Sains Malaysiana 49(6)(2020): 1245-1272
http://dx.doi.org/10.17576/jsm-2020-4906-04

Identification and Analysis of microRNAs Responsive to Abscisic Acid and Methyl Jasmonate Treatments in *Persicaria minor*

(Pengenalpastian dan Analisis Gerak Balas mikroRNA kepada Rawatan Asid Absisik dan Metil Jasmonat dalam *Persicaria minor*)

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ABSTRACT

Persicaria minor *has been recognised as a plant with high content of volatile organic compounds (VOC) especially terpenoid and green leaf volatile (GLV). Previous finding had showed signaling molecules such as abscisic acid (ABA) and methyl jasmonate (MeJA) can increase the VOC content in plant. In this study, we performed next generation sequencing (NGS) of small RNA to uncover miRNAs roles and their response to both phytohormones (ABA and MeJA) in* P. minor. *For both ABA and MeJA treated* P. minor*, small RNA libraries containing 17,253,566 and 40,437,576 reads were generated, respectively. In addition, 18,634,904 reads were generated in plant treated with sterile distilled water which served as control. In these libraries, a total of 88 miRNAs were identified, comprising 41 known and 47 novel miRNAs. It was observed that 21 and 38 miRNAs were significantly regulated in ABA and MeJA libraries, respectively. Four selected miRNAs related to VOC pathways were subjected to RT-qPCR analysis and found to display diverse expression patterns with their targets. This study provides the initial framework for further exploration of miRNA roles in ABA and MeJA responses.*

Keywords: Abscisic acid; methyl jasmonate; microRNA; Persicaria minor*; volatile organic compound*

ABSTRAK

Persicaria minor *telah dikenal pasti sebagai tumbuhan yang mempunyai kandungan sebatian organik meruap (VOC) yang tinggi terutama terpenoid dan sebatian daun hijau meruap (GLV). Kajian lepas menunjukkan molekul pengisyaratan seperti asid absisik (ABA) dan metil jasmonat (MeJA) boleh meningkatkan kandungan VOC dalam tumbuhan. Dalam kajian ini, kami menjalankan penjujukan generasi terkini (NGS) RNA kecil untuk merungkai peranan miRNA dan tindak balasnya terhadap kedua-dua fitohormon (ABA dan MeJA) dalam* P. minor. *Bagi kedua-dua rawatan ABA dan MeJA terhadap* P. minor*, perpustakaan kecil RNA masing-masing telah menjana sejumlah 17,253,566 dan 40,437,576 bacaan. Tambahan lagi, sejumlah 18,634,904 bacaan telah dijana daripada tumbuhan terawat air suling steril yang bertindak sebagai kawalan. Dalam perpustakaan tersebut, sejumlah 88 miRNA telah dikenal pasti yang terdiri daripada 41 miRNA yang telah diketahui fungsinya dan 47 miRNA novel. Sejumlah 21 dan 38 miRNA masing-masing telah dicerap dikawal atur secara signifikan dalam perpustakaan ABA dan MeJA. Sebanyak empat miRNA yang berkait dengan tapak jalan VOC telah dikaji melalui analisis RT-qPCR dan didapati menunjukkan corak pengekspresan yang pelbagai terhadap transkrip sasaran masing-masing. Kajian ini menyediakan rangka kerja awal untuk penerokaan selanjutnya mengenai peranan miRNA dalam tindak balas ABA dan MeJA.*

Kata kunci: Asid absisik; metil jasmonat; mikroRNA; Persicaria minor*; sebatian organik meruap*

INTRODUCTION

Persicaria minor or known as 'kesum' is a medicinal plant with high content of secondary metabolites (Ee et al. 2014). These secondary metabolites are responsible for its pharmaceutical properties, such as its antioxidant, antiviral, antifungal, antiulcer and antimicrobial activities (Christapher et al. 2015). Additionally, due to its unique aroma, this plant is commonly used as food additives in local dishes in Southeast Asia countries (Christapher et al. 2015). Among these secondary metabolites, flavonoid and terpenoid were dominant (Baharum et al. 2010; Roslan et al. 2012). For example, *β-caryophyllene is the highest terpenoid compound in P. minor* essential oil (Baharum et al. 2010). In addition, other volatile compounds were also detected in *P. minor* for example decanal and dodecanal which belong to aliphatic aldehyde group (Christapher et al. 2015).

Phytohormones are signaling molecules which are essential in regulating plant growth and stress responses. In addition, their ability to act as a messenger in plant cell make them suitable candidates for mediating biosynthesis of particular product (Liang et al. 2013). ABA is a recognised elicitor that induces plant secondary

metabolite. Previously, ABA treatments on *Salvia miltiorrhiza* have led to the highlevel production of the active compound, tashinones (Yang et al. 2012). Similarly, jasmonic acid or its derivatives, methyl jasmonate (MeJA) participates in a variety of growth processes, stress response and secondary metabolite induction (Yan & Xie 2015). For instance, exogenous application of MeJA enhanced taxol formation in *Taxus cuspidata* suspension culture (Lenka et al. 2015). Based on previous study, both phytohormones, ABA and MeJA were able to alter gene expression which leads to the production of a particular compound at the downstream level.

Gene expressions are coordinated through multilayers level, beginning at epigenetic, transcriptional and post-transcriptional levels to ensure precise control. At post-transcriptional level, a group of small RNA, miRNA, is known to be involved in various biological processes in plant (Samad et al. 2017). miRNA acts as gene silencer by binding to the target gene to induce cleavage or translational inhibition (Samad et al. 2017). Latest miRBase version (version 22) showed a total of 38,589 miRNA that had been discovered in animals, plants and viruses, and the number is expected to be increasing in the future (Kozomara et al. 2019). This is an indicator that miRNA has already gained researchers attention due to its regulatory role and subsequently recognised as potential tool for manipulating gene expression to produce plant with desirable traits. Furthermore, the public database will facilitate the discovery of miRNA in other plant species especially for plant with no genome information available.

To date, several approaches had been carried out at transcriptional level to explore the elicitation effect of MeJA towards *P. minor*. Those approaches include construction of subtracted cDNA library and transcriptomic library. Among the induced genes were peroxidase and defense related genes (Gor et al. 2011; Rahnamaie-Tajadod et al. 2017). However, at present, not much information is known about the post-transcriptional regulation in *P. minor* represented by miRNA. Hence, this study focused on characterisation of miRNA and their response in *P. minor* under ABA and MeJA treatments.

Materials and Methods

PLANT MATERIALS AND TREATMENTS

P. minor plants were grown and propagated in controlled condition at Rumah Tumbuhan, Universiti Kebangsaan Malaysia. Approximately, 6 weeks old plants were selected for MeJA and ABA treatments. The treatments were carried out as mentioned in previous report (Nazaruddin et al. 2017). Two sets of *P. minor* plants were sprayed with 100 µM of MeJA and 100 µM of ABA, while the control plants were sprayed with distilled water. Two biological replicates were prepared for each treatment. For MeJA-treated plants, leaf samples were harvested after 2 days while ABA-treated plants were harvested after 3 days of treatment. These periods of treatments were selected based on the changes in leaf morphology of the *P. minor*. Prior to RNA extraction, *P. minor* leaves were harvested and immediately stored in -80 °*C* freezer for further use.

TOTAL RNA EXTRACTION AND SMALL RNA LIBRARY CONSTRUCTION

Approximately 0.1 g of leaves were ground to extract total RNA from mock-inoculated (K) leaves, and ABA and MeJA treated leaves using PureLink® Plant RNA reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA integrity number (RIN) from each sample was measured using Nanodrop 1000 (ThermoFisher Scientific Inc., USA), gel electrophoresis and Agilent 2100 Bioanalyzer (Agilent Technology, USA). Total RNA with RIN of at least 7 was selected for small RNA library construction. Then, the small RNA libraries were sequenced using Ilumina platform (HiSeq 2500) in *Rapid Run* mode.

DIFFERENTIAL GENE EXPRESSION

Prior to identification of differentially expressed miRNA, the data from each library was normalised to transcript per million (TPM). The analysis was carried out using Baggerley's test from CLC Genomics software (Baggerly et al. 2003). A threshold of a P-value < 0.05 and a fold-change ≥ 2 were used to determine significant changes of miRNA expression (Audic & Claverie 1997). Additionally, the false discovery rate (FDR < 0.05) correction method was deployed to correct the P-value which then referred to determine the significantly expressed miRNA (Benjamini & Hochberg 1995). Transcriptomic sequence was retrieved from GeneBank under accession number SRX669305 (Loke et al. 2016).

PREDICTION OF PUTATIVE NOVEL miRNA

Novel miRNA identification was carried out using homology search of unannotated small RNA sequences against *P. minor* transcriptomes. The potential transcript was investigated based on the ability of the sequence to form secondary structure and value of Minimum Folding Energy Index (MFEI) (Zhang et al. 2006). Sequence folding was carried out using mFold software (http://unafold.rna.albany.edu/) (Markham & Zuker 2008). The parameters for determination of MFEI were described in previous report (Samad et al. 2018).

miRNA TARGET PREDICTION AND GENE ONTOLOGY ENRICHMENT

PsRobot(http://omicslab.genetics.ac.cn/psRobot/) was employed to predict the target for miRNA (Wu et al. 2012). Since *P. minor* genome is still not available, previous transcriptomic library was used in this analysis. This analysis used overall score 4.0 to allow more detection of miRNA targets. In addition, gene ontology analysis was

carried out using WEGO software (http://wego.genomics.org.cn/) (Ye et al. 2018).

EXPRESSION ANALYSIS USING RT-QPCR

cDNA for each sample was synthesised using RevertAid Reverse Transcriptase (Thermofisher, USA) according to the manufacturer's protocols. RT-qPCR analysis for ABA and MeJA treated samples were carried out in series of timeline for three consecutive days. A set of mock treated plants with sterile distilled water were prepared as control (Day 0). The RT-qPCR was carried out using Thermo Scientific Maxima SYBR Green qPCR Master Mix (Thermofisher, USA). miRNA mature sequence was used as miRNA forward primer (Table 1) and universal primer from miScript SYBR® *Green PCR Kit (Qiagen, Germany) was used as reverse primer. PrimerQuest Tool Integrated DNA Technologies (*https://sg.idtdna.com/) was used to design forward and reverse primers for target genes (Table 2). For reference genes, 5.8s rRNA was used for miRNA and tubulin was used for target genes. Relative gene expression was analysed and calculated according to Livak and Schmittgen (2001).

TABLE 1. List of miRNA primers

miRNA	Primer sequence
pmi-miR396a	5'-GTT CAA TAA AGC TGT GGG A-3'
pmi-miR396b	5'-GGG GTT CAA TAA AGC TGT TGG AA-3'
pmi-miR6173	5'-GGG GGA GCC GTA AAC GAT GGA TA-3'
pmi-miR6300	5'-GGG GGT CGT TGT AGT ATA GTG GA-3'
pmi-miRNew-27	5'-CGT GTT ATC GTG TCG GAT A-3'

TABLE 2. List of target primers

Target genes	Primer sequence
Peroxidase	5'-GGA ACC CAA ACC ACA ACT TTC-3' (Forward) 5'-CTG TCG CCA ATC TTT CAT CAA TC-3' (Reverse)
ADH1	5'-TAC TTGTTC AGC AAA TCT CTC CA-3' (Forward) 5'-CTC TTC AGG TTG ATG TGT CCT T-3' (Reverse)
Sesquiterpene synthase	5'-AGA CGT AGT GAG CAA CCA AC-3' (Forward) 5'-CTT GGC ATA CCC TTG TGG TAA-3' (Reverse)
HMGR	5'-GCC AAC ATT GTG TCT GCT ATC-3' (Forward) 5'-ATG GTC ACG GAG ATG TGA AG-3' (Reverse)

RESULTS AND DISCUSSION

DEEP SEQUENCING ANALYSIS OF SMALL RNA

To investigate the miRNAs that had responded to ABA and MeJA treatments, three types of small RNA libraries (K, ABA, and MeJA) were constructed. The high-throughput sequencing generated around 18,634,904, 17,253,566, and 40,437,576 reads in three libraries, respectively. After removing adaptor sequences, low quality reads and filtering sequences into 18-30 nt, K, ABA and MeJA libraries produced 10,973,180, 11,571,770 and 21,458,916 sequences, respectively. The annotation and statistics of *P. minor* small RNAs was documented in Table 3.

TABLE 3. Statistics of small RNA in K, ABA and MeJA libraries

	Total reads	Percent (%)	Unique reads	Percent (%)
K library				
Raw reads	18,634,905± 10,481,749			
Clean reads (18-30nt)	10,973,181± 6,171,438	100.0	1,852,647± 931,365	100.0
miRNA	28,193±9,808	0.26	1,124±600	0.06
Rfam	694,910± 309,740	6.33	84,125± 13,931	4.54
Unannotated	10,250,078± 5,871,505	93.41	1,767,398± 918,034	95.40
ABA library				
Raw reads	17,253,566± 18,826,895			
Clean reads (18-30nt)	11,571,771± 12,886,302	100.0	1,580,735± 153,3613	100
miRNA	22,049± 26,830	0.19	538± 434	0.03
Rfam	579,375± 624,828	5.00	68,857± 45,657	4.36
Unannotated	10,970,347± 12,234,644	94.8	1,511,349± 1,487,522	95.61
MeJA library				
Raw reads	40,437,576 ±9,816,458			
Clean reads (18-30nt)	21,458,917± 3,343,499	100.0	2,163,212± 339,378	100.0
miRNA	143,282± 51,799	0.67	2,773±179	0.13
Rfam	1,089,945± 21,209	5.08	119,792± 12,669	5.54
Unannotated	20,225,690 ±3,374,089	94.25	2,040,647 ±326,888	94.33

The results showed that 28,193 (0.26%), 22,049 (0.19%), and 143,282 (0.67%) of miRNA were discovered in K, ABA and MeJA libraries, respectively. In addition, for K and ABA libraries, small RNAs with 22 nt in length were most abundant while small RNA with 20 nt in length was most abundant in MeJA library (Figure 1). Previous study showed that small RNAs with 21 nt in length was the most abundant miRNA in *A. thaliana* (Pontes et al. 2009). Around 694,910 (6.33%), 579,375 (5.00%) and 1,089,945 (5.08%) sequences were mapped against Rfam database in K, ABA and MeJA libraries, respectively. The rest of the unmapped sequences were used to find the potential novel miRNA in *P. minor*.

