miRNA alteration is an important mechanism in sugarcane response to low-temperature environment

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Abstract

Background: Cold is a major abiotic stress limiting the production of tropical and subtropical crops in new production areas. Sugarcane (Saccharum spp.) originates from the tropics but is cultivated primarily in the sub-tropics where it frequently encounters cold stress. Besides regulating plant growth, miRNAs play an important role in environmental adaption.

Results: In this study, a total of 412 sugarcane miRNAs, including 261 known and 151 novel miRNAs, were obtained from 4 small RNA libraries through the Illumina sequencing method. Among them, 62 exhibited significant differential expression under cold stress, with 34 being upregulated and 28 being downregulated. The expression of 13 miRNAs and 12 corresponding targets was validated by RT-qPCR, with the majority being consistent with the sequencing data. GO and KEGG analysis indicated that these miRNAs were involved in stress-related biological pathways. To further investigate the involvement of these miRNAs in tolerance to abiotic stresses, sugarcane miR156 was selected for functional analysis. RT-qPCR revealed that miR156 levels increased in sugarcane during cold, salt and drought stress treatments. Nicotiana benthamiana plants transiently overexpressing miR156 exhibited better growth status, lower ROS levels, higher anthocyanin contents as well as the induction of some cold-responsive genes, suggesting its positive role in the plant cold stress response.

Conclusions: This study provides a global view of the association of miRNA expression with the sugarcane response to cold stress. The findings have enriched the present miRNA resource and have made an attempt to verify the involvement of miR156 in plant response to cold stress.

Keywords: Saccharum spp., High-throughput sequencing, miR156, Cold, Transcriptome

Background

Small non-coding RNAs of 18–40 nucleotides (nt) in length, including microRNAs (miRNAs) and small interference RNAs (siRNAs), can regulate gene expression by posttranscriptional mechanisms and epigenetic modifications to influence a number of biological processes [1]. These endogenous 18–25 nt non-coding RNAs [2] can target mRNA cleavage or translational repression by guiding the RNA-induced silencing complex (RISC) to bind to the target mRNA [3]. Primary miRNAs (pri-miRNAs) are transcribed as long precursors in the nucleus, and can be transferred to the cytoplasm to form mature miRNAs after a series of processing steps [4, 5]. The physiological significance of miRNAs in plants has been demonstrated in various biological processes [6, 7]. In order to understand the molecular mechanism of miRNAs in non-model organisms, more miRNAs need to be identified and characterized. With the advantages of identifying large amounts of miRNAs effectively in a short time and at low cost [8], high-throughput sequencing has become the main method for obtaining miRNAs [9, 10].

Cold stress, including chilling (<20 °C) and freezing (<0 °C), is one important limiting factor that can affect the geographical distribution and planting season of crops, especially for tropical and subtropical crops [11]. Low temperature can suppress plant growth and development.
by inhibiting metabolic reactions, leading to osmotic, oxidative and other stresses [12]. Plants usually possess a series of strategies to respond to temperature fluctuations, such as the remodeling of cell and tissue structures and gene expression, and metabolism reprogramming [11–13]. Cold-related gene expression can be regulated at the transcriptional, post-transcriptional and post-translational levels [14]. During low temperature stress, C-repeat binding factors (CBFs), which bind to dehydration-responsive elements in gene promoters to activate the downstream cold-responsive (COR) genes, is regulated by the inducer of C-repeat binding factor expression 1 (ICE1). This is known as the ICE-CBF-COR pathway [14, 15]. Furthermore, reports have shown that some miRNAs also take part in the plant response to cold stress and play crucial roles in this process [16, 17]. In sugarcane (Saccharum spp.), the up-regulation of miR319 and down-regulation of its targets, including a Myb transcription factor (GAMYB) and a TCP transcription factor (PCF5), were observed in both cold-tolerant and -sensitive sugarcane varieties under treatment at 4 °C [18]. However, their expression changes were delayed in cold-tolerant varieties [18]. Similar results were found in Oryza sativa L., whereby the overexpression of osa-miR319 led to enhanced cold tolerance in transgenic rice seedlings [19]. In addition, some other cold-related miRNAs, such as miR156k from rice [20] and miR394 from Arabidopsis [21], have also been reported.

Sugarcane accounts for ~80% of the global sugar production and has successfully been developed to be one of the most promising energy biofuels in Brazil [22]. However, sugarcane growth and development is affected by various factors, and due to its tropical origin, domestic varieties are restricted to tropical and subtropical regions [23, 24]. Although low temperature is beneficial for sucrose accumulation in sugarcane [25], it also leads to bud browning, resulting in poor growth [26]. Sugarcane harvest normally begins in winter and continues until spring.

In the present study, a high-throughput small RNA deep sequencing method was used to obtain cold-related miRNAs in sugarcane. The interaction networks of these miRNAs and their targets were investigated based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. In addition, the roles of several miRNAs, such as miR156, miR168, miR169 and miR408, in the plant response to abiotic stress, were explored. Our aim was to gain insight into the molecular mechanism of sugarcane cold tolerance and the role of miRNAs in this process.

**Methods**

**Samples and small RNA library preparation**

The nodes of ROC22 (relative cold sensitivity) and FN39 (relative cold tolerance) sugarcane cultivars (Saccharum spp. hybrid) were harvested from the field in the Key Laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture in Fuzhou City, China. The related information of cold tolerance is indicated in Additional file 10: Fig. S1. These nodes were firstly treated with flowing water for 2 days to promote the sprouting of buds and remove impurities, and then they were cultivated in an incubator (Dongqi, Ningbo, China) at 28 °C in the dark and at 65% relative humidity for 4 days for sprouting using the moisture culture method [26]. The cold treatment (4 °C) was performed on corresponding buds from both cultivars for 0 (before the cold treatment), 3, 12, 24 and 48 h. Three biological replications were used for each time point and 6 buds for each sample. The total RNA of all the collected samples was extracted using Trizol™ Reagent (Invitrogen, Carlsbad, CA), qualified by electrophoresis with a 1% agarose gel and quantified using a NanoVue Plus (GE healthcare, Little Chalfont, UK). Usually, gene expression changes occur early upon cold stress, as attested by already published reports [27, 28] and previous results obtained in our lab (unpublished data). For this reason, samples collected at 0 and 3 h of cold stress treatment were assumed to be suitable for target molecular analyses. Therefore, the total RNA (10 μg) of the 0 and 3 h samples of FN39 and ROC22 cultivars was sent to BGI (Shenzhen, China) on dry ice for high-throughput small RNA deep sequencing using the Illumina HiSeq high-throughput sequencing platform.

