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*  

ABDUL FATAH A. SAMAD, NAZARUDDIN NAZARUDDIN, JAERYES JANI & ISMANIZAN ISMAIL*

**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**

**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, antialulcer and antimicrobial activities (Christopher et al. 2015). Additionally, due to its unique aroma, this plant is commonly used as food additives in local dishes in Southeast Asia countries (Christopher 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, tashinone (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; Rahnamae-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 Free 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(https://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).

| miRNA      | Primer sequence          |
|------------|--------------------------|
| pmi-miR396a| 5’-GTT CAA TAA AGC TGT GGG A-3’ |
| pmi-miR396b| 5’-GGG GTT CAA TAA AGC TGT TGA AA-3’ |
| pmi-miR6173| 5’-GGG GGA GCC GTA AAc GAT GGA TA-3’ |
| pmi-miR6300| 5’-GGG GGT CGT TGT ATG ATA GTG GA-3’ |
| pmi-miRNew-27| 5’-CGT GTT ATC GTG TCG GAT A-3’ |

| 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 TTGTT ACG AAA TCT TCT CA-3’ (Forward) 5’-CTC TTC AGG TGG TGT CCT T-3’ (Reverse) |
| Sesquiterpenesynthase | 5’-AGA CGT AGT GAG CAA CCA AC-3’ (Forward) 5’-CTT GGC ATA CCC TGG TGG TAA-3’ (Reverse) |
| HMGR         | 5’-GCC AAC ATT GTG TCT GTC 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±   | 100.0       | 1,852,647±    | 100.0       |
| miRNA                | 28,193±9,808  | 0.26        | 1,124±600     | 0.06        |
| Rfam                 | 694,910±      | 6.33        | 84,125±       | 4.54        |
| Unannotated          | 10,250,078±   | 93.41       | 1,767,398±    | 95.40       |
| **ABA library**      |               |             |               |             |
| Raw reads            | 17,253,566±   |             | 18,826,895    |             |
| Clean reads (18-30nt)| 11,571,771±   | 100.0       | 1,580,735±    | 100.0       |
| miRNA                | 22,049±       | 0.19        | 538±          | 0.03        |
| Rfam                 | 579,375±      | 5.00        | 68,857±       | 4.36        |
| Unannotated          | 10,970,347±   | 94.8        | 1,511,349±    | 95.61       |
| **MeJA library**     |               |             |               |             |
| Raw reads            | 40,437,576±   |             | ±9,816,458    |             |
| Clean reads (18-30nt)| 21,458,917±   | 100.0       | 2,163,212±    | 100.0       |
| miRNA                | 143,282±      | 0.67        | 2,773±179     | 0.13        |
| Rfam                 | 1,089,945±    | 5.08        | 119,792±      | 5.54        |
| Unannotated          | 20,225,690±   | 94.25       | 2,040,647     | 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 sequence (5'-3') | Sequence length | Conserved miRNA | Plant species         |
|--------------|----------|-------------------------------|-----------------|-----------------|-----------------------|
| 156          | pmi-miR156 | TTGACAGAAGAGAGTGAGCACA         | 22              | tae-miR156      | *Triticum aestivum*   |
|              | pmi-miR156a | TGACAGAAGAGAGTGAGCACA         | 22              | bna-miR156a     | *Brassica napus*      |
|              | pmi-miR156b | TGACAGAAGAGAGTGAGCATA         | 21              | cca-miR156b     | *Cynara cardunculus*  |
|              | pmi-miR156c | TTGACAGAAGAGAGTGAGCATA         | 21              | gna-miR156c     |                      |
|              | pmi-miR156d-3p | GCTCTCTGTGCTTCGTCATCA     | 22              | stu-miR156d-3p  | *Solanum tuberosum*   |
|              | pmi-miR156f | TTGACAGAAGAGAGAGTGGCA       | 22              | gma-miR156f     |                      |
|              | pmi-miR156i-3p | TGACAGAAGAGAGTGGCA       | 21              | mtr-miR156i-3p  | *Medicago truncatula* |
|              | pmi-miR156j | TTGACAGAAGAGAGTGGCA         | 20              | mtr-miR156j     |                      |
|              | pmi-miR156k | TTGACAGAAGAGAGTGGCA         | 20              | gma-miR156k     |                      |
|              | pmi-miR156l-3p | GCTACCTCTCTCTCTGTCAGCA   | 23              | osa-miR156l-3p  | *Oryza sativa*        |
|              | pmi-miR156p | TTGACAGAAGAGAGAGTGGCA       | 20              | mdm-miR156p     | *Malus domestica*     |
|              | pmi-miR156q | TTGACAGAAGAGAGAGTGGCA       | 22              | gma-miR156q     |                      |
|              | pmi-miR156r | TTGACAGAAGAGAGAGTGGCA       | 22              | gma-miR156r     |                      |
| 157          | pmi-miR157b | CTGACAGAAGAGAGAGAGAGCACACTA | 23              | smo-miR157b     | *Selaginella moellendorffii* |
|              | pmi-miR157c-5p | TTGACAGAAGAGAGAGAGAGACATCA | 23              | aly-miR157bc-5p | *Arabidopsis lyrata*  |
|              | pmi-miR157d-3p | GCTCTCTGTGCTTCGTCATCA   | 21              | aly-miR157bd-3p |                      |
159  pmi-miR159  TTTGAGTCGAGGAGCTCTCA  21  atr-miR159  Amborella trichopoda
  pmi-miR159a  TTTGAGTCGAGGAGCTCTTTA  24  ath-miR159a  Arabidopsis thaliana
  pmi-miR159b-3p  TTTGAGTCGAGGAGCTCTTCA  23  aly-miR159b-3p  Arabidopsis lyrata
  pmi-miR159c  CTGGAGTCGAGGAGCTCTCA  21  sof-miR159c  Saccharum officinarum
  pmi-miR159f  CTGGAGTCGAGGAGCTCTCTCA  22  osa-miR159f  Oriza sativa

