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Full Length Article

Co-Expression Analysis of miRNAs and Target NBS-LRR Genes in *Cucumis sativus*

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Abstract

Plants react against their biological enemies by activating the innate immune system. Their defense system comprises of various R-protein, which usually contain NBS-LRR domain. MicroRNAs (miRNAs) are important molecules of 2nd layer of plant defense and play pivotal role behind the scene. To support such endeavors, this study was carried out to dissect the co-expression of miRNAs and NBS-LRR genes upon viral infection. Bioinformatics prediction of miRNAs which were targeting the NBS-LRR genes was conducted by TAPIR and miRU softwares. After analyzing the results from two software tools only those miRNAs were selected for co-expression analysis, whose interaction with NBS-LRR genes was predicted by both softwares. The results revealed that the complementation of only ten miRNAs with NBS-LRR genes was affirmed by two tools used in this study. The expression of miRNAs and NBS-LRR genes was evaluated through RT-PCR from CMV (cucumber mosaic virus) infected and non-infected plants. All NBS-LRR genes showed high level of expression in response to viral infection, while miRNA couldn't be detected by stem loop RT-PCR in the infected plants. But mature miRNAs were detected in the control plants with no expression of NBS-LRR genes. After analyzing the results, it was concluded that these miRNAs might be involved in downregulating/silencing the target NBS-LRR genes in the absence of any infection. This tight control of transcription by miRNAs might be relaxed upon infection, so that a plant can combat its enemy with the help of resistance genes. © 2018 Friends Science Publishers

Keywords: Resistance genes; CMV; Innate immune system; Biotic stress; Stem loop RT-PCR

Introduction

Micro RNAs (miRNAs) play a major role in many biological processes such as growth, development, signaling pathways of hormones, biotic and abiotic stress responses (Bartel, 2004; Sunkar *et al.,* 2007; Jin, 2008; Rubio-Somoza, 2009). It has also been reported that small RNAs play a critical role in provoking the resistance mechanism against certain diseases (Navarro, 2006; Katiyar-Agarwal, 2006, 2007; Jin, 2008; Padmanabhan *et al*., 2009; Gibbings and Voinnet, 2010). miRNAs are usually involved in the regulation of genes at post transcriptional level. They target R-proteins and regulate growth and development in plants (Perez *et al.,* 2010). Moreover, using bioinformatics approaches, it was shown that miRNAs are also involved in defense system of plants against certain viruses (Pérez-Quintero *et al.,* 2010). Hence, the plants exhibited a catalog of miRNA with potential targets in the viral genome. Viruses are targeted by conserved miRNAs families located in different regions of plant genome.

A strong barrier of plants defense system comprises of a large number of proteins with leucine rich repeats and a nucleotide binding sites (NBS-LRR), which are encoded by R genes (resistance genes). These genes are activated against any pathogen trying to invade the plant's territory (Li *et al*., 2012). Researchers have observed a conserved role of miRNAs and secondary siRNA in attenuating NBS-LRR mediated immune response against pathogens. Cucumber plant is a model plant in cucurbiteceae family and its production is subject to infections by a wide range of pathogens (Hamid *et al.,* 2002). Cucurbit plants have been reported to be infected with as many as 60 plant viruses globally (Amer, 2015). R-genes were found to be involved in inciting cucumber plant against powdery mildew disease (Wan *et al.,* 2010). Four RGAs of cucumber plant were isolated and CsRGA23 was related to defense response against powdery mildew disease. Martinez *et al.* (2011) identified the conserved regions of specific miRNAs and reported an efficient method of bioinformatics to analyze miRNAs in cucumber (*C. sativus*). As a result, 19 conserved

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and 6 non-conserved miRNAs families were found in sRNA dataset in cucumber. The bioinformatics prediction was confirmed by northern blot analysis of these miRNA. In another study, two small RNAs libraries were constructed from leaves and roots of cucumber and high throughput sequencing identified 2 novel families of miRNAs, containing 64 miRNAs (Mao *et al.,* 2012). The quantitative RT-PCR (real-time PCR) analysis uncovered that some of miRNAs were expressed in certain tissues of cucumber plant and 21 miRNAs were identified for the first time by degradome analysis. The interaction between miRNAs and their targets was also indicated in cucumber.

The role of miRNA in attenuating the NBS-LRR innate immune receptors in legumes and Solanacea plants was also observed (Zhai *et al.,* 2011; Li *et al.,* 2012). A striking finding was revealed by Shivaprasad *et al.,* 2012. They showed that miRNA482 targeted the mRNA of LRR resistance proteins having CC domain, which resulted in the decay of mRNA. The decay of NBS-LRR mRNAs by miRNA further induced the mechanism of short interfering RNA mediated gene silencing of other members of disease resistant gene. It was also demonstrated that silencing of disease resistance mRNAs mediated by miRNA was lost upon infection with bacteria and viruses leading to the pathogen induced expression of NBS-LRR genes (Shivaprasad *et al.,* 2012). This study proposed that pathogen inducible expression of NBS-LRR genes mediated by miRNA ultimately contribute to the defense against pathogen.

