavage. The RGA (Resistance Gene Analog) protein provides plant protection against pathogens by interacting

directly or indirectly with avirulence proteins found in the pathogens. This interaction triggers a defense response that inhibits pathogen growth[24]. The proteins At4g19060 and At4g27220 belong to the NLR (nucleotide-binding, leucine-rich repeat) protein family, which detect pathogens' presence and activity. NLR proteins can directly recognize pathogen effectors by binding to them through the LRR domain or indirectly detect modifications in host proteins caused by effector actions. In certain cases, host protein domains targeted by pathogen effectors have become integrated or fused into NLR proteins[25,26].

In addition to their targeting of plant resistance genes, the four upregulated miRNAs, miR3627a, Novel-miR6, miR3954a, and Novel-miR34, are also predicted to target seven L-type lectin-domain containing receptor kinases. Wang et al. [27] have reported the involvement of L-type lectin receptor kinases (LecRK) in the resistance response against oomycete pathogens such as *Phytophthora infestans* and *Phytophthora capsici.*

Several miRNAs, including Novel-miR34, Novel-miR6, miR3954a, Novel-miR18, Novel-miR59, and miR156b, are predicted to target cellulose synthase-like protein genes and expansin genes, which are essential for cell wall synthesis and elongation. These processes play a crucial role in plant defense against pathogen infection. Specifically, cellulose synthase-like proteins are necessary for synthesizing cell wall polysaccharides required for root hair elongation in rice. They are considered functional orthologs of CSLD3/KOJAK in the Arabidopsis[28]. On the other hand, expansin genes contribute to the loosening and extension of plant cell walls by disrupting non-covalent bonds between cellulose microfibrils and the glucan matrix.

In addition to the upregulated miRNAs, the downregulated miRNAs identified in this study also exert significant regulatory roles in modulating the expression of various genes associated with plant defense against pathogens. Notably, seven downregulated miRNAs, namely Novel-miR2, Novel-miR39, Novel-miR9, Tca-MIR472a, Tca-MIR482a, Tca-MIRN9, tcc-miR399a, and tcc-miR396c, were predicted to target disease resistance genes, receptor protein kinases, and cell wall structural components. The downregulation of these miRNAs leads to decreased repression activity, enabling their target transcripts to be regulated normally without any specific treatment. This finding suggests that the reduced expression levels of these miRNAs may contribute to the maintenance of proper gene expression patterns associated with plant resistance mechanisms.

Identifying miRNAs that target their mRNA within the genome provides potential sources of plant resistance by eliminating specific miRNAs, leading to the continuous improvement of resistance genes. Silencing miRNAs using methods such as employing long noncoding RNA (lncRNA) represents an approach to weaken the repressive effects of miRNAs on their target genes[7,8]. For example, the elimination of miR482/2118s by lncRNAs acting in a 'zig-zag-zig' model of the plant immune system increases the levels of the target gene NBS-LRR which is an important component of ETI[29].

Another strategy to inhibit miRNA expression and alleviate gene repression is the utilization of CRISPR/Cas9-based genome editing. In a study conducted by Hong et al. [30], the MIR482 family, which targets the NBS-LRR protein, was successfully knocked out, resulting in enhanced plant resistance to *Phytophthora infestans* in tomato plants. Interestingly, his study also observed a decrease in reactive oxygen species (ROS) activity in response to plant stress. These findings are consistent with previous report by Hong et al. [31], which demonstrated that upregulation of MIR482c in tomato led to an increase in ROS levels and a decrease in the expression of POD and SOD genes, indicating the involvement of miRNA-mediated regulation in plant stress responses.

Another form of plant defense against pathogen infection is Cross Kingdom RNAi. This defense mechanism involves the transfer of small regulatory RNA molecules into the invading pathogen, subsequently initiating the silencing of virulence genes within the pathogen's genome[32]. The silencing of these virulence genes critically undermines the pathogen's ability to establish infection, thereby suppressing infection[32].