Healthy sugarcane plantlets exhibiting constant growth status derived from the tissue culture of the ROC22 cultivar were grown under 16 h light/8 h dark conditions at 28 °C for 1 week in an incubator. Then, the plantlets were treated with 250 mM NaCl and 25% PEG8000 as the salt and drought stresses, respectively [29]. The samples subjected to salt and drought treatments for 0, 6, 12 and 24 h were collected for RNA extraction. Three biological replications were setup and 5 plantlets were used for each sample.

**Bioinformatics analysis**

Following size fractionation of the 18–30 nucleotide small RNAs and 5′ adaptor ligation and 3′ adaptor ligation, the small RNAs were reversely transcribed into cDNA, amplified and sequenced. The clean reads were obtained from the raw data from small RNA deep sequencing by removing the low-quality reads and impurities, including the reads without insertions; without 3′-primer; with 5′-primer contaminants; those with poly A tails as well as small fragment sequences. The clean reads were firstly used to analyze the common/specific sequences and length distributions [30]. The ribosomal RNA (rRNA), small cytoplasmic RNA (scRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA) and transfer RNA (tRNA) were annotated and removed
from the clean reads via a BLASTn search on the NCBI GenBank database and Rfam (10.1) database (e = 0.01). The remaining clean reads were aligned with the known miRNAs in miRBase (http://www.mirbase.org/) using the procedure and parameter blastall -p blastn -F F -e 0.01. The detail criterion is that firstly align the clean reads to the miRNA precursor in miRBase with no mismatch. Secondly, the obtained tags align with the mature miRNAs in miRBase with at least 16 nt overlap allowing offsets. Those miRNAs satisfying both criteria will be counted to get their expression, and will be analyzed to get base bias on the first position of the identified miRNAs with certain length, and base bias on each position of all identified miRNAs respectively. The novel miRNAs were predicted using the Mireap software (http://sourceforge.net/projects/mireap/) developed by BGI through considering the biological characteristics of miRNA, such as the secondary structure, the location of mature miRNA on its precursor and the minimum free energy [31]. Specific parameters were set as follows: minimal miRNA sequence length was 18; maximal miRNA sequence length was 25; minimal miRNA reference sequence length was 20; maximal miRNA reference sequence length was 23; maximal copy number of miRNAs on reference was 20; maximal free energy allowed for a miRNA precursor was −18 kcal/mol; maximal space between miRNA and miRNA* was 300, minimal base pairs of miRNA and miRNA* was 16; maximal bulge of miRNA and miRNA* was 4; maximal asymmetry of miRNA/miRNA* duplex was 4; flank sequence length of miRNA precursor was 20 [31].

The comparison analysis between the control and the cold-treated sugarcane was performed according to the following steps. The miRNA expression quantity was normalized using the formula: Normalized expression = \( \frac{\text{actual miRNAcount}}{\text{total count of clean reads}} \times 1,000,000 \). Then, the normalized expression quantity was used to calculate the fold change using the formula: Fold change = \( \log_2 \left( \frac{\text{treatment}}{\text{control}} \right) \).

Mireap software was also used to predict the potential target genes according to the rules as previously reported [32, 33]. In order to identify the functions of potential targets of differentially expressed miRNAs, the number of targets mapping to each gene ontology term in the Gene Ontology database was calculated. Then, the significantly enriched GO terms were obtained using the super geometry inspection method. The GO enrichment analysis can provide clues for the identification of the main biological functions of the potential targets [34]. To further understand the biological functions, KEGG pathway analysis was also conducted, which provides information on the main biochemical pathways and signal transduction pathways the potential targets involved [35]. The GO enrichment analysis was used on predicted target gene candidates of miRNAs. This method firstly map all target gene candidates to GO terms in the database (http://www.geneontology.org/), and calculate gene numbers for each term, and then use hypergeometric test to find significantly enriched GO terms in target gene candidates comparing to the reference gene background. The calculating formula is: \( P = 1 - \sum_{i=0}^{m-1} \frac{\binom{n-i}{m-i} \binom{N-n}{m-n}}{\binom{N}{m}} \), within which N is the number of all genes with GO annotation; n is the number of target gene candidates in N; M is the number of all genes that are annotated to a certain GO term and m is the number of target gene candidates in M. The Bonferroni Correction for the p-value is used to obtain the corrected p-value. GO terms with corrected p-value <0.05 are defined as significantly enriched in target gene candidates. KEGG pathway analysis was also used for the target gene candidates. The calculating formula is the same as that in GO analysis. In this formula, N is the number of all genes with KEGG annotation, n is the number of target gene candidates in N, M is the number of all genes annotated to a certain pathway, and m is the number of target gene candidates in M. Genes with FDR(false discovery rate) <0.05 are considered as significantly enriched in target gene candidates. The KEGG analysis reveals the main pathways which the target gene candidates are involved in.

Expression validation of miRNAs and potential target genes by RT-qPCR
The cold responsive genes, CBF1 (C-repeat binding factor gene), CBF3 and NAC23 (NAM-ATAF-CUC gene), have been reported to be induced by cold stress and were used to verify the efficiency of cold treatment in this study by the reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) method. In order to validate the presence and expression levels of miRNAs, 13 miRNAs were randomly detected by RT-qPCR method. Total RNA was extracted from the samples of 0, 3, 12, 24 and 48 h cold-treated sugarcane buds using Trizol® Reagent (Invitrogen, CA), followed by DNase (Promega, USA) treatment to remove the genomic DNA. For the miRNA, the cDNA synthesis was performed according to the instruction of the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA). For each miRNA, the specific stem loop primer (Additional file 1: Table S1) was added to the reaction system. The reaction was performed at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 s. All cDNA samples were 25-fold diluted and then used as a template in the miRNA RT-qPCR analysis. For the potential target genes, the cDNA synthesis was performed
according to the instruction of the PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). The reaction procedure included reverse transcription at 37°C for 15 min and the inactivation of reverse transcriptase with heat treatment at 85°C for 5 s. The expression patterns of miRNAs and potential targets were detected according to the SYBR Green Master (ROX) (Roche, China) manufacturer instruction on a 7500 real time PCR system (Applied Biosystems, USA) (Additional file 2: Table S2). 185 ribosomal RNA (18S rRNA) [26] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [18] were used as the internal control for miRNA and potential targets, respectively. The expression was calculated using the 2^(-ΔΔCt) method [36].

