Pak. J. Agri. Sci., Vol. 60(2), 337-346; 2023
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI:10.21162/PAKJAS/23.41
http://www.pakjas.com.pk

In-Silico prediction and profiling of novel non-coding miRNAs and their target proteins in muskmelon (*Cucumis melo*)

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MicroRNAs are small non-coding regulatory RNAs that regulate gene expression after transcription by suppressing translation or degrading mRNA. MiRNAs play an important role in the regulatory systems such as biological processes in plants. By investigating miRNAs in muskmelon the current study will focus on miRNA prediction, profiling and expression. The *Cucumis melo* Expressed Sequence Tags (ESTs) were used in the current investigation to identify 22 novel miRNAs with Minimum Folding Energy (MFE) values ranging from -10.7 to -49.13 kcal/mole. The newly predicted *C. melo* miRNAs have the greatest number of target genes using psRNA-Target tool connected with Gene Ontology (GO) enrichment analysis. As a result, a total of roughly 78 targets were predicted in response to abiotic and biotic stress. In addition, miR157 has been found to target the receptor, cell signalling, gene expression and transcriptional activation. Consequently, cme-miR2657 targets high mobility group protein while miR2657 and miR5215 participate in positive control of gene silencing post-transcriptionally via RNA for plant hormones.

Keywords: Conserved non-coding miRNAs, blast, psRNA-target, phylogeny, cladogram.

INTRODUCTION

MicroRNAs are small single stranded RNAs containing 18-26 nucleotides (nt) found in animals, plants and in some viruses. They play key role in RNA gene silencing and post-transcriptional control of gene expression. MicroRNAs can perform their roles in base pairing with complementary sequences found in messenger RNAs (mRNA) molecules (Riolo *et al*., 2020). MicroRNAs have been described as negatively regulating gene expression by targeting mRNAs for cleavage (Khvorova *et al*.,2007) or translational inhibition (Zhang *et al*. (2006). MiRNA synthesis in both plants and mammals has been extensively discussed (Mlotshwa *et al*., 2015). A MIR gene is initially translated into pri-miRNAs (primary miRNAs) by RNA polymerase II. The 70–500 base-pair (bp) length of pri–miRNA then forms an improperly paired hairpin structure that is processed by RNAse III-like DICER–like I (DCL1) to produce precursors miRNA (pre–miRNAs) (Meyers *et al*., 2008). The pre-miRNA is further broken down by DCLI, releasing a miRNA/miRNA* duplex. The canonical mature miRNA of 18–26 nucleotides is then specifically integrated into the RNA–induced silencing complex (RISC) linked to Argonaute 1 after being translocated into the cytoplasm by HASTY (plant homolog of exportin 5 (AGO1). In RISC complex, the miRNAs try to link to mRNA perfectly or near perfectly manner and subjugate gene expression. Recent research on plants reveals that MIR genes are bi-functional since they produce both long miRNA (miRNA, 23–27 nucleotides) and conventional miRNA (20–22 nucleotides). In rice and moss the miRNAs produced from MIR genes are guided DNA methylation at certain of their target loci in Trans by DCL3 and AGO4 associations respectively (Bologna and Voinnet, 2014). The importance of post-transcriptional alterations of miRNA precursors was recently and significantly discovered understanding the complexity of miRNA synthesis and miRNA function (Wyman *et al*., 2011). It has been observed in plants RNA editing events or a combination of miRNA 5 prime (5') and 3 prime (3') uridylation modify the targeting and preference of Argonaut association (Yu *et al*., 2017). Model species with genomes have been sequenced including Arabidopsis, Oryza, Populus, Physcomitrella and Vitis to identify most miRNAs. Several other plants comprising *Glycine max* (Joshi *et al*., 2010), *Arachis hypogaea* (Chi *et al*., 2011), *Solanum lycopersicum* (Din and Barozai, 2014), *Brassica napus* (Xie, 2007), *Phaseolus vulgaris* (Pelaez *et al*., 2012) and the

Ghaffar, A., N. Khan, A. Baqi, I. Ali, N.U.D. Baloch and W.A. Shah. 2023. *In-Silico* prediction and profiling of novel non-coding miRNAs and their target proteins in muskmelon (*Cucumis melo*). Pakistan Journal of Agricultural Sciences 60:337-346.
[Received 3 Mar 2023; Accepted 10 Jun 2023; Published 27 Jun 2023]

euphorbiaceous species *Ricinus communis* (Xu *et al*., 2013) have also had several miRNAs discovered in them.

Plant's stem, leaf, root and flower development are influenced by miRNA which affect the actions of abiotic and biotic pressures from vegetative to reproductive development (Barozai and Baloch, 2012; Matsui and Nguyen, 2013). MicroRNAs have been found in a variety of model and agricultural plant species (Jagadeeswaran *et al*., 2009; Lu *et al*., 2005; Subramanian *et al*., 2008) but less known in the Cucurbitaceae family. Cucurbitaceae has 90 genera and 700 species including watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), muskmelon (*Cucumis melo*), bottle gourd (*Lagenaria siceraria*) and squash and pumpkin (Cucurbita) (Jeffrey, 1975; Jeffrey, 1980). *Cucumis sativus* (Cucumber), *Cucumis melo* (Muskmelon) and *Citrullus lanatus* (watermelon) are important food and agricultural commodities around the world. Understanding the regulation of growth and development are crucial given the importance of the cucurbit species for foods and health goods. Cucumber (Mao *et al*., 2012), melon (Garcia-Mas *et al*., 2012) and watermelon (Liu *et al*., 2013) have all recently been identified using high-throughput sequencing. However, only a few miRNAs were predicted in these investigations and only a few experiments were confirmed and no attempts were made to realize the role of ncRNAs in development and growth in Cucurbitaceae family. MiRNAs from numerous other Cucurbitaceae species such as *Cucurbita pepo* and *Siraitia grosvenorii* have also been reported on a rare basis.

Several comparative genome-based homolog search tools were employed to predict novel miRNAs in muskmelon. For instance, miRbase was used to acquire different reference sequences for new miRNAs analysis. Consequently, Blastn at NCBI was employed to obtain similar genomics sequences after eliminating false sequences. The secondary stem-loop structure having minimum MFE values were made by Mfold tool which was noted and saved for further analysis. Their various protein targets were achieved using the psRNA target tool. As a result, almost 78 different protein targets were found. In addition, multiple sequence alignment and weblogo tools were used to generate conservation and ancestral studies. Hence, our findings will pave the way for more research into the physiological function and regulatory roles of miRNAs particularly in muskmelon growth and development.

MATERIALS AND METHODS

The "Bioinformatics Algorithms" for homology investigation was used to perform computational recognition and expressional assessments of novel miRNAs in muskmelon. The basic steps for this study are shown in the flow chart diagram (Fig. 1).

