Article

Deciphering microRNAs and Their Associated Hairpin Precursors in a Non-Model Plant, *Abelmoschus esculentus*

Kavitha Velayudha Vimala Kumar †, Nagesh Srikakulam †, Priyavathi Padbhanabhan and Gopal Pandi *

Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu 625021, India
†- Equal contribution
Kavitha Velayudha Vimala kumar- kavithabt28@gmail.com
Nagesh Srikakulam- bioinagesh@gmail.com
Priyavathi Padbhanabhan- priyapadbhanabhan@gmail.com

* Correspondence: pgp79@yahoo.com or pgopal.biotech@mkuniversity.org; Tel.: +914522458230

Abstract: MicroRNAs (miRNAs) are crucial regulatory RNAs, originated from hairpin precursors. For the past decade, researchers are focusing extensively on miRNA profiles in various plants. However studies on precursor miRNAs (pre-miRNAs) global profiling stay static even in model plants. Here, for the first time in a non-model plant, *Abelmoschus esculentus* with negligible genome information, we are reporting the global profiling to characterize the miRNAs and their associated pre-miRNAs by applying next generation sequencing approach. Preliminarily we performed sRNA (small RNA) sequencing with five biological replicates of leaf samples to attain 207,285,863 reads and data analysis using miRPlant keyed out 128 known and 845 novel miRNA candidates. With the objective to seize their associated hairpin precursors, we accomplished pre-miRNA sequencing to attain 83,269,844 reads. The paired end reads are merged, adaptor trimmed and the resulting 40-241 nt (nucleotide) sequences were picked out for analysis by using perl scripts from miRGrep tool and in-house built shell script for Minimum Fold Energy Index (MFEI) calculation. Applying stringent criteria of dicer cleavage pattern and perfect stem loop structure revealed precursors for 57 known miRNAs of 15 families and 18 novel miRNAs. Quantitative Real Time (qRT) PCR was performed to determine the expression of selected miRNAs.

Keywords: miRNAs; pre-miRNAs sequencing; *Abelmoschus esculentus*; Next generation sequencing; non-model plant

1. Introduction

MicroRNAs (miRNA) are short of size 21-24 nucleotides (nt) in plants and comprises a crucial component of small RNAs (sRNAs). Mostly, miRNAs are transcribed by RNA Polymerase II to produce primary miRNAs (pri-miRNA). The stem-loop structure of pri-miRNA directs DCL1 (Dicer like 1) proteins to process near at the base of the stem [1], ending in the formation of precursor miRNA (pre-miRNA). Elimination of pre-miRNA loop is subsequently followed by continuous stretch of two or multi step cleavage at 21- nucleotide intervals alongside the stem, resulting in the formation miRNA:*miRNA duplex. The precise miRNA recognition from the pri-miRNA relies on the precursor composition comprising processing signals like 14- to 15-bp nucleotides paired in stem proximal, specific length of nucleotides paired in stem distal to the miRNA/miRNA* duplex and a terminal loop[2,3]. In addition, an alternative biogenesis pathway has been proposed, the loop-to-stem processing of pri miRNAs [4]. The miRNA-*miRNA duplexes are methylated at their 3'end by HUA ENHANCER 1[5, 6] to maintain their size and to defend from polyuridylation and consequent

degradation [7]. The duplexes are channelized into cytoplasm, associated with Argonaute (AGO) protein to organize the RNA-induced silencing complex (RISC) where the * miRNA is sliced by the AGO at 10th or 11th nt position [8]. The mature miRNA in association with AGO silences the target transcript either by direct cleavage (slicing) or by destabilization through slicer independent turnover mechanisms and translational repression [9].

The emerging trend of next generation sequencing technologies (NGS) revealed various unknown genes and small RNAs recently in model and non-model plants [10, 11]. Small RNA sequencing in *Arabidopsis* exposed differentially expressed miRNAs along with other small RNAs during phosphate deficiency [12]. A report on the function of miRNAs in rice grain development by deep sequencing is available [13]. However most of the studies are on mature miRNA profiling, efforts to explore on the pre-miRNAs are limited even in model plants. Unlike mature miRNA profiling, precursor miRNA profiling is not demanding due to its complicated hairpin structure and its low abundance [14]. Generally, northern blot, *in situ* hybridization and qRT-PCR had been used to resolve pre-miRNAs expression pattern. To gain more insights on pre-miRNA sequences and secondary structure formation, rather than using sequencing approaches, researchers often presume from the genome sequences of corresponding miRNA-*miRNA. However there is a possibility to end in puzzlement when *miRNA is absent.

Abelmoschus esculentus (lady's finger or *Abelmoschus*), belongs to the *Malvaceae* family, allopolyploid in nature is cultivated around the globe in tropical, subtropical and warm temperate regions. *A. esculentus* is a vital vegetable crop, rich in fiber and vitamins with limited genome sequence information. sRNAs have proven to be regulators, targeting a large number of regulatory proteins. Apart from targeting various transcription factors, they also regulate protein stability, development, signaling pathways and plant pathogen resistance [15]. Hence discovering the miRNAs role in *A. esculentus* will help us to understand nexus between the miRNAs and their target genes, throw light to control the *A. esculentus* associated begomoviruses [16]. In this paper, to gain more insights into the miRNAs and their hairpin precursors, we executed NGS for miRNAs with five different *A. esculentus* leaf tissues. Data analysis applying miRPlant [17] identified 128 known miRNAs conserved across the plant kingdom and 845 novel miRNA candidates. To resolve pre-miRNAs for the known and novel predicted candidates, for the first time, we performed pre-miRNA sequencing by selecting libraries of size 160-300 (includes adaptors) nt . For precursor data analysis, we devised a strategy with stringent criteria and parameters by applying minimal fold energy index (MFEI) and dicer cleavage pattern with perfect stem loop structure. Eventually, we determined precursors for 18 novel miRNAs and 57 known miRNAs with high assurance.

2. Results

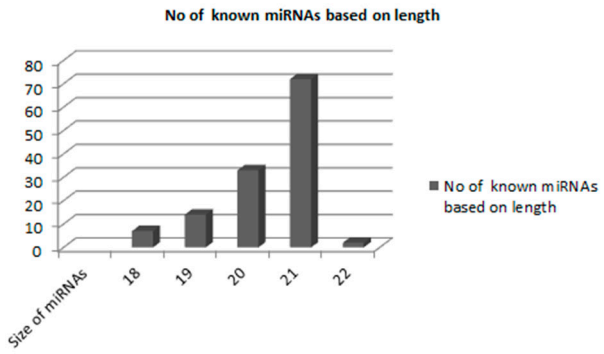
2.1. sRNA sequencing reveals known as well as novel miRNA candidates:

For the identification of conserved and novel miRNAs in *A. esculentus*, five small RNA libraries were formed from the leaves by applying Illumina and Ion torrent sequencing platforms. A total of 207,285,863 raw reads were gathered from all the 5 libraries. After adapter trimming, quality filtration was executed employing fastqc with phred score 30. Among the 5 datasets, 2 datasets were of poor quality and consequently had been discarded. We preceded analysis with the quality passed 3 datasets. Quality filtration retained 99,229,831 reads, out of these reads, 8,188,118 reads were unique. The reads were subjected to tRNA, rRNA, sn and sno RNA removal and eventually retained 7,384,997 reads for further analysis on miRplant for miRNA prediction. The reads were mapped to the reference genome such as *G. raimondii*, *Arabidopsis*, *O. sativa* by not allowing any mismatch. miRPlant finds the flanking sequence around the reads and determines whether the genomic region forms a hairpin based on the RNA secondary structure algorithm. The score for individual miRNAs are calculated by measuring the strength of the prediction. If the score for a predicted miRNA is higher, the probability for that miRNA to be a true candidate is increasing [17]. Accordingly miRNAs with positive scores were considered for miRNA candidates. miRPlant predicted 1023 novel miRNAs

