RESEARCH ARTICLE

Novel MicroRNAs and their Functional Targets from *Phytophthora infestans* and *Phytophthora cinnamomi*

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Abstract: Background: Even though miRNAs play vital roles in developmental biology by regulating the translation of mRNAs, they are poorly studied in oomycetes, especially in the plant pathogen *Phytophthora*.

Objective: The study aimed to predict and identify the putative miRNAs and their targets in *Phytophthora infestans* and *Phytophthora cinnamomi*.

Methods: The homology-based comparative method was used to identify the unique miRNA sequences in *P. infestans* and *P. cinnamomi* with 148,689 EST and TSA sequences of these species. Secondary structure prediction of sRNAs for the 76 resultant sequences has been performed with the MFOLD tool, and their targets were predicted using psRNATarget.

Results: Novel miRNAs, miR-8210 and miR-4968, were predicted from *P. infestans* and *P. cinnamomi*, respectively, along with their structural features. The newly identified miRNAs were identified to play important roles in gene regulation, with few of their target genes predicted as transcription factors, tumor suppressor genes, stress-responsive genes, DNA repair genes, etc.

Conclusion: The miRNAs and their targets identified have opened new interference and editing targets for the development of *Phytophthora* resistant crop varieties.

Keywords: Data mining, fungus, genome annotation, miRNA, oomycete, siRNA, target prediction.

1. INTRODUCTION

Oomycete *Phytophthora* infects a wide range of crops, causing enormous economic losses [1]. These microbes have a large genome enriched in genes coding for effector proteins that promote plant infection by secretion into the apoplastic and cytoplasmic spaces of the host tissue [2]. *Phytophthora infestans* causes late blight in potatoes, whereas *P. cinnamomi* causes root rot, dieback, and pink disease in many crops [3]. This plant pathogen is highly invasive, having spread to over 70 nations [4].

RNA polymerase-II transcribed miRNAs or dsRNAs are converted into 21-22 nucleotide small RNAs (sRNAs) in plants. Through translational and post-translational suppression of target transcripts, sRNAs regulate the expression of genes. Additionally, they are involved in numerous processes, including development and stress responses [5]. MicroRNA research in *Phytophthora* genus is at the primitive stage, with only five miRNAs belonging to three species available at miRBase. Compared to the plants and animals, miRNAs finding from the protists has been difficult using conventional tools [6]. Potato lines transformed with artificial microRNAs (amiRNAs) were shown to suppress the effector genes of *Phytophthora infestans*, developing resistance to late blight disease [7].

Several computational techniques have been developed to supplement experimental approaches for identifying and validating new miRNAs [8]. Generally, they use prediction algorithms and pipelines with appropriate computational infrastructures to find novel miRNAs from NGS data [9]. Even though 38,589 miRNAs belonging to 271 species are available at miRBase, only five miRNAs are reported from this devastating plant pathogen. Since the pre-miRNAs, in general, possess a conserved secondary hairpin structure, EST-based miRNA mining is a common strategy [10]. Through a comparative genome-based homologue search in EST and Transcription Shotgun Assembly (TSA) databases at NCBI, we report two novel miRNAs belonging to separate families, from *P. infestans* and *P. cinnamomi*. The structural features and the targets predicted for these miRNAs suggest their vital roles in biological functions.

2. METHODS

2.1. ESTs *Phytophthora* sp. and Reference sets of miRNAs

A total of 38,589 mature non-redundant, protein non-coding miRNA sequences from various species, including five from *Phytophthora* sp., were retrieved from miRBase (Re-
lese 22.1: October 2018) and used for searching the miRNAs in Phytophthora. Previous studies had defined these miRNAs as the reference set of miRNA sequences [10]. NCBI dbEST and TSA database had 111,106 cDNA and EST sequences from Phytophthora infestans and 37578 TSA and five EST sequences from Phytophthora cinnamomi. Thus, a total of 148,689 Phytophthora sequences were used in this study.

2.2. Prediction of Putative miRNAs and their Precursors

The strategy followed in this work to search for the putative miRNAs is presented in Fig. (1). Initially, using BLAST+ [11], the matched patterns between all known mature miRNAs and Phytophthora sequences were analyzed. ESTs with a cut-off E-value of 0.001 and the possibility of a maximum of two mismatches (one deletion and one insertion) were identified. Subsequently, CD-HIT [12] was employed for removing the redundant miRNA and EST sequences. CD-HIT suite and CD-HIT-EST web tool were run with a sequence identity cut-off of 0.99, representing 99% identity. A total of 400 nucleotides from 5’ and 3’ flanking regions were taken from the matching ESTs as candidate sequences for each pattern hit.