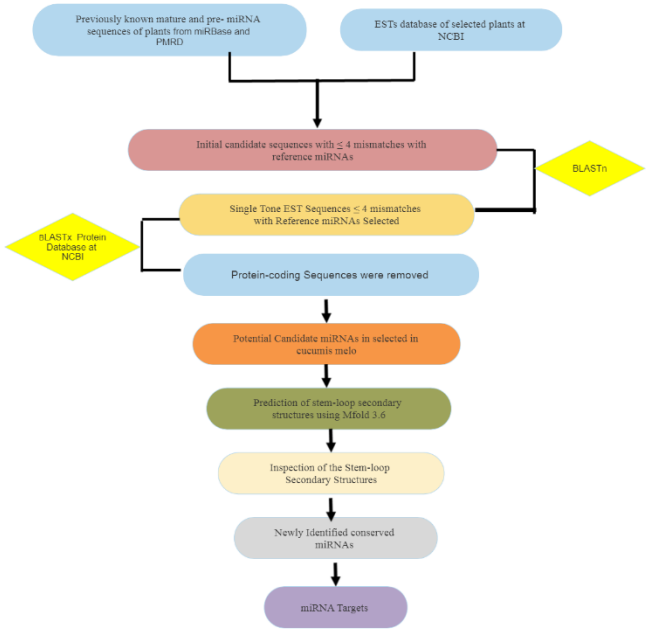

Figure 1. The whole steps in methodology are indicated in flow chart diagram.

Prediction of Candidate's pre-miRNAs and Reference miRNAs: Using the methods previously described by Barozai (2012), the miRNAs in the muskmelon were predicted. Plant pre-miRNA sequences from the miRbase were compared with sequences of muskmelon by using the Blastn programme (Version 22, released on March 2018) (Jones and Griffiths, 2004). The Blastn parameters were set as: expect threshold values set as 1000, low as the sequence filter, database programme selection to make in a relatively similar sequence and all other parameters to be left at their default values. With a maximum of four mismatches, all of the initial potential candidates' pre-miRNA sequences saved in FASTA format. It is crucial to confirm novel miRNAs as non-protein coding RNA. The pre-miRNA sequences of the initial potential candidates will be added to BALST against the NCBI protein database using Blastx with default value to eliminate protein-coding miRNAs (Larkin, 2007).

Creation of Hairpin Structures: The original candidate's sequences were used to create the hairpin secondary structure using the Zuker folding method and MFOLD (version 3.2) (Zuker, 2003). The structures expected to have the lowest free energy will undergo a physical inspection. The identified miRNAs will undergo a manual screening procedure to look for mature sequences that are 10 base pairs or fewer than the source miRNAs involved in Watson-Crick (G/U model) matching between the mature miRNA and its opposing strand (miRNA*) in the duplex. The thresholds for miRNA choice were identical as reported by Zhang *et al* (2006).

Targets Anticipation of miRNAs: Several methods were used to predict the miRNAs targets in muskmelon. The newly

predicted melon miRNAs were introduced into psRNA-Target tool, a plant-based miRNA target prediction tool, connected with GO enrichment analysis (Dai and Zhao, 2011). Using psRNA-Target, miRNAs from the melon that did not yield potential targets were exposed to the second method, which Barozai has described (2012). The second approach briefly checked the mature miRNA sequences from muskmelon using the NCBI BLASTn programme. This programme was used with some modifications in parameters.

***Minimizing False Positives*:** Keep the number of false positives to a low for the validation of bioinformatics-based novel miRNA identification. Many other methods were used to get rid of them (Barozai *et al*., 2012).

RESULTS

By utilizing publicly available database of ESTs (dbEST) at (http://blast.ncbi.nlm. nih.gov/Blast.cgi), it became easy to predict 22 conserved novel miRNAs and 78 target genes in muskmelon on the basis of homology search-based computational techniques (Griffiths-Jones, 2008). These results showed that the muskmelon genus contains numerous miRNAs and further proves that several miRNAs have undergone significant evolutionary conservation among plant species. The candidate sequence features and characterization of the recently predicted miRNAs are explained thoroughly (Table 1).

***Characterization of miRNAs in the Muskmelon*:** The 22 muskmelon miRNAs have been predicted which belong to 20 families. Each family of miRNAs has single member except in the case of miR157 and miR5161 which have two members. They have mature sequences on the 5' arm and on the 3' arm of the strand (Fig. 2). It has been established that mature miRNA sequences are found on either side of putative pre-secondary miRNA's stem-loop structure. Furthermore, the hairpin secondary structure of predicted muskmelon miRNAs contained 13 (59%) on the 5' arm and 09 (41%) on the 3' arm respectively. The average length of muskmelon miRNAs is 21 nt although they can be 18 minimum and 25 nt maximum (Fig. 2). The majority (45%) of miRNAs or 10 out of 22 are 21 nt in length followed by 27% of 20 nt, 5% for 18, 22, 23, 24 and 25 nt. Muskmelon pre-miRNA has a length that ranges from 42 to 167 nt with an average of 90 nt. Similarly, the earlier reports in other plant species were shown in mature miRNA arm positions, miRNA length division and their precursor sequences (Din *et al*., 2014; Zhang *et al*., 2006b, 2008, 2007b).

Minimum Folding Energy (MFE) is crucial for the formation of stem-loop hairpin structures in miRNAs. The secondary structure of a miRNA sequence is often more stable with low MFE values. The range of MFEs average values was -24.03 having lowest -49.13 and highest -10.7 kcal mol^{-1} (Table 1). For newly conserved muskmelon miRNAs, the acceptable range of mismatches between the reference miRNA and

Table 1. Annotation of recently profiled muskmelon miRNAs. The muskmelon predicted miRNAs are characterized in terms of source miRNAs, MS (mature sequences), PL (precursor miRNA length), NM (number of mismatches represented in bold and red) MFE (minimum free energy) ML (mature sequence length) SO (strand orientation), MSA (mature sequence arm) SE (source EST) OE (organ of expression) and GC guanine and cytosine percentage.