Schaefer et al. [33] also showed that suppression of SvrPm3a1/f1, Bgt_Bcg-6, and Bgt_Bcg-7 effectors in wheat with RNAi can reduce effector expression and increase plant resistance to infection of powdery mildew. Wang et al. [34] also found that the Botrytis can uptake exogenous sRNA which in this study is an sRNA specifically targeting the Bc-DCL1 and Bc-DCL2 genes involved in synthesizing pathogenic effectors. The uptake will then cause repression of effector production thereby suppressing infection by the pathogen. On the Contrary, pathogens do the same by sending sRNAs to plants in self-defense. Wang et al. [35] showed that *Botyris cinerea* sends Bc-siR37 sRNA into Arabidopsis plants to suppress the immunity. Bc-siR37 is predicted to target several important Arabidopsis genes, such as WRKY transcription factors, receptor-like kinases, and cell wall-modifying enzymes.

Target analysis on *P. palmivora* transcripts revealed that 9 upregulated miRNAs were predicted to regulate 694 genes. Among them, 12 were identified as RxLR effector genes, which regulated by Novel-miR59, miR3627a, Novel-miR6, miR156b, and miR167a. RXLR effectors represent a group of proteins characterized by RXLR (Arginine-any amino acid-leucine-arginine) motifs highly conserved at the N-terminal while displaying greater diversity at the C-terminal region. The N-terminal segment facilitates the translocation of the effector into the host cell, whereas the C-terminal region is involved in the effector's activity and function[36]. Pathogens release effectors to counter the plant PTI response. Within the host cell, RxLR effectors will target diverse components within the plant defense system. This can be achieved by disrupting signal transduction

pathways or modifying specific proteins functionality[37]. Upon recognition by host R proteins, RxLR proteins elicit host effector-triggered immunity (ETI) by triggering a hypersensitive response (HR).

Novel-miR6 and Novel-miR18 are also predicted to target Crinkler (CRN) family protein effector genes. CRN effectors are characterized by an N terminus containing a highly conserved LxLFLAK (leucine-any amino acid-phenylalanine-leucine-alanine-lysine) motif, while their C terminus exhibits more sequence diversity. These CRN effectors mainly induce necrosis in host cells[38,39]. Silencing of these effector genes can suppres P. palmivora infection. Hou et al. [40] demonstrated that targeting effector PSR2 of *Phytophthora capsici* using siRNA can effectively suppress the infection of *P. capsici* in *Arabidopsis thaliana.*

Other miRNAs were also predicted to target important genes in *P. palmivora*, including genes involved in respiration, glycolysis, protein transport, protein phosphorylation, transcription factors, transmembrane proteins and mRNA processing. These genes play critical roles in various metabolic activities of *P. palmivora*. Silencing these genes can disrupt the growth of *P. palmivora*. The study conducted by Hu et al. [41] demonstrated that targeting genes encoding acetyl-CoA, 40S ribosomal protein, and glycine cleavage system in *Phakopsora pachyrhizi* using dsRNA can effectively suppress the growth of *P. pachyrhizi*.

miRNAs possess notable environmentally friendly characteristics and exhibit high specificity towards targets, making them a promising candidate to be used in developing plant protection methods with the RNAi approach[42]. Currently, many RNAi approaches rely on transgenic plants expressing double-stranded RNA[43], However, public reaction towards transgenic plants has been unfavorable. Koch et al. [11] demonstrate that applying CYP3-dsRNA that targets *Fusarium graminearum* CYP3 gene through spraying or Spray-Induced Gene Silencing (SIGS) can effectively repress the infection of *F. graminearum* on barley. This finding highlights the potential of SIGS as an environmentally friendly alternative for plant protection methods. Therefore, identifying miRNAs that target pathogen effectors provides potential sources of plant resistance by repressing the expression of effector gene, thereby suppressing infection and improving plant resistance against pathogen.

In general, this study also holds potential in contributing to miRNA-based breeding strategies aimed at improving crop traits. miRNAs are known to play a role in various functions in plants, including response to stress, disease resistance regulation, flower development, and grain yield [44]. Identification and elimination of disease- and pathogen-associated miRNAs, as well as identification and overexpression of miRNAs associated with crop yield and quality, can serve as effective strategies in molecular breeding. Hong et al. [30] showed that elimination of miR482b and miR482c, which are known to contribute to tomato susceptibility to *P. infestans*, resulted in increased resistance of tomato plants to *P. infestans* infection. Zhang et al. [45] also showed that overexpression of miRNA OsmiR397 could increase grain size and promote panicle branching, hence promoting rice yield.

