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Genome-wide investigation of microRNAs and their targets in response to freezing stress in *Medicago sativa* L, based on high-throughput sequencing

Running title: MicroRNAs and their targets determining freezing tolerance in alfalfa

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SUMMARY

In alfalfa, freezing tolerance is a complex trait determined by interactions among many genes, which is a major limitation of its exploitation. Understanding the complex regulation mechanisms of freezing tolerance is imperative for alfalfa genetic breeding. The authors identified and characterized the important roles of the miRNAs and their targets in the cold and/or freezing stress response by small RNA sequencing and degradome sequencing. The results provide valuable information for determining molecular mechanisms of freezing tolerance, which will aid future efforts to improve freezing tolerance in alfalfa.

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ABSTRACT

Winter damage, especially in northern climates, is a major limitation of the utilization of perennial forages such as alfalfa. Therefore, improving freezing tolerance is imperative in alfalfa genetic breeding. However, freezing tolerance is a complex trait that is determined by many genes. To understand the complex regulation mechanisms of freezing tolerance in alfalfa, we performed small RNA sequencing analysis under cold (4°C) and freezing stress (–8°C). The sequencing results revealed that 173 known and 24 novel miRNAs were expressed, and that the expressions of 35 miRNAs were affected by cold and/or freezing stress. Meanwhile, 105 target genes cleaved by these miRNAs were characterized by degradome sequencing. These targets were associated with biological regulation, cellular process, metabolic process and response to stress. Interestingly, most of them were characterized as transcription factors (TFs), including auxin response factors (ARFs), SBP, NAC, AP2/ERF and GRF, which play important roles in plants' abiotic responses. In addition, important miRNAs and mRNAs involved in nodulation were also identified, for example, the relationship between miR169 and the TF CCAAT (also named as NF-YA/HAP2), which suggested that nodulation has an important function in freezing tolerance in alfalfa. Our results provide valuable information to determine the molecular mechanisms of freezing tolerance in alfalfa, which will aid the application of these miRNAs and their targets in the improvement of freezing tolerance in alfalfa and related plants.

Keywords *Medicago sativa*; cold acclimation; freezing stress; microRNA; degradome sequencing

INTRODUCTION

Lack of tolerance to freezing is a major environmental limitation of survival, productivity and ecological distribution of plants. However, freezing tolerance is a complex trait that is determined by numerous factors from plants and the environment. Among these factors, cold acclimation, the exposure of plants to low, sub-freezing temperatures, plays an important role in conferring freezing tolerance (Thomashow 2010). During the cold accumulation process, several biochemical and physiological modifications occur, including accumulation of soluble sugars, free amino acids and the expression of cold-regulated (COR) genes, which potentially improving freezing tolerance in plants. To date, the identification and characterization of C-repeat (CRT)-binding factors (CBFs) have shown that they play critical roles in the cold acclimation process (Gilmour *et al.* 1998; Thomashow 2010), by regulating downstream functional genes, such as COR genes (Hajela *et al.* 1990; Thomashow 2010). Regulation of COR genes by CBFs constitutes the central component of cold signaling pathways that confer freezing tolerance on plants. In addition, the CBF signaling pathway is also regulated by other factors, for example, ICE1 (Inducer of CBF Expression 1) (Chinnusamy *et al.* 2003; Lee *et al.* 2005; Miura and Hasegawa 2008), and HOS1 (High Expression of Osmotically Responsive Gene 1) (Jung and Park 2013; Lee *et al.* 2001), which are important components in the plant cold acclimation process.

MicroRNAs are a class of non-coding small RNAs of approximately 21-24 nucleotides, which bind to complementary sequences in the mRNAs of target genes

45 (Mallory and Vaucheret 2006). This binding results in the regulation of gene
46 expression at the posttranscriptional level by cleavage-induced degradation of the
47 mRNA or suppression of its translation (Mallory and Vaucheret 2006). In recent years,
48 many miRNAs have been demonstrated to have important regulatory functions in
49 plant growth, development and stress responses (Babar *et al.* 2008; Khraiwesh *et al.*
50 2012; Sunkar *et al.* 2012). A number of miRNAs are involved in the cold response
51 process in *Arabidopsis* (Liu *et al.* 2008; Sunkar and Zhu 2004; Zhou *et al.* 2008a),
52 poplar (Chen *et al.* 2012; Lu *et al.* 2008), *Brachypodium distachyon* (Zhang *et al.*
53 2009), rice (Lv *et al.* 2010), and wheat (Tang *et al.* 2012; Tang *et al.* 2011), including
54 miR156/157, miR169, miR393, miR396, miR394 and miR398 (Rajwanshi *et al.*
55 2014). With the development of high-throughput sequencing technology, numerous
56 miRNAs have been identified and characterized in plants. MiRNAs biological
57 functions have been deduced by the identification and characterization of their target
58 genes. Degradome sequencing, also termed parallel analysis of RNA ends (PARE),
59 was developed based on high-throughput sequencing technology for the genome-wide
60 identification of miRNA target genes (Folkes *et al.* 2012). Using high throughput
61 sequencing methods, many new miRNA-mRNA target pairs have been identified
62 successfully in *Arabidopsis* (Addo-Quaye *et al.* 2008), rice (Sun *et al.* 2015), wheat
63 (Chen *et al.* 2015a), soybean (Shamimuzzaman and Vodkin 2012), maize (Zhao *et al.*
64 2013), and grapevine (Pantaleo *et al.* 2010), which helped to elucidate the regulatory
65 relationships between miRNAs and their target genes.

66 Alfalfa (*Medicago sativa* L.) is a highly productive perennial forage species, with the

67 capacity for biological fixation of atmospheric nitrogen, which is grown worldwide.
68 However, because of insufficient freezing tolerance, hard winters (with extremely low
69 temperatures) are a major limitation to alfalfa production (Castonguay *et al.* 2013;
70 Pennycooke *et al.* 2008). Thus, improvement of freezing tolerance is an important
71 breeding aim for high yield and longer production periods in alfalfa, especially in
72 northern climates, for example, in the USA, Canada and China (Chen *et al.* 2015b).
73 *M. sativa* L. cv. Zhaodong was domesticated and bred from wild *M. sativa* by the
74 Institute of Animal Husbandry of Heilongjiang Province (IAH-HLJ, China), and has
75 high tolerance to freezing stress, enabling it to survive during the winter season in the
76 field of Heilongjiang Province, China (average temperature -35°C). The high freezing
77 tolerance of *M. sativa* L. cv. Zhaodong is determined by its specific gene regulation
78 network (Luo *et al.* 2004). Determining the specific expression patterns of miRNAs
79 and mRNAs would be helpful to understand the complex molecular mechanism of
80 freezing tolerance in this species.