Cucumis melo miRNAs	Source miRNAs	PL	MFE	MS	NM	ML	SE	MSA	GC%	SO	OE
cme-miR157	aly-miR157	167	-49.13	UGCUCUCUAUGCUUCUGUCAUC	2	22	AM734232	3’	45.5	+	cotyledon
cme-miR157	aly-miR157	167	-49.13	UUGACAGAAGAUAGAGAGCAC	0	21	AM734232	5’	42.9	+	cotyledon
cme-miR847	ath-miR847	50	-13.00	CUCCUCUU**U**UUCUUGAUG	1	18	JG552076	3’	38.9	+	Callus
cme-miR2657	mtr-miR2657	79	-13.10	UGUUAUUUC**U**UCGAUUUUGUU**U**	2	22	AM714970	3’	22.7	+	Root
cme-miR5064	bdi-miR5064	56	-10.70	**C**GAAUUUGU**U**CAUAGCAUCAG	2	21	JG469125	5’	35.0	-	Callus
cme-miR5161	osa-miR5161	97	-32.83	UCUGGAUCAGAGGGAGU**AUA**	3	20	AM722643	3’	52.9	+	Root
cme-miR5161	osa-miR5161	97	-32.83	UCUGGAUCAGAGGGAGU**AUA**	3	20	AM722643	5’	52.9	+	Root
cme-miR5168	ata-miR5168	88	-37.30	UCGGACCAGGCUUCA**U**UCCC**U**	2	21	JG506718	3’	44.4	+	Root
cme-miR5204	mtr-miR5204	123	-37.0	**GC**UGGAAGGUUUUGU**U**G**A**AAC	4	21	JG465070	5’	42.9	+	Callus
cme-miR5215	mtr-miR5215	61	-15.10	AGGAGGAUGAGCUA**U**CU**U**CU	2	20	AM738041	3’	45.0	+	Fruit
cme-miR5298b	mtr-miR5298b	134	-32.50	UGAUGGAGAUGAU**G**AUGA**U**GAUGAA	2	25	AM718177	5’	36.0	-	Root
cme-miR6180	hvu-miR6180	59	-19.60	AGGGUGGA**G**G**GU**GGAGGGCG	4	20	AM742533	5’	75.0	-	Fruit
cme-miR6182	hvu-miR6182	59	-15.00	UGA**A**UGUGU**C**AUGGAUGG**G**UU	3	21	AM742289	5’	42.9	+	Fruit
cme-miR6214	hvu-miR6214	51	-11.80	GGACGACGACGAG**G**ACGAC**A**	2	20	AM720149	3’	68.4	-	Root
cme-miR7698	mtr-miR7698	89	-19.20	CAGA**A**AACUUUGAUGAAA**G**GC	2	21	DV634859	5’	38.1	+	Fruit
cme-miR7732	bdi-miR7732	88	-18.84	**UU**CCUCCAAGAUCU**U**GGAG**U**CUC	4	23	JG470927	5’	52.4	+	Callus
cme-miR7740	bdi-miR7740	119	-26.40	**UU**UGAACAACCUCG**U**UCUC**U**U	4	21	AM722620	5’	42.1	+	Root
cme-miR7743	bdi-miR7743	98	-23.90	UUUGA**G**CUUUUGUAUU**C**GAUCU**C**U	3	24	JG551705	3’	33.3	-	Fruit
cme-miR9674c	ata-miR9674c	56	-10.70	GAAUUU**GU**UCAUAGCAUCAG	2	20	JG469125	5’	35.0	-	Callus
cme-miR9776	ata-miR9776	59	-20.20	GCA**G**AUCCAUGUCCAAG**U**UC**U**	3	21	HO867557	3’	50.0	-	Stem
cme-miR11425	pab-miR11425	143	-29.20	UUUACUGCUA**GG**UAUUUU**U**CU	3	21	JG466057	5’	28.6	+	Callus
cme-miR11534	pab-miR11534	42	-11.20	UGAGAUUGUUGGAGAGG**C**UCA	1	21	JG464300	5’	47.6	-	Callus

newly identified miRNAs were also described (Zhang *et al*., 2006a). Mismatches were observed to range from 0 to 4 nt, with a mean of 2 nt. The miRNAs with the most mismatches with their homologs had a maximum of 2 = 10 (45%), followed by 3 = 6 (37%), 4 = 4 (18%) and 1 = 2 (13%) mismatches while accurately matched is 1 which is 4%. Newly identified novel conserved mature miRNA secondary hairpin structures are shown (Fig. 2).

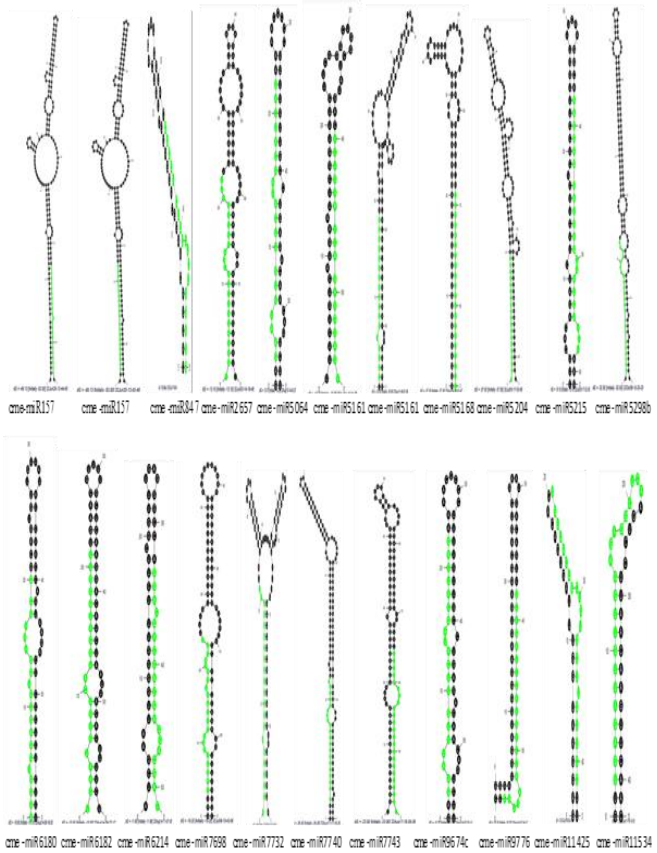

Figure 2. The secondary structures of 22 novel *C. melo* miRNAs. These 22 stem loop structures, i.e, cme-miR157a, 157b, 847, 2657, 5064, 5161a, 5161b, 5168, 5204, 5215, 5298b, 6180, 6182, 6214, 7698, 7740, 7742, 9674c, 9776, 11425 and 11534 are formed by the help of mfold algorithm. All of them have MFE less than -10 kcal/mol. In the stem loop structures, the mature sequences are shown in green in the stem section with little bulges.

According to the secondary structures of the muskmelon, the mature miRNA and the opposing arms (miRNAs*) in stem portion involved nearly 17 nucleotides in Watson-Crick or G/U base pairings, apart from some instances where the miRNAs* also have fewer base pairings and stem loop structures were not predicted with significant internal loops or bulges. The muskmelon miRNAs were therefore classified according to the organs in which they were expressed such as root expression being 32% (7 out of 22), callus expression being 32% (7 out of 22), fruit expression being 22% (5 out of 22), cotyledon 9% (2 out 22) and stem expression being 5% (1 out of 22). According to different researchers' chilli (Din *et al*., 2016), *Artemisia annua* (Barozai, 2013), eggplant (Din *et al*., 2014a), tomato (Din *et al*., 2014b) and potato all exhibit similar MFE ranges, mismatch counts, Watson-Crick or G/U pairings and expression in definite organs (Din *et al*., 2014). Number and length of precursor and mature miRNAs are shown in Fig. 3.