160  pmi-miR160  CCATGAGGACCAAGACATA  22  csi-miR160  Citrus sinensis
  pmi-miR156a-3p  TGCCCTGGCCTCTGATGCGGA  21  gma-miR156a-3p  Glycine max
  pmi-miR156c  CCTGAGCCTCTGATGCGGACTTA  22  mes-miR156c  Manihot esculenta

162  pmi-miR162  TCGAATACCTCCTCAGCTCA  21  aau-miR162  Acacia auriculiformis
  pmi-miR162-5p  TGGAGGACAGAGCTGTCATTA  23  csi-miR162-5p  Citrus sinensis
  pmi-miR162a  TCGAATACCTCCTCAGCTCAGA  20  gma-miR162a  Glycine max
  pmi-miR162b  TCGAATACCTCCTCAGCTCAGA  22  osa-miR162b  Oriza sativa
  pmi-miR162b-5p  TGGAGGACAGAGCTGTCATCCAA  22  aly-miR162b-5p  Arabidopsis lyrata

164  pmi-miR164a  TGAGAAGCAGGAGCACGTGA  20  hci-miR164a  Helianthus ciliaris
  pmi-miR164b-5p  TGAGAAGCAGGAGCACGTGCA  21  ata-miR164b-5p  Aegilops tauschii
  pmi-miR164e-5p  TGGAGGACAGAGCTGTCATCCCA  22  bra-miR164e-5p  Brassica rapa
  pmi-miR164g-3p  CACGTCGCTCCCTTCATCCACCA  22  zma-miR164g-3p  Zea mays

165  pmi-miR165a-3p  TGGAGGACAGAGCTGTCATCCCCA  21  ath-miR165a-3p  Arabidopsis thaliana
  pmi-miR165b  TGGAGGACAGAGCTGTCATCCCCA  21  ath-miR165b  Arabidopsis thaliana
  pmi-miR165c-5p  GGAATGTTGTCTGTCGAGGA  22  mtr-miR165c-5p  Medicago truncatula
  pmi-miR166  TCGAAGAGGCTCTCATCCACCA  22  ata-miR166b-3p  Aegilops tauschii
  pmi-miR166a-5p  TGGAGGACAGGAGCACGTGGA  22  aly-miR166a-5p  Arabidopsis lyrata
  pmi-miR166b  TGGAGGACAGGAGCACGTGGA  21  mtr-miR166b  Medicago truncatula
  pmi-miR166b-3p  TGGAGGACAGGAGCACGTGGA  22  aly-miR166b-3p  Arabidopsis lyrata
  pmi-miR166c  TGGAGGACAGGAGCACGTGGA  22  aly-miR166c  Arabidopsis lyrata

166  pmi-miR166d  TCGAAGAGGCTCTCATCCACCA  23  ctr-miR166  Citrus trifoliata
  pmi-miR166e  TCGAAGAGGCTCTCATCCACCA  22  aly-miR166e  Arabidopsis lyrata
  pmi-miR166f-5p  TCGAAGAGGCTCTCATCCACCA  22  aly-miR166f-5p  Arabidopsis lyrata
  pmi-miR166h-5p  TGGAGGACAGGAGCACGTGGA  22  osa-miR166h-5p  Oriza sativa
  pmi-miR166i  TCGAAGAGGCTCTCATCCACCA  20  cme-miR166i  Cucumis melo
  pmi-miR166j-3p  TCGAAGAGGCTCTCATCCACCA  23  gma-miR166j-3p  Glycine max
  pmi-miR166l-3p  TCGAAGAGGCTCTCATCCACCA  21  ppt-miR166l-3p  Physcomitrella patens
  pmi-miR166m  TCGAAGAGGCTCTCATCCACCA  22  zma-miR166m  Zea mays
  pmi-miR166n  TCGAAGAGGCTCTCATCCACCA  22  zma-miR166n-5p  Zea mays
  pmi-miR166p  TCGAAGAGGCTCTCATCCACCA  20  ptc-miR166p  Populus trichocarpa
  pmi-miR166q  TCGAAGAGGCTCTCATCCACCA  23  ptc-miR166q  Populus trichocarpa