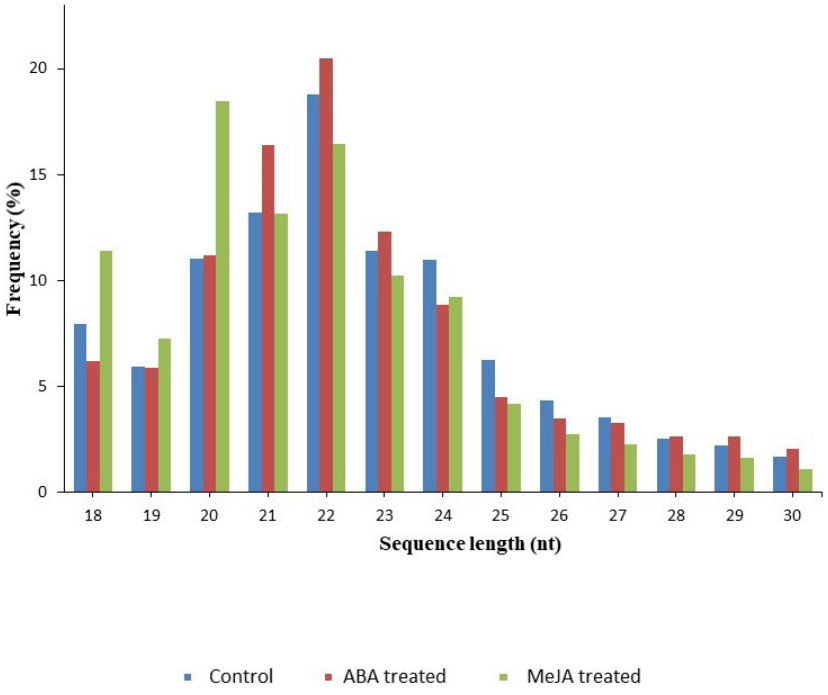


FIGURE 1. Length distribution of small RNA in each library. Distribution of small RNA sequence derived from K, ABA and MeJA treated libraries. Majority of the generated reads were 22 (> 20%), 20 (> 15%), and 21 (> 15%) nucleotides

Analysis of miRNA base compositions revealed that uracil was the dominant first base while cytosine was the most dominant at the 19th base (Figure 2). This finding was similar with previous study in soybean which indicated that these two bases may have crucial role in miRNA biogenesis and/or miRNA-mediated gene regulation (Zhang et al. 2008). In total, 173 conserved miRNAs which belong to 62 families were identified (Table 4). In order to unravel novel miRNA in *P. minor*, the unannotated sequences of K, ABA and MeJA libraries were searched against transcriptome for the potential miRNA precursors. After the folding prediction and MFEI calculation, 47 unique sequences of putative novel miRNA were discovered in *P. minor* (Table 5). Based on parameters established by Zhang et al. (2006), a secondary structure must have MFEI at least 0.85 to be recognised as precursor miRNA. Table 5 shows all the miRNA precursors that had been discovered in this study that possessed MFEI of at least 0.85. In addition, all the structures of miRNA precursors were documented in Table 6.

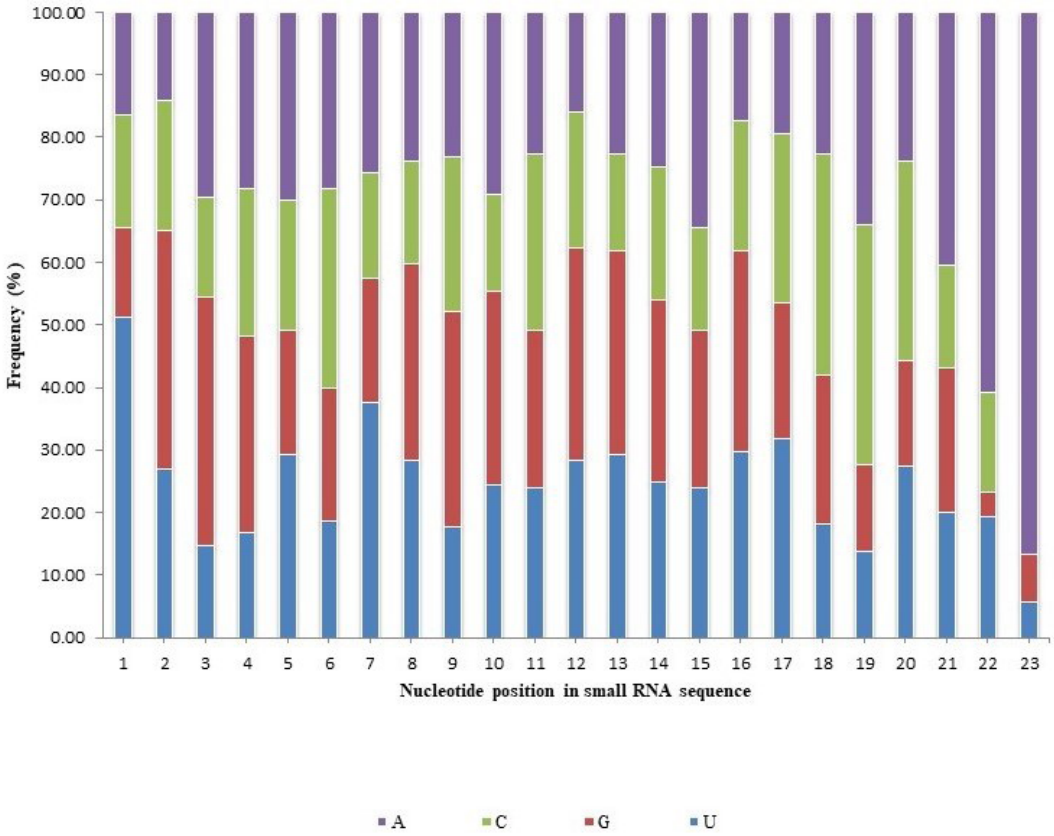


FIGURE 2. First nucleotide bias in small RNA libraries

TABLE 4. List of conserved miRNAs identified in *P. minor*

miRNA family	miRNA	miRNA mature sequce (5'-3')	Sequence length	Conserved miRNA	Plant species
156	pmi-miR156	TTGACAGAAGAGAGTGAGCACA	22	tae-miR156	*Triticum aestivum*
	pmi-miR156a	TGACAGAAGAGAGTGAGCACAA	22	bna-miR156a	*Brassica napus*
	pmi-miR156b	TGACAGAAGAGAGTGAGCATA	21	cca-miR156b	*Cynara cardunculus*
	pmi-miR156c	TTGACAGAAGATAGAGAGCGA	21	gma-miR156c	*Glycine max*
	pmi-miR156d-3p	GCTCTCTGTGCTTCTGTCATCA	22	stu-miR156d-3p	*Solanum tuberosum*
	pmi-miR156f	TTGACAGAAGAGAGAGAGCATA	22	gma-miR156f	*Glycine max*
	pmi-miR156i-3p	TGCTCACTTCTCTTTCTGTCA	21	mtr-miR156i-3p	*Medicago truncatula*
	pmi-miR156j	TTGACAGAAGAGGGTGAGCA	20	mtr-miR156j	*Medicago truncatula*
	pmi-miR156k	TTGACAGAAGAGAGTGAGCA	20	gma-miR156k	*Glycine max*
	pmi-miR156l-3p	GCTCACTTCTCTTTCTGTCAGCA	23	osa-miR156l-3p	*Oryza sativa*
	pmi-miR156p	CTGACAGAAGATAGAGAGCA	20	mdm-miR156p	*Malus domestica*
	pmi-miR156q	TGACAGAAGAGAGTGAGCACTA	22	gma-miR156q	*Glycine max*
	pmi-miR156r	CTGACAGAAGATAGAGAGCATA	22	gma-miR156r	*Glycine max*
157	pmi-miR157b	CTGACAGAAGATAGAGAGCACTA	23	smo- miR157b	*Selaginella moellendorffii*
	pmi-miR157c-5p	TTGACAGAAGATAGAGAGCACTA	23	aly- miR157bc-5p	*Arabidopsis lyrata*
	pmi-miR157d-3p	GCTCTCTGTGCTTCTGTCATA	21	aly- miR157bd-3p	*Arabidopsis lyrata*

159	pmi-miR159	TTTGGATCGAAGGGAGCTCTA	21	atr-miR159	*Amborella trichopoda*
	pmi-miR159a	TTTGGATTGAAGGGAGCTCTATTA	24	ath-miR159a	*Arabidopsis thaliana*
	pmi-miR159b-3p	TTTGGATTGAAGGGAGCTCTTCA	23	aly-miR159b-3p	*Arabidopsis lyrata*
	pmi-miR159c	CTTGGATTGAAGGGAGCTCTA	21	sof-miR159c	*Saccharum officinarum*
	pmi-miR159f	CTTGGATTGAAGGGAGCTCCTA	22	osa-miR159f	*Oryza sativa*
160	pmi-miR160	GCGTATGAGGAGCCAAGCATA	22	csi-miR160	*Citrus sinensis*
	pmi-miR156a-3p	TGCCTGGCTCCCTGTATGCCGA	21	gma-miR160a-3p	*Glycine max*
	pmi-miR156c	CCTGGCTCCCTGTATGCCATTA	22	mes-miR160c	*Manihot esculenta*
162	pmi-miR162	TCGATAAACCTCTGCATCCAA	21	aau-miR162	*Acacia auriculiformis*
	pmi-miR162-5p	TGGAGGCAGCGGTTCATCGATCA	23	csi-miR162-5p	*Citrus sinensis*
	pmi-miR162a	TCGATAAACCTCTGCATCCA	20	gma-miR162a	*Glycine max*
	pmi-miR162b	TCGATAAGCCTCTGCATCCAGA	22	osa-miR162b	*Oryza sativa*
	pmi-miR162b-5p	GGAGGCAGCGGTTCATCGATCA	22	aly-miR162b-5p	*Arabidopsis lyrata*
164	pmi-miR164a	TGGAGAAGCAGGGCACGTGA	20	hci-miR164a	*Helianthus ciliaris*
	pmi-miR164b-5p	TGGAGAAGCAGGGCACGTGCA	21	ata-miR164b-5p	*Aegilops tauschii*
	pmi-miR164e-5p	TGGAGAAGCAGGGCACGTGCAA	22	bra-mir164e-5p	*Brassica rapa*
	pmi-miR164g-3p	CACGTGCTCCCCTTCTCCACCA	22	zma-miR164g-3p	*Zea mays*
165	pmi-miR165a-3p	TCGGACCAGGCTTCATCCCCA	21	ath-miR165a-3p	*Arabidopsis thaliana*
	pmi-miR165b	TAGGACCAGGCTTCATCCCCA	21	ath-miR165b	*Arabidopsis thaliana*
	pmi-miR165c-5p	GGAATGTTGTCTGGTGCGAGGA	22	osa-miR165c-5p	*Oryza sativa*
166	pmi-miR166	TCGGACCAGGCTTCATTCCCCCA	23	ctr-miR166	*Citrus trifoliate*
	pmi-miR166a-5p	GGAATGTTGTCTGGCTCGAGGA	22	aly- miR166a-5p	*Arabidopsis lyrata*
	pmi-miR166b	TCGGACCAGGCTTCATTCCTA	21	mtr- miR166b	*Medicago truncatula*
	pmi-miR166b-3p	TCGGACCAGGCTTCATTCCCCA	22	ata- miR166b-3p	*Aegilops tauschii*
	pmi-miR166c	TCGGACCAGGCTTCATTCCTTA	22	aqc- miR166c	*Aquilegia caerulea*
	pmi-miR166d	TCGGACCAGGCTTCATTCCCTA	22	csi- miR166d	*Citrus sinensis*
	pmi-miR166e	TCGGACCAGGCTTCATTCCTCA	22	cme- miR166e	*Cucumis melo*
	pmi-miR166f-5p	TGAATGTTGCCTGGCTCGACA	21	aly- miR166f-5p	*Arabidopsis lyrata*
	pmi-miR166h-5p	GGAATGTTGGCTGGCTCGAGGTA	23	osa- miR166h-5p	*Oryza sativa*
	pmi-miR166i	TCGGACCAGGCTTCATTCTA	20	cme- miR166i	*Cucumis melo*
	pmi-miR166j-3p	TCGGACCAGGCTTCATTCCCGCA	23	gma- miR166j-3p	*Glycine max*
	pmi-miR166	CCGGACCAGGCTTCATTCCCA	21	ppt-miR166j/k/l	*Phsycomitrella patens*
	pmi-miR166l-3p	TCGGACCAGGCTTCATCCCTCAA	23	zma-miR166l-3p	*Zea mays*
	pmi-miR166m	CGGACCAGGCTTCATTCCCCA	21	gma- miR166m	*Glycine max*
	pmi-miR166m-5p	GGAATGTTGGCTGGCTCGAGTCA	23	zma- miR166m-5p	*Zea mays*
	pmi-miR166p	TCGGACCAGGCTCCATTCCA	20	ptc- miR166p	*Populus trichocarpa*
	pmi-miR166q	TCGGACCAGGCTTCATTCCTTCA	23	ptc- miR166q	*Populus trichocarpa*
167	pmi-miR167-5p	TGAAGCTGCCAGCATGATCTTTA	23	ahy-miR167-5p	*Arachis hypogaea*
	pmi-miR167a	TGAAGCTGCCAGCATGATCTCA	22	lus-miR167a	*Linum usitatissimum*
	pmi-miR167b	TGAAGCTGCCAGCATGATCTAAA	23	cme-miR167b	*Cucumis melo*
	pmi-miR167c	TGAAGCTGCCAGCATGATCTA	21	ata-miR167c	*Aegilops tauschii*

