Bioinformatics Analysis of Sugarcane Cold Tolerance-Related miRNAs and Target Gene Prediction (Postprint)

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Abstract

To elucidate the molecular mechanisms underlying the response of different sugarcane (Saccharum officinarum) genotypes to low temperature stress and to identify cold tolerance-related miRNAs and their associated target genes, this study employed leaves from three sugarcane varieties with contrasting cold tolerance after 24 h of 4 °C treatment as materials for Illumina HiSeqTM2000 high-throughput sequencing, constructing 18 sRNA libraries before and after low temperature stress. The results showed: (1) A total of 322 known miRNAs belonging to 84 families and 110 novel miRNAs were obtained, among which 100 differentially expressed miRNAs (61 up-regulated, 39 down-regulated) were identified from the known miRNAs, and 37 differentially expressed miRNAs (15 up-regulated, 22 down-regulated) from the novel miRNAs. (2) Target gene prediction for the obtained differentially expressed miRNAs was performed using psRNATarget, TargetFinder, and Tapirhybrid software, yielding 1,844 target genes, and GO analysis revealed three major functional categories of these target genes: molecular function, cellular component, and biological process. (3) To validate the reliability of the high-throughput sequencing data, 14 miRNAs and their target genes were selected for qRT-PCR verification, which demonstrated that all these miRNAs were detected and most expression patterns were consistent with the sequencing results. (4) Additionally, target genes of some differentially expressed miRNAs were identified that are involved in plant growth, development, and low temperature stress responses. In summary, the results indicate that miRNAs in cold-tolerant sugarcane directly or indirectly regulate target genes to modulate expression of related metabolic pathways, playing crucial regulatory roles in key agronomic traits.

Full Text

Preamble

Bioinformatics Analysis of Cold Tolerance-Related miRNAs and Prediction of Their Target Genes in Sugarcane

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Abstract

To understand the molecular mechanisms underlying low-temperature stress responses in different sugarcane (*Saccharum officinarum*) genotypes and to identify cold tolerance-related miRNAs and their target genes, this study performed Illumina HiSeqTM2000 high-throughput sequencing on leaves from three sugarcane varieties with different cold tolerance levels after 24 h of cold stress at 4 °C, constructing 18 sRNA libraries before and after low-temperature treatment. The results showed: (1) A total of 322 known miRNAs belonging to 84 families were obtained, along with 110 predicted novel miRNAs. Among these, 100 differentially expressed miRNAs (61 up-regulated and 39 down-regulated) were screened from known miRNAs, and 37 differentially expressed miRNAs (15 up-regulated and 22 down-regulated) were screened from novel miRNAs. (2) Using psRNATarget, TargetFinder, and Tapirhybrid software, 1,844 target genes were predicted for the differentially expressed miRNAs. GO analysis revealed three major functional categories: molecular function, cellular component, and biological process. (3) To verify the reliability of the high-throughput sequencing data, 14 miRNAs and their target genes were selected for qRT-PCR validation, confirming that these miRNAs were detected and that most expression patterns were consistent with the sequencing results. (4) Several target genes of differentially expressed miRNAs were identified that are involved in plant growth, development, and low-temperature stress responses. Overall, these results indicate that miRNAs in cold-tolerant sugarcane directly or indirectly regulate target gene expression in related metabolic pathways, playing a crucial regulatory role in key agronomic traits.

Keywords: sugarcane, low-temperature stress, cold tolerance, miRNAs, bioinformatics

MicroRNAs are a class of small endogenous non-coding RNAs of 20–24 nucleotides with high conservation, temporal specificity, and tissue specificity (Ambros, 2004). miRNAs primarily regulate gene expression at the post-transcriptional level by mediating target gene mRNA cleavage or translational repression, playing multiple regulatory roles in biological metabolism, including plant organ morphogenesis (Sunkar, 2004, 2012), growth and development

(Thiebaut et al., 2012), hormone secretion, signal transduction, and responses to environmental stresses (Xiong, 2003). Studies on low-temperature responses in *Arabidopsis* and rice have revealed that miRNA families such as miR-167, miR-169, miR-319, and miR-171 play important biological roles in low-temperature responses (Sunkar, 2005; Wang et al., 2010). Notably, the target gene of miR-169 is CBF, a key gene induced by low temperature (Sunkar et al., 2007). Research on rice responses to low-temperature stress has demonstrated that the target genes of miRNA-319 and miRNA-171 belong to MYB transcription factors, and their expression levels show an inverse relationship, illustrating the regulatory role of miRNAs in rice low-temperature tolerance pathways (Sunkar et al., 2005; Lü et al., 2010). Therefore, accurate and efficient isolation and identification of miRNAs and their target genes, along with functional analysis to precisely understand miRNA regulatory mechanisms in plant stress responses, represents an important focus in plant miRNA research.

