

1 **MiRNAs profiling and degradome sequencing between the CMS-line N816S and**
2 **its maintainer line Ning5m during anther development in pepper (*Capsicum***
3 ***annuum* L.)**

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9

10 **Abstract**

11 Utilization of cytoplasmic male sterility (CMS) is significant for agriculture.
12 MiRNAs are a class of endogenously non-coding small RNAs (21-24 nt) that play key
13 roles in the regulation of various growth and developmental processes in plants. The
14 knowledge miRNA-guided CMS regulation is rather limited in pepper. To better
15 understand the miRNAs involvement and regulatory mechanism of CMS, miRNA
16 libraries from anther of CMS-line N816S and its maintainer line Ning5m were
17 generated by miRNAome sequencing in pepper. A total of 76 differentially expressed
18 miRNAs were detected, of which 18 miRNAs were further confirmed by quantitative
19 real-time PCR (qRT-PCR). In addition, miRNA targets were identified by degradome
20 sequencing. The result showed that 1292 targets that were potentially cleaved by 321
21 miRNAs (250 conserved miRNAs and 71 novel miRNAs). Gene Ontology (GO) and
22 KEGG pathway analysis indicated that 35 differentially expressed miRNAs might
23 play roles in the regulation of CMS sterility, by cleaving 77 target transcripts, such as
24 *MYBs*, *SPLs*, and *AFRs*, of which targeted by miR156, miR167, miRNA858 family.
25 Nineteen miRNA-cleaved targets were selectively examined by qRT-PCR, and the
26 results showed that there were mostly negative correlations between miRNAs and
27 their targets on the expression level. These findings provide a valuable information to
28 understand miRNAs mechanism during anther development and CMS occurrence in
29 pepper.

30

31 **Keywords:** CMS; miRNAome; degradome sequencing; miRNA regulation; Pepper

32 **Introduction**

33 MicroRNAs (miRNAs) are a class of endogenous non-coding small RNAs
34 (sRNAs) with 21-24 nucleotides (nt) in animals and plants(Zhou et al., 2020). In
35 plants, mature miRNA sequences were generated from primary miRNA transcripts
36 (pri-miRNA) by Dicer-like (DCL1)(Zhang et al., 2015). The mature miRNAs are

37 incorporated into the RNA-induced silencing complex (RISC) to guiding the cleavage
38 of specific complementary mRNAs(Schuck et al., 2013). MiRNAs are widely
39 considered as negative-regulated gene expression at the post-transcription or
40 translation process via degrading target mRNAs or repressing mRNA
41 translation(Stepien et al., 2017). Previous studies have manifested that miRNAs
42 involved in various plant growth and developmental processes, such as hormone
43 homeostasis, flower development, embryogenesis and stress responses(Jha and
44 Shankar, 2011;Pei et al., 2013).

45 Recently, a large number of studies have revealed miRNAs as important
46 regulators of gene expressions to be involved in the developmental transition from
47 vegetative growth to reproductive growth(Lin et al., 2013). MiR156 and miR172, two
48 prime participators in flowering regulation, have been shown to regulate the floral
49 transition in plant(Zhu and Helliwell, 2011;Yu et al., 2012;Diaz-Manzano et al., 2018).
50 MiR156 could target SQUAMOSA Promoter Binding Protein-like (SPL)
51 Transcription Factors (TF) and decreases with increasing life spans of the plant(Zheng
52 et al., 2019). Overexpression of miR156 could delay flowering and prolong vegetative
53 stage in many species, including Arabidopsis, rice and tomato(Zheng et al., 2019).
54 The miR172, which targets APETALA2-like (*AP2*) transcription factors, has the
55 opposite effect to the miR156 on the regulation of flowering time and increases with
56 phase development and induces floral organogenesis and promote flowering(Tripathi
57 et al., 2018). In addition, miR397 has been reported promoting panicle branching,
58 increasing grain size, and resulting in improving yield in rice(Zhang et al., 2013).
59 These studies showed that miRNAs play important roles in a number of
60 developmental processes and pathways which regulate flower development related
61 process.

62 Pepper (*Capsicum annuum* L.) is one of the most economically important
63 worldwide vegetable crops(Barrajon-Catalan et al., 2020). Hybrid breeding has made
64 a tremendous contribution to pepper yield, increasing seed production efficiency and
65 protection of the varieties patent(Jifon et al., 2019). The utilization of male sterility in
66 hybrid pepper is mainly based on three-line systems, which including cytoplasmic
67 male sterile line (CMS), a maintainer line and restorer line(Bohra et al., 2016). In the
68 CMS pepper, an ORF named *orf456*, was found at the 3'-end of the *coxII* gene, which
69 is concluded that the *orf456* may represent a candidate gene, from mitochondrial
70 genes, for determining the male-sterile phenotype of CMS(Kim et al., 2007).
71 Furthermore, several CMS-related sterile genes and fertility restorer genes also have
72 been cloned from various plants(Bohra et al., 2016). In Arabidopsis, miR167
73 overexpression has been reported leading to male fertility defects(Ru et al., 2006),
74 whereas miR159a overexpression results in decreased expression of *MYB33* and
75 *MYB65*, leading to male sterility and delays flowering time(Anthony, 2005). With the
76 development of miRNAome sequencing technology, differential expression patterns
77 of miRNAs between the cytoplasmic male sterility (CMS) line and its maintainer line
78 have been reported in many vegetable crops, such as Brassica juncea(Yang et al.,
79 2013), Chinese cabbage(Wei et al., 2015) and Radish(Zhang et al., 2016b). A large
80 number of miRNAs related to flowering and flower development have been identified

81 and characterized in above species. Nevertheless, there are no reports on systematic
82 identification and characterization of CMS-related miRNAs in pepper.

83 To explore the roles of miRNA in CMS, we identified the miRNAs via a
84 high-throughput sequencing approach from pepper anthers at the early uninucleate
85 stage of the sterile line N816S and its maintainer line Ning5m. Differential expression
86 patterns of miRNAs were analyzed and compared between N816S and Ning5m.
87 Targets were predicted by degradome sequencing. These results may provide insights
88 into clarification of the molecular mechanisms underlying the regulation of miRNAs
89 during pollen development.

90

91 **Materials and Methods**

92 **Pepper materials**

93 The CMS sterile line N816S and its maintainer line Ning5m were used in this
94 study. The plants were grown in greenhouse of the vegetable institute of Wuhan
95 academy of agricultural sciences under normal conditions. In general, anthers at the
96 uni-nucleate stage were manually collected. Anthers were harvested from three
97 individual plants of each cultivar, immediately frozen in liquid nitrogen, stored at
98 -80°C , and then used for RNA isolation. The microspore development was judged by
99 both the floret length as described by Parra- Vega et al (Parra-Vega et al., 2013).

100

101 **Small RNA library Construction, sequencing, and miRNA analysis**

102 Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA). RNA
103 quantity was detected by Qubit Fluorometer (Invitrogen, CA, USA), and RNA purity
104 was assayed by NanoDrop spectrophotometer (BioRad, PA, USA) and Agilent2100
105 bioanalyzer (Agilent Technologies, CA, USA). According to the manufacturer's
106 instructions, sRNA libraries were constructed using the Small RNA Sample Prep Kit
107 (Illumina, CA, US) and meanwhile the sRNA passed quality test(Yeri et al., 2018).
108 Briefly, the small RNA (18-30 nt) were ligated to a 3' adaptor and a 5' adaptor
109 sequentially and then converted to cDNA by RT-PCR. The purified cDNAs after
110 reverse transcription reaction were sequenced by the Illumina Hiseq2000 (Illumina,
111 CA, USA). After the Illumina sequencing, the raw sequences were obtained through a
112 quality control process to generate high quality reads, and clean reads were directly
113 used for further bioinformatics analysis with ACGT101-v4.2-miR (LC Sciences, TX,
114 USA) to remove adaptor sequences, junk reads, short reads, common RNA families
115 (rRNA, tRNA, snRNA and snoRNA), repeats(Jeyaraj et al., 2019). Because of lacking
116 known miRNA records of pepper in miRNA database (miRBase 21 released), unique
117 sRNA sequences (18-25 nt) were mapped to specific species precursors in the
118 miRBase 21 and the pepper reference sequence to identify conserved miRNAs by
119 BLAST search. New miRNAs were identified by extracting flanking genome
120 sequence of unique sRNAs using MIREAP (<http://sourceforge.net/projects/mireap/>)

121 (Huang et al., 2010), followed by the prediction of secondary structures by Mfold
122 program (<http://unafold.rna.albany.edu/?q=mfold>) (Reuter and Mathews, 2009).