	pmi-miR167c-5p	TAAGCTGCCAGCATGATCTTA	21	aly-miR167c-5p	*Arabidopsis lyrata*
	pmi-miR167d	TAAGCTGCCAGCATGATCTGGA	22	ath-miR167d	*Arabidopsis thaliana*
	pmi-miR167f-5p	TAAGCTGCCAGCATGATCTGCTA	23	ata-miR167f-5p	*Aegilops tauschii*
	pmi-miR167h	TAAAGCTGCCAGCATGATCTTA	22	mdm-miR167h	*Malus domestica*
168	pmi-miR168	TCGCTTGGTGCAGGTCGGGAA	21	atr-miR168	*Amborella trichopoda*
	pmi-miR168a	TCGCTTGGTGCAGGTCGGGAACA	23	cca-miR168a	*Cynara cardunculus*
	pmi-miR168a-3p	CCCGCCTTGCATCAACTGAATCA	23	aly-miR168a-3p	*Arabidopsis lyrata*
	pmi-miR168b-3p	CCCGCCTTGCATCAACTGAATA	22	sly-miR168b-3p	*Solanum lycopersicum*
	pmi-miR168c-5p	TCGCTTGGTGCAGGTCGGGATA	22	bra-miR168c-5p	*brassica rapa*
169	pmi-miR169f	TAGCCAGGGATGACTTGCCGGA	22	mes-miR169f	*Monihot esculenta*
	pmi-miR169h	AGGCAGTCTCCTTGACTATTA	21	aly-miR169h-3p	*Arabidopsis lyrata*
	pmi-miR169i	TAGCCAAGGACGACTTGCCTGA	22	aly-miR169i	*Arabidopsis lyrata*
171	pmi-miR171	TGATTGAGCCGCGCCAATATCA	22	ccl-miR171	*Citrus clementina*
	pmi-miR171a	TGAGCCGCGCCAATATCA	18	csi-miR171a	*Citrus sinensis*
	pmi-miR171c-3p	TTGAGCCGTGCCAATATCA	19	ata-miR171c-3p	*Aegilops tauschii*
	pmi-miR171c-5p	GGATATTGGTGCGGTTCAATCA	22	osa-miR171c-5p	*Oryza sativa*
	pmi-miR171d	TTGAGCCGTGCCAATATCACGA	22	bna-miR171d	*Brassica napus*
172	pmi-miR172a	AGAATCTTGATGATGCTGCAGTA	23	lja-miR172a	*Lotus japonicas*
	pmi-miR172a-3p	AGAATCTTGATGATGCTGCAA	21	csi-miR172a-3p	*Citrus sinensis*
	pmi-miR172b	AGAATCTTGATGATGCTACACA	22	vvi-miR172b	*Vitis vinifera*
	pmi-miR172c	GGAGCATCATCAAGATTCACA	21	aly-miR172c	*Arabidopsis lyrata*
	pmi-miR172d	AGAATCTTGATGATGCTGCAGCA	23	gma-miR172d	*Glycine max*
	pmi-miR172d-5p	GGAGCATCATCAAGATTCACATA	23	stu-miR172d-5p	*Solanum tuberosum*
	pmi-miR172f	AGAATCTTGATGATGCTGCATCA	23	nta-miR172f	*Nicotiana tabacum*
	pmi-miR172h	GCAGCAGCATCAAGATTCACA	21	gma-miR172h-5p	*Glycine max*
	pmi-miR172i	AGAATCTTGATGATGCTGCATTA	23	nta-miR172i	*Nicotiana tabacum*
	pmi-miR172m	AGAATCTTGATGATGCTGCAGCA	23	mdm-miR172m	*Malus domestica*
319	pmi-miR319	TTGGACTGAAGGGAGCTCCCTA	22	aqc-miR319	*Aquilegia caerulea*
	pmi-miR319a	CTTGGACTGAAGGGAGCTCCA	21	ppt-miR319a	*Phsycomitrella patens*
	pmi-miR319b	TTGGACTGAAGGGAGCTCCCTTA	23	mdm-miR319b	*Malus domestica*
	pmi-miR319c	CTTGGACTGAAGGGAGCTCCCA	22	ppt-miR319c	*Phsycomitrella patens*
	pmi-miR319c-3p	TTGGACTGAAGGGAGCTCCCA	21	mtr-miR319c-3p	*Medicago truncatula*
	pmi-miR319e	CTTGGACTGAAGGGAGCTCCCAA	23	ppt-miR319e	*Phsycomitrella patens*
	pmi-miR319i	TTGGGCTGAAGGGAGCTCCCA	21	ptc-miR319i	*Populus trichocarpa*
390	pmi-miR390a-5p	AAGCTCAGGAGGGATAGCGCCA	22	aly-miR390a-5p/b	*Arabidopsis lyrata*
	pmi-miR390b	AAGCTCAGGAGGGATAGCGCCCA	23	ppt-miR390b	*Phsycomitrella patens*
	pmi-miR390d	AAGCTCAGGAGGGATAGCACCA	22	gma-miR390d	*Glycine max*
391	pmi-miR391-5p	CTTCGCAGGAGCGATGGCGCCA	22	ath-miR391-5p	*Arabidopsis thaliana*
393	pmi-miR393	TCCAAAGGGATCGCATTGATCTA	23	ghr-miR393	*Gossypium hirsutum*
	pmi-miR393a-5p	TCCAAAGGGATCGCATTGATCCA	22	ath-miR393a-5p	*Arabidopsis thaliana*
	pmi-miR393c-3p	ATCATGCTATCCCTTTGGATTA	22	gma-miR393c-3p	*Glycine max*

394	pmi-miR394	TTGGCATTCTGTCCATCTCCA	21	cca-miR394	*Cynara cardunculus*
	pmi-miR394a	TTGGCATTCTGTCCATCTCCTTA	23	vvi-miR319a	*Vitis vinifera*
	pmi-miR394b-5p	TTGGCATTCTGTCCACCTCCTA	22	ptc-miR394b-5p	*Populus trichocarpa*
395	pmi-miR395	CTGAAGCGTTTGGGGGAACGA	21	ppt-395	*Physcomitrella patens*
	pmi-miR395a	TGAAGTGTTTGGGGGAACTCCA	22	sly-miR395a	*Solanum lycopersicum*
	pmi-miR395b	TGAAGTGTTTAGGGGAACTCGA	22	tea-miR395b	*Triticum aestivum*
	pmi-miR395d	TGAAGTGTTTGGGGGAACTCTA	22	rco-miR395d	*Ricinus communis*
396	pmi-miR396	TTCCACAGCTTTCTTGAACTGCA	23	aau-miR396	*Acacia auriculiformis*
	pmi-miR396a	GTTCAATAAAGCTGTGGGA	19	vvi-miR396a	*Vitis vinifera*
	pmi-miR396b-3p	GTTCAATAAAGCTGTTGGAA	20	zma-miR396b-3p	*Zea mays*
	pmi-miR396b-5p	TTCCACAGCTTTCTTGAACTTTA	23	ath-miR396b-5p	*Arabidopsis thaliana*
	pmi-miR396c	TTCAAGAAAGCTGTGGGAAAA	21	cca-miR396c	*Cynara cardunculus*
	pmi-miR396e-3p	TTCAATAAAGCTGTGGGAAA	19	ata-miR396e-3p	*Aegilops tauschii*
397	pmi-miR397	TCATTGAGTGCAGCGTTGACGA	22	pab-miR397	*Picea abies*
	pmi-miR397a	TCATTGAGTGCAGCGTTGATGTA	23	bna-miR397a	*Brassica napus*
	pmi-miR397b	TTATTGAGTGCAGCGTTGATGA	22	osa-miR397b	*Oryza sativa*
398	pmi-miR398	TGTGTTCCCAGGTCGCCCCTGA	22	atr-miR398	*Amborella trichopoda*
	pmi-miR398	GGAGCGACCTGAGATCACATGA	22	hbr-miR398	*Hevea brasiliensis*
	pmi-miR398a	TGTGTTCTCAGGTCGCCCCTGCA	23	cme-miR398a	*Cucumis melo*
	pmi-miR398b	TGTGTTCTCAGGTCGCCCCTG	21	osa-miR398b	*Oryza sativa*
	pmi-miR398c-5p	GGAGCGACCTGAAACCACATGA	22	ptc-miR398c-5p	*Populus trichocarpa*
	pmi-miR398f	GGTGTTCTCAGGTCGCCCCTAA	22	lus-miR398f	*Linum usitatissimum*
399	pmi-miR399	TGCCAAAGGAGAGTTGCCCTA	21	aqc-miR399	*Arabidopsis thaliana*
	pmi-miR399b	TGCCAAAGGAGAGTTGCCCTGA	22	ath-miR399b	*Arabidopsis thaliana*
	pmi-miR399f	TGCCAAAGGAGATTTGCCCGGA	22	ath-miR399f	*Arabidopsis thaliana*
	pmi-miR399h-5p	GGGCAAGATCTCTATTGGCAGGA	23	aly-miR399h-5p	*Arabidopsis lyrata*
	pmi-miR399j	TGCCAAAGGAGAGTTGCCCTAA	22	osa-miR399j	*Oryza sativa*
408	pmi-miR408	TGCACTGCCTCTTCCCTGGCTA	22	cca-miR408	*Cynara cardunculus*
	pmi-miR408-3p	ATGCACTGCCTCTTCCCTGGCA	22	ath-miR408-3p	*Arabidopsis thaliana*
	pmi-miR530b	TGCATTTGCACCTACACCTTA	21	cme-miR530b	*Cucumis melo*
535	pmi-miR535	TGACAATGAGAGAGAGCACA	20	csi-miR535	*Citrus sinensis*
	pmi-miR535a	TGACAATGAGAGAGAGCACGT	21	mes-miR535a	*Monihot esculenta*
	pmi-miR535b	TGACAATGAGAGAGAGCACGGA	22	mes-miR535b	*Monihot esculenta*
	pmi-miR535d	TGACGATGAGAGAGAGCACGA	21	mdm-miR535d	*Molus domesticus*
828	pmi-miR828a	TCTTGCTCAAATGAGTATTCCA	22	vvi-miR828a	*Vitis vinifera*
833	pmi-miR833a-5p	GTTTGTTGTGCTCGGTCTA	19	ath-miR833a-5p	*Arabidopsis thaliana*
845	pmi-miR845a	CGGCTCTGATACCAATTGTTA	21	ath-miR845a	*Arabidopsis thaliana*
	pmi-miR845c	AGGCTCTGATACCAATTGAAGCA	23	vvi-miR845c	*Vitis vinifera*
	pmi-miR845d/e	TGGCTCTGATACCAATTGACGCA	23	vvi-miR845d/e	*Vitis vinifera*
858	ath-miR858b	TTCGTTGTCTGTTCGACCTTGA	22	ath-miR858b	*Arabidopsis thaliana*
894	ppt-miR894	CGTTTCACGTCGGGTTCACCAA	22	ppt-miR894	*Phsycomitrella patens*
1127	pmi-miR1127b-3p	ACATGTATTTTTGGACGGAGGGA	23	tae-miR1127b-3p	*Triticum aestivum*
1128	pmi-miR1128	CTACTACCTCCGTCTCAAAAA	21	ssp-miR1128	*Saccharum* sp.
1436	pmi-miR1436	ATTATGGAACGGAGGGAGTA	20	hvu-miR1436	*Hordeum vulgare*
1439	pmi-miR1439	TTTTGGAACGGAGAGAGTA	19	osa-miR1439	*Oryza sativa*

1511	pmi-miR1511-3p	ACCTGGCTCTGATACCATA	19	ppe-miR1511-3p	*Prunus persica*
1863	pmi-miR1863	AAGCTCTGATACCATGTTAGATTTA	25	cme-miR1863	*Cucumis melo*
	pmi-miR1863a	AAGCTCTGATACCATGTTAGATTA	24	osa-miR1863a	*Oryza sativa*
1874	pmi-miR1874-5p	AGGGCTACTATAACATCCATA	21	osa-miR1874-5p	*Oryza sativa*
2673	pmi-miR2673a/b	CTCTTTCTCTTCCTCTTCCAA	21	mtr-miR2673a/b	*Medicago truncatula*
2916	pmi-miR2916	TGGGGGCTCGAAGACGATCAGATA	24	peu-miR2916	*Populus euphratica*
3627	pmi-miR3627a	CTTCGCAGGAGCGATGGCACTA	22	mdm-miR3627a	*Malus domestica*
3630	pmi-miR3630-3p	TGGGAATCTCTCTGATGCACA	21	vvi-miR3630-3p	*Vitis vinifera*
4995	pmi-miR4995	TAGGCAGTGGCTTGGTTAAGGGAA	24	gma-miR4995	*Glycine max*
5049	pmi-miR5049c	AGACAATTATTGTGGGACGGAGGAA	25	hvu-miR5049c	*Hordeum vulgare*
5054	pmi-miR5054	TCCCCACGGACGGCGCCAA	19	bdi-miR5054	*Brachypodium distachyon*
5056	pmi-miR5056	GAGGAAGAACCGGTAATAGACA	22	bdi-miR5056	*Brachypodium distachyon*
5077	pmi-miR5077	TTCGCGTCGGGTTCACCAA	19	osa-miR5077	*Oryza sativa*
5083	pmi-miR5083	CAGACTACAATTATCTGATCAA	22	osa-miR5083	*Oryza sativa*
5139	pmi-miR5139	AAAACTTGGCTCTGATACCA	20	rgl-miR5139	*Rehmannia glutinosa*
5174	pmi-miR5174d-3p	CAATCTTTTTGGATCGGAGAGAGTA	25	bdi-miR5174d-3p	*Brachypodium distachyon*
	pmi-miR5174e-5p	ACTCCCTCTGTTCCATAA	18	bdi-miR5174e-5p.2	*Brachypodium distachyon*
5181	pmi-miR5181-3p	ACACTTATTTTGGAACAGAGGGA	23	ata-miR5181-3p	*Aegilops tauschii*
5368	pmi-miR5368	GGACAGTCTCAGGTAGACA	19	gma-miR5368	*Glycine max*
5532	pmi-miR5532	ATGGAATATATGACAAGGGTGTA	23	osa-miR5532	*Oryza sativa*
5538	pmi-miR5538	CTACTGAACTCAATCACTTGCTA	23	osa-miR5538	*Oryza sativa*
5658	pmi-miR5658	TGATGATGAAGATGATGAA	19	ath-miR5658	*Arabidopsis thaliana*
6173	pmi-miR6173	GAGCCGTAAACGATGGATA	22	hbr-miR6173	*Hevea brasiliensis*
6300	pmi-miR6300	GTCGTTGTAGTATAGTGGA	18	gma-miR6300	*Glycine max*
6478	pmi-miR6478	CCGACCTTAGCTCAGTTGGTGA	22	ptc-miR6478	*Populus trichocarpa*
6485	pmi-miR6485	AGGATGTAGAAGATCATAACA	21	hbr-miR6485	*Hevea brasiliensis*
7729	pmii-miR7729a/b-3p	CAATGGTGGTGGTTGGGAGGA	21	bdi-miR7729a/b-3p	*Brachypodium distachyon*
7767	pmi-miR7767-5p	CCCCAAGATGAGTGCTCTCCCA	22	bdi-miR7767-5p	*Brachypodium distachyon*
8175	pmi-miR8175	CGATCCCCGGCAACGGCGCCAA	22	ath-miR8175	*Arabidopsis thaliana*
9670	pmi- miR9670-3p	AGGTGGAAAACCTGAAGAAGA	21	tae-miR9670-3p	*Triticum aestivum*