Cucumber is the world's leading greenhouse crop, which is prone to the attack of various diseases. It is also used as a model plant to study various molecular and physiological mechanisms coupled with the advantage of its genome sequence availability (Huang *et al.,* 2009; Del Valle-Echevarria *et al.,* 2015; Chandrasekaran *et al.,* 2016). Hence, the current study was performed to decipher the role of miRNA in attenuating the plant response against disease infection in cucumber plants. For this, the bioinformatics analysis of all available sequences of cucumber's miRNAs and their targets was carried out and stem loop RT-PCR was conducted to validate their transcription in response to infection in the resistant and susceptible genotypes.

Materials and Methods

Sample Collection

Cucumber plants were grown in the green house in triplicate fashion. Treated plants were infected with Cucumber Mosaic Virus (CMV) inoculum. Control plants were infected with mock solution. Fresh leaf samples from cucumber plant were collected for total RNA extraction from infected and non-infected genotype (P1-618950) of each replication at the same position and timing as was carried out for RNA isolation to conduct the transcript profiling of NBS-LRR genes.

Retrieving miRNAs of *C. Sativus*

To understand the involvement of miRNAs and resistance genes to provoke immune response against biotic stresses, almost 40 miRNAs were retrieved from miRBase database.

Prediction of miRNA Targets in NBS-LRR Genes of *C. Sativus*

Sequences of NBS-LRR genes (Table 1) were retrieved from phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). To understand the biological functions of the retrieved miRNAs, putative target among NBS-LRR genes were searched by using two plant target prediction tool named TAPIR (http://bioinformatics.psb.ugent.be/webtools/tapir/) and miRU (http://bioinfo3.noble.org/miRU.htm). The complementation of only 10 miRNAs with NBS-LRR genes was confirmed by both target prediction software tool (Table 2).

Stem Loop-RT Primer Designing for miRNAs

Primers were designed following the methodology by Chen (2005) with some modifications (Varkonyi *et al.,* 2007). The specificity to individual miRNA was performed using specific stem-loop RT primers through a 6 nucleotide extension at 3' end which is a reverse complement of the last six nucleotides at the 3' end (Chen, 2005). The stem-loop RT primers have a universal backbone with specific extension and specifically designed to form a stem-loop structure because of the complementarity between 5′ and 3′ end nucleotides. Forward primers were specific to the miRNA sequence excluding the last six nucleotides at 3' end. At 5' end, extension of 5–7 nucleotides was added to increase the melting temperature for each forward primer. These randomly chosen sequences were relatively rich in GC contents. The universal primer 5′GTGCAGGGTCCGAGGT 3′ was used as a reverse primer (Table 4).

RNA Extraction from *C. Sativus*

For co-expression analysis, the same RNA sample was used to:

i. Synthesize cDNA for RT-PCR of NBS-LRR genes
ii. Synthesize cDNA for stem loop RT-PCR of miRNAs

Total RNA from each of the triplicates of infected and non-infected plants of resistant genotype (P1-618950) was extracted by grinding 100 mg of fresh leaf tissue with 500 µL of plant RNA reagent. The homogenate was incubated at room temp. for 5 min and then laid down horizontally to maximize surface area. Then centrifugation of the mixture was performed for 2 min at 12,000 x g and the supernatant was transferred to an RNase-free tube. 0.1 mL of 5 M NaCl was added and thoroughly mixed followed by an addition of 0.3 mL of chloroform and mixed thoroughly by inversion.