Sugarcane is a crucial sugar crop in tropical and subtropical regions, where low temperature is a major factor limiting its cultivation expansion and high-yield stability (Wu et al., 2010). Occasional large-scale freezing rain, snow, or severe frost events cause substantial economic losses in China's major sugarcane production areas (He et al., 2009; Kuang et al., 2009). Guangxi's unique geographical environment, characterized by persistent low temperatures in winter and frequent "late spring coldness" with rain and frost, leads to extensive cold damage in sugarcane, manifested as leaf withering, stalk necrosis, and reduced sucrose content and yield (Li et al., 2011). Analysis of over 40 years of meteorological data from major sugarcane-producing counties, combined with indicators such as planting area, yield, and sucrose production, has identified low-temperature frost damage as the primary environmental factor affecting sugarcane cultivation and production (He et al., 2008; Gu et al., 2011). Therefore, understanding the molecular mechanisms of low-temperature tolerance is essential for breeding cold-tolerant sugarcane varieties suitable for the tropical marginal climate in China.

Based on observations of agronomic traits in different sugarcane varieties under field conditions and physiological-biochemical studies under simulated low-temperature stress, this study selected cold-tolerant sugarcane varieties as experimental materials. Using high-throughput sequencing technology and bioinformatics methods, we identified miRNAs associated with sugarcane responses to low-temperature stress, analyzed their differential expression patterns, clarified the relationships between miRNAs and their target genes, and performed gene ontology (GO) analysis on the predicted target genes to identify low-temperature stress response genes. This work provides a theoretical basis and technical support for breeding excellent new sugarcane varieties with enhanced cold tolerance.

1.1 Materials

The experimental materials included 'Guitang 28' (GT28) bred by the Sugarcane Research Institute of Guangxi Academy of Agricultural Sciences, the widely cultivated variety 'Xintaitang 22' (ROC22) in Guangxi sugarcane regions, and the newly bred 'Gure 2' (GR2) variety from the Guangxi Subtropical Crops Research Institute.

1.2.1 Experimental Design and Management

Disease-free and uniformly sized stalks were selected and cut into single-bud segments. After washing with water and drying, the segments were disinfected by soaking in 1,000-fold diluted 50% carbendazim wettable powder for 12 h, followed by a 1-min rinse with distilled water. The segments were then wrapped in distilled water-moistened cotton cloth, labeled, and placed in a constant temperature incubator at 25 °C for germination. When buds sprouted and young roots emerged, they were transplanted into plastic pots containing nutrient soil, with one segment per pot (pot height: 17.5 cm, width: 16 cm). During the seedling period, each plant was treated with complete nutrient solution twice (10 mL each time). At the two-leaf-and-one-heart stage, uniformly growing seedlings were selected for low-temperature treatment. The low-temperature stress treatment was conducted at 4 °C with a light intensity of 5,000 lx for 24 h, while the control (CK) was maintained at 28 °C with the same light intensity.

1.2.2 RNA Extraction and Illumina Sequencing

Leaves from both low-temperature treated and control samples were collected, and RNA was extracted using the TRNzol Reagent kit (Tiangen Biotech, Beijing). High-quality RNA from both treatment and control groups was sent to BGI Tech (Shenzhen) for library construction, with three replicates per treatment. RNA libraries passing quality control were sequenced using the Illumina HiSeqTM 2000 platform (Ali et al., 2008; Wang et al., 2009). After data processing (adapter removal, low-quality read filtering, and contamination removal), clean reads were obtained. Bowtie2 software was used to annotate small RNAs (sRNAs) by sequentially aligning them to MiRbase, pirnabank, snoRNA (human/plant), Rfam, and other sRNA databases to obtain unannotated RNA fragments.

