

In silico analysis of chili encoded miRNAs targeting *Chili leaf curl begomovirus* and its associated satellite

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ABSTRACT

Chili leaf curl virus (ChiLCV), a member of the *Geminiviridae* family (Genus: *Begomovirus*), is one of the most destructive plant viruses. Micro (mi) RNAs (miRNAs) are the endogenous non-coding small RNAs that play significant roles in plant growth and stress resistance by degrading targeted mRNA or repressing mRNA translation. Computational methods have identified numerous miRNAs in many plant species, whereas there is no report of chili miRNAs targeting essential genes of the ChiLCV genome and associated satellites. In this study, we have predicted chili-encoded miRNAs that could be used for silencing against *chili leaf curl virus* (Accession no. MF737343) infection. We predicted several potential mir-miRNAs, exhibited high complementarities with V1 coat protein and C1 (Rep) genes of ChiLCV. Other overlapping genes, such as V2, C2, and C3 were also targeted by mir-miRNAs.

1. INTRODUCTION

The chili (*Capsicum annum*) is an economically important and widely cultivated vegetable cum spice crop of the family *Solanaceae*. Various pathogens affect the chili crop and limit its production [1–3]. Viruses are the main cause of loss in chili production. Around 65 viruses are known to cause leaf curl disease in chilies (ChiLCD) throughout the world, among which begomoviruses (family-*Geminiviridae*) are one of the most destructive plant viruses [4]. ChiLCD is characterized by curling of leaves, thickening, and swelling of veins, reduced leaf size, and stunted plant growth [5,6]. Geminiviruses are non-enveloped, circular, single-stranded DNA viruses having one (DNA-A, monopartite) or two (DNA-A and B, bipartite) DNA molecule of 2.5–3.0 kb size with nine genes, namely, *Becurtovirus*, *Begomovirus*, *Capulavirus*, *Curtovirus*, *Eragrovirus*, *Grabovirus*, *Mastrevirus*, *Topocovirus*, and *Turncurtovirus* [7]. The transmission vectors of begomoviruses are Whiteflies (*Bemisia tabaci*) [8]. *Begomovirus* has bidirectionally organized six open reading frames (ORFs) on virion sense and complementary strand and an Intergenic region with the origin of replication [9]. Sense

strand has V1 (CP: Coat Protein), V2 (Pre-CP: Pre Coat Protein) ORFs and C1 (Rep: Replication associated protein), C2 (TrAP: Transcriptional activator protein), C3 (REN: Replication Enhancer protein), and C4 ORFs are present on complementary strand [10].

RNA interference (RNAi) induces RNA silencing either by transcriptional silencing or by post-transcriptional gene silencing through the interaction of endogenous or externally introduced small RNAs (sRNAs) with target RNA. Thus, RNAi can work as a significant defense strategy to hinder virus attack in plants [11]. These sRNAs include micro RNAs (miRNAs) and small-interfering RNAs (siRNAs). miRNAs are involved in only post-transcriptional gene regulation and are derived from endogenous single-stranded hairpin precursors. Hairpin precursor or double-stranded RNA is processed by Dicer or a Dicer-like protein into around 22-nt double-stranded RNA. Later, one strand of this 22-nt RNA, called “guide” strand, loaded into the RNA-induced silencing complex helps binding it to a complementary strand of the target, leading to cleavage or translational repression of the target [12].

Sequence-specific interaction of miRNA and target mRNA transcript leads to its cleavage or translational repression by proteins involved in RNAi mechanism [13]. Many computational tools have been developed to predict target genes of these miRNAs, as prediction is crucially important for

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down-sizing potential genes–miRNA interactions for further downstream interaction analysis using wet-lab methods, and for understanding their effects on gene expression [14]. These *in silico* target prediction tools use various miRNA–target interaction features for prediction such as complementarity between miRNA and target site, target site accessibility, and thermodynamic stability of miRNA–target duplex using MFE calculations [15]. These miRNAs play a critical role in regulating the growth and development of plants' cell development and cell differentiation, regulation of development processes, biotic, and abiotic stress responses [16,17]. In this study, we have predicted the chili-encoded miRNAs that could be used for silencing against *chili leaf curl virus* (ChiLCV) infection. ChiLCV was isolated, sequenced, and submitted to the National Center for Biotechnology Information GenBank database under the accession number MF737343. An *in silico* analysis was applied to predict chili miRNAs that could target genes present in the ChiLCV genome (Fig. 1). It may serve as an initial approach for understanding the viral gene expression regulation by host miRNAs and could pave a way into design strategies for defense in chili (*C. annuum*) plant against ChiLCV infection. This is the first study reporting the identification of potential chili miRNAs capable of targeting essential genes of the ChiLCV genome and associated satellites.