167  pmi-miR167-5p  TGAAGTGCTGCCAGCAGATCTTTTA  23  ahy-miR167-5p  Arachis hypogaea
  pmi-miR167a  TGAAGTGCTGCCAGCAGATCTTCA  22  lus-miR167a  Linum usitatissimum
  pmi-miR167b  TGAAGTGCTGCCAGCAGATCTTCA  23  cme-miR167b  Cucumis melo
  pmi-miR167c  TGAAGTGCTGCCAGCAGATCTTCA  21  aly-miR167c  Arabidopsis lyrata
pmi-miR167c-5p TAAGCTGCCAGCAGTACCTTA 21 aly-miR167c-5p Arabidopsis lyrata
pmi-miR167d TAAGCTGCCAGCAGTACCTGA 22 ath-miR167d Arabidopsis thaliana
pmi-miR167f-5p TAAGCTGCCAGCAGTACCTCTA 23 ata-miR167f-5p Aegilops tauschi
pmi-miR167h TAAGCTGCCAGCAGTACCTTAA 22 mdm-miR167h Malus domestica

168 pmi-miR168 TCGCTTTGAGCTTAGTCGGAA 21 atr-miR168 Amborella trichopoda
pmi-miR168a TCGCTTTGAGCTTAGTCGGAAA 23 cca-miR168a Cynara cardunculus
pmi-miR168a-3p CCGCCTTGTCACTACATGACA 22 aly-miR168a-3p Arabidopsis thaliana
pmi-miR168b-3p CCGCCTTGTCACTACATGACAA 22 sly-miR168b-3p Solanum lycopersicum
pmi-miR168c-5p TCGCTTTGAGCTTAGTCGGATA 22 bra-miR168c-5p brassica rapa

169 pmi-miR169f TAGCCAGGAGTACTTGCCGGA 22 mes-miR169f Monhot esculenta
pmi-miR169h AGGCAGACTCTTAGACTGACTA 21 aly-miR169h-3p Arabidopsis lyrata
pmi-miR169i TAGCCAGGAGTACTTGACATTAA 22 aly-miR169i Arabidopsis lyrata

171 pmi-miR171 TGATTGAGCTAGCCACATATCA 22 ccl-miR171 Citrus clementina
pmi-miR171a TGATTGAGCTAGCCACATATCA 18 csi-miR171a Citrus sinensis
pmi-miR171c-3p TGGAGCTAGCCACATATCA 19 aly-miR171c-3p Aegilops tauschi
pmi-miR171c-5p TGGAGCTAGCCACATATCA 22 gma-miR171c-5p Oryza sativa
pmi-miR171d TGGAGCTAGCCACATATCA 22 bna-miR171d Brassica napus

172 pmi-miR172a AGAATCTTGTAGATGCTCAGTA 23 lja-miR172a Lotus japonicas
pmi-miR172a-3p AGAATCTTGTAGATGCTCAGTA 21 csi-miR172a-3p Citrus sinensis
pmi-miR172b AGAATCTTGTAGATGCTCAGTA 22 vvi-miR172b Vitis vinifera
pmi-miR172c GGAGCATTCAAGATCCTCACA 21 aly-miR172c Arabidopsis lyrata
pmi-miR172d AGAATCTTGTAGATGCTCAGTA 23 gma-miR172d Glycine max
pmi-miR172d-5p GGAGCATTCAAGATCCTCACA 23 stu-miR172d-5p Solanum tuberosum
pmi-miR172f AGAATCTTGTAGATGCTCAGTA 23 nta-miR172f Nicotiana tabacum
pmi-miR172h GCAGACCATCAAGATCCTCACA 21 gma-miR172h-3p Glycine max
pmi-miR172i AGAATCTTGTAGATGCTCAGTA 23 nta-miR172i Nicotiana tabacum
pmi-miR172m AGAATCTTGTAGATGCTCAGTA 23 mdm-miR172m Malus domestica