81 In present study, miRNAs and their targets that are involved in cold and/or freezing
82 stress were investigated using high-throughput sequencing. Freezing stress responsive
83 miRNAs were selected and validated by quantitative real-time reverse transcription
84 (qRT-PCR) experiments. Meanwhile, the potential miRNA targets were predicted and
85 confirmed by degradome sequencing.

86 MATERIALS AND METHODS

87 Plant growth and treatment

88 Seeds of *M. sativa* (cv. Zhaodong), kindly provided by Prof. Hong Li (IAH-HLJ,
89 China), were germinated and transferred onto a mix of perlite and sand (3:1, V/V). All

90 seedlings were grown in a growth chamber (Conviron E15, Canada) at a temperature
91 between 18°C (night) and 24°C (day), with humidity ranging from 60% to 80%, and a
92 light period of 14h/10h (daytime, 06:00–20:00). The seedlings were irrigated with
93 half-strength Hoagland solution once every other day, and after eight weeks they were
94 randomly divided into three groups for stress treatments. For the control group
95 (untreated, A group), the seedlings continued to grow at 18°C (night) to 24°C (day).
96 For cold stress (B group) and freezing stress (C group), the seedlings were transferred
97 into another chamber with the temperature set at 4°C or −8°C, respectively. According
98 to our previous research (Shu *et al*. 2015), all seedlings were harvested at 3 h after
99 stress treatments; five whole seedlings from each group were bulked separately. All
100 samples were frozen in liquid nitrogen, and stored at −80 °C until use.

101 **Small RNA library construction and sequencing**

102 Small RNAs were extracted from samples from the three treatments (control, cold and
103 freezing) using the TRIzol method (Invitrogen, CA, USA), according to the
104 manufacturer's instructions. Small RNAs were ligated sequentially to 5′ and 3′
105 RNA/DNA chimeric oligonucleotide adaptors, and the resulting ligation products
106 were gel purified by 15% denaturing PAGE and reverse-transcribed to produce
107 cDNAs. The cDNAs were sequenced using a Genome Analyzer IIx System, according
108 to the manufacturer's instructions (BGI-Shenzhen Co. Ltd., Shenzhen, China).

109 **Identification of conserved and novel miRNAs**

110 Raw data were first processed by filtering out low quality reads, trimming the

111 adaptors and removing other noise reads, to obtain clean reads. The clean reads were
112 then aligned to the Rfam database to remove other noncoding RNAs, including rRNA,
113 tRNA and snRNA. The remaining reads were mapped to assembled transcriptome
114 sequences from *M. sativa* cv. Zhaodong (as described in the following transcriptome
115 sequencing section). Mapping reads were retrieved and aligned to plant miRNA
116 sequences from miRBase V21 (Kozomara and Griffiths-Jones 2014) using Bowtie
117 (Langmead and Salzberg 2012), which identified and annotated conserved miRNA
118 genes. To identify novel miRNA genes, firstly, miRDeep-P retrieved the flanking
119 assembled transcriptome sequences of mapping reads, which were identified as
120 candidate precursors of miRNA (Yang and Li 2011). Secondly, the novel miRNAs
121 were identified using MIREAP, based on the precursors, whose secondary structures
122 were verified by software RNAfold, as previously reported (Denman 1993). In
123 addition, plant miRNA criteria mentioned for Arabidopsis (Meyers *et al.* 2008) and
124 rice (Jeong *et al.* 2011) were also considered for alfalfa novel miRNA identification.

125 All miRNA abundances were evaluated and normalized using the tags per million
126 reads (TPM) method, based on BLAST mapping results. The TPM values were
127 calculated as follows: TPM = number of mapped miRNA reads*10^6/number of clean
128 sample reads. The normalized expression was adjusted to 0.01 when miRNA
129 expression (TPM) was zero to avoid negating the subsequent calculation of fold
130 change. The miRNA expression fold changes between stress and control groups
131 (Cold/Control, Freezing/Control) were computed, and chi-squared tests were
132 performed to determine the significance of miRNA expression for each comparison

133 using the R software. The miRNAs with fold change (TPM ratios) ≥ 2 or ≤0.5, and
134 p-value ≤ 0.05 were deemed differentially expressed in response to cold and/or
135 freezing stresses, as described by Xie *et al.* (2014).

136 **Quantitative real-time reverse transcription PCR (qRT-PCR) analysis of**
137 **miRNAs expression**

138 The expressions of 12 selected miRNAs from the three conditions were assayed using
139 stem-loop quantitative reverse transcription PCR (qRT-PCR). Primers for all miRNAs
140 and the reference gene (U6) were designed as shown in Table S1. Total RNA was
141 extracted from alfalfa grown under the three conditions (control, cold and freezing)
142 with TRIZOL reagent, according to the manufacturer's instructions (Invitrogen).
143 These RNAs were reverse transcribed to cDNA, according to the manufacturer's
144 protocol. qRT-PCR was performed using the LightCycler 480II Detection System
145 (Roch) with a total reaction volume of 20 μL, containing 1 μL of cDNA templates, 8
146 μM of primer mix, 10.0 μL of 2×SYBR Green Mix, and 8.7 μL ddH_2O. The PCR
147 conditions were set as follows: 95°C for 2 min; 40 cycles of 95°C for 10 s and 60°C
148 for 30 s; and 60°C for 45 s. The miRNA expression abundances were determined
149 based on the $2^{-\Delta\Delta CT}$ method, and relative changes in miRNA expression from the
150 qRT-PCR experiments were calculated. Three biological replicates for each group
151 were run, and each reaction was performed with three technical replicates.

152 **Degradome library construction and target identification**

153 To investigate the potential target mRNAs, two degradome libraries from cold stress

154 and freezing stress samples were constructed, as previously described (Folkes *et al.* 2012). In brief, poly(A)-enriched RNAs were isolated and ligated to an RNA oligo-nucleotide adaptor containing a 3′ *Mme*I recognition site, and the ligated products were used to synthesize first-strand cDNA. A short PCR (five cycles) reaction was used to amplify the cDNA, and the product was ligated to a double-stranded DNA adaptor, before being subjected to gel purification again for PCR amplification. The final cDNA library was purified and sequenced on an Illumina GAII by BGI-Shenzhen Co. Ltd (Shenzhen, China).

162 Adaptor sequences and low quality sequencing reads were removed from the raw reads, and the clean reads were used to identify potentially cleaved targets based on *M. sativa* assembled transcriptome sequences (described below) by the CleaveLand4 pipeline (Addo-Quaye *et al.* 2009). Meanwhile, the psRNATarget tool was also used to predict miRNA targets using a set of default parameters (Dai and Zhao 2011). MiRNA target genes were also predicted from *M. sativa* assembled transcriptome sequences. Prediction targets were used to cross-check the degradome sequencing results.

