Probing the mitochondrial genomes of *Phytophthora* spp. for the presence of mature miRNAs

Rahul C.U.[1] and Rajesh M.K.[2*]
ICAR-Central Plantation Crops Research Institute, Kasaragod 671124, Kerala, INDIA
*mkraju.cpcri@gmail.com

Abstract
MicroRNAs (miRNAs) serve as gene regulators in a multitude of biological processes. The study of the differential expression of miRNAs present in any microorganism upon infection of its host could help in better understanding the process as well as provide a methodology to possibly mitigate the functioning of that particular entity. Phytophthora is an oomycete that belongs to the Stramenophile kingdom which is a major pest of plants worldwide. With more than 80 species identified worldwide, Phytophthora has the ability to attack a variety of crops. miRNA are a class of small non-coding RNA that regulate gene expression by the degradation of RNA or inhibiting protein translation. They play a major role in many biological processes which can influence an organism both positively and negatively.

In this communication, we aim to identify any potential miRNAs that may be present in the mitochondrial genome of various species of Phytophthora. Disruption of the mitochondrial processes (which helps in maintaining cell homeostasis) of this pathogen, by manipulating mitochondrial miRNA molecules, could serve as a potential deterrent to the spread of this disease.

Keywords: miRNA, mitochondria, *Phytophthora*, RNAFold, RNAhybrid.

Introduction

Phytophthora is a genus of oomycete that causes millions of dollars' worth of crop loss every year. In the current scenario where there are unpredictable weather patterns, population explosion in many parts of the world and a shortage of arable land, it is of predominant interest that we find ways to control and mitigate this destructive plant disease.

Over the past few years, miRNAs have started to emerge as a promising approach for the detection and propagation of various diseases. MicroRNAs (miRNAs) are single stranded, ~22nt RNAs that play regulatory roles in animals and plants through the translational repression or cleavage of messenger RNAs (mRNAs). This mechanism of gene regulation is termed RNA silencing[1]. Experiments with plants yielded the first clue to gene silencing. Genetic experiments conducted on petunias to study flower pigmentation by introducing genes coding for a deep purple colour had surprisingly no effect on floral pigmentation[2].

In another similar study[3], it was observed that introducing a chimeric petunia CHS gene reduced the level of mRNA produced by this gene by almost 50-fold when compared with wild type levels. More insight into this phenomenon took almost a decade to come about, when a study by Fire and his research team[4] reported that double stranded RNA was more effective in causing gene interference rather than each strand individually through a study in the nematode *Caenorhabditis elegans*. This potency in gene silencing through RNA interference led to intensive research over the next few years thus granting us the knowledge we possess now. The first known miRNA was *lin-4* through the study of heterochronic gene *lin-4* in worms and it controls the timing of *Caenorhabditis elegans* larval development[5]. Ruvkun's group was the first to propose that small RNAs are found in a wide range of species through their study of *let-7* RNA expression. Each miRNA could regulate multiple genes and the huge number of miRNAs present suggests that they might form the majority of the gene regulatory machinery[6].

More than half of the currently known miRNAs are found in the introns of coding genes or non-coding RNA transcripts. miRNA biogenesis in eukaryotes involves both cytoplasmic and nuclear processing helped along by ribonuclease III (RNAse – III) endonucleases. miRNAs are initially expressed as part of long primary transcripts consisting of an imperfect RNA hairpin of ~80 nt length. These initial transcripts are cleaved by Drosha (RNAse – III enzyme) releasing pre-miRNAs which are about 60 to 70 nt in length[7]. These pre-miRNAs are further exported to the cytoplasm mediated by a RanGTP-dependent exportin (exportin 5). In the cytoplasm, further processing is performed by Dicer enzyme (another RNAse – III enzyme) thus giving us the mature ~ 22 nt miRNA.

Mitochondrion have major role in cellular processes like inflammation and cell death well beyond their primary function of energy metabolism. A number of protein import functions have also been found in the mitochondria and proteins form the major regulatory mechanism in molecular biology[8].The up-regulation or the down-regulation of gene expression influenced by miRNAs can be detrimental to the functioning of any organism. The objective of this study was to identify potential miRNAs present in the mitochondrial sequence of three different *Phytophthora* species.

Material and Methods

All known mature miRNA sequences were retrieved from miRBase database (http://www.mirbase.org/) in the FASTA format. Whole mitochondrial sequences were obtained for *Phytophthtora infestans*, *P. ramoram* and *P. sojae* oomycetes via NCBI (National Centre for Biotechnology Information) database, also in the FASTA format. Standalone Blast version of 2.2.29+ was installed and a database was constructed with the retrieved mature miRNA sequences after removing redundant sequences. A BLASTN search was conducted with the database query of mature miRNA sequences against the retrieved mitochondrial sequences with the parameters set at a word length of minimum 7. The output format parameter was set at 8 and the results were obtained in an excel file.

In order to check the reliability of a matched miRNA being present in the mitochondrial genome, a custom PERL script was used which was used to retrieve 70 nucleotides both upstream and downstream of the matched area as per the BLAST output. This was done so as to acquire a probable precursor sequence and further check if this precursor sequence is capable of forming a secondary hair-pin loop structure which is a characteristic of pre-cursor miRNA sequences. These obtained sequences were further used as an input in the preprocessing and information of sequences tool (PRINSEQ v0.20.4) and all duplicate sequences were removed.