Additionally, miRNAs can also serve as molecular markers in plant breeding programs. Due to their tissue and developmentally-specifics expression patterns, miRNAs can be used as reliable markers for trait selection and identification of elite germplasms. Sihag et al. [46] succeed in developing heat responsive miRNA-SSE markers for characterization of wheat germplasm for terminal heat tolerance breeding. Mondal et al. [47] also successfully identified salt responsive miRNA-SSR markers in rice. The development of miRNA-based molecular markers allows for efficient and precise selection of desired traits, reducing the time and resources required for traditional breeding methods.

In our study, we also identified seven promising miRNAs (Table 3) with potential for further research and development. Among them, five miRNAs, namely Tca-MIR3954a, Novel-miR34, tcc-miR396c, Tca-MIR472a, and Tca-MIR482a, were identified as targets for elimination in *T. cacao* transcripts. These miRNAs play important roles in pathogen resistance and cell wall formation in cacao plants. By eliminating the suppressive effects on their target genes, it is expected that deletion of these five miRNAs in cacao plants will improve their resistance to pathogens. In addition, two miRNAs, Novel-miR6 and tcc-miR399a, were identified as potential candidates for biocontrol agents against *P. palmivora*. These miRNAs are predicted to target effector proteins in the pathogen. By applying these synthetic miRNAs to cacao plants, the expression of effector genes in *P. palmivora* can be targeted and repressed, thus suppressing the infection caused by *P. palmivora* in cacao plants.

Table 3
Differentially expressed miRNAs that target both their own transcripts and genes in *P. palmivora* without contradicting each other.

miRNA ID	Regulation	Target on *Theobroma cacao* transcripts		Target on *Phytopthora palmivora* transcripts	
	Target prediction		Protein Function	Target Prediction	Protein Function
Tca-MIR3954a	Up	Protein chromatin remodeling 19 (LOC18609933) mRNA	Plant pathogen resistance	CAMK/CDPK protein kinase complete cds	Protein phosphorilation
Novel-miR34	Up	Cellulose synthase-like protein D3 (LOC18599512) mRNA	Synthesis of a cell wall polysaccharide essential	CMGC/DYRK protein kinase complete cds	Protein phosphorilation
tcc-miR396c	Down	G-type lectin S-receptor-like serine/threonine-protein kinase LECRK1 (LOC18611782) mRNA	Involved in innate immunity.	Pre-mRNA-splicing factor SYF1-like protein (PITG_11158) mRNA, complete cds	Pre-mRNA splicing
Tca-MIR472a	Down	Putative disease resistance protein At3g14460 (LOC108662475) mRNA	Potential disease resistance protein.	Transmembrane protein, putative (PITG_19640) complete cds	Transmembrane protein
Tca-MIR482a	Down	Probable disease resistance protein At1g12280 (LOC18595262) mRNA	Potential disease resistance protein.	Cytochrome oxidase subunit I (coxi) gene, partial cds; mitochondrial	Cellular respiration
Novel-miR6	Up	RING finger protein 5 (LOC18591332) mRNA	Protein ubiquitination	Secreted RxLR effector peptide protein, putative (PITG_13593) complete cds	Effector that acts as an elicitor of hypersensitive response (HR)
tcc-miR399a	Down	Probable ubiquitin-conjugating enzyme E2 24 (LOC18596849) mRNA	Protein ubiquitination	Secreted RxLR effector peptide protein, putative (PITG_14984) complete cds	Effector that acts as an elicitor of hypersensitive response (HR)

The selection of these seven miRNAs from a total of 17 differentially expressed miRNAs was based on their targets in *T. cacao* transcripts and genes in *P. palmivora* that are not contradictory in enhancing resistance. This discovery potentially advances our understanding of the molecular mechanisms at the miRNA level in the interactions between cacao plants and the pathogen *P. palmivora*.

Conclusion

In conclusion, the identification and characterization of miRNAs in cacao plants provide valuable insights into the mechanisms governing plant defense against black pod disease caused by *P. palmivora* in infected cacao fruits compared to healthy ones. Our study identified five miRNAs, Tca-MIR3954a, Novel-miR34, tcc-miR396c, Tca-MIR472a, and Tca-MIR482a with promising potential for future engineering, such as CRISPR/Cas9 or LncRNA methods, to suppress the expression of miRNAs targeting their own resistance genes during pathogen response. Additionally, we propose synthesizing two miRNAs, Novel-miR6 and tcc-miR399a, that can be utilized as biocontrol agents to suppress expression of pathogenesis related mRNAs in *P. palmivora*.