123 **Degradome Library Construction, Data analysis and Target identification**

124 The degradome library, a mixed samples' library, was constructed according to
125 the method described previously using sliced ends of polyadenylated
126 transcripts (German et al., 2009). In brief, poly-A-containing mRNA was purified from
127 total RNA mixture of N816S and Ning5m, then ligated to 5' RNA adaptor containing
128 a *MmeI* recognition site. Subsequently, RT-PCR was performed to first-strand cDNA,
129 followed by digestion with *MmeI*, and then ligated to 3' adaptor. Finally, ligation
130 product was amplified, purified and subjected to Illumina sequencing.

131 Raw reads were performed to remove adaptor sequence and low-quality reads
132 resulting in clean reads. The high-quality specific sequences of were collected for
133 subsequent degradome tags analysis. To identified potentially sliced targets of
134 miRNAs, degradome sequence analysis were processed using the Cleveland 3.0
135 software package and the ACGT301-DGE program (LC Sciences, TX, USA) (Gong et
136 al., 2015). The tags, which mapped to sense cDNA, were used to predict cleavage
137 sites. Height of the degradome peak at each occupied transcript position was placed
138 into five possible categories.

139 **Quantitative real-time PCR (qRT-PCR) validation**

140 Total RNA was extracted from pepper anthers, and RNA-free DNase I
141 (Fermentas, USA) was used to remove DNA contamination for 15 min at 37°C.
142 Stem-loop qRT-PCR were carried out to validate differential expressional levels of
143 miRNAs. The mRNA template for the miRNA target was reverse transcribed using
144 the OligodT₂₀ primer for qRT-PCR. All miRNA detection primers were designed and
145 synthesized based on the mature miRNA sequences. For each miRNA, approximately
146 1 µg of total RNA was reverse-transcribed by reverse transcriptase using
147 miRNA-specific stem-loop primers and a Fermentas Revert Aid First Strand cDNA
148 Synthesis Kit (Fermentas, USA). Relative expression analysis of the miRNA and its
149 target were performed using the ABI Step One Plus™ Real Time PCR System
150 (Applied Biosystems, USA) and SYBR Green Master Mix (Roche, Germany). All
151 reactions were run with three individual biological replicates, and *18S rRNA* was used
152 as the internal control gene refer to Hwang et al (Hwang et al., 2013). The relative
153 expression of miRNA and mRNA were used quantified the $2^{-\Delta\Delta Ct}$ method to calculate
154 the fold change between N816S and Ning5m (Asha et al., 2016). The primers used are
155 listed in Table S1.

156

157 **Results**

158 **Overview of Small RNAs Libraries Sequencing Date**

159 To determine the involvement regulatory roles of miRNAs in the fertility of sterile
160 and maintainer lines during anther development in pepper. Six small RNA libraries,
161 including three biological replicates from N816S (N816S_1, N816S_2, N816S_3) and
162 Ning5m (Ning5m_1, Ning5m_2, Ning5m_3), were constructed for deep sequencing.
163 An average of 14,567,767 and 14,274,100 raw reads were obtained from N816S and
164 Ning5m anthers, respectively, which after length, Junk reads, Rfam, Repeat, mRNA,
165 rRNA, tRNA, snoRNA and snRNA reads filtering, an average of 10,780,898 (72.21%)
166 valid reads representing 6,882,786 (84.68%) unique sequences and 10,442,445
167 (74.25%) valid reads representing 6,666,094 (86.4%) unique reads, respectively
168 (Table 1). The proportion of the valid reads in the corresponding raw reads was more
169 than 70%, which suggested that the quality of the sequencing data was high (Table1).
170 The length of total sRNAs and ranged from 18 to 25 nt, and in both the N816S and
171 Ning5m libraries, the 24 nt category was most abundant (average of 55.83% and
172 59.92% in N816S and Ning5m libraries, respectively) (Figure 1A-B). The length of
173 unique sRNAs, in both the N816S and Ning5m libraries, the 24 nt category was most
174 abundant (average of 59.15% and 62.90% in N816S and Ning5m libraries,
175 respectively) (Figure 2C-D). This is consistent with the typical lengths of plant
176 sRNAs reported in other studies(Asha et al., 2016;Hu et al., 2016).

177 **Conserved miRNAs and novel miRNAs identified in Pepper**

178 These unique sequences were subsequently used to identify conserved and novel
179 miRNAs by alignment against miRBase (Version 22), the pepper genome and
180 expressed sequence tag (EST) or pre-miRNA sequences. Unique miRNA transcripts
181 identified from the mappable sequences are divided into five group types (Table
182 S2)(Gong et al., 2015): (1) gp1a type: Unique reads map to specific
183 miRNAs/pre-miRNAs in miRbase and the pre-miRNAs further map to the genome
184 and EST. Total 77 miRNAs from the six samples corresponding to 50 known pepper
185 pre-miRNAs (Table S2-1). (2) gp1a type: Reads map to selected (except for specific)
186 miRNAs/pre-miRNAs in miRbase and the pre-miRNAs further map to the genome
187 and EST. 122 miRNAs, which correspond to 94 known pepper pre-miRNAs, which
188 cannot be mapped to genome or EST (Table S2-2). (3) gp2b type: Unique reads map
189 to selected miRNAs/pre-miRNAs in miRbase. The mapped pre-miRNAs do not map
190 to the genome, but the reads (and of course the miRNAs of the pre-miRNAs) map to
191 genome. The extended genome sequences from the genome loci may form hairpins.
192 261 miRNAs corresponding to 314 others known miRbase plant pre-miRNAs, which
193 are mapped to genome or EST (Table S2-3). (4) gp3a type: Unique reads map to
194 selected miRNAs/pre-miRNAs in miRbase. The mapped pre-miRNAs do not map to
195 the genome, and the reads do not map to the genome; Forty miRNAs corresponding to
196 35 others known miRbase plant pre-miRNAs, which cannot be mapped to genome or
197 EST (Table S2-4). (5) gp4a: Unique reads do not map to selected pre-miRNAs in

198 miRBase, but the reads map to genome and the extended genome sequences from
199 genome may form hairpins; 411 miRNAs corresponding to 421 candidate
200 pre-miRNAs, which are predict RNA hairpins derived from genome or EST, and these
201 miRNAs are novel miRNA, which are labeled PC (pepper candidate) (Table S2-5).