319 pmi-miR319 TTGGACTGAAGGAGCTCCCTA 22 aqc-miR319 Aquilegia caerulea
pmi-miR319a TTGGACTGAAGGAGCTCCCTA 21 ppt-miR319a Physcomitrella patens
pmi-miR319b TTGGACTGAAGGAGCTCCCTA 23 mdm-miR319b Malus domestica
pmi-miR319c TTGGACTGAAGGAGCTCCCTA 22 ppt-miR319c Physcomitrella patens
pmi-miR319c-3p TTGGACTGAAGGAGCTCCCTA 21 mtr-miR319c-3p Medicago truncatula
pmi-miR319e TTGGACTGAAGGAGCTCCCTA 23 ppt-miR319e Physcomitrella patens
pmi-miR319i TTGGACTGAAGGAGCTCCCTA 21 ptc-miR319i Populus trichocarpa

390 pmi-miR390a-5p AAACCTAGGAGGATAGCCCA 22 aly-miR390a-5p Arabidopsis lyrata
pmi-miR390b AAGCTACGAGGATAGCCCA 23 ppt-miR390b Physcomitrella patens
pmi-miR390d AAAGCTACGAGGATAGCCCA 22 gma-miR390d Glycine max

391 pmi-miR391-5p TTTCGAGGAGCGATGCCC 22 ath-miR391-5p Arabidopsis thaliana

393 pmi-miR393 TCCAAAGGATCGATGATCTA 23 ghr-miR393 Gossypium hirsutum
pmi-miR393a-5p TCCAAAGGATCGATGATCTA 22 ath-miR393a-5p Arabidopsis thaliana
pmi-miR393c-3p ATCATGCTATCTGTGGAGT 22 gma-miR393c-3p Glycine max
| PMI-miR394 | TTGGCATCTGGTCCATCTCCA | 21 | cca-miR394 | Cynara cardunculus |
| PMI-miR394a | TTGGCATTCTGCTACCCTCTTA | 23 | vvi-miR319a | Vitis vinifera |
| PMI-miR394b-5p | TTGGCATCTGGTCCACCTCCTTA | 22 | ptc-miR394b-5p | Populus trichocarpa |
| PMI-miR395 | CGTAAGCCTTTGCGGAACACG | 21 | ppt-395 | Physcomitrella patens |
| PMI-miR395a | TGAAGCTTTTGGGGGAACTCCA | 22 | sly-miR395a | Solanum lycopersicum |
| PMI-miR395b | TGAAGCTTCTGGGAAGACTGGA | 22 | tea-miR395b | Triticum aestivum |
| PMI-miR395d | TGAAGCTTTTGGGGAAACTCTA | 22 | reo-miR395d | Ricinus communis |
| PMI-miR396 | TCCACAGCTTTCCAGTGA | 23 | aau-miR396 | Acacia auriculiformis |
| PMI-miR396a | TGTGTTATTTTTGGGAACTTGT | 19 | vvi-miR396a | Vitis vinifera |
| PMI-miR396b-3p | TCCACAGCTTTCCAGTGA | 20 | zma-miR396b-3p | Zea mays |
| PMI-miR396b-5p | TCCACAGCTTTCCAGTGA | 20 | cca-miR396b-5p | Arabidopsis thaliana |
| PMI-miR396c | TTAATAATTTTTGGGAACTTGT | 21 | ath-miR396c | Arabidopsis thaliana |
| PMI-miR396e-3p | TTAATAATTTTTGGGAACTTGT | 19 | cme-miR396e-3p | Cucumis melo |
| PMI-miR397 | TTGGTCCACAGTCCCTTTGGA | 22 | pab-miR397 | Picea abies |
| PMI-miR397a | TGTGTTCTCTCTGCTTTTGGGAACTTGT | 21 | bna-miR397a | Brassica napus |
| PMI-miR397b | TGTGTTCTCTCTGCTTTTGGGAACTTGT | 21 | cme-miR397b | Oryza sativa |
| PMI-miR397c | TGTGTTCTCTCTGCTTTTGGGAACTTGT | 22 | mes-miR397c | Oryza sativa |
| PMI-miR398 | TGTGTTGCTCCGAGAAAAGGAAGAGCAACG | 22 | aly-miR398 | Arabidopsis lyrata |
| PMI-miR398a | TGTGTTGCTCCGAGAAAAGGAAGAGCAACG | 22 | cme-miR398a | Arabidopsis thaliana |
| PMI-miR398b-3p | TGTGTTGCTCCGACCACTT | 21 | mes-miR398b-3p | Arabidopsis thaliana |
| PMI-miR398f | TGTGTTGCTCCGAGAAAAGGAAGAGCAACG | 22 | cme-miR398f | Arabidopsis thaliana |
| PMI-miR399 | TGTGTTGCTCCGAGAAAAGGAAGAGCAACG | 22 | mes-miR399 | Arabidopsis thaliana |
| PMI-miR408 | TGCAATGAGAGACAGACGGGAA | 22 | cca-miR408 | Cynara cardunculus |
| PMI-miR408-3p | TGCAATGAGAGACAGACGGGAA | 22 | ath-miR408-3p | Arabidopsis thaliana |
| PMI-miR530b | TGTGTTGCTCCGAGAAAAGGAAGAGCAACG | 21 | cme-miR530b | Arabidopsis thaliana |
| PMI-miR535 | TGCAATGAGAGACAGACGGGAA | 22 | ath-miR535 | Arabidopsis thaliana |