Furthermore, PRINSEQ (http://edwards.sdsu.edu/cgi-bin/prinseq/prinseq.cgi) was used to retrieve only those sequences with GC content between 30% and 70% for further analysis so as to ensure the stability of the predicted hair-pin loop structures of these sequences. Those sequences which gave hits with BLASTX were also removed since miRNAs do not code for proteins. RepeatMasker v4.0.5 (http://www.repeatmasker.org/) was used to screen sequences for low complexity regions. miPred and RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) were used to predict if the sequences formed secondary hairpin loop structures and to distinguish between significant and pseudo-miRNAs. A minimum free energy value (MFE) of ~20 kcal/mole was considered to be optimum for the predicted structures. Target prediction was done using RNA hybrid which is a tool that hybridizes a target to a query sequence based on minimum free energy.

Table 1
Potential pre-miRNAs with their precursor sequences.

S. N.	miRNAs	Potential pre-miRNA sequences	*Phytophthora spp.*
1.	miR-9760	AUGAUGUAGUUUAAUGGUAGAGCGUGGGAAU CAUAAUCCUAAUGUUGUAGGUUCAAAUCCUA CCAUCAUUAUUAUUUCUUAA	*P. infestans*
2.	miR-5106	CCUGCAUGAAUGGUGUAACGACAUCCCCGCU GUCUCCAAUAUAGACUCAGUGAAUUUGAAUU AUCCGUGAAGAUGCGGAUUU	*P. infestans*
3.	miR-8-5p	ACUAAUAAGAUGAUGUAGUUUAAUGGUAGAG CGUGGGAAUCAUAAUCCUAAUGUUGUAGGUU CAAAUCCUACCAUCAUUAAA	*P. ramoram*
4.	miR-9760	GUUCAAAUCCCACUUCGACUUAUUUUACUAA UAAGAUGAUGUAGUUUAAUGGUAGAGCGUGG GAAUCAUAAUCCUAAUGUUG	*P. ramoram*
5.	miR-9760	UCCCACUUCGACUUAUUUUAUUAAUUUUUAA UAAGAUGAUGUAGUUUAAUGGUAGAGCGUGG GAAUCAUAAUCCUAAUGUUG	*P. sojae*

Table 2
Potential pre-miRNAs MFE and p-values after secondary structure prediction

S.N.	Matching miRNA	MFE value	P-Value
1.	miR-9760	-17.20	0.001
2.	miR-5106	-16.90	0.001
3.	miR-8-5p	-15.40	0.001
4.	miR9760	-15.60	0.001
5.	miR9760	-16.30	0.001

Results and Discussion

After an evaluation of the results, five potential miRNA hairpin loops (Table 1) were detected among the mitochondrial sequences of all three organisms. It was found that there was a match of the miR-9760 family against all the three mitochondrial genomes. An RNA fold analysis put their hairpin loop structure MFE value consistently below and around the previously decided value of 20 kcal/mole (Table 2 and 3). From a previous study, miR-9760 has been identified to be present in the cotyledons of *Glycine max*[9](Table 4).

Through an analysis using the psRNATarget tool miR-9760 was also found to possibly target rubredoxin and transporter membrane proteins while it was found viable that miR-8-5p could be targeting trans-splicing factors and superoxide dismutase.Various studies have documented miR-8-5p targeting the mRNAs responsible for chitin biosynthesis, atrophin synthesis, growth factor receptor binding etc.[10,11] miR-5106 was found to possibly target small nuclear ribonucleoprotein-associated proteins and flagellar associated proteins. miRNAs have been found to be extensively conserved among species as documented by a study in which human miRNAs were found to have a close relationship with miRNA families in *C. elegans* and *D. melanogaster*[12].Also, due to the relatively small size of miRNAs, even a single nucleotide change is sufficient to bring about a major change in the function, structure, species specificity or target of a particular miRNA[13]. Hence, the matches that were detected can safely be said to be conserved, novel or closely related to the detected miRNAs. Further analysis could be conducted through in vitro studies to confirm the results of this study.

Table 3
Target prediction for each of the predicted conserved miRNAs in the mitochondrial genome of each species

S. N.	miRNA	MFE value (kcal/mol)	Species	Target Predicted by RNAhybrid
1.	mir-9760	-2.3	*P. infestans*	target 5' N 3' UA AU miRNA 3' AAAAA UCAUGACAGUAAAAG 5'
2.	mir-5106	-2.1	*P. infestans*	target 5' N 3' UA AU miRNA 3' CAGCA GUGGUAAGUACGUCC 5'
3.	gsa-miR-8-5p	-2.4	*P. ramoram*	target 5' N 3' UA AU miRNA 3' AUUUGAUGUAGUAGA AAUCA 5'
4.	mir-9760	-1.8	*P. ramoram*	target 5' N A 3' A U U A miRNA 3' A UC GCUUCACCCUAAACUUG 5'
5.	mir-9760	-2.1	*P. sojae*	target 5' N 3' UA AU miRNA 3' UU UUUAUUCAGCUUCACCCU 5

Table 4
Possible targets for the predicted miRNAs with their methods of inhibition predicted using the psRNATarget tool and literature studies.

S. N.	miRNA	Possible targets	Method of inhibition
1.	miR-9760	chitin biosynthesis, atrophin synthesis, growth factor receptor binding, rubredoxin and transporter membrane proteins	Translation and Cleavage
2.	miR-8-5p	targeting trans-splicing factors and superoxide dismutase	Translation and Cleavage
3.	miR-5106	small nuclear ribonucleoprotein-associated proteins and flagellar associated proteins	Cleavage

Conclusion

Mitochondria have a huge role in many cellular functions and this is regulated by proteins encoded from both nuclear and mitochondrial genome. The alteration of protein expression in the mitochondria organelle can lead to a change in the physiology of cell. In the present investigation, set protocol was used to analyze the presence of conserved miRNAs in the mitochondrial sequence of *Phytophthora* spp. with the conclusion that conserved miRNAs do exist which could be targeted for *Phytophthora* disease control.

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