202 In the present study, identified mature miRNAs are divided into two type, including
203 conserved miRNA and novel miRNA (Table 1), referring to the research of Maize(Li
204 et al., 2017). The length of the mature miRNAs ranged from 18 to 25 nt. The 24 nt
205 and 21 nt miRNA category was most abundant, 41.85% and 27.41%, respectively
206 (Figure 2A). This is consistent with the typical lengths of plant sRNAs reported in
207 other studies(Gao et al., 2016;Zhang et al., 2016a). Of the conserved miRNA 21-nt
208 miRNAs were most abundant (37.32%) (Figure 2B), representing the dominant size
209 of mature miRNAs in plants. The 5' terminal nucleotides of sRNA sequences
210 influence classification of their AGO complexes and is an important feature affecting
211 function(Schuck et al., 2013). Most miRNAs are incorporated into the AGO1 effector
212 complex, resulting in sequence specificity that either cleaves or translationally
213 represses their targets(Dalmadi et al., 2019). Therefore, we examined the 5' nucleotide
214 distribution of conserved miRNAs, and 42.49% started with uridine at 5' -end, and
215 26.17% started with adenine (Figure 2C).

216

217 **Differentially expressed miRNAs analysis between pepper sterile line N816S and** 218 **its maintainer line Ning5m**

219 MiRNAs are play an important role in plant development and apoptosis(Jovanovic
220 and Hengartner, 2006;Wang et al., 2007). To determine differential expression
221 between N816S and Ning5m anthers. MiRNA expression was normalized to
222 transcripts per million and simplified as normalized expression (Norm)(Gong et al.,
223 2015). A miRNA was considered if the Norm value was greater than one in the all
224 given replicated samples. Based on this criterion, 525 miRNAs were detected (Figure
225 3A), including a total of 350 conserved miRNAs (38.42% of the total miRNAs)
226 belonging to 55 families were observed in at least one of the group samples (Table
227 S3). In correlation analysis, Norm values of the N816S and Ning5m anthers were
228 found to be highly correlated between repeats ($r>0.99$), indicating good
229 reproducibility of the miRNAome results (Figure S1).

230 A one-tailed t-test was used to identify differentially expressed miRNAs with p
231 value < 0.05 (Xing et al., 2012). The hierarchical clustering of the differential
232 expression mRNAs was made and showed different expression patterns between the
233 N816S and Ning5m (Figure 5A). As a result, a total of 76 miRNAs (59 conserved
234 miRNAs and 17 novel miRNAs) were found to be differentially expressed between
235 the two phenotypes (Table 3). Compared with the Ning5m, 44 miRNAs (34 conserved
236 miRNAs and 10 novel miRNAs) were found to be down-regulated and 32 miRNAs
237 (25 conserved miRNAs and 7 novel miRNAs) up-regulated in the N816S (Figure 3B).
238 Nta-miR156g_L+1 and stu-miR156a-5p had high expression abundance in
239 MiRNA156 family, were down-regulated in sterile line N816S. However, miRNA390
240 family, including hex-MIR390a-p5_1ss21GT, hex-MIR390b-p5_2ss10TC21GA and

241 hex-MIR390b-p5_1ss21GT, were up-regulated in sterile line N816S. Five miRNAs
242 (stu-miR393-3p, stu-miR399j-3p_1ss21GA, nta-miR6149a_L+1R-1_1ss21GC and
243 stu-miR398b-3p ath-miR8175_L+4) were specifically expressed in Ning5m. Five
244 miRNA(nta-MIR172e-p3_2ss15CG19TA,ppe-MIR399a-p3_2ss5AT18TC,ppe-MIR39
245 9a-p5_2ss5AT18TC, stu-miR3627-5p_R-1 and sly-MIR10528-p3_2ss9GA19TC)
246 were specifically expressed in N816S. To validate conserved miRNAs identified and
247 novel miRNAs predicted, we selected 18 differential expressed miRNAs for
248 stem-loop qRT-PCR. The expression trends of these miRNAs were consistent with the
249 high-throughput sequencing results. As showed in Figure 3C, expression of these
250 miRNAs from qRT-PCR displayed a similar tendency with those from small RNA
251 sequencing.

252

253 **Targets analysis of miRNAs in anthers of Pepper.**

254 To understand the function of the mature miRNAs from anthers of Pepper,
255 targets of miRNAs were detected using degradome sequencing technology. A total of
256 34233673 (99.24%) mappable reads from raw reads were obtained, including
257 12320360(99.19%) unique mappable reads, while 20851831 (60.45%) transcript
258 mapped reads, including 6635426(53.42%) unique transcript mappable reads, were
259 obtained (Table S4). The target transcripts were sorted into five categories according
260 to the relative abundance of the tags at the target mRNA sites(Gong et al., 2015):
261 category “0” is defined as >1 raw read at the position, with abundance at a position
262 equal to the maximum on the transcript and with only one maximum on the transcript;
263 category “1”, the expected cleavage signature was equal to the maximum on the
264 transcript and more than one maximum position on the transcript; category “2”,
265 abundance at the position was less than the maximum but higher than the median for
266 the transcript; category “3”, the abundance at the position equal to or less than the
267 median for the transcript; category “4”, abundance at the position was only one raw
268 read. Figure 6 showed the typical five categories of the target transcripts.

269 A total of 1292 reliable targets (p -value <1) that were potentially cleaved by
270 321 miRNAs (250 conserved miRNAs and 71 novel miRNAs) were identified (Table
271 S5). A total of 77 target genes were identified to be targeted by 35 differentially
272 expressed miRNAs (27 conserved miRNAs and 8 novel miRNAs) (Table S6). Many
273 targets of miR156, miR167, miRNA858 families were identified as transcription
274 factors, such as SPL, ARF, and MYB, respectively, which have been experimentally
275 validated by the previous studies.

276 **Gene Ontology (GO) and KEGG Pathway Analysis of Targets.**

277 The targets were annotated using the GO annotations analysis, which is
278 commonly used to describe the function of genes and gene products, meanwhile the
279 KEGG analysis is used to provide the pathway of annotated targets. Figure 5B

280 showed the GO functional classification of miRNA targets in the pepper anthers.
281 Targets covered a broad range of functional categories. However, targets of DNA
282 binding, regulation of transcription-, and DNA-templated genes, transcription factor
283 activity-, and sequence-specific DNA binding, transcription-, and DNA-templated
284 genes were mostly enriched. This suggested that those target genes may play an
285 important role in pepper anthers. The pathways of all the miRNAs and targets
286 involved were listed (Table S7). There were 107 pathways that targets involved, while
287 carbohydrate metabolism, translation, folding sorting and degradation pathways
288 owned most targets (Figure 5C). Furthermore, the 77 miRNA targets from 35
289 differentially expressed miRNAs were found to be involved in multiple pathways,
290 including plant hormone signal transduction, purine metabolism, starch and sucrose
291 metabolism, oxidative phosphorylation, and others (Table S8).
292

293 **Expression profiles of miRNA targets examined by qRT-PCR**

294 To examine the correlation between the targets and the corresponding miRNAs,
295 the expression levels of 19 selected targets were examined by qRT-PCR analysis.
296 MiRNAome sequencing result showed that miRNA156a/g (stu-miR156a and
297 nta-miR156g_L+1), miRNA167a/h (stu-miR167a-5p, stu-miR167a-5p_1ss21AT and
298 mdm-miR167h_1ss22AT), stu-miR393-5p and nta-miR6149a_L+1R-1_1ss21GC
299 were down-regulated in N816S compare to Ning5m (Table 3). The degradome
300 sequencing results indicate that miRNA156a/g target SPL family genes (SPL3, SPL6
301 and SPL9), miRNA167a/h (aly-miR167a-5p_R+1_1ss21AT, gma-miR167a_1ss21AT,
302 mdm-miR167h_1ss22AT, stu-miR167a-5p_1ss21AT) targets an AUXIN RESPONSE
303 FACTOR gene (ARF8), MiRNA393 targets two AUXIN SIGNALING F-box
304 genes (TIR1 and AFB2) (Table S8). QRT-PCR analysis showed that the expression
305 profiles of the above miRNA targets were up-regulated in N816S (Figure 6A).

