

RESEARCH ARTICLE

# Cold stress responsive microRNAs and their targets in *Musa balbisiana*

Jingyi WANG<sup>1</sup>, Juhua LIU<sup>1</sup>, Caihong JIA<sup>1</sup>, Hongxia MIAO<sup>1</sup>, Jianbin ZHANG<sup>1</sup>, Zhuo WANG<sup>1</sup>,  
Biyu XU (✉)<sup>1</sup>, Zhiqiang JIN (✉)<sup>1,2</sup>

<sup>1</sup> Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture/Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China

<sup>2</sup> Key Laboratory of Genetic Improvement of Bananas, Haikou Experimental Station, Chinese Academy of Tropical Agricultural Sciences, Haikou 570101, China

**Abstract** Cold stress is an environmental factor affecting plant development and production. Recently, microRNAs (miRNAs) have been found to be involved in several plant processes such as growth regulation and stress responses. Although miRNAs and their targets have been identified in several banana species, their participation during cold accumulation in banana remains unknown. In this study, two small RNA libraries were generated from micropropagated plantlets of *Musa balbisiana* grown at normal and low temperature (5°C). A total of 69 known miRNAs and 32 putative novel miRNAs were detected in the libraries by Solexa sequencing. Sixty-four cold-inducible miRNAs were identified through differentially expressed miRNAs analysis. Among 43 miRNAs belonging to 26 conserved miRNA families with altered expression, 18 were upregulated and 25 downregulated under cold stress. Of 21 putative novel miRNAs with altered expression, four were downregulated and 17 upregulated. Furthermore, eight miRNAs were validated by stem-loop qRT-PCR and their dynamic differential expression was analyzed. In addition, 393 target genes of 58 identified cold-responsive miRNAs were predicted and categorized by function. These results provide important information for further characterization and functional analysis of cold-responsive miRNAs in banana.

**Keywords** cold stress, microRNA, *Musa balbisiana*

RNAs, which post-transcriptionally regulate gene expression in many organisms by cleavage or translation suppression of their target mRNAs<sup>[1–4]</sup>. Increasing evidence demonstrates that miRNAs have important roles in many biological and metabolic processes including regulation of plant growth, development and response to biotic and abiotic stresses<sup>[5,6]</sup>. Cold temperature exposure is a major abiotic stress that affects plant development and growth and significantly impacts crop production. Plants have evolved various processes to cope with cold stress at the cellular and developmental levels. Not unexpectedly, the involvement of miRNAs in response of plants to cold has been evaluated in several plant species such as *Arabidopsis thaliana*<sup>[7–9]</sup>, poplar<sup>[10]</sup>, *Brachypodium*<sup>[11]</sup>, and rice<sup>[12]</sup>. In *Arabidopsis*, the expression of miR165/166, miR319c, miR169, miR393 and miR396 is induced by cold stress<sup>[7–9]</sup>. In *Brachypodium*, poplar, and *Arabidopsis*, miR397 and miR169 were found to be upregulated in response to cold stress<sup>[11]</sup> and miR168 was upregulated in *Arabidopsis*<sup>[9]</sup> and poplar<sup>[10]</sup>, although it was downregulated in rice<sup>[12]</sup> during cold stress. Recently, an analysis of deep sequencing data from a wheat thermo-sensitive genic male sterile line showed that 78 unique miRNAs may play a regulatory role in male sterility during cold stress<sup>[13]</sup>. miR319 has also been shown to regulate cold responses in both sugarcane<sup>[14]</sup> and poplar<sup>[10]</sup>.

Banana (*Musa* spp.), one of the most important staple crops widely cultivated in the tropics and subtropics, is a tropical giant perennial herb belonging to the Musaceae family of the order Zingiberales<sup>[15]</sup>. Cultivated bananas have evolved from the hybridization of wild species of *Musa acuminata* (AA) and *Musa balbisiana* (BB)<sup>[16]</sup>. Since they are mainly cultivated in tropical and subtropical regions, bananas are quite sensitive to cold. However, cold spells often occur during winter or early spring in banana planting areas in China, which can have a serious impact on banana development, fruit quality and yield. *Musa*

## 1 Introduction

MicroRNAs (miRNAs) are a class of non-coding small

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Correspondences: [biyuxu@126.com](mailto:biyuxu@126.com), [jinzhiqiang@itbb.org.cn](mailto:jinzhiqiang@itbb.org.cn)

*balbisiana*, native to South Asia with a geographical distribution ranging from India to south China, is immune to several diseases and pests, such as Fusarium wilt and Black leaf streak disease, and represent excellent sources of natural resistance<sup>[17]</sup>. The “B” genome is used in breeding programs for its traits of vigor and cold resistance and could also be interesting for tolerance to pests and pathogens<sup>[16]</sup>. Furthermore, genotypes with the “B” genome are more tolerant to abiotic stresses than those solely based on the “A” genome<sup>[18]</sup>.

Although miRNAs in bananas were identified in previous studies<sup>[15,19–23]</sup>, compared with the number of identified miRNAs in other plants such as rice and maize, the number of known miRNAs in banana is still very low. Moreover, there is no report on cold-responsive miRNAs from *M. balbisiana*, a wild banana species. In the present study, high-throughput sequencing, which has been widely used for miRNA research, was used to identify conserved and novel miRNA of *M. balbisiana*. The altered expression levels of these miRNAs under cold treatment were analyzed and compared with controls, and the potential roles of their target genes were investigated. In addition, the expression patterns of several miRNAs were examined by RT-qPCR methods. This is the first study to prove that miRNAs are widely involved in regulating the cold-induced process of banana. These data provide a new insight to cold-tolerance molecular mechanisms of banana under cold stress.

## 2 Materials and methods

### 2.1 Plant material and stress treatment

Micropropagated plantlets of *M. balbisiana* (BB) were obtained from the Bioversity International Musa Germplasm Transit Centre (ITC) in Leuven, Belgium [Musa Germplasm Information System (MGIS) accession no. ITC0545]. These plantlets were grown in a growth chamber at 25°C and 80% air humidity under a 16/8 h (light/dark) photoperiod. Five-leaf stage plantlets were used in the study. For the cold-stress treatments, leaf samples were first collected from plants at 25°C (CK), then, the temperature was lowered to 5°C and samples collected at 4, 8, and 24 h. The leaves from these three sampling times were cut into pieces and mixed well (CS). The collected leaf tissues were immediately frozen in liquid nitrogen and stored at – 80°C until used.