170 **RNA-seq library construction, sequencing and analysis**

171 Total RNAs were extracted from three samples using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), and transcriptome sequencing libraries were constructed according to the manufacturer's instructions. In brief, short fragments were purified using a MinElute PCR Purification Kit (Qiagen) and eluted in 10 μL of EB buffer (Qiagen). The short fragments were ligated with sequencing adapters, and

176 the desired fragments (around 250 bp) were separated by agarose gel electrophoresis
177 and purified using a gel extraction kit. Finally, the sequencing library was constructed
178 by linear PCR amplification (15 cycles) and sequenced using the Illumina GAII
179 platform by BGI-Shenzhen Co. Ltd (Shenzhen, China), generating 100 bp pair-end
180 reads. Processing of raw data, removal of adapter sequences, base-calling, and quality
181 value calculations were performed to produce clean data. Clean reads from three
182 libraries were assembled *de novo* into contigs using Trinity software with the
183 following parameter: "min_kmer_cov 2" (Haas *et al.* 2013). To remove redundancy
184 among the Trinity-generated contigs, they were further assembled *de novo* using
185 iAssembler, with the minimum percent identify (–p) set to 97 (Zheng *et al.* 2011). The
186 resulting unique transcripts were identified as *M. sativa* transcriptome unique
187 assembled mRNA sequences. These assembled transcripts were BLAST searched
188 against combined databases of Arabidopsis, rice, soybean, and *Medicago truncatula*
189 protein sequences for functional annotation (the e-value was set at 1E-5). Gene
190 ontology (GO) annotations were assigned to the targets based on the GO terms
191 annotated to their corresponding homologs in the combined database, and the GO
192 enrichment analysis of miRNA targets was performed using package topGO on the R
193 platform. Meanwhile, plant transcription factors (TFs) from *M. sativa* were identified
194 and classified into different families using the iTAK pipeline
195 (http://bioinfo.bti.cornell.edu/tool/itak) (Jin *et al.* 2014).

196 Clean reads from the three samples were mapped to the *M. sativa* assembled
197 transcripts generated by RNA-seq, as previously described, using the TopHat software

198 (Trapnell *et al.* 2009), and mRNA target gene expressions (as estimated by the
199 fragments per kilobase of exon per million fragments mapped (FPKM) method)
200 across the control, cold and freezing samples were evaluated using the Cufflinks
201 software (Trapnell *et al.* 2012). Differential expression analysis was performed using
202 the edgeR package (Robinson *et al.* 2010) on the R platform, and target genes with
203 fold changes ≥2 or ≤0.5, with an adjusted p-value ≤0.01, were identified as
204 differentially expressed in response to cold and/or freezing stress.

205 **RESULTS**

206 **High-throughput sequencing of small RNA libraries**

207 Raw reads of three libraries were obtained by high-through sequencing, and they were
208 deposited into NCBI SRA with accession number: SRP064230. After removing
209 low-quality reads, poly(A) reads, oversized insertions, reads shorter than 18-nt, and
210 adaptor contaminated reads, 10823011, 10833023, and 10781132 clean reads were
211 generated from the control, cold and freezing libraries, respectively. These reads
212 included unique reads, and the length distribution of small RNA reads from the three
213 libraries ranged from 18 to 28 nt (see Figure S1). The majority of small RNA reads
214 were 20–24 nt sequences, comprising over 80% of the reads. The 21 and 24 nt small
215 RNA comprised the two major classes, which was consistent with previous
216 publications (Fan *et al.* 2014; Tang *et al.* 2012; Zhang *et al.* 2009). Other non-coding
217 RNAs, including rRNA, tRNA and snRNA, were removed by mapping reads to the
218 Rfam database. The remaining reads were mapped to the alfalfa transcriptome

219 assembled sequences; about 41% of the reads were mappable (Table 1). To identify
220 conserved miRNAs in alfalfa, the mappable reads were aligned to known plant
221 miRNAs in the miRBase database, using Bowtie with no more than one mismatch. In
222 total 1597215, 1287494 and 987515 reads were identified as homologous to known
223 miRNAs from the control, cold and freezing libraries, respectively. They were
224 identified as 173 conserved miRNA genes from 112 miRNA families (see Table S2).
225 The flanking sequences of the remaining mappable reads were retrieved and analyzed
226 by MIREAP using plant default parameters. In total, 24 novel miRNAs among the
227 predicted RNA hairpins were identified in alfalfa, most of them were 21 and 24 nt in
228 length (Table S3).

229 To identify miRNAs involved in alfalfa response to cold and freezing stress, the
230 miRNA expressions of three groups were evaluated and normalized (see Table S4).
231 Compared with the control group, miRNAs with a log2 fold change higher than 1 or
232 less than -1, combined with a p-value less than 0.05, were identified as differentially
233 expressed miRNAs. There were 35 differentially expressed miRNAs in response to
234 cold and/or freezing stress (Table 2). Among these miRNAs, twelve were regulated by
235 cold stress, while 30 responded to freezing stress. Nine miRNAs responded to cold
236 and freezing stress, three miRNAs were specifically regulated by cold stress, and 23
237 miRNAs specifically responded to freezing stress. In addition, most (29 miRNAs)
238 were down-regulated by cold and/or freezing stress; only six miRNAs were induced
239 by cold and/or freezing stress.

240 **qRT-PCR validation of miRNA expression**

241 To validate miRNAs' function in alfalfa in response to cold and freezing stress, 12
242 differentially expressed miRNAs were selected for qRT-PCR detection. The means of
243 the correlation coefficients of the qRT-PCR validations and the high-through
244 sequencing results for the miRNAs were as high as 0.80 and 0.83 under cold and
245 freezing stress, respectively, which implied that our miRNA sequencing results were
246 highly reliable (Figure 1). For example, miR167a, miR172c-3p, miR396a-5p and
247 miR5231 were identified as downregulated by both cold and freezing stress by
248 Illumina sequencing, and qRT-PCR showed the same expression profile in response to
249 cold and freezing stress (Figure 2). Similarly, the qRT-PCR results also validated the
250 expression patterns of miR160e and miR166f, which were up-regulated by
251 cold/freezing stress. Notably, the expressions of three novel miRNAs (NmiR0018,
252 NmiR0026 and NmiR0051) were characterized and confirmed by qRT-PCR detection.
253 However, there were some miRNAs that showed qRT-PCR results that were
254 inconsistent with the sequencing results, i.e. miR156i-5p, miR398a-5p and miR5037c.
255 This possibly reflected the different sensitivities of high-throughput sequencing and
256 qRT-PCR detection method for specific miRNAs.