306 In contrast, miRNA159_R3, nta-MIR169j-p3_2ss8GA19CT,
307 stu-miR319b_L+1R-1, hex-MIR390a-p5_1ss21GT, ppe-MIR399a-p3_2ss5AT18TC,
308 ppe-MIR858-p5_1ss4GA, cst-MIR11334-p5_2ss9TG18TC and PC-5p-154497_29
309 were up-regulated in N816S (Table 3). MiRNA159_R3 targets a plasma membrane
310 H⁺-ATPase gene (*ACA13*), a γ -TuC Protein 3 (GCP3)-Interacting Protein gene (*GIP1*),
311 MiRNA169j-p3 targets a heat shock protein gene (*HSP70*), MiRNA319b targets four
312 TEOSINTE BRANCHED/CYCLOIDEA/PCF transcription factor genes (*TCP2*,
313 *TCP4*, *TCP24*) and a Aldehyde dehydrogenase gene (*ALDH6B2*), MiRNA390a-p5
314 targets a GDP dissociation inhibitor gene (*GDI1*), miRNA399a-p3 targets a
315 ceramide-1-phosphate transfer protein gene (*ACD11*), miRNA858-p5 targets several
316 MYB protein genes (*MYB3*, *MYB4*, *MYB12* and *MYB80*), miR11334-p5 targets a
317 phospholipase D alpha 1-like gene (*PLD1*) and a Acyl-CoA oxidase gene (*ACX1*), and
318 a novel miRNA of PC-5p-154497_29 targets a UDP-galactose-dependent
319 digalactosyldiacylglycerol synthase gene (*DGD2*) (Table S8). Most of above targets
320 were down-regulated in N816S (Figure 6B).

321 Most negative correlations were found between the expression levels of the

322 target genes and their corresponding miRNAs in anthers of the sterile line N816S and
323 its maintainer line Ning5m, implying that miRNA-mediated mRNA silencing
324 occurred during anther development.
325

326 **Discussion**

327 Several studies have shown that miRNAs regulate anther development in
328 plants (Yang et al., 2013; Gong et al., 2015). However, few studies on the relationships
329 between miRNA biogenesis and CMS occurrence during anther development in
330 pepper were very limited. MiRNAome and degradation sequencing technology
331 provides an effective way to identify and evaluate the expression profiles of miRNAs
332 and targets associated with CMS occurrence in plant tissues during anther
333 development (Wei et al., 2015; Hu et al., 2016; Bai et al., 2017). To explore the roles of
334 miRNAs during the occurrence of CMS, anthers at the early uninucleate stage were
335 used to examine the expression profiles of the miRNAs in the sterile line N816S and
336 its maintainer line Ning5m using high-throughput sequencing. To the best of our
337 current knowledge, this study is the first report on identification and characterization
338 of miRNAs, and their targets between a sterile line and its maintainer line during
339 anther development in pepper.

340 In this study, a total of 76 miRNAs (59 conserved miRNAs and 17 novel
341 miRNAs) were identified as differentially expressed between the sterile line N816S
342 and its maintainer line Ning5m. To understand the function of the miRNAs from
343 anthers of Pepper, targets of miRNAs were detected using degradome sequencing
344 technology. From those targets, 80 target genes were identified to be targeted by 35
345 differentially expressed miRNAs (27 conserved miRNAs and 8 novel miRNAs). The
346 qRT-PCR results indicated that most negative correlations were found between the
347 expression levels of the target genes and their corresponding miRNAs in the anthers
348 of the sterile line N816S and its maintainer line Ning5m. KEGG analysis showed that
349 most gene targets of the 35 differentially expressed miRNAs were involved in
350 pathways, including plant hormone signal transduction, starch and sucrose
351 metabolism, oxidative phosphorylation, purine metabolism, and others.

352 Intriguingly, a number of genes in hormone signaling were confirmed or
353 predicted as targets of miRNAs. Auxin regulates anther dehiscence, pollen maturation,
354 and filament elongation in *Arabidopsis*. The miR167-guided cleavage of auxin
355 response factor *ARF8*, miR393-guided cleavage of auxin receptor *TIR1* and *AFB2*,
356 and miR319-guided cleavage of jasmonate acid biosynthesis related transcription factor
357 genes *TCPs*, which were reported in previous study (Liu and Chen, 2009). In
358 *Arabidopsis*, loss of miR167 regulation in *mARF6* and *mARF8* expression caused
359 arrested ovule development and anther indehiscence (Wu et al., 2006). Four auxin
360 receptor-encoding genes, *TIR1*, *AFB1*, *AFB2*, and *AFB3*, are transcribed in anthers
361 only during late stages of development starting at the end of meiosis (Shimizu-Mitao
362 and Kakimoto, 2014). Up-regulation of *TIR1* enhances auxin sensitivity, and causes

363 altered leave phenotype and delayed flowering in *Arabidopsis*(Chen et al., 2011).
364 MicroRNA319-regulated TCPs can activate CO transcription and control flowering
365 time, shaping flower structure, leaf morphology, and plant architecture in
366 *Arabidopsis*(Chen et al., 2011). In this study, up-regulation *AFR8* was targeted by
367 down-regulated miRNA393a/h, up-regulated *TIR1* and *AFB2* were targeted by
368 down-regulated miR393a, and down-regulated *TCPs* (*TCP2* and *TCP4*) were targeted
369 by up-regulated miRNA393b, indicating might modulate the hormone response to
370 play roles in the pollen development and CMS occurrence.

371 Targets of these differentially expressed miRNAs containing important
372 transcription factors (TFs) and functional proteins are involved in many biological
373 processes, including signal transduction, floral organ development, and organellar
374 gene expression(Liu and Chen, 2009). For instance, miR156 targets *SPL* transcription
375 factor family, which is regulatory functions throughout the growth and development
376 stages in plants. Previous report showed that miR156 regulates the timing of flower
377 formation vis *SPL3/4/5*, activating the expression of *LEAFY*, *FRUITFULL* and
378 *APETALA*(Jung et al., 2011). In *Arabidopsis*, multiple *SPL* genes regulate cell
379 division, differentiation and can result in fertile flower(Zheng et al., 2019). In this
380 study, *SPL3*, *SPL6* and *SPL9* were identified as potentially targets of miR156 miRNA
381 family members (stu-miR156a, stu-miR156a, nta-miR156g_L+1) were identified as
382 approximately triple up-regulated in N816S compared with Ning5M (Figure 6A),
383 leading to disordered floral organ development in pepper, indicating that miR156 may
384 participate in fertility regulation. Furthermore, miR858 targets *MYB* transcription
385 factor family, which is involved in the control of plant development, determination of
386 cell fate and identity, primary, and secondary metabolism. In rice, anther and pollen
387 defect in floral organ development are found in the loss-of-function mutations of *MYB*.
388 In *Arabidopsis*, *AtMYB3*, *AtMYB4*, *AtMYB7* and *AtMYB32* encode transcriptional
389 repressors, and *AtMYB4* controls sinapate ester biosynthesis, whereas *AtMYB32*
390 regulates pollen wall composition, and *AtMYB12* is involved in the regulation of
391 flavonoid biosynthesis control flavonol biosynthesis(Dubos et al., 2010). The
392 transcriptional activity of the PtMYB4 protein is positively regulated by the
393 mitogen-activated protein kinase (MAPK) PtMAPK6 in *Pinus taeda*, which
394 phosphorylates a Ser in the C terminal activation domain. In addition, *AtMYB80*
395 regulated exine formation and acts downstream of *AtMYB35* to control anther
396 development and/ or functionalities. In this present study, up-regulated miRNA858
397 might via reducing transcript expression of *MYB3*, *MYB4*, *MYB12* and *MYB80*,
398 leading to anther and pollen defect. In addition, MIR169j targets a heat shock protein
399 *HSP70*, which has been widely involved in the protein peptides folding, assembly and
400 transports, and the degradation of abnormal proteins. Studies have found that *HSP70*
401 is associated with male sterility in plant and animal. Down regulation of *HSP70*
402 expression level prolongs the duration of heat-induced male sterility in *Drosophila*
403 *buzzatii*(Sarup et al., 2004). *HSP70* antisense RNA expression leads to male sterility
404 in rice(Liu et al., 2008). In this study, up-regulated miRNA169j may through via
405 reducing transcript expression of *HSP70*, leading to pollen abortion.