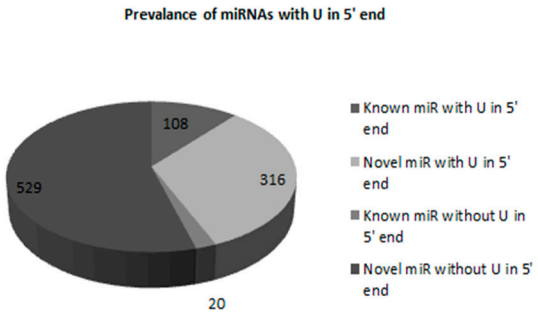
Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

and 128 conserved miRNAs. Selection of unique sequences amidst the repeated sequences based on their lengths yielded 845 novel miRNAs. The 128 conserved miRNAs are grouped under 28 families. For most of the conserved miRNAs predicted by miRPlant, the mature miRNA sequence and its complimentary sequence (*miRNA) is also predicted to be found. All the novel miRNAs predicted were subjected to similarity searching with miRNAs submitted in miRBase (http://www.mirbase.org/, Version 21) to ensure their novelty. The read count summary of small RNA data analysis was mentioned in Table-S1. Overall, 21 nucleotide miRNAs dominated in conserved as well as novel candidates followed by 20 nucleotide miRNAs (Fig. 1A, B). Among the 128 conserved miRNAs, 108 miRNAs carried U residue in their 5′ end and among the 845 novel miRNAs predicted, 316 miRNAs showed U residue in their 5′end. (Fig. 1C)

1A



1C



1B

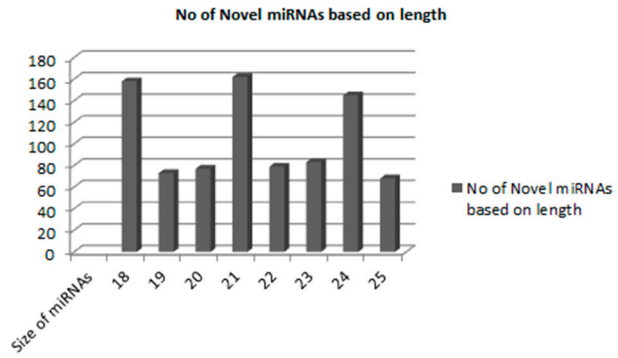


Figure.1: sRNA sequencing data analysis in *A. esculentus*. sRNA sequencing was performed and quality filtered data was analysed with miRPlant by using cotton, *Arabidopsis* and rice plants without allowing any mismatch. The filtered miRNAs were studied by investigating the terminal nucleotides (1C), length for conserved (1A) and novel (1B) miRNAs.

2.2. Pre-miRNAs profiling and Determination of pre-miRNAs for known and novel miRNAs:

As we identified 128 known and putative 845 novel miRNA candidates, we intend to reveal the sequence of pre-miRNAs for the identified miRNAs. Therefore we designed a strategy to sequence the pre-miRNAs as mentioned in methods section. The 160-300nt libraries are size selected and subjected to Illumina sequencing in paired ends to attain 83,269,844 reads. Pre-miRNA mapping could not be performed due to deficiency of *A. esculentus* genome sequence information.

Paired-end pre-miRNA sequencing reads were assembled as mentioned in the methods section, followed by quality filtration. Assembly yielded 41,194,311 reads. Quality filtration with phred score cut off 30 and adapters trimming retained 13,620,056 reads. 386,003 reads were found to be unique. We carried out rRNA, tRNA, sn and snoRNA elimination and retained 147,028 reads. The 40-241nt adapter trimmed sequences were considered as long reads for pre-miRNAs determinations.

As we mapped the short reads on the long reads, we found a sliding pattern of short reads within a single long read at 1 nt interval ensuing puzzlement, imparting more hassle to analyse the data (Fig.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 March 2017 doi:10.20944/preprints201703.0081.v1

Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

S1). Hence, we decided to analyse the data by retrieving known and novel miRNA reads from Plant micro RNA Database (PMRD) and miRPlant by executing the below mentioned strategy (Fig. 2A):

I) In order to predict the precursors for conserved miRNAs, initially, 15,041 known pre-miRNA sequences retrieved from PMRD were considered as long reads. The short reads of *A. esculentus* were mapped with the long reads from PMRD to analyse the over lapping position by not allowing any mismatches, speculating that only conserved miRNA/*miRNA will get aligned with the PMRD long reads. 5069 short reads showing alignment were extracted and mapped to *A. esculentus* pre-miRNA data to determine the pre-miRNA reads precisely. 549 mapped long reads were retrieved and remapped with short reads for complete cluster formation. The precursors predicted had been subjected to manual analysis for filtering out the incomplete precursors and precursors whose mature miRNA falls in the loop region. Manual analysis retained only 25 complete precursors with perfect stem-loop structure holding the mature miRNA in their stem region. Mapping summary for the three small RNA datasets are mentioned in the Table 1 and mapping pattern of the miRNA 156 with its precursor is shown in the Fig. 2B.

Table-1: Mapping summary of precursors with the small RNA datasets

S.No	Precursors	Small RNA dataset 1	Small RNA dataset 2	Small RNA dataset 3
1	No of precursors for known miRNAs	309	305	241
2	No of common precursors(for Known miRNAs) among the 3 datasets		219	
3	No of Unique precursors(for known miRNAs) among the 3 datasets		330	
4	No of precursors for novel miRNAs	4863	4115	3509
5	No of common precursors(for novel miRNAs) among the 3 datasets		2003	
6	No of Unique precursors (for novel miRNAs) among the 3 datasets		6793	

Number of reads obtained during precursor data analysis was shown Table 1. The precursor reads were mapped with small RNA reads from datasets 1, 2 and 3.

II) For predicting the novel pre-miRNA candidates, the pre-miRNA reads mapped with the miRPlant predicted 845 novel miRNAs were retrieved and aligned with *A. esculentus* small RNA dataset to see the mature miRNA and its complimentary sequence clusters. The final results were subjected to manual analysis to reveal the mapping pattern of the novel miRNAs as depicted in Fig. 2C.

We predicted stem and loop structure for the selected long reads using RNAfold [18] and p value by Randfold [19]. We set the following criteria for sorting out the perfect precursors:

a. The long reads which showed unambiguous secondary structure are considered as possible pre-miRNA candidates.

b. Minimum length of the long reads chosen was 40nt.

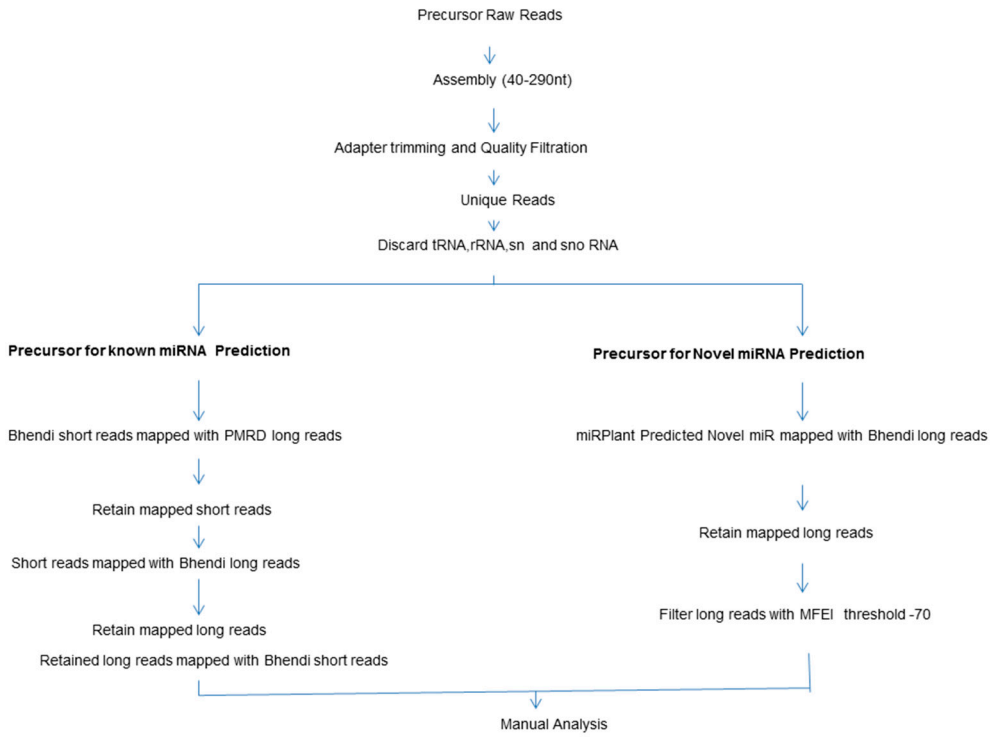
Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 March 2017 doi:10.20944/preprints201703.0081.v1

Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

c. We calculated the minimal fold energy (MFE) for the reads using RNA Fold and Randfold. Minimum fold energy index (MFEI) was calculated [20] as follows.

MFEI= (100*MFE)/Length of RNA/G+C)) %

2A



2B

```
TTGACAGAAGATAGAGAGCACCCTCTCTCTCTCCCTGTCTGCCTTTCTGTCTGTCTTATTATTGACACGGCTGATGACTTGTAAATTCTCCATGAGAATCAGTT
..((((((.(((.((((((.....)))))).))).))))).....((.(((((((.........)))).))).)).........((((((...)))))).....  (-27.40)
TTGACAGAAGATAGAGAG                                                                                          323 MIR156
TTGACAGAAGATAGAGAGC                                                                                         151 tcc-MIR156f
```

2C

```
GACCGACGCGGATCGAGGTGGCGGCCCAAGCCCGGGCCTTCGATACGCCCGCGGAGACGTCGTCTCCTCGATCTGCGTTGATGAAGCAATTTTATTGTGGACTAGAGTTTCTGATCTGG
...(((((((((((((.((((((.....(((((((.........)))))).)).....)))))).)))))))))))).....(((((...)))))...(((((........)))))  (-54.50)
      CGCGGATCGAGGTGGCGGC                                                                                                   8
```

Figure 2. Pre-miRNA data analysis. In order to reveal the pre-miRNA sequences for the known and novel miRNAs, pre-miRNA sequencing was carried out and data was analysed by two ways (2A) by mapping the short reads (sRNA data) with the long reads (pre-miRNA data). The mapping patterns were studied by modifying miRGrep protocol for known miRNAs (2B) and novel miRNAs (2C).

The possible pre-miRNA candidates had many splicing variants and incomplete precursors along with perfect stem loop structured precursors. To sort out the perfect precursors we carried out manual analysis using the following criteria.

- The base pairing between the mature miRNA and its complimentary sequence includes not more than 4 mismatches.
- The asymmetric bulges in between the duplex region are less frequent and should contain less than 4 bases. However the total number of mismatches in the duplex region is not more than four.

Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

- The precursors which carry the mature miRNA in its loop region are discarded. Also the precursors with bigger loops in between the duplex region are also discarded.
- For novel precursors, we preceded with sequences holding the MFEI index -70 as their minimum threshold to get rid of the other RNA species contamination like rRNAs and tRNAs. Moreover we manually checked the individual precursors by similarity searching in NCBI to ensure that the sequences are devoid of these contaminating RNAs.
- In the case of novel precursors, some novel miRNAs are predicted based on their expression as well as their mapping pattern with the precursor.

Overall, we located precursors for 18 novel miRNAs and 57 conserved miRNAs of 15 families. The secondary structures of some of the known and novel miRNAs are depicted in Fig. 3A and 3B.

2.3. Precursors of Conserved miRNAs and Novel miRNAs

Among the precursors for known and novel miRNAs, many precursors for a particular miRNA showed similarity in their 5′ end and slight variation in their 3′ end indicating that they were splicing variants obtained during gene duplication events. Apart from these variants, we observed different precursors with perfect stem loop structure with the mature miRNA and its complimentary sequence falling on the stem region. Precursors were abundant for the conserved miRNAs like miRNA159, 6300 and 482 and also for the novel miRNA 3. Many of them were fragments of precursors and possible splicing variants. We chose only the perfect precursors by applying above mentioned criteria and found precursors for 15 conserved miRNA families, which include miRNA 157, 159, 482 and so on. We found many precursors for a single miRNA and vice versa. miRNA 482 and miRNA 159 has 3 and 4 precursors respectively . In addition each of the miRNAs such as miRNA 6300, 396, 168 and 408 have two precursors separately as like novel miRNA 4. miRNA 157 and 156 share the same precursor, which is obvious as they share a high degree of sequence similarity. But still there is a separate precursor for miRNA 156 alone which does not give rise to miRNA157. For most of the miRNAs like miRNA 166, 159, 156 and 160 a single precursor gave rise to many members in a particular family. Apart from these precursors, there are precursors particularly meant for miRNA159a, 482a, 159b and 166a devoid of their other family members. Known miRNAs along with their precursors are listed out in Table. 2. We found precursors for 7 novel miRNAs predicted by miRPlant. In addition we predicted 11 novel miRNAs based on the mapping pattern with their respective precursors as well as their expression from the small RNA data. Surprisingly we found that three of the novel miRNA precursors have their origin in the chloroplast DNA which demands further experimental validation. The novel miRNAs along with their precursors are listed in Table. S2.

Minimum fold energy index (MFEI) of the precursor sequences ranges from -0.36 to -0.94 with an average of -0.72. The p value of the precursors ranges from 0.005- 0.3 with an average of 0.04. Altogether we found 44 precursors which include 25 precursors for known miRNAs and 19 precursors for novel miRNAs. The length of the precursor ranges from 42-114 nucleotides (Fig. 3C). Similar to mature miRNAs, we noted the prevalence of U residues in the 5′ end of the precursor sequences also. Among the 25 known miRNA precursors, 21 precursors showed U in their 5′ end. Among the 19 novel miRNA precursors, 10 precursors showed U in their 5′ end (Fig. 3D)

Table-2: List of known miRNAs and their precursors

miRNA ID	**miRNA Sequence**	**Precursor Sequence**	**MFEI**
miR156	TTGACAGAAGATAGAGAG	TTGACAGAAGATAGAGAGCACCCTCTCTCTCTCTCCCTGTCTGCCTTTCTGTCTGTCTTATTATTGACACGGCTGATGACTTGTAAATTCTCCATGAGAATCAGTT	-0.696
miR482a	TCTTTCCTACTCCTCCCA	TCTTTCCTACTCCTCCCATTCCAGGGACGGAGGAGGCTAGGT	-0.750

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 March 2017 doi:10.20944/preprints201703.0081.v1

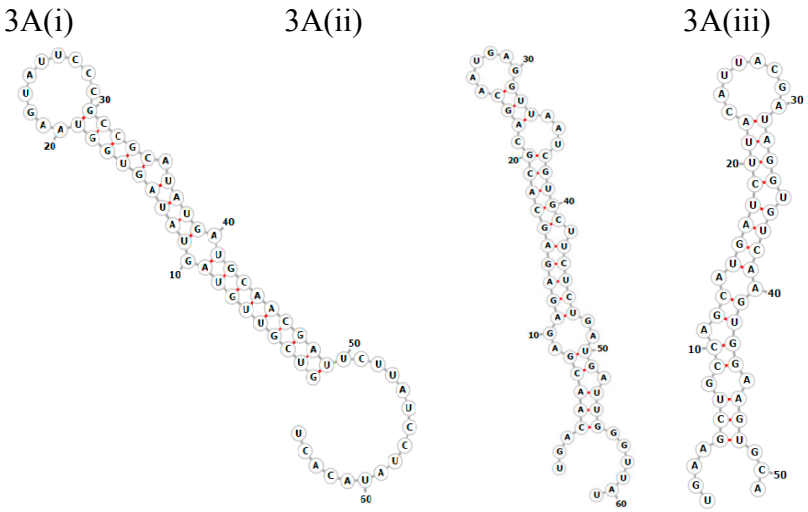
Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