For structure identification, secondary structures were created using the Mfold 3.5 software [13], and the hairpin structures were judged. Finally, these raw hairpin candidates were used as queries against the non-redundant protein sequence database using BLASTx [14] with a cut-off E-value of 0.001. Protein coding sequences were removed, and non-coding sequences were carried forward for further analyses.

Three parameters, minimum fold energies (MFEs), minimal free energy indices (MFEIs), and G+C content, were employed to filter miRNAs from coding and other non-coding RNAs. MFEs of all candidate miRNA precursors were generated by the Mfold 3.5 program. The adjusted minimal folding free energy (AMFE), which is the MFE of a 100-nt RNA sequence and the MFEI were determined as per the protocol given by Zhang et al. [15]. AMFEs (%) were calculated as (MFE of the pre-miRNA/Length of the pre-miRNA)*100 and MFEIs (%) as (MFE of the pre-miRNA/Number of G+C in the pre-miRNA)*100.

Criteria followed to deem an RNA sequence as potential miRNA were as follows: (i) RNA sequence could fold into an appropriate stem-loop hairpin secondary structure, (ii) predicted mature miRNAs show no more than three nucleotide substitutions when compared to known mature miRNAs, (iii) mature miRNA sequence site is present in one arm of the hairpin structure, (iv) mature miRNA sequence has less than six mismatches with the opposite miRNA* sequence (miRNA* refers to the small RNAs processed from the hairpin arm opposite the mature miRNA) in the other arm, (v) no loops or breaks found in the miRNA or miRNA* sequences, and (vi) predicted secondary structures have higher MFEIs and negative MFEs. Since the sequences surrounding miRNAs can form hairpin structures that distinguish them from other RNAs, secondary structures of all the putative pre-miRNAs were used for miRNA evaluation.

2.3. Target Prediction for Identified miRNAs

The psRNATarget [16] analysis server was used to screen potential target genes to predict the possible functions of the identified miRNAs. As the transcriptomic information of Phytophthora infestans is unavailable in psRNA-Target software, the transcriptomic information from Arabidopsis thaliana (transcript, removed miRNA gene, TAIR, v.10, 2010 release) was selected as alternative target libraries. Parameters followed in the screening were: the maximum expectation of 3 and target accessibility of 20. Subsequently, miRNA-target duplexes were checked manually.

3. RESULTS

3.1. Identification of miRNAs

Homology search-based computational methods were used to predict new miRNAs in Phytophthora. Sequences of all the known mature miRNAs in miRBase were used as candidates and screened against the EST sequence database of P. cinnamomi and P. infestans. BLASTn and BLASTx analyses of sequences of mature reference miRNA were performed against 148,689 EST and TSA sequences, with E-value set to 0.001 to predict only the highly significant matching regions. The 22 EST sequences identified from P. infestans and 54 from P. cinnamomi had 100% similarity with a maximum of two mismatches and were non-coding for proteins. Manual determination of the secondary structures predicted from those EST sequences, following the approved criteria, has revealed the sequences of miRNA precursors and appropriate stem-loop structures. Lengths of the identified mature sequences of the P. infestans and P. cinnamomi miRNAs were 19 and 21 nucleotides, respectively. One putative miRNA belonging to the mir-8210 family and another belonging to the miR-4968 family were found in P. infestans and P. cinnamomi, respectively (Table 1). The structures of pre-miRNA mir-8210 in P. infestans and miR-4968 in P. cinnamomi are presented in Figs. (2 and 3), respectively, and their sequence information is available in Supplementary File 1.

3.2. Targets and Putative Roles of miR-8210 in P. infestans

A total of 160 genes in A. thaliana were targeted by the putative miRNA found from P. infestans, whereas the putative miRNA found from P. cinnamomi targeted 204 genes. Altogether, 364 targets for the two miRNAs family were identified, which target several genes with diverse biological functions (Supplementary File 2).

The most significant target genes of this miRNA are presented in Table 2. Some of the targets found in Phytophthora infestans were identified as potential targets for C2 calcium/lipid-binding plant phosphoribosyl transferase family protein, cyclin B1, calmodulin 5, DNA glycosylase superfamily protein, Ribosomal protein L19 family protein, as well as auxin-responsive proteins. Other important targets were DNA glycosylase superfamily protein, SUAR-like auxin-responsive protein family gene, MLO6 gene, which is a
Fig. (1). Strategy followed to search for the putative miRNAs in Phytophthora genomes.
Fig. (2). Structure of pre-miRNA miR-8210 in *P. infestans*. 
Fig. (3). Structure of pre-miRNA miR-4968 in *P. cinnamomi*. 
Table 1. *P. infestans* and *P. cinnamomi* miRNAs identified by homolog search and secondary structure.