Materials & Methods

Plant Material and Sample Preparation

The cacao samples were obtained from the Indonesian coffee and cacao research institute plantation in Jember, East Java, Indonesia. Two varieties, namely TSH858 (resistant, healthy) and Sulawesi 1 (susceptible, infected), were selected for the study. Each variety was collected in two replicates, labeled as R1 and R2 for the resistant samples, and S1 and S2 for the susceptible samples.

Disease scoring was performed on two samples, R and S, by measuring the length and width of the spots that appeared on the surface of the cacao pod. The obtained measurements were then used in the cacao pod rot area formula to calculate the average and determine the disease score or the resistance parameter of the cacao pod against *P. palmivora*[48]. Following the disease scoring, the cacao pod samples were thinly sliced and freeze-dried using liquid nitrogen to preserve the integrity of the samples. Afterward, the samples were stored at -80 C until further analysis.

The presence of *P. palmivora* on cacao was confirmed through the amplification of the ITS2 and 5.8S rDNA genes of *P. palmivora*. The specific primers utilized for ITS2 amplification were designed based on the accession number KU172528.1. The forward primer sequence was 5

$- \top CGCGGTATGG \top GGC \top C- 3$, while the reverse primer sequence was $5- G\mathbb{C}G \forall GCATACAC \forall G \top \mathbb{C}- 3$. The selection of the 5.8S rDNA primer was based on the research conducted by Darmono et al. [49] where the forward primer (5'-3') employed was $5 - C \top AG \top GGGGGTCTC \top TC- 3$ and the reverse primer (5'-3') was $5- G \top CA\mathbb{C} \forall TCATA\mathbb{C}A\mathbb{C}- 3$. The PCR process involved the following steps: preheating the sample to 95°C for 3 minutes and denaturation at 95°C for 15 seconds. The annealing step was performed at 59°C for *ITS2* and 54°C for 5.8S *rDNA*, each for 15 seconds. Elongation of the DNA strands occurred at 72°C for 10 seconds, followed by a final elongation at 72°C for 3 minutes. The amplified products were then sequenced to confirm the presence of *P. palmivora.*

Small RNA Extraction and Illumina Sequencing

Total small RNA was extracted using mirPremier® microRNA Isolation Kit by Sigma-Aldrich, Merck, Germany, per manufacture instruction. The isolates were measured for purity and concentration using a nanodrop spectrophotometer (ThermoFisher Scientific, USA) and band quality was visualized using a 4% agarose gel. Total small RNAs were then sequenced on the Illumina Novoseq 6000 platform (Novogene, Singapore) and library preparation followed the guidelines the manufacturers provided. After passing the library preparation quality assessment, the library was sequenced on the illumina Novoseq 6000 platform with a read length of 50 bp.

Reads Quality Check and Filtering

The output of small RNA sequencing was checked for quality reads using the FastQC v0.11.9[50]. The presence of contaminating adapters and overrepresented sequences was eliminated using the Trimmomatic v0.39[51]. Then the reads were filtered to obtain miRNAs with a minimum length of 18 bp and a maximum of 28 bp. The targeted miRNA length was then subjected to further analysis.

Small RNA annotation and miRNA identification

After removing the low-quality reads that contain 5′ adapter contaminants and overrepresented sequences from raw data, the clean reads obtained were mapped to reference the cacao genome using Rsubread v2.10.5[52]. For known miRNAs, reads were quantified using feature counts from Rsubread based on two cacao miRNA databases, pmiREN (https://www.pmiren.com/) and miRbase (https://www.mirbase.org/). The remaining unannotated reads were then subjected to novel miRNA prediction using mirdeep2 v0.1.3[15]. The predicted miRNA obtained was then quantified using feature counts from Rsubread. pre-miRNAs structure and their minimum free energy (MFE) were also predicted with RNAfold [53].