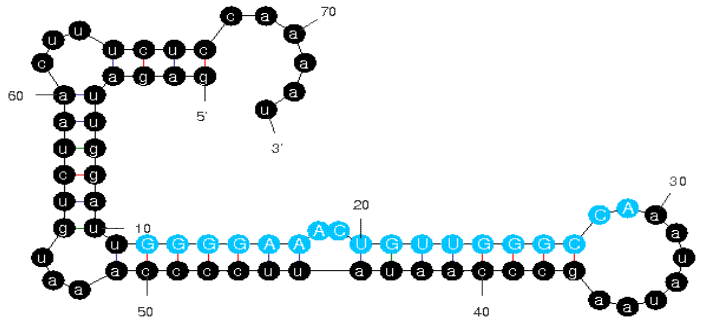
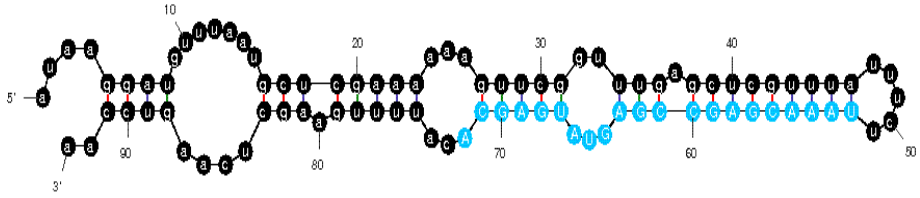
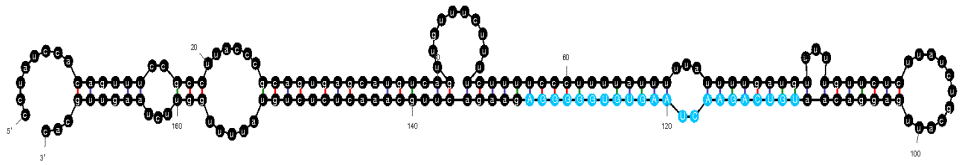
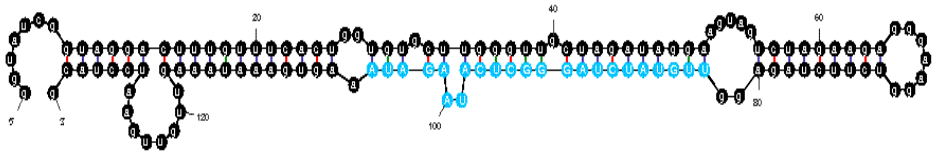
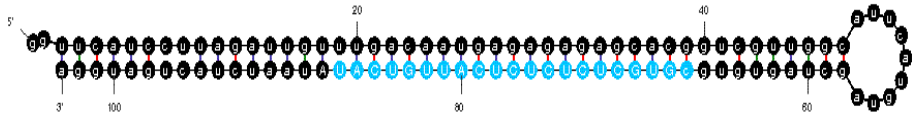
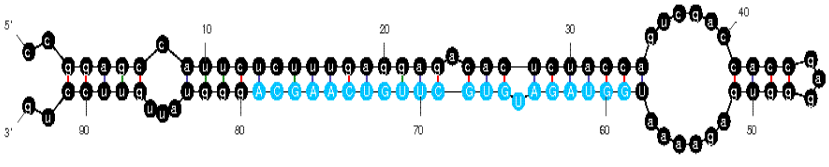
TABLE 5. List of putative novel miRNAs that had been discovered in *P. minor*

Novel miRNA	Mature sequences	LM	LP	Side Arm	ΔG	A+U (%)	G+C (%)	AMFE	MFEI
pmi-miRNew-01	GGGGAAACTGTTGGGCCA	18	73	5’	-28.6	60.27	39.73	39.18	1.01
pmi-miRNew-02	TAAACGAGCCGAGTATGAGCA	21	93	3’	-29.5	62.37	37.63	31.72	1.19
pmi-miRNew-03	TGTCAGAACTAAGTGTGGGGGA	22	172	3’	-43.4	60.47	39.53	25.23	1.57
pmi-miRNew-04	TTGTATCTAGGGCTCATAAGATA	23	133	3’	-46.5	57.89	42.11	34.96	1.20
pmi-miRNew-05	GTGCTCTCTCTCATTGTCATA	20	103	3’	-57.2	56.31	43.69	55.53	0.99
pmi-miRNew-06	TGGTAGATGTGCTTGTCAAGCA	22	93	5’	-35.7	48.39	51.61	38.39	1.34
pmi-miRNew-07	CGTCTCGTCGCCCTTAGATCGA	22	103	5’	-64.3	40.78	59.22	62.43	0.95
pmi-miRNew-08	GGAGCGACCTTAGACCACATGA	22	143	5’	-59.0	47.55	52.45	41.26	1.27
pmi-miRNew-09	CCTTTGTCGCATTTGGGGAAA	21	143	3’	-76.1	58.04	41.96	53.22	0.99
pmi-miRNew-10	CATTTCTGGTGGTAGCTCATA	21	73	5’	-19.9	63.01	36.99	27.26	1.36

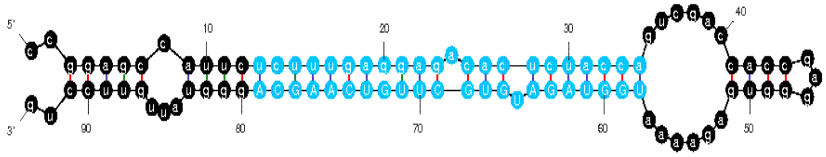
pmi-miRNew-11	CGGGGAAGAGGCTGAGCAAGGA	22	103	5’	-57.5	50.49	49.51	55.83	0.89
pmi-miRNew-12	TGAATTGTGTGTGAATGA	18	83	3’	-18.6	73.49	26.51	12.77	2.08
pmi-miRNew-13	TGTATTTTTGGACGGAGGTAGTA	23	82	3’	-18.8	67.07	32.93	16.83	1.96
pmi-miRNew-14	GTGCTCTCTCTCATTGTCAA	20	123	3’	-48.7	56.91	43.09	39.59	1.09
pmi-miRNew-15	GTGGTGGTATTGTGGACAGCA	21	123	5’	-35.3	50.41	49.59	28.70	1.73
pmi-miRNew-16	GGAATTATGGCTGTATCGCATA	21	123	5’	-22.2	67.48	32.52	18.05	1.80
pmi-miRNew-17	TTCTGATTTGTGATGTAATCCA	22	93	3’	-59.7	59.14	40.86	64.19	0.85
pmi-miRNew-18	GCTGAGATTGTAAAGGCTTTTTA	23	103	3’	-29.4	67.96	32.04	28.54	1.12
pmi-miRNew-19	CTGTTGGGCTTGCTCTTA	18	82	5’	-27.0	50.00	50.00	32.93	1.52
pmi-miRNew-20	TCCCACTCTCAACACCAA	18	83	5’	-28.6	44.58	55.42	34.46	1.61
pmi-miRNew-21	AACGGTGAAACGAATGAATATTG	23	114	5’	-34.7	72.81	27.19	30.44	0.89
pmi-miRNew-22	AAGAAGATCAACGGATGAGATTA	23	83	5’	-20.4	61.45	38.55	24.58	1.57
pmi-miRNew-23	TGATTGAAATGGTTCTCGACGA	22	63	3’	-23.2	58.73	41.27	36.83	1.12
pmi-miRNew-24	ATGGACAGCACTGTATTGGCA	21	83	3’	-21.9	54.22	45.78	26.39	1.74
pmi-miRNew-25	TTGCAGAGATTGCCGGTAACA	21	63	5’	-18.3	55.56	44.44	24.29	1.83
pmi-miRNew-26	CGAGGCAAGAACTTTGGAGCA	21	103	3’	-43.1	53.40	46.60	41.84	1.11
pmi-miRNew-27	CGTGTTATCGTGTCGGATA	19	63	3’	-33.6	50.79	49.21	53.33	0.92
pmi-miRNew-28	GACAGGACCTTTGAAGTAGCA	21	93	3’	-24.1	49.46	50.54	25.91	1.95
pmi-miRNew-29	TCAAACACGGGAGTACAACTA	21	123	3’	-49.5	66.67	33.33	40.24	0.85
pmi-miRNew-30	TGGGATTTGAGCCACAGATAA	21	113	5’	-31.6	51.33	48.67	27.96	1.74
pmi-miRNew-31	CCGGAAGACCTAGAGCTA	18	83	5’	-24.7	57.83	42.17	29.76	1.42
pmi-miRNew-32	GATTAATCCGGCATGAGCTA	20	83	5’	-29.2	50.60	49.40	35.18	1.40
pmi-miRNew-33	CAGAGGTTAATCGTACTCTGGCA	23	83	5’	-20.0	60.24	39.76	24.10	1.65
pmi-miRNew-34	TGGCTCAATGCATGCAACTCA	21	103	5’	-50.0	54.37	45.63	48.54	0.94
pmi-miRNew-35	CTGTGACTCAAGAGGGGCA	19	143	3’	-70.9	60.84	39.16	49.58	0.99
pmi-miRNew-36	AGGTCACAAATGGACGGTTGA	21	113	5’	-60.2	49.56	50.44	53.27	0.95
pmi-miRNew-37	GTCTGTTTATTACATTTTGAA	21	93	5’	-21.1	68.82	31.18	22.69	1.37
pmi-miRNew-38	CCAAATCTGAGTTATCTGTCA	21	173	3’	-53.6	51.45	48.55	30.98	1.57
pmi-miRNew-39	TTCTCGTAGGATAATTGTACTA	22	133	3’	-55.1	59.40	40.60	41.43	0.98
pmi-miRNew-40	AGAGATGTTGGCTAAGCAAGA	21	133	3’	-56.1	60.90	39.10	42.18	0.93
pmi-miRNew-41	CGATCTGTATGAGAATCTTGA	22	123	3’	-59.8	59.35	40.65	48.62	0.85
pmi-miRNew-42	AATGTGCAAATTTGAGCA	18	63	3’	-24.6	55.56	44.44	39.05	1.14
pmi-miRNew-43	CTCGAAGAGGAACACAAGATA	21	153	3’	-28.0	61.44	38.56	18.30	2.11
pmi-miRNew-44	AGAGATGTGAATGAGACCA	19	123	3’	-29.6	57.72	42.28	24.07	1.76
pmi-miRNew-45	TTTTTACTGTTGTCAACTA	19	72	5’	-18.2	62.50	37.50	22.50	1.67
pmi-miRNew-46	ACAGAGACGGTCGGGGGTA	19	83	3’	-36.7	55.42	44.58	44.22	1.01
pmi-miRNew-47	AGCTAATTGGTTGTTCAAACA	21	103	3’	-37.0	58.25	41.75	35.92	1.16

LM = Length of mature sequence, LP = Length of precursor sequence, ΔG = Free energy, AMFE = Adjusted minimum folding energy, MFEI = Minimum folding energy index

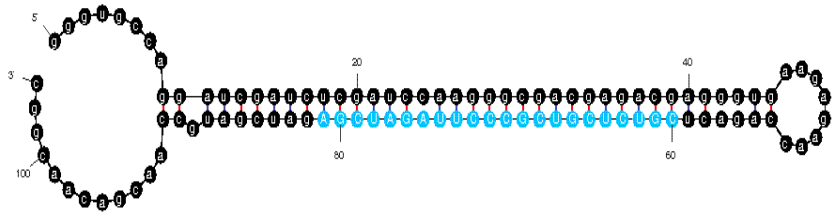
TABLE 6. List of precursors of putative novel miRNAs

Novel miRNA	miRNA precursor
pmi-miRNew-01	
pmi-miRNew-02	
pmi-miRNew-03	
pmi-miRNew-04	
pmi-miRNew-05	
pmi-miRNew-06	