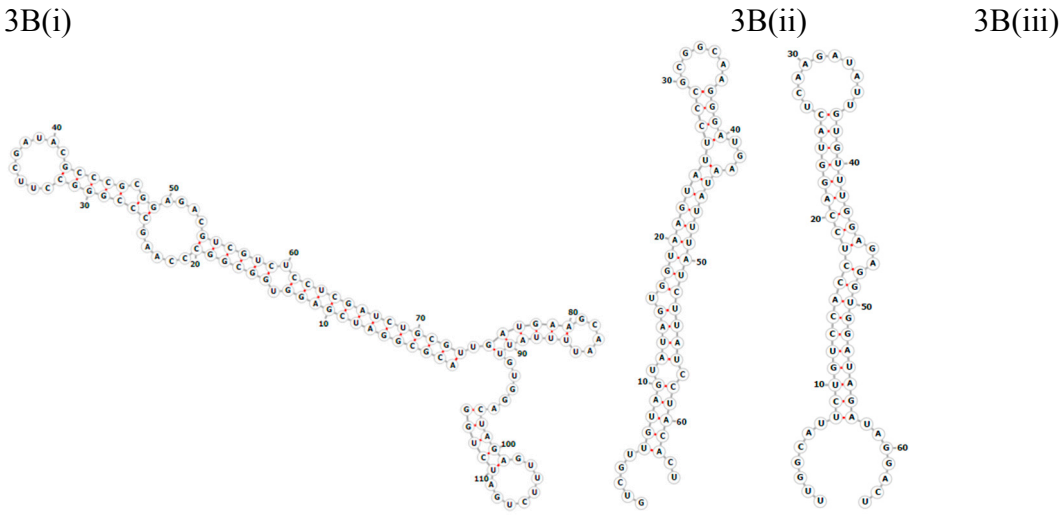
miR6300	GTCGTTGTAGTATAGTGGT	GTCGTTGTAGTATAGTGGTAAGTATTCCCGCCGCATATGATGCAACGATTCTTATCCTATACACT	-0.659
miR157	TTGACAGAAGATAGAGAGCA	TTGACAGAAGATAGAGAGCACCCTCTCTCTCTCTCCCTGTCTGCCTTTCTGTCTGTCTTATTATTGACACGGCTGATGACTTGTAAATTCTCCATGAGAATCAGTT	-0.696
miR159	GGATTGAAGGGAGCTCTA	TTTGGATTGAAGGGAGCTCTATTCTGTGATGAAGCAATTTTATTGTGGACTAGAGTTTCTGATCTGG	-0.769
miR396	CACAGCTTTCTTGAACTT	TTCCACAGCTTTCTTGAACTTGCGAGTCAACGGGTTAGCAAACCCGCAAGGCGCAAGGAAGCTGATTGGCGGGATCCCTCGCGGGTGCACCGCCGA	-0.750
miR6424	TGGTGCCACGCTGTGTGCG	AGAGCTGAATGTGGTGTTTTGGGCTCATGCTTGCGTGGTGCCATCAAAACTTGGCATTGGAGAAGCGGTGATGGTGCCACGCTGTGTGCGACTGA	-0.696
miR168	TCGCTTGGTGCAGGTCGG	TCGCTTGGTGCAGGTCGGGAAATTACGATAGGTGTCAAGTGGAAGTGCA	-0.604
miR160	TGCCTGGCTCCCTGTATG	TGCCTGGCTCCCTGTATGCCACAATGTAGGCAAGGGAAGTCGGCAAAATGG	-0.775
miR530	TGCATTTGCACCTGCACC	TGCATTTGCACCTGCACCTTCTCATTACGATAGGTGTCAAGTGGAAGTGCA	-0.878
miR166	TCTCGGACCAGGCTTCAT	TCTCGGACCAGGCTTCATTCCCGAAGCCTGCCCAGCAGAACGACCCGCGAACGTGTTATCGAAAAAC	-0.463
miR535	CAACGAGAGAGAGCACGC	TGACAACGAGAGAGAGCACGCAGCAATGAGGTTAATCGTGCTTCTCTGATGATTGGGTTAT	-0.571
miR162	TCGATAAACCTCTGCATC	TCGATAAACCTCTGCATCCAGGAGCAATGAGGATAATCTGCTCTTGTGATGATAGGGTTATC	-0.881
miR408	CACTGCCTCTTCCCTGGCT	TGCACTGCCTCTTCCCTGGCTTTCAGGTCTCCAAGGTGAACAGCCTCTGGTCGATGGAACAATGTAGGCAAGGGAAGTCGGCAAAATG	-0.646
miR167	GCTGCCAGCATGATCTTA	TGAAGCTGCCAGCATGATCTTACATTACGATAGGTGTCAAGTGGAAGTGCA	-0.339

The known miRNA sequences along with their corresponding precursor sequences are shown.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 March 2017 doi:10.20944/preprints201703.0081.v1

Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019





3C

3D

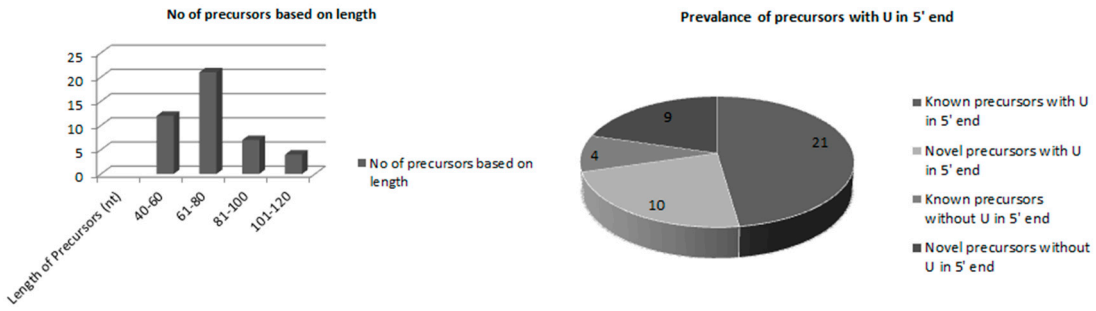


Figure. 3. Secondary structure of pre- miRNAs. The selected pre-miRNAs were mapped to the rRNAs, tRNAs, sn and snoRNAs to ensure that they did not align with them, The pre-miRNAs MFE, MFEI were calculated and subjected to secondary fold formation for the known (3Ai-iii) and novel (3Bi-iii) miRNAs by using Forna view from RNAFold (http://rna.tbi.univie.ac.at/cgi-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 March 2017 doi:10.20944/preprints201703.0081.v1

Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

bin/RNAfold.cgi). In addition, pre-miRNAs candidates were studied based on their length (3C) and the presence of U terminal nuleotide (3D).