| PMI-miR535a | TGCAATGAGAGACAGACGGGAA | 22 | mes-miR535a | Arabidopsis thaliana |
| PMI-miR535b | TGCAATGAGAGACAGACGGGAA | 22 | mes-miR535b | Arabidopsis thaliana |
| PMI-miR535d | TGCAATGAGAGACAGACGGGAA | 21 | cme-miR535d | Arabidopsis thaliana |
| PMI-miR828a | TGGTCTTCGACCACTT | 21 | vvi-miR828a | Vitis vinifera |
| PMI-miR833a-5p | TGTTTGTGCTCTCGTCA | 19 | ath-miR833a-5p | Arabidopsis thaliana |
| PMI-miR845a | CGCGTCTGATACAAATTGTTA | 21 | ath-miR845a | Arabidopsis thaliana |
| PMI-miR845c | CGCGTCTGATACAAATTGTTA | 23 | ath-miR845c | Arabidopsis thaliana |
| PMI-miR845d/e | CGCGTCTGATACAAATTGTTA | 23 | ath-miR845d/e | Arabidopsis thaliana |
| PMI-miR858 | TGGTATGCTCTGCTTCGATTTACC | 22 | vvi-miR858 | Vitis vinifera |
| PMI-miR858a | TGGTATGCTCTGCTTCGATTTACC | 22 | vvi-miR858a | Vitis vinifera |
| PMI-miR1127b-3p | ACACTATGTGTTGACGAGAGCAAGGAGG | 23 | tae-miR1127b-3p | Triticum aestivum |
| PMI-miR1128 | CACTACCTCCGTCCTCAAAAA | 21 | ssp-miR1128 | Saccharum sp. |
| PMI-miR1436 | ATATGGAACCGAGGAGGGA | 20 | hvu-miR1436 | Hordeum vulgare |
| PMI-miR1439 | TTTTGGGAAACCGAGGAGGGA | 19 | osa-miR1439 | Oryza sativa |
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-miRN1974-3p   | ACTCCCTCTGGTTCACCA                   | 18 |    | 5'       | -28.6 | 60.27   | 39.73   | 39.18 | 1.01 |
| pmi-miRN1983-3p   | TAAACCTTTTTGGAACAGGGGA               | 23 |    | 3'       | -29.5 | 62.37   | 37.63   | 31.72 | 1.19 |
| pmi-miRN1992-3p   | TGTcAGAAcTAAGTGTGGGGGA               | 22 |    | 3'       | -43.4 | 60.47   | 39.53   | 25.23 | 1.57 |
| pmi-miRN2003-3p   | TTGTATcTAGGGcTcATAAGATA              | 23 |    | 3'       | -46.5 | 57.89   | 42.11   | 34.96 | 1.20 |
| pmi-miRN2013-3p   | GTGcTcTcTcTcATTGTcATA               | 20 |    | 3'       | -57.2 | 56.31   | 43.69   | 55.53 | 0.99 |
| pmi-miRN2023-3p   | TGGTAGATGTGcTTGTcAAGcA              | 22 |    | 3'       | -35.7 | 48.39   | 51.61   | 40.78 | 1.34 |
| pmi-miRN2033-3p   | CCCCAGGATGAGTGCTCTCCCA              | 22 |    | 3'       | -59.0 | 47.55   | 52.45   | 41.26 | 1.27 |
| pmi-miRN2043-3p   | cATTTcTGGTGGTAGcTcATA              | 21 |    | 5'       | -19.9 | 63.01   | 36.99   | 27.26 | 1.36 |
| Name               | Sequence                | LM  | LP  | ΔG  | AMFE | MFEI |
|--------------------|-------------------------|-----|-----|-----|------|------|
| pmi-miRNew-11      | CCGGAAGAGGCTGAGCAAGGA   | 22  | 103 | -57.5 | 50.49 | 49.51 | 55.83 | 0.89 |
| pmi-miRNew-12      | TGAATTTGTGTTGGAATAGA    | 18  | 83  | -18.6 | 73.49 | 26.51 | 12.77 | 2.08 |
| pmi-miRNew-13      | TGATTTTGGAAGGAGAGTATA   | 23  | 82  | -18.8 | 67.07 | 32.93 | 16.83 | 1.96 |
| pmi-miRNew-14      | GTGCCTCTCTCTATTGTCAA    | 20  | 123 | -48.7 | 56.91 | 43.09 | 39.59 | 1.09 |
| pmi-miRNew-15      | GTTGGTTAATTGTTGGACAGCA | 21  | 123 | -35.3 | 50.41 | 49.59 | 28.70 | 1.73 |
| pmi-miRNew-16      | GGAATTATGCTGTAATCGCA    | 21  | 123 | -22.2 | 67.48 | 32.52 | 18.05 | 1.80 |
| pmi-miRNew-17      | TTCTGATTGTTGTAATATCCA  | 22  | 93  | -59.7 | 59.14 | 40.86 | 64.19 | 0.85 |
| pmi-miRNew-18      | GCTGAGATTTGAAAGGCTTTTA | 23  | 103 | -29.4 | 67.96 | 32.04 | 28.54 | 1.12 |