1.2.3 Identification of Known miRNAs and Prediction of Novel miRNAs

miRDeep2 software was used to align unannotated sRNA sequences to the reference genome [*Saccharum spontaneum* genome] (Marc, 2008; Maurits et al., 2015) to identify known sugarcane cold tolerance-related miRNAs. Unmapped sequences were further aligned to reference sequences, and novel miRNAs were predicted through base extension and miRNA structure prediction.

1.2.4 Identification of Differentially Expressed miRNAs

The expression levels of known miRNAs in cold-tolerant sugarcane samples were analyzed to identify differentially expressed miRNAs before and after low-temperature stress. The screening criteria were $|\log_2(\text{FoldChange})| \geq 1$ and $P < 0.05$.

1.2.5 miRNA Target Gene Prediction

psRNATarget (Wu et al., 2012), TargetFinder (Fahlgren & Carrington, 2010), and Tapirhybrid (Peer, 2010) software were used to predict target genes of differentially expressed miRNAs. Predicted target genes were subjected to GO database homology searches to determine their involvement in signal transduction and metabolic pathways.

1.2.6 qRT-PCR Validation

Fourteen differentially expressed miRNAs and their target genes were selected for validation. Specific forward primers for mature miRNAs, universal reverse primers, and stem-loop primers were designed using Premier 5.0 (Table 4). RT-PCR amplification was performed using the LightCycler® 480 Instrument II, with negative controls (no cDNA template) to monitor potential contamination. Sugarcane GAPDH was used as the reference gene, with three technical replicates per sample. Relative gene expression was calculated using the 2^(-ΔΔCt) method.

2.1 High-Throughput Sequencing Data Analysis

Seedlings of three sugarcane varieties with different cold tolerance levels were subjected to low-temperature stress, with three replicates each for control (CK) and treatment (T) groups. Leaves were collected from 18 samples for high-throughput sequencing to construct sRNA libraries before and after cold stress. Raw sequencing data underwent quality control processing (3' adapter removal, low-quality trimming, and fragment size selection) to obtain high-quality clean reads (Table 1). For GR2, 24,310,558 and 23,925,673 raw reads were obtained from CK and T leaves, respectively, yielding 21,343,194 and 21,576,594 clean reads that accounted for 87.83% and 90.25% of total sequences. Alignment of these libraries to the reference genome showed mapping rates of 86.71% (CK) and 84.32% (T). For GT28, 23,915,244 and 23,528,103 raw reads were obtained, yielding 22,045,186 (92.18%) and 21,481,129 (91.28%) clean reads, with genome mapping rates of 71.75% (CK) and 84.1% (T). For ROC22, 27,069,867 and 23,631,208 raw reads were obtained, yielding 21,007,371 (79.5%) and 20,640,357 (87.35%) clean reads, with mapping rates of 92.62% (CK) and 85.67% (T).

sRNA length is associated with different functions, with 21–22 nt sRNAs primarily involved in mRNA cleavage and post-transcriptional gene silencing, while 24 nt sRNAs are mainly associated with RNA-directed DNA methylation and

transcriptional gene silencing. Statistical analysis of total clean reads revealed that sRNAs were predominantly concentrated in the 21–24 nt range, though differences existed among materials with varying cold tolerance, and sequencing frequencies varied by length (Figure 1 [Figure 1: see original paper]).

Pearson correlation coefficients were calculated based on gene expression levels between replicate samples and visualized as a heatmap (Figure 2 [Figure 2: see original paper]). The correlation coefficients between replicate samples of the same variety under the same treatment were high (0.667–0.990), indicating consistent expression profiles across all samples and meeting the standards for replicate experiments, thus enabling subsequent differential expression analysis.

2.2 Analysis of Known miRNAs and Prediction of Novel miRNAs

Using AASRA software, nucleotide sequences were aligned to the reference genome and miRBase database, identifying 322 known miRNAs belonging to 84 families. The control group contained 297 miRNAs from 69 families, while the low-temperature treatment group contained 305 miRNAs from 74 families. Among these families, miR169 had the most members (32), followed by miR166, miR171, miR167, miR156, and miR396 (Figure 3 [Figure 3: see original paper]).

Based on the high conservation of mature plant miRNA sequences and the characteristic hairpin structure of miRNA precursors, homology-based searches were performed in the *Saccharum spontaneum* miRBase database. Homologous miRNA sequences were extracted, and the most highly expressed candidates were selected as conserved miRNAs responsive to low temperature in sugarcane, resulting in the identification of 110 novel miRNAs.