257 **Analysis of alfalfa transcriptome sequences**

258 Three transcriptome libraries were sequenced, and 19808866, 23870694, 22350578
259 reads were collected from the control, cold and freezing samples, respectively. After
260 discarding the low quality raw reads, the remaining reads were assembled *de novo*
261 using the Trinity software. We obtained 124821 assembled transcripts, with an N50 of
262 1392 bp and an average length of 828 bp; the detailed information is provided in

263 Table 3. The assembled alfalfa transcripts were annotated by BLASTX analysis
264 against Arabidopsis, rice, soybean, and *M. truncatula* proteins, revealing 73993
265 (59.3%) transcripts with significant hits. The results showed that the percentage of
266 genes that could be annotated was positively correlated with the length of the genes,
267 as shown in Figure S2. In addition, a BLASTN search was performed against the
268 Mt4.0v1 mRNAs (http://jcvi.org/medicago/) to estimate possible differences in
269 transcript sequences between *Medicago sativa* and the model plant *Medicago*
270 *truncatula*. The results showed that 54.1% (67492/124821) of assembled alfalfa
271 transcripts had significant matches with *M. truncatula* transcripts, most of them have
272 high identity percentages, as shown in Figure S3, which indicated high genetic
273 similarity between the two species.

274 **Identification of miRNA targets in alfalfa**

275 To determine the function of miRNAs in alfalfa, degradome sequencing was used to
276 identify the miRNA targets. After removing reads without adaptor sequences,
277 10878175 and 10849379 clean reads were obtained from the two degradome libraries
278 respectively (cold and freezing libraries), most of them were 20 and 21 nt in length
279 (Figure S4). The reads were aligned to the alfalfa transcriptome sequences: 3008989
280 and 2441179 reads mapped to the assembled transcriptome sequences, respectively.
281 The CleaveLand pipeline was used for further analysis, and 105 target mRNAs that
282 were potentially cleaved by known miRNAs and novel miRNAs were identified from
283 the two degradome libraries (Figure 3 and Table S5). Among these genes, 75 targets
284 were identified in cold stress library, and 66 targets present in freezing stress, and 36

285 targets were cleaved during both cold and freezing stress (Figure 4). According to the
286 relative abundance of reads at the target sites, they were classified into five categories,
287 0–4, (see Figure S5). Category 0 has more than one raw read at the position, and the
288 abundance at the position is equal to the maximum on the transcript, only one.
289 Category 1 has a similar definition to Category 0, but there are more than one
290 maximum on the transcript. Category 2 has more than one raw read at the position.
291 The abundance at the position is less than the maximum, but higher than the median
292 for the transcript. Category 3 has more than one raw read at the position. The
293 abundance at the position is equal or less than the median for the transcript. Category
294 4 has only one raw read at the position. In this study, Category 0 had the most
295 members under both cold and freezing stress, providing high confidence in the
296 degradome sequencing results. To validate target genes from degradome sequencing,
297 miRNA and transcripts were submitted to psRNATarget to identify cleavage using
298 default parameters. There were 65 (65/105, 61.9%) targets from the degradome that
299 were present in the psRNATarget results list (Figure 4). The results indicated that
300 degradome sequencing was an effective method to identify cleaved targets of
301 miRNAs.

302 **GO analysis and expression analysis of target genes**

303 To further understand the functions of miRNAs, the target genes were BLAST
304 searched against a combined database of Arabidopsis, rice, soybean, and *M.*
305 *truncatula*. In total, 94 target genes were identified as homologous to functional genes
306 from model plants, and enrichment analysis of GO annotation was performed using

307 the topGO package (Figure 5 and Table S6). As expected, GO terms in the biological
308 process category were highly enriched, including GO:0006355 (regulation of
309 transcription), GO:0031323 (regulation of cellular metabolic process), GO:0050896
310 (response to stimulus) and GO:0009725 (response to hormone). The most enriched
311 molecular function was GO:0003677 (DNA binding), which is consistent with the
312 results in biological process. Similarly, the most enriched cellular components were
313 GO:0005634 (nucleus), GO:0044424 (intracellular part), and GO:0044464 (cell part).
314 These results implied the possible function of miRNAs in the regulation of
315 transcription during cold and freezing stresses. To investigate transcriptional
316 regulation in detail, we used the iTAK pipeline to scan for TFs: we identified 28 TFs
317 as miRNA target genes (Table 4), which were highly enriched (hypergeometric test,
318 p-value is 6.7E-20). The results were consistent with and confirmed the results of the
319 GO enrichment analysis.

320 Based on transcriptome sequencing, we evaluated the expressions of the target genes
321 under cold and freezing stresses. Among them, six target genes are regulated by four
322 differentially expressed miRNAs (Table S7). Compared with control conditions, the
323 expressions these four miRNAs were depressed under cold and freezing stress, and
324 their targets (except MsUN037724, cleaved by miR396a-5p) were remarkably
325 upregulated under cold and freezing stresses, i.e. MsUN007721 and MsUN018812
326 (Figure 6). In addition, correlation coefficients between miRNAs and their target
327 genes were computed. Negative results (mean value close to -0.41) implied repressive
328 regulation between an miRNA and its target gene. In particular, the expression of

329 transcript MsUN007721 (an AUX/IAA TF), targeted by miRNA167a, was
330 significantly induced under cold and freezing stress, which is consist with observed
331 repression of miRNA167a. These results suggested that miRNA167 plays an
332 important role by regulating the functions of genes of the auxin signaling pathway
333 (i.e. MsUN007721) in alfalfa in response to cold and freezing stresses. A similar
334 regulation function between miR167 and ARF TF has been confirmed as essential for
335 soybean nodulation (Wang *et al.* 2015).

336 **DISCUSSION**

337 Plant growth and development are threatened by extreme environmental condition
338 worldwide. In response to stress, plants employ complex systems to adapt to
339 environmental stress, undergoing physiological and biochemical changes in response
340 to unfavorable conditions. MiRNAs are important regulators involved in various
341 stress responses, which have received increasing attention, especially with the
342 development of high-throughput sequencing technology. Freezing stress is a common
343 environmental stress of plants that affects plant growth, development and survival,
344 and particular limits perennial plants. Recently, miRNAs involved in freezing stress
345 have been investigated in many plants, mainly in model plant, such as *Arabidopsis*
346 (Mallory and Vaucheret 2006; Sunkar and Zhu 2004), rice (Lv *et al.* 2010), wheat
347 (Tang *et al.* 2012; Tang *et al.* 2011) and *M. truncatula* (Formey *et al.* 2014;
348 Lelandais-Briere *et al.* 2009; Reynoso *et al.* 2013; Zhou *et al.* 2008b); however, few
349 reports have been published in alfalfa, a perennial legume forage (Fan *et al.* 2014;
350 Long *et al.* 2015). Here, we presented a comprehensive analysis of miRNAs and their

351 targets in the freezing tolerant alfalfa cultivar Zhaodong under cold and freezing
352 stress, using high-throughput sequencing. The results revealed that miRNAs are
353 indeed affected by freezing stress, implying their role in this process. In addition,
354 targets of miRNAs, including TFs, enzymes and nodulation genes, were identified by
355 degradome sequencing. Taken together, these results provide novel insights into the
356 regulatory mechanism of cold acclimation and freezing tolerance in alfalfa mediated
357 by miRNAs.