All the statistical analysis was conducted using the Statistical Product and Service Solutions (SPSS20.0 version) software. Data was expressed as the mean ± standard error (SE). Significance (p-value <0.05) was calculated using one-way Analysis of Variance (ANOVA) followed by Duncan’s multiple range test.

The transient expression of miRNA in Nicotiana benthamiana

The precursor of miRNA, MIRNA, cloned from sugarcane was sub-cloned with BamH I and Xba I sites into the overexpression vector pCAMBIA 1301, and then transferred into the Agrobacterium tumefaciens strain EHA105. After inoculation in LB medium containing kanamycin (50 μg/mL) and rifampicin (34 μg/mL) twice, the cells of the EHA105 strain were collected and diluted to OD₆₀₀ = 0.8 with Murashige and Skoog (MS) medium (containing 200 mM acetosyringone). The Agrobacterium-mediated transformation was performed according to a previously described method [37]. The 1 mL syringe was used to infiltrate diluted bacterial suspension into the leaves of tobacco plants at the 6–8-leaf stage. After cultivation for 48 h under 16 h light/8 h dark at 23°C conditions, the injected tobacco plants were treated with 4°C stress for 12, 24 and 48 h in an incubator (Dongqi, Ningbo, China). The collected injected tobacco leaves were collected for RNA extraction and DAB staining. Six biological replications were used.

3, 3′-diaminobenzidine (DAB) solution staining was used to assess H₂O₂ production [37]. The collected leaves were put into DAB solution (1 mg/mL, pH = 5.8) overnight under dark condition for staining. After boiling in 95% alcohol until the green color faded, the leaves were rinsed in 95% alcohol and photographed.

Anthocyanin quantification was performed according to a previously described method [38]. Briefly, 0.2 g fresh injected tobacco leaves were excised with MeOH: HCl: H₂O (80:5:15) at 4°C overnight in the dark. After centrifugation at 15,000 x g for 5 min, the anthocyanin levels in the extracts were qualified at 530 nm (T6 spectrophotometer, Beijing Purkinje General Instruments).

Results

Data analysis of small RNA libraries

In order to investigate the expression of miRNAs in sugarcane under cold stress, samples were collected at 0, 3, 12, 24 and 48 h over the 4°C treatment. To validate the cold treatment, the expression of three cold responsive genes (CBF1, CBF3 and NAC23) was examined in these collected samples by RT-qPCR. The results showed that the transcripts of the three genes were all increased compared to those under control condition (Additional file 11: Figure S2), indicating that the cold treatment to sugarcane was effective in this study. In order to identify the miRNAs and other small RNAs involved in cold tolerance, two sugarcane cultivars including FN39 (relative cold tolerance) and ROC22 (relative cold sensitivity) were used to generate four small RNA libraries: F0 (samples of FN39 cultivar without cold treatment), F3 (samples of FN39 cultivar with 3 h cold treatment), R0 (samples of ROC22 without cold treatment) and R3 (samples of ROC22 cultivar with 3 h cold treatment). These libraries were sequenced by Illumina sequencing, which generated between 20 and 25 million clean reads after filtering out the low quality reads and contaminations, which accounted for 0.78% (F0), 0.97% (F3), 0.78% (R0) and 0.90% (R3) of the raw reads, respectively (Table 1). In all the above libraries, the reads with 24 nt and 21 nt lengths were the most abundant (Fig. 1). The analysis of 18–30 nt miRNA nucleotide bias showed that the first base of 21 nt and 22 nt miRNAs was uracil (U), and the miRNA nucleotide bias at the tenth-eleventh position, usually as the cleavage site, was an adenine (A) in all these libraries.

Identification of known miRNAs in sugarcane

To identify the known miRNAs, the clean reads were aligned with known miRNAs in miRBase. There were 219, 207, 210 and 209 known miRNAs identified in the F0, F3, R0 and R3 small RNA libraries, respectively (Additional file 3: Table S3). Compared with F0, F3 had 24 differentially expressed miRNAs (|log₂ ratio| ≥ 1, p ≤ 0.05, Fig. 2a, Fig. 3a) including 17 upregulated and seven downregulated (Table 2). Similarly, 15 differentially expressed miRNAs (all upregulated) were identified upon comparison of R3 with R0 (Table 2). Among these differentially expressed miRNAs, there were eight common miRNAs (miR2199, miR5054, miR5059, miR5072, miR5077, miR5152-3p, miR5813 and miR8155) in both cultivars; all of which were upregulated (Fig. 2a). Furthermore, some known differentially expressed miRNAs were identified in either FN39 or ROC22. Among them, 16 known miRNAs, including nine upregulated and seven downregulated miRNAs, were only found in FN39 libraries, accounting for 66.7% (Table 2), including miR157a, miR160a, miR169b, miR6300, miR6196 and...
Table 1: Small RNA categorization in sugarcane

| Categorization | Unique sRNA (%) | Total sRNA (%) |
|----------------|----------------|----------------|
|                | F0 (%)         | F3 (%)         | R0 (%)         | R3 (%)         |
| miRNA          | 67,373 (0.53)  | 57,249 (0.56)  | 59,960 (0.54)  | 58,772 (0.58)  |
|                | 2,386,352 (9.41) | 1,638,016 (7.89) | 2,635,873 (10.12) | 2,302,177 (9.52) |
| rRNA           | 56,395 (0.44)  | 80,912 (0.79)  | 81,039 (0.73)  | 98,210 (0.97)  |
|                | 729,453 (2.88) | 1,613,788 (7.78) | 1,453,854 (5.58) | 2,451,555 (10.14) |
| repeat         | 412 (0)        | 345 (0)        | 447 (0)        | 376 (0)        |
|                | 957 (0)        | 799 (0)        | 1019 (0)       | 774 (0)        |
| snRNA          | 4811 (0.04)    | 4600 (0.05)    | 4096 (0.04)    | 4259 (0.04)    |
|                | 12,260 (0.05)  | 12,838 (0.05)  | 13,164 (0.05)  | 13,164 (0.05)  |
| snoRNA         | 2152 (0.02)    | 2197 (0.02)    | 2584 (0.02)    | 2396 (0.02)    |
|                | 4967 (0.02)    | 4583 (0.02)    | 5840 (0.02)    | 5522 (0.02)    |
| srpRNA         | 0 (0)          | 2 (0)          | 0 (0)          | 0 (0)          |
|                | 0 (0)          | 2 (0)          | 0 (0)          | 0 (0)          |
| tRNA           | 9007 (0.07)    | 13,069 (0.13)  | 13,810 (0.12)  | 15,819 (0.16)  |
|                | 214,125 (0.84) | 170,843,377 (1.94) | 467,304 (1.79) | 696,897 (2.88) |
| unann          | 12,602,337 (98.8) | 10,044,731 (98.45) | 10,917,735 (98.54) | 9,907,437 (98.22) |
|                | 22,010,746 (86.8) | 17,084,337 (1.94) | 21,461,967 (82.42) | 18,702,763 (77.37) |
| Clean reads    | 12,742,487 (100) | 10,203,105 (100) | 11,079,671 (100) | 10,087,269 (100) |
|                | 25,358,652 (100) | 20,755,724 (100) | 26,038,695 (100) | 24,172,852 (100) |
| Raw reads      | –              | –              | –              | –              |
|                | 25,556,975     | 20,959,989     | 26,243,698     | 24,392,550     |