2.3 Analysis of Differentially Expressed miRNAs

By comparing miRNA expression levels between control and treatment groups, differential expression patterns of miRNAs in sugarcane varieties with different cold tolerance under low-temperature stress were identified. Using $|\log_2(\mathrm{FC})| \geq 2$ and $P < 0.05$ as screening criteria, 100 differentially expressed known miRNAs (61 up-regulated, 39 down-regulated) and 37 differentially expressed novel miRNAs (15 up-regulated, 22 down-regulated) were identified (Table 2). Down-regulated miRNA families included miR8175, miR5564, miR444, and miR166 among 21 families, while up-regulated families included miR156, miR169, miR172, miR393, miR397, and miR408 among 10 families. These differentially expressed miRNAs may perform specific functions in sugarcane responses to low-temperature stress.

2.4 Prediction and Analysis of miRNA Target Genes

Based on the sequences of differentially expressed miRNAs and corresponding gene information, psRobot, TargetFinder, and Tapurhybrid software were used

for target gene prediction (Figure 4 [Figure 4: see original paper]). The three methods collectively predicted 1,844 potential target genes, including 1,696 targets of known miRNAs and 148 targets of novel miRNAs. GO analysis of predicted target genes revealed 13 functional subcategories in biological process, 11 in cellular component, and 7 in molecular function (Figure 4). In the biological process category, target genes were primarily enriched in cellular processes and metabolic processes. In the cellular component category, enrichment was mainly in cell, organelle, and membrane. In the molecular function category, enrichment was primarily in binding, catalytic activity, and transporter activity. Most target gene functions were related to these binding activities. Most miRNAs respond to low-temperature stress by directly or indirectly mediating target gene expression to regulate related metabolic pathways, and these miRNA-regulated target genes play key regulatory roles in sugarcane cold tolerance (Table 3).

2.6 qRT-PCR Validation

qRT-PCR was used to validate the differential abundance of miRNAs and target genes obtained from sequencing. Primers for miRNAs and target genes are shown in Table 4. Fourteen differentially expressed miRNAs and their target genes were selected for RT-PCR validation, including miR156, miR160g_1, miR167d, miR169a-3p_3, miR171b-3p_3, miR172d-5p_4, miR393-3p_1, miR396b, miR397a_3, miR398b, miR399k_1, miR408d, novel_{mir36}, and novel_{mir89} (Figure 5 [Figure 5: see original paper]).

The results showed that, except for novel-miR36, the expression patterns of the other 13 miRNAs in qRT-PCR experiments were consistent with those detected by high-throughput sequencing (Figure 5A), confirming the reliability and authenticity of the sequencing data. Among the 14 selected miRNAs, all except novel-miR36 showed negative regulatory relationships with their target genes (Figure 5B), demonstrating that miRNAs typically regulate plant growth, development, and environmental stress responses through negative regulation or silencing of target genes.

3 Discussion and Conclusions

miRNAs are a class of small non-coding RNA molecules widely distributed in plants that play crucial regulatory roles in plant growth, development, and environmental stress responses (Liu et al., 2007; Guo et al., 2011). *Saccharum officinarum* originates from tropical regions and is thermophilic, with modern sugarcane cultivars primarily based on this species' germplasm. As a traditional leading industry in Guangxi, low temperature not only restricts cultivation expansion and high-yield stability but also affects farmers' income and the stable development of the sugar industry (Su et al., 2006). To understand the intrinsic molecular mechanisms of sugarcane responses to low temperature and identify cold tolerance-related miRNAs and target genes, this study subjected different sugarcane genotypes to low-temperature stress and systematically analyzed

their responses using high-throughput sequencing and bioinformatics.