358 **Different expression patterns of miRNAs involving in freezing stress in alfalfa**

359 Based on our sequencing results, 35 miRNAs were differentially regulated by cold
360 and/or freezing stress in alfalfa. Notably, most miRNAs (29/35, 83%) were
361 downregulated by cold and/or freezing stress, which is consist with previous reports in
362 other plants (Chen *et al.* 2012; Long *et al.* 2015; Lv *et al.* 2010; Zhang *et al.* 2009;
363 Zhou *et al.* 2008a). Among these miRNAs, miRNAs responsive to cold stress were
364 more conserved across plants, for instance, miR156, miR159, miR167, miR172,
365 miR396, and miR398, implying their consistent function in cold stress. For example,
366 the Cu/Zn superoxide dismutase-encoding gene, which is cleaved by miR398 in
367 plants, acts as ROS scavenger, playing an important role in plant abiotic stress (Dugas
368 and Bartel 2008; Sunkar *et al.* 2006). Other miRNAs that were specifically regulated
369 by freezing stress, and which were mainly Medicago-specific, included miR5231,
370 miR5232, miR5234, miR5239, miR5287, and novel miRNAs (NmiR0019,
371 NmiR0026, NmiR0028, NmiR0029, NmiR0043, NmiR0047, NmiR0053). The results
372 suggested that they play important roles in freezing tolerance of alfalfa. However,

373 their functions have been reported rarely in Medicago or other plants, some were even
374 identified in the present study for the first time. Thus their regulatory functions need
375 to be determined in depth, which would be valuable for breeding alfalfa with
376 improved freezing tolerance. In addition, there were also some miRNAs that were
377 upregulated by cold and/or freezing stress, such as miR160, miR166, miR171,
378 miR2119 and miR5037, which might regulate genes that negatively involved in the
379 freezing tolerance of alfalfa.

380 **TF targeted by alfalfa miRNAs involved in freezing tolerance**

381 With development of high-throughput sequencing technology, degradome sequencing
382 has been used widely to identify targets of miRNAs in plants. In the present study, we
383 identified 105 targets from cold stress and freezing stress libraries. Based on the
384 targets' functional annotations, we found that conserved miRNAs were more likely to
385 target TFs involved in regulating plant growth and stress response, which was
386 consisted with previous reports in other plants (Aung *et al.* 2015; Fang *et al.* 2014;
387 Tang *et al.* 2012; Wang *et al.* 2015) (see Table 4). Degradome sequencing identified
388 TF members from families characterized as targets of miRNAs, including SBP, ARF,
389 NAC and GRF. In *Arabidopsis* and rice, miR156 participates in plant growth and
390 development by cleaving SBP TFs (Ling and Zhang 2012; Xing *et al.* 2010).
391 Similarly, miR160 and miR167 target auxin response factors (ARF and AUX/IAA),
392 involved in plant development process (Rubio-Somoza *et al.* 2009; Wang *et al.* 2015;
393 Yang *et al.* 2006). In this study, eight SBP, ARF and AUX/IAA TFs were identified
394 by high-throughput sequencing, which indicated that the conserved miRNAs might

395 regulate alfalfa cold and freezing responses by controlling alfalfa developmental
396 process, which would correlate with their functions in other model plants. Similarly,
397 NAC TFs are plant-specific, with important roles in plant development and stress
398 response processes. Two NAC TFs were identified as targets of miR164, which was
399 slightly suppressed by cold stress, implying that NAC TFs were positively regulated
400 during the cold response (Fang *et al.* 2014). In addition to targeting TFs, some genes
401 involved in stress responses and metabolic processes were also cleaved by miRNAs in
402 alfalfa. For instance, the pentatricopeptide repeat (PPR) protein is a negative regulator
403 of abscisic acid (ABA) signaling, which has a positive on the plant's response to
404 abiotic stresses (Jiang *et al.* 2014; Sechet *et al.* 2015). In total, eight PPR genes were
405 identified as targets of NmiR0018 and NmiR0041 (see Table S8), implying that these
406 miRNA function in cold and freezing stress by controlling the expression of PPR
407 genes.

408 **Freezing response miRNAs and their targets involved in the nodulation process**

409 As a legume crop, alfalfa is able to establish a symbiotic association with nitrogen
410 fixing bacteria, resulting in development of a novel plant organ, termed the root
411 nodule (Soyano and Hayashi 2014). Within nodules, the symbiotic nitrogen fixation
412 relationship between the plant and bacteria is sensitive to environmental stress, such
413 as salt, drought and cold stress (Kaur *et al.* 2014; Zhang *et al.* 2014). In particular,
414 nodules of alfalfa are perennial, and are capable of continuous growth, helping alfalfa
415 to undergo dormancy and to survive hard winters. However, the molecular
416 mechanisms of symbiotic nitrogen fixation (SNF) in response to cold stress have

417 received relatively little attention. In Medicago, some TFs were characterized as being
418 involved in nodulation, including GRAS (Cerri *et al.* 2012; Heckmann *et al.* 2006;
419 Hirsch *et al.* 2009; Liu *et al.* 2011), AP2/ERF (Middleton *et al.* 2007) and CCAAT
420 TFs (HAP2-1/NF-YA) (Battaglia *et al.* 2014; Laporte *et al.* 2014), etc. Recently,
421 miRNAs were also demonstrated to participate in the nodulation process, such as
422 miR169 and miR172, which target the NF-YA/HAP2 and AP2 TFs (Combier *et al.*
423 2006; Reynoso *et al.* 2013; Wang *et al.* 2014). In this study, we confirmed the
424 relationship between miRNAs and mRNAs, for instance, GRAS, AP2/ERF and
425 CCAAT TFs were regulated by NmiR0007, miR5205 and miR169. Significantly, six
426 CCAAT TFs were cleaved by miR169, as identified by degradome sequencing (see
427 Table 2). The results confirmed function of miR169 as regulating the expression of
428 CCAAT TFs (NF-YA/HAP2), which implied that miR169 responded to cold stress by
429 via the SNF process (Lelandais-Brière *et al.* 2009; Zhang *et al.* 2014).

430 **CONCLUSIONS**

431 In summary, we investigated miRNAs and their targets by high-throughput
432 sequencing technology during the cold and freezing response of alfalfa. We identified
433 the expression profiles of 197 miRNAs (173 known miRNAs and 24 novel miRNAs);
434 35 miRNAs were identified as cold and/or freezing responsive miRNAs. Using
435 degradome sequencing, 105 functional genes were found to be cleaved by these
436 miRNAs; most of them were transcription factors involved in plant development and
437 abiotic response processes, which implied important roles in alfalfa freezing
438 tolerance. Some differentially expressed miRNAs and their targets were identified as

439 participating in SNF, which indicated that SNF might aid freezing tolerance in alfalfa.
440 These findings provided valuable information for exploring the molecular
441 mechanisms of the cold and freezing response, and also represent a foundation for
442 future application of miRNAs to improve freezing tolerance in alfalfa or related
443 plants.