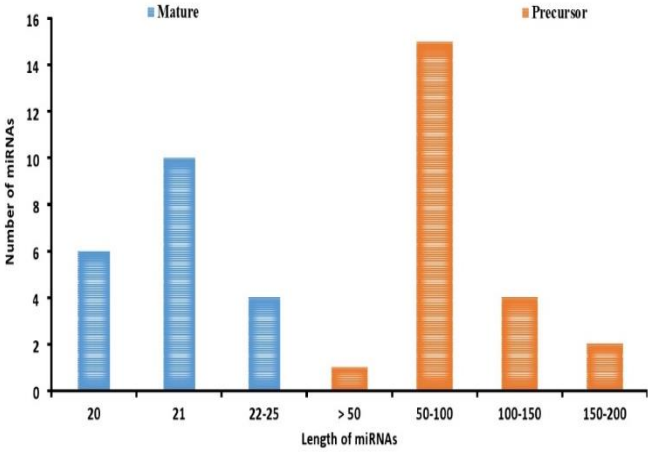

Figure 3. Mature and Precursor microRNAs length and number. The mature sequences shown in orange color ranges from 20-25 nt. While the precursors sequences are shown in blue that range from 50-200 nt. Majority of mature miRNAs consist of 21 nt i.e., 10 out of 22 (45%) followed by 27% of 20 nt, 5% for 18, 22, 23, 24 and 25 nt.

***Validation of genus Muskmelon miRNAs Through BLASTx*:** An essential first step is to confirm that muskmelon miRNAs are noncoding RNAs. The NCBI's tool BLASTx (Altschul *et al*., 1997) programme used to analyse the muskmelon miRNAs. The fact that muskmelon miRNAs are noncoding RNAs and identified to have any homology to known proteins.

***Conservation and Phylogenetic Studies of Genus Muskmelon miRNA*:** Due to its conservation in plants the miRNA from *C. melo* (miR157) was chosen and put through phylogenetic and conservation analysis. As seen in Fig. 4a, *C. melo* miR157 displayed conservation with *Brassica oleracea* (bol), *Arabidopsis thaliana* (ath), *Camelina sativa* (cas) and *Gossypium raimondii* (gra). The phylogenetic study of the identical miRNA (miR157) sequences indicated that *C. melo* is nearer to *B. oleracea* than *A. thaliana* and *C. sativa* as indicated in Fig. 4a. Similarly, the phylogenetic studies and the cladogram of 20 novel *C. melo* miRNAs are also generated and displayed in Fig. 4b.

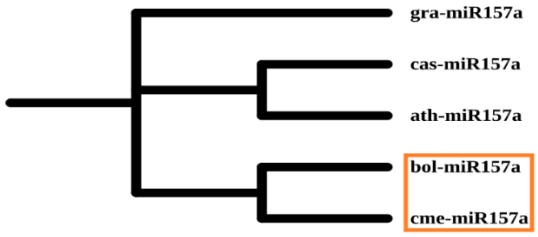

Figure 4a. Cladogram for *C. melo* miRNAs. It shows that *B. Oleracea* is more closely related to *C. melo* than to *A. thaliana, C. sativa* and *G. raimondii* which are displayed in the red box.

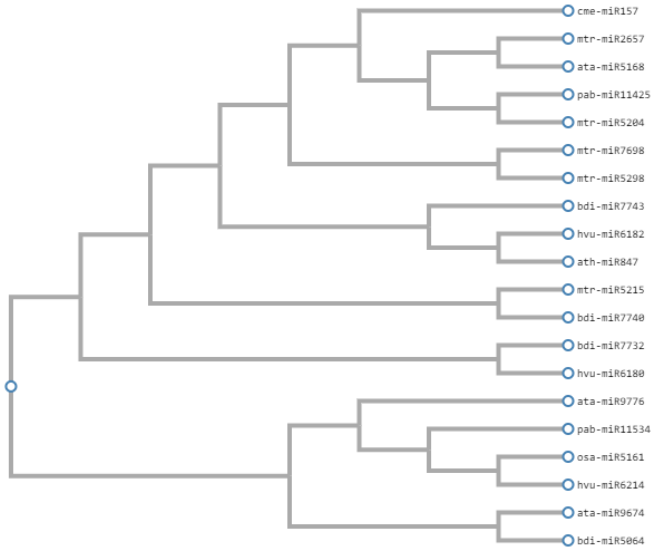

Figure 4b. Shows the cladogram of 20 novel *C. melo* miRNAs

Plant miRNAs are substantially preserved among related species according to the findings of the current study. When homologous sequences were not discovered, miRNAs displayed an independent evolutionary relationship. MicroRNA conservation studies of *C. melo* was created via Clustal omega and WebLogo. Alignment of *C. melo* miRNA (cme-miR847) with *A. thaliana* and *A. layrata*. A sequence logo (WebLogo) was created to demonstrate the conserved nature of mature miRNA sequences. The red box exhibited the mature sequences as shown in Fig. 5.

Figure 5. Conservation studies of *C. melo* sequences created via WebLogo. Three sequences i.e., *A. lyrata, A. thaliana* and *C. melo* are used in the conservation study. The mature conserved area is displayed in the red box portion.

***Potential Targets of the Genus Muskmelon*:** To determine the probable targets for the 22 predicted miRNAs in muskmelon, we used an appropriate standard (Zhang *et al*., 2006a) that was modified by Din *et al*., (2014). We identified 78 probable targets after carefully analysing the alignment data (Table 2). We found several miRNA targets in this research that are shared by numerous plant species having rice, Arabidopsis, tomato, poplar, cotton, eggplant, potato, corn and chilli. MiRNAs target numerous transcription factors that aid in the management of plant growth as shown by various studies using computational and/or experimental methods. This class of targets was also shown by melon genome. Targets are listed below for muskmelon genome in Fig. 6 and Table 2.

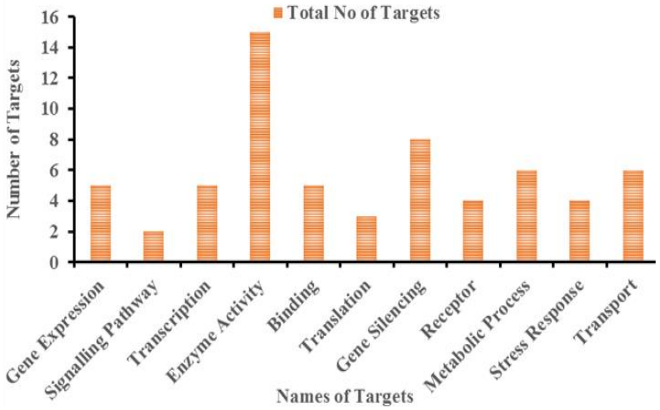

Figure 6. Targets of *C. melo* potential miRNAs obtained via psRNA Target Tool. The graph shows that majority of targets are presented by enzyme activity (24%) followed by gene silencing (12%), metabolic process (10%), transport (10%), binding (08%), gene expression 08%), transcription (08%), stress response (06%), receptor (06%), translation (05%) and signalling pathway (03%).