Differential expression analysis and target prediction

The known and novel miRNA quantification results were then analyzed for differential expression using the Deseq2 v1.36.0[54]. A fold change > 1 with *p*adj < 0.005 was considered as differentially expressed miRNAs (DE miRNA) that were upregulated, and fold change < 1 with *p*adj < 0.005 was considered as DE miRNAs that were downregulated. DE miRNAs were then visualized in EnhancedVolcano v1.14.0[55] and heatmap v1.0.12[56]. The target gene of significantly upregulated miRNAs in the cacao and *P. palmivora* genomes were then predicted using psRNATarget v2[57], BLAST[58] and uniport[59]. Prior to BLAST, miRNA sequences were reverse complemented using FastX Toolkit program[60]. This process converts the miRNA sequences into their corresponding target sequences.

Declarations

Data Availability

The miRNA sequencing data of 4 samples reported in this study have been deposited into the NCBI Geo Expression Omnibus with GEO accession GSE244955 that are publicly accessible at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE244955

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Author contributions

Authors have contributed to this work in the following roles: P.S., S.P., and K.M. devised the study and supervised the project. Y.P. and D.U.N. performed the experiment. P.S., Y.P., D.U.N., S.P. and K.M. analyzed the data. Y.P. and D.U.N. drafted the original manuscript. P.S., Y.P., D.U.N., S.P. and K.M reviewed & revised the manuscript.

Additional Information

Declaration of competing Interest: The authors declare thay they have no conflict of interest.