### 2.2 Small RNA sequencing and bioinformatics analysis

Total RNA was extracted from banana leaves using plant total RNA isolation kit (Tiandz Inc., Beijing, China). RNA degradation and contamination was monitored on 1% agarose gels; RNA purity was checked using a Nano-Photometer spectrophotometer (Implen, Westlake Village,

CA, USA) and RNA integrity was assessed using the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). A total of 3 µg RNA per sample was used for the construction of small RNA libraries. Sequencing libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (New England Biolabs, Ipswich, MA, USA) following the manufacture’s recommendations and index codes were added to attribute sequences to each sample. Briefly, total RNA was purified and fractionated by 15% TBE urea denaturing polyacrylamide gel electrophoresis (PAGE) and small RNA regions corresponding to the 18–30 nt bands in the marker lane were excised and recovered. Then 18–30 nt small RNAs were ligated to a 5’-adaptor and a 3’-adaptor sequentially by T4 RNA ligase. The adapter-ligated small RNAs were subsequently transcribed into cDNA by SuperScript III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and then PCR-amplified for 17 cycles using the adaptor primers. The PCR products were purified for high-throughput sequencing. The sequencing was performed by Novogene (Beijing, China).

After sequencing, clean reads were obtained by removing reads containing the following: poly-N, poly A/T/G/C, adapter-contaminated tags, low quality reads and reads less than 18 nt in length from the raw data. All of the high quality sequences were considered significant and analyzed further. The small RNA tags were mapped to a reference sequence by Bowtie (<http://banana-genome-hub.southgreen.fr/>)<sup>[15]</sup>. The sequences matching non-coding RNAs (rRNA, scRNA, snoRNA, snRNA and tRNA) deposited in the Rfam 10.1 database were removed from the sequences. The remaining unique sequences that mapped to known mature rice miRNAs in miRBase 21 (<http://www.mirbase.org/>)<sup>[24]</sup> were considered to be known miRNAs. The remaining unannotated sRNA sequences were analyzed by an integrated combination of miREvo<sup>[25]</sup> and mirDeep2<sup>[26]</sup> software to predict potential novel miRNAs through exploring hairpin structure, Dicer cleavage sites and the minimum free energy. The criteria used for novel miRNA were based on the work of Meyers et al.<sup>[27]</sup>.

### 2.3 Expression analysis of miRNA under cold stress

The differential expression analyses of miRNA were based on sequence reads generated from cold treatment and control libraries. The expression of miRNAs in the two libraries was normalized to obtain the number of miRNAs per million reads [normalized expression = (number of miRNA reads/total number of clean reads) × 10<sup>6</sup>]. Normalized miRNA reads with values less than one were excluded from the differential analysis. Differential expression analysis of two samples was performed using the DEGseq<sup>[28]</sup>. *P*-values were adjusted using *q*-value<sup>[29]</sup>. *q*-value < 0.05 and fold change > 1 or < –1 were set as

the threshold for significant differential expression by default.

#### 2.4 Quantitative real-time PCR analysis of miRNA expression

Identified *M. balbisiana* miRNAs were experimentally validated using quantitative real-time PCR (qRT-PCR). For the cold treatments, the plantlets were treated for 0, 4, 8 and 24 h at 5°C in a growth chamber. Untreated plantlets were used as controls. qRT-PCR were carried out as previously described<sup>[20]</sup>, with 5S rRNA used as an internal control (endogenous reference). All reactions were run in triplicate for each sample. Validated miRNAs and primers are listed in Table S1 (Appendix A). qRT-PCR analysis was performed using FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH, Mannheim, Germany, Cat. No. 04913850001) on a Mx3005P™ Real-Time PCR System (Stratagene, La Jolla, CA, USA). The relative expression quantification was calculated using the  $2^{-\Delta\Delta C_T}$  method<sup>[30]</sup>.

#### 2.5 Target gene prediction

Target predictions were performed by the online software psRNATarget (<http://plantgrn.noble.org/psRNATarget>)<sup>[31]</sup> with default parameters. Gene ontology (GO) enrichment analysis was used for the target gene candidates of differentially expressed miRNAs. Goseq based Wallenius non-central hypergeometric distribution<sup>[32]</sup>, which could adjust for gene length bias, was implemented for GO enrichment analysis.

### 3 Results

#### 3.1 Deep sequencing of *Musa balbisiana* sRNAs

A total of 13151282 (CK) and 17315104 (CS) raw reads were generated from Solexa Sequencing of the two libraries. After processing, 12198214 (CK) and 16738624 (CS) clean reads were obtained. Analysis of these sequences resulted in identification of 914848 (CK)

and 12677356 (CS) sequences ranging in size from 18 to 30 nt in length. About 683275 (CK) and 6615966 (CS) sequence reads (about 53% to 60% of 18–30 nt reads), could be mapped to the reference *Musa* genome<sup>[15]</sup> (Table 1). The majority of the small RNAs are 19–22 nt in size in the two libraries, with the 21 (CK) and 20 (CS) nt size classes predominant (Fig. 1).

#### 3.2 Identification of known miRNAs in *M. balbisiana*

To identify the known miRNAs in banana leaves, small RNA sequences were mapped to the known rice miRNAs from the miRBase database. After Blastn search and further sequence analysis, a total of 69 known miRNAs were identified (52 and 68 miRNAs in control and cold treatments, respectively), which belonged to 28 miRNA families (Appendix A, Table S2). Among these miRNAs, 51 known miRNAs (56%) were detected in both libraries, while 17 miRNAs were only detected in the cold treatment library (Fig. 2a). MiR396 was the largest family with seven members, followed by miR166, miR159, miR169 and miR171, with 6, 5, 5 and 5 members, respectively. Of the remaining 23 miRNA families, 10 comprised two to four members, and the others had only one member (Fig. 3). Read numbers based on expression level analysis showed significant differences in known miRNA families, which ranged from one to more than 100000 reads. Five miRNA families (miR156, miR159, miR164, miR166 and miR167) were strongly expressed, with more than 10000 reads detected in the CS library (Appendix A, Table S2).

