miRNAs play important roles in aroma weakening during the shelf life of ‘Nanguo’ pear after cold storage

Fei Shi¹, Xin Zhou¹, Miao-miao Yao, Zhuo Tan, Qian Zhou, Lei Zhang, Shu-juan Ji*

Department of Food Science, Shenyang Agricultural University, Shenyang 110866, PR China

Running title: miRNAs play important roles in aroma loss

¹These authors contributed equality to this study.

Fei Shi: 2604826491@qq.com; Xin Zhou: 573259732@qq.com; Miao-miao Yao: 342458541@qq.com; Zhuo Tan: 2545761354@qq.com; Qian Zhou: 309504752@qq.com; Lei Zhang: 573572011@qq.com.

*Corresponding author: Dr Shujuan Ji, Department of Food Science, Shenyang Agricultural University, No. 120 Dongling Road, Shenyang 110866, P.R. China. Tel: +86 (0) 24 88498337; Fax: +86 (0) 24 88498337.

E-mail: jsjsyau@yeah.net; jsjsyau@sina.com

Figures number: 5; Tables number: 4; Word count: 4639; Pages number: 23

Submission Date: 15 Januray 2017

22 Abstract

23 Cold storage is commonly employed to delay senescence in ‘Nanguo’ pears after harvest.
 24 However, this technique also causes fruit aroma weakening. MicroRNAs play important roles in
 25 plant development and in eliciting responses to abiotic environmental stressors. In this study, the
 26 miRNA transcript profile of the fruit at the first day (C0, LT0) move in and out of cold storage and
 27 the optimum tasting period (COTP, LTOTP) during shelf life at room temperature were analyzed,
 28 respectively. More than 300 known miRNAs were identified in ‘Nanguo’ pears; 176 and 135
 29 miRNAs were significantly differentially expressed on the C0 vs. LT0 and on the COTP vs.
 30 LTOTP, respectively. After prediction the target genes of these miRNAs, *LOX2S*, *LOX1_5*, *HPL*,
 31 and *ADH1* were found differentially expressed, which were the key genes during aroma formation.
 32 The expression pattern of these target genes and the related miRNAs were identified by RT-PCR.
 33 Mdm-miR172a-h, mdm-miR159a/b/c, mdm-miR160a-e, mdm-miR395a-i, mdm/ppe-miR399a,
 34 mdm/ppe-miR535a/b, and mdm-miR7120a/b negatively regulated target gene expression. These
 35 results indicate that miRNAs play key roles in aroma weakening in refrigerated ‘Nanguo’ pear and
 36 provide valuable information for studying the molecular mechanisms of miRNAs in the aroma
 37 weakening of fruits due to cold storage.

38 **Keywords:** ‘Nanguo’ pears; Low temperature storage; Aroma weakening; High-throughput
 39 sequencing; Differentially expressed miRNAs; Target genes.

40 **Abbreviations:** RT, room temperature; LT, low temperature; OTP, optimum tasting period; SSC,
 41 soluble solid content; miRNAs, microRNAs; LOX, lipoxygenase; HPL, hydroperoxide lyase;
 42 ADH, alcohol dehydrogenase; AAT, alcohol acyltransferase; FC, fold change; RT-qPCR, reverse
 43 transcription quantitative PCR.

44 Introduction

45 ‘Nanguo’ pear (*Pyrus ussuriensis* Maxim.), which is mainly produced in Liaoning Province, China,
 46 has soft flesh, a rich aroma, dietary fiber, sugar, protein, and various other nutrients when ripe. Its
 47 fruits are usually consumed fresh and are believed to provide health benefits. During the optimal
 48 taste period (OTP) of the fruit at room temperature (RT, $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$), the pear has soft and smooth
 49 flesh, a sour-sweet taste, and an attractive aroma. Together with fruit color, shape, total soluble
 50 solid content, and titratable acidity, aroma is an important factor affecting fruit quality and final
 51 consumer acceptance (Infante et al., 2008). The aroma of ‘Nanguo’ pear is mainly due to aroma
 52 esters (Ji et al., 2012). The fruit ripens and rapidly deteriorates at RT after harvest and thus has a
 53 short shelf life. Low temperature (LT, $0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) is an effective method for prolonging storage
 54 life by slowing fruit ripening and reducing decay. However, fruit aroma also weakens with cold
 55 storage, which in turn affects its quality and commodity value, especially following long-term LT
 56 storage (Zhou et al., 2014; Shi et al., 2017).

57 MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs (18–24 nt in length) that
 58 mainly negatively regulate gene expression at the post-transcriptional level by binding to
 59 complementary sequences within target mRNAs (Jones-Rhoades et al., 2006; Mallory and
 60 Vaucheret, 2006; Meyers et al., 2008). MiRNA binding leads to either cleavage-induced
 61 degradation of the mRNA or suppression of its translation. In plants, the complementarity between
 62 miRNAs and their targets is very high, and miRNAs play critical roles in the regulation of plant
 63 growth (Mallory et al., 2004), development (Chen, 2004), and responses to abiotic stresses such as
 64 high salinity (Liu et al., 2008), high or low temperature (Zhang et al., 2009; Lv et al., 2010). LT
 65 storage is known as a major abiotic stress that affects plant development and growth and impacts

66 aroma production (Josine et al., 2011; Zhou et al., 2014).

67 High-throughput degradome library sequencing has provided a new strategy for validating
68 splicing targets at the whole-genome level and has been successfully employed in identifying
69 various miRNAs and their target genes in plants (Addo-Quaye et al., 2008; Bartel, 2004a; Song et
70 al., 2016; Xu et al., 2012, 2013). High-throughput sequencing has identified numerous critical
71 miRNAs, such as miR156, miR159, miR160, miR164, miR172, miR319, miR393, miR394,
72 miR395, miR399, and miR408, that are expressed in response to abiotic stresses in plants
73 (Jones-Rhoades et al., 2006; Schommer et al., 2008; Stief et al., 2014; Sun, et al., 2015; Zhang et
74 al., 2016). MiR156/157 negatively regulates the MADS-RIN to induce the transcription of
75 ripening-related genes in tomato (Dalmay 2010), and the known ripening regulators, the CNR and
76 AP2 TFs, were also identified as targets of miR156 and miR172, respectively (Chung et al., 2008;
77 Karimi et al., 2016). In addition, miRNAs negatively regulate their targets in *Arabidopsis* roots
78 after infection with cyst nematodes, leading to the downregulation of miRNAs (Xing et al., 2010).
79 In *Arabidopsis thaliana*, *Populus trichocarpa*, and other plant species, miR156, miR159, miR172,
80 and miR394 exhibit altered expression under cold stress (Liu et al., 2008; Lu et al., 2008; Zhou et
81 al., 2008). To date, more than 30,000 miRNAs have been identified, but the function of most
82 miRNAs remain unclear; miRNAs have not been reported in ‘Nanguo’ pear and their functions in
83 fruit aroma weakening after cold storage remain unknown.