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677 **Figure legends**

678 Figure 1 Comparison of the expressions of 12 miRNAs between small RNA
679 sequencing and qRT-PCR in response to cold and freezing stress. Red dots are
680 plot-based fold changes of each miRNA gene between the abundance from RNA
681 sequencing and qRT-PCR detection. The line correction relationship was computed
682 based the expressions of twelve miRNA genes (blue line).

683 Figure 2 Validation of the expression of 12 miRNAs in alfalfa using qRT-PCR.
684 Expressional abundances of each miRNA gene in the control sample were set as 1,
685 and fold changes of each miRNA gene relative to the control sample were calculated.
686 Fold change values greater than 2 or less than 0.5 indicate up-regulated or
687 down-regulated miRNAs. The blue plots are small RNA sequencing results, and pink
688 represents the qRT-PCR results.

689 Figure 3 Target plots (t-plots) of miRNA targets identified by degradome sequencing
690 in alfalfa. The values of the Reads axis indicate signature abundances of cleavage
691 sites. Red circles on the Position axis indicate predicted cleavage sites, and red lines
692 indicate signatures produced by miRNA-directed cleavage-based analysis by the
693 CleaveLand4 software.

694 Figure 4 Distribution of targets genes identified by two degradome sequencing
695 libraries and psRNATarget. Target genes of miRNAs from cold and freezing libraries
696 were identified by degradome sequencing, while target genes of psRNATarget were
697 mined by scanning assembled alfalfa transcripts using psRNATarget software with

698 default parameters.

699 Figure 5 Enrichment analysis results of biological processes involving miRNA target
700 genes in alfalfa. Biological processes were scanned by Fisher's test using the topGO
701 package, and each GO term was shown in yellow or red according to the p-value
702 (yellow for a high p-value; red for a low p-value). The rectangular boxes represent top
703 five significant GO terms.

704 Figure 6 Differential expression of miRNAs and their target genes in response to cold
705 and/or freezing stresses. (A) miRNA167a *vs*. MsUN007721; (B) miRNA396a-5p *vs*.
706 MsUN018812. The miRNA expression abundances were evaluated by small RNA
707 sequencing using the TPM method, while target genes were estimated by RNA-seq
708 data using the FPKM method. Their expression levels were adjusted by comparing
709 them with control samples, and relative fold change (FC) values were explored. FCs
710 greater than 2 or less than 0.5 were identified as up- or down-regulated, respectively.

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Tables

Table 1 Overview of small RNA sequences in three alfalfa libraries.

Table 2 Differential expression of miRNA genes in alfalfa in response to cold and/or freezing stresses. The miRNA abundance was evaluated and normalized using the TPM method, and miRNAs whose expression values were zero were adjusted to 0.01. The fold changes were computed by the formula, log2(Treatment/Control) for cold sample and freezing sample based their expressions. Meanwhile, chi-squared tests were performed for the significance of each comparison using the R software. A p-value no more than 0.01 was set as the extremely significant level (**), while a p-value greater than 0.01 and no more than 0.05 was defined as significant (*).

Table 3 Summary of the de novo assembled alfalfa transcriptome.

Table 4 TF targets of miRNAs identified by degradome sequencing. The assembled alfalfa transcript sequences were submitted to iTAK software for TF identification using default parameters. Twenty-eight TFs from thirteen families were identified, and the result of hypergeometric testing showed that TF genes were highly enriched in these targets.

Table 1. Overviews of small RNA sequences in three alfalfa libraries

Data type	Control		Cold		Freezing	
	Total reads	Unique reads	Total reads	Unique reads	Total reads	Unique reads
Clean reads	10823011	4415425	10833023	4746716	10781132	4296444
Rfam	229925	18696	400604	28495	1947224	38460
Transcriptome	4933169	1201107	4509305	1232313	3892472	1219439
Known miRNA	1597215	24334	1287494	27292	987515	24278
Novel miRNA	172273	1355	82573	1121	62290	944

Table 2. The differential expression of miRNA genes in alfalfa in response to cold and/or freezing stresses. The miRNA abundance was evaluated and normalized using the TPM method, and the expression values of miRNAs whose expression was zero were adjusted to 0.01. The fold changes were computed from cold (log_2(Cold/Control)) and freezing (log_2(Freezing/Control)) based their expressions. Meanwhile, chi-squared tests were also performed to test the significance of each comparison, using the R software. A p-value no more than 0.01 was set as the extremely significant level (**), while a p-value greater than 0.01 and no more than 0.05 was defined significant (*).

miRNA	Fold change		Statistical significance	
	Cold	Freezing	Cold	Freezing
miR156c-3p	-0.62	-1.02	**	**
miR156i-5p	-0.78	-1.43	**	**
miR159a	-1.85	-1.15	**	**
miR159b	0.00	9.80		**
miR160e	0.11	1.50		**
miR166e-5p	-0.58	-1.64		**
miR166f	1.52	2.81	**	**
miR166g-5p	-0.75	-1.97	**	**
miR167a	-1.11	-1.53	**	**
miR167b-5p	-0.94	-1.99	**	**
miR168c-3p	-0.19	-1.04		**
miR171a	-0.99	1.43		**
miR172a	-1.77	-2.30	**	**
miR172c-3p	-1.21	-1.82	**	**
miR172d-3p	-1.31	-0.69	**	**
miR2119	-0.90	2.08		**
miR396a-5p	-1.74	-2.31	**	**
miR396b-3p	-0.71	-1.16	*	**
miR398a-5p	-2.28	0.17	**	
miR5037c	2.01	1.63	**	*
miR5231	-0.93	-1.94	**	**
miR5232	0.10	-1.01		*
miR5234	-0.63	-1.37	*	**
miR5239	-0.85	-1.58	**	**
miR5287b	-0.30	-1.83		**
NmiR0018	-1.01	-0.28	**	*
NmiR0019	-0.62	-3.60	**	**
NmiR0026	-0.69	-1.37	**	**
NmiR0028	-0.84	-1.07	**	**
NmiR0029	-0.98	-1.85	**	**
NmiR0043	-0.51	-1.92		**
NmiR0047	-0.45	-2.18	*	**
NmiR0049	-1.79	-2.58	**	**

NmiR0051	-2.04	-2.21	**	**
NmiR0053	-0.52	-2.20		*

Table 3. Summary of the *de novo* assembled alfalfa transcriptome

Data type	Number
Total sequence	124821
Number of sequences in 200-500 bp	64650
Number of sequences in 500-1000 bp	26072
Number of sequences more than 1000 bp	34099
Minimal length (bp)	201
Maximal length (bp)	14177
N50 (bp)	1392
Average length (bp)	828