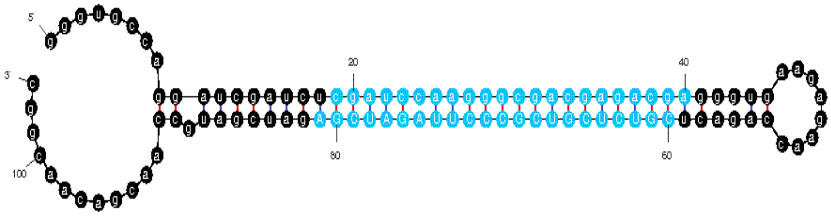
pmi-miRNew-06*



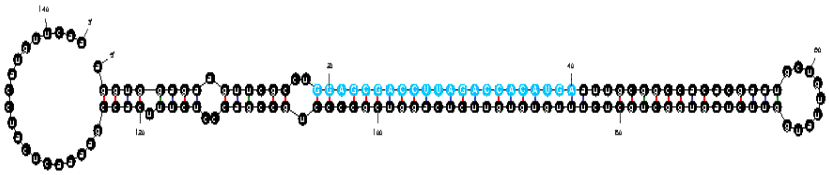
pmi-miRNew-07



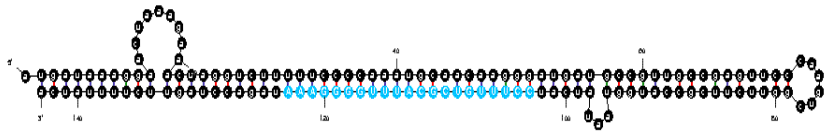
pmi-miRNew-07*



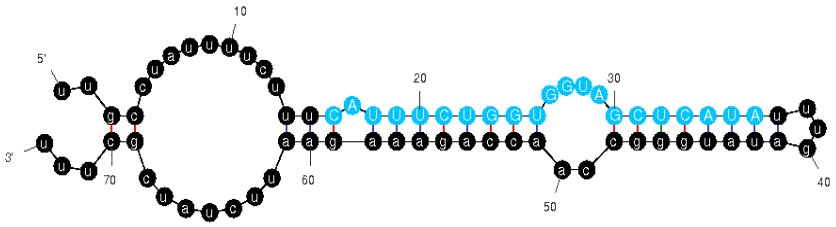
pmi-miRNew-08



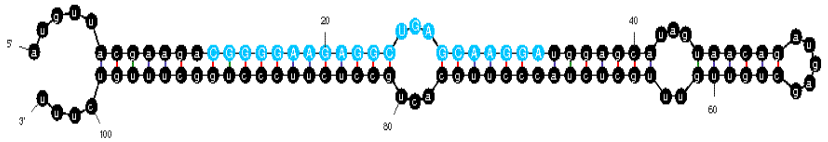
pmi-miRNew-09



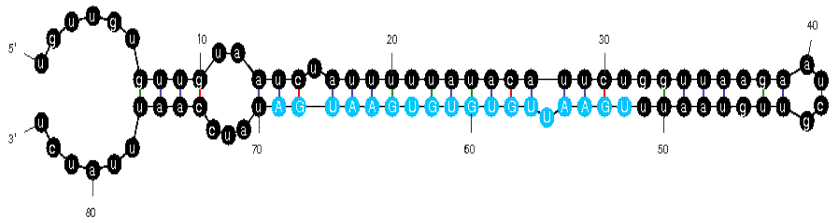
pmi-miRNew-10



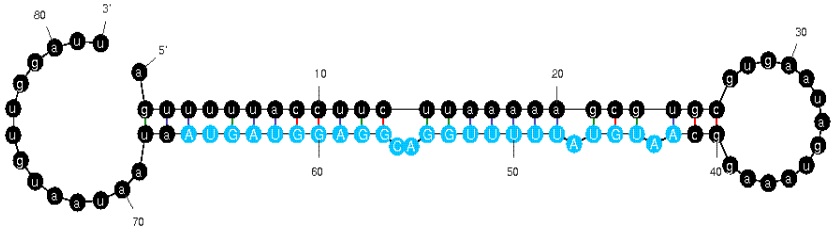
pmi-miRNew-11



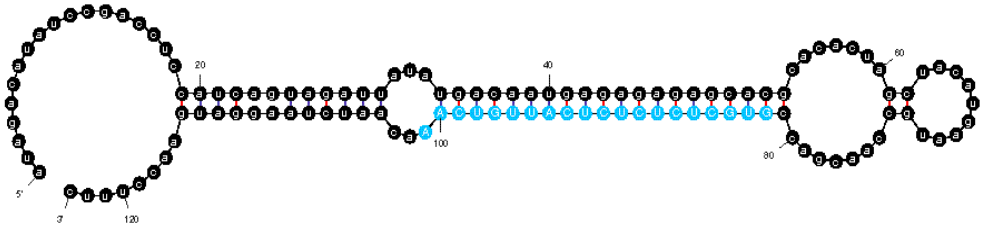
pmi-miRNew-12



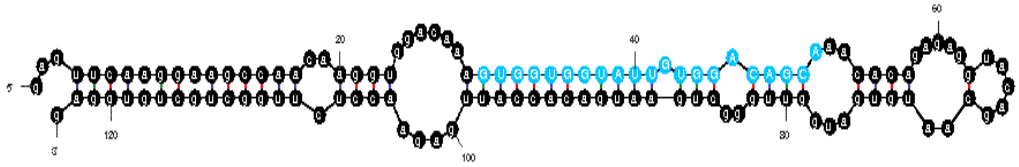
pmi-miRNew-13



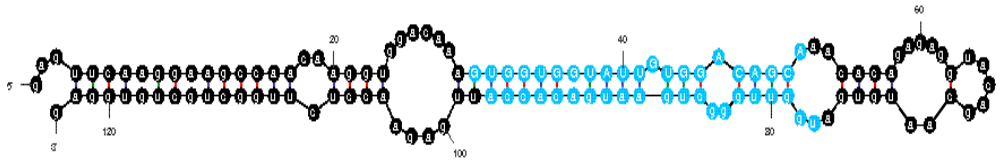
pmi-miRNew-14



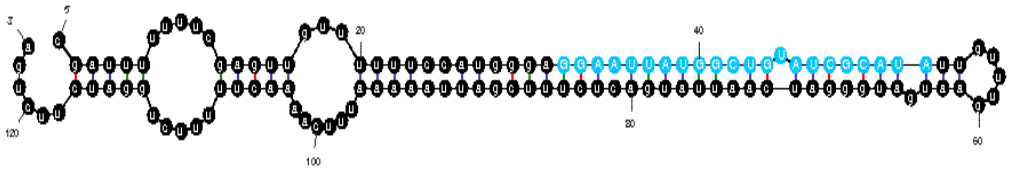
pmi-miRNew-15



pmi-miRNew-15*



pmi-miRNew-16



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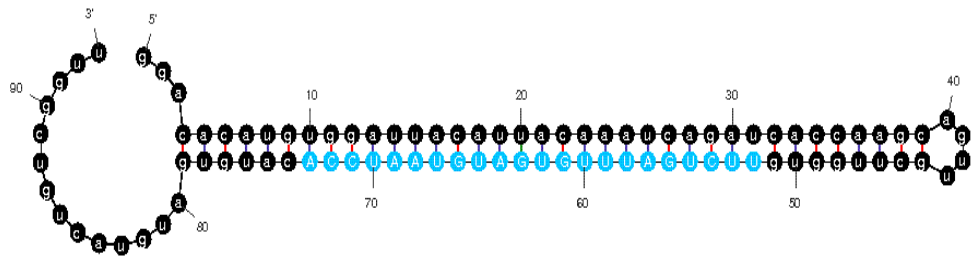
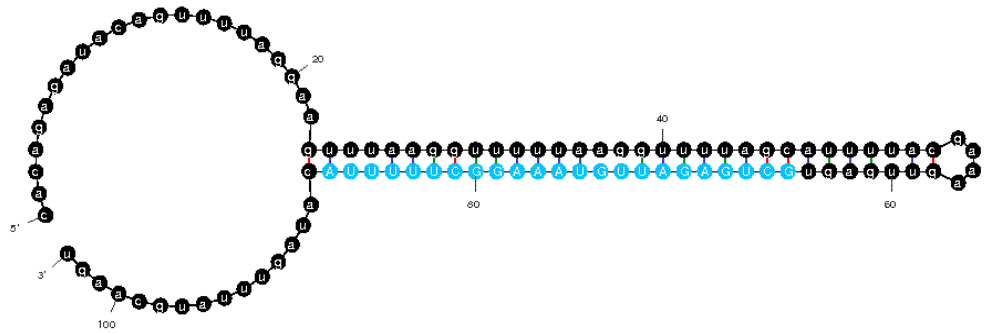


pmi-miRNew-18



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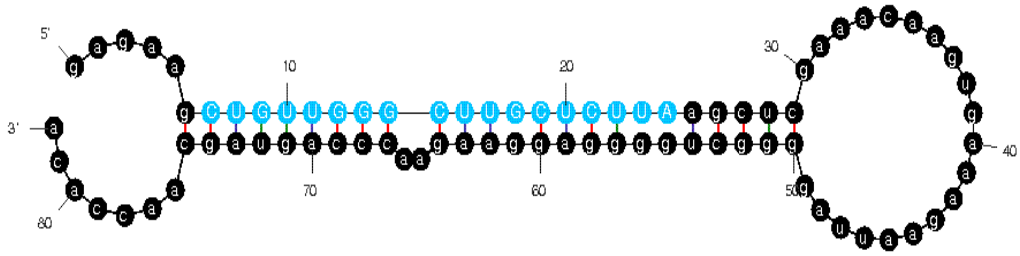

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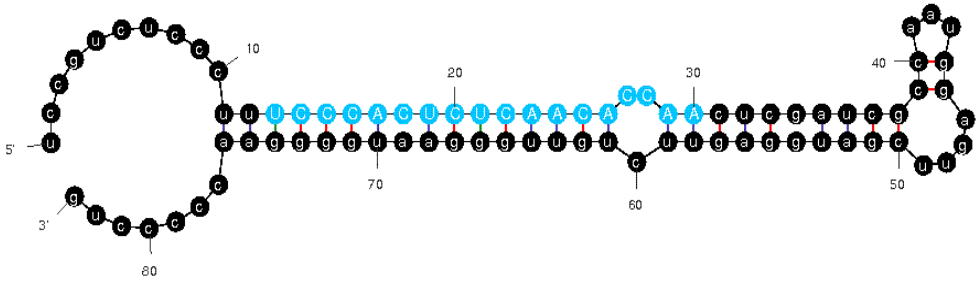

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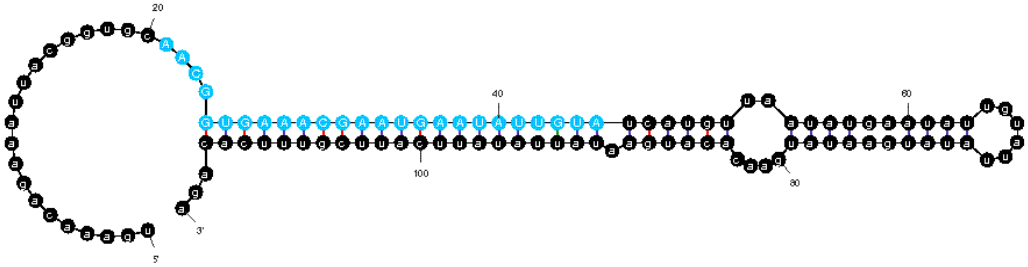

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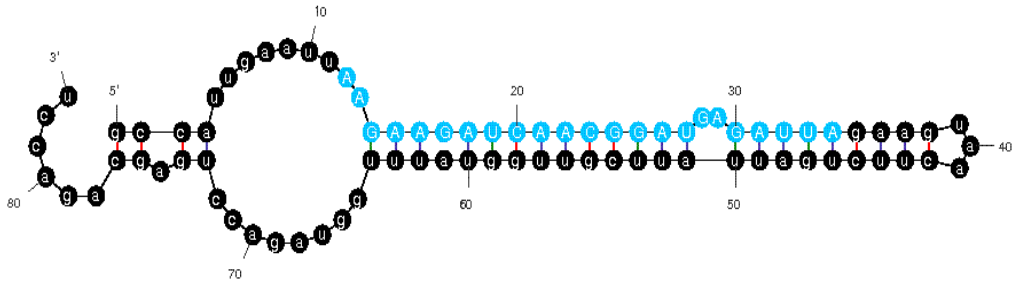
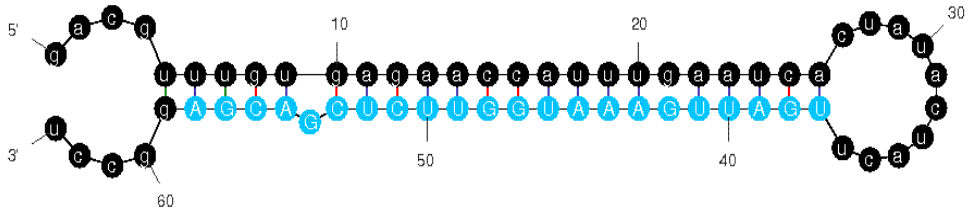
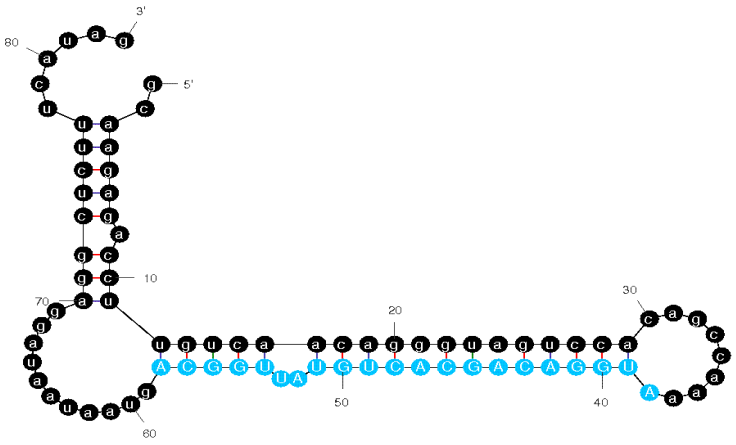