Table 1: Primers for RT-PCR of NBS-LRR genes, retrieved from Phytozome

S. No	Acc. No	Primer name	Primer sequence	Amplicon Size (bp)
1	Cucsa.017460	Cuc.1- F	5′ TCCTCGTCTTGAATGTCTGC 3′	100
		Cuc.1- R	5′ACTTCTGACCTATCTCACTTTC 3′	
2	Cucsa.094660	Cuc.3- F	5′GAACCTGGAGTCCCTCTTAAT3′	125
		Cuc.3- R	5′GCAGTTTCTGATGTCCACTTT 3′	
3	Cucsa.094670	Cuc.5- F	5′AAAGCAGAGGAAGAAGAGGAAA 3′	132
		Cuc.5- R	5′ GCATTTGTCTATCCTCAACGTATC3′	
4	Cucsa.102240	Cuc.6- F	5′ GGAGTTTCAATTGCCCAGAG 3′	140
		Cuc.6- R	5′ GGTTTGTGGCAAACTTTGAA 3′	
5	Cucsa.128030	Cuc.7- F	5′CCCTTCTCTTGGATCCCTTAATC 3′	200
		Cuc.7- R	5′CACCACCTTGAACTCCGTATT3′	
6	Cucsa.155730	Cuc.8- F	5′AAAGATTCACCGAAACCCTAAGA 3′	250
		Cuc.8- R	5′TCCTCACAGCTCCATGAATTG3′	
7	Cucsa.237520	Cuc.9- F	5′ TGATTTGAATGTGTTGGAAGGG 3′	150
		Cuc.9- R	5′TTTCAACAAAGAGGCCATTAGG3′	
8	Cucsa.132370	Cuc.10- F	5′GAGATCACTGCATATTTCGCATTT 3′	135
		Cuc.10- R	5′GACGCCATGTCTTTCAAGTTTAC3′	
9	Cucsa.178360	Cuc.12- F	5′TCTACCAGAATGGTTGGGAAAC 3′	146
		Cuc.12- R	5′GTTAATCGTTGTATGGCCTCTCT3′	
10	Cucsa.249360	Cuc.13- F	5′ ACCTCTTGCACTGAAGGTAATC 3′	125
		Cuc.13- R	5′AAATATGGAGCCACTTCTAGCC3′	
11	Cucsa.251930	Cuc.14- F	5′ACAAGTAAGGATCTTCTTCTCCAA 3′	400
		Cuc.14- R	5′ CTTATCATCATTCCTCCCGATCA3′	
12	Cucsa.277260	Cuc.15- F	5′ GATTGGTTTGGTCGAGGTAGTA 3′	200
		Cuc.15- R	5′ GAGCATGGTCTTGATCCAATTC3′	
13	Cucsa.337180	Cuc.16- F	5′ TCACCTTCATGATTTTGCCA3′	156
		Cuc.16- R	5′ GTCTTCAACGCAACCCAAGT3′	
14	Cucsa.094670	Cuc.11- F	5′ TCCAATGCATCCTTTGCCTGA3′	145
		Cuc.11- R	5′ ATCTTTTGTTGTCACATTCGTA3′	
15	AB698859.1*	Act-F	5′ GTACCACTGGCATCGTGCT 3′	340
		Act- R	5′ GCAACGGAATCTCTCAGCTC 3′	

*****Gene sequence of control actin gene was retrieved from GenBank

Centrifugation for 10 min at 12,000 x g and 4°C helped separate the phases of sample. The top aqueous phase in tube was then transferred to new RNase-free tube. An equal volume of isopropyl alcohol was added. After 10 min the sample was centrifuged at 4ºC for 10 min at 12,000 x g decanted the supernatant taking care not to lose the pellet and added 1 mL of 75% ethanol to the pellet. Then the samples were centrifuged at room temp. 1 min at 12,000 x g. The liquid was decanted carefully taking care not to lose the pellet. The samples were briefly centrifuged to collect the residual liquid and removed it with a pipette. The pellet was dissolved in 10–30 µL RNase free water and stored at 80ºC. Treatment of RNA with DNase I (Thermo scientific, USA) was performed to remove the plant DNA following instruction of manufacturer. RNA was electrophoresed on 1% agarose gel to check its integrity. The concentration of RNA was determined by spectrophotometer. 1 µL of each sample was used to measure OD values at 260 nm or 240 nm after taring to zero using distilled water. An OD260 of 1 is equivalent to 50 µg/mL of DNA and an OD240 of 1 is equivalent to 50 µg/mL of RNA. The concentration of RNA was noted.

cDNA Synthesis for RT-PCR of NBS-LRR Genes

Primers of the retrieved NBS-LRR genes were designed by using Primer3 software (Table 1). For reverse transcription (RT)-PCR the Super Script™ III First Strand Synthesis Super mix (Invitrogen) was used. Two micro gram of total RNA, 30 ng/µg of oligo (dT) primers and annealing buffer were added to RNase/DNase-free water to make the volume 8 µL. The mixture was incubated at 65°C for 5 min and placed on ice for at least 1 min. After a short spin, 10 µL of 2X First-Strand Reaction Mix was added along with 2 µL of RNase inhibitor Enzyme Mix. This reaction mixture was incubated at 45°C for 60 min and the reaction was terminated by heating the mixture at 70°C for 5 min. 2–3 µL cDNA was used for the RT-PCR of NBS-LRR genes and remainder was stored at -80°C. RT-PCR product was resolved at 2% agarose gel and visualized at gel documentation system.

Stem Loop-RT Reaction for miRNAs

Stem-loop RT reaction was performed as per described by Chen (2005). Stem-loop RT primer (1 µM) was denatured by heating to 65ºC for 5 min and then incubated on ice for 2 min was centrifuged slightly to let the solution at the bottom of tube and incubated on ice. The prepared reactions were loaded in thermal cycler and incubated for 30 min at 16ºC followed by pulsed RT of 60 cycles at 30ºC for 30 sec, 42ºC for 30 sec and 50ºC for 1 sec. This mixture was incubated at 85ºC for 5 min for reverse transcriptase inactivation.