Table 4. TFs targets of miRNAs identified by degradome sequencing. The assembled alfalfa transcript sequences were submitted to the iTAK software for TF identification using default parameters. Twenty-eight TFs from thirteen families were identified, and result of hypergeometric test showed that TF genes were highly enriched among these targets.

miRNA family	Target gene	TF family
miR156	MsUN014236	SBP
	MsUN046666	SBP
	MsUN050762	SBP
	MsUN011072	SBP
	MsUN049469	SBP
	MsUN086910	SBP
miR160	MsUN041965	ARF
	MsUN097717	ARF
miR164	MsUN043080	NAC
	MsUN045895	NAC
miR167	MsUN007721	AUX/IAA
miR169	MsUN004554	CCAAT
	MsUN014711	CCAAT
	MsUN030848	CCAAT
	MsUN044017	CCAAT
	MsUN096784	CCAAT
	MsUN102122	CCAAT
miR396	MsUN007041	GRF
	MsUN088040	GRF
	MsUN104762	C3HC4
	MsUN104763	C3HC4
	MsUN101411	MADS-box
miR2645	MsUN102295	bZIP
miR5205	MsUN046011	AP2-EREBP
mtr-miR5249	MsUN104998	bHLH
miR530	MsUN047511	NAC
NmiR0007	MsUN030863	GRAS
NmiR0028	MsUN045647	G2-like

Fold change of miRNA expression (Cold VS Control)

y=1.46*x-0.18

rho: 0.7997; pval: 0.0018

miR166f
miR160e
miR5037c
NmiR0018
miR398a-5p
miR156i-5p
miR396a-5p
NmiR0026
miR172c-3p
NmiR0051
miR167a
miR5231

Fold change by qRT-PCR

Fold change by small RNA sequencing

Fold change of miRNA expression (Freezing VS Control)

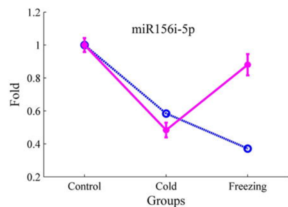
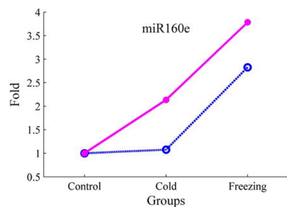
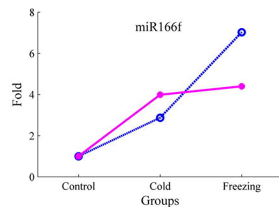
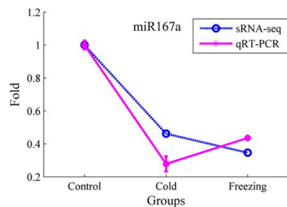
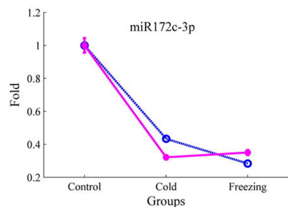
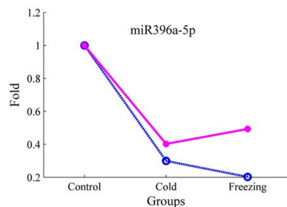
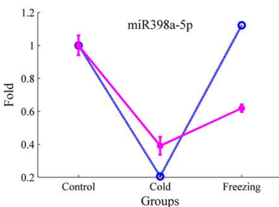
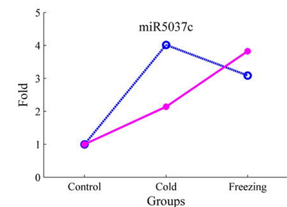
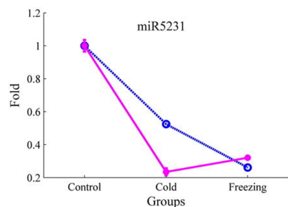
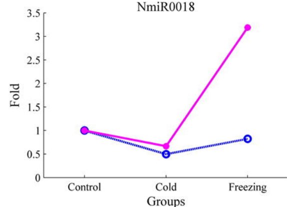
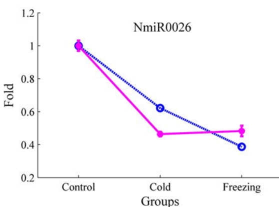
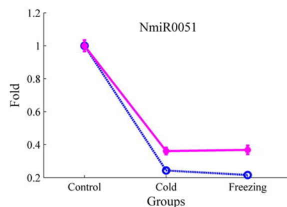
y=1.25*x+0.09

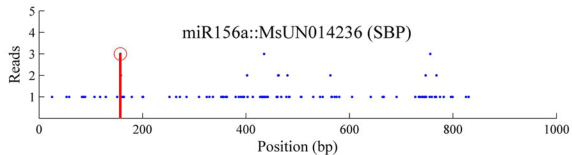
rho: 0.8294; pval: 8.4853e-04

miR166f
miR160e
miR5037c
NmiR0018
miR396a-5p
miR172c-3p
miR156i-5p
NmiR0026
miR398a-5p
NmiR0026
NmiR0051
miR172c-3p
miR5231
miR5231

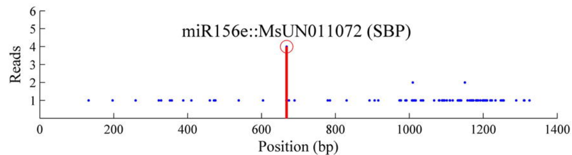
Fold change by qRT-PCR

Fold change by small RNA sequencing

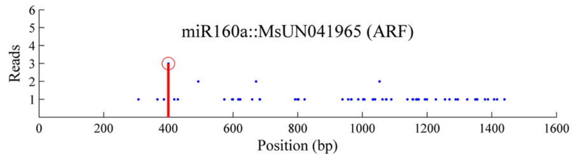

miR156i-5p
Fold
Control
Cold
Freezing
Groups

miR160e
Fold
Control
Cold
Freezing
Groups

miR166f
Fold
Control
Cold
Freezing
Groups

miR167a
sRNA-seq
qRT-PCR
Fold
Control
Cold
Freezing
Groups

miR172c-3p
Fold
Control
Cold
Freezing
Groups

miR396a-5p
Fold
Control
Cold
Freezing
Groups

miR398a-5p
Fold
Control
Cold
Freezing
Groups

miR5037c
Fold
Control
Cold
Freezing
Groups

miR5231
Fold
Control
Cold
Freezing
Groups

NmiR0018
Fold
Control
Cold
Freezing
Groups

NmiR0026
Fold
Control
Cold
Freezing
Groups

NmiR0051
Fold
Control
Cold
Freezing
Groups



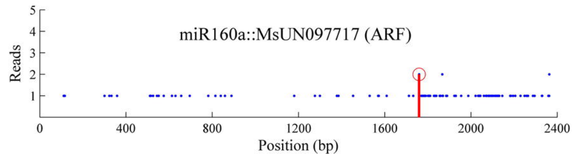
miR156a::MsUN014236 (SBP)
Reads
Position (bp)