Through high-throughput sequencing and bioinformatics analysis, we found that sRNAs were mainly concentrated in the 21–24 nt range, with differences among materials with varying cold tolerance and varying sequencing frequencies for different lengths. Many studies have shown that sRNA length distribution differs among species: *Arabidopsis* (Pasquinelli et al., 2000), wheat (Meng et al., 2013), and cotton (Sripathi et al., 2014) show peak distribution at 24 nt, while poplar (Li et al., 2014), soybean (Turner et al., 2012), and tomato (Pilcher et al., 2007) show peak distribution at 21 nt, consistent with our findings. We identified 137 differentially expressed miRNAs before and after low-temperature stress, including 100 known miRNAs (61 up-regulated, 39 down-regulated) and 37 novel miRNAs (15 up-regulated, 22 down-regulated). Numerous studies have demonstrated that plants regulate miRNA expression levels under low-temperature stress, thereby modulating target gene expression and triggering changes in related metabolic and signal transduction pathways to cope with adversity. These pathways 主要包括 extracellular signaling, intracellular second messengers, transcription factors, and functional genes. Wu & Poethig (2006) found that low-temperature stress caused miR156 down-regulation and increased target gene expression, thereby regulating extended vegetative growth and slowed metabolism in *Arabidopsis* to cope with adverse environments, consistent with our miR156 down-regulation results. Wang et al. (2017) analyzed miR160 responses to low-temperature stress in *Arabidopsis*, wheat, and rice. In our study, miR160g-1 was down-regulated after low-temperature stress, with its target gene suppressed and acting in the auxin signaling pathway to confer cold resistance. Pourcel et al. (2005) showed that miR397 target genes are related to laccase and involved in cell wall lignin synthesis, disease resistance, and environmental adaptation. We found that miR397a-3 was up-regulated in sugarcane leaves after low-temperature stress, with its target gene related to L-ascorbate oxidase, potentially participating in regulating ascorbate oxidase expression to enhance low-temperature response capacity. Li et al. (2014) studied miRNA responses to low-temperature stress in celery and found differential expression of miR160, miR164, miR394, miR395, and miR408. Sunkar & Zhu (2004) identified stress-regulated miRNAs in *Arabidopsis*, finding that miR393 was induced by low temperature, drought, and high salinity. Gupta et al. (2014) studied wheat miRNA responses to low temperature, salt, and osmotic stress, finding that miR168 and miR397 were down-regulated under salt and cold stress, while miR172 was up-regulated; miR393 increased under osmotic and salt stress but decreased under cold stress. Sun et al. (2015) found that miR169 was up-regulated in grape under cold stress but down-regulated in *Arabidopsis* and almond. Combined with previous research, we conclude that specific miRNA responses to low-temperature stress may vary depending on plant species, genotype, tissue type, and stress duration.

This study used three analytical methods (psRNATarget, TargetFinder, and Tapirhybrid) to predict target genes. GO analysis revealed that in the biological process category, target genes were primarily enriched in cellular and metabolic

processes; in the cellular component category, enrichment was mainly in cell, organelle, and membrane; and in the molecular function category, enrichment was primarily in binding, catalytic activity, and transporter activity. Under stress conditions, plants regulate miRNA expression levels to modulate target gene expression, thereby triggering changes in related metabolic and signal transduction pathways to cope with adversity. Therefore, we further performed qRT-PCR validation on target genes involved in plant hormone signal transduction, photosynthetic pigment synthesis, antioxidant enzyme systems, ubiquitin-mediated protein hydrolysis, and starch and sucrose metabolism. We found that miR156 targeted SBP transcription factors; under low-temperature stress, down-regulated miR156 negatively regulated SBP transcription factors, slowing sugarcane growth and metabolism while enhancing cold tolerance. Mei (2007) found that in winter wheat, the target gene of miR160, ARF17, showed significantly decreased expression in *Arabidopsis* overexpressing miR160f. Studies in *Arabidopsis*, rice, and maize have confirmed that the target genes of miR160 and miR167 are ARFs (Auxin Response Factors) that primarily cope with adversity by regulating the auxin signaling pathway. Our study also found that miR160g-1 was up-regulated after low-temperature stress and acted on ARF transcription factors. Zhang et al. (2012) found that miR169ac was down-regulated in *Populus tomentosa* under low-temperature stress, negatively regulating its target gene NAC transcription factor. Dang (2013) found that miR169a showed no significant expression under cold stress in *Chorispora bungeana*, while miR169 was down-regulated, indicating differential responses among miR169 family members. In our study, miR169a-3p_3 was up-regulated after low-temperature stress, down-regulating nuclear transcription factor expression, suggesting that miR169a-3p_3 is closely related to regulating sugarcane low-temperature tolerance.