2. MATERIALS AND METHODS

2.1. Source of miRNA and Genomic Data

To search potential conserved miRNA in chili, genome sequence of *C. annuum* cv. CM334 published by Kim et al. [18] and 15,041 mature miRNA of plants available in the Plant Non-Coding RNA (PNRD) database were downloaded [19]. Redundant miRNA sequences were filtered using a custom Perl script. A non-redundant set of 30,721 unique miRNA sequences was selected for further processing.

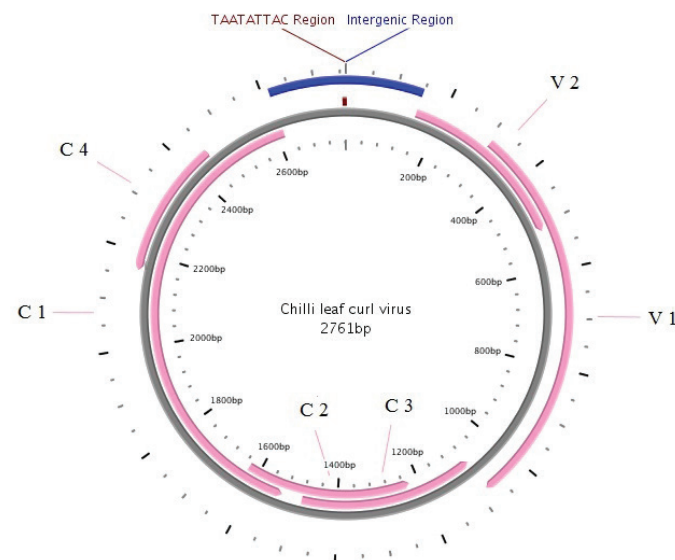


Figure 1: Genome of ChiLCV, showing all 6 ORFs.

2.2. Aligning miRNA to Chili Genome and Secondary Structure Prediction

To get the location of potential pre-miRNA sequences, Bowtie was used to align the selected unique miRNA sequences to the chili genome [20]. Two mismatches were allowed on the full length of miRNA. After alignment sequences within 150 nucleotides upstream and downstream of the first and last nucleotide alignment position of the mature miRNA were extracted using bedtools [21]. These sequences were examined for the presence of characteristic hairpin secondary structure present in pre-miRNA using Mirinho [22]. The following are the parameters: length of the stem-arm: 33; minimum length of the terminal loop: 5nt; maximum length of the terminal loop: 20 nt; and free-energy threshold: -20.6 kcal/mol.

2.3. Potential miRNA Prediction

The criteria for miRNA identification were according to the latest guidelines by Axtell and Meyers [23,24]. Custom Perl scripts were written using the following criteria:

- Minimum length of the hairpin structure is 70 nucleotides.
- Must be folded into an appropriate stem-loop structure necessary for it to get processed into mature miRNA.
- Mature miRNA must be placed in either arm of the pre-miRNA hairpin and not in the loop.
- Maximum number of unpaired nucleotides in 5' and 3' arm of mature miRNA are 6.
- Maximum number of bulge ≤ 2 consecutive nucleotides.
- The A+U content should be 30%–70%.

2.4. Prediction of Targets of Chilli miRNA in ChiLCV Genome

These selected miRNAs after the prediction of potential pre-miRNA were used to predict their target in virus genome using psRNATarget server [25] with the following parameters:

top targets: 200; penalty for G:U pair: 0.5; penalty for other mismatches: 1; seed region: 2–13 nt; extra weight in seed region: 1.5; mismatched allowed in seed region: 2; HSP size: 19; penalty for opening gap: 2; and translational inhibition range: 10–11 nt.