The targets of mature miRNAs were predicted in order to comprehend their regulatory functions. As previously established, miRNA can bind to mRNA at one or more complementary binding sites from where they either cleave or suppress the translation of target mRNA. On the basis of their mode of action, the microRNAs targets were classified into three categories. The miRNAs target which involved growth, reproduction and pigmentation are known as biological process (BP). In molecular function (MF), the miRNAs are involved in catalysis and binding activities. If the anticipated targets of miRNAs were involved in cellular structures such as transmembrane and extra-cellular functions are named as cellular components (CC). MF, BP and CC functions were predicted using psRNA-Target tool connected with GO enrichment analysis. Fig.7 shows the putative targets of muskmelon.

Table 2. Muskmelon targets enrichment analysis in psRNAs-Target tool and GO-terms. Where (BP) Biological Process (MF) Molecular Function (CC) Cellular Component and (GO) Gene Ontology.

Cucumis melo miRNA	Target Acc	GO Annotation	GO	Target Description	Gene No.	Alignment
cme-miR157	MELO3C012177	GO:0046961	MF	proton-transporting ATPase activity, rotational mechanism	2319	miRNA 22 CUACUGUCUUCGUAUCUCUCGU 1 :: :: ::::::::.:.: Target 31 CUAGAAAGUAGCAUAGAGGGUA 52
		GO:0019288	BP	isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway		
		GO:0098855	CC	HCN channel complex		
		GO:0008137	MF	NADH dehydrogenase (ubiquinone) activity		
		GO:2001289	BP	lipid X metabolic process		
		GO:0003849	MF	3-deoxy-7-phosphoheptulonate synthase activity		
		GO:0032933	BP	SREBP signalling pathway		
		GO:1903800	BP	positive regulation of miRNA maturation		
	MELO3C017479	GO:1990407	MF	calcitonin gene-connected with peptide binding	925	miRNA 22 CUACUGUCUUCGUAUCUCUCGU 1 : :::: ..: :::::::::: Target 432 CAGGACAAGGGAAUAGAGAGCA 453
		GO:0070058	BP	tRNA gene clustering		
		GO:0140053	BP	mitochondrial gene expression		
		GO:0035195	BP	miRNA-mediated gene silencing		
		GO:1990406	CC	CGRP receptor complex		
		GO:0060966	BP	Gene silencing regulation by RNA		
		GO:0090625	BP	siRNA-mediated gene silencing by mRNA destabilization		
		GO:0007131	BP	reciprocal meiotic recombination		
cme-miR847	MELO3C034490	GO:0050082	MF	maltose phosphorylase activity	690	miRNA 18 GUAGUUCUUUUUCUCCUC 1 ::::::::::::.::.: Target 426 CAUCAAGAAAAAGGGGGG 443
		GO:0034551	BP	mitochondrial respiratory chain complex III assembly		
		GO:1990724	CC	galectin complex		
cme-miR2657	MELO3C030609	GO:1990715	MF	mRNA CDS binding	540	miRNA 22 UUUGUUUUAGCUUCUUUAUUGU 1 ::...:::::.::::::::..::
		GO:0060962	BP	regulation of ribosomal protein gene transcription by RNA polymerase II		Target 352 AAGUGAAAUUGAAGAAAUGGCA 373
		GO:1900368	BP	post-transcriptional gene silencing and positive regulation by RNA		
	MELO3C034111	GO:0140869	MF	miRNA inhibitor activity	629	miRNA 22 UUUGUUUUAGCUUCUUUAUUGU 1 : :.:::::.:::::::: ::
		GO:0006311	BP	meiotic gene conversion		Target 23 ACAUAAAAUUGAAGAAAUAUCA 44
		GO:0006349	BP	regulation of gene expression by genomic imprinting		
		GO:1990406	CC	CGRP receptor complex		
cme-miR5064	MELO3C020277	GO:0008422	MF	beta-glucosidase activity	452	miRNA 21 GACUACGAUACUUGUUUAAGC 1 :::::: :::::...::::
		GO:1903800	BP	positive regulation of miRNA maturation		Target 217 GCGAUGCUUUGAACGGGUUCG 237
		GO:0120216	CC	matrilin complex		
		GO:0008334	BP	histone mRNA metabolic process		
		GO:0006355	BP	regulation of DNA-templated transcription		
cme-miR5161	MELO3C028927	GO:0040033	BP	sRNA-mediated gene silencing	1662	miRNA 20 AUAUGAGGGAGACUAGGUCU 1 :: .:::::::::::::
		GO:0098795	BP	global gene silencing by mRNA cleavage		Target 172 UCAACAUCCUCUGAUCCAGA 191
cme-miR5168	MELO3C016982	GO:0071270	BP	1-butanol metabolic process	294	miRNA 21 UCCCUUACUUCGGACCAGGCU 1 : ::::::::::::::.:
		GO:0070595	BP	(1->3)-alpha-glucan metabolic process		Target 224 UUGUAAUGAAGCCUGGUCUGU 244
		GO:0010690	BP	Gene transcription from RNA polyerase II and negative regulation of ribosomal protein against response to stress		
cme-miR5204	MELO3C013991	GO:0047705	MF	bilirubin oxidase activity	1446	miRNA 21 CAAAGUUGUUUUGGAAGGUCG 1 ::::::.:.:.::::::.:.
		GO:0046391	BP	5-phosphoribose 1-diphosphate metabolic process		Target 485 CAUUCAACGAGAUCUUCCGGU 505
		GO:1990724	CC	galectin complex		
cme-miR5215	MELO3C016542	GO:0017075	MF	syntaxin-1 binding	1796	miRNA 20 UCUUCUAUCGAGUAGGAGGA 1 ::.:::::.:.::::::.:..:
		GO:0016441	BP	post-transcriptional gene silencing		Target 960 AGGAGAUGGUUCAUCUUUUU 979
		GO:0035976	CC	transcription factor AP-1 complex		
cme-miR5298b	MELO3C002454	GO:0043798	MF	glycerate 2-kinase activity	969	miRNA 25 AAGUAGUAGUAGUAGUAGAGGUAGU 1 ::.:::::::::::::.:::::
		GO:0033223	BP	2-aminoethylphosphonate transport		Target 829 GAGAUUAUCAUCAUCAUCUUCAUCA 853
		GO:0070390	CC	transcription export complex 2		
cme-miR6180	MELO3C009094	GO:0008877	MF	glucose-1-phosphatase activity	1152	miRNA 20 GCGGGAGGUGGGAGGUGGGA 1 : ::::::::::::::::
		GO:0019080	BP	viral gene expression		Target 1020 CUCCCUCCACCCUCCACCCU 1039
		GO:0071012	CC	catalytic step 1 spliceosome		
cme-miR6182	MELO3C012347	GO:0002113	MF	interleukin-33 binding	631	miRNA 21 UUGGGUAGGUACUGUGUAAGU 1 :..:::::.:::...::::::
		GO:0140053	BP	mitochondrial gene expression		Target 84 UAUUCAUCUAUGGUGCAUUCA 104
		GO:1990406	CC	CGRP receptor complex		
cme-miR6214	MELO3C035112	GO:0038036	MF	sphingosine-1-phosphate receptor activity	507	miRNA 20 ACAGCAGGAGCAGCAGCAGG 1 :::::.::::::::::::
		GO:1902389	BP	ceramide 1-phosphate transport		Target 117 CUUCGUCUUCGUCGUCGUCC 136
		GO:0000110	CC	nucleotide-excision repair factor 1 complex		
cme-miR7698	MELO3C032266	GO:0004034	MF	aldose 1-epimerase activity	1116	miRNA 21 CGGAAAGUAGUUUCAAAAGAC 1 .: ::::::::::::::::
		GO:0070555	BP	response to interleukin-1		Target 1077 UUCCUUCAUCAAAGUUUUCUG 1097
		GO:0005853	CC	eukaryotic translation elongation factor 1 complex		