#### 3.3 Identification of novel miRNAs in *M. balbisiana*

Based on the plant miRNA annotation criteria<sup>[27]</sup>, the formation of a stable hairpin structure is the primary criterion of a miRNA. In total, 32 potential novel miRNAs were predicted from the two libraries (Appendix A, Table S3). Twenty-one of the 32 novel miRNAs candidates were shared by the two libraries, and 10 predicted miRNAs candidates only existed in the cold treatment library, but only one of the 32 novel miRNA candidates was unique to the control library (Fig. 2b). In this study, the length of

**Table 1** Statistics of sRNA sequences for control (CK) and cold-stress (CS) libraries

Category	CK		CS	
	Total sRNAs	Unique sRNAs	Total sRNAs	Unique sRNAs
Raw reads	13151282		17315104	
Clean reads	12198214		16738624	
Sequences of 18 to 30 nucleotides	914848		12677356	
Sequences mapped to the genome	683275	338261	6615966	63348
Known miRNAs	17912	175	321534	434
Novel miRNAs	510	69	10019	214

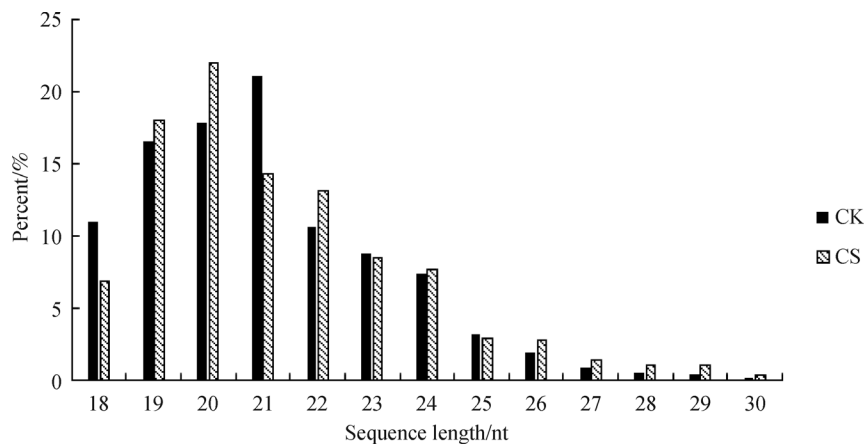


Fig. 1 The size distribution of small RNAs

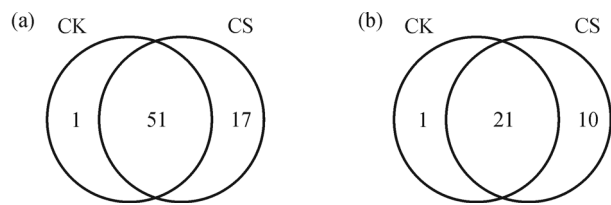


Fig. 2 Unique and overlapping banana miRNAs in control and cold-stress libraries. (a) Known unique and overlapping banana miRNAs; (b) novel unique and overlapping banana miRNAs.

these 32 potential novel miRNA precursors ranged from 44 to 273 nt, which was close to the length of miRNA precursors in Cavendish banana fruits<sup>[22]</sup>. Except for mba-miR19 and mba-miR22, the minimum free energy (MFE) folding value of these miRNA precursors ranged from  $-92.5.1\text{ kJ}\cdot\text{mol}^{-1}$  to  $-841.0\text{ kJ}\cdot\text{mol}^{-1}$ , with an average of  $282.2\text{ kJ}\cdot\text{mol}^{-1}$ , which was consistent with the previously

published low MFE characteristics of miRNA<sup>[33]</sup> and much higher than the published sequencing data for miRNA precursors from banana fruit ( $194.6\text{ kJ}\cdot\text{mol}^{-1}$ )<sup>[22]</sup>. In addition, Meyers et al. reported the existence of complementary sequences increases the authenticity of predicted novel miRNAs<sup>[27]</sup>. In our study, 15 of the 32 novel miRNAs have complementary miRNAs. Moreover, all of the novel complementary miRNAs exhibited lower expression levels than the corresponding mature miRNAs (Appendix A, Table S3), which might be due to the rapid degradation of complementary strands during the biogenesis of mature miRNAs<sup>[34]</sup>.

3.4 Differential expression analysis of miRNAs involved in cold stress in *M. balbisiana*

To detect the effect of cold stress on *M. balbisiana* miRNA expression, the relative abundances of untreated (CK) and cold-treated (CS) *M. balbisiana* miRNA sequences were compared. The details of differentially expressed miRNAs

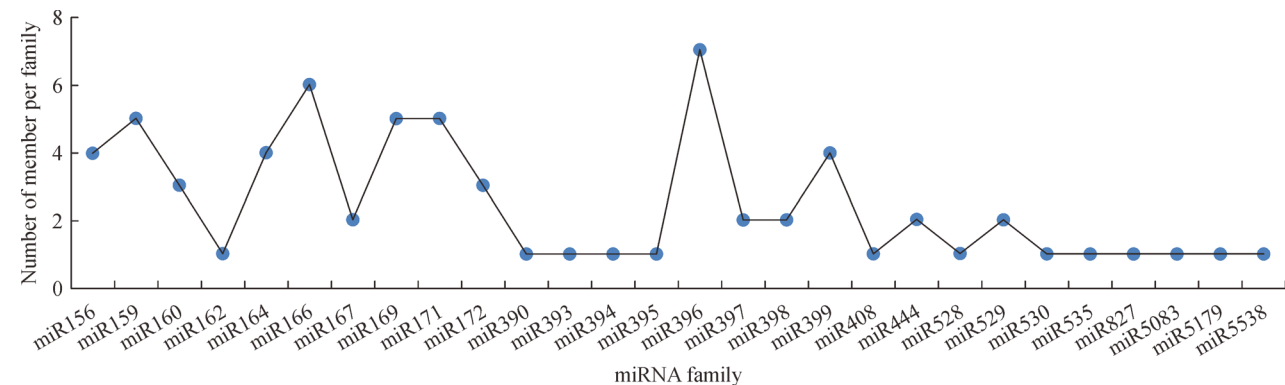


Fig. 3 Family size distribution of conserved miRNA in banana

are shown in Tables S2 and S3 (Appendix A). Based on the selected criteria (fold change >1 or < -1 and a *P*-value < 0.05), 43 known miRNAs and 21 novel miRNAs showed differential expression in response to cold stress (Table 2).