84 In our previous study, to understand the biological functions of miRNAs and to investigate
85 the mechanism of fruit aroma weakening after LT storage, 12 mRNA libraries of ‘Nanguo’ pear at
86 RT and after storage were constructed and sequenced on an Illumina HiSeq 4000 platform, and
87 numerous mRNAs and TFs involved in aroma formation were identified. In the present study, to

88 understand the role of miRNAs on fruit aroma weakening in response to cold storage, 12 miRNA
89 libraries (at 0 d and OTP at RT and after LT storage, respectively, with three biological replicates
90 each) from ‘Nanguo’ pears were constructed and sequenced on an Illumina Hiseq 4000 platform.
91 Compared to the RT fruit, 176 miRNAs and 135 miRNAs were differentially expressed in
92 refrigerated fruit at 0 d and at OTP, respectively. Prediction of the target genes of the differentially
93 expressed miRNAs showed that these are related to aroma weakening after LT storage, and their
94 possible roles were analyzed.

95 **Materials and Methods**

96 **Plant materials and treatment**

97 ‘Nanguo’ pears were harvested at a commercial orchard in Anshan, Liaoning Province, China and
98 transported to the laboratory on the day of harvest. Fruits without visible signs of defects or decay
99 were selected and randomly divided into two groups (N = 400 fruits in each group), and stored at
100 RT for 5 d premature. The fruits were subpackaged in 0.04-mm thick polyethylene (PE) bags to
101 maintain a relative humidity of about 80%–85%, and then one group was stored at 20°C and
102 monitored at time points 0 d, 3 d, 6 d, 9 d, and 12 d. The other group was stored at 0°C; the bag
103 was opened for precooling 24 h, then the bag was and stored for 150 d, and subsequently
104 transferred to RT and monitored at time points 0 d, 3 d, 6 d, 9 d, and 12 d. The fruit samples on the
105 first day (C0, LT0) move in and out cold storage, and on the OTP (COTP, LTOTP) during shelf
106 life were as the sequencing samples. The test comprised three replicates of 30 fruits for each
107 treatment. The fruit peels of each sample at various time points were immediately frozen in liquid
108 nitrogen and stored at -80°C.

109 **Evaluation of the fruit ripening**

110 Fruit firmness was measured with a texture analyzer using a 2-mm plunger tip (TA-XT2i Plus,
111 Stable Micro System, UK). The test rate was 3 mm·s⁻¹ to a 5-mm depth on opposite sides of the
112 equator of 18 fruits after removal of a slice of skin. Ethylene was collected from the box
113 headspace and analyzed using a gas chromatograph (CP-3800, Varian, USA) equipped with a
114 flame ionization detector (the injector, detector, and oven temperatures were 110°C, 140°C, and
115 90°C, respectively). Four fruits from each treatment were weighed and placed in the 1.2-L
116 fresh-keeping box for 5 h at RT. Soluble solid content (SSC) were measured using a digital
117 hand-held refractometer (PAL-13810, Atago, Tokyo, Japan) and expressed as percentage (%). The
118 test was performed using the flesh of 18 fruits from the two opposing sides.

119 **miRNA Illumina sequencing and library construction**

120 MiRNAs was extracted from fruit samples on the day move in and out of the cold storage (C01-03,
121 LT01-03) and at OTP (COTP1-3, LTOTP1-3), with three biological replicates. Extraction was
122 performed using an miRNA isolation kit (CWBIO, Beijing, China) following the instructions
123 provided by the manufacturer. RNA quality was monitored by gel electrophoresis and an
124 OD₂₆₀/OD₂₈₀ ratio. High-quality RNA samples were used for miRNA library preparation and
125 massive sequenced by an HiSeq 4000 SBS kit (300 cycles) (Illumina, San Diego, CA, USA).
126 Multiplexing was applied during sequencing. Libraries were constructed according to TruSeqTM
127 Small RNA sample prep kit (Illumina, USA). Briefly, total RNA was purified by electrophoretic
128 separation on a 15% TBE-urea denaturing polyacrylamide gel electrophoresis (PAGE) gel, and
129 miRNA regions corresponding to the 18–32 nt bands in the marker lane were excised and
130 recovered. Then, the 18–32 nt miRNAs were ligated to a 5'-adaptor and a 3'-adaptor sequentially
131 (TruSeqTM Small RNA sample prep kit). The adapter-ligated miRNAs were subsequently

transcribed into cDNA and then PCR amplified for 12 cycles using the adaptor primers. The PCR products were purified for high-throughput sequencing.

Analysis of miRNA sequencing data

The raw data were assessed in terms of quality and base count. Then, the raw data were extracted from unknown and low-quality base sequences and then screened in terms of length. The remaining data were then designated as clean, small RNA sequences, and their length distribution and base preference were analyzed using *Fastx-Toolkit* (http://hannonlab.cshl.edu/fastx_toolkit/). To analyze the conserved miRNAs, unique small RNAs were aligned with mature plant miRNAs from miRBase. Using the Rfam (12.1, <http://Rfam.sanger.ac.uk/>) database to annotate the measured small RNAs, the non-miRNA sequences were identified using BLASTn (<http://blast.ncbi.nlm.nih.gov/>). The miRNA sequences were compared to reference genomes using Bowtie (<http://bowtie-bio.sourceforge.net/index.shtml>). Small maize RNA sequences with one mismatched bases were identified as known plant miRNAs.

Identification of differently expressed miRNAs and prediction of their target genes

Differentially expressed miRNAs were analyzed using the software DESeq2. Transcripts per million (TPM) reads were used to evaluate the relative expression levels of each miRNA in four small RNA libraries. If the read count of a given miRNA was zero, then the TPM was modified to 0.01 and excluded from analysis of differential expression. Differences in fold-change among the four libraries were calculated using the following equation: Fold-change = $\log_2$ (FC). The miRNAs with FCs > 1 or < -1 and *p*-values ≤ 0.05 were respectively considered upregulated or downregulated in response to low temperature.