406 Many miRNA-targeted genes were involved in lipid transport and metabolism,

407 such as *PLD1/ACX1*, *ACA13/GIP*, *ALDH6*, *GDI1* and *DGD2*, which are cleaved by
408 miR11334, miR159_R-3, miR319b, miRNA390b and PC-5p-154497_29, respectively.
409 Those miRNAs may throughout their targets be involved in CMS sterile process.
410 Phospholipase D alpha 1-like (*PLD1*) has been identified as cytosolic protein, which
411 regulated cytosolic lipid droplet formation(Andersson et al., 2006). Acyl-CoA oxidase
412 (*ACX*) was the first and the key step controlling enzyme involved in fatty acid
413 β -oxidation, and mutation of *ACX1* led to petal degeneration in Chinese
414 Cabbage(Zheng et al., 2019). In plant, *ALDH6B2* encodes a methylmalonyl
415 semialdehyde dehydrogenase, of which involved in the degradation of valine to
416 propionyl CoA(Brocker et al., 2013). In addition, Plant cells have multiple plasma
417 membrane (PM)-localized calcium ATPases (*ACAs*) pumping calcium ions out of the
418 cytosol. The loss of *ACA13* combination with a reduction in function of other *ACAs*
419 leads to seedling death at bolting, revealing the essential role of their collective
420 function in plant growth(Yu et al., 2018). Moreover, the γ -tubulin complex (γ -TuC)
421 Protein 3 (*GCP3*)-Interacting Protein 1 (*GIP1*) is the smallest γ -TuC component
422 identified. In Arabidopsis, *AtGIP1* and its homologous protein *AtGIP2* mutants are
423 impaired in establishing a fully functional mitotic spindle and exhibit severe
424 developmental defects(Batzenschlager et al., 2013). The GDP dissociation inhibitor
425 protein *GDI1* relates to control vesicle number and transport in an amelioration of
426 zinc toxicity, allowing yeast to survive in the presence of toxic(Ezaki and Nakakihara,
427 2012). Furthermore, Digalactosyl-diacylglycerol (*DGD*) is one of the major lipids
428 found predominantly in the photosynthetic membrane of higher plants. *OsDGD2 β*
429 is the sole *DGDG* synthase gene highly expressed in anther, and its mutation confers
430 male sterility with pale yellow and shrunken anther, devoid of starch granules in
431 pollen, and delayed degeneration of tapetal cells in rice(Basnet et al., 2019). All above
432 related miRNAs are up-regulated in sterile line N816S compare to its maintainer
433 Ning5m and those disorder miRNA-targeted genes perhaps leading to
434 membrane-disruptive effects and cell apoptosis.

435

436 **Conclusion**

437 In the present study, small RNA libraries from anther of CMS-line N816S and its
438 maintainer line Ning5m were generated by small RNA sequencing in pepper. A total
439 of 76 differentially expressed miRNAs were discovered, of which 18 were further
440 confirmed by real-time quantitative PCR (qRT-PCR). Furthermore, targets of
441 miRNAs were identified by degradome sequencing. A total of 1292 targets that were
442 potentially cleaved by 321 miRNAs (250 conserved miRNAs and 71 novel miRNAs)
443 were identified. Gene Ontology (GO) and KEGG pathway analysis of target
444 transcripts indicated that 77 target genes cleaved by 35 differentially expressed
445 miRNAs might play roles in the regulation of CMS sterility, such as MYB, SPL, and
446 AFR family proteins targeted by miR156, miR167, miRNA858 family. Nineteen

447 targets were selectively examined by qRT-PCR, and the results showed that there was
448 a negative correlation on the expression patterns between miRNAs and their targets.
449 These findings provide valuable information to understand the roles of miRNAs
450 during anther development and CMS occurrence in pepper.

451 **Author contributions**

452 Min Zhang designed the study. Hongyuan Zhang, Shuping Huang and Jie Tan
453 carried out the experiment, data analysis, interpretation of the results. Hongyuan
454 Zhang drafted the manuscript. Xia Chen supervised the work and revised the
455 manuscript. All authors have read and approved the final version of this submission.
456

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461

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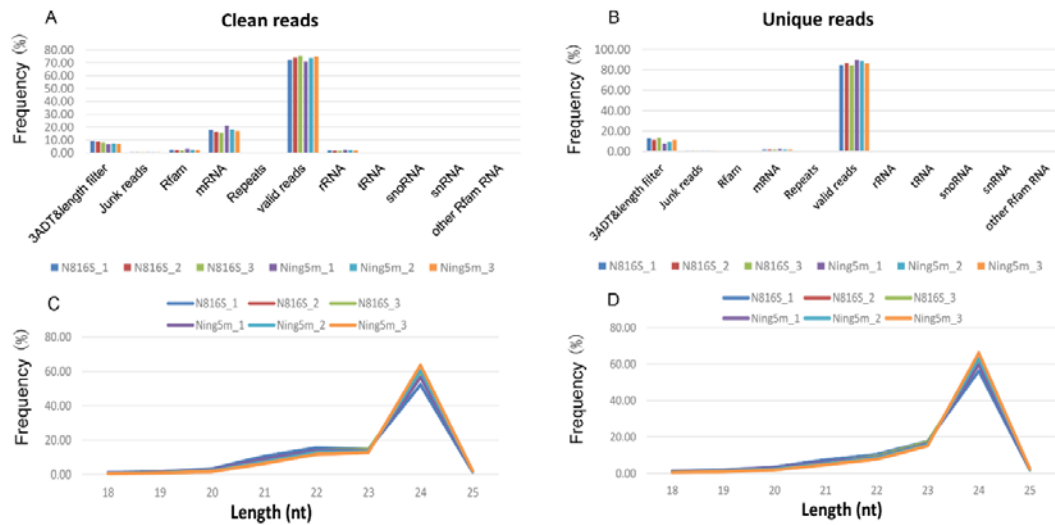
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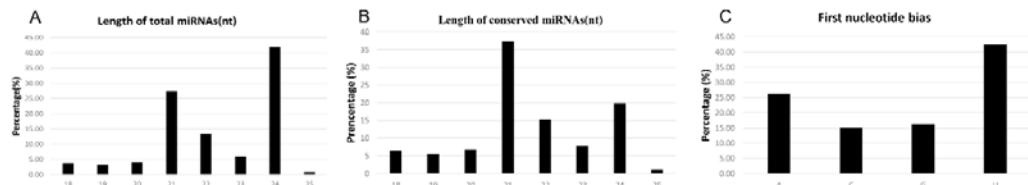
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Figures and Tables:



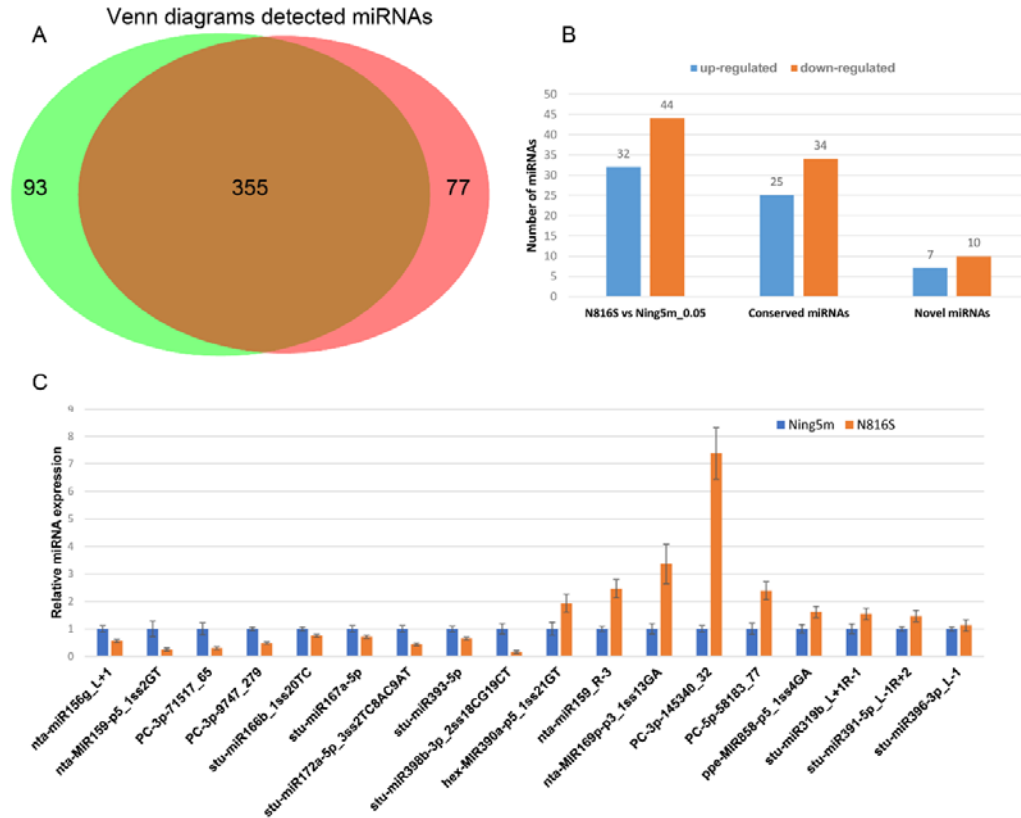
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Figure 1. Frequency percent of Clean reads (A) and Unique reads (B) sequencing reads, and the length distribution of the clean reads (C) and unique reads (D) from anther of *Capsicum annuum* L.



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Figure 2. Size distribution of miRNAs and characterization of converted miRNAs detected by deep sequencing. (A) Length distribution of the total miRNAs; (B) Distribution of obtained unique converted miRNAs (gp1-gp3); (C) Percentage of first nucleotide bias in the identified converted miRNAs(gp1-gp3).

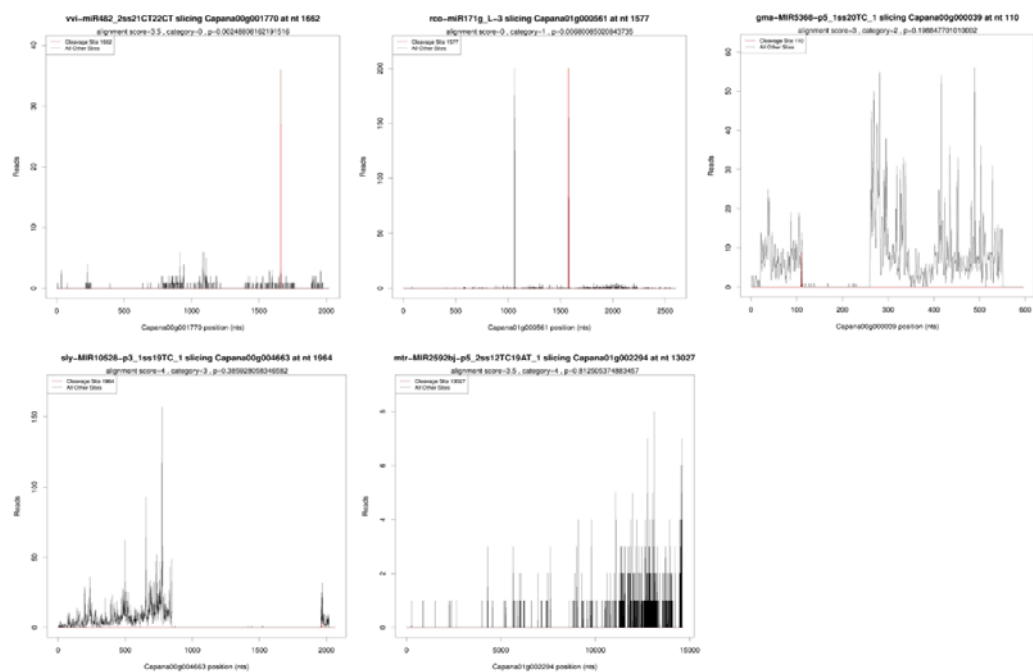


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656 Figure 3. Venn diagrams detected miRNA, the number statistics of differential
 657 expression miRNAs, and qRT-PCR for verifying deep sequencing. (A) Detected
 658 miRNAs in N816S and Ning5m; (B) The number statistics of up-regulated and
 659 down-regulated differential expression miRNAs in conserved miRNAs and novel
 660 miRNAs. (C) Detection of selected miRNA expression in N816S and Ning5m anthers
 661 using qRT-PCR.

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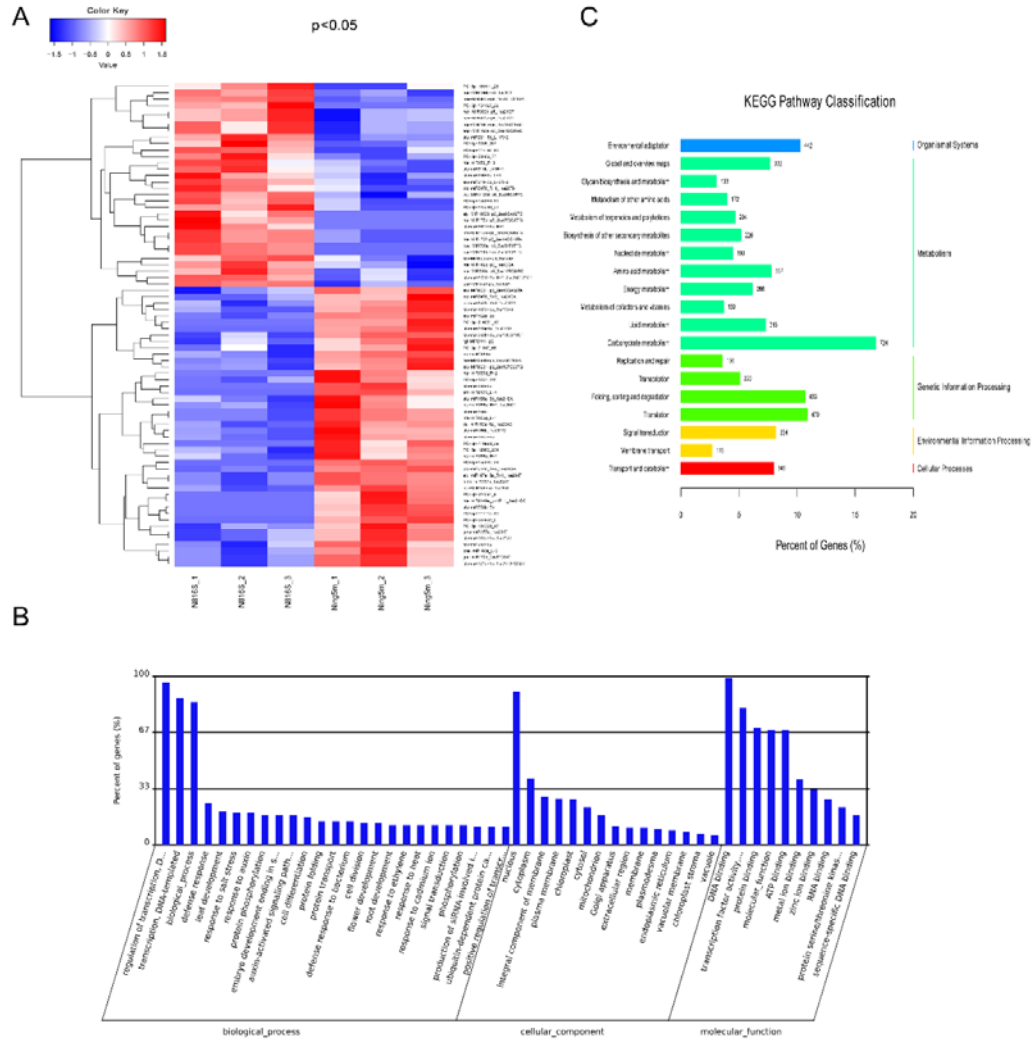
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Figure 4. Typical categories of the target transcript according to the relative abundance of the tags at the target mRNA sites.