Table 2: Bioinformatic prediction of miRNAs target sites in NBS-LRR genes by TAPIR and miRU

miRNAs	Target Genes	Target site complementation	Mode of inhibition/ suppression	Prediction tool
csa-miR156a	Cucsa132370	miRNA 19CUGUCUCUCUCUUCA - CUCG 1 : . : :: : : : : : : ::: : : : : Target 381AAUAGAGAGUGAAGUUGAGC 400	Translation	miRU
		miRNA_3' AGACUGUCUCUCUCUUC - ACUCG aln . . . . |o | | | | | | . | | | | . | | | | | target_5' AAAAAAUAGAGAGUGAAGUUGAGC	Translation	TAPIR
	Cucsa251930	miRNA_3' AGACUGUCUCUCUCUUCACUCG aln | o | || . o | |. . | | | | o . | | | | . target_5' UUUGAAGGAUUGAGAGAUGAGA	Translation	TAPIR
		miRNA_3 ACGUGCACGGGACGAAGAGGU aln . | . | . . | . o o | | | | | | o | | | . target_ AGAAAUUCUUCUGCUUUUCCU	Cleavage	TAPIR
	Cucsa155730	miRNA 19 CUGUCUCUCUCUUCAC - UCG 1 . : . : : : : : : . : : : : : . : : : : Target 3381 AAUAGAGAGUGAAGUUGAGC 4000	Cleavage	miRU
		miRNA_3' AGACUGUCUCUCUCUUC - ACUCG aln . . . . |o | | | | | | . | | | | . | | | | | target_5' AAAAAAUAGAGAGUGAAGUUGAGC	Cleavage	miRU
	Cucsa292710	miRNA 19 UGUC - UCUCUCUUCACU - CG 1 :. . . . : : : . : : : : : .. .: : Target 273 AAUAGAGAGUGAAGUUGAGC 295	Translation	miRU
		miRNA_3' AGACUGUCUCUCUCUUCACUCG aln | o | | | . o | | . . | | | | o . | | | | . target_5' UUUGAAGGAUUGAGAGAUGAGA	Translation	TAPIR
	Cucsa094660	miRNA 19 CUGUCUCUCUCUUCACUCG 1 . : . : : : : : : : : : : : . : : : Target 71 AAUAGAGAGUGAAGUUGAC 90	Translation	miRU
		miRNA_3' AGACUGUCUCUCUCUUCACUCG aln . . | | . . | . | | | | | | | | | . | | . . target_5' CAUGUGAUAGAGAGAAGAGAUG	Translation	TAPIR
csa-miR157b	Cucsa102240	miRNA_3' ACACGAGAGAUAGAAGACAGU aln . . . . o | | | o . | | | | | | . | | | | target_5' CUGAUUCUUAAUCUUCAGUCA	Cleavage	TAPIR
		miRNA 21 ACACGAGAGAUAGAAGACAGU 1 :. : : . : . : :: : :: : : : : . ::: : Target2393 UCUGUUGUCUACCUUCUCUCA 2413	Translation	miRU
	Cucsa337180	miRNA_3' ACACGAGAGAUAGAAGACAGU aln | | . |o | | | | | | |o | | o . |. o | target_5' UGCGUUCUCUAUUUUUGGAUA	Cleavage	TAPIR
		miRNA 21 ACACGAGAGAUAGAAGACAGU 1 : :: . :: : : : :: : : : : : : :.:: : Target 2393 UCUGUUGUCUACCUUCUCUCA 2413	Translation	miRU
csa-miR159a	Cucsa178360	miRNA 20 CCUAGGUAUCGAAUCGUCGA 1 :: : : :: : :: : : : : : : :. : . :: Target 3538 GUAUCCAUAGCUAAGGAUCU 3557	Translation	miRU
		miRNA_3' CCCUAGGUAUCGAAUCGUCGA aln . | . | | | | | | | | | | . | | . | . | | target_5' UGUAUCCAUAGCUAAGGAUCU	Translation	TAPIR
csa-miR162a	Cucsa337180	miRNA 21 CCAGCUACUUGGCGACGGAGG 1 : : : : : :: : :: . :: . .: . : : . : Target 219 GGUGGAUGAAUCGAGGUCUUC 239	Cleavage	miRU
		miRNA_3' CCAGCUACUUGGCGACGGAGG aln | .| | .| . | | . | | . | | | | | | | | target_5' GCUCCAAGAUCCACUGCCUCC	Translation	TAPIR
	Cucsa102240	miRNA_3' CCAGCUACUUGGCGACGGAGG aln | .| | .| . | | . | | . | | | | | | | | target_5' GCUCCAAGAUCCACUGCCUCC	Cleavage	TAPIR
		miRNA 21 CCAGCUACUUGGCGACGGAGG 1 : : : : : :: : :: . :: . .: . : : . : Target 1550 GGUGGAUGAAUCGAGGUCUUC 1570	Translation	miRU