Furthermore, through bioinformatics mining of sugarcane low-temperature-responsive miRNAs, we identified not only genes involved in antioxidant enzyme systems, plant hormone signal transduction, and genetic information but also genes related to carotenoid metabolism, porphyrin and chlorophyll metabolism, and starch and sucrose metabolism. Studies have shown that carotenoids in chloroplasts can act as accessory pigments to transfer energy to chlorophyll a and safely dissipate excess light energy under continuous stress to protect photosynthetic apparatus (Barbara et al., 1996; Liu et al., 2004; Holt et al., 2005). Our previous research also found that sugarcane can effectively utilize and distribute light energy by increasing dissipation of excess excitation energy from photosynthetic apparatus and adjusting leaf photosynthetic pigment content and composition under low-temperature stress. Moreover, qRT-PCR validation of some differentially expressed miRNAs and their target genes revealed that miR167d involved in carotenoid metabolism negatively regulated its target gene. Therefore, future in-depth studies on the molecular regulatory mechanisms of miRNAs involved in photosynthetic physiological processes under low-temperature conditions will provide important theoretical foundations and experimental basis for cold tolerance breeding.

References

AMBROS V. 2004. The function of animal MicroRNAs[J]. Nature, 431: 350-55.

BONNET E, HE Y, BILLIAU K, et al., 2010. TAPIR, a web server for the prediction of plant microRNA targets, including target mimics[J]. Bioinformatics, 26: 1566-1568.

DANG YC. 2013. Expression analysis of chilling-stress regulated miRNAs and their targets in *Chorispora bungeana* [J]. Lanzhou: Lanzhou University.

DEMMIG-ADAMS B, ADAMS WW. 1996. The role of xanthophyll cycle carotenoids in the protection of photosynthesis[J]. Trends Plant Sci, 1: 21-26.

EVERS M, MICHAEL H, DUECK A, et al., 2015. miRA: adaptable novel miRNA identification in plants using small RNA sequencing data[J]. Bmc Bioinform, 16: 370.

FAHLGREN N, CARRINGTON JC. 2010. miRNA target prediction in plants[J]. Meth Mol B, 592: 51-57.

FRIEDLANDER MR, CHEN W, ADAMIDI C, et al., 2008. Discovering microRNAs from deep sequencing data using miRDeep[J]. Nat Biotechnol, 26: 407–415.

GU L, HUANG ZG, LI WB, et al., 2011. Analysis on climatic factors affecting sugarcane meteorological yield in Nanning area during 1980-2007 [J]. J S Agric, 42(5): 492-95.

GUO T, LI GL, WEI Q, et al., 2011. The function of plant MicroRNA [J]. Acta Bot Boreal-Occidental Sin, 31(11): 2237-2254.

GUPTA O, MEENA N, SHARMA I, et al., 2014. Differential regulation of microRNAs in response to osmotic, salt and cold stresses in wheat[J]. Mol Biol Rep, 41: 4623–4629.

HE Y, TAN ZK, DING MH, et al., 2008. Key meteorological factors controlling sugarcane yield and sucrose content in Guangxi [J]. J Anhui Agric Sci, 36(8): 3181-84.

HE Y, TAN ZK, DING MH, et al., 2009. Infrequent disaster of the cold and freezing disaster and its impacts on sugarcane production in Guangxi [J]. J Catastrophol, 24(1): 68-72.

HOLT NE, ZIGMANTAS D, VALKUNAS, et al., 2005. Carotenoid cation formation and the regulation of photosynthetic light harvesting[J]. Science, 307: 433-436.

KUANG ZM, LI Q, RAO YM, et al., 2009. Application of EOS/MODIS data to monitoring sugarcane cold damage [J]. J Appl Meteorol Sci, 20(3): 360-64.

LI MH, LONG RC, YANG QC, et al., 2014. Cloning and function analysis of a salt-stress-induced HD-Zip transcription factor MsHB2 from Alfalfa [J]. Plant

Physiol J, 47(4): 622-32.

LI MY, WANG F, XU ZS, et al., 2014. High throughput sequencing of two celery varieties small RNAs identifies microRNAs involved in temperature stress response[J]. Bmc Genom, 15: 242.

LI YR, FANG FX, WU JM, et al., 2011. Survey of frost and cold damage on sugarcane production in Guangxi in 2010/2011 milling season and countermeasures [J]. J S Agric, 42(1): 37-42.

LIU YH, LIU ZZ, LUO LJ, et al., 2007. Plant miRNA and its potential role in plant developmental process and environmental stress responses [J]. Plant Physiol J: 987-992.