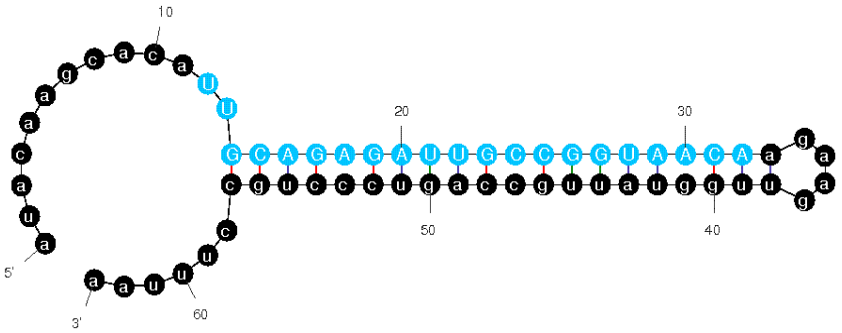
pmi-miRNew-23



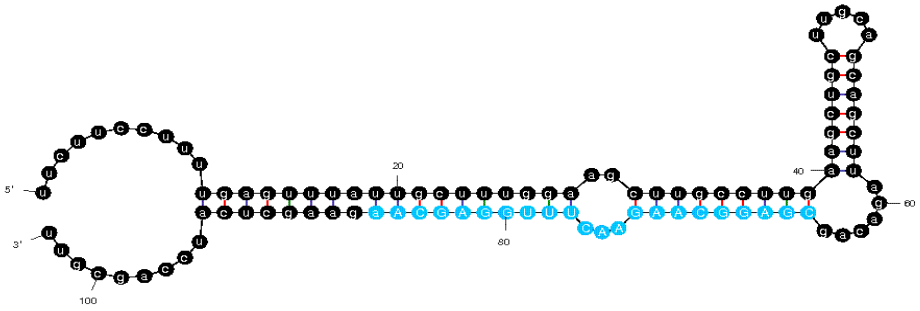
pmi-miRNew-24



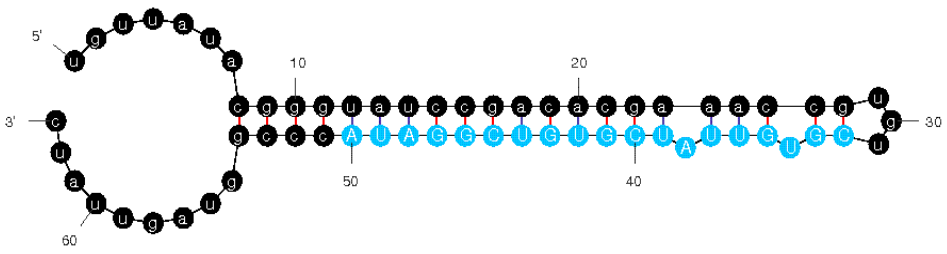
pmi-miRNew-25



pmi-miRNew-26



pmi-miRNew-27



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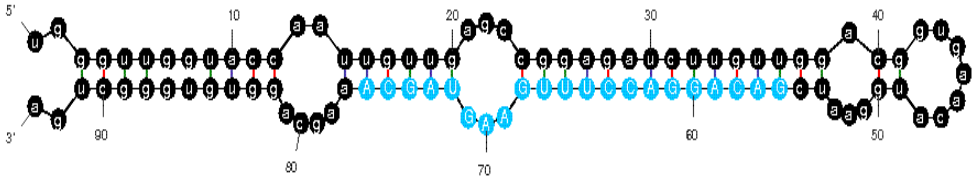
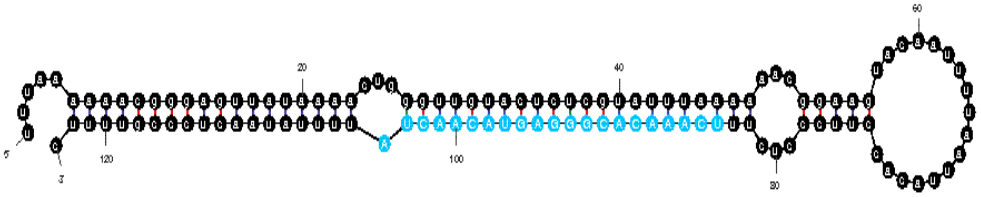
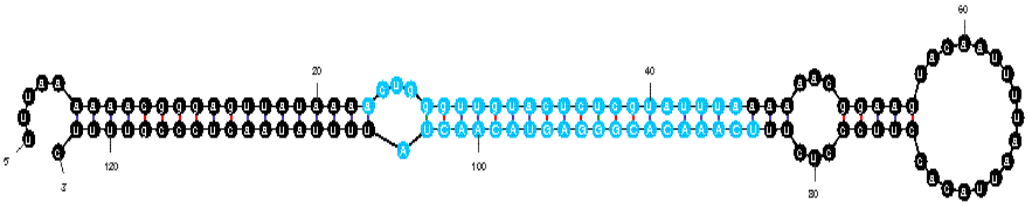


pmi-miRNew-29



pmi-miRNew-29*



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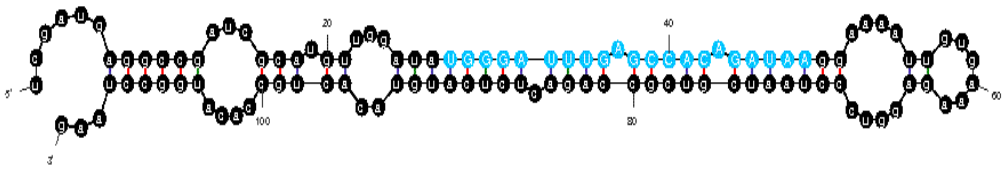
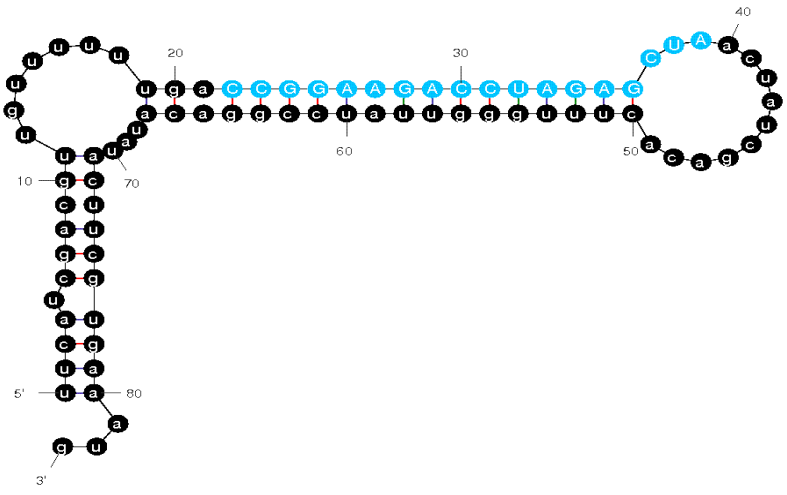
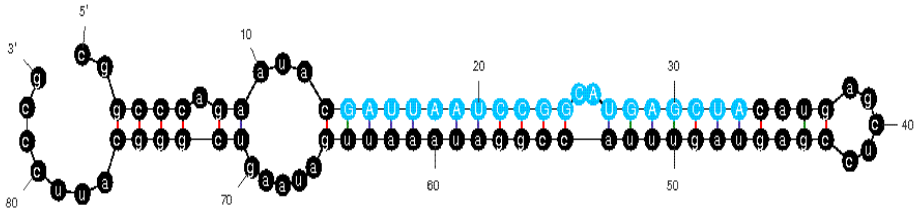


pmi-miRNew-31



pmi-miRNew-32



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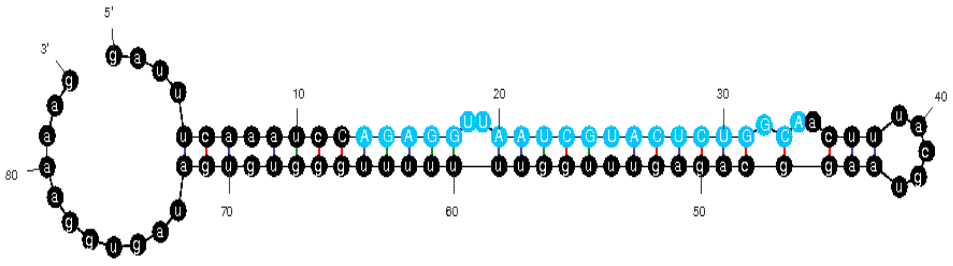
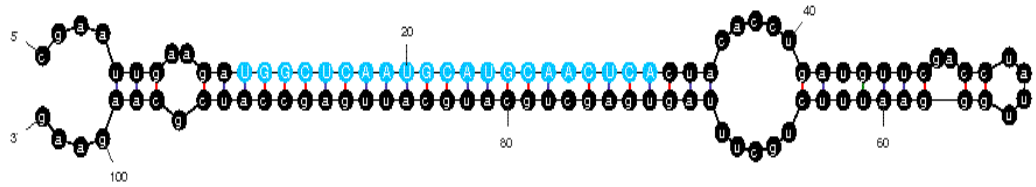
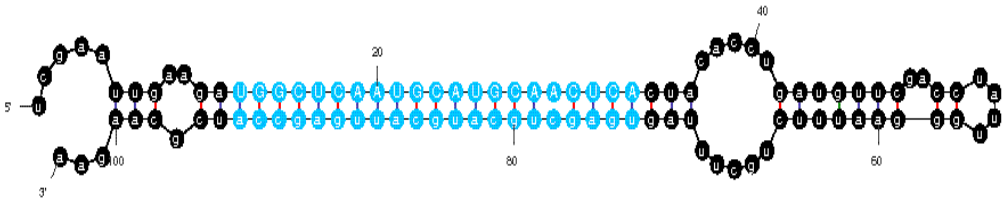


pmi-miRNew-34



pmi-miRNew-34*



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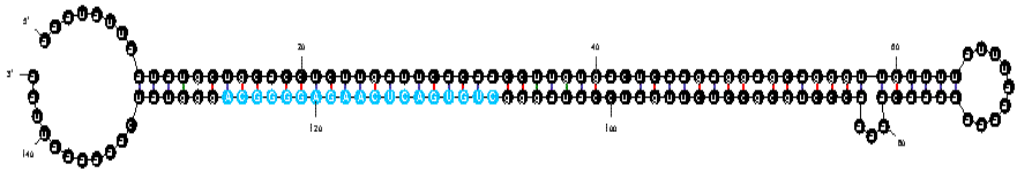

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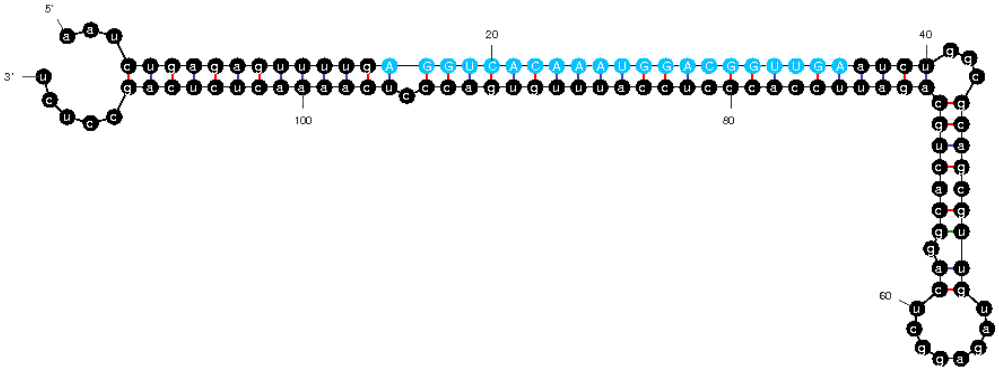

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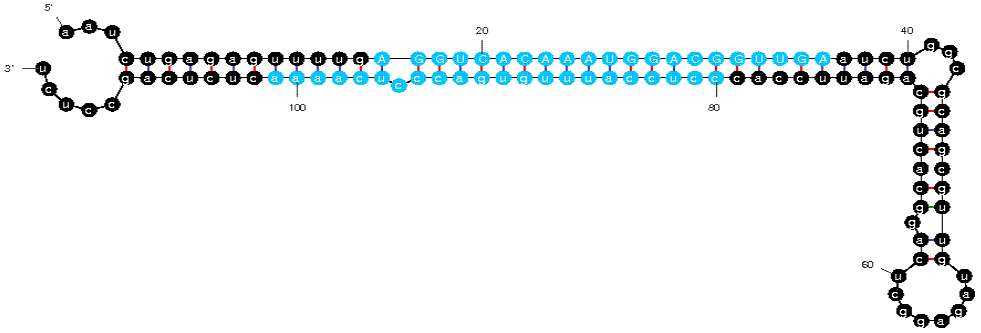
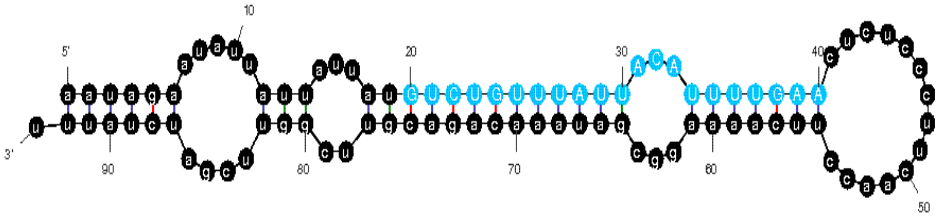


pmi-miRNew-37



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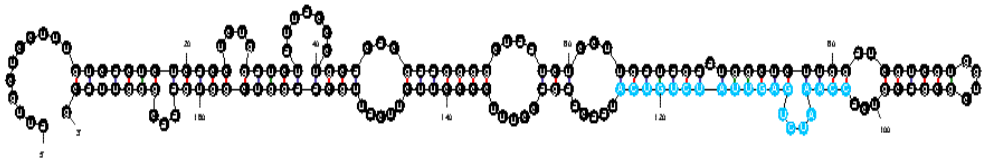

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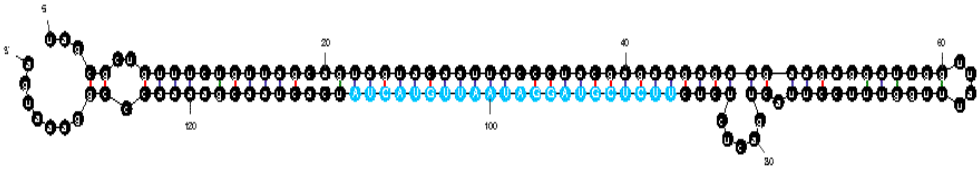
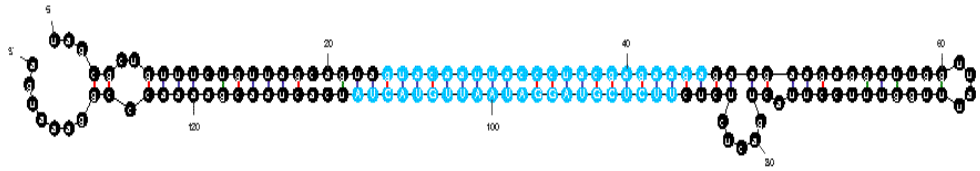
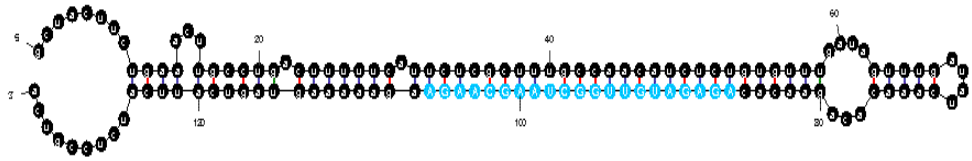