Table 2: Continued

Table 2: Continued

	Cucsa337180	miRNA 21 ACCGUUAAGUAGGAACCGAA 1 . : . : . . . : : . : . . . . . : : . : :	Translation	miRU
csa-miR169a		Target 2218 AUCGGUUUCAACAAAAUGGUU 2238		
		miRNA_3' GACCGUUAAGUAGGAACCGAA	Cleavage	TAPIR
		aln . | . | | | o | | o . | | | | | | . | | |		
		target_5' GUUGCAGUUUUUCCUUGUCUU		
	Cucsa178360	miRNA_3' GACCGUUAAGUAGGAACCGAA	Cleavage	TAPIR
		aln . | . | | | o | | o . | | | | | | . | | |		
		target_5' GUUGCAGUUUUUCCUUGUCUU		
		miRNA 20 ACCGUUAAGUAGGAACCGAA 1 . : . : . . . : : . : . . . . . : : . : :	Translation	miRU
		Target 3174 AUCGGUUUCAACAAAAUGGUU 3193		
csa-miR169c	Cucsa337190	miRNA 22 UGUCCGUUCAGUAGAAACCGAU 1 : . : : : : : : : : . : .. : . : : : : : : :	Translation	miRU
		Target 625 AUAGGCAAGACGACCUUGGCUA 646		
		miRNA_3' UGUCCGUUCAGUAGAAACCGAU	Translation	TAPIR
		aln . o | | | . | | . . | | | | | | | | | . |		
		target_5' CUAGGAAAAACAUCUUUGGCGA		
	Cucsa155730	miRNA_3' UGUCCGUUCAGUAGAAACCGAU	Translation	TAPIR
		aln . o | | | . | | . . | | | | | | | | | . |		
		target_5' CUAGGAAAAACAUCUUUGGCGA		
		miRNA 22 UGUCCGUUCAGUAGAAACCGAU 1 : . : : : : : : : : . : .. : . : : : : : : :	Translation	miRU
		Target 46 AUAGGCAAGACGACCUUGGCUA 67		
		miRNA 22 UGUCCGUUCAGUAGAAACCGAU 1 : . : : : : : : : : . : .. : . : : : : : : :	Cleavage	miRU
		Target 28 AUAGGCAAGACGACCUUGGCUA 49		
csa-miR169e	Cucsa251930	miRNA 21 UCCGUUCAGUAGGAACCGAGU 1 : : : : : : : . : . . : : : : : : : . :	Translation	miRU
		Target 627 AGGCAAGACGACCUUGGCUAA 647		
		miRNA_3' UCCGUUCAGUAGGAACCGAGU	Translation	TAPIR
		aln | | | . | | | . | | . | o | | | | | | . |		
		target_5' AGGAAAGACAGCUUUGGCUAA		
		miRNA 21 UCCGUUCAGUAGGAACCGAGU 1 : : : : : : : . : . . : : : : : : : . :	Cleavage	miRU
		Target 827 AGGCAAGACGACCUUGGCUAA 847		
csa-miR172d	Cucsa178360	miRNA 19 ACUUAGAACUAU - UACGAUG 1 : : : : : . : . : : : : : . : : : : : . :	Translation	miRU
		Target 1736 UGAAUUUGGAUAGAUGCUGC 1755		
		miRNA_3' ACACUUAGAACUAUUACGAUG	Translation	TAPIR
		aln | | | . . | | | | | | | | . | | | . | . | |		
		target_5' UGUACAUCUUGACAAUACAAC		
	Cucsa337180	miRNA_3' ACACUUAGAACUAUUACGAUG	Cleavage	TAPIR
		aln | | | . . | | | | | | | | . | | | . | . | |		
		target_5' UGUACAUCUUGACAAUACAAC		
		miRNA 20 ACUUAGAACUAU - UACGAUG 1 : : : : : . : . : : : : : . : : : : : . :	Translation	miRU
		Target 307 UGAAUUUGGAUAGAUGCUGC 325		
	Cucsa277260	miRNA_3' ACACUUAGAACUAUUACGAUG	Cleavage	TAPIR
		aln | | | . . | | | | | | | | . | | | . | . | |		
		target_5' UGUACAUCUUGACAAUACAAC		
		miRNA 20 ACUUAGAACUAU - UACGAUG 1 : : : : : . : . : : : : : . : : : : : . :	Cleavage	miRU
		Target 1380 UGAAUUUGGAUAGAUGCUGC 1399		
csa-miR390b	Cucsa337180	miRNA 20 CUUUGAGUCCUACCUAUCGC 1 : : : : : : . : : : : : : : : : : : . :	Translation	miRU
		Target 2412 GAUACUUUGCAUGGAUAGUG 2431		
		miRNA_3' CCUUUGAGUCCUACCUAUCGC	Translation	TAPIR
		aln . | | | | | | | | . | . | . | | | | | o .		
		target_5' UGAAACUCAAGCUUGAUAGUA		
csa-miR396c	Cucsa094660	miRNA_3' GAAGGGUGUCGAAAUAACUUG	Cleavage	TAPIR
		aln | | . | | . . | | . | | |. | | | | | | .		
		target_5' CUGCCAUCAUCUUCAUUGAAG		
		miRNA 20 ACGGGAGAUAGAAGACAGUU 1 : . : : . . : : : : : . : : . : : : : :	Translation	miRU
		Target 2235 UUCCUCUUAUCUGUUGUUAA 2254		

Note: The out put of target site complementation in miRU and TAPIR was represented by
miRU ":"= complementation, "."= loose complementation and "space" =no complementation;
TAPIR "|"= complementation, "o"-loose complementation and "."= no complementation.