| pmi-miRNew-19      | CTGGTTGCTTTGCTCTTTA    | 18  | 82  | -27.0 | 50.00 | 50.00 | 32.93 | 1.52 |
| pmi-miRNew-20      | TCCACTCTCAACACCAA       | 18  | 83  | -28.6 | 44.58 | 55.42 | 24.58 | 1.61 |
| pmi-miRNew-21      | AAGGGTAAACGGAATATCGA    | 23  | 114 | -34.7 | 72.81 | 27.19 | 30.44 | 0.89 |
| pmi-miRNew-22      | AAGAAAGATCAGGGATGAGATTA | 23  | 83  | -20.4 | 61.45 | 38.55 | 24.58 | 1.57 |
| pmi-miRNew-23      | TTGATTGAAATGGcTGTATA    | 22  | 63  | -23.2 | 58.73 | 41.27 | 36.83 | 1.12 |
| pmi-miRNew-24      | ATGGACAGCCTCTATTGGCA    | 21  | 83  | -21.9 | 54.22 | 45.78 | 26.39 | 1.74 |
| pmi-miRNew-25      | TTGCAGAGATGTCGGGATCAA  | 21  | 63  | -18.3 | 55.56 | 44.44 | 24.29 | 1.83 |
| pmi-miRNew-26      | CGAGCGAAGACCTTGGGACA    | 21  | 103 | -43.1 | 53.40 | 46.60 | 41.84 | 1.11 |
| pmi-miRNew-27      | CGTGTATCGTGGGATATA     | 19  | 63  | -33.6 | 50.79 | 49.21 | 53.33 | 0.92 |
| pmi-miRNew-28      | GACAGGACCTTGGAAATGAGCA | 21  | 93  | -24.1 | 49.46 | 50.54 | 25.91 | 1.95 |
| pmi-miRNew-29      | TCAAACAGGGAGTACACTA    | 21  | 123 | -49.5 | 66.67 | 33.33 | 40.24 | 0.85 |
| pmi-miRNew-30      | TGGAATTTGAGCCACACGATA  | 21  | 113 | -31.6 | 51.33 | 48.67 | 27.96 | 1.74 |
| pmi-miRNew-31      | CCGGAAGACCTAGAGCTA     | 18  | 83  | -24.7 | 57.83 | 42.17 | 29.76 | 1.42 |
| pmi-miRNew-32      | GATTTACGGGAACTGAGCTA   | 20  | 83  | -29.2 | 50.60 | 49.40 | 35.18 | 1.40 |
| pmi-miRNew-33      | CAGAGGTAAATCGTACTTGGCA | 23  | 83  | -20.0 | 60.24 | 39.76 | 24.10 | 1.65 |
| pmi-miRNew-34      | TGCTCAATGCTAGCAACTCA   | 21  | 103 | -50.0 | 54.37 | 45.63 | 48.54 | 0.94 |
| pmi-miRNew-35      | CTGTGAATCAGAGGGGCA     | 19  | 143 | -70.9 | 60.84 | 39.16 | 49.58 | 0.99 |
| pmi-miRNew-36      | AGTTCACCAATGGCAGCTGGA  | 21  | 113 | -60.2 | 49.56 | 50.44 | 53.27 | 0.95 |
| pmi-miRNew-37      | GTCTGTTATACATTGGTAAA   | 21  | 93  | -21.1 | 68.82 | 31.18 | 22.69 | 1.37 |
| pmi-miRNew-38      | CCAAATCTGATTATCCTGCA   | 21  | 173 | -53.6 | 51.45 | 48.55 | 30.98 | 1.57 |
| pmi-miRNew-39      | TTCTGATTGAAATATGACTA   | 22  | 133 | -55.1 | 59.40 | 40.60 | 41.43 | 0.98 |
| pmi-miRNew-40      | AGAGATGTTGGCTAAGCGAAGA | 21  | 133 | -56.1 | 60.90 | 39.10 | 42.18 | 0.93 |
| pmi-miRNew-41      | CGATCTGTATGAAGAATCTGTA | 22  | 123 | -59.8 | 59.35 | 40.65 | 48.62 | 0.85 |
| pmi-miRNew-42      | AATGTCGAATTTTGGCAGCA   | 18  | 63  | -24.6 | 55.56 | 44.44 | 39.05 | 1.14 |
| pmi-miRNew-43      | CTCGAGAGGAGCAACAGATA   | 21  | 153 | -28.0 | 61.44 | 38.56 | 18.30 | 2.11 |
| pmi-miRNew-44      | AGAGATGTAATGAGACCA     | 19  | 123 | -29.6 | 57.72 | 42.28 | 24.07 | 1.76 |
| pmi-miRNew-45      | TTTTACTGTTGTCAACTA     | 19  | 72  | -18.2 | 62.50 | 37.50 | 22.50 | 1.67 |
| pmi-miRNew-46      | ACAGAGACGCTGGGCGGTA    | 19  | 83  | -36.7 | 55.42 | 44.58 | 44.22 | 1.01 |
| pmi-miRNew-47      | AGCTATGGTTGTCTCAACA    | 21  | 103 | -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-miRN-01 | ![Diagram](image1) |
| pmi-miRN-02 | ![Diagram](image2) |
| pmi-miRN-03 | ![Diagram](image3) |
| pmi-miRN-04 | ![Diagram](image4) |
| pmi-miRN-05 | ![Diagram](image5) |
| pmi-miRN-06 | ![Diagram](image6) |
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.