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669 Figure 5. (A) Differentially expressed miRNAs cluster, (B) GO functional

670 classification of miRNA targets, (C) KEGG Pathway Classification; Notes: miRNAs

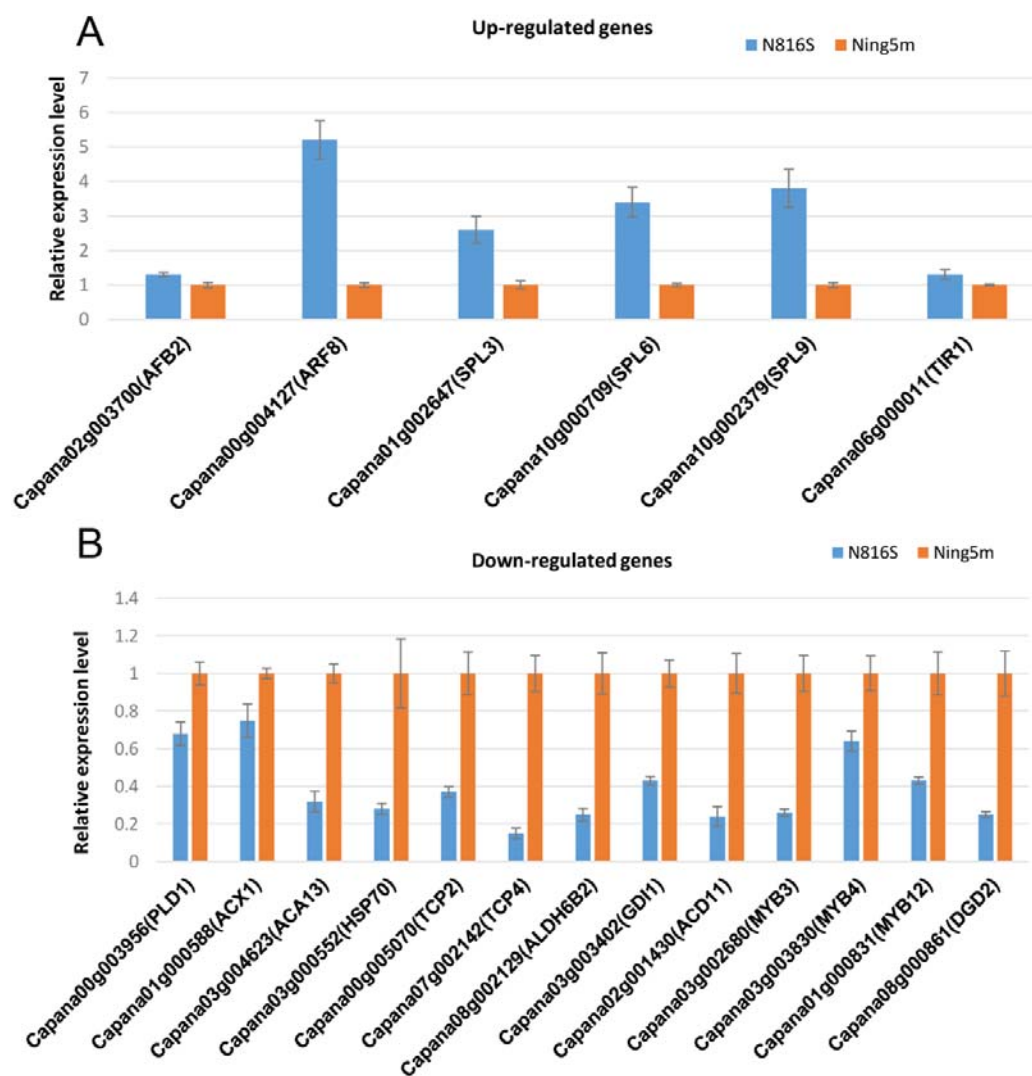
671 cluster columns are different samples; rows are different miRNAs. Clustering with

672 p -value, red is up-expressed miRNA, blue is down-expressed miRNA.

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677 Figure 6. Quantitative real-time PCR analysis of the relative expression of
678 miRNA targets in the CMS-line N816S and its maintainer line Ning5m. (A)
679 up-regulated targets in N816S; (B) down-regulated targets in N816S.

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Table 1. Overview of reads from raw data to cleaned sequences.

Category	N816S_mean		Ning5m_mean	
	Total sRNAs (%)	unique sRNAs	Total sRNAs (%)	unique sRNAs
Raw reads	14567767 (100%)	8074144 (100%)	14274100 (100%)	7568101 (100%)
		1003388		
3ADT&Length filter	1255570 (8.62%)	(12.43%)	1012608 (7.09%)	707822 (9.35%)
Junk reads	74827 (0.51%)	54461 (0.67%)	74748 (0.52%)	54565 (0.72%)
Rfam	328527 (2.26%)	10516 (0.13%)	382951 (2.68%)	11080 (0.15%)
mRNA	2437774 (16.73%)	132754 (1.64%)	2728370 (19.11%)	138920 (1.84%)
Repeats	8437 (0.06%)	306 (0.00%)	8898 (0.06%)	293 (0.00%)
	10780898	6882786	10442445	6666094
Valid reads	(74.01%)	(85.24%)	(73.16%)	(88.08%)
rRNA	254459 (1.75%)	8362 (0.10%)	323537 (2.27%)	9018 (0.12%)
tRNA	47680 (0.33%)	833 (0.01%)	32260 (0.23%)	786 (0.01%)
snoRNA	2925 (0.02%)	191 (0.00%)	2198 (0.02%)	165 (0.00%)
snRNA	2671 (0.02%)	182 (0.00%)	1554 (0.01%)	115 (0.00%)
other Rfam RNA	20793 (0.14%)	948 (0.01%)	23402 (0.16%)	997 (0.01%)

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Table 2. Summary of conserved and predicted miRNA.

Groups	Pre-miRNA	Unique miRNA	miRNA type
gp1	50	77	Conserved miRNA
gp2a	94	122	Conserved miRNA
gp2b	314	261	Conserved miRNA
gp3	35	40	Conserved miRNA
gp4	421	411	Novel miRNA

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Table 3. Summary of the differentially expressed conserved and novel miRNAs.