The number represents the raw data generated directly from small RNA deep sequencing. F0 and F3 represent the bud sample from sugarcane cultivar FN39 with 0 h and 3 h cold (4 °C) treatment, respectively, while R0 and R3 represent the similar samples from cultivar ROC22, respectively.
miR64785. Conversely, there were seven known differentially expressed miRNAs that existed only in ROC22, accounting for 46.7%, including miR5272, miR863-5p, miR5242, miR5072, miR5221, miR395o-3p and miR1861d.

Identification of novel miRNAs in sugarcane

A total of 151, 109, 140 and 132 novel miRNAs were discovered from the F0, F3, R0 and R3 libraries respectively using the Mireap software (Additional file 3: Table S3). There were 18 (five upregulated and 13 downregulated miRNAs) and 18 (six upregulated and 12 downregulated miRNAs) novel miRNAs expressed differentially in FN39 and ROC22 respectively after cold treatment for 3 h (|log2 ratio| ≥ 1, p ≤ 0.05, Fig. 2b, Fig. 3b, Table 3). Compared with known miRNAs, the novel miRNAs exhibited greater differential expression (a 10-fold up-regulation of novel_mir_117 in R3 and 11-fold down-regulation of novel_mir_72 in F3). A proportion of novel miRNAs also showed cultivar-specificity, being expressed only in FN39 (13 novel, four upregulated and nine downregulated) or in ROC22 (13 novel, 5 upregulated and 8 downregulated).

Functional analysis of target genes of differential expressed miRNAs

A total of 2805 target genes for 202 known expressed miRNAs were predicted using the Mireap software from the sugarcane EST database and the sugarcane unigene database (Additional file 4: Table S4). In order to provide an insight into their biological functions, GO and KEGG analyses of differentially expressed miRNAs were performed.

GO analysis of the predicted targets of differentially expressed miRNAs includes three ontologies: biological process, cellular component and molecular function (Additional file 5: Table S5). On the biological process, the result of the GO analysis indicated that the targets of the differentially expressed known and novel miRNAs were mostly involved in cellular and metabolic processes (Fig. 4). On the cellular component, the predicted targets of differentially expressed known miRNAs were mainly associated with cell and cell part, while for the novel miRNAs, their targets were mainly related with the macromolecular complex (Fig. 4). The molecular function of these targets was mainly related to the binding and catalytic activity (Fig. 4).

KEGG analysis of target genes was performed (Additional files 6, 7, 8 and 9: Table S6–S9). In our study, six pathways related to cold stress were analyzed, including plant hormone signal transduction, ABC transporters, peroxisomes, phosphatidylinositol signaling systems, ubiquitin mediated proteolysis and calcium signal pathways (Fig. 5). There were a number of miRNAs that are associated with the regulation of hormone signal pathways (Fig. 6). These miRNAs could affect plants in a number of ways, including cell enlargement, stomatal closure, stem growth, germination, cell division, shoot initiation, fruit ripening and senescence through regulating their target genes.
Expression analysis of miRNAs and their targets by RT-qPCR

RT-qPCR was used to detect the expression levels of miRNAs and their targets. A total of 13 miRNAs and 12 corresponding targets including four transcription factors and eight protein coding genes, were tested in the samples of the two sugarcane cultivars collected at different time points under cold stress (Fig. 7). In total, there were 11 miRNAs, sharing a similar expression profile with those in the deep sequencing data when detected by RT-qPCR. As shown in Fig. 8, the miRNA expression patterns (up- or down-regulation) as measured...
by quantitative analysis and the sequencing results of the miRNAs were largely consistent, except miR394 and miR5177. From the expression profiles detected by RT-qPCR, there were several patterns among these 13 miRNAs. The transcripts of miR160, miR167 and miR319 were all induced by cold treatment in FN39 and ROC22. In contrast, the expressions of miR169, miR5177 and miR5564 were all suppressed. MiR168 was induced at the early stage, but was inhibited afterwards. The other six miRNAs showed different expression patterns in FN39 and ROC22. MiR156 showed a trend of increase firstly, then decrease in FN39, while it was downregulated in ROC22. The expression levels of miR397 and miR398 showed first decrease then increase in FN39, while the opposite trend was observed in ROC22. In FN39, the expression levels of miR393, miR394 and miR408 were all induced by the cold stress, while in ROC22, the expression levels of miR393 and miR408 were first increased then decreased, and the miR394 was inhibited all the time. The different expression patterns of miRNAs in relative cold tolerance FN39 and relative cold sensitivity ROC22 may indicate their important