![Venn diagram showing the common and specific sequence of significantly regulated miRNA in ABA and MeJA libraries](image)

**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 | GCTCTCTGTGCTTCTGTGCTCA | -∞                     | 7.79  |      |
| pmi-miR156j | TTGACAGAGAGAGACAGTA | -                     | ∞     |      |
| pmi-miR157d | TGCTCTCTGTGCTTCTGTAC | -                     | ∞     |      |
| pmi-miR159  | TGGATGGAAGGGAGCTCTA | -9.70                 | 7.79  |      |
| pmi-miR159a | TGGATGGAAGGGAGCTCTACA | -                     | ∞     |      |
| pmi-miR160a | TGCTCTGCTCCCTGTATGCTTA | -                     | ∞     |      |
| pmi-miR162  | TCGATAACCTCTGCATCTCA | -∞                    | ∞     |      |
| pmi-miR165a/b | TCGGACAGGCTGCATCCCA | -∞                    | -     |      |
| pmi-miR166  | TCGGACAGGCTGCATCCCA | -∞                    | -     |      |
| pmi-miR166a | GAATGGTGTCTTGCTGAGGA | -6.40                 | ∞     |      |
| pmi-miR166b | CCGGACAGGCGCTTCATCCCA | -∞                    | -     |      |
| pmi-miR166c | TCGGACAGGCTGCATCCCA | -∞                    | -     |      |
| pmi-miR166d | TCGTACAGGCTTCATCCCA | -∞                    | -     |      |
| pmi-miR167a | TAAGCTGCACGATGATCAGCA | -13.58               | ∞     |      |
| pmi-miR167b/d | TAAAGCTGCTAGCATGATCCTGA | -                    | -     |      |
| pmi-miR168  | TCGTTTGCTGCAGGCTGGGAA | -∞                    | -     |      |
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).

| miRNA    | Score | ID transcript         | Target annotation               |
|----------|-------|-----------------------|----------------------------------|
| pmi-miR168b | 2.8   | comp53688_c0_seq1     | Photosystem II                   |
| pmi-miR169i/j/l | 2.8   | comp59110_c2_seq1     | Agrogenate dehydratase           |
| pmi-miR172a | 2.2   | comp53137_c1_seq1     | SPL                              |
| pmi-miR319 | 3.0   | comp53825_c0_seq1     | F-box protein CPR30              |
| pmi-miR319b/d/e | 3.2   | comp59318_c1_seq1     | Probable ion channel POLLUX      |
| pmi-miR390 | 3.8   | comp48954_c1_seq1     | 60S ribosomal protein L14-2      |
| miRNA          | expression (fold change) | target ID             | gene or protein function                                    |
|---------------|--------------------------|-----------------------|-------------------------------------------------------------|
| pmi-miR159    | 1.0                      | comp57600_c3_seq1     | Transcription factor GAMYB                                  |
| pmi-miR159a   | 1.0                      | comp57600_c3_seq1     | Transcription factor GAMYB                                  |
| pmi-miR160a   | 0.8                      | comp55762_c0_seq1     | Auxin response factor                                       |
| pmi-miR67380_c0_seq2 | 2.0      |                        | Putative disease resistance protein RGA3                    |
| pmi-miR166a   | 0.0                      | comp62276_c1_seq14    | Vacuolar protein sorting-associated protein 35A             |
| pmi-miR166b   | 2.5                      | comp62276_c1_seq14    | 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/c | 2.0                 | comp57600_c3_seq1     | Transcription factor GAMYB                                  |
| pmi-miR319b/d/c | 2.0                 | 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-miR399a/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                                                        |
| pmi-miR6478   | N/A                      | N/A                   | Proteasome subunit beta type-2-A                            |
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.
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 hmg1 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).