3. RESULTS AND DISCUSSION

The in-silico study enabled us to identify the potentially known miRNA in the chili genome and their target genes in ChiLCV. The known miRNAs were identified by mapping PNRD miRNAs to the chili genome then taking 150 nucleotide sequences from both ends of aligned miRNA position. This sequence was folded to identify pre-miRNA hairpin-like structure. The miRNAs were selected based on the criteria described in the material and method section. Only these miRNA were used for predicting target ChiLCV genes using psRNATarget parameters described in the material and method section. Seven different miRNAs were identified to target all six genes of ChiLCV genome (Fig. 2). V1 (coat protein) gene was observed being targeted by a maximum number of miRNA (mir-98, mir-231, mir-113, and mir-68).

3.1. Aligning miRNA to Chilli Genome and Secondary Structure Prediction

Out of 30,721 initial unique miRNAs, 14,998 miRNAs were mapped to the chili genome with maximum two nucleotide mismatches. A total of 150 nucleotide sequences from upstream and downstream of aligned miRNA were extracted using a custom script. These sequences were treated as precursor microRNAs (pre-miRNAs) and searched for the presence of a hairpin-like secondary structure by folding them using mirinho. Mirinho folded 60415 unique pre-miRNA sequences were selected.

3.2. Potential miRNA Prediction

A total of 60,415 pre-miRNA sequences were used to predict the hairpin-like secondary structure present in the sequence using mirinho tool [22]. 6,007 unique hairpin-like structures were predicted by mirinho. By using some criteria of mature miRNA in the hairpin-like structure, we selected 262 unique miRNA as potential known miRNA in the chili genome. The selected miRNAs varied from 19 to 24 nucleotides in length with an average of 21 nucleotides and the average A+U% was observed ~55%. The range was (−18.9)–(−26.8) kCal/mol. Potential miRNA was observed to be located on both the arms of the hairpin structure. In our study, more potential miRNA was reported to be located on the forward strand. These miRNAs were used for further target prediction analysis. By applying all the filtering criteria, a total of eight mature miRNAs were selected for further analysis.

3.3. Prediction of Targets of Chilli miRNA in ChiLCV Genome

The predicted chili miRNAs were uploaded to psRNA target server along with ChiLCV genes. We observed all the six genes

of ChiLCV were being targeted by selected mature miRNA by the prediction server. miRNAs and their targets are shown in Table 1. The mode of targeting was either cleavage of target or translation inhibition. In most of target cases, the mode was cleaved from the target genes. Only 4 out of 10 predicted targets were due to translation inhibition. Coat protein gene (V1) had the highest targeting miRNA (mir98, mir231, mir113, and mir68). The possible reason for this may be that V1 is the most conserved region of the *Begomovirus* genome and plays a crucial role in symptom development and observed free energy may be the more suitable for the folding and stability of respective miRNAs [26]. Out of 10 targets in virus genome, 2 were identified for Rep protein (C1) by mir257 and mir67.

The formation of the stem-loop hairpin pre-miRNA secondary structure is very important in miRNA biogenesis with mature miRNA present in its one arm. A potential hairpin structure containing ~22-nt mature miRNA sequence within one of its arm is a primary pre-condition for new miRNA synthesis. The prediction of ChiLCV targets of these miRNA was anticipated to help us to understand the regulatory roles of these miRNA in plant defense. Plant miRNAs generally show perfect or near perfect complementarity with their targets [27]. In this study, the host miRNAs predicted were able to target ChiLCV in chili plant. A complete complementarity between miRNA and mRNA sequences leads to cleavage of the targeted mRNA. On the contrary, partial complementarity typically decreases gene expression by suppressing translation of target mRNA [28]. We found that several potential mir miRNAs exhibited high complementarities with V1 (CP) and C1 (Rep) genes of ChiLCV. Both the proteins are involved in virus infection and symptom development.