Target gene predictions were performed based on the criteria described by Enright et al.

(2003). Previous reports have shown that miRNAs are capable of binding to both coding sequences (CDs) and the 3' untranslated regions (3'UTRs) of a transcript, regulating the expression of the target gene (Bartel, 2004b; Ren and Yu, 2012).

RT-PCR analysis of differentially expressed miRNAs and their target genes

'Nanguo' pears were sampled on 0 d, 3 d, 6 d, 9 d, and 12 d of shelving at room temperature and after cold storage for RT-PCR of miRNAs and their target genes. MiRNA and RNA were extracted using an miRNA isolation and OminiPlant RNA kit according to the manufacturer's instructions. The miRNAs and RNA were subsequently converted into cDNA using an miRNA and RNA cDNA synthesis kit (CWBIO, Beijing, China), which was then used as template for RT-PCR. RT-PCR amplification and analysis were performed using QuantStudio 5 Flex (Life Technologies, USA) with a miRNA and RNA RT-PCR assay kit. The RT-PCR conditions were based on the instructions of the RealMasterMix kit (SYBRGreen, CWBIO, Beijing, China). The levels of miRNA and target genes were calculated using the $2^{-\Delta\Delta C_t}$ method, with the actin gene (PuActin, JN684184) used as internal control (Zhou et al., 2014). Three biological repeats were used in the RT-PCR assay. The primer sequences used in this study are shown in Table 4.

Statistical analysis

All data were analyzed by one-way ANOVA. Mean separations were performed by Duncan's multiple range test at the 5% level, standard errors ($\pm$ SE) were calculated using Microsoft Excel (3 replicates; Redmond, WA, USA).

Results

Maturity of 'Nanguo' pears during postharvest storage

Fruit maturity was evaluated by measuring the firmness, ethylene production, and SSC during

shelf life at RT and after cold storage, as well as changes in fruit aroma relative to stages of maturation (Ji et al., 2012). Table 1 shows that the firmness values of ‘Nanguo’ pears after release from LT storage for 150 d were significantly lower than those recorded on the day before cold storage was initiated, and firmness rapidly decrease to below that at RT, which both decreased during shelving. A 6.5-7.5 N firmness value was considered indicative of moderate softness and the optimal time for tasting ‘Nanguo’ pears (Zhou et al., 2014; Shi et al., 2017). Ethylene production was significantly higher after cold storage for 150 d ($P < 0.05$); it peaked on the sixth day, which was three days earlier than that observed in fruits stored at RT. No changes in TSS during shelving were observed in the two fruit groups, which initially increased, peaking on at 9 d and 6 d at RT and LT, respectively, and then subsequently decreased. The results indicated that rate of fruit ripening increased after cold storage, and the OTP was on the 9 d and 6 d in fruits stored at RT and LT, respectively.

Sample collection and overview of the miRNA sequencing of ‘Nanguo’ pears

To investigate the function of miRNAs in aroma weakening in ‘Nanguo’ pears during cold storage, fruits stored at RT and LT were sampled on the first day and at OTP, respectively. Twelve libraries (C01-03, COTP1-3, LT01-03, and LTOTP1-3) of small RNAs were constructed for the samples by Illumina sequencing. Table 2 shows that an average of 16,490,952 raw reads were obtained from the 12 libraries of the small RNA sequence of ‘Nanguo’ pears. After quality control of the raw reads, 7.95-19.4 million clean-read distinct sequences were obtained, with sizes ranging from 18 nt to 32 nt; with two major peaks at 21 nt and 24 nt (Figure 1A). The number of reads for the 21-nt sequences was higher in the RT samples; however, the reads of the 24-nt was higher in the LT samples. As shown in other plants, the 21-nt and 24-nt miRNAs may play a role in maintaining

198 genome integrity and stabilization via heterochromatin formation.

199 Identification of known miRNAs

200 After removing the rRNAs, snRNAs, tRNAs, and non-miRNA sequences, approximately
201 656,407,32 reads were matched in the all 153,641,399 reads, and 292,720,11 reads (19.05%) were
202 matched to miRNAs (Figure 1B). To identify known miRNAs in the 12 libraries of ‘Nanguo’
203 pears, these sequences were mapped to the genome of *Prunus persica* and *Malus domestica* using
204 bowtie, allowing one mismatch with less than two substitutions to known miRNAs from miRBase.
205 By querying the remaining sequences against the currently known plant precursor or mature
206 miRNA sequences in miRBase21 (<http://www.mirbase.org/>), more than 300 known miRNAs
207 belonging to 46 miRNA families were identified in ‘Nanguo’ pear that mainly matched those of *M.*
208 *domestica* and *P. persica*. Comparison of family sizes showed that the number of miRNA
209 members widely varied among miRNA families. The number of members in each miRNA family
210 in ‘Nanguo’ pear ranged from 1 to 380, with families miR156, miR166, MIR172, and miR395
211 showing the highest number of members (Figure 1C).

212 Identification of the differentially expressed miRNAs in response to LT storage

213 To identify differentially expressed miRNAs related to aroma formation during cold storage, the
214 miRNAs identified in the LT0 and LTOTP groups were compared to those of the C0 and COTP
215 groups, whereas those of the C0 and COTP groups were used as control. The number of
216 differentially expressed miRNAs of the CO vs. COTP and LT0 vs. LTOTP ‘Nanguo’ pears were
217 analyzed, using those from the C0 and LT0 groups as control. Figure 2B shows that 12 miRNAs,
218 including mdm-miR396g/f, mdm-miR397a/b, mdm-miR398b/c, ppe-miR398b/a-3p, and
219 mdm-miR408b/c/d were downregulated, and 14 miRNAs consisting of ppe/mdm-miR858,