Among the known miRNAs with altered expression, 18 upregulated and 25 downregulated were detected. As for novel miRNAs, 17 upregulated and 4 downregulated were detected in the novel miRNAs.

**Table 2** Identification of differently expressed miRNAs during cold treatment in *Musa balbisiana*

miRNAs	Normalized count (TPM)		Fold change	<i>P</i> -value
	Cold stress	Control		
mba-miR156a	73224.56	22330.05	1.71	0.0000
mba-miR156c-3p	0.00	151.13	- 8.24	0.0000
mba-miR159f	11415.16	2644.85	2.11	0.0000
mba-miR160a-3p	12.02	0.00	4.59	0.0024
mba-miR160a-5p	128.26	831.24	- 2.70	0.0000
mba-miR160e-5p	72.15	415.62	- 2.53	0.0000
mba-miR164a	5066.28	1360.21	1.90	0.0000
mba-miR164c	53227.99	7972.32	2.74	0.0000
mba-miR166b-5p	80.16	340.05	- 2.08	0.0000
mba-miR166d-5p	11920.18	4193.97	1.51	0.0000
mba-miR167d-5p	246997.12	49647.53	2.31	0.0000
mba-miR169e	132.27	0.00	8.05	0.0000
mba-miR169h	16.03	0.00	5.00	0.0003
mba-miR171a	6096.37	869.02	2.81	0.0000
mba-miR171d-5p	20.04	0.00	5.32	0.0000
mba-miR171i-3p	7523.26	2871.55	1.39	0.0000
mba-miR172b	12.02	0.00	4.59	0.0024
mba-miR172c	4.01	37.78	- 3.24	0.0000
mba-miR390-5p	112.23	37.78	1.57	0.0004
mba-miR393a	160.33	0.00	8.32	0.0000
mba-miR394	3138.37	831.24	1.92	0.0000
mba-miR395b	505.02	75.57	2.74	0.0000
mba-miR396a-3p	573.16	264.48	1.12	0.0000
mba-miR396a-5p	10481.26	31851.50	- 1.60	0.0000
mba-miR396c-3p	16.03	37.78	- 1.24	0.0000
mba-miR396e-5p	19359.28	79496.50	- 2.04	0.0000
mba-miR396f-5p	38085.27	83917.17	- 1.14	0.0000
mba-miR396g	1218.47	6989.95	- 2.52	0.0000
mba-miR397a	40.08	113.35	- 1.50	0.0000
mba-miR397b	16.03	113.35	- 2.82	0.0000
mba-miR398a	56.11	0.00	6.81	0.0000
mba-miR398b	132.27	566.75	- 2.10	0.0000
mba-miR399a	124.25	415.62	- 1.74	0.0000
mba-miR399e	52.11	0.00	6.70	0.0000
mba-miR444a-3p.2	8.02	37.78	- 2.24	0.0000
mba-miR444f	16.03	151.13	- 3.24	0.0000
mba-miR5083	168.34	340.05	- 1.01	0.0000
mba-miR5179	24.05	0.00	5.59	0.0000
mba-miR528-5p	13090.56	2758.20	2.25	0.0000

(Continued)