Stem Loop RT-PCR Amplification

The mature miRNAs were amplified by PCR using a reaction mixture of 50 µL containing 10 pg 1 µg template DNA, 5 µL 10X *Taq* polymerase buffer, 5 µL 2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM primer and 1.25 units of *Taq* DNA polymerase (Fermentas, USA) was prepared in a 0.25 mL PCR tube. The reaction mixture was incubated in thermal cycler (Eppendorff master cycler) programmed for a preheat treatment of 94ºC for 5 min followed by 35 cycles of 94ºC for 1 min, 55ºC for 30 sec and 72ºC for 30 sec, followed by a final incubation of 10 min at 72ºC. PCR product was then stored at 4ºC.

Polyacrylamide Gel Electrophoresis (PAGE) for miRNA

Amplified PCR products of miRNAs were resolved on 6% polyacrylamide gel (19:1, acrylamide: bisacrylamide) using Omni PAGE Maxi Vertical Electrophoresis system. Before loading the samples the gel was pre-run for about 20 min. About 3 µL of PCR product was mixed with 4 µL (6X) gel loading dye (Fermentas, USA) and loaded on gel along with 50 bp gene ruler (Fermentas, USA). The gel was submerged in 1 L of TBE buffer containing Ethidium bromide at a concentration of 0.5 µg/mL of distilled water with gentle shaking for 20 min. After staining, the gel was washed with distilled water for 10 min. The amplified products were viewed under ultraviolet transilluminator and documented using gel documentation system.

Results

Bioinformatics Analysis of the NBS-LRR Genes Targeted by miRNAs

When 14 NBS-LRR genes having CC domain of *C. sativus* were bioinformatically checked, using TAPIR and miRU software tool for the target sites of csa-miRNAs, it was found that all genes were targeted by different miRNAs (Table 2 and 3). In many cases, same gene was targeted by different miRNAs.

RT-PCR for NBS-LRR Genes

RT-PCR was performed to analyze the expression level of NBS-LRR genes in infected and non-infected genotypes. Fourteen NBS-LRR genes were amplified only in those plant samples, which were infected with CMV, while control plants didn't show any amplification. This showed the upregulation of these NBS-LRR genes in response to viral infection and their strong relation with plant's immune response. (Fig. 1).

miRNA Target Prediction for NBS-LRR Genes with TAPIR and miRU

To explore the role of miRNAs in attenuating the gene expression of these R-genes, bioinformatics tools were used. Out of these 14 genes, only 7 NBS-LRR genes (Cucsa337180, Cucsa102240, Cucsa155730, Cucsa251930, Cucsa178360, Cucsa277260, Cucsa094660) were predicted to be targeted by 10 miRNAs, as was confirmed by both TAPIR and miRU. These 10 miRNAs (csa-miR156a, csa-miR157b, csa-miR159a, csa-miR162a, csa-miR169a, csa-miR169c, csa-miR169e, csa-miR172d, csa-miR390b and csa-miR396c) showed incomplete complementarity to these target genes (Table 2). Target prediction results further showed that some genes have more than one target site for a certain miRNA. Two miRNAs; csa-miR159d and csa-156a, targeted the same gene (Cucsa155730), while csa-miR159c targeted a single gene (Cucsa094660) at more than one site. Similarly, csa-miR156a targeted the Cucsa251930 and csa-miR-157a targeted the Cucsa155730, Cucsa277260 at more than one site. The mode of inhibition of all the genes was not same, some genes indicated translational inhibition and others showed the cleavage of target mRNAs (Table 2). Based on these results, only those 10 miRNAs were further studied for co-expression analysis, which were predicted to target the NBS-LRR genes by both prediction tools.

Detection of Mature miRNAs through Stem Loop RT-PCR

The miRNAs are key regulators of gene expression in development and stress responses in most of the eukaryotes. Amplification of mature miRNAs from infected plants was observed through stem loop RT-PCR. The plants not infected with pathogen did not show any amplification. It showed that miRNAs target the messenger RNA of NBS-LRR genes under control condition. But the infection releases this control and the target genes keep on expressing. The miRNAs amplification size was very small like 60 bp. Therefore, the reaction was first run on 2% agarose gel and then reconfirmed by PAGE (Fig. 2A, 2B and 2C).