mdm-miR168a/b, mdm-miR156t/u/v/w, ppe/mdm-miR394a/b, and mdm-miR827 were upregulated between C0 and COTP. However, 36 miRNAs, including mdm-miR156a-o, ppe-miR156c/d/e, mdm-miR1511, mdm-miR172a-h, ppe-miR169f-j, and ppe/mdm-miR858 were upregulated, and only four miRNAs, including mdm-miR399a/b/c and ppe-miR399b, were downregulated in LT0 vs. LTOTP (Figure 2C). These results show that miRNAs are significantly differentially expressed during aroma formation before and after cold storage. Compared to the miRNA expression in fruits stored at RT, 117 miRNAs, including mdm-miR167b-g, mdm-miR162a/b, mdm-miR172a-o, ppe/mdm-miR394a/b, mdm-miR399a/b/c, mdm-miR164b-f, mdm-miR160a-e, mdm-miR7120a/b, mdm-miR7121a/b/c, mdm-miR159a/b, mdm-miR156t/u/v/w, mdm-miR396b, mdm/ppes-miR395a-f, mdm-miR171a/b/f/h, mdm-miR167i/j, and mdm/ppes-miR535a were upregulated, and 59 miRNAs, including ppe/mdm-miR166a-i, mdm-miR482a-5p, ppe-miR398b, mdm-miR171i, mdm-miR156a-o, mdm-miR398b/c, mdm-miR397a/b, mdm-miR408a/b/c/d, and mdm-miR159c were downregulated between C0 and LT0 (Figure 2D), and 108 miRNAs, including ppe-miR398b, mdm-miR172a-o, mdm-miR167b-g, mdm-miR156t/u/v/w, mdm/ppes-miR394a/, mdm-miR159a/b, mdm/ppes-miR395b-i, mdm-miR160a-e, mdm-miR7120a/b, mdm/ppes-miR535a, mdm-miR164b-f, mdm-miR2118a/b/c, and mdm-miR168a/b were upregulated, and 27 miRNAs consisting of ppe/mdm-miR166a-i, mdm-miR408a, mdm-miR393a/b/c, mdm-miR7121d-g, mdm-miR398b/c, and mdm-miR319a/b were downregulated between COTP and LTOTP (Figure 2E). These findings show that the miRNA expression is significantly altered during cold storage, particularly mdm-miR172a-o, mdm-miR167b-g, mdm/ppes-miR395a-i, mdm-miR160a-e, mdm-miR7120a/b, mdm-miR159, mdm-miR164b-f, and mdm-miR396b, which were only significantly upregulated after LT storage.

242 Identification of the target genes of the differentially expressed miRNAs

243 MiRNAs regulate their target genes, some miRNAs may have multiple target genes, and the same
244 target gene may also be regulated by multiple miRNAs. Several target genes of the differentially
245 expressed miRNAs were identified, and some of the target genes were key enzyme genes of aroma
246 formation such as *LOX2S*, *LOXI_5*, *HPL*, and *ADHI*. As shown in Figure 3, *LOX2S* may be
247 regulated by mdm-miR535a/b, mdm-miR160a/b/c, mdm-miR156a-o/t-w, mdm-miR408a-d,
248 mdm-miR159a/b/c, mdm-miR168a/b, mdm-miR172a-o, mdm-miR394a/b, mdm-miR395a-h,
249 mdm-miR396a/c/e, and mdm-miR7120a/b. *LOXI_5* may be regulated by mdm-miR408b/c/d,
250 mdm-miR159a/c, mdm-miR399a/b/d, mdm-miR7120a/b, mdm-miR169b, and
251 mdm-miR172c/e/f/g/h. *HPL* is regulated by mdm-miR7120a/b. *ADHI* may be mainly regulated by
252 mdm-miR394a/b, mdm-miR395a-h, mdm-miR827, mdm-miR156a-o/t-w, mdm-miR168a/b, and
253 mdm-miR408b/c/d. The four target genes are involved in the fatty acid metabolism pathway, and
254 are key genes in aroma formation. Other target genes included signal transduction genes in the
255 plant hormone signal transduction pathway and transcription factors in response to chilling injury.
256 Correlation analysis indicated that the *LOX2S* gene is mainly regulated by mdm-miR160a-e,
257 mdm-miR172a-h, mdm-miR535a/b, mdm-miR394a/b, mdm-miR395a-i, and mdm-miR159a/b/c;
258 the *LOXI_5* gene is mainly regulated by mdm/ppe-miR399a and mdm-miR159a/b/c; and the
259 *ADHI* gene is mainly regulated by mdm-miR156a-o (Tables 3a and b).

260 RT-PCR expression profiling of differentially expressed miRNAs and their target genes

261 To understand the role of various miRNAs in aroma weakening in 'Nanguo' pears during cold
262 storage, the expression pattern of key genes related to aroma formation, namely, *LOX2S*, *LOXI_5*,
263 *HPL*, and *ADHI*, were analyzed by RT-PCR (Figure 4A, 4B, 4C, 4D), simultaneous with those of

264 miRNAs mdm-miR7120a/b, mdm-miR172a-h, mdm/ppe-miR394a/b, mdm-miR395a-h,
265 mdm-miR535a, mdm-miR160a/b/c, mdm-miR156a-o, mdm-miR408a-d, mdm-miR159a-c, and
266 mdm/ppe-miR399a. Figure 4 shows that compared to fruits stored at RT, the *LOX2S* and *HPL*
267 genes were significantly downregulated on the day these were moved out of cold storage, and the
268 *LOX2S* and *LOXI_5* genes were significantly downregulated at the OTP. And mdm-miR172a-o,
269 mdm-miR395a-h, mdm/ppe-miR394a/b, mdm/ppe-miR399a, mdm-miR159a-c,
270 mdm-miR160a/b/c, mdm-miR7120a/b and mdm-miR535a were significantly upregulated, and
271 mdm-miR408a-d was significantly downregulated in the fruits compared to that stored at RT.
272 MiRNAs negatively regulate the expression of target genes; therefore, mdm-miR172a-h,
273 mdm-miR159a/b/c, mdm-miR160a-e, mdm-miR395a-h, mdm/ppe-miR399a,
274 mdm/ppe-miR535a/b, and mdm-miR7120a/b may play important roles in aroma weakening in
275 ‘Nanguo’ pears after cold storage by regulating these target genes.

276 Discussion

277 ‘Nanguo’ pears are characterized by aroma esters, which influence their commercial value. Studies
278 have shown that maturity and senescence are critical factors affecting the abundance of aroma
279 compounds in fruits (Lara, 2003), and that these processes induce the synthesis and degradation of
280 aroma volatiles (Manriquez et al., 2007; Tietel et al., 2011). ‘Nanguo’ pears evolve through the
281 stages of ripening, full ripening, and overripening during postharvest RT and LT storage. To
282 ensure consistency in maturation stage at OTP before and after cold storage, the present study
283 assessed fruit maturity under various storage conditions in relation to maturation. Here, firmness,
284 ethylene production, and SSC were used as indices of fruit maturity during storage at room
285 temperature and after cold storage. The results showed that fruit firmness was lower than that

286 before cold storage, and ethylene production and the SSC increased upon removal from cold
287 storage. Fruits stored at RT reached the OTP on the 9th day, and the cold storage fruits reached the
288 OTP three days earlier, whereas the stages in fruit maturity on the OTP with RT and after cold
289 storage showed no significant differences.