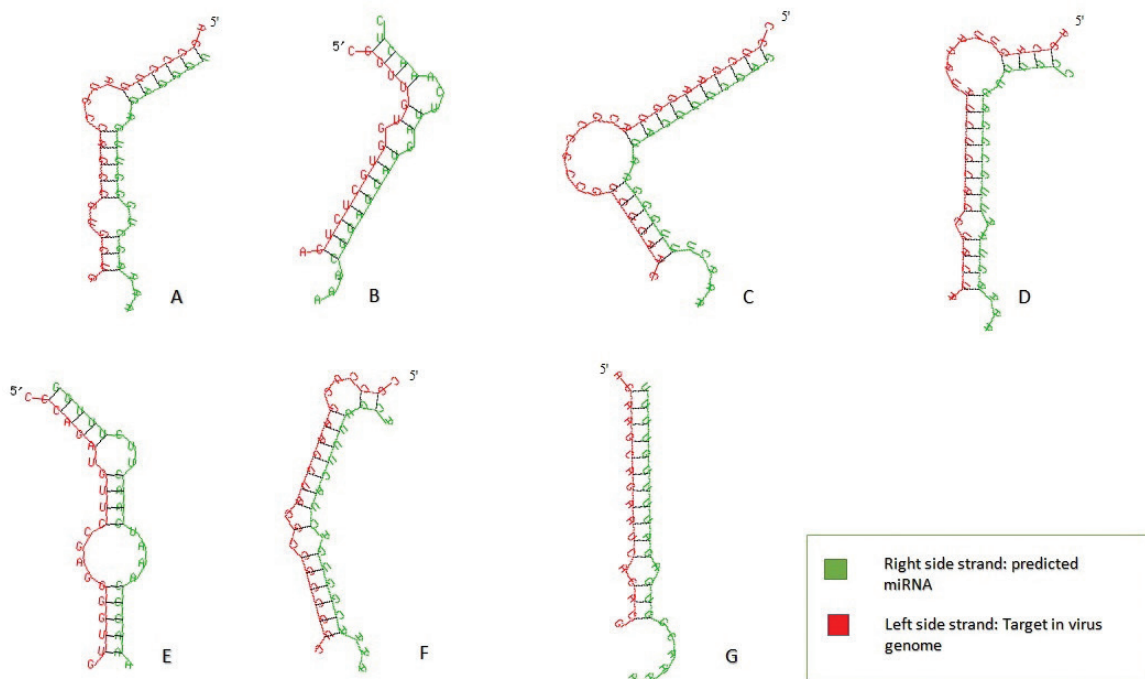


Figure 2: Selected miRNA-target pair obtained from RNA hybrid version 2.2. (A) mir-98, (B) mir-231, (C) mir-257, (D) mir-113, (E) mir-202, (F) mir-67, and (G): mir-68.

Table 1: The characteristics of predicted miRNAs. Minimum free energy (MFE) was calculated by RNAhybrid program and psRNATarget tool was used for mode of inhibition.

Chili miRNA	miRNA sequence	Target binding position	Target gene of ChiLCV	Inhibition type	MFE (kCal/mol)
can-miR-98	AAAAGCUGGGUUGAGAGGGU	309–1,082	V1	Cleavage	–26.8
can-miR-231	AAACGGAGCAUGAUCAAACUC	309–1,082	V1	Translation	–20.7
can-miR-257	AAACUUUGGGAUACUUUCGAG	1,531–2,616	C1	Translation	–21.6
can-miR-257	AAACUUUGGGAUACUUUCGAG	2,166–2,459	C4	Translation	–21.6
can-miR-113	AAAAGUAAUUGCGGAUUUUGCC	309–1,082	V1	Cleavage	–21.4
can-miR-202	AAACCCAAUGAACUUCUUUGG	1,079–1,483	C3	Cleavage	–20.3
can-miR-202	AAACCCAAUGAACUUCUUUGG	1,224–1,628	C2	Cleavage	–20.3
can-miR-67	AAAACGGUGACUACUUUUGCA	1,531–2,616	C1	Translation	–21.5
can-miR-68	AAAACGGUGAGAUUUUGUUUU	309–1,082	V1	Cleavage	–18.9
can-miR-68	AAAACGGUGAGAUUUUGUUUU	149–514	V2	Cleavage	–18.9

4. CONCLUSION

In the present study, these miRNAs can further be used to make artificial miRNAs (amiRNAs) and to provide immunity to chili crops against ChiLCV. Other overlapping genes V2, C2, and C3 were also targeted by mir-miRNAs. A broad information of these viral gene involved in disease development via miRNA induced RNAi would significantly facilitate in development of effective strategies to control the spread of ChiLCV infection.

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