Cucumis melo miRNA	Target Acc	GO Annotation	GO	Target Description	Gene No.	Alignment
cme-miR7732	MELO3C024340	GO:0043115	MF	precorrin-2 dehydrogenase activity	1260	miRNA 23 CUCUGAGGUUCUAGAACCUCCUU 1
		GO:0040029	BP	epigenetic regulation of gene expression		.:. :.::.:::.::::::::
		GO:0043676	CC	Tectum		Target 342 CGGGGUUCAGGAUUUUGGAGGAA 364
cme-miR7740	MELO3C032486	GO:0004430	MF	1-phosphatidylinositol 4-kinase activity	439	miRNA 21 UUCUCUUGCUCCAACAAGUUU 1
		GO:1902820	BP	1-undecene metabolic process		::::::: ::::::::::::.
		GO:0030121	CC	AP-1 adaptor complex		Target 80 AAGAGAAAGAGGUUGUUCAAG 100
cme-miR7743	MELO3C029544	GO:0031764	MF	type 1 galanin receptor binding	852	miRNA 24 UCUCUAGCUUAUGUUUUCGAGUUU 1
		GO:1902389	BP	ceramide 1-phosphate transport		.:: :::::::...::::::::::
		GO:0005853	CC	eukaryotic translation elongation factor 1 complex		Target 784 GUGGAACGAAUAUGGAAGCUCAAA 807
cme-miR9674c	MELO3C011007	GO:0008889	MF	glycerophosphodiester phosphodiesterase activity	932	miRNA 20 GACUACGAUACUUGUUUAAG 1
						.:::: .:.:::::..:::::
		GO:1905617	BP	bsolete negative regulation of miRNA-mediated gene silencing by inhibition of translation		Target 720 UUGAUUUUGUGAAUGAAUUC 739
		GO:0052466	BP	obsolete induction by organism of symbiont resistance gene-dependent defense response		
cme-miR9776	MELO3C021498	GO:0004454	MF	ketohexokinase activity	396	miRNA 21 UCUUGAACCUGUACCUAGACG 1
		GO:0051214	BP	RNAi-mediated antiviral immunity against RNA virus		::.:::::::::: ::.:: Target 249 UGAGCUUGGACAUGCAUUUGG 269
		GO:0140621	CC	type I pilus		
cme-miR11425	MELO3C030707	GO:0004032	MF	alditol:NADP+ 1-oxidoreductase activity	423	miRNA 21 UCUUUUUAUGGAUCGUCAUUU 1
						::.:: :.::::::::.:.
		GO:1901239	MF	malonate(1-) transmembrane transporter activity		Target 15 CCAAGAAAAUCUAGCAGUGAG 35
		GO:0071271	BP	1-butanol biosynthetic process		
cme-miR11534	MELO3C028553	GO:0003954	MF	NADH dehydrogenase activity	1068	miRNA 21 ACUCGGAGAGGUUGUUAGAGU 1
						:: :: :::::::::::::::
		GO:1900753	BP	doxorubicin transport		Target 106 AGAACCGCUCCAACAAUCUCA 126
		GO:0070021	CC	transforming growth factor beta ligand-receptor complex		

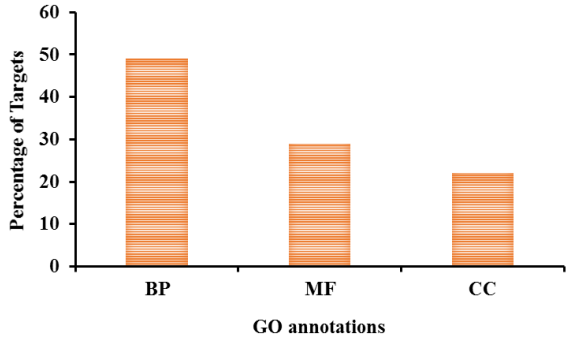

Figure 7. Percentage composition of muskmelon targets assigned on the basis of their mode of action such as Molecular Functions, Biological Process and Cellular Components. The highest percentage is indicated by BP (48%), followed MF (29%) and CC (21%).

DISCUSSION

In this study, the 22 novel potential miRNA homologs were identified and present in both dicots and monocots whereas only a few are unique to one or the other. This Research shows that 22 candidate potential miRNA identified in 20 miRNA families are shared by both monocot and dicot plants. Numerous sequence features of the predicted miRNAs were found to be consistent with other findings. The structural feature filtering method suggests using the negative minimal fold energy (MFE) and adjusted minimal fold energy (AMFE) as two criteria to distinguish miRNAs from other non-coding and coding RNAs. (Vishwakarma and Jadeja, 2013; Rebijith *et al*., 2014). The mature muskmelon miRNA sequences that were found in the stem region of the hairpin structures are shown in green. All the recently identified muskmelon miRNA precursors have negative MFE values ranging between -49.13 and -10.7. Additionally, it has already been observed that lower the MFE values, greater the thermodynamic stability of secondary hairpin structure (Altschul *et al*., 1990). Since, most of the sequences in the current analysis had low MFE values and therefore regarded thermodynamically stable.

Many of the projected targets were transcription factor-coding genes which are primarily involved in cell differentiation, plant growth and developmental patterning. This conclusion is in line with past studies. The numerous miRNA targets could fall under several categories such as those predicted to encode transcription factors, proteins and enzymes crucial for plant metabolism, cell signaling, transferring groups, defense response proteins and proteins related to stress response (Vishwakarma and Jadeja, 2013). As opposed to what was previously hypothesized in the case of *Hordeum vulgare* L. miR6180 predicted in the present study has been found to target various genes involved in stress response and defense. We can therefore draw the conclusion that a single miRNA may target many genes in various plant species.