| miRNA   | sequence                      | F0-std | F3-std | R0-std | R3-std | Fold change |
|---------|-------------------------------|--------|--------|--------|--------|-------------|
| miR1310 | GAGGCATCCGGGGCGCAAGGCC       | 8.32   | 29.24  | 15.78  | 30.98  | 1.81**      |
| miR1520d| ATCAGAACTGTGACGACGAA         | 574.08 | 1917.98| 1131.16| 2620.83| −1.74**     |
| miR157a | TGGACAGAAGATAGAGAGCAC         | 4.65   | 9.68   | 4.34   | 4.26   | 1.06**      |
| miR2199 | TGGATACCTTGACGACGTCGC        | 594.2  | 179.46 | 129.61 | 272.82 | 1.59**      |
| miR2916 | GGGGCTCGAAGACGTGACGATCATGAT| 46.30  | 198.06 | 128.65 | 255.78 | −2.10**     |
| miR5054 | TCCCCACGGACGGCGCCA           | 50.00  | 117.65 | 81.84  | 164.77 | 1.23**      |
| miR5059 | CGTTCCTGGGCAGCAACACCA        | 35.92  | 97.80  | 84.02  | 201.88 | 1.44**      |
| miR5072 | GTTCCTGGCGGAGCTGCCA          | 2.16   | 7.70   | 5.34   | 20.97  | 1.82**      |
| miR5077 | TCCACGTCCGGGTTCCACCA         | 104.90 | 321.31 | 321.30 |        | 1.62**      |
| miR5152-3p| AGTCCCGTCTACCCACCA          | 0.59   | 2.02   | 1.30   | 3.76   | 1.77**      |
| miR5575 | TGGACAGAAGATAGAGAGCAC        | 4.65   | 9.68   | 4.34   | 4.26   | 1.06**      |
| miR5671 | CATCGTGATCGCGGTGACG          | 32.73  | 70.96  | 72.74  | 126.88 | 1.12**      |
| miR5813 | ACACCGAGCCGTGGTGTATCAGG     | 63.41  | 206.30 | 132.57 | 269.48 | 1.70**      |
| miR6196 | GGAGACAGAAGATAGAGAGCA        | 5.12   | 11.12  | 17.93  | 21.15  | 1.12        |
| miR6300 | GTTCTGTATAGTATGTGTTG         | 101.38 | 217.53 | 216.10 | 284.36 | 1.10**      |
| miR6478 | CCGCCTTATGCTAGTGGTA          | 14.70  | 32.52  | 30.53  | 51.46  | 1.14**      |
| miR8155 | CGTACTGCTTGCTGCCGTA          | 4.14   | 9.20   | 8.10   | 16.96  | 1.15**      |
| miR894  | GTTCTGGCTCCGGTCCACCA         | 334.04 | 1108.12| 1040.41| 2131.64| 1.72**      |
| miR160a | TGCTGGCTCCGTAAGGCCCA         | 13.92  | 4.43   | 4.64   | 8.89   | −1.65**     |
| miR169b | CACCGAGAGTGATAGTTCGCGG       | 43.54  | 20.76  | 38.28  | 39.38  | −1.06**     |
| miR169e-3p| GCCAGCTCTTGCTGCTAGC         | 73.19  | 31.02  | 14.24  | 21.84  | −1.24**     |
| miR397-5p| TTGACTGCTGCGGCTGACG         | 9.82   | 12.34  | 2.30   | 3.64   | 1.12**      |
| miR528-5p| TGGAAAGGCGATCAGCAGAGG        | 123.62 | 49.19  | 303.86 | 262.15 | −1.32**     |
| miR5532 | ATGGAAATGCATGACAGAGGG        | 1.58   | 0.77   | 1.46   | 1.20   | −1.03 *     |
| miR1861d| TGCGTGGCTCCGGTCCACCA         | −      | −      | 3.30   | 15.34  | −           |
| miR3950-3p| TGAAGTGTTGCTGGTACCT         | 2.60   | 1.64   | 0.88   | 2.56   | −0.66       |
| miR5221 | AACCGAGATGGTTTCTTACTT       | −      | −      | 3.00   | 14.23  | −2.24**     |
| miR5242 | TATTTGGAACAGCGGTGTCA         | 1.26   | 1.49   | 3.68   | 13.24  | 0.24        |
| miR5272 | GGATTGTTGCTGGTGGATAAATT     | 3.27   | 2.64   | 0.80   | 1.78   | −0.30       |
| miR394a | TGGGCATTTGCTCCACCT          | 4.26   | 7.78   | 0.88   | 0.74   | 1.03**      |
| miR863-5p| TTATGGCTGCTTGGATCAAAAT      | −      | −      | 0.23   | 1.53   | −           |

F0 and F3 represent the bud sample from sugarcane cultivar FN39 with 0 h and 3 h cold (4 °C) treatment, respectively, while R0 and R3 represent the similar samples from cultivar ROC22, respectively. ** and * indicate a significant difference at p-value <0.01 and p-value <0.05, respectively. "−" indicates no expression.
roles in plant response to cold stress. Most of the above 13 miRNAs exhibited a negative correlation with their predicted targets in both cultivars (FN39 and ROC22) with the exception of miR168 and miR394 (Fig. 7). The inaccuracy of target prediction or regulatory mechanism other than miRNAs modulating the expression of targets in sugarcane may explain this result.

Transient expression analysis of miR156 in tobacco
Homologues of miR156, miR168, miR169 and miR408 have been implicated in abiotic stresses [39, 40]. In addition to cold stress (Fig. 7), the expression profiles of these four miRNAs were detected under drought and salt stresses using stem loop RT-qPCR (Fig. 9). Compared with the other three miRNAs, miR156 showed the largest differential expression, with 4-fold and 3-fold up-regulation respectively under drought and salt stresses. The other three miRNAs exhibited smaller differences compared to the control. This suggests a role for miR156 in plant response to abiotic stress in sugarcane.