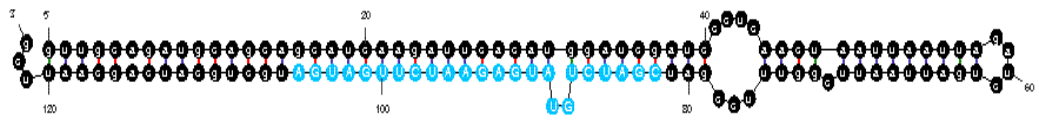
pmi-miRNew-39*



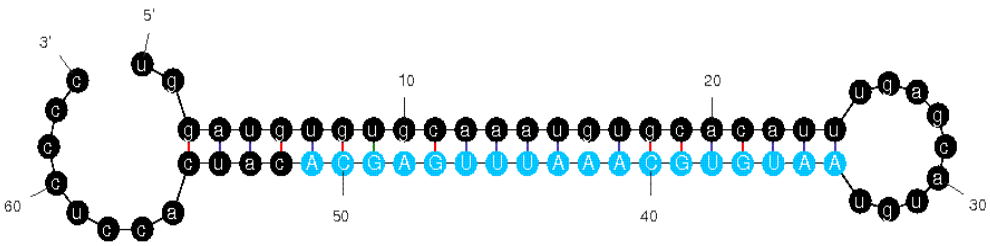
pmi-miRNew-40



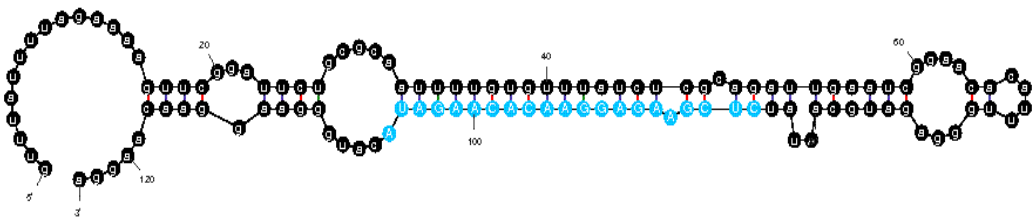
pmi-miRNew-41



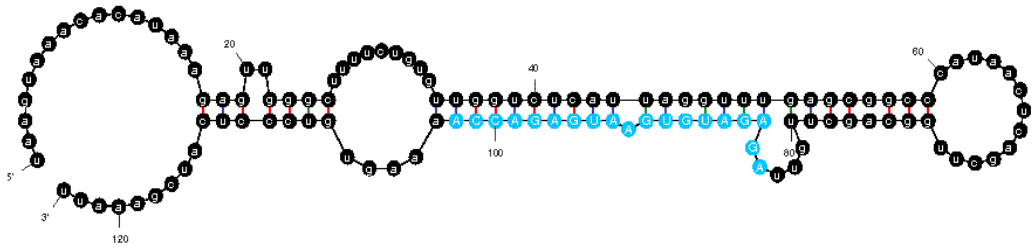
pmi-miRNew-42



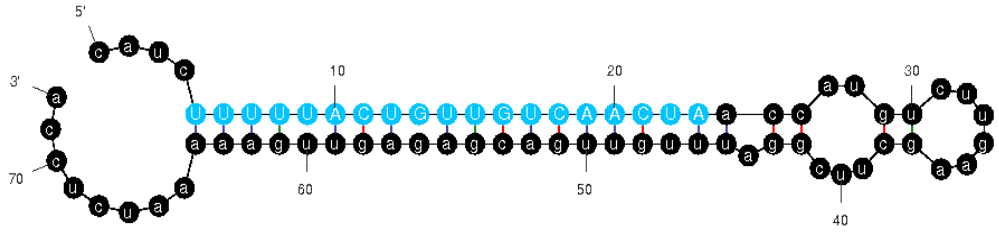
pmi-miRNew-43



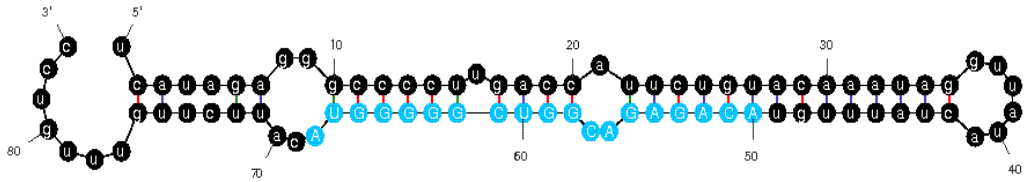
pmi-miRNew-44



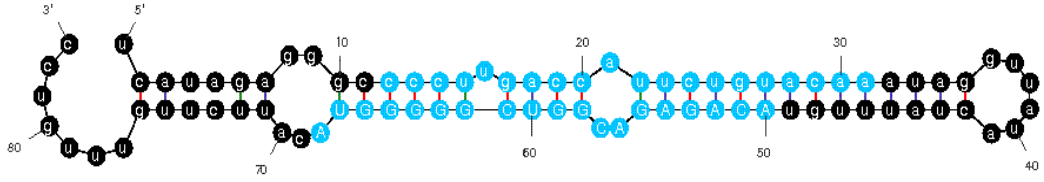
pmi-miRNew-45



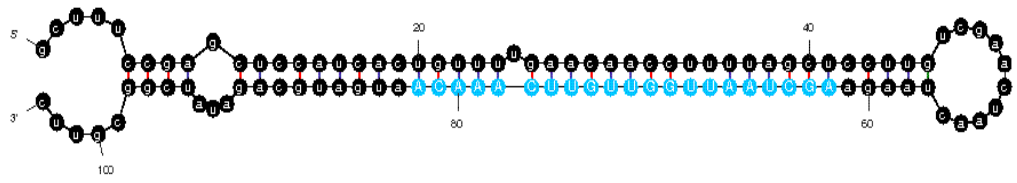
pmi-miRNew-46



pmi-miRNew-46*



pmi-miRNew-47



DIFFERENTIAL EXPRESSION OF miRNA UNDER ABA AND MeJA TREATMENTS

Differential expression was carried out by comparing the normalized expression of miRNAs in the treatments (ABA and MeJA) against control libraries (K). In ABA treated plants, it was observed that 21 miRNAs were differentially regulated where two miRNAs were up-regulated and 19 miRNAs were down-regulated. In MeJA treated plants, 38 miRNAs were differentially regulated which involved 24 up-regulated and 14 down-regulated miRNAs. This result demonstrated that majority of the miRNAs were more responsive towards MeJA (42%) than ABA treatments (7%) (Figure 3). Meanwhile, 51% of miRNA were significantly regulated in both libraries. All the significantly regulated miRNA were shown in Table 7.

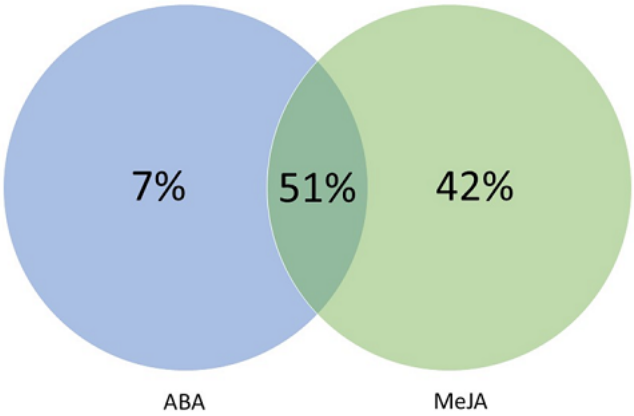


FIGURE 3. Venn diagram showing the common and specific sequence of significantly regulated miRNA in ABA and MeJA libraries

TABLE 7. List of significantly regulated miRNA under ABA and MeJA treatments. Negative and positive values indicated down- and up-regulated expressions, respectively. Minus sign (-) indicated no miRNA expression detected in the particular library

miRNA	Mature sequence	Normalized Fold Change	
		ABA	MeJA
pmi-miR156d	GCTCTCTGTGCTTCTGTCGTCA	-∞	7.79
pmi-miR156j	TTGACAGAAGAGAGTGAGTA	-	∞
pmi-miR157d	TGCTCTCTGTGCTTCTGTCATCA	-	∞
pmi-miR159	TTGGATTGAAGGGAGCTCTA	-9.70	∞
pmi-miR159a	TTTGGATTGAAGGGAGCTCTACA	-	∞
pmi-miR160a	TGCCTGGCTCCCTGTATGCTTA	-	∞
pmi-miR162	TCGATAAACCTCTGCATCCTA	-∞	∞
pmi-miR165a/b	TCGGACCAGGCTGCATCCCCA	-∞	-
pmi-miR166	TCGGACCAGGCTTCATCCCA	-∞	-
pmi-miR166a	GAATGTTGTCTGGCTCGAGGA	-6.40	∞
pmi-miR166b	CCGGACCAGGCCTCATTCCCCA	-∞	-∞
pmi-miR166c	TCGGACCAGGCTTCATTCCATA	-∞	-
pmi-miR166d	TCGTACCAGGCTTCATTCCCTA	-	∞
pmi-miR167a	TAAGCTGCCAGCATGATCGCA	-	∞
pmi-miR167b/d	TAAAGCTGCTAGCATGATCTGA	-	-13.58
pmi-miR168	TCGTTTGGTGCAGGTCGGGAA	-	∞

pmi-miR168b	CCCGCCTTGCACCAACTGAATA	-	∞
pmi-miR169i/j/l	TAGCCAAGGACGACTTGCCTGA	-∞	-5.06
pmi-miR172a	AGAATCTTGATGATGCTGCAGGA	-	∞
pmi-miR319	CTTGGACTGAAGGGAGCTCCTTA	-4.91	-8.05
pmi-miR319b/d/e	TGGACTGAAGGGAGCTCCTA	-∞	-11.08
pmi-miR390	CGCTATCTATCCTGAGCA	-	-∞
pmi-miR393c	TCCAAAGGGATCGCATTGATTCA	-	∞
pmi-miR396a	GTTCAATAAAGCTGTGGG	-∞	-∞
pmi-miR396b	GTTCAATAAAGCTGTTGGAA	-	∞
pmi-miR397a/b	TCATTGAGTGCAGCGTGGATGA	-	7.78
pmi-miR398	GGAGCGACCTGAGACCACATA	4.46	3.72
pmi-miR398b	CGTGTTCGCAGGTCGCCCCTGA	-∞	∞
pmi-miR399	TGCCAAAGGAGAGTTGCCCTA	-	-6.24
pmi-miR408	TGCACTGCCTCTTCCCTGGCAA	-∞	∞
pmi-miR535	TGACAATGAGAGAGAGCACTA	5.36	-8.05
pmi-miR535a	TGACAATGAGAGAGAGCACGT	-	-8.05
pmi-miR858	TTCGTTGTCTGTTCAACCTTA	-	9.03
pmi-miR894	GATTCACGTCGGGTTCACCAA	-4.90	6.19
pmi-miR2916	GGGGCTCGAAGACGATCAGATA	-∞	-4.08
pmi-miR4995	AGGCAGTGGCTTGGTTAAGGA	-7.36	-4.17
pmi-miR5077	TCACGTCGGGTTCACCAG	-	6.79
pmi-miR5368	AGGGACAGTCTCAGGTAGACAGCA	-	8.48
pmi-miR6173	AGCCGTAAACGATGGATA	-∞	-16.30
pmi-miR6300	GTCGTTGTAGTATAGTGGA	-	-∞
pmi-miR6478	CCGACCTTAGCTCAGTTGGTACA	-∞	∞

ANALYSIS OF miRNA TARGET GENES

miRNA function is closely related to its target gene. In this study, we employed psRNA Robot software to search for the miRNA targets. Table 8 showed a total of 37 potential target genes predicted in *P. minor*. Some miRNAs were identified to target the same genes (Table 8). Based on miRNA target prediction result, four miRNAs and targets were selected to be further explored due to their involvement in plant defense system and volatile compound biosynthesis pathway. The targets involved were peroxidase targeted by pmi-miR396a, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) targeted by pmi-miR6300, sesquiterpene synthase targeted by pmi-miR6173 and alcohol dehydrogenase 1 (ADH1) targeted by pmi-miR396b. Additionally, analysis of target genes via gene ontology showed most of the targets belong to cellular component (35%), followed by molecular function (34%) and biological process (31%) (Figure 4).

TABLE 8. List of predicted target genes

miRNA	Score	ID transcript	Target annotation
pmi-miR156d	2.8	comp53688_c0_seq1	Photosystem II
	2.8	comp59110_c2_seq1	Agrogenate dehydratase
pmi-miR156j	2.2	comp53137_c1_seq1	SPL
	3.0	comp53825_c0_seq1	F-box protein CPR30
pmi-miR157d	3.2	comp59318_c1_seq1	Probable ion channel POLLUX
	3.8	comp48954_c1_seq1	60S ribosomal protein L14-2

pmi-miR159	1.0	comp57600_c3_seq1	Transcription factor GAMYB
	2.0	comp67380_c0_seq2	Putative disease resistance protein RGA3
pmi-miR159a	1.0	comp57600_c3_seq1	Transcription factor GAMYB
	4.0	comp10554_c0_seq1	Growth-regulating factor 3
pmi-miR160a	0.8	comp55762_c0_seq1	Auxin response factor
	3.5	comp63097_c0_seq14	Auxin-responsive protein IAA9
pmi-miR162	N/A	N/A	N/A
pmi-miR165a/b	2.5	comp52509_c0_seq3	Wall-associated receptor kinase 2
pmi-miR166	0.2	comp62172_c1_seq10	Homeobox-leucine zipper protein HOX32
	2.5	comp67610_c2_seq1	Probable WRKY transcription factor 19
pmi-miR166a	0.0	comp62276_c1_seq14	Vacuolar protein sorting-associated protein 35A
pmi-miR166b	2.5	comp62172_c1_seq1	Homeobox-leucine zipper protein HOX32
pmi-miR166c	1.2	comp62276_c1_seq14	Vacuolar protein sorting-associated protein 35A
pmi-miR166d	1.8	comp62276_c1_seq14	Vacuolar protein sorting-associated protein 35A
pmi-miR167a	2.5	comp64807_c0_seq2	Putative ABC transporter B family member 8
pmi-miR167b/d	N/A	N/A	N/A
pmi-miR168	N/A	N/A	N/A
pmi-miR168b	N/A	N/A	N/A
pmi-miR169i/j/l	2.5	comp67132_c1_seq1	Protein MEI2-like2
pmi-miR172a	1.5	comp63292_c0_seq4	Floral homeotic protein APETALA 2
pmi-miR319	2.5	comp64847_c0_seq10	Transcription factor GAMYB
pmi-miR319b/d/e	2.0	comp57600_c3_seq1	Transcription factor GAMYB
	2.5	comp50465_c0_seq1	Transcription factor TC4
pmi-miR390	2.5	comp53986_c0_seq1	Cellulose synthase A catalytic subunit 6
pmi-miR393c	N/A	N/A	N/A
pmi-miR396a	2.5	comp60490_c0_seq1	Peroxidase
pmi-miR396b	2.5	comp63431_c1_seq16	ADH1
pmi-miR397a/b	2.0	comp67947_c0_seq1	Laccase-4
pmi-miR398	2.0	comp61311_c1_seq2	Cytochrome c oxidase subunit 5b-2, mitochondrial
pmi-miR398b	N/A	N/A	N/A
pmi-miR399	2.0	comp50399_c1_seq1	Probable inorganic phosphate transporter
pmi-miR408	2.2	comp43803_c0_seq1	Putative disease resistance protein At4g19050
pmi-miR535	2.5	comp58725_c1_seq1	GDP-mannose 3,5-epimerase 2
pmi-miR535a	2.5	comp58725_c1_seq1	GDP-mannose 3,5-epimerase 2
pmi-miR858	2.0	comp55943_c0_seq1	50S ribosomal protein L34
pmi-miR894	N/A	N/A	N/A
pmi-miR2916	1.8	comp58044_c0_seq1	Probable DNA primase large subunit
pmi-miR4995	2.2	comp62773_c1_seq3	E3 ubiquitin ligase
pmi-miR5077	2.5	comp60152_c0_seq2	Cell division protein FtsZ homolog 2-1
pmi-miR5368	1.0	comp40772_c0_seq1	Uncharacterized protein ORF91
pmi-miR6173	3.0	comp46206_c0_seq1	Probable sesquiterpene synthase
pmi-miR6300	3.2	comp55945_c0_seq1	HMGR
	3.5	comp59913_c0_seq1	Proteasome subunit beta type-2-A
pmi-miR6478	N/A	N/A	N/A