2.4. Precursors of miRNAs from PMRD not in miRBase

In addition to the conserved miRNAs which have been submitted in miRBase, we found some of the miRNAs which have been no longer submitted in miRBase but available in PMRD (Plant micro RNA database), aligned with our precursor data. We found precursors for 7 such miRNAs, which includes miRNA35-npr, miRNAf10238-npr, miRNAf10239-npr, miRNAf11010-npr, miRNAf11025-akr, miRNAf10082-akr and miRNAf10271-akr. Mostly these miRNAs descended along with other miRNAs like miRNA160, 168, 530, 396 and 159 from their respective precursors. For example miRNAf 10238-npr arising along with miRNA 160 precursor and miRNAf 10239-npr produced along with miRNA 168 precursor. Others include miRNAf 11025-akr and miRNAf 10082-akr with miRNA 530, miRNAf 11010-npr with miRNA 396 and miRNAf 10271-akr with miRNA 159. Apart from these precursors, there is a separate precursor for miRNA 35-npr which is particular to that miRNA alone. These miRNAs along with their precursors are listed in Table.S3.

2.5 miRNAs from 5′ and 3′ positions of precursors

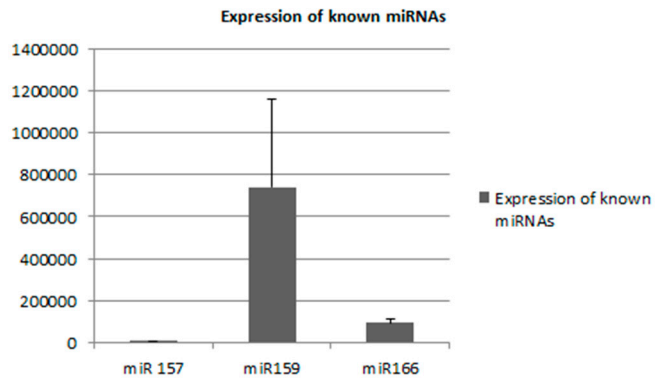
In any precursor, the mature miRNA descends either from the 5′ or 3′ position of the precursor. Most of our known miRNAs are produced from the 5′ position of their corresponding precursors except the known miRNA 6424 whose position is in the 3′ end of the precursor. Among the novel miRNAs, 12 miRNAs have their position in the 5′ end and 6 miRNAs have their position in the 3′ end of their respective precursors. Though we had more than one precursor for some of the known and novel miRNAs, all the precursors for a particular miRNA produced the mature miRNA from a single position either 5′ or 3′ and not both.

2.6 qRT- PCR:

Initially we examined the specificity of primers for each miRNA by performing reverse transcription and PCR. Negative controls like RNA control, minus template control ensured that the PCR product is not a primer dimer and also DNase treatment is appropriate. The PCR products were digested with specific restriction enzymes to confirm the presence of specific miRNAs. *Hinc*II digestion of miRNA 157 released 39bp&7bp fragments. *BstN*I digestion of miRNA 166 produced fragments of size 33bp&8bp. *Sac*I digestion of miRNA 159 produced fragments of size 30bp&17bp. NmiRNA 19 is digested with the enzyme *Sau3A*I to confirm the PCR product (Fig. S2).

qRT-PCR was performed for three known miRNAs, miRNA 166, 157 and 159 and for two novel miRNAs. miRNA 166 showed higher expression than the other 2 conserved miRNAs. NmiRNA 9 showed lower expression and on the other hand the NmiRNA 19 showed higher expression (Fig. 4A, B). The expression of miRNAs was normalised using 5.8srRNA.

4A



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 14 March 2017 doi:10.20944/preprints201703.0081.v1

Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

4B

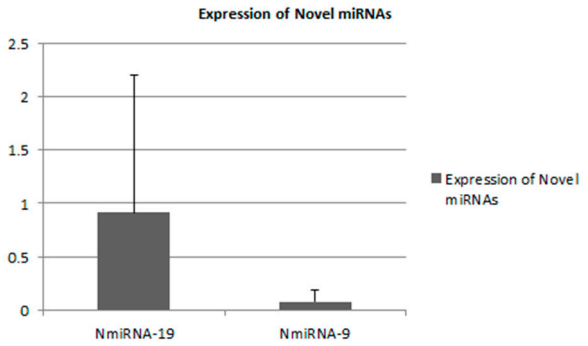


Figure 4. qRT-PCR of miRNAs. Randomly selected miRNAs , miRNA-157, miRNA-159 and 166 (4A) and two novel candidates (4B) are reverse transcribed and confirmed the specific amplification by restriction digestions (Supplementary figures 2) . Once genuine amplification is ensured, selected known and novel miRNAs were quantified by qRT-PCR.

2.7 Target prediction: A

Prediction of targets for novel miRNAs is necessary for its validation. Most of the targets predicted for miRNAs were transcription factors and genes involved in development, differentiation and metabolism. Targets are anticipated, both for conserved as well as novel miRNAs. Some of the targets predicted for known and novel miRNAs are mentioned in Table-3

Table 3-List of some known and novel miRNAs targets predicted from *A. esculentus* transcriptome data (Schafleitner *et al.*, 2012)

S.No	miRNA	Target
1	miR-169	1. mitogen-activated protein kinase kinase kinase 1-like transcript
2	miR-166	1. Homeobox-leucine zipper family protein 2. U-box domain-containing protein 26-like transcript 3. calcium-transporting ATPase 10, plasma membrane-type-like, transcript variant 4. casein kinase I-like transcript
3	miR-157	1. squamosa promoter-binding-like protein 2 2. LIGULELESS1 protein 3. serine/threonine protein phosphatase 2A 55 kDa regulatory subunit B beta isoform-like transcript 4. ubiquitin carboxyl-terminal hydrolase 9-like transcript 5. Cyclin b1,5 isoform 1 transcript
4	miR-159	1. ABC transporter A family member 2-like 2. transcription factor GAMYB-like

Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

		3. U-box domain-containing protein 44-like 4. forkhead box protein P2-like 5. CBL-interacting serine/threonine-protein kinase 14-like
5	NmiRNA- 4	1.ABC transporter A family member 2-like transcript 2.transcription factor GAMYB-like transcript
6	NmiRNA- 1	1.E3 ubiquitin-protein ligase RHA2A , mRNA
7	NmiRNA- 7	1. copper-transporting ATPase RAN1, mRNA
8	NmiRNA- 18	1.GBF-interacting protein 1-like , mRNA

3. Discussion

In recent times, the miRNAs emerged as powerful regulators of gene expression at post-transcriptional or chromatin levels [21] which are exploited to reveal the fundamental mechanism of genes [22] and in the production of pathogen resistant plants [23]. With the advent of Next generation sequencing technology, researchers were habituated to find the conserved as well as novel miRNAs in an astronomically immense number of plants including the non-model plants Some of them include *Arabidopsis* [10], tomato [24], cucumber [25], maize [26], rice [13], *Medicago* [27], *Citrus trifoliate* [28], *Hevea brasiliensis* [29], and potato [11]. miRPlant was used for the identification of conserved and novel miRNAs in cotton, which is taxonomically close to *Abelmoschus* [30]. In line with other studies, we performed sRNA sequencing and found 128 conserved known miRNAs and 845 novel miRNAs in *A. esculentus* . From the genome known plants, the maximum predicted miRNA number is 576 from the soybean and rice. *Gossypium raimondii* is having only 294 miRNA candidates (miRBase, Version 21) .Therefore identifying 128 conserved miRNAs candidates from the sRNA sequencing data is comparable and may be reliable.