290 Cold storage is an important method of maintaining fruit quality during postharvest storage.
291 However, the fruits are subjected to chilling injury after long-term storage at LT. Chilling injury
292 also induces aroma weakening, browning of the pericarp, and a reduction in shelf life (Zhou et al.,
293 2014; Sheng et al., 2016; Wang et al., 2017). Previous studies have shown that cold storage
294 reduces aroma production in peaches, tomatoes, and mangoes (Brian et al., 2015; Velu et al., 2017;
295 Zhang et al., 2011). In the present study, RT was used as the control temperature, and LT was
296 employed as the treatment temperature of 'Nanguo' pears to study the effect of LT storage on the
297 expression of miRNAs that are related to fruit aroma volatile synthesis. The current study showed
298 that 150 d of LT storage accelerates fruit ripening and senescence in 'Nanguo' pears. Furthermore,
299 the aroma ester levels of the fruits were significantly lower during the OTP after cold storage than
300 those at RT. These findings provide additional information on the mechanism underlying aroma
301 weakening, which was previously associated with changes in the expression of regulatory genes
302 related to aroma formation (Zhou et al., 2014; Shi et al., 2017).

303 Aroma volatiles are biosynthesized mainly through a sequence of enzyme reactions involving
304 the lipoxygenase (LOX) pathway (Schwab et al., 2008). LOX is involved in the biosynthesis of
305 9-hydroperoxide and 13-hydroperoxide, which are derived from C6 and C9 aldehydes and
306 catalyzed by HPL. These aldehydes are reduced to the corresponding alcohols by alcohol
307 dehydrogenase (ADH) (EI Hadi et al., 2013). The availability of alcohols and acyl-CoA, and the

properties of alcohol acyltransferases (AATs), determine the composition of volatile esters in fruits. AAT transfers acyl-CoA into the corresponding alcohol, and esters are generated by ester linkages (Tieman et al., 2006). *PuLOX1*, *PuLOX8*, *PuADH3*, and *PuAAT* may play important roles in aroma ester formation during pear ripening (Li et al., 2014). The roles of the *LOX*, *HPL*, *ADH*, and *AAT* genes in aroma weakening in ‘Nanguo’ pears during storage at RT after LT storage have been identified (Zhou et al., 2014; Shi et al., 2017). In the present study, the key genes *LOX2S* and *HPL* were significantly downregulated in fruits on the day they were removed from cold storage, and the *LOX2S*, *LOX1_5*, and *ADH1* genes were significantly downregulated in the fruits at the OTP after cold storage compared to those at the OTP at RT, and these genes showed no significant changes during aroma formation in fruits stored at RT. Moreover, the four key genes were predicted as the target genes of known significantly differentially expressed miRNAs in ‘Nanguo’ pears after cold storage.

In recent years, high-throughput sequencing technology has been widely used to identify miRNAs in plants, opening up a new area for research investigations on various molecular mechanisms. LT stress, a common abiotic stress that severely affects normal plant growth, development, and quality, has also received increasing attention, and significant progress has been made in understanding the role of miRNAs in response to LT stress (Zhang et al., 2009; Zhang et al., 2014). MiRNAs have been increasingly studied in response to LT stress in the past few years. However, no studies involving ‘Nanguo’ pears have been conducted to date. In the current study, to identify LT-responsive miRNAs related to aroma weakening in ‘Nanguo’ pears, four miRNA libraries of the fruit were established, which consisted on average of 10,878,552 (C0), 10,675,515 (COTP), 17,058,667 (LT0), and 12,601,065 (LTOTP) clean reads that were generated by

high-throughput sequencing. Additionally, the differentially expressed miRNAs in fruits stored in the cold were identified, and mdm-miR172a-o, mdm-miR167b-g, mdm/ppe-miR395a-i, mdm-miR160a-e, mdm-miR7120a/b, mdm-miR159mdm-miR164b-f, and mdm-miR396b were most significantly differentially expressed in the fruits after LT storage. These low temperature-related miRNAs have also been identified in other plants (Chinnusamy et al., 2007; Josine et al., 2011; Karimi et al., 2016).

MiRNAs are post-transcriptional regulators of responses to abiotic stresses in plants and have been found to be multi-responsive to multi-environmental conditions individually or together with their target genes (Liu et al., 2008; Zhang et al., 2009; Lee et al., 2010). The miRNA families and their target genes in plants responding to LT stress include miR394, miR399, miR395, miR408, miR169, miR172, miR167, miR160, and miR159 (Cao et al., 2014; Xu et al., 2013; Karimi et al. 2016). MiR169 was differentially expressed by the NFYA gene, and the expression of miR160 and miR167 was regulated by auxin-response factors (ARFs) that were related to cold stress (Chen et al., 2012; Karimi et al. 2016). MiR156 and miR172 are the most important cold-responsive miRNAs (Xing et al., 2010), and the effect of miR156 and miR172 on LT has been reported in rice, wheat, and peach (Wang et al., 2014; Zhu et al., 2011). Although different species express different sets of miRNAs that respond to LT, there is a set of core miRNAs that is shared by most species. In the current study, miRNAs that mainly include mdm-miR172a-o, mdm-miR395a-i, mdm/ppe-miR399a, mdm-miR159a-c, mdm-miR160a-e, mdm-miR7120a/b, and mdm-miR535a were uniquely significantly differentially expressed during aroma formation in ‘Nanguo’ pears after cold storage, and the predicted target genes of these significantly differentially expressed miRNAs include the key aroma formation genes *LOX*, *HPL*, and *ADH*. Furthermore, we found

that the expression of these target genes is related to these miRNAs. RT-PCR analysis indicated that mdm-miR172a-h, mdm-miR159a/b/c, mdm-miR160a-e, mdm-miR395a-i, mdm/ppe-miR399a, mdm/ppe-miR535a/b, and mdm-miR7120a/b are negatively regulated by the *LOX2S*, *LOX1_5*, *HPL*, and *ADH1* genes. Therefore, these miRNAs may be key factors in the aroma weakening of ‘Nanguo’ pears after LT storage.