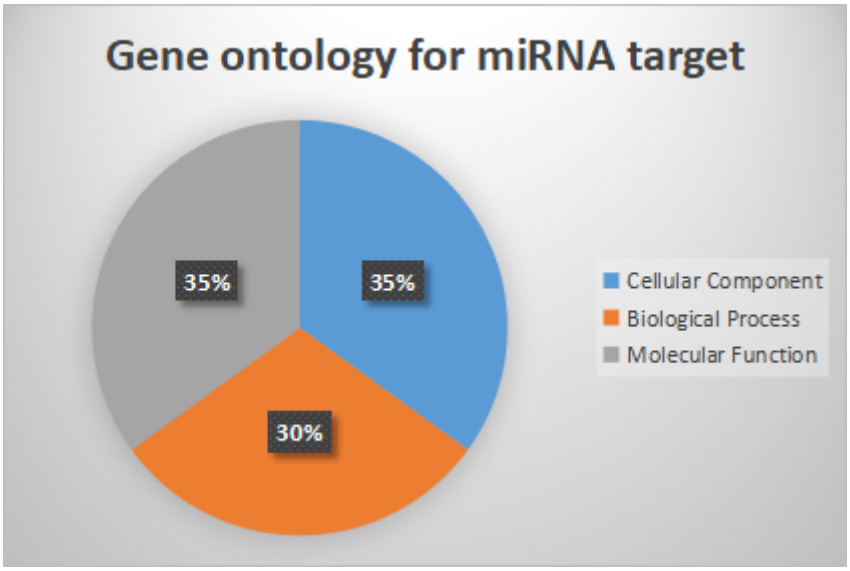
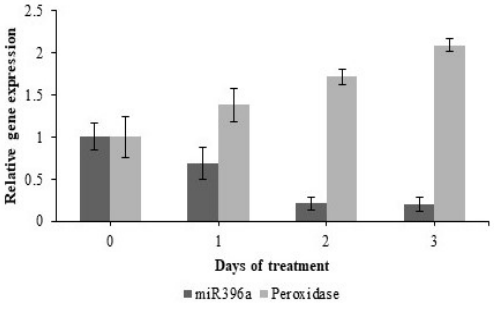
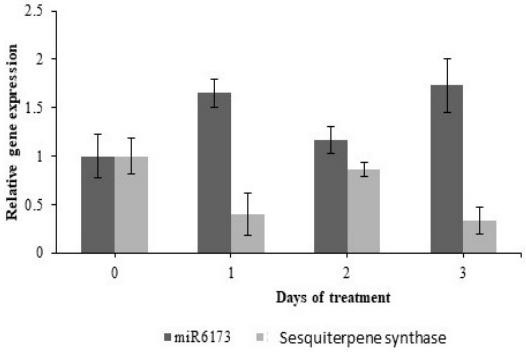


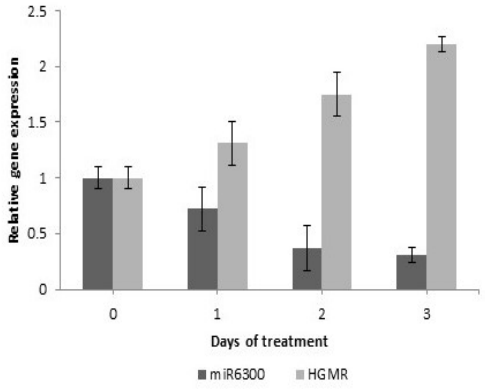
FIGURE 4. Pie chart showing the distribution of miRNA targets according to WEGO analysis

EXPRESSION PROFILE OF SELECTED miRNAS AND THEIR TARGETS USING RT-qPCR

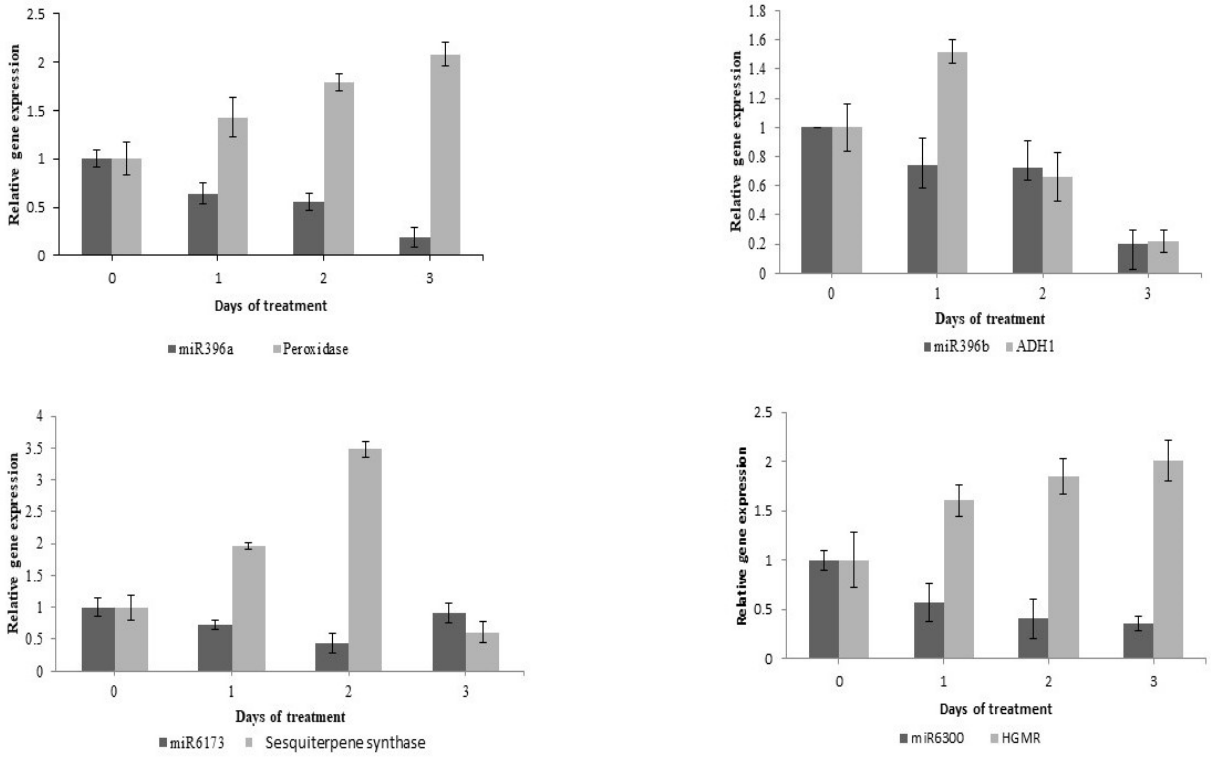
Four conserved miRNAs (pmi-miR396a, pmi-miR396b, pmi-miR6173, and pmi-miR6300) were selected for RT-qPCR analysis. Based on high throughput sequencing, the selected miRNAs were detected in ABA and MeJA libraries except pmi-miR396b which was observed in MeJA library only. The RT-qPCR analysis was carried out to identify the expression of selected miRNAs throughout the treatments. The analysis results were shown in Figure 5 (A) and (B) for ABA and MeJA, respectively. Pmi-miR396a, pmi-miR6300 and pmi-New27 showed decreasing pattern in both ABA and MeJA treatments. Pmi-miR396b also showed similar pattern under MeJA treatments. In contrast, all the target genes showed increasing pattern. Pmi-miR6173 exhibited increasing pattern in ABA treatment while decreasing pattern under MeJA treatment. For its target genes, sesquiterpene synthase showed decreasing pattern under ABA treatment, while in MeJA treatment the expression was increased to two- fold and more than three-fold on Day 1 and Day 2, respectively. However, the expression of the target decreased on Day 3. In general, most of the miRNAs were down regulated under both treatments. In MeJA treatments, pmi-miR6173 and pmi-miR6300 had shown similar pattern (decreasing) with our previous study which involved *P. minor* treated with pathogenic fungi. In the study, both pmi-miR6173 and pmi-miR6300 were down regulated in *Fusarium*-treated compared to the control libraries (Samad et al. 2019). Our current study together with our recent study showed that the targets of pmi-miR6173 and pmi-miR6300 might play essential role in biotic and abiotic stresses in *P. minor*.







(A)



(B)

FIGURE 5. Relative expression of selected miRNAs and their targets in response to ABA (A) and MeJA (B) treatments

In this study, we observed that most miRNAs were being significantly regulated in MeJA than ABA libraries. This indicated that MeJA signaling pathway is way more diverse since previous studies revealed it could interact with other hormones, such as salicylic acid (SA), gibberellin (GA), ethylene (ET), auxin,

brassinosteroid (BR) and even abscisic acid (ABA), to regulate gene expression in regulatory networks (Liu et al. 2015). These interactions led to major adjustments in plant biological processes including seed germination, root growth, flowering, senescence and stimulation of various secondary metabolite to counter insects and pathogen invasion (Huang et al. 2017). In contrast, ABA role is more focused in seed germination, stomatal closure and various abiotic stresses (Rai et al. 2011; Sah et al. 2016). In this study, we discovered a total of 41 conserved miRNAs that were responsive to ABA and MeJA treatments in *P. minor*. The targets involved were peroxidase targeted by pmi-miR396a, HMGR targeted by pmi-miR6300, sesquiterpene synthase targeted by pmi-miR6173 and ADH1 targeted by pmi-miR396b. In ABA and MeJA treatments, the expression of miRNA and their targets were similar. These might happen because the crosstalk between the ABA and MeJA signaling pathways lead to similar changes in gene expression (Riemann et al. 2015). Previous findings showed both hormones contributed towards plant stress response by modulating the gene expression to synthesise secondary metabolites such as terpenoid indole alkaloid in *Catharanthus roseus* and anthocyanins in *Arabidopsis thaliana* (El-Sayed & Verpoorte 2004; Loreti et al. 2008). The target of miR396a, peroxidase, is an enzyme involved in cell elongation, lignification, seed germination, and defense response (Shigeto & Tsutsumi 2016). The up-regulation of peroxidase is consistent with the ABA and MeJA roles as signal transduction pathway during plant stress (Almagro et al. 2009). High expression of peroxidase may induce the plant VOC as a response to the environmental stresses especially herbivore attack (War et al. 2011). The targets of pmi-miR6173 and pmi-miR6300, HMGR, and sesquiterpene synthase, respectively, are both involved in terpenoid biosynthesis pathway (Tholl 2015). HMGR is a rate limiting enzyme in MVA pathway which is required for accumulation of sesquiterpene (Chappell et al. 1991; Tholl 2015). In *A. thaliana*, loss of function for *hmg*1 showed a 65% reduction in triterpene compound accumulation compared to the wild type (Ohyama et al. 2007). Moreover, mutant *hmg1* also led to dwarfing, early senescence and male sterility, and reduced sterol levels (Suzuki et al. 2004). HMGR enzyme catalyses the conversion of HMG-CoA to mevalonate, which is later converted into mevalonate-5-phosphate through the enzyme MVK. High expression of HMGR gene induced by elicitor and wounding could enhance the sesquiterpene production (Chappell et al. 1991; Kondo et al. 2003). Similarly, sesquiterpene synthase is a type of terpene synthase required for sesquiterpene biosynthesis at downstream level (Tholl 2015). Functional analysis of *P. minor* sesquiterpene synthase led to the production of β-sesquiphellandrene in transgenic *A. thaliana* (Ee et al. 2014). Another study showed two novel sesquiterpene genes (PmSTPS1 and PmSTPS2) isolated from *P. minor* were responsible for the production of β-farnesene, α-farnesene and farnesol. Additionally, PmSTPS2 was found to produce nerolidol as an additional product compared to PmSTPS1 (Rusdi et al. 2018).

For pmi-miR396b target, ADH1 is involved in GLV biosynthesis pathway by catalysing the conversion of aldehydes to alcohols (Ul Hassan et al. 2015). GLVs have emerged as major players in plant defense, plant-plant interactions and plant-insect interactions. Some GLVs inhibit the growth and proliferation of plant pathogens, including bacteria, fungi, and viruses. Furthermore, GLVs emitted from plants under herbivore attack can serve as aerial messengers to neighbouring plants and to attract parasitic or parasitoid enemies of the herbivores (Ul Hassan et al. 2015). In general, ADH are classified into two main superfamilies, medium-chain dehydrogenase/reductase (MDR) and short-chain dehydrogenase/reductase (SDR) which consist of 370 and 250 amino acid residues, respectively (Jörnvall 2008). In *P. minor*, this enzyme was reported to have two family members, PmADH1a and PmADH1b. Both of them were up-regulated under drought stress and involved in ABA signaling pathway (Abd Hamid et al. 2018).

Conclusion

High throughput sequencing and advance computational approaches have resulted in the accumulation of huge data on miRNAs. Therefore, exploration of miRNAs role in biological system becomes relatively easy than before. Investigation on miRNAs and their targets at each step of a particular pathway and identifying their significance are current approaches to decipher the functions of miRNAs in plant system. In this study, we managed to characterise miRNA in *P. minor* and their response under ABA and MeJA treatments. Four miRNAs related to volatile compound biosynthesis were selected to be further studied. However, lack of genome information resulted in the limitation of miRNA discovery in *P. minor.* We believe more miRNA related to various biological processes could be discovered with the availability of *P. minor* genome sequence. However, this study was essentially an attempt to provide the fundamental relationship between miRNAs and their response towards ABA and MeJA.

Acknowledgements

We would like to thank Institute of Bioscience, Universiti Putra Malaysia (UPM) and Malaysian Genome Institute (MGI) for providing the Bioanalyzer services. We also would like to extend our gratitude to anonymous reviewers for their comments on this manuscript. This work was funded by University Research Grant (DIP-2015-018).

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Received: 9 August 2019
Accepted: 14 February 2020