In this study, miR157 has been found to have the same targets that have been linked to receptor, cell signaling, gene expression and transcriptional activation (Rebijith *et al*., 2014). Additionally, it is also involved in NADH dehydrogenase (ubiquinone) activity, siRNA-mediated gene silencing by mRNA destabilization and lipid X metabolic process (Lu *et al*., 2011; Achard *et al*., 2004). The present

study identified miR847 has been found to target the several genes such as maltose phosphorylase activity and mitochondrial respiratory chain complex III assembly that is different from what was described previously by Rajagopalan *et al*., (2006). In the case of *A. thaliana*, miR847 was involved in cyclophilin–RNA-interacting protein. Therefore, it follows that a single miRNA may target distinct genes in various plant species. Hence, miR5168 was induced in response to biotic and abiotic stress which is consistent with findings from prior research reviewed by Jia *et al*., (2013).

Furthermore, it is found in the current study that cme-miR2657 targets high mobility group protein. Additional target genes found by psRNA-Target utilizing the melon ESTs database revealed the involvement of the genes in a variety of functions, including regulation of ribosomal protein, gene transcription by RNA polymerase II, positive regulation of post-transcriptional gene silencing by RNA, developmental processes, regulation of gene expression by genomic imprinting, calcitonin gene-related peptide (CGRP) receptor complex and biosynthetic activities. One interesting discovery from the GO investigation was the involvement of multiple different miRNAs in targeting the same pathway. Additionally, miR2657 participates in positive control of post-transcriptional gene silencing through RNA. MiR5215 play a similar role in regulating the post-transcriptional gene silencing for plant hormones.

These results suggest that several targets interact to have a particular effect on the plant growth. This research makes it evident that one miRNA may target numerous pathways. MiR157 has been found to have targets in the production of carotenoids, amino acids, secondary metabolites and the tricarboxylic acid cycle. These compounds were found to be implicated in several pathways. Intriguingly, the recently identified miR157 is also involved in the synthesis of the terpene backbone which is one of the major phytonutrients in melon known as terpenoids. The results of this study support the notion that miRNAs play a crucial role in controlling a wide range of biological activities in plants. Similarly, galectin complex is a crucial carbohydrate-protein that miR5204 targets and is involved in many physiological functions, such as immune responses, inflammation, autophagy, cell migration, signaling and immunology, which helps us control cell signaling in plants via miRNAs (Mithoe *et al*., 2016).

The fact that several miRNAs target the same pathway was a noteworthy finding of the GO analysis. MiR5064, miR5168, miR5204 and miR7740 all control the metabolism process in plants. The secondary metabolite biosynthetic process shared by miR157 and miR11425 was same. Likewise, miR6214, miR7743, miR11425 and miR11534 frequently control the transport pathway for plant hormones. These findings imply that different targets interact to have a specific effect on plants.

Research on miRNAs has provided a rapid and accurate method for the prediction of novel and conserved microRNAs in both plants and mammals. Although a sizable number of microRNAs have been identified in model monocot and dicot plants. The primary phytochemicals found in muskmelons including beta-carotene, flavonoids, terpenoids, carbohydrates, volatile compounds and fatty acids have several medical effects like antioxidant, analgesic, antiulcer, antidiabetic, anticancer and hepato-protective characteristics. Muskmelon is a notable plant variety that is readily available in commercial scale. Given the importance of melon structural feature, the novel miRNAs were predicted using ESTs biased computational technique. According to the results of this study, researchers will have a better knowledge on regulatory system of the muskmelon that how it works. To better understand the molecular mechanism of microRNA-mediated post-transcriptional and gene silencing in melon, the next critical step will be the experimental confirmation of the predicted candidate's potential miRNAs.

***Conclusion*:** In this research, the 22 novel candidate potential miRNAs belonging to 20 families have been predicted via operating different bioinformatics tools. These new miRNAs are described for the first time to the best of our knowledge based on a review of the literature. Given the significance of melon, a computational method was used to predict novel miRNAs. The results revealed 22 possible miRNAs in muskmelon with 78 particular protein targets. The discovered targets have been demonstrated to be essential for plant development and growth, metabolism, ageing, disease resistance, salt stress, cold stress, oxidative stress and several other stress responses. As a result, this research will smooth the way to understand the genome of muskmelon and the role of miRNAs as well as to control the environment for increasing the production and the yield of melons.

***Conflict of Interest*:** The Authors declare that there is no conflict of interest.

***Authors' Contribution Statements*:** AG and NK write the manuscript, AG and AB executed research, AG and IA conceived the idea. NK, NB and WAS supervised the work.

REFERENCES

Achard, P., A. Herr, D.C. Baulcombe and N.P. Harberd. 2004. Modulation of floral development by a gibberellin-regulated microRNA. The Company of Biologists 131:3357-3365.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

Altschul, S.F., T.L Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman. 1997. Gapped

BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Barozai M.Y.K. 2013. Identification of microRNAs and their targets in *Artemisia annua* L. Pakistan Journal of Botany 45:461-465.

Barozai M.Y.K., I.A. Baloch and M. Din. 2012. Identification of MicroRNAs and their targets in Helianthus. Molecular Biology Reports 39:2523-2532.

Bartel D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281-97.

Bologna, N.G and O. Voinnet. 2014. The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis. Annual Review of Plant Biology 65:473-503.

Chi, X., Q. Yang, X. Chen, J. Wang, L. Pan, M. Chen and S. Yu. 2011. Identification and characterization of microRNAs from peanut (*Arachis hypogaea* L.) by high-throughput sequencing. PloS One 6:e27530.

Dai, X and P.X. Zhao. 2011. PsRNA Target: a plant small RNA target analysis server. Nucleic Acids Research 39:155-159.

Din, M and M.Y.K. Barozai. 2014. Profiling and characterization of eggplant (*Solanum melongena* L.) microRNAs and their targets. Molecular Biology Reports 41:889-894.

Din, M and M.Y.K. Barozai. 2014. Profiling microRNAs and their targets in an important fleshy fruit: Tomato (Solanum lycopersicum). Gene 53:198-203.

Din, M., M.Y.K. Barozai and I.A. Baloch. 2014. Identification and functional analysis of new conserved microRNAs and their targets in potato (*Solanum tuberosum* L.). Turkish Journal of Botany 38:1199-1213.

Din, M., M.Y.K Barozai and I.A. Baloch. 2016. Profiling and annotation of microRNAs and their putative target genes in chilli (*Capsicum annuum* L.) using ESTs. Gene Reports 5:62-69.

Garcia-Mas, J., A. Benjak, W. Sanseverino, M. Bourgeois, G. Mir, V.M. Gonzalez and P. Puigdomenech. 2012. The genome of melon (*Cucumis melo* L.). The Proceedings of the National Academy of Sciences 109:11872-11877.

Griffiths-Jones, S., H.K Saini, S. Van Dongen and A.J. Enright. 2007. miRBase: tools for microRNA genomics. Nucleic Acids Research 36:D154-D158.