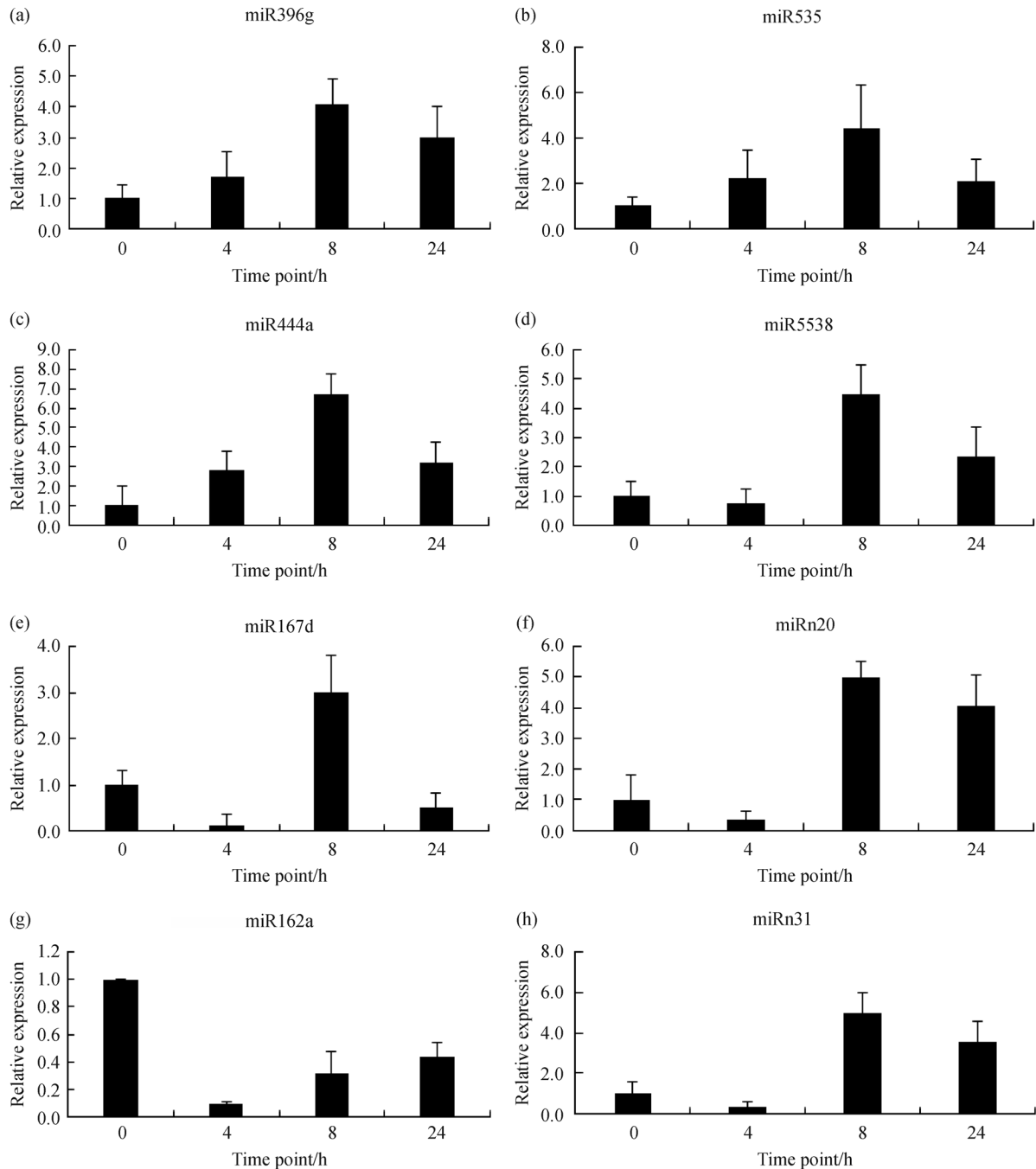
miRNAs	Normalized count (TPM)		Fold change	P-value
	Cold stress	Control		
mba-miR529a	68.14	0.00	7.09	0.0000
mba-miR530-5p	52.11	113.35	– 1.12	0.0000
mba-miR535-5p	39764.68	16775.88	1.25	0.0000
mba-miR5538	8.02	0.00	4.00	0.0197
mba-miRn02	76.15	0.00	7.25	0.0000
mba-miRn03	4.01	37.78	– 3.24	0.0000
mba-miRn04	32.07	0.00	6.00	0.0000
mba-miRn05	12.02	37.78	– 1.65	0.0000
mba-miRn06	8.02	0.00	4.00	0.0197
mba-miRn07	8.02	0.00	4.00	0.0197
mba-miRn08	0.00	113.35	– 7.82	0.0000
mba-miRn10	200.41	37.78	2.41	0.0000
mba-miRn11	6352.89	2795.98	1.18	0.0000
mba-miRn14	949.93	113.35	3.07	0.0000
mba-miRn17	424.86	0.00	9.73	0.0000
mba-miRn19	168.34	0.00	8.40	0.0000
mba-miRn20	2288.64	377.84	2.60	0.0000
mba-miRn22	10886.09	1133.51	3.26	0.0000
mba-miRn23	8.02	3400.52	– 8.73	0.0000
mba-miRn25	1551.15	528.97	1.55	0.0000
mba-miRn27	3230.55	75.57	5.42	0.0000
mba-miRn28	16.03	0.00	5.00	0.0003
mba-miRn29	320.65	0.00	9.32	0.0000
mba-miRn30	148.30	37.78	1.97	0.0000
mba-miRn31	88.18	0.00	7.46	0.0000

### 3.5 Validation of *M. balbisiana* miRNAs and dynamic analysis of differential expression

To confirm the expression of identified miRNAs and detect their dynamic response to cold stress at different treatment stages, the expression of six conserved and two novel miRNAs (miR162a, miR167d, miR396g, miR444a, miR535, miR5538, miRn20 and miRn31) with or without significantly altered expression following treatment were analyzed by stem-loop qRT-PCR after 0, 4, 8 and 24 h of cold stress at 5°C. Eight potential miRNAs were expressed and exhibited different expression patterns at different treatment stages (Fig. 4). The eight miRNAs were divided into three groups according to their expression pattern. Group I included three miRNAs (miR396g, miR444a and miR535), and their expression increased rapidly and reached maximum level at 8 h, then decreased. The expression of Group II miRNAs (miR167d, miR5538,

miRn20 and miRn31) decreased rapidly and reached a minimum level at 4 h, then increased at 8 h. The expressions of Group I and II were upregulated in 5°C cold stress. During the cold stress, the average expression level of these miRNAs was higher than the control (0 h). Group III had only one miRNA (miR162a), and its expression was reduced during the whole period of the cold treatment.

Deep sequencing results showed that miR162a, miR167d, miR535, miR5538, miRn20 and miRn31 were upregulated, whereas miR396g and miR444a were down-regulated (Table 2). qRT-PCR analysis indicated that the expression of six miRNAs showed the same variation trend with deep sequencing in spite of variation in the absolute change between the two methods. However, opposite results were detected in miR396g and miR444a. Such inconsistency between deep sequencing and qRT-PCR verification has also been reported by other researchers<sup>[35,36]</sup>.



**Fig. 4** Real-time RT-PCR validation of the cold-responsive mba-miRNAs

### 3.6 Target prediction for cold-responsive miRNAs and function annotation

For the 64 differentially expressed miRNAs, the target genes were predicted in the psRNATarget database to illustrate the putative mRNAs regulated by miRNAs under cold stress. The detailed annotation results of the prediction are shown in the Table S4 (Appendix A). Among these 64 miRNAs, six miRNAs (miR171d,

miR398a, miR5083, miRn11, miRn14 and miRn36) had no predicted targets. A total of 393 target mRNAs were predicted from the 58 cold-responsive miRNAs.

GO analysis showed that the predicted targets were classified into three main categories: biological processes, cellular components, and molecular functions (Fig. 5). Of these, cellular and metabolic process in biological process, cell and organelle part in cellular component, and binding and catalytic activity in molecular function were the most



**Fig. 5** Gene ontology analysis of mba-miRNA target genes

established categories. According to the identified miRNA targets results, many were homologous to conserved target genes existing in other plants species: these targets included squamosa promoter binding-like protein (miR156), auxin response factor (miR160 and miRn10), MADS-box transcription factor (miRn17, miR444 and miR5179), MYB family (miRn07 and miR159), AP-2 and ethylene-responsive transcription factor (miR172), basic helix–loop–helix transcription factor (miR5179), WRKY transcription factor (miRn07), NAC transcription factor (miRn20, miRn25, miRn31 and miR164), TCP family transcription factor (miRn14) and homeobox-leucine zipper protein (miRn27). In addition, other predicted target genes are involved in diverse metabolic and physiological processes, including catalytic activities such as dehydrogenase, decarboxylase, helicase, synthase, protein kinases, oxidoreductase, binding protein, lipase, transporters, disease resistance protein, f-box proteins, zinc finger protein, heat shock protein and laccase (Appendix A, Table S4).