In conclusion, the present study has identified differentially expressed miRNAs in response to LT during aroma formation in ‘Nanguo’ pears, which included mdm-miR172a-h, mdm-miR159a/b/c, mdm-miR160a-e, mdm-miR395a-i, mdm/ppe-miR399a, mdm/ppe-miR535a/b, and mdm-miR7120a/b. Furthermore, their predicted target genes play important roles in the aroma weakening of ‘Nanguo’ pears that were subjected to cold storage. The findings of the present study may be potentially utilized in future studies on the molecular mechanisms underlying the chilling injury of fruits subjected to cold storage. This study also provides valuable information for better understanding the roles of miRNAs in mediating aroma formation in fruits stored at LT.

Conflicts of interest: None.

Acknowledgments

The National Natural Science Foundation of China (Grant number 31570687) supported this study.

References

- Addo-Quaye C, Eshoo TW, Bartel DP, Axtell MJ.** 2008. Endogenous siRNA and miRNA targets identified by sequencing of the Arabidopsis degradome. *Current Biology* **18**, 758–762.
- Bartel DP.** 2004a. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Molecular Cells* **14**, 787–799.

374 **Bartel DP.** 2004b. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**,
375 281–297.

376 **Brian F, Fotios G, Simona M, Frans JM.** 2015. Chilling-Induced changes in aroma volatile
377 profiles in tomato. *Food Bioprocess Technology* **8**, 1442-1454.

378 **Cao X, Wu Z, Jiang F, Zhou R, Yang Z.** 2014. Identification of chilling stress-responsive tomato
379 microRNAs and their target genes by highthroughput sequencing and degradome analysis. *BMC*
380 *Genomics* **15**, 1130.

381 **Chen L, Zhang Y, Ren Y, Xu J, Zhang Z, Wang Y.** 2012. Genome-wide identification of
382 cold-responsive and new microRNAs in *Populus tomentosa* by high-throughput sequencing.
383 *Biochemical and Biophysical Research Communications* **417**, 892–896.

384 **Chinnusamy V, Zhu J, Zhu JK.** 2007. Cold stress regulation of gene expression in plants. *Trends*
385 *in Plant Science* **12**, 444–451.

386 **Chung S, Parish RW.** 2008. Combinatorial interactions of multiple ciselements regulating the
387 induction of the Arabidopsis XERO2 dehydrin gene by abscisic acid and cold. *The Plant Journal*
388 **54**, 15–29.

389 **Dalmay, T.** 2010. Short RNAs in tomato. *J. Integr. Plant Biology* **52**, 388–392.

390 **EI Hadi MA, Zhang FJ, Wu FF, Zhou CH, Tao J.** 2013. Advances in fruit aroma volatile
391 research. *Molecules* **18**, 8200–8229.

392 **Enright, Anton J.** 2003. MicroRNA targets in *Drosophila*. *Genome Biology* **5**, 1.

393 **Infante R, Farcuh M, Meneses C.** 2008. Monitoring the sensorial quality and aroma through an
394 electronic nose in peaches during cold storage. *Journal Science Food Agriculture* **88**, 2073-2078.

395 **Josine TL, Ji J, Wang G, Guan CF.** 2011. Advances in genetic engineering for plants abiotic

396 stress control. African Journal of Biotechnology **10**, 5402–5413.

397 **Karimi M, Ghazanfari F, Fadaei A, Ahmadi L, Shiran B, Rabei M, Fallahi H.** 2016. The

398 small-RNA profiles of almond (*Prunus dulcis* Mill.) reproductive tissues in response to cold stress.

399 PloS One **11**, e0156519.

400 **Jones-Rhoades MW, Bartel DP, Bartel B.** 2006. MicroRNAs and their regulatory roles in plants.

401 Annu Rev Plant Biology **57**, 19–53.

402 **Lara I, Miró RM, Fuentes T, Sayez G, Graell J, López ML.** 2003. Biosynthesis of volatile

403 aroma compounds in pear fruit stored under long-term controlled atmosphere conditions.

404 Postharvest Biology Technology **29**, 29–39.

405 **Lee H, Yoo SJ, Lee JH, Kim W, Yoo SK, Fitzgerald H, Carrington JC, Ahn JH.** 2010. Genetic

406 framework for flowering-time regulation by ambient temperature-responsive miRNAs in

407 Arabidopsis. Nucleic Acids Research **38**: 3081–3093.

408 **Lester G.** 2006. Consumer preference quality attributes of melon fruit. Acta Horti, 175–181.

409 **Li GP, Jia HJ, Li JH, Wang Q, Zhang, MJ, Teng YW.** 2014. Emission of volatile esters and

410 transcription of ethylene and aroma-related genes during ripening of ‘Pingxiangli’ pear fruit

411 (*Pyrus ussuriensis* Maxim). Scientia Horticulture **170**, 17–23.

412 **Liu HH, Tian X, Li YJ, Wu CA, Zheng CC.** 2008. Microarray based analysis of stress regulated

413 microRNAs in Arabidopsis thaliana. RNA **14**, 836–843.

414 **Llave C, Kasschau KD, Rector MA, Carrington JC.** 2002. Endogenous and

415 silencing-associated small RNAs in plants. Plant Cell **14**, 1605–1619.

416 **Lu S, Sun YH, Chiang VL.** 2008. Stress-responsive microRNAs in Populus. The Plant Journal **55**,

417 131–151.

418 **Lv DK, Bai X, Li Y, Ding XD, Ge Y, Cai H, Zhu YM.** 2010. Profiling of cold-stress-responsive
419 miRNAs in rice by microarrays. *Gene* **459**, 39–47.

420 **Manriquez D, Flores FB, El-Sharkawy I, Latche A, Pech JC.** 2007. Molecular control of fruit
421 ripening and sensory quality of Charentais melon. *Acta Hort.* 413–419.

422 **Mallory AC, Vaucheret H.** 2006. Functions of microRNAs and related small RNAs in plants. *Nat.*
423 *Genet* **38**, S31–S36.

424 **Meyers BC, Axtell MJ, Bartel B, Bartel DP, Baulcombe D, Bowman JL, Cao X, Carrington**
425 **JC, Chen X, Green PJ.** 2008. Criteria for annotation of plant MicroRNAs. *Plant Cell*, **20**,
426 3186–3190.

427 **Ren G, Yu B.** 2012. Post-transcriptional control of miRNA abundance in Arabidopsis. *Plant*
428 *Signalling and Behavior* **7**, 1443–1446.

429 **Schommer C, Palatnik JF, Aggarwal P, Chetelat A, Cubas P, Farmer EE, Nath U, Weigel D.**
430 2008. Control of jasmonate biosynthesis and senescence by miR319 targets. *PLoS Biology* **6**,
431 e230.