MiRNA family	MiRNA name	N816S(Norm)	Ning5m(Norm)	p_value(t_test)	up/down
miR156	nta-miR156g_L+1	3732.40	6752.02	0.003	down
	han-MIR156b-p3_1ss3CT	107.99	67.05	0.003	up
MIR159	stu-miR156a	3732.40	6752.02	0.003	down
	nta-MIR159-p5_1ss2GT	21.74	90.63	0.002	down
	nta-miR159_R-3	384.38	160.79	0.047	up
miR162	csi-miR159a-5p_1ss20AC	36.01	74.86	0.008	down
	stu-miR162a-3p	5177.75	7769.93	0.001	down
miR166	stu-miR166a-3p_1ss21CA	1369.44	2506.50	0.041	down
	stu-miR166b_1ss20TC	52460.31	71297.86	0.029	down
	bna-miR166a_L+2	49.73	83.04	0.013	down
	aqc-MIR166a-p3_2ss1GC20AC	26.72	19.50	0.025	up
	aqc-MIR166a-p5_2ss1GC20AC	26.72	19.50	0.025	up
	nta-miR166a_R+1	62.57	97.20	0.033	down
	nta-miR166a_R+1_1ss20CT	11.51	26.44	0.045	down

miR167	stu-miR167a-5p_1ss21AT	44.40	62.92	0.010	down
	gma-miR167a_1ss21AT	44.40	62.92	0.010	down
	aly-miR167a-5p_R+1_1ss21AT	0.97	6.41	0.001	down
	nta-miR167d_R+2	17.46	30.80	0.040	down
	mdm-miR167h_1ss22AT	0.97	6.41	0.001	down
	stu-miR167a-5p	3533.81	5033.84	0.019	down
MIR169	nta-MIR169p-p3_1ss13GA	15.55	4.98	0.033	up
MIR172	nta-MIR172e-p5_2ss14CG18TA	8.43	0.64	0.002	up
	nta-MIR172e-p3_2ss15CG19TA	5.31	0.00	0.048	up
	stu-miR172a-5p_3ss2TC8AC9AT	21.28	49.93	0.010	down
	nta-MIR172f-p5_2ss14CG18TA	8.43	0.64	0.002	up
	gra-miR172b_2ss8TC9AT	21.28	49.93	0.010	down
miR319	stu-miR319-3p_L+2R-2	25.71	11.69	0.018	up
	stu-miR319b_L+1R-1	4345.23	2869.03	0.029	up
MIR390	hex-MIR390a-p5_1ss21GT	186.32	99.94	0.030	up
	hex-MIR390b-p5_2ss10TC21GA	10.01	4.27	0.001	up
	hex-MIR390b-p5_1ss21GT	186.32	99.94	0.030	up
miR391	stu-miR391-5p_L-1R+2	726.92	508.13	0.009	up
miR393	stu-miR393-5p	84.78	133.01	0.020	down
	stu-miR393-3p	0.00	41.28	0.029	down
miR396	stu-miR396-3p_L-1	1503.77	1367.94	0.038	up
miR398	stu-miR398b-3p_2ss18CG19CT	1.86	12.21	0.020	down
	stu-miR398b-3p	0.00	2.70	0.021	down
MIR399	ppe-MIR399a-p5_2ss5AT18TC	7.11	0.00	0.006	up
	fve-MIR399b-p5_2ss2GT20GA	4.89	33.61	0.005	down
	mtr-MIR399b-p5_2ss11TC20TG	22.65	18.22	0.019	up
	ppe-MIR399a-p3_2ss5AT18TC	7.11	0.00	0.006	up
	stu-miR399j-3p_1ss21GA	0.00	2.84	0.015	down
miR482	stu-miR482a-5p_1ss12AG	158.27	274.34	0.008	down
MIR858	ppe-MIR858-p5_1ss4GA	611.66	386.05	0.013	up
miR1885	bra-miR1885b	14.61	18.27	0.007	down
miR3627	stu-miR3627-5p_R-1	8.89	0.00	0.048	up
MIR10528	sly-MIR10528-p3_2ss9GA19TC	7.81	0.00	0.048	up
MIR11334	cst-MIR11334-p5_2ss9TG18TC	22.54	13.15	0.025	up
MIR5141	rgl-MIR5141-p5	16.53	40.47	0.023	down
miR6024					
	stu-miR6024-3p_R-1_2ss20CT21CT	20.40	9.84	0.021	up
miR6149	nta-miR6149a_L+1R-1_1ss21GC	0.00	4.06	0.034	down
miR6478	ptc-miR6478_R-1_1ss20TA	4.45	0.77	0.035	up

	ptc-miR6478_R+3_1ss21GA	3.68	11.02	0.042	down
	ptc-miR6478_R+2_1ss21GA	198.84	273.50	0.000	down
	ptc-miR6478_R+1_1ss21GA	13.40	23.60	0.013	down
MIR8005	stu-MIR8005a-p3_1ss5GA	11.39	3.39	0.036	up
MIR8021	stu-MIR8021-p3_2ss15GA22TA	9.40	13.95	0.021	down
	stu-MIR8021-p3_2ss16TC22TG	26.11	35.95	0.013	down
miR8175	ath-miR8175_L+4	0.00	3.83	0.013	down
	PC-3p-155334_29	0.00	5.53	0.001	down
	PC-3p-216061_19	0.00	6.33	0.021	down
	PC-3p-71517_65	5.84	20.24	0.050	down
	PC-3p-9747_279	24.16	51.14	0.034	down
	PC-5p-101290_47	3.77	6.09	0.020	down
	PC-5p-117714_40	0.00	13.55	0.025	down
	PC-5p-119988_39	0.00	14.12	0.040	down
Novel	PC-5p-12980_234	62.33	116.97	0.018	down
miRNAs	PC-5p-347691_9	0.00	7.12	0.040	down
	PC-5p-567932_5	0.00	5.04	0.014	down
	PC-3p-145340_32	11.33	1.54	0.014	up
	PC-3p-9335_287	78.11	53.49	0.041	up
	PC-5p-117747_40	11.26	1.96	0.023	up
	PC-5p-153350_30	14.73	2.06	0.010	up
	PC-5p-154497_29	4.86	0.00	0.038	up
	PC-5p-160111_28	9.37	1.96	0.049	up
	PC-5p-58183_77	17.38	7.49	0.030	up

686

687 Note: The miRNA name is composed of the 1st known miRNA name in a cluster, a
688 underscore, and a matching annotation: such as L-n/+n means the detected miRNA is n base
689 less or more than known rep_miRSeq in the left side; R-n/+n means the detected miRNA is n
690 base less or more than known rep_miRSeq in the right side; 2ss5TC13TA means 2
691 substitution (ss), which are T->C at position 5 and T->A at position 13.

692

693 **Supporting Information:**

694 Figure S1. Small RNAs Pearson correlation between the six libraries

695 Table S1. Primers used in this study. S1-1, Primers designed for conserved
696 miRNAs and novel miRNAs; S1-2 Primers designed for miRNA targets.

697 Table S2. Summary of five types of miRNA in this study. S2-1, gp1a type; S2-2,
698 gp2a type; S2-3, gp2b type; S2-4, gp3 type; S2-5, gp4 type.

699 Table S3. Primers used in this study. S1-1, Primers designed for conserved
700 miRNAs and novel miRNAs; S1-2, Primers designed for miRNA targets.

701 Table S4. Overview of degradome sequencing reads from raw data to mapping
702 sequences.

703 Table S5. The overview of reliable identified targets of miRNAs.

704 Table S6. Transcript annotation of targets of differential expression conserved
705 and novel miRNAs in this study.

706 Table S7. The pathways of all the miRNAs and targets involved in this study.
707 Table S8. The transcript annotation, GO terms and KEGG pathways of
708 differential expression miRNAs with those targets in pepper.
709