## 4 Discussion

In recent years, increasing evidence has demonstrated that miRNAs have critical roles in both biotic and abiotic stress responses. However, there has been no systematic identification of cold-responsive miRNAs from banana reported. Taking advantage of the sequenced genome of *M. acuminata* cv. Pahang (AA) and the high-throughput sequencing technology, the present study reports the first identification of cold-inducible miRNAs in *M. balbisiana*. We constructed two small RNA libraries and identified 69 known miRNAs and 32 novel miRNAs (Appendix A, Tables S2 and S3). The 69 known miRNAs belonged to 28 conserved miRNA families. Previous studies indicate that 25 conserved miRNA families exist in both monocot and dicotyledonous species<sup>[37]</sup>. Notably, three miRNA families (miR444, miR5179 and miR5538) are conserved in monocots but not in *Arabidopsis* or poplar, suggesting that these are monocot-specific miRNAs. Except for the miR169 family, the other conserved miRNA families were detected in the two libraries. The 32 novel miRNAs significantly increased the number of miRNAs in *M. balbisiana*.

Relative expression of the conserved miRNAs varied widely, based on the sequence data in our study. Based on the bioinformatics analysis, 43 conserved miRNAs were identified as cold-responsive (Table 2). The most striking ones with 8-fold changes were miR156c, miR169e and miR393a. The remaining changes in the expression of miRNAs ranged up to 7-fold (Table 2). In this study, the expression of 13 conserved miRNAs, including miR164, miR167, miR169, miR171, miR390, miR393, miR394, miR395, miR5179, miR528, miR529, miR535 and miR5538, was upregulated, while miR166, miR396, miR397 and miR444 showed significant downregulation

after cold stress. However, miR169 in rice<sup>[12]</sup>, wheat<sup>[38]</sup>, poplar<sup>[39]</sup> and *Poncirus*<sup>[40]</sup> was found to be downregulated under cold stress. MiR166 in rice<sup>[12]</sup> and miR396 in *Arabidopsis*<sup>[7–9]</sup> and *Poncirus*<sup>[40]</sup> were upregulated under cold stress. These results indicate these miRNAs have a species-specific role in response to cold stress in plants. Moreover, we also found miRNA members in the same miRNA family, such as miR160a and miR160e, miR397a and miR397b, and miR398a and miR398b, exhibited reverse expression patterns. This suggests that they may have disparate roles in regulating expressions of the stress-responsive genes or that their downstream target genes are diversified.

The prediction of miRNA target genes is important for understanding miRNA gene-regulation. In this study, targets of 64 cold-responsive miRNAs were predicted (Appendix A, Table S4). The targets of conserved miRNAs, such as miR156, miR171 and miR172, have been investigated in several banana cultivars, and their functions were in accordance with most of the previous studies<sup>[20–23]</sup>. For the three conserved and three novel mba-miRNAs, our results revealed that the functions of most target genes are unknown. However, some mba-miRNA could target genes that belong to MYB, bHLH and bZIP transcription factor (TF) families, which have been reported as important regulators of cold stress responses<sup>[41–43]</sup>. As TFs are crucial master switches for biotic and abiotic stress response through controlling a wide range of downstream genes, it is conceivable that the miRNAs may exert an extensive impact on gene expression by regulating these TFs<sup>[44]</sup>. NACs are plant-specific TFs with diverse roles in development and stress regulation<sup>[45]</sup>. In this work, NAC was predicted to be the target of miR164, miRn20, miRn25, and miRn31, which was suppressed by cold, implying that the NAC gene may be activated under cold. The enhanced NAC expression may help the plant to fight against the cold stress, as overexpression of a NAC gene, *SNAC2*, has been reported to confer cold tolerance in rice<sup>[46]</sup>.

In addition, miRNAs of the same family may target the same genes. For instance, miR396a, miR396e, miR396f and miR396g have the same target genes (growth-regulating factor, GRF). It was reported that GRF7 functions as a repressor of a broad range of osmotic stress-responsive genes to prevent growth inhibition under normal conditions<sup>[47]</sup>. MiR396 was also detected to target a zinc ion binding protein family. Metal ion (copper, zinc, or iron) binding is important in normal respiratory function and the process of protein damage during oxidative stress in *A. thaliana*<sup>[48]</sup>. Zinc ion binding increases the structural complexity of abscisic acid stress ripening proteins, which is associated with DNA binding<sup>[49]</sup>. The functional analysis of these candidate target genes, combined with research on miRNA-mediated post-transcriptional regulation, will finally help in the illustration of the roles of miRNAs under cold stress in banana.

## 5 Conclusions

The present study is the first to examine the miRNA expression profile and evaluate miRNA function under cold stress in banana. We identified 69 conserved and 32 putative novel miRNAs from the banana leaf under cold treatment using high-throughput sequencing. The majority of the identified miRNAs were significantly responsive to cold stress in banana. Furthermore, eight miRNAs were validated by stem-loop qRT-PCR and their dynamic differential expressions were analyzed. In addition, 393 target genes of 58 identified cold-responsive miRNAs were predicted and categorized by function. These results provide important information for further characterization and functional analysis of cold-responsive miRNAs in banana.

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**Compliance with ethics guidelines** Jingyi Wang, Juhua Liu, Caihong Jia, Hongxia Miao, Jianbin Zhang, Zhuo Wang, Biyu Xu, and Zhiqiang Jin declare they have no conflicts of interest or financial conflicts to disclose.