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Figures

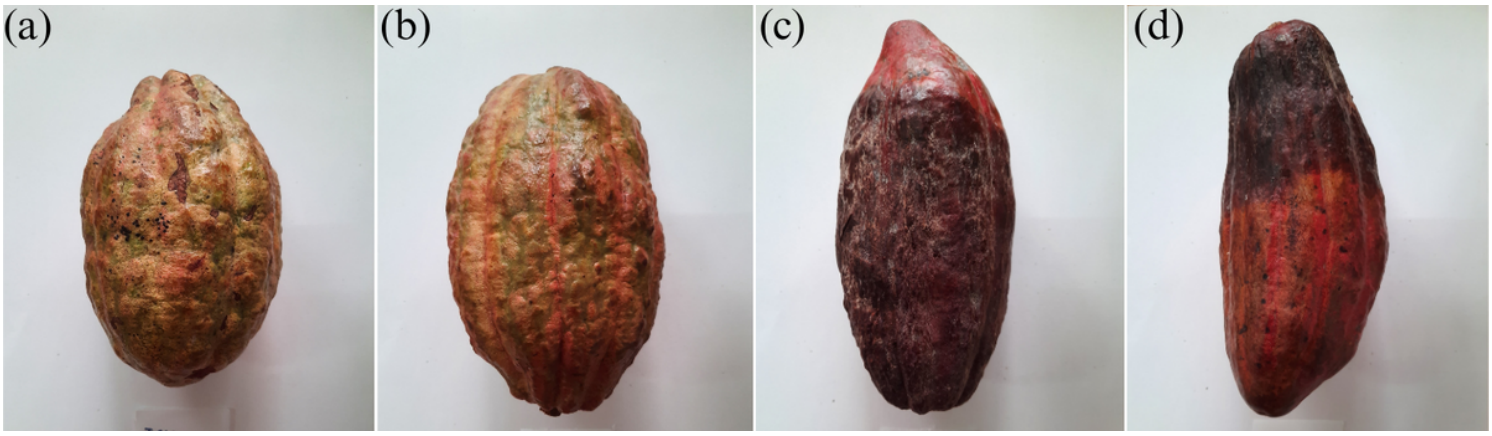

Figure 1

T. Cacao pod used in miRNA extraction. (a, b) Resistant (TSH858) and (c, d) susceptible (Sulawesi 1) varieties.

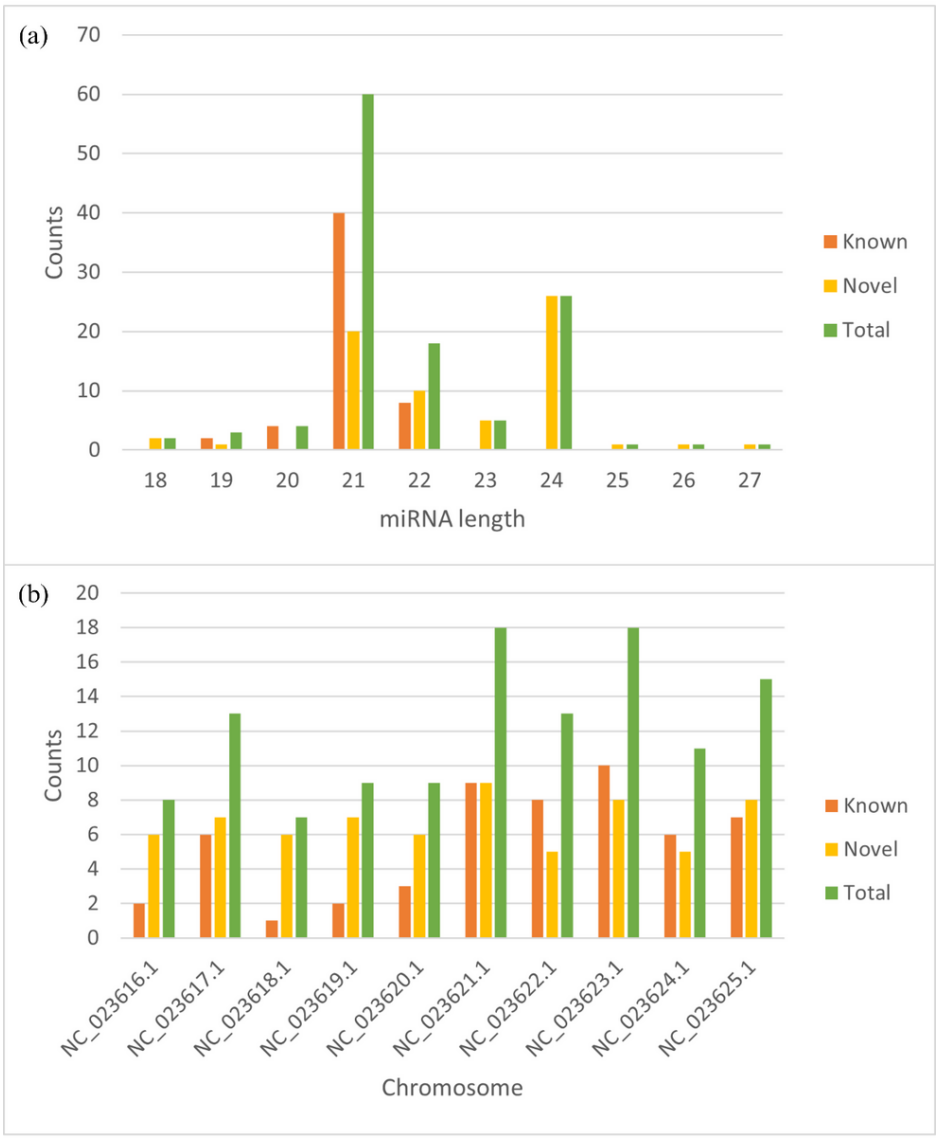

Figure 2

(a) Distribution of known and novel miRNAs length; and (b) Distribution of miRNAs in *T. cacao* chromosomes.