**Table 3** Novel miRNAs with significant differential expression in sugarcane buds following cold treatment

| miRNA     | sequence               | F0-std | F3-std | R0-std | R3-std | Fold change F3-F0 | R3-R0  |
|-----------|------------------------|--------|--------|--------|--------|------------------|--------|
| nov_mir_107 | AGGGATGGTATGCACTGAAGC  | 0.01   | 3.71   | 0.01   | 4.55   | 8.54**           | 8.83** |
| nov_mir_117 | AAGAGGAAGAGAGAGAGGGTG  | 0.01   | 0.01   | 0.01   | 12.33  | –                | 10.27**|
| nov_mir_134 | GAGGCAAGGGTGAAGATGAC  | 0.01   | 3.90   | –      | –      | 8.61**           | –      |
| nov_mir_136 | AAGCAATGTTGGGATGGCTAA | 0.01   | 1.30   | –      | –      | 7.02**           | –      |
| nov_mir_142 | TGAACCGTACGGCTGATTGTT  | 0.01   | 1.06   | –      | –      | 6.73**           | –      |
| nov_mir_21  | TAAATATATAAATACAGGACC | 1.10   | 0.01   | –      | –      | –6.70**          | –      |
| nov_mir_34  | GTACCACCCGTCGGAGCTAA  | 1.14   | 0.01   | –      | –      | –6.84**          | –      |
| nov_mir_37  | TCCAAATGACACCTGTTTCTAA | 3.94   | 0.01   | 5.03   | 0.01   | –8.62**          | –8.97**|
| nov_mir_41  | CCGAGAAAGAGGCGGTGGC  | 6.55   | 0.01   | 12.83  | 10.34  | –9.35**          | –      |
| nov_mir_54  | TCAAAATTAAGACGGTTTTG  | 1.03   | 0.34   | 1.57   | 0.79   | –1.60**          | –1.00  |
| nov_mir_57  | GGGCTGGCTGCGGGGTGGAA | 1.10   | 0.01   | –      | –      | –6.79**          | –      |
| nov_mir_68  | ATAAGACGTTTTTGCTTTTCTT | 3.90   | 0.01   | –      | –      | –8.61**          | –      |
| nov_mir_72  | AAGAGGAAGAGAGAGAGGGTG  | 16.09  | 0.01   | 16.17  | 0.01   | –10.65**         | –10.66**|
| nov_mir_74  | TTTGGAGGTGAGGCTGGGCC  | 1.18   | 0.01   | –      | –      | –6.89**          | –      |
| nov_mir_79  | CGGCCACGCCCGGCCCGGCCC  | 2.64   | 0.01   | 2.19   | 0.01   | –8.05**          | –7.77**|
| nov_mir_80  | TGGTTGTCGGCGGCAATGCTG  | 5.44   | 5.93   | 6.57   | 0.01   | –9.36**          | –      |
| nov_mir_81  | GCTAGGAGCCACACAGCTCATA | 9.94   | 0.01   | –      | –      | –9.96**          | –      |
| nov_mir_89  | CAATCGTTGACCACTAGCTTTT | 4.85   | 0.01   | 3.53   | 4.01   | –8.92**          | 0.18   |
| nov_mir_91  | CCTGCGTCGACCGGATTTCTG  | 1114.45| 3231.35| –      | –      | 1.54**           | –      |
| nov_mir_97  | GTGCTGGCGGCTGCGGCTGCTG | 1.10   | 0.34   | 1.19   | 0.79   | –1.71**          | –0.60  |
| nov_mir_101 | GGAATGTTGTCTGGTCGGAGA  | 9.31   | 7.37   | 10.48  | 0.01   | –0.34            | –10.03**|
| nov_mir_130 | GGCATGGGAACATGTAGGAAGG | –      | –      | 0.01   | 2.81   | –8.14**          | –      |
| nov_mir_171 | TTTGAATAGACAGCTGGTCA  | –      | –      | 10.33  | 0.01   | –10.01**         | –      |
| nov_mir_183 | TAAGTGGCTGAGTCGAGTCTT | –      | –      | 1.65   | 0.01   | –7.37**          | –      |
| nov_mir_216 | TCGCGGGAACTGTTAGAGG  | –      | –      | 0.01   | 1.53   | –7.26**          | –      |
| nov_mir_228 | TTTTGTGATTGTGACAAAC  | –      | –      | 0.01   | 2.90   | –8.18**          | –      |
| nov_mir_28  | AGGTGCTCGTAGTTTCTATC | –      | –      | 1.30   | 3.02   | –1.21**          | –      |
| nov_mir_3   | GGCATGAGTGGGTCATGAGT  | 0.75   | 1.49   | 7.18   | 2.40   | 1.00             | –1.58**|
| nov_mir_58  | CTCGGCGCTGCGCTGCGCTG  | 2.29   | 1.64   | 1.11   | 0.50   | –0.48            | –1.16  |
| nov_mir_64  | AGGGTGTTGGGCGGGGTCGCC | –      | –      | 1.42   | 0.01   | –7.15**          | –      |
| nov_mir_67  | AAAACATTGTATACGCTAA  | –      | –      | 2.27   | 0.01   | –7.82**          | –      |

F0 and F3 represent the bud sample from sugarcane cultivar FN39 with 0 h and 3 h cold treatment, respectively; R0 and R3 represent the bud sample from sugarcane cultivar ROC22 with 0 h and 3 h, respectively. ** and * indicate a significant differences at p-value <0.01 and p-value <0.05, respectively. "—" indicates no expression.
Fig. 4 GO analysis of the predicted target genes for known and novel differentially expressed miRNAs

Fig. 5 Predicted regulation networks between miRNAs and their target genes involved in six biological pathways in the sugarcane response to cold stress
**MIR156.** Significant levels (p-value <0.05) of the MIR156 transcript were detected (Fig. 10a), but not in the control. After 24 h cold treatment, the tobacco plants showed a visual phenotype. Compared with the control, the leaves overexpressing MIR156 exhibited better growth status (Fig. 10b, c). A number of assays were performed on the tobacco leaves. The extent of H$_2$O$_2$ accumulation under cold stress was assayed using the DAB staining method. A deeper staining was produced in mock leaves, indicating lower H$_2$O$_2$ accumulation in leaves overexpressing MIR156 (Fig. 10d). Three members of the early-responsive dehydration (ERD) gene family, ERD10B, ERD10C and ERD10D, which are downstream genes in the ICE-CBF-COR pathway [41], and the LEA gene, whose encoded protein protects cells from water stress [42], were upregulated in pCAMBIA1301-MIR156 overexpressing tobacco leaves after 12 h cold treatment (Fig. 10e). Additionally, under cold...
stress, anthocyanin levels in leaves overexpressing pCAMBIA1301-MIR156 were higher than those in the control (Fig. 10f). These results suggest that tobacco leaves overexpressing MIR156 have enhanced cold tolerance. In addition, the transient expression of the other three miRNAs, miR168, miR169 and miR408, produced no obvious phenotype or difference in reactive oxygen species (ROS).

**Discussion**

Small RNA analysis of sugarcane response to cold stress

Cold stress is a major limiting factor in the distribution, yield and quality of crops [11]. Although the cold signaling pathway has been well studied [11, 14], the role of small RNAs in low temperature sensing in plants remains unclear. In recent years, several researchers have found a critical role of miRNA in abiotic stress [43, 44].
Cold-related miRNAs in plants have been identified using various methods. Zhou et al. (2008) [16] found 19 upregulated miRNA genes of 11 miRNA families in Arabidopsis thaliana under cold stress using a computational, transcriptome-based approach. In Brachypodium distachyon, 27 conserved and 129 predicted miRNAs involved in the cold stress response were identified by high-throughput sequencing and whole-genome-wide data mining [17]. In Poncirus trifoliate (L.) Raf., a total of 107 conserved and five potentially novel miRNAs were characterized before and after cold treatment through deep sequencing [45]. In this study, a total of 412 sugarcane miRNAs, including 261 known and 151 predicted novel miRNAs, were discovered in two cultivars exhibiting differences in cold tolerance by small RNA deep sequencing and bioinformatics analysis, enriching the present miRNA resources in sugarcane. This is far more than previously identified in other species, except for the possibility of the parameters used were different, it is most probably related to the larger genome and complex genetic background of sugarcane.