Intriguingly, we predicted 845 novel candidates when we used cotton as a reference genome which is closely related to the *A. esculentus* . The high numbers of novel miRNA predictions may depend on the chromosomal number as *A.esculentus* has 130 while cotton is having 52. Though sRNA sequencing is common in plants, up to our knowledge we did not find any precursors sequencing until today. Precursor miRNA sequencing has been done in mouse with locked nucleic acids to abstract other non-coding RNAs [31]. Moreover precursor RNA sequencing was performed utilizing pre-miRNA specific primers to enrich the pre-miRNAs [32]. In order to identify the precursor miRNAs for known and novel candidates in *A. esculentus* plants, we carried out pre-miRNA sequencing by limiting 40-241nt. The pre-miRNA length was restricted from 40-241nt, keeping in mind the end goal to stay away from mRNA degradation products. Moreover in plants, the stems of the precursor miRNAs are stable and conserved, diversity is observed with the loop region and bulges [33]. Since the mature miRNA and * miRNA lies in the stem region, we aligned the reads gathered by small RNA sequencing to the precursor miRNA sequencing reads. Among the precursors of conserved miRNAs, we found several precursors sharing common sequences in the 5′ position but differs slightly in their 3′ position. These may be splicing variants of a particular miRNA gene. There are several miRNAs arising from a specific precursor. For example miRNA 157 and 156 arise from the same precursor. On the other hand, we obtained more than one precursor for the miRNAs 482, 159 etc. as reported in rice [34]. In addition, we observed single precursor gave rise to two distinct miRNAs. Although we got numerous precursors for a single miRNA, we selected those precursor candidates having not only the most effective mature miRNA sequence, but additionally

Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

must form the precise stem loop structure with the desired minimal fold energy index and p value. We discarded the reads which do not fulfil the criteria as mentioned within the methods section. For half of the known miRNAs from small RNA data predicted in miRPlant, we obtained their corresponding precursors from the precursor data showing their relevance. Although we used novel miRNAs from miRPlant for getting the precursors, we predicted some novel miRNAs from the precursor data as their mapping pattern shows they fall in the stem region of the precursor with mismatches less than 4.

Prediction of secondary structure of pre-miRNAs and calculation of free energy are required for false positive reduction of precursor miRNAs [19]. The minimum fold energy index (MFEI) is an important criteria used in differentiating the precursors from the other RNAs, both coding and noncoding. In a precursor study in Asiatic cotton, it was found that the MFEI of the identified precursors fall in the range of 0.29-1.85 [35]. Most of our precursors have their MFEI value around -0.72, since for predicting the novel precursors we set a stringent criterion for discarding all the other precursors which falls beyond the minimum threshold limit of -0.70. In a study in *Cassava*, the MFEI of the precursors were reported to be in the range of -0.84 to -1.20. It was reported that MFEI for tRNA is 0.64, for mRNA is 0.65 and for rRNA is 0.59 [36]. Although a few of our precursor sequences fall in the above mentioned MFEI, we ruled out that it is not a contaminating RNA by manually checking in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Thus by following stringent criteria, we determined pre-miRNAs sequence for 18 novel miRNAs candidates from the 845 and further characterization of those candidates should be carried out. It is noteworthy to say that, out of 41 million assembled pre-miRNA reads, we observed less than 1% pre-miRNAs. The feasible logic may be the rapid processing of pre-miRNA by Dicer and its co-proteins since pre-miRNAs is serving as an intermediate during mature miRNA processing. In addition, the secondary hairpin structure of the precursor may affect adapter ligation during library preparation resulting in limited number of reads.

Conclusion:

A. esculentus is an important vegetable crop with little genome information. Using NGS technology, we have identified *A. esculentus* associated miRNAs and their intermediate products which are known to play key role in the gene expression and plant resistance. This data will laid a foundation to unravel on miRNA: miRNA relationship which could be useful to develop pathogen resistant plants.

4. Materials and Methods:

4.1. RNA extraction:

RNA was extracted from the leaves of *A. esculentus* by slight modifications in Trizol method to get rid of the mucilaginous content.100mg of sample was minced in liquid nitrogen to a fine powder and 1.5ml of Trizol, 250µl of 100mM Sodium citrate (pH-6) and 250µl of 5M sodium chloride were added. After brief mixing, the samples were allowed to stand for 5min at room temperature followed by 200µl of chloroform addition. The sample was shaken vigorously for 15 seconds and allowed to stand for 10 min at room temperature. The mixture was centrifuged at 14,000 rpm for 30 min at 4˚C and transferred the upper aqueous phase into a fresh tube. Precipitation was done with 100% isopropanol and RNA quality was checked with Nanodrop ND-1000 (Thermo Scientific, USA) followed by electrophoresis. After DNase (Macherey Nagel, Germany) treatment, the quality was rechecked by the above mentioned methods and the RNA was used for NGS or qRT-PCR.

4.2. sRNA sequencing libraries preparation:

Libraries of sRNAs were prepared by using True Seq Illumina sRNA library kits. Before library preparation, RNA quality was checked by electrophoresis and Nano Drop ND-1000 (Thermoscientific, USA) and RNA integrity was assured by Bioanalyzer (Agilent, California, United States).We isolated leaves from five different plants to have biological replicates. The good quality

Peer-reviewed version available at Non-Coding RNA 2017, 3, 0019; doi:10.3390/ncrna3020019

RNA from five *A. esculentus* leaf samples were size fractionated ranging from 10-40nts. Using RNA ligase (New England Biolabs, M0242), artificial adapters were ligated at the 3′ and 5′end, reverse transcribed and amplified using Illumina sequencing primers or Ion Torrent primers. The first strand cDNA synthesis was carried out for 50 minutes (min) at 65°C using SuperScript III reverse transcriptase (Invitrogen, 18064014). PCR was performed for fifteen cycles (98°C for 10 s, 60°C for 30 s and 72°C for 15 s) and amplified products were subsequently cleaned and enriched by polyacrylamide gel electrophoresis (PAGE). Size selection of the library in the range of 140 – 160 bp was followed by overnight gel elution and salt precipitation utilizing Glycogen (Invitrogen, 10814-010), 3M Sodium Acetate (Sigma, S7899) and absolute ethanol (Merck, 100983) and the resulting precipitate was re-suspended in Nuclease free water. The libraries were quantified and quality validated utilizing Qubit Fluorometer and High Sensitivity Bioanalyzer Chip (Agilent, California, United States) respectively. For small RNA sequencing, a total of five samples were processed. Three samples were processed by Illumina sequencing platform (Genotypic Pvt Ltd, Bangalore, India) and two samples by Ion torrent sequencing platform (Shrimpex Biotech, Chennai, India).

4.3 sRNA sequence read mapping:

Small RNA raw reads were trimmed for their adapters followed by quality checking by FastQC [37]. Adapter trimming and quality filtration was executed using Trimmomatic [38]. FAST X Artifacts filter (http://hannonlab.cshl.edu/fastx_toolkit/index.html) has been used for filtering sequencing artefacts. We used fq trim (http://ccb.jhu.edu/software/fqtrim/) for phred score conversion. After quality filtration, other contaminating RNAs like rRNA, tRNA, sn and snoRNAs are removed by alignment with bowtie [39]. The filtered small RNA sequences were analysed through miRPlant [17]. Since *A. esculentus* genome sequence is not available, we used *Gossypium raimondii*, *Arabidopsis* and *Oryza sativa* as reference genomes to find out the conserved as well as novel miRNAs by adopting the default parameters.

4.4 Precursor miRNA library preparation:

Adopting, Illumina TruSeq Small RNA library protocol outlined in TruSeq Small RNA Sample Preparation Guide, the precursors RNA sequencing library was constructed. To 1µg of total RNA 3′ adaptors were ligated followed by 5′ adaptor ligation. Reverse transcription of the ligated products were carried out by Superscript III Reverse transcriptase (Invitrogen, 18064014) after priming with Reverse transcription primers. cDNA enrichment and barcoding by PCR (15 cycles, 98°C for 10 s, 60°C for 30 s and 72°C for 15 s) were performed subsequently and products were cleaned by polyacrylamide gel. Libraries in the range of 160-300bp (including adaptors) were size selected and gel eluted. Salt precipitation, quantity and quality checking were performed as mentioned in sRNA library preparation. Illumina sequencing (Illumina Next Seq 500, Genotypic Pvt Ltd Bangalore) was performed for two libraries in paired ends to cover the 160-300 nucleotides sequences.