REFERENCES
Abd Hamid, N.A., Zainal, Z. & Ismail, I. 2018. Two members of unassigned type of short-chain dehydrogenase/reductase superfamily (SDR) isolated from Persicaria minor show response towards ABA and drought stress. Journal of Plant
Samad, A.F.A., Rahnamaie-Tajadod, R., Sajad, M., Jani, J., Murad, A.M.A., Noor, N.M. & Ismail, I. 2019. Regulation of terpenoid biosynthesis by miRNA in Persicaria minor induced by Fusarium oxysporum. BMC Genomics 20(1): 586.

Samad, A.F.A., Nazaruddin, N., Murad, A.M.A., Jani, J., Zainal, Z. & Ismail, I. 2018. Deep sequencing and in silico analysis of small RNA library reveals novel miRNA from leaf Persicaria minor transcriptome. 3 Biotech 8(3): 136.

Samad, A.F.A., Sajad, M., Nazaruddin, N., Fauzi, I.A., Murad, A.M.A., Zainal, Z. & Ismail, I. 2017. MicroRNA and transcription factor: Key players in plant regulatory network. Frontiers in Plant Science 8: 565.

Shigeto, J. & Tsutsumi, Y. 2016. Diverse functions and reactions of class III peroxidases. New Phytologist 209(4): 1395-1402.

Suzuki, M., Kamide, Y., Nagata, N., Seki, H., Ohyama, K., Kato, H., Masuda, K., Sato, S., Kato, T., Tabata, S., Yoshida, S. & Muranaka, T. 2004. Loss of function of 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (HMG1) in Arabidopsis leads to dwarfing, early senescence and male sterility, and reduced sterol levels. The Plant Journal 37(5): 750-761.

Tholl, D. 2015. Biosynthesis and biological functions of terpenoids in plants. Advances in Biochemical Engineering/ Biotechnology 148: 63-106.

Ul Hassan, M.N., Zainal, Z. & Ismail, I. 2015. Green leaf volatiles: Biosynthesis, biological functions and their applications in biotechnology. Plant Biotechnology Journal 13(6): 727-739.

War, A.R., Sharma, H.C., Paulraj, M.G., War, M.Y. & Ignacimuthu, S. 2011. Herbivore induced plant volatiles: Their role in plant defense for pest management. Plant Signaling & Behavior 6(12): 1973-1978.

Wu, H.J., Ma, Y.K., Chen, T., Wang, M. & Wang, X.J. 2012. PsRobot: A web-based plant small RNA meta-analysis toolbox. Nucleic Acids Research 40: 22-28.

Yan, C. & Xie, D. 2015. Jasmonate in plant defence: Sentinel or double agent? Plant Biotechnology Journal 13(9): 1233-1240.

Yang, D., Ma, P., Liang, X., Wei, Z., Liang, Z., Liu, Y. & Liu, F. 2012. PEG and ABA trigger methyl jasmonate accumulation to induce the MEP pathway and increase tanshinone production in Salvia miltiorrhiza hairy roots. Physiologia Plantarum 146(2): 173-183.

Ye, J., Zhang, Y., Cui, H., Liu, J., Wu, Y., Cheng, Y., Xu, H., Huang, X., Li, S., Zhou, A., Zhang, X., Bolund, L., Chen, Q., Wang, J., Yang, H., Fang, L. & Shi, C. 2018. WEGO 2.0: A web tool for analyzing and plotting GO annotations, 2018 update. Nucleic Acids Research 46(W1): W71-W75.

Zhang, B.H., Pan, X.P., Cox, S.B., Cobb, G.P. & Anderson, T.A. 2006. Evidence that miRNAs are different from other RNAs. Cellular and Molecular Life Sciences CMLS 63(2): 246-254.

Zhang, B., Pan, X. & Stellwag, E.J. 2008. Identification of soybean microRNAs and their targets. Planta 229(1): 161-182.

Ye, J., Zhang, Y., Cui, H., Liu, J., Wu, Y., Cheng, Y., Xu, H., Huang, X., Li, S., Zhou, A., Zhang, X., Bolund, L., Chen, Q., Wang, J., Yang, H., Fang, L. & Shi, C. 2018. WEGO 2.0: A web tool for analyzing and plotting GO annotations, 2018 update. Nucleic Acids Research 46(W1): W71-W75.

Zhang, B.H., Pan, X.P., Cox, S.B., Cobb, G.P. & Anderson, T.A. 2006. Evidence that miRNAs are different from other RNAs. Cellular and Molecular Life Sciences CMLS 63(2): 246-254.

Zhang, B., Pan, X. & Stellwag, E.J. 2008. Identification of soybean microRNAs and their targets. Planta 229(1): 161-182.