432 **Schwab W, Davidovich R, Lewinsohn E.** 2008. Biosynthesis of plant-derived flavor compounds.
433 *Plant Journal* **54**, 712–732.

434 **Sheng L, Zhou X, Liu ZY, Wang JW, Zhou Q, Wang L, Zhang Q, Ji SJ.** 2016. Changed
435 activities of enzymes crucial to membrane lipid metabolism accompany pericarp browning in
436 ‘Nanguo’ pears during cold storage and subsequent shelf life at room temperature. *Postharvest*
437 *Biology Technology* **117**, 1-8.

438 **Shi F, Zhou X, Zhou Q, Tan Z, Yao MM, Wei BD, Ji SJ.** 2017. Membrane lipid metabolism
439 changes and aroma ester loss in low temperature stored ‘Nanguo’ pears, *Food Chemistry*,

doi:<https://doi.org/10.1016/j.foodchem.2017.10.091>.

Song JB, Gao S, Wang Y, Li BW, Zhang YL, Yang ZM. 2016. miR394 and its target gene LCR are involved in cold stress response in Arabidopsis. *Plant Gene* **5**, 56–64.

Stief A, Altmann S, Hoffmann K, Pant BD, Scheible WR, Bäurle I. 2014. Arabidopsis miR156 regulates tolerance to recurring environmental stress through SPL transcription factors. *The Plant Cell* **26**, 1792–1807.

Sun X, Fan G, Su L, Wang W, Liang Z, Li S, Xin H. 2015. Identification of cold-inducible microRNAs in grapevine. *Frontiers in Plant Science*. <https://doi.org/10.3389/fpls.2015.00595>.

Tieman D, Taylor M, Schauer N, Fernie AR, Hanson AD, Klee HJ. 2006. Tomato aromatic amino acid decarboxylases participate in synthesis of the flavor volatiles 2-phenylethanol and 2-phenylacetaldehyde. *Proc. Natl. Acad. Science* **103**, 8287–8292.

Tietel Z, Feldmesser E, Lewinsohn E, Fallik E, Porat R. 2011. Changes in the transcriptome of 'Mor' mandarin flesh during storage: emphasis on molecular regulation of fruit flavor deterioration. *J. Agric. Food Chemistry* **59**, 3819–3827.

Velu S, Itay M, Oleg F, Dalia M, Noam A. 2017. Chilling stress upregulates α -Linolenic acid-oxidation pathway and induces volatiles of C6 and C9 aldehydes in mango fruit. *J. Agric. Food Chemistry* **65**, 632–638.

Voinnet, O. 2009. Origin, biogenesis, and activity of plant microRNAs. *Cell* **136** (4), 669–687.

Wang B, Sun YF, Song N, Wei JP, Wang XJ, Feng H, Yin ZY, Kang ZS. 2014. MicroRNAs involving in cold, wounding and salt stresses in *Triticum aestivum* L. *Plant Physiology and Biochemistry*, **80**, 90–96.

Wang, JW, Zhou, X, Zhou, Q, Cheng, SX, Wei, BD, Ji, SJ. 2017. Low temperature

462 conditioning alleviates peel browning by modulating energy and lipid metabolisms of ‘Nanguo’
 463 pears during shelf life after cold storage. *Postharvest Biology Technology* **131**, 10–15.

464 **Wang ST, Sun XL, Hoshino Y, Yu Y, Jia B, Sun ZW, Zhu YM.** 2014. MicroRNA319 positively
 465 regulates cold tolerance by targeting OsPCF6 and OsTCP21 in rice (*Oryza sativa* L.). *PloS One* **9**,
 466 e91357.

467 **Xing S, Salinas M, Hohmann S, Berndtgen R, Huijser P.** 2010. miR156-targeted and
 468 nontargeted SBP-box transcription factors act in concert to secure male fertility in *Arabidopsis*.
 469 *Plant Cell* **22**, 3935–50.

470 **Xu MY, Dong Y, Zhang QX, Zhang L, Luo YZ, Sun J, Fan YL, Wang L.** 2012. Identification
 471 of miRNAs and their targets from *Brassica napus* by high-throughput sequencing and degradome
 472 analysis. *BMC Genomics* **13**, 421.

473 **Xu XB, Yin LL, Ying QC, Song HM, Xue DW, Lai TF, Xu MJ, Shen B, Wang, HZ, Shi, XQ.**
 474 2013. High-Throughput sequencing and degradome analysis identify miRNAs and their targets
 475 involved in fruit senescence of *Fragaria ananassa*. *PLoS One* **8**, e70959.

476 **Yamaguchi-Shinozaki, K, Shinozaki, K.** 2006. Transcriptional regulatory networks in cellular
 477 responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biology* **57**, 781–803.

478 **Zhang B, Xi WP, Wei WW, Shen JY, Ferguson I, Chen KS.** 2011. Changes in aroma-related
 479 volatiles and gene expression during low temperature storage and subsequent shelf life of peach
 480 fruit. *Postharvest Biology Technology* **60**, 7-16.

481 **Zhang J, Xu Y, Huan Q, Chong K.** 2009. Deep sequencing of *Brachypodium* small RNAs at the
 482 global genome level identifies microRNAs involved in cold stress response. *BMC Genomics* **10**,
 483 449.

484 **Zhang S,Wang Y, Li K, Zou Y, Chen L, Li X.** 2014. Identification of cold responsive miRNAs
485 and their target genes in nitrogen-fixing nodules of soybean. *International Journal of Molecular*
486 *Sciences* **15**, 13596–13614.

487 **Zhang X, Wang W, Wang M, Zhang HY, Liu JH.** 2016. The miR396b of *Poncirus trifoliata*
488 functions in cold tolerance by regulating accoxidase gene expression and modulating
489 ethylene-polyamine homeostasis. *Plant Cell Physiology* **57**, 1865–1878.

490 **Zhou, X, Dong, L, Li, R, Zhou, Q, Wang, JW, Ji, SJ.** 2014. Low temperature conditioning
491 prevents loss of aroma-related esters from ‘Nanguo’ pears during ripening at room temperature.
492 *Postharvest Biology Technology* **100**, 23–32.

493 **Zhou X, Wang G, Sutoh K, Zhu JK, Zhang W.** 2008. Identification of cold inducible
494 microRNAs in plants by transcriptome analysis. *Biochimical Biophysica Acta* **1779**, 780–788.