| Properties                      | *P. infestans* | *P. cinnamomi* |
|---------------------------------|----------------|----------------|
| EST/TSA ID                      | GR300526.1     | HAC001004026.1 |
| Length of EST/TSA sequences     | 813            | 457            |
| Mature miRNA length             | 19             | 21             |
| MFEI                            | 0.653          | 0.67           |
| miRNA family                    | miR-8210-3p    | miR-4968-3p    |
| miRNA                           | pin-miR-8210   | pci-miR-4968   |

Table 2. The predicted targets of miR-8210 in *P. infestans*.

| Target Accession | Inhibition | Target Description |
|------------------|------------|--------------------|
| AT5G06850.1      | Cleavage   | C2 calcium/lipid-binding plant phosphoribosyl transferase family protein |
| AT2G27030.2      | Cleavage   | CAM5, calmodulin 5 |
| AT1G15970.1      | Cleavage   | DNA glycosylase superfamily protein |
| AT1G28310.1      | Cleavage   | DoF-type zinc finger DNA-binding family protein |
| AT2G26760.1      | Cleavage   | CYCB1;4, Cyclin B1;4 |
| AT4G35860.2      | Cleavage   | ATRABB1B, ATGB2, ATRAB2C, GB2, GTP-binding 2 |
| AT4G17560.1      | Cleavage   | Ribosomal protein L19 family protein |
| AT4G35860.1      | Cleavage   | ATRABB1B, ATGB2, ATRAB2C, GB2, GTP-binding 2 |
| AT2G01540.1      | Cleavage   | Calcium-dependent lipid-binding (CaLB domain) family protein |
| AT2G27030.1      | Cleavage   | CAM5, ACAM-2, calmodulin 5 |
| AT5G48040.1      | Cleavage   | Ubiquitin carboxyl-terminal hydrolase family protein |
| AT3G08340.1      | Cleavage   | SAUR-like auxin-responsive protein family |
| AT1G61560.2      | Cleavage   | MLO6, Seven transmembrane MLO family protein |
| AT1G58330.1      | Cleavage   | ZW2, transcription factor-related |
| AT1G55210.2      | Cleavage   | Disease resistance-responsive (dirigent-like protein) family protein |
| AT3G21250.2      | Cleavage   | MRP6, ABCC8, multidrug resistance-associated protein 6 |

Table 3. Predicted targets of miR-4968 in *P. cinnamomi*.

| Target Accession | Inhibition | Target Description |
|------------------|------------|--------------------|
| AT4G32551.2      | Cleavage   | LUG, LisH dimerisation motif, WD40/YVTN repeat-like-containing domain |
| AT2G41990.1      | Cleavage   | CCCH-type zinc finger protein with ARM repeat domain |
| AT2G45620.1      | Cleavage   | Nucleotidytransferase family protein |
| AT5G28640.1      | Cleavage   | AN3, G1F, G1F1, ATGIF1, SSXT family protein |
| AT2G03070.1      | Cleavage   | MED8, mediator subunit 8 |
| AT4G06726.1      | Cleavage   | Transposable element gene |
| AT2G27050.1      | Cleavage   | EIL1, AeIL1, ETHYLENE-SENSITIVE3-like 1 |
| AT3G43156.1      | Cleavage   | Transposable element gene |
| AT2G30590.1      | Cleavage   | WRKY21, WRKY DNA-binding protein 21 |
| AT1G16610.1      | Cleavage   | SR45, RNPS1, arginine/serine-rich 45 |
| AT2G45530.1      | Cleavage   | RING/U-box superfamily protein |
| AT1G30330.2      | Cleavage   | ARF6, auxin response factor 6 |
| AT1G06070.1      | Cleavage   | Basic-leucine zipper (bZIP) transcription factor family protein |

protein that contains seven transmembrane helices. ZW2 gene, which is a transcription factor-related gene, acts in sequence-specific DNA binding. MRP6 gene, which is also called the ABCC6 gene, was another noteworthy target found in *A. thaliana*, which is found to be involved in multidrug resistance. Basic-leucine zipper (bZIP) gene, AHL22, auxin-responsive factor ARF6, Transcription initiation factor EER4, Ethylene response factor ATERF-7, and transcriptional co-regulator SEUSS were also identified as targets. These results illustrate the significance of identified miRNA in the plant pathways.
3.3. Targets and Putative Roles of miR-4968 in *P. cinnamomi*

Similar to the results obtained from *P. infestans*, the miRNA found from *P. cinnamomi* also revealed a variety of target hits. The most significant target genes of this miRNA are presented in Table 3. The targets identified in *P. cinnamomi* are transcription factors like WRKY and bZip, LUG, auxin-responsive genes, and many others. Apart from the transcription factors, CCCH-type zinc finger protein, Nucleotidyltransferase family protein, transcription co-activator AN3, mediator of RNA polymerase II transcription MED8, ethylene response gene EIL1, serine/arginine-rich splicing factor SR45 genes were also found to be significant targets. Some other gene targets worth mentioning were RNA-binding PEP gene, phosphate deficiency response gene PEP, RNA-binding gene AML5, alcohol dehydrogenase gene, vacuolar transporter IREG2, and so on. Interestingly, genes such as MRp6, ZW2, MRp6, ARF6 were found to be common in *P. infestans* and *P. cinnamomi*.