These small RNAs obtained in this study were divided into several classes. Due to the limited knowledge of sugarcane genome information, the unannotated small RNAs occupied the majority, which was consistent with the halophyte Halostachys caspica [30] and Morus alba L. [46]. According to a previous report, the 21 nt and 24 nt length sRNA sequences are generated by different DCL proteins [6]. Small RNAs with 20–22 nucleotides are generated by DCL1. Longer small RNAs, 23–25
Fig. 10 Functional analysis of miR156 in tobacco infiltrated by *Agrobacterium tumefaciens* strain EHA105. a) The transcript analysis of MIR156 in infiltrated tobacco leaves; b) and c) the phenotype observed in infiltrated tobacco leaves overexpressing pCAMBIA1301 and pCAMBIA1301-MIR156 with 4 °C treatment for 24 h; d) DAB staining of tobacco leaves with 4 °C treatment for 24 h; e) expression analysis of cold response genes in infiltrated tobacco leaves with 4 °C treatment for 12 h; f) quantification of anthocyanin in tobacco leaves with 4 °C treatment for 24 h. A data point represents the mean ± SE (n = 3) (a, e, f). Lowercase letters in each graph attest significant differences within the same gene/miRNA over the treatment period, as determined by Duncan’s new multiple range test (p-value <0.05).
nucleotides in length, are processed by DCL3. The DCL2 and DCL4 generate the 22 and 24 nucleotides respectively [47]. Usually, the length of miRNAs corresponds to 21 nt or 22 nt, while the siRNAs are distributed in the 24 nt [6]. The 24 nt and 21 nt small RNA length distribution in sugarcane was consistent with the phenomenon in *Panicum virgatum* [48], *Fragaria ananassa* [49], rice [50] and *H. caspica* [30], which verified our data.

Among the total 412 identified miRNAs, 62 miRNAs exhibited significant expression differences ([log2 ratio] ≥ 1, p ≤ 0.05) between the cold-treated and the control samples. Differentially expressed miRNAs were further validated by RT-qPCR. According to previous reports, *miR160/167* and *miR393* were involved in the auxin response by targeting of auxin response factors (*ARF*) and transport inhibitor response (*TIR*) genes, respectively [51, 52]. In this study, *miR167* and *miR393* were upregulated in both cultivars, while *miR160* was upregulated in FN39 (relative cold tolerance cultivar) and downregulated in ROC22 (relative cold sensitivity cultivar). In *Lycopersicon esculentum* Mill studies using stem-loop RT-qPCR, *miR167* and *miR393* increased at 4 °C at early time points (0, 1, 4 and 16 h) [53]. In 2-week-old *Arabidopsis* seedlings, *miR393* was strongly induced by cold, NaCl, dehydration and ABA treatments [54]. The up-regulation of *miR167* was found in *Triticum aestivum*, suggesting that *miR167* and the trans-acting short-interfering RNA-auxin response factor (tasiRNA-ARF) have a role in regulating the auxin signaling pathway and the developmental response to cold stress [55]. Under un-stressed conditions, low levels of these miRNAs may be sufficient for the fine-tuning of their targets. Conversely, under stress conditions, upregulated miRNAs can reduce ARF and TIR transcript levels (Fig. 7), suppressing ARF-mediated auxin responsive gene expression, leading to plant growth and development attenuation and eventually enhancing plant stress tolerance.

There was up-regulation of *miR168* and *miR408* under cold stress in both the cold tolerant and less cold tolerant sugarcane cultivars. Liu et al. (2008) [39] identified 14 stress-inducible miRNAs using microarray data in *Arabidopsis*, and found *miR168* to be responsive to cold stress. The increased expression of *miR408* could lead to the improved tolerance of *Arabidopsis* to cold and oxidative stresses and the enhancement of cellular antioxidant capacity, manifested by the lower level of ROS and induction of genes related to anti-oxidative function [56]. *MiR319* showed upregulated expression in both cultivars in this study, which was consistent with previous research which identified its positive role in the response of sugarcane to cold stress [18]. Some miRNAs exhibited differential expression in the two cultivars: *miR156*, *miR160* and *miR394* were upregulated in the cold tolerant FN39, but downregulated in the cold sensitive ROC22, while *miR397* and *miR398* were downregulated in FN39 and upregulated in ROC22. The opposite expression changes of these miRNAs in these cultivars may be a key factor leading to difference in cold tolerance. *MiR156* is a conserved miRNA in many plant species and its function in plant growth and development has been studied during events such as lateral root development [57] and floral transition [58]. *MiR156* also has a role in plant response to abiotic stress, such as drought and salt stresses [59].

As the function of miRNAs is performed by targeting of miRNAs, the target genes were predicted with certain described criteria. Several targets of conserved miRNAs in this study, such as the SQUAMOSA promoter-binding protein-like gene (*SPL*, target of *miR156*) and nuclear factor Y gene (*NF-Y*, target of *miR169*), have been reported in some other plant species, indicating the miRNAs and its targets may have a similar role in different plant species [60]. In our study, a total of 4002 targets were predicted for the 259 miRNAs, showing that some miRNAs have more than one target, which is consistent with earlier reports in *Glycine max* seeds [61] and trifoliate orange [45]. Another reason for the large amount of predicted targets may be the lack of sufficient sugarcane genome sequences and annotation information, leading to inaccuracies in prediction. Among the targets, transcription factors were included and involved in the response of sugarcane to cold stress, such as the *ARF*, *TIR*, *MYB* and *NF-Y*. Transcription factors control a series of downstream genes by binding cis-elements in promoter regions [62]. It is conceivable that miRNAs regulating the transcription factors will exert a more extensive influence on gene expression. However, the function of miRNAs and their targets needs to be validated through further experiments.

**Functional analysis of miR156 in tobacco under abiotic stress**

Several miRNAs, including *miR156*, *miR168*, *miR169* and *miR408*, which have been studied in plant response to abiotic stresses [39, 40], were selected for further functional investigation. The highly conserved *miR156* family has been reported as a key factor in the regulation of plant growth and development by targeting the *SPL* genes. In *Gossypium hirsutum* L., the *miR156* was upregulated under 0.25% NaCl or 2.5% PEG stresses in roots [63]. In *Zea mays* L., *miR156* showed a modest up-regulation in two inbred lines under both salt and drought stresses, but also a strong higher expression in the hybrid lines based on northern blot detection. *SPL*, a target of *miR156*,
showed opposite expression patterns under abiotic stresses, indicating the role of miR156 as a negative regulator in maize during this process [64]. A similar positive role of miR156 in sugarcane was shown in our RT-qPCR results, with a 3.7-fold and 4.5-fold up-regulation under salt and drought stresses, respectively.