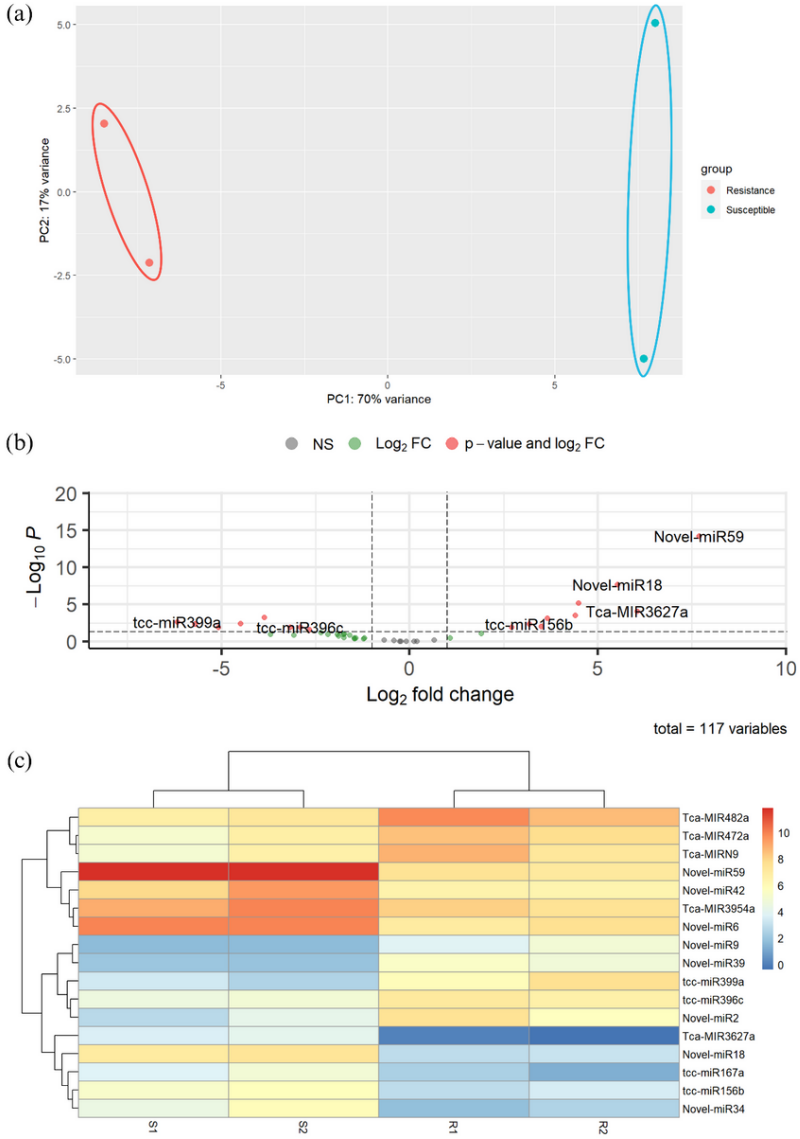

Figure 3

Visualization of differentially expressed miRNAs (a) PCA plot visualization of the two samples of resistant and susceptible varieties shows the clustering differences between the two groups of samples, (b) Volcano plot shows differentially expressed miRNAs are represented by red plots, and (c) Heatmap illustrating differentially expressed miRNAs with a gradient of red color indicating miRNA upregulation and a gradient of blue color indicating miRNA downregulation.

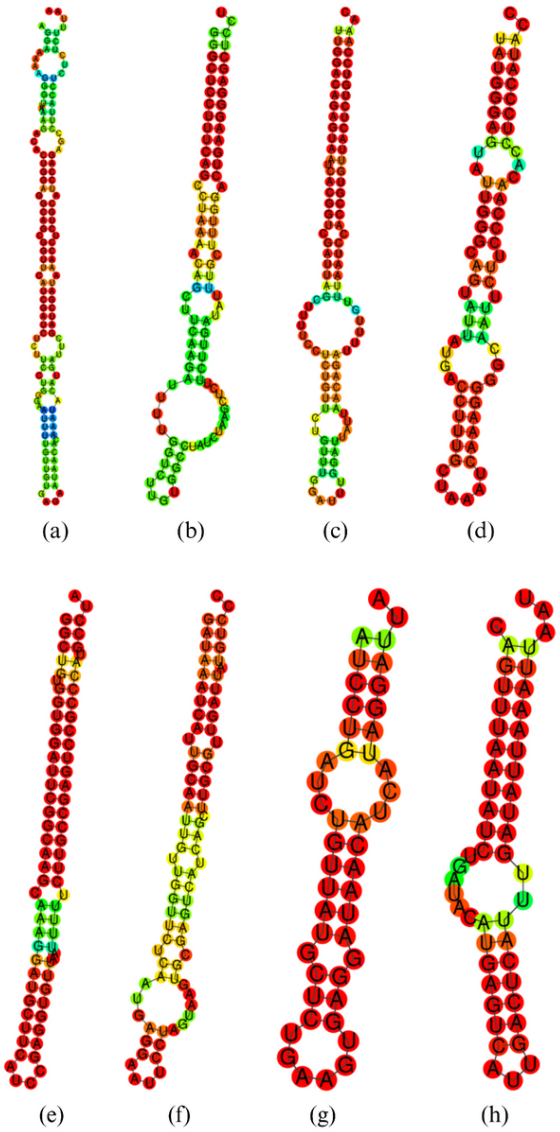

Figure 4

Secondary structure of some known miRNA (a) tcc-miR162; (b) tca-MIR159d; (c) Tca-MIR3954a; (d) Tca-MIR482a and novel miRNA (e) Novel-miR1; (f) Novel-miR6; (g) Novel-miR42; and (h) Novel-miR59.

Supplementary Files

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