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## References

1. Bartel D P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 2004, **116**(2): 281–297
2. Park M Y, Wu G, Gonzalez-Sulser A, Vaucheret H, Poethig R S. Nuclear processing and export of microRNAs in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 2005, **102**(10): 3691–3696
3. Papp I, Mette M F, Aufsatz W, Daxinger L, Schauer S E, Ray A, van der Winden J, Matzke M, Matzke A J. Evidence for nuclear processing plant microRNA and short interfering precursors. *Plant Physiology*, 2003, **132**(3): 1382–1390
4. Valencia-Sanchez M A, Liu J D, Hannon G J, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes & Development*, 2006, **20**(5): 515–524
5. Dong Q H, Han J, Yu H P, Wang C, Zhao M Z, Liu H, Ge A J, Fang J G. Computational identification of microRNAs in strawberry expressed sequence tags and validation of their precise sequences by miR-RACE. *Journal of Heredity*, 2012, **103**(2): 268–277
6. Sunkar R, Li Y F, Jagadeeswaran G. Functions of microRNAs in plant stress responses. *Trends in Plant Science*, 2012, **17**(4): 196–203
7. Sunkar R, Zhu J K. Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell*, 2004, **16**(8): 2001–2019
8. Zhou X, Wang G, Sutoh K, Zhu J K, Zhang W. Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochimica et Biophysica Acta*, 2008, **1779**(11): 780–788
9. Liu H H, Tian X, Li Y J, Wu C A, Zheng C C. Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA*, 2008, **14**(5): 836–843
10. Lu S, Sun Y H, Chiang V L. Stress-responsive microRNAs in *Populus*. *Plant Journal*, 2008, **55**(1): 131–151
11. Zhang J, Xu Y, Huan Q, Chong K. Deep sequencing of Brachypodium small RNAs at the global genome level identifies microRNAs involved in cold stress responses. *BMC Genomics*, 2009, **10**(1): 449–465
12. Lv D K, Bai X, Li Y, Ding X D, Ge Y, Cai H, Ji W, Wu N, Zhu Y M. Profiling of cold-stress-responsive miRNAs in rice by microarrays. *Gene*, 2010, **459**(1–2): 39–47
13. Tang Z, Zhang L, Xu C, Yuan S, Zhang F, Zheng Y, Zhao C. Uncovering small RNA-mediated responses to cold stress in a wheat thermosensitive genic male-sterile line by deep sequencing. *Plant Physiology*, 2012, **159**(2): 721–738
14. Thiebaut F, Rojas C A, Almeida K L, Grativol C, Domiciano G C, Lamb C R, De Almeida Engler J, Hemerly A S, Ferreira P C G, De Almeida Engler J, Hemerly A S, Ferreira P C. Regulation of miR319 during cold stress in sugarcane. *Plant, Cell & Environment*, 2012, **35**(3): 502–512
15. D'Hont A, Denoeud F, Aury J M, Baurens F C, Carreel F, Garsmeur O, Noel B, Bocs S, Droc G, Rouard M, Da Silva C, Jabbari K, Cardi C, Poulain J, Souquet M, Labadie K, Jourda C, Lengellé J, Rodier-Goud M, Alberti A, Bernard M, Correa M, Ayyampalayam S, Mckain M R, Leebens-Mack J, Burgess D, Freeling M, Mbéguié-A-Mbéguié D, Chabannes M, Wicker T, Panaud O, Barbosa J, Hribova E, Heslop-Harrison P, Habas R, Rivallan R, Francois P, Poirion C, Kilian A, Burthia D, Jenny C, Bakry F, Brown S, Guignon V, Kema G, Dita M, Waalwijk C, Joseph S, Dievart A, Jaillon O, Leclercq J, Argout X, Lyons E, Almeida A, Jeridi M, Dolezel J, Roux N, Risterucci A M, Weissenbach J, Ruiz M, Glaszmann J C, Quétier F, Yahiaoui N, Wincker P. The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature*, 2012, **488**(710): 213–217
16. Baurens F C, Bocs S, Rouard M, Matsumoto T, Miller R N, Rodier-Goud M, MBéguié-A-MBéguié D, Yahiaoui N, MBéguié-A-MBéguié D, Yahiaoui N. Mechanisms of haplotype divergence at the RGA08 nucleotide-binding leucine-rich repeat gene locus in wild banana (*Musa balbisiana*). *BMC Plant Biology*, 2010, **10**(1): 149–165
17. Subbaraya U. Potential and constraints of using wild Musa. In: Subbaraya U, eds. Farmers' knowledge of wild Musa in India. *Food and Agriculture Organization of the United Nations*, 2006, 33–36
18. Ravi I, Uma S, Vaganan M M, Mustaffa M M. Phenotyping bananas for drought resistance. *Frontiers in Physiology*, 2013, **4**: 9
19. Davey M W, Gudimella R, Harikrishna J A, Sin L W, Khalid N, Keulemans J. A draft *Musa balbisiana* genome sequence for molecular genetics in polyploid, inter- and intra-specific *Musa* hybrids. *BMC Genomics*, 2013, **14**(1): 683–703
20. Chai J, Feng R J, Shi H R, Ren M Y, Zhang Y D, Wang J Y. Bioinformatic identification and expression analysis of banana