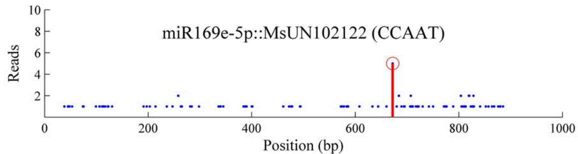
miR156e::MsUN011072 (SBP)
Reads
Position (bp)



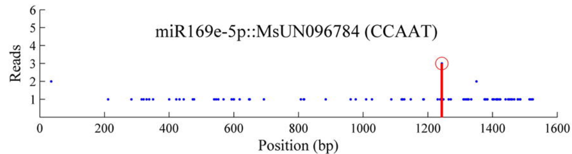
miR160a::MsUN041965 (ARF)
Reads
Position (bp)



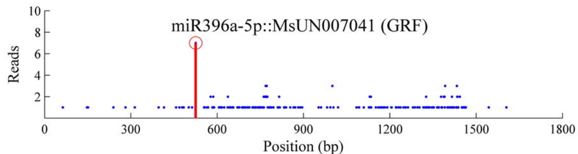
miR160a::MsUN097717 (ARF)
Reads
Position (bp)



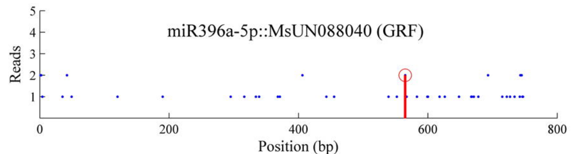
miR169e-5p::MsUN102122 (CCAAT)
Reads
Position (bp)



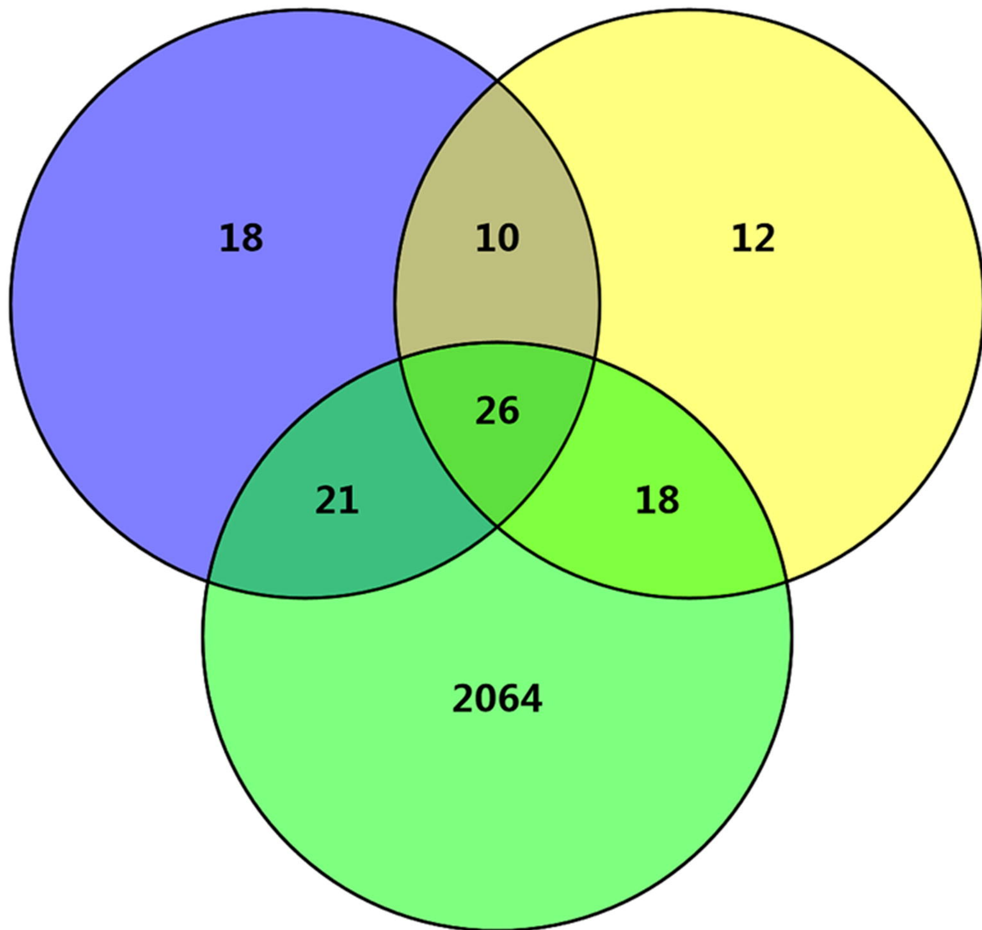
miR169e-5p::MsUN096784 (CCAAT)
Reads
Position (bp)



miR396a-5p::MsUN007041 (GRF)
Reads
Position (bp)



miR396a-5p::MsUN088040 (GRF)
Reads
Position (bp)


Cold
Freezing
18
10
12
26
21
18
2064
psRNATarget

GO:0008150
1.000000

GO:0008152
0.061587

GO:0009987
0.004204

GO:0065007
3.18e-09

GO:0071704
0.126078

GO:0009058
3.20e-06

GO:0044237
0.000755

GO:0006807
3.35e-05

GO:0050789
7.36e-09

GO:1901576
9.24e-07

GO:0044249
7.11e-07

GO:1901360
1.35e-05

GO:0046483
8.95e-06

GO:0006725
1.01e-05

GO:0034641
8.94e-06

GO:0044238
0.091709

GO:0019222
1.07e-09

GO:0050794
1.36e-09

GO:0043170
0.006385

GO:1901362
8.42e-10

GO:0018130
3.60e-10

GO:0019438
3.96e-10

GO:0044271
3.68e-10

GO:0006139
4.14e-06

GO:0009889
3.87e-11

GO:0080090
9.55e-11

GO:0051171
7.25e-11

GO:0031323
1.17e-10

GO:0010467
1.04e-06

GO:0009059
1.87e-08

GO:0060255
1.26e-10

GO:0044260
0.003523

GO:0090304
6.84e-07

GO:0034654
1.02e-10

GO:0031326
3.82e-11

GO:0019219
6.93e-11

GO:0010468
9.40e-11

GO:0034645
1.80e-08

GO:0010556
3.64e-11

GO:0016070
5.98e-09

GO:2000112
3.64e-11

GO:0032774
2.71e-11

GO:0051252
2.56e-11

GO:0006351
2.35e-11

GO:2001141
2.75e-11

GO:0006355
2.78e-11

(A)

Control
Cold
Freezing

Fold

0
1
2
3
4
5
6
7

miR167a
MsUN007721

(B)

Control
Cold
Freezing

Fold

0
0.5
1
1.5
2
2.5
3

miR396a-5p
MsUN018812