4. DISCUSSION

Even though the mining and characterization of miRNAs is a major research objective in all the life forms, miRNA research *Phytophthora* is in its infancy. Only a few miRNAs are identified and characterized in this genus. Fahlgren et al. [17] used a pipeline to identify candidate miRNA genes using high-throughput small RNA sequencing data from *P. infestans*, *P. sojae*, and *P. ramorum* and identified a single miRNA locus, MIR8788. So far, no attempts have been made in this genus to mine the miRNAs from ESTs. Our studies revealed two potential miRNAs that fulfill all the criteria described by Wang et al. [18]. The normal frequency of miRNA from EST is reported to be 0.01% [15] compared to the 0.0011% we obtained in *P. infestans*. Non-representation of the complete coding regions by the ESTs used in this study might be the reason for this difference.

4.1. Targets of miR-8210 in *P. infestans*

The miRNAs identified are shown to play important roles in this oomycete. One of the targets identified was cyclin B1, a key component in cell cycle progression from G2 to M phase, which bears implications in tumorigenesis and malignancy development [19]. Another important target CAM5 (Calmodulin 5) gene identify regulate miRNA genes using high-throughput small RNA sequencing data from *P. infestans*, *P. sojae*, and *P. ramorum* and identified a single miRNA locus, MIR8788. So far, no attempts have been made in this genus to mine the miRNAs from ESTs. Our studies revealed two potential miRNAs that fulfill all the criteria described by Wang et al. [18]. The normal frequency of miRNA from EST is reported to be 0.01% [15] compared to the 0.0011% we obtained in *P. infestans*. Non-representation of the complete coding regions by the ESTs used in this study might be the reason for this difference.

4.2. Targets of miR-4968 in *P. cinnamomi*

In *P. cinnamomi*, WRKY and bZIP transcription factors were found to be the miRNA targets. WRKY TFs can mediate several abiotic responses as well as biotic stress responses. They are shown to regulate the production of several secondary metabolites such as phenolic compounds along with lignin, flavanols, and tannins. These transcription factors also regulate plant defense response and development by altering the secondary metabolite biosynthesis. WRKY stimuli-based overexpression systems can enhance secondary metabolism [30]. In plants, bZIP TFs regulate many processes, including pathogen defence, light and stress signalling, seed maturation, and flower development [31]. Protein kinase superfamily proteins have diverse functions, including central roles in metabolic signalling and stress responses, regulation of mitosis and cytokinesis, expansion, and involvement in plant-specific processes [32].

The P-type ATPases, also known as E1-E2 ATPases, are a large group of evolutionarily related ion and lipid pumps that are found in bacteria, archaea, and eukaryotes [33]. P-type ATPases make up a large superfamily of ATP-driven pumps involved in the transmembrane transport of charged substrates [34]. Another target protein in the RING/U-box superfamily represents a type of E3 ligases, which has been implicated as a regulator of fundamental cellular processes ranging from cellular growth, damage responses, and apoptosis [35]. Sun et al. [36] reported that AtSZF1 and AtSZF2, two closely related CCCH-type zinc finger proteins, are involved in salt stress responses in *Arabidopsis*.

Thus, this study has revealed two miRNAs carrying out vital roles in the systems biology of this economically very important oomycete. These findings shall be very important while designing breeding and gene regulation strategies to develop the crop varieties resistant to this pathogen.
CONCLUSION

MicroRNAs are small non-coding RNAs with crucial roles in RNA silencing and post-transcriptional regulation of gene expression. Following an in-silico pipeline, miRNAs were mined from the EST and TSA sequences of Phytophthora cinnamomi and P. infestans, and two novel miRNAs were identified. The targets of these miRNAs were predicted using psRNATarget software, and their functions were predicted. Genes with vital functions such as tumour suppression, DNA repair, and stress tolerance were predicted to be targeted by the newly identified miRNAs, demanding further studies to develop the mechanisms to regulate their expression within the cells of target plants.

AUTHORS’ CONTRIBUTIONS

BV and DM conceived the research hypothesis. BV and RV performed EST and TSA sequence mining and target prediction. BV and DM wrote and revised the manuscript. All authors reviewed the manuscript and agreed with the content.

ETHICS APPROVAL AND CONSENT TO PARTICIPE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the finding of the study are available within the article and it’s supplementary material.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIALS

Supplementary material is available on the publisher’s website along with the published article.

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