Jagadeeswaran, G., Y. Zheng, Y.F. Li, L.I. Shukla, J. Matts, P. Hoyt and R. Sunkar. 2009. Cloning and characterization of small RNAs from Medicago truncatula reveals four novel legume-specific microRNA families. New Phytologist 184:85-98.

Jeffrey, C. 1975. Further notes on Cucurbitaceae: III: Some southern African taxa. Kew Bulletin 475-493.

Jeffrey, C. 1980. A review of the Cucurbitaceae. Biological Journal of the Linnean Society Soc8:233-247.

Jia, J., S. Zhao, X. Kong, Y. Li, G. Zhao, W. He and J. Wang. 2013. Aegilops tauschii draft genome sequence reveals a gene repertoire for wheat adaptation. Nature 496:91-95.

Joshi, T., Z. Yan, M. Libault, D.H. Jeong, S. Park, P.J Green and G. Stacey. 2010. Prediction of novel miRNAs and associated target genes in Glycine max. BMC Bioinformatics 11:1-9.

Khvorova, A., A. Reynolds and S.D. Jayasena. 2007. Functional siRNAs and miRNAs exhibit strand bias. Cell 131:41-49.

Kozomara, A., M. Birgaoanu and S. Griffiths-Jones. 2019. miRBase: from microRNA sequences to function. Nucleic Acids Research 47:D155-D162.

Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna P.A. McGettigan, H. McWilliam, and D.G. Higgins. 2007. Clustal W and Clustal X version 2.0. Journal of Bioinformatics 23:2947-2948.

Liu, N., J. Yang, S. Guo, Y. Xu and M. Zhang. 2013. Genome-wide identification and comparative analysis of conserved and novel microRNAs in grafted watermelon by high-throughput sequencing. PloS One 8:e57359.

Lu Y and Z. Zhang. 2011. Bioinformatic identification of microRNAs and their targets in Aquilegia formosa x Aquilegia pubescens. African journal of biotechnology10:11345-11359.

Lu, C., S.S. Tej, S. Luo, C.D. Haudenschild, B.C. Meyers and P.J Green. 2005. Elucidation of the small RNA component of the transcriptome. Sciences 309:1567-1569.

Mao, W., Z. Li, X. Xia, Y. Li and J. Yu. 2012. A combined approach of high-throughput sequencing and degradome analysis reveals tissue specific expression of microRNAs and their targets in cucumber. PloS one 7:e33040.

Matsui, A., A.H. Nguyen, K. Nakaminami and M. Seki. 2013. Arabidopsis Non-Coding RNA Regulation in Abiotic Stress Responses. International Journal of Molecular Sciences 14:22642-22654.

Meyers B.C., M.J Axtell, B. Bartel, D.P Bartel, D. Baulcombe, J.L Bowman, X. Cao, J.C. Carrington, X. Chen, P.J. Green et al. 2008. Criteria for annotation of plant microRNAs. Plant Cell 20:3186-3190.

Mithoe, S. C., C. Ludwig, M.J Pel, M. Cucinotta, A. Casartelli, M. Mbengue and F.L Menke. 2016. Attenuation of pattern recognition receptor signaling is mediated by a MAP kinase kinase kinase. European Molecular Biology reports 17:441-454.

Mlotshwa, S., G.J Pruss, J.L. MacArthur, M.W. Endres, C. Davis, L.J Hofseth and V. Vance. 2015. A novel chemopreventive strategy based on therapeutic microRNAs produced in plants. Cell Research 25:521-524.

Pelaez, P., M.S.Trejo, L.P. Iniguez, G. Estrada-Navarrete, A.A Covarrubias, J.L. Reyes and F. Sanchez. 2012. Identification and characterization of microRNAs in

Phaseolus vulgaris by high-throughput sequencing. BMC Genomics 13:1-18.

Rajagopalan, R., H. Vaucheret, J. Trejo and D.P Bartel. 2006. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Genes and Development 20:3407-3425.

Chakraborty, S., K.J Devi, B. Deb and R. Rajwanshi. 2016. Identification and characterization of novel microRNAs and their targets in Cucumis melo L.: an insilico approach. Focus Science 2:1-11.

Rebijith, K.B., R. Asokan, H.H. Ranjitha, V. Krishna and Nirmalbabu. 2013. In silico mining of novel microRNAs from coffee (Coffea arabica) using expressed sequence tags. ournal of Horticultural Science and Biotechnology 88:325-337.

Riolo, G., S. Cantara, C. Marzocchi, and C. Ricci. 2020. miRNA targets: from prediction tools to experimental validation. Methods and Protocols 4:1-20.

Subramanian, S., Y. Fu, R. Sunkar, W.B. Barbazuk, J.K. Zhu and O. Yu. 2008. Novel and nodulation-regulated microRNAs in soybean roots. BMC Genomics 9:1-14.

Vishwakarma N.P and V.J. Jadeja. 2013. Identification of miRNA encoded by Jatropha curcas from EST and GSS. Plant Signaling and Behavior 8:e23152.

Wyman, S. K., E.C. Knouf, R.K. Parkin, B.R. Fritz, D.W. Lin, L.M. Dennis and M. Tewari. M. 2011. Post-transcriptional generation of miRNA variants by multiple nucleotidyl transferases contributes to miRNA transcriptome complexity. Genome Research 21:1450-1461.

Xie, F. L., S.Q. Huang, K. Guo, A.L. Xiang, Y.Y. Zhu, L. Nie and Z.M Yang. 2007. Computational identification of novel microRNAs and targets in Brassica napus. Federation of European Biochemical Societies 581:1464-1474.

Xu, W., Q. Cui, F. Li and A. Liu. 2013. Transcriptome-wide identification and characterization of microRNAs from castor bean (*Ricinus communis* L.). PLoS One 8:e69995.

Yu, Y., L. Ji, B.H. Le, J. Zhai, J. Chen, E. Luscher and X. Chen. 2017. ARGONAUTE10 promotes the degradation of miR165/6 through the SDN1 and SDN2 exonucleases in Arabidopsis. PLoS Biology 15:e2001272.

Zhang, B. H., X.P. Pan, S.B. Cox, G.P. Cobb and T.A. Anderson. 2006. Evidence that miRNAs are different from other RNAs. Cellular and Molecular Life Sciences 63:246-254.

Zhang, B., X. Pan and E.J. Stellwag. 2008. Identification of soybean microRNAs and their targets. Planta 229:161-182.

Zhang, B., X. Pan, C.H. Cannon, G.P Cobb and T.A. Anderson. 2006. Conservation and divergence of plant microRNA genes. The Plant Journal 46:243-259.

Zhang, B., Q. Wang, K. Wang, X. Pan, F. Liu, T. Guo and T.A. Anderson. 2007. Identification of cotton microRNAs and their targets. Gene 397:26-37.

Zuker, M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic acids Research 31:3406-3415.