4.5 Data analysis:

Paired-end pre-miRNA sequencing reads were assembled by using Paired-End reAd merger (PEAR) [40]. Assembled pre-miRNA reads of length between 40 – 290 nt were retained. This contained sequences with or without adaptor sequences either in a single end or in both ends. After assembly, we performed adapter trimming and quality filtration with phred score cutoff value 30, followed by removal of rRNAs, tRNAs, sn and snoRNAs by mapping using bowtie2 [41]. The adapter trimmed 40–241 nt long reads are considered as possible pre-miRNA candidates on which we analysed sRNA reads (18-34 nt) alignment by soap.short without allowing any mismatch. From the miRGrep tool we have used the unique_reads_for_mapping.pl, S1_reversed.pl and S0_print.pl scripts [14] to generate the mapping pattern files. We used RNA Fold and Randfold to calculate the minimum fold energy and infoseq [42] to calculate the GC percentage. The possible pre-miRNA

candidates were selected based on their length, minimum fold energy index (MFEI), dicer cleavage pattern and their perfect stem loop structure. The criteria for pre-miRNA determination and parameters were discussed in detail in the results section.

4.6. qRT PCR :

To quantify and validate sRNA sequencing, we did qRT-PCR [43]. Specific primers were designed for three known miRNAs, miRNA 166, 157 and 159 and two novel candidates to determine the expression. The primer list was mentioned in Table 4. For qRT-PCR, RNA was isolated by modified Trizol method and DNase (Ambion, USA) treated at 37°C for 30min.RNA quality was checked in Nanodrop 1000 (Thermo Scientific, USA) and by electrophoresis. cDNA conversion was done as follows. Briefly, 100 ng of RNA was dissolved in a final volume of 10µl including 0.1 mM of ATP, 1 µM of RT-primer, 0.1 mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 100 units of MuLV reverse transcriptase (New England Biolabs, USA) and 1 unit of poly (A) polymerase (New England Biolabs, USA). The sample was incubated at 42°C for 1 hour and enzyme inactivation at 95°C for 5 minutes. Fast start Universal SYBR Green master (Roche, Switzerland) was used for quantification. qRT PCR was performed with 4 biological and experimental triplicates.

Table 4-List of primers used for quantitative Real Time PCR

S.No	miRNA	Forward Primer	Reverse Primer
1	miRNA159a	GCAGTTTGGATTGAAGGGA	AGTCCAGTTTTTTTTTTTTTTAGAGC
2	miRNA 166a	GTCGGACCAGGCTTCAT	CCAGTTTTTTTTTTTTTTGGGGA
3	miRNA 157a	CGCAGTTGACAGAAGATAGAG	TCCAGTTTTTTTTTTTTTTGTGCT
4	NmiRNA 19	GGCGCAGAGTTACTAATTCATGA	GTCCAGTTTTTTTTTTTTTTCAGAT
5	NmiRNA 9	CGCAGGGTGGCTGTAGTTTA	GTCCAGTTTTTTTTTTTTTTACCAC

The forward and reverse primers for the corresponding known and novel miRNAs are shown.

Before performing qRT-PCR, RT-PCR was performed with various controls to ensure that there was no primer dimer in the amplification. The amplified products of miRNA166, 167 and 159 were digested with *Sac*I, *Hinc*II and *BstN*I respectively and the novel miRNA (NmiRNA), NmiRNA 19 was digested with *Sau*3A I to confirm specific amplification. Once ensuring the genuine amplification, qRT-PCR was performed. Relative expression of miRNAs was quantified using $2^{-\Delta\Delta c}$T method [44].

4.7 Prediction of miRNA target genes

Target prediction was done using psRNATarget tool [45] (http://plantgrn.noble.org/psRNATarget/). *A. esculentus* transcriptome data [46], was used as the reference sequence. Targets were predicted using default tool parameters for known and novel miRNAs.

4.8 Accession numbers

All the small RNA and precursor RNA raw data are submitted in NCBI under the BioProject accession ID -PRJNA352593

Supplementary Materials: Supplementary data are available online at www.mdpi.com/link, Figure S1: title, Table S1: title, Video S1: title.

Acknowledgments: This work was supported by grants from DBT (Ref:No. BT/PR2061/AGR/36/707/2011 dated 25-06-2013), SERB (Ref:No. SB/EMEQ-070/2013 dated 05-07-2013) and DST-INSPIRE for Kavitha (Ref no: IF110736). We are thankful to DST-PURSE, UGC-SAP for the equipment's grants. We are acknowledging our gratitude to Dr.R.Usha, Dr. K.Veluthambi, Dr. S. Krishnaswamy, all retired professors from School of Biotechnology, Madurai Kamaraj University for their valuable suggestions and lab facilities. We are thankful to Dr.Velmurugan and Mr.Shanmugarajan from School of Biological Sciences, Madurai Kamaraj University for their support in Bioinformatic analysis.

Author Contributions: Gopal.P and Kavitha.V conceived and designed the experiments. Kavitha .V and Priyavathi.P performed the experiments. Data Analysis was done by Nagesh.S, Kavitha.V and Gopal.P. Kavitha.V, Gopal.P and Nagesh.S wrote the paper

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bartel, D.P. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*. **2004**,116, 281-297.
2. Mateos, J.L.; Bologna, N.G.; Chorostecki, U.; Palatnik, J.F. Identification of MicroRNA Processing Determinants by Random Mutagenesis of Arabidopsis MIR172a Precursor. *Curr. Biol*. **2010**, 20, 49-54.
3. Werner, S.; Wollmann, H.; Schneeberger, K.; Weigel, D. Structure Determinants for Accurate Processing of miR172a in Arabidopsis thaliana. *Curr. Biol*. **2010**, 20, 42-48.
4. Czech, B.; Hannon, G.J. Small RNA sorting: matchmaking for Argonautes. *Nat. Rev. Genet*. **2011**, 12 (1), 19-31.
5. Li, J.; Yang, Z.; Yu, B.; Liu, J.; Chen, X. Methylation Protects miRNAs and siRNAs from a 3′-End Uridylation Activity in Arabidopsis. *Curr. Biol*. **2005**, 15, 1501-1507.
6. Yu, B.; Yang, Z.; Li, J.; Minakhina S.; Yang M.; Padgett R.W.; Steward R.; Chen X. Methylation as a crucial step in plant microRNA biogenesis. *Science*. **2005**, 307, 932-935.
7. Zhao, Y.; Mo, B.; Chen, X. Mechanisms that impact microRNA stability in plants. *RNA. Biol*. **2012**, 9:10, 1218-1223.
8. Mallory, A.; Vaucheret, H. Form, Function, and Regulation of ARGONAUTE Proteins. *Plant Cell*. **2010**, 22, 3879-3889.
9. Voinnet, O. Origin, Biogenesis, and Activity of Plant MicroRNAs. *Cell*. **2009**, 136, 669-687.
10. Fahlgren, N.; Howell, M.D.; Kasschau, K.D.; Chapman, E.J.; Sullivan, C.M.; Cumbie, J.S.; Givan, SA, Law, T.F.; Grant, S.R.; Dangl, J.L; et al. High-Throughput Sequencing of Arabidopsis microRNAs:Evidence for Frequent Birth and Death of MIRNA Genes. *PLoS One* 2(2). **2007**, e219.
11. Zhang, R.; Marshall, D.; Bryan, G.J.; Hornyik, C. Identification and Characterization of miRNATranscriptome in Potato by High-Throughput Sequencing. *PLoS One*. **2013**, 8, e57233.
12. Hsieh, L.C.; Lin, S.I.; Shih, A.C.C.; Chen, J.W.; Lin, W.Y.; Tseng, C.Y.; Li, W.H.; Chiou, T.J. Uncovering Small RNA-Mediated Responses to Phosphate Deficiency in Arabidopsis by Deep Sequencing. *Plant.Physiol*. **2009**, 151, 2120-2132.
13. Zhu, Q.H.; Spriggs, A.; Matthew, L.; Fan, L.; Kennedy, G.; Gubler, F.; Helliwell, C. A diverse set of microRNAs and microRNA-like small RNAs in developing rice grains. *Genome Res*. **2008**, 18, 1456-1465.
14. Li, N.; You, X.; Chen, T.; Mackowiak, S.D.; Friedlander, M.R.; Weigt, M.; Du, H.; Gogol-Doring, A.; Chang, Z.; Dieterich, C.; et al. Global profiling of miRNAs and the hairpin precursors: insights into miRNA processing and novel miRNA discovery. *Nucleic Acids Res*. **2013**, 41, 3619-3634.
15. Rhoades, M.W.J.; Bartel, D.P.; Bartel, B. Micro RNAs and Their Regulatory roles in Plants. *Annu.Rev.Plant Biol.* **2006**, 57, 19-53.
16. Priyavathi, P.; Kavitha, V.; Gopal, P. Complex nature of infection associated with yellow vein mosaic disease in Bhendi (Abelmoschus esculentus). *Curr.Sci.* **2016**, 111, 1511-1515.
17. An, J.; Lai, J.; Sajjanhar, A.; Lehman, M.L.; Nelson, C.C. miRPlant: an integrated tool for identification of plant miRNA from RNA sequencing data. *BMC Bioinformatics*. **2014**, 15, 275.
18. Hofacker, I.L. Vienna RNA secondary structure server. *Nucleic Acids Res*. **2003**, 31, 3429-3431.
19. Bonnet, E.; Wuyts, J.; Rouzé, P.; Peer, Y.V. Evidence that microRNA precursors, unlike other non-coding RNAs, have lower folding free energies than random sequences. *Bioinformatics*. **2004**, 20, 2911-2917.
20. Zhang, B.H.; Pan, X.P.; Cox, S.B.; Cobb, G.P.; Anderson, T.A. Evidence that miRNAs are different from other RNAs. *Cell. Mol. Life. Sci*. **2006**, 63, 246-254.