495 **Zhu QH, Helliwell CA.** 2011. Regulation of flowering time and floral patterning by miR172.
496 *Journal of Experimental Botany* **62**, 487-495.

1 **Figure 1**

2
3 Fig. 1 A: Length distribution of sRNA reads and unique sequences in the room temperature fruit
4 (RT) and low temperature storage fruit (LT) twelve libraries constructed from 'Nanguo' pears. B:
5 Comparison the unique sequence of all samples to the Rfam database. C: Distribution of known
6 miRNA family size in 'Nanguo' pears.

7
8
9
10

Figure 2

Fig.2 The number of up-regulated and down-regulated miRNAs between the four compared groups is summarized in A, and the expression pattern of the significantly differentially expressed miRNAs in different compared libraries are shown in B, C, D and E. B: C0 VS COTP, C: LT0 VS LTOTP, D: C0 VS LT0, E: COTP VS LTOTP.

23 **Figure 3**

24
25 Fig. 3 The network map of the significantly differentially expressed miRNAs and the target genes
26 LOX2S, LOX1_5, HPL and ADH1. Red is the target genes, green is the miRNAs. LOX2S:
27 c62400_g2, c63403_g2, c65860_g1, c65968_g1, c66100_g1; LOX1_5: c54748_g2, c63599_g3;
28 HPL: c64295_g2; ADH1: c27994_g3, c66564_g1.

52 **Figure 4**
53

54
55 **Fig. 4** Expression analysis of significantly differentially expressed miRNAs and their predicted

target genes in 'Nanguo' pear by RT-PCR. The symbols * and ** in the graph show the significant differences at $p < 0.05$ and $p < 0.01$, respectively.

Table 1

Changes of firmness, ethylene and soluble solid content (SSC) during shelf life of 'Nanguo' pears at room temperature (RT) and after 150 d low temperature storage (LT).

Index		0 d	3 d	6 d	9 d	12 d
Firmness (N)	RT	16.23a	15.58a	12.48a	6.895a	3.67a
	LT	15.57a	13.63b	6.875b	4.07b	2.175b
Ethylene ($\mu\text{L}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$)	RT	12.9b	16.72b	32.45b	46.55b	24.74b
	LT	28.68a	38.27a	74.56a	58.56a	41.47a
SSC (%)	RT	11.73a	12.55a	13.18a	14.28a	13.72a
	LT	12.22a	12.95a	14.08a	13.72b	12.45b

136

137 **Table 2**

138 Summary of the raw data of all microRNAs in the room temperature fruit (C0 and COTP) and the
139 cold storage fruit (LT0 and LTOTP).

Sample-ID	Total Reads	Total Bases	Error%	Q20%	Q30%	GC%	Clean reads
C01	13303737	678490587	0.0108	99.3	97.89	51.74	15886825
C02	13070747	666608097	0.0109	99.3	97.87	51.83	13962727
C03	11614705	592349955	0.0103	99.56	98.46	52.34	7953644
COTP1	15036061	766839111	0.0107	99.38	98.08	52.23	14197822
COTP2	12337321	629203371	0.0103	99.57	98.47	52.86	19403640
COTP3	12040228	614051628	0.0103	99.57	98.46	53.43	17574539
LT01	18091065	976917510	0.0099	99.37	97.77	50.29	12454217
LT02	25532143	1378735722	0.0099	99.36	97.73	49.75	9974659
LT03	23318692	1259209368	0.01	99.3	97.61	49.87	9597669
LTOTP1	22473351	1213560954	0.01	99.33	97.65	50.12	11915780
LTOTP2	20576979	1111156866	0.0101	99.25	97.45	49.87	11503596
LTOTP3	10496389	566805006	0.01	99.31	97.61	50.02	9216281

140

141

142

143

144

145

146

147

148

149

150

151

152

153

154

155

156

157

158

159

160

161

162

163

164

165

Table 3

The correlation coefficients between the change of the significantly differentially expressed miRNAs and the change of their predicted target genes LOX2S, LOX1_5, HPL, ADH1 on the 0 d and the OTP in 'Nanguo' pears after low temperature storage.

a

C0 VS LT0	LOX2S	LOX1_5	HPL	ADH1
mdm-miR160a-e	-0.852*	-	-	-
mdm-miR172a-h	-0.833*	-0.768	-	-
mdm-miR535a/b	-0.812*	-0.761	-	-
mdm-miR394a/b	-0.842*	-	-	0.907**
mdm-miR159a/b/c	-0.883*	-0.834*	-	0.988**
mdm-miR399a/b/c	-	-0.820*	-	-
mdm-miR7120a/b	-0.823*	-0.753	-0.677	-
mdm-miR395a-i	-0.673	-0.623	-	0.817*
mdm-miR408b/c/d	0.468	0.483	-	-0.776
mdm-miR156a-o	0.711	-	-	-0.916**

b

COTP VS LTOTP	LOX2S	LOX1_5	HPL	ADH1
mdm-miR160a-e	-0.955**	-	-	-
mdm-miR172a-h	-0.881*	-0.674	-	-
mdm-miR535a	-0.842*	-0.712	-	-
mdm-miR394a/b	-0.979**	-	-	0.632
mdm-miR159a/b/c	-0.931**	-0.699	-	0.676
mdm-miR399a/b/c	-	-0.705	-	-
mdm-miR7120a/b	-0.912*	-0.677	-0.862*	-
mdm-miR395a-i	-0.980**	-0.671	-	0.766
mdm-miR408b/c/d	0.573	0.348	-	-0.694
mdm-miR156a-o	0.673	-	-	-0.816*

*, Correlation is significant at the 0.05 level, **, Correlation is significant at the 0.01 level.

182 **Table 4**

183 The primers of miRNAs for RT-PCR analysis.

Name	Primer
mdm-miR160a-e	TGCCTGGCTCCCTGTATGCCA
mdm-miR172a-h	AGAATCTTGATGATGCTGCA
mdm-miR535a	TGACAACGAGAGAGAGCACGC
mdm-miR394a/b	TTGGCATTCTGTCCACCTCC
mdm-miR159a/b/c	CTTGGATTGAAGGGAGCTCC
mdm-miR399a/b/c	TGCCAAAGGAGAATTGCCCTG
mdm-miR7120a/b	TGTTATATTGTCAGATTGTCA
mdm-miR395a-i	CTGAAGTGTTTGGGGGAAGTC
mdm-miR408b/c/d	ACAGGGAAGAGGTAGAGCATG
mdm-miR156a-o	TGACAGAAGAGAGTGAGCAC

184