- microRNAs and their targets. *PLoS One*, 2015, **10**(4): e0123083
21. Ghag S B, Shekhawat U K S, Ganapathi T R. Small RNA profiling of two important cultivars of banana and overexpression of miRNA156 in transgenic banana plants. *PLoS One*, 2015, **10**(5): e0127179
  22. Bi F C, Meng X C, Ma C, Yi G J. Identification of miRNAs involved in fruit ripening in Cavendish bananas by deep sequencing. *BMC Genomics*, 2015, **16**(1): 776–791
  23. Lee W S, Gudimella R, Wong G R, Tammi M T, Khalid N, Harikrishna J A. Transcripts and microRNAs responding to salt stress in *Musa acuminata colla* (AAA group) cv. berangan roots. *PLoS One*, 2015, **10**(5): e0127526
  24. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Research*, 2014, **42**(D1): 68–73
  25. Wen M, Shen Y, Shi S, Tang T. miREvo: an integrative microRNA evolutionary analysis platform for next-generation sequencing experiments. *BMC Bioinformatics*, 2012, **13**(1): 140–150
  26. Friedlander M R, Mackowiak S D, Li N, Chen W, Rajewsky N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Research*, 2012, **40**(1): 37–52
  27. Meyers B C, Axtell M J, Bartel B, Bartel D P, Baulcombe D, Bowman J L, Cao X, Carrington J C, Chen X, Green P J, Griffiths-Jones S, Jacobsen S E, Mallory A C, Martienssen R A, Poethig R S, Qi Y, Vaucheret H, Voinnet O, Watanabe Y, Weigel D, Zhu J K. Criteria for annotation of plant MicroRNAs. *Plant Cell*, 2008, **20**(12): 3186–3190
  28. Wang L, Feng Z, Wang X, Wang X, Zhang X. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics*, 2010, **26**(1): 136–138
  29. Storey J D. The positive false discovery rate: a Bayesian interpretation and the  $q$ -value. *Annals of Statistics*, 2003, **31**(6): 2013–2035
  30. Livak K J, Schmittgen T D. Analysis of relative gene expression data using realtime quantitative PCR and the  $2^{-\Delta\Delta C_T}$  method. *Methods*, 2001, **25**(4): 402–408
  31. Dai X B, Zhao P X. psRNAtarget: a plant small RNA target analysis server. *Nucleic Acids Research*, 2011, **39**(suppl. 2): W155–W159
  32. Young M D, Wakefield M J, Smyth G K, Oshlack A. Goseq: gene ontology testing for RNA-seq datasets. *ResearchGate*, 2012, 1–26
  33. Bonnet E, Wuyts J, Rouze P, Van de Peer Y. Evidence that microRNA precursors, unlike other non-coding RNAs, have lower folding free energies than random sequences. *Bioinformatics*, 2004, **20**(17): 2911–2917
  34. Rajagopalan R, Vaucheret H, Trejo J, Bartel D P. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes & Development*, 2006, **20**(24): 3407–3425
  35. Sunkar R, Zhou X F, Zheng Y, Zhang W X, Zhu J K. Identification of novel and candidate miRNAs in rice by high throughput sequencing. *BMC Plant Biology*, 2008, **8**(1): 25–42
  36. Xu Q, Liu Y L, Zhu A D, Wu X M, Ye J L, Yu K Q, Guo W W, Deng X X. Discovery and comparative profiling of microRNAs in a sweet orange red-flesh mutant and its wild type. *BMC Genomics*, 2010, **11**(1): 246–263
  37. Jones-Rhoades M W, Bartel D P, Bartel B. MicroRNAs and their regulatory roles in plants. *Annual Review of Plant Biology*, 2006, **57**(1): 19–53
  38. Wang B, Sun Y F, Song N, Wei J P, Wang X J, Feng H, Yin Z Y, Kang Z S. MicroRNAs involving in cold, wounding and salt stresses in *Triticum aestivum* L. *Plant Physiology and Biochemistry*, 2014, **80**: 90–96
  39. Chen L, Zhang Y, Ren Y, Xu J, Zhang Z, Wang Y. Genome-wide identification of cold-responsive and new microRNAs in *Populus tomentosa* by high-throughput sequencing. *Biochemical and Biophysical Research Communications*, 2012, **417**(2): 892–896
  40. Zhang X N, Li X, Liu J H. Identification of conserved and novel cold-responsive microRNAs in trifoliate orange (*Poncirus trifoliata* (L.) Raf.) using high-throughput sequencing. *Plant Molecular Biology Reporter*, 2014, **32**(2): 328–341
  41. Agarwal M, Hao Y, Kapoor A, Dong C H, Fujii H, Zheng X, Zhu J K A. R2R3type MYB transcription factor is involved in the cold regulation of *CBF* genes and in acquired freezing tolerance. *Journal of Biological Chemistry*, 2006, **281**(49): 37636–37645
  42. Liu C, Wu Y, Wang X. bZIP transcription factor OsbZIP52/RISBZ5: a potential negative regulator of cold and drought stress response in rice. *Planta*, 2012, **235**(6): 1157–1169
  43. Huang X S, Wang W, Zhang Q, Liu J H. A basic helix-loop-helix transcription factor, PtrbHLH, of *Poncirus trifoliata* confers cold tolerance and modulates peroxidase-mediated scavenging of hydrogen peroxide. *Plant Physiology*, 2013, **162**(2): 1178–1194
  44. Giacomelli J I, Weigel D, Chan R L, Manavella P A. Role of recently evolved miRNA regulation of sunflower HaWRKY6 in response to temperature damage. *New Phytologist*, 2012, **195**(4): 766–773
  45. Olsen A N, Ernst H A, Leggio L L, Skriver K. NAC transcription factors: structurally distinct, functionally diverse. *Trends in Plant Science*, 2005, **10**(2): 79–87
  46. Hu H H, You J, Fang Y J, Zhu X Y, Qi Z Y, Xiong L Z. Characterization of transcription factor gene SNAC2 conferring cold and salt tolerance in rice. *Plant Molecular Biology*, 2008, **67**(1): 169–181
  47. Kim J S, Mizoi J, Kidokoro S, Maruyama K, Nakajima J, Nakashima K, Mitsuda N, Takiguchi Y, Ohme-Takagi M, Kondou Y, Yoshizumi T, Matsui M, Shinozaki K, Yamaguchi-Shinozaki K. *Arabidopsis* growth-regulating factor7 functions as a transcriptional repressor of abscisic acid- and osmotic stress-responsive genes, including DREB2A. *Plant Cell*, 2012, **24**(8): 3393–3405
  48. Tan Y F, O'Toole N, Taylor N L, Millar A H. Divalent metal ions in plant mitochondria and their role in interactions with proteins and oxidative stress-induced damage to respiratory function. *Plant Physiology*, 2010, **152**(2): 747–761
  49. Goldgur Y, Rom S, Ghirlando R, Shkolnik D, Shadrin N, Konrad Z, Bar-Zvi D. Desiccation and zinc binding induce transition of tomato abscisic acid stress ripening 1, a water stress-and salt stress-regulated plant-specific protein, from unfolded to folded state. *Plant Physiology*, 2007, **143**(2): 617–628