The role of miR156 in cold stress has been shown in rice [20]. Transgenic rice with overexpressed OsmiR156k exhibited a lower survival rate, proline content, chlorophyll II, and down-regulation of cold-sensitive genes under cold stress, indicating decreased cold tolerance. They concluded that miR156k worked as a negative regulator of plant tolerance to cold stress [20]. In our work, miR156 showed a positive role in the response of tobacco to cold stress, supported by better growth status and lower ROS accumulation. Additionally, 4 cold-related genes (ERD10B, ERD10C, ERD10D and LEA genes) all showed higher expression levels in tobacco leaves when MI156 was overexpressed than those in the control. This may be due to the fact that miR156 exerts various effects in different plant species.

Anthocyanin level has been shown to be responsive to many stresses including cold, salt, UV-B and biotic stresses, and protect plants from damage by scavenging the free radicals [65, 66]. Gou et al. (2011) [38] concluded that increased miR156 could promote the accumulation of anthocyanins, while reduced miR156 would be conducive to flavonol accumulation in Arabidopsis. During this process, SPL9, one of the miR156 targets, inhibits the expression of anthocyanin synthesis genes by interacting with the production of the anthocyanin pigments1 (PAP1) component of the MYB-BHLH-WD40 complex, resulting in the interference with the regulation of anthocyanin accumulation [38]. Due to the fact that the greater accumulation of anthocyanins in miR156 transiently overexpressed tobacco leaves was observed, it is possible that the overexpression of sugarcane miR156 leads to the accumulation of anthocyanins to prevent the over production and accumulation of ROS in tobacco leaves. This deduction was consistent with the observation of growth status and DAB staining, in which the MI156 overexpressing tobaccos displayed better growth status and lower ROS accumulation. Considering the better growth status, lower ROS level, higher anthocyanins level with the higher expression levels of four cold-related genes in MI156 overexpressing tobaccos than those in control, it indicated that the miR156 plays a positive role in plant response to cold stress.

**Conclusions**

In this study, small RNA sequencing of sugarcane with cold treatment and comprehensive analysis of the cold-responsive miRNAs and their targets were performed. The results showed that miRNAs played an important role in sugarcane under cold stress by regulating several biological processes, including plant hormone signal transduction, ABC transporter function, peroxisomes, phosphatidylinositol signaling systems, ubiquitin mediated proteolysis and calcium signaling pathways. In addition, the role of miR156 in plant response to cold stress was also studied. These findings provide the valuable information for further functional characterization of miRNAs in sugarcane under cold stress.

**Additional files**

Additional file 1: Table S1. The stem loop primers of miRNAs in sugarcane (DOCX 14 kb)
Additional file 2: Table S2. The real time PCR primers of miRNAs and target gene in sugarcane (DOCX 15 kb)
Additional file 3: Table S3. The known and novel miRNAs in 4 small RNA libraries (F0, F3, R0 and R3) (XLSX 30 kb)
Additional file 4: Table S4. Prediction of target genes of the known and novel miRNAs (XLSX 208 kb)
Additional file 5: Table S5. GO analysis of the predicted targets of known and novel miRNAs (XLSX 243 kb)
Additional file 6: Table S6. KEGG analysis of the predicted targets of known miRNAs in F3/F0 (XLSX 12 kb)
Additional file 7: Table S7. KEGG analysis of the predicted targets of known miRNAs in R3/R0 (XLSX 11 kb)
Additional file 8: Table S8. KEGG analysis of the predicted targets of novel miRNAs in F3/F0 (XLSX 15 kb)
Additional file 9: Table S9. KEGG analysis of the predicted targets of novel miRNAs in R3/R0 (XLSX 12 kb)
Additional file 10: Figure S1. The stem longitudinal section of sugarcane FN39 (relative cold tolerance) and ROC22 (relative cold sensitivity) in plant tip part after cold stress, indicating that more serious damage was observed in ROC22 in its stem tissues and growing point (TIF 31806 kb)
Additional file 11: Figure S2. Validation of the cold-stress treatment in sugarcane. The expression patterns of three cold responsive genes, CBF1, CBF3 and NAC23, were detected by RT-qPCR in sugarcane FN39 (A) and ROC22 (B) cultivars. The relative expression at 0 h is equal to 1. GAPDH was used as internal control. Error bars show the range of duplicate analysis of sample in RT-qPCR. Lower case letters in each graph attest significant differences within the same gene/miRNA over the treatment period, as determined by Duncan’s new multiple range test (p-value <0.05) (TIF 2258 kb)

**Abbreviations**

18S rRNA: 18S ribosomal RNA; ARF: auxin response factor gene; CBF: C-repeat binding factor gene; DAB: 3, 3′-diaminobenzidine; F0: samples of FN39 cultivar without cold treatment; F3: samples of FN39 cultivar with 3 h cold treatment; GA4β: GA3-β-glucuronidase; ICE1: inducer of C-repeat binding factor expression 1 gene; KEGG: Kyoto Encyclopedia of Genes and Genomes; NF-Y: nuclear factor Y gene; PAP1: production of the anthocyanin pigments1; PCLS: TCP transcription factor gene; R0: samples of ROC22 cultivar without cold treatment; R3: samples of ROC22 cultivar with 3 h cold treatment; RISC: RNA-induced silencing complex; ROS: reactive oxygen species; RNAi: ribosomal RNA; RT-qPCR: reverse transcription quantitative real-time polymerase chain reaction; scRNA: small cytosolic RNA; snoRNA: small nucleolar RNA; snRNA: small nuclear RNA; SPL: SQUAMOSA promoter-binding protein-like gene; taizmRNA-ARF: trans-acting short-interfering RNA-auxin response factor; TIR: transport inhibitor response gene; trRNA: transfer RNA
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Availability of data and materials
The data supporting the conclusions of this article are within the paper and its additional files. All sequencing reads are deposited in the National Center for Biotechnology Information under the BioProject number PRJNA388815 with the Sequence Read Archive (SRA) study accession SRR108561.

Authors’ contributions
YTY, LPX and YXQ conceived, designed and initiated the project. YTY drafted the manuscript. LPX prepared materials. YTY, XZ, YCS, JKZ and ZTW performed experiments and contributed to data analysis and validation. YTY, LPX and YXQ helped to revise the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable. This is to confirm that no specific permits were needed for the described experiments, and this study did not involve any endangered or protected species.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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