Discussion

The role of miRNAs in regulating the plant's developmental machinery has been well established (Bartel, 2004; Sunkar *et al.,* 2007; Jin, 2008; Rubio-Somoza, 2009) but, however, little is known about their role in preparing plant to cope up with biotic stress. In the current study, 40 miRNAs were retrieved from miRBase data base to analyse them through bioinformatics tools. Different miRNAs and their targets were predicted by two different software tools. The reason of not having 100% same target prediction might lie in the default criteria of target prediction in these tools (Rhoades *et al.,* 2002). The results showed that there are only 10 miRNAs, whose complementation to 7 NBS-LRR genes (accession numbers) and putative targeting was confirmed by both softwares. This strategy of choosing miRNAs to further analyze their role in regulating gene expression of the target genes enhances the chances of their subsequent validation in gene regulation mechanism (Mao *et al.,* 2012). In addition, it also circumvents the time and economic loss

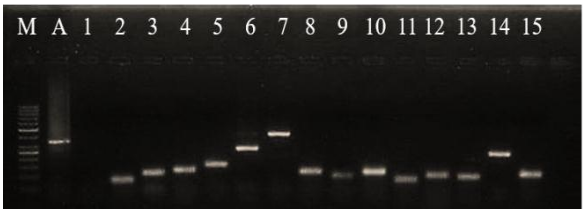

Fig. 1: RT-PCR of 14 NBS-LRR genes, amplified from CMV infected cucumber plants

M- 50bp ladder, A-Actin (340 bp), 1- negative control, 2- Cucsa.017460 (100bp), 3- Cucsa.094660 (125 bp), 4- Cucsa.102240 (140 bp), 5- Cucsa.128030 (200 bp), 6-Cucsa.155730 (250bp), 7-Cucsa.251930 (400 bp), 8- Cucsa.237520 (150 bp), 9- Cucsa.178360 (146), 10- Cucsa.337180 (156 bp), 11- Cucsa.132370 (135 bp), 12- Cucsa.094670 (145 bp), 13- Cucsa.249360 (125 bp), 14- Cucsa.277260 (200 bp)

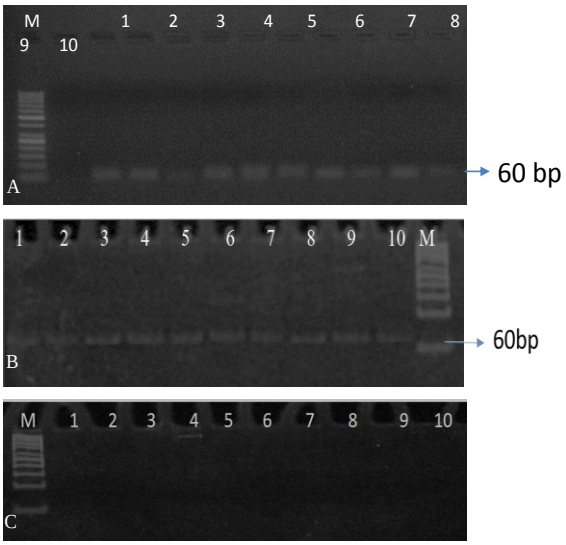

Fig. 2: Expression analysis of selected ten miRNAs. A) Agarose gel electrophoresis of stem loop RT-PCR amplicons of miRNAs from mock infected samples (control plants); B) PAGE analysis of the same amplicons to further confirm the small sized miRNA amplicons; C) Stem loop RT-PCR of miRNAs from infected plants

M- 50bp ladder, 1- miR156a, 2- miR157b, 3- miR159a, 4- miR162a, 5- miR169a, 6- miR169c, 7- miR169e, 8- miR172d, 9- miR390b, 10- miR396c

of experimenting with those molecules which are not yet predicted by trained simulations of advanced prediction tools. It has been described that miRNAs regulate the expression of genes either by translational inhibition or cleavage of the target mRNA. Usually miRNAs guide the RISC to target complementary sites of mRNAs to direct its cleavage through Argonaute (AGO) endonuclease activity. In animals, complementation between miRNAs and their corresponding targets occurs at 5 or 3' UTRs (Doench and Sharp, 2004). But in the present study, bioinformatical prediction of miRNA's target site in NBS-LRR showed that the partial complementation is located within the coding sequence of the gene. This observation is supported by the fact that most of the plant miRNAs repress the gene expression by partial complementation within gene sequence and subsequent AGO mediated cleavage (Aukerman and Sakai, 2003; Chen, 2005; Brodersen *et al.*, 2008; Lanet *et al.*, 2009).