LIU Z, YAN H, WANG K, et al., 2004. Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution[J]. Nature, 428 (6980): 287.

LV, DE KANG, XI BAI, YONG LI, et al., 2010. Profiling of cold-stress-responsive miRNAs in rice by microarrays[J]. Gene, 459: 39-47.

MEI L, 2016. Cloning four kinds of MiRNAs and analysis of expression pattern with cold related in *Triticum aestivum* L. [D]. Northeast Agricultural University.

MENG F, LIU H, WANG K, et al., 2013. Development-associated microRNAs in grains of wheat (*Triticum aestivum* L.) [J]. Bmc Plant Biol, 13: 140.

MORTAZAVI A, WILLIAMS BA, MCCUE K, et al., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq[J]. Nat methods, 5: 621-628.

PASQUINELLI AE, REINHART BJ, SLACK F, et al., 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA[J]. Nature, 408 (6808): 86-89.

PILCHER RLR, MOXON S, PAKSERESHT N, et al., 2007. Identification of novel small RNAs in tomato (*Solanum lycopersicum*) [J]. Planta, 226: 709-17.

POURCEL L, ROUTABOUL JM, KERHOAS L, et al., 2005. Transparent TESTA10 encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in Arabidopsis seed coat[J]. Plant Cell, 17: 2966-80.

SRIPATHI, VR, CHOI Y, CHAN AP, et al., 2014. Small RNA transcriptome profiles of four cotton species, *Gossypium hirsutum*, *G. herbaceum*, *G. arboreum* and *G. raimondii*[C]. San Diego: International Plant and Animal Genome Conference XXII.

SU YX, LI Z, SUN H. 2006. Climate division of sugarcane planting based on GIS in Guangxi [J]. Chin J Agrometeorol, (3): 987-992.

SUN X, FAN G, SU L, et al., 2015. Identification of cold-inducible microRNAs in grapevine[J]. Front Plant Sci, 6: 595.

SUNKAR R, CHINNUSAMY V, ZHU J, et al., 2007. Small RNAs as big players in plant abiotic stress responses and nutrient deprivation[J]. Trends Plant Sci,

12: 301-309.

SUNKAR R, GIRKE G, JAIN PK, et al., 2005. Cloning and characterization of microRNAs from rice[J]. The Plant Cell Online, 17: 1397-1411.

SUNKAR R, LI YF, JAGADEESWARAN G. 2012. Functions of microRNAs in plant stress responses[J]. Trends Plant sci, 17: 196-203.

SUNKAR R, ZHU JK. 2004. Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis[J]. Plant Cell Online, 16: 2001-2019.

THIEBAUT F, ROJAS CA, ALMEIDA KL, et al., 2012. Regulation of miR319 during cold stress in sugarcane[J]. Plant Cell Environ, 35: 502-512.

TURNER M, YU O, SUBRAMANIAN S. 2012. Genome organization and characteristics of soybean microRNAs[J]. Bmc Genom, 13: 169.

WANG LL, ZHAO TL, GE JT, et al., 2017. Application prospects of plant cold-stress-responsive miRNAs in cold resistance research of plants [J]. Acta Agric Shanghai, 33(6): 129-134.

WANG Z, GERSTEIN M, SNYDER M, et al., 2009. RNA-Seq: a revolutionary tool for transcriptomics[J]. Nat Rev Genet, 10: 57-63.

WANG X, TONG Y, WANG S. 2010. Rapid and accurate detection of plant miRNAs by liquid northern hybridization[J]. Int J Mol Sci, 11: 3138-3148.

WU G, POETHIG RS. 2006. Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3[J]. Development, 133: 3539-47.

WU HJ, MA YK, TONG C, et al., 2012. PsRobot: a web-based plant small RNA meta-analysis toolbox[J]. Nucl Acid Res, 40: 22-28.

WU MG, LIN YQ, ZHANG H. 2010. Research status and prospect on industrial standard of sugarcane in China[J]. Subtrop Agric Res, 6: 209-212.

XIONG L, ZHU JK. 2003. Regulation of abscisic acid biosynthesis[J]. Plant Physiol, 133: 29-36.

ZHANG YY, REN YY, CHEN L, et al., 2017. Differential expression analysis of 12 MicroRNAs under cold stress in *Populus tomentosa* [J]. Chin Agric Sci Bull, 28(7): 1-7.

Note: Figure translations are in progress. See original paper for figures.