21. Moazed, D. Small RNAs in transcriptional gene silencing and genome defence. *Nature*. **2009,** 457(7228), 413–420.
22. Aukerman, M.J.; Sakai, H. Regulation of Flowering Time and Floral Organ Identity by a MicroRNA and Its APETALA2 like Target Genes. *The Plant Cell*. **2003**, 15, 2730–2741.
23. Qu, J.; Ye, J.; Fang, R. Artificial Micro- RNA mediated Virus Resistance in Plants. *J. Virol*. **2007**, 81, 6690-6699.
24. Moxon, S.; Jing, R.; Szittya, G.; Schwach, F.; Pilcher, R.L.R.; Moulton, V.; Dalmay, T. Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening. *Genome.Res*. **2008**, 18, 1602-1609.
25. Martinez, G.; Forment, J.; Llave, C.; Pallas, V.; Gomez, G. High-Throughput Sequencing, Characterization and Detection of New and Conserved Cucumber miRNAs. *PLoS One*. **2011**, 6, e19523.
26. Wang, L.; Liu, H.; Li, D.; Chen, H. Identification and characterization of maize microRNAs involved in the very early stage of seed germination. *BMC Genomics*. **2011**, 12, 154.
27. Briere, CL.; Naya, L.; Sallet, E.; Calenge, F.; Frugier, F.; Hartmann, C.; Gouzy, J.; Crespi, M. Genome-Wide Medicago truncatula Small RNA Analysis Revealed Novel MicroRNAs and Isoforms Differentially Regulated in Roots and Nodules. *Plant Cell*. **2009**, 21, 2780-2796.
28. Song, C.; Wang, C.; Zhang, C.; Korir, N.K.; Yu, H.; Ma, Z.; Fang, J. Deep sequencing discovery of novel and conserved microRNAs in trifoliate orange (Citrus trifoliata). *BMC Genomics*. **2010**, 11, 43.
29. Gebelin, V.; Argout, X.; Engchuan, W.; Pitollat, B.; Duan, C.; Montoro, P.; Leclercq, J. Identification of novel microRNAs in Hevea brasiliensis and computational prediction of their targets. *BMC Plant Biol*. **2012**, 12, 18.
30. Naoumkina, M.; Thyssen, G.N.; Fang, D.D.; Hinchliffe, D.J.; Florane, C.B.; Jenkins, J.N. Small RNA sequencing and degradome analysis of developing fibers of short fiber mutants Ligon-lintles-1 (Li1) and −2 (Li2) revealed a role for miRNAs and their targets in cotton fiber elongation. *BMC Genomics*. **2016**, 17, 360.
31. Burroughs, A.M.; Kawano, M.; Ando, Y.; Daub, C.O.; Hayashizaki, Y. pre-miRNA profiles obtained through application of locked nucleic acids and deep sequencing reveals complex 5′/3′ arm variation including concomitant cleavage and polyuridylation patterns. Nucleic Acids Res. 2011, 40, 1424–1437.
32. Newman, M.A.; Mani, V.; Hammond, S.M. Deep sequencing of microRNA precursors reveals extensive 3′ end modification. RNA. 2011, 17, 1795–1803.
33. Xuan, P.; Guo, M.Z.; Wang, J.; Wang, C.Y.; Liu, X.Y.; Liu, Y. Genetic algorithm-based efficient feature selection for classification of pre-miRNAs. Genet. Mol. Res. 2011, 10 (2), 588-603.
34. Lacombe, S.; Nagasaki, H.; Santi, C.; Duval, D.; Piegu, B.; Bangratz, M.; Breitler, J.; Guiderdoni, E.; Brugidou, C.; Hirsch, J.; Cao, X.; Brice, C.; Panaud, O.; Karlowski, W.M.; Sato, Y.; Echeverria, M. Identification of precursor transcripts for 6 novel miRNAs expands the diversity on the genomic organisation and expression of miRNA genes in rice. BMC Plant Biol. 2008, 8, 123.
35. Wang, M.; Wang, Q.; Wang, B. Identification and Characterization of MicroRNAs in Asiatic Cotton (Gossypium arboreum L.). PLoS One. 2012, 7, e33696.
36. Rogans, SJ.; Rey, C. Unveiling the Micronome of Cassava (Manihot esculenta Crantz). PLoS One. 2016,11(1), e0147251.
37. Andrews, S. FastQC: A quality control tool for high throughput sequence data, http://www.bioinformatics.babraham.ac.uk ? /projects/fastqc/.2010.
38. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina Sequence Data, Bioinformatics.2014, btu170.
39. Langmead, B.; Trapnell, C.; Pop, M.; Salzberg, S.L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome. Biol. 2009, 10, R25.
40. Zhang, J.; Kobert, K.; Flouri, T.; Stamatakis, A. PEAR: A fast and accurate Illumina Paired-End reAd merger. Bioinformatics. 2013, btt593.
41. Langmead, B.; Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. Nature Methods. 2013, 9(4), 357–359.
42. Rice, P.; Longden, I.; Bleasby, A. EMBOSS: The European Molecular Biology Open Software Suite. Trends.Genet. 2000, 16, 276-277.
43. Balcells, I.; Cirera, S.; Busk, P.K. Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers. BMC Biotechnol. 2011, 11, 70.

44. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using RealTime Quantitative PCR and the 2-ΔΔcT Method. Methods. 2001, 25, 402–408.
45. Dai, X.; Zhao, P.X. psRNATarget: a plant small RNA target analysis server. Nucleic Acids Res. 2011, 39, W155–W159.
46. Schafleitner, R.; Kumar, S.; Lin, C.; Hegde, S.G.; Ebert, A.The okra (Abelmoschus esculentus) transcriptome as a source for gene sequence information and molecular markers for diversity analysis. Gene. 2013, 517, 27-36.