The role of miRNAs in regulating the biotic stress response in plants has been extensively described (Navarro, 2006; Katiyar-Agarwal, 2006, 2007; Jin, 2008; Padmanabhan *et al.*, 2009; Gibbings and Voinnet, 2010). Thus, finding miRNA's cleavage targets in NBS-LRR genes was not surprising. The more interesting part is to explore its role in various signaling pathways. There were seven NBS-LRR genes (Cucsa.094660, Cucsa.155730, Cucsa.237520, Cucsa.178360, Cucsa.249360, Cucsa.251930 and Cucsa.368510) predicted to be targeted by both of these softwares. These NBS-LRR genes belong to the R-gene family, which is involved in defense system of *C. sativus*. Targeting of this gene family by miRNA suggests the role of these small molecules in regulating the expression of resistance genes and thus attenuating the innate immune system of cucumber. It has also been reported that small RNAs play a critical role in disease resistance responses (Navarro, 2006; Katiyar-Agarwal, 2006, 2007; Jin, 2008; Padmanabhan *et al.*, 2009; Gibbings and Voinnet, 2010). In *Brassica*, it is shown that miR1885 target TIR-NBS-LRR class genes and widely reported as upregulated in plants upon Turnip mosaic virus infection (He *et al.*, 2008). During the last few years, molecular genetics tools have greatly enriched the strategies and approaches for the discovery of small RNA molecules. These tiny regulators interact with their target genes leading to the site-specific cleavage or translational repression of the mRNA transcripts as are predicted in our study. The molecular mechanism of miRNA function requires base-pairing between miRNAs and their target mRNA genes. Since only limited complementarity is sufficient for target recognition in animals the animal miRNAs tend to have hundreds of targets which are typically inhibited at the translational level (Brennecke *et al.*, 2005; Lewis *et al.*, 2005). While, perfect complimentary base pairing mostly exists in plants and it is believed that most of plant miRNAs trigger mRNA degradation by cleavage (Axtell and Bowman, 2008; Mallory and Bouche, 2008). Nevertheless, our findings showed partial complementation, pointing towards translation repression of the target molecules as was observed in other plants (Brodersen *et al.*, 2008). It is worth mentioning that to validate miRNA and its involvement in the regulation of a particular gene or set of genes co-expression strategy is widely used (Kuhn *et al.*, 2008; Tang *et al.*, 2015). The co expression analysis in our findings showed that mature miRNAs were present only in infected samples while no amplicon was detected in non-infected samples.

On the other hand, NBS-LRR genes were expressed only in infected leaf samples as detected by RT-PCR. This suggests that miRNA might be involved in downregulating/silencing the target NBS-LRR genes in the

Table 3: miRNAs of cucumber retrieved from http://www.mirbase.org/and their predicted NBS-LRR target genes

Sr. No.	miRNAs	miRNAs sequences 5'-3'	Target genes
1	csa-miR156a	GCUCACUUCUCUCUCUGUCAGA	Cucsa132370, Cucsa251930, Cucsa155730, Cucsa292710, Cucsa094660
2	csa-miR157b	UGACAGAAGAUAGAGAGCACA	Cucsa102240, Cucsa337180
3	csa-miR159a	AGCUGCUAAGCUAUGGAUCCC	Cucsa178360
4	csa-miR162a	GGAGGCAGCGGUUCAUCGACC	Cucsa337180, Cucsa102240
5	csa-miR169a	AAGCCAAGGAUGAAUUGCCAG	Cucsa337180, Cucsa178360
6	csa-miR169c	UAGCCAAAGAUGACUUGCCGGU	Cucsa337190, Cucsa155730
7	csa-miR169e	UGAGCCAAGGAUGACUUGCCU	Cucsa251930
8	csa-miR172d	GUAGCAUUAUCAAGAUUCACA	Cucsa178360, Cucsa337180, Cucsa277260
9	csa-miR390b	CGCUAUCCAUCCUGAGUUUCC	Cucsa337180
10	csa-miR396c	GUUCAAUAAAGCUGUGGGAAG	Cucsa094660

Table 4: Stem loop RT primers for the amplification of mature miRNAs

miRNA	RT-primer (5′- 3′)	F-primer (5′- 3′)
csa-miR156a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTGAC	TCGGAAGGCTCACTTCTCTCTCT
csa-miR157b	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTGCT	GCGGCTTTGACAGAAGATAGAG
csa-miR159a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGATC	GCGTTCAAGCTGCTAAGCTATG
csa-miR162a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGTCGA	TTTCAAAGGAGGCAGCGGTTCA
csa-miR169a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGGCA	GCGGCTTAAGCCAAGGATGAAT
csa-miR169c	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGGC	GCCGTCTTAGCCAAAGATGACTT
csa-miR169c	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGGC	GCGGTTCTAGCCAAAGATGACTT
csa-miR169e	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGCAA	GTTCGGTTGAGCCAAGGATGAC
csa-miR172d	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTGAA	GCCGGTCGTAGCATTATCAAGA
csa-miR390b	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAAAC	GCGTTAACGCTATCCATCCTGA
csa-miR396c	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTCCC	GCGGCCAGTTCAATAAAGCTGT

absence of any infection or threat. However, the tight control of transcription by miRNAs was released upon infection in a way that plant can combat its enemy with the help of resistance genes. This suggested role is also in accordance with Shivaprasad *et al.* (2012). The current findings suggested a potential role of identified miRNAs in regulating the expression of target genes. It further affirms that the target prediction tools are a useful source to sort out the potential target miRNAs and their target for future studies. The identified relationship between miRNAs and their targets need further validation by transformation experiments in future.

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