Analysis of microRNA Expression Characteristics Related to Low Temperature Stress in Chewing Cane

Minghui CHEN[1,2*], Shiping CHENG[1], Jiansheng WANG[1], Junqing WANG[1]

1. Pingdingshan University, Henan Province Key Laboratory of Germplasm Innovation and Utilization of Eco-economic Wood Plant, Pingdingshang 467000, China;
2. Guangxi Key Laboratory of Sugarcane Genetic Improvement, Nanning 530000, China

Abstract [**Objectives**] To explore microRNA expression characteristics related to low temperature stress in chewing cane. [**Methods**] The research on miRNA under abiotic stress of sugarcane at home and abroad mainly focused on the types and regulation of miRNA under cold, heat, drought, high salt, and mechanical stress. However, there are few studies on miRNA under low temperature stress in chewing cane. The target genes of miR394 and miR825 in chewing cane were predicted and functionally analyzed by bioinformatics technology. [**Results**] The results showed that the target genes of miR394 and miR825 were mainly members of the WRKY transcription factor family, involved in plant growth, development and stress resistance. Real-time fluorescence quantitative PCR analyzed the expression characteristics of target miRNA in different tissues of chewing cane at different periods of low temperature stress. [**Conclusions**] The results showed that the expression of chewing cane miR394 and miR408 had temporal and spatial specificity and tissue specificity, both of which could respond to low temperature stress with significant differential expression.

Key words Chewing cane, Low temperature stress, microRNA, Target gene

1 Introduction

Low temperature is the main factor limiting crop productivity and geographical distribution, causing significant losses worldwide. miRNA is a type of small single-stranded non-coding RNA with a length of 20 - 24 nt, which can regulate plant growth and development, cell differentiation, metabolism, and response to biotic and abiotic stresses at the post-transcriptional level.

Chewing cane is one of the most important cash crops in tropical and subtropical regions. The main producing areas in China are Guangdong, Guangxi, Hainan, Fujian provinces. In recent years, with the heat deficit in the south and the north, the introduction and domestication of varieties and the improvement of cultivation techniques, the sugarcane area has moved northward year by year, and has now expanded to Henan, Hebei, Shandong and other provinces. Chewing cane originates from tropical and subtropical regions, and is more sensitive to low temperature and chilling damage during the growth cycle. Temperature, especially the low temperature in early spring, is one of the important limiting factors for the introduction and production of chewing cane. Therefore, identifying and screening the key genes for cold resistance in chewing cane and exploring the molecular mechanism of cold resistance can provide a theoretical basis for the selection and breeding of cold-resistant varieties.

The miRNAs are small endogenous non-coding RNAs of 20 to 24 nucleotides in length. In 1994, Lee *et al.* [1] discovered miRNA lin-4 in *Caenorhabditis elegans*. In 2002, Reinhart *et al.* [2] first discovered plant miRNA in the model plant *Arabidopsis thaliana*. Plant miRNAs mainly regulate downstream gene expression through two ways of post-transcriptional gene silencing, that is, cutting target mRNA that is reverse complementary to its sequence or inhibiting the translation of target mRNA[3]. On the one hand, when the sequence of the plant miRNA and the target mRNA is completely or almost completely complementary (only 0 - 5 mismatch bases), the miRNA cuts the downstream target mRNA under the action of RISC, HASTY, *etc.* to degrade it[4]. On the other hand, when the sequence of the plant miRNA and the target miRNA is not completely complementary, the miRNA regulates the expression of downstream genes by inhibiting the translation of the target mRNA. In addition to the above two main mechanisms of action, the third mechanism of action of plant miRNAs on their target genes is based on the methylation of DNA and histones, which regulates them at the transcriptional level, thereby causing post-transcriptional silencing of target genes[5]. In 2015, Sun et al used the low temperature stress of *grapevine* seedlings for 24 h at 0 ℃ for the first time to prove that miRNA is involved in the regulation of cold stress[6]. With the advancement of research technology, high-throughput and next-generation sequencing methods have become the first choice for miRNA analysis under low temperature stress. miRNAs and their target genes are the main regulators in response to various stresses. miRNAs almost regulate all biological and metabolic processes of plants, and are of great significance for regulating the expression of endogenous resistance

Received: May 3, 2023 Accepted: July 19, 2023

Supported by Science and Technology Research Project of Henan Provincial Science and Technology Department (222102110448); Key Scientific Research Projects of Colleges and Universities in Henan Province (21B210007); Open Research Project of Guangxi Sugarcane Genetic Improvement Key Laboratory (19-185-24-K-01-01).

* Corresponding author. Minghui CHEN, PhD., associate professor, research fields: crop breeding.

genes.

Our laboratory used second-generation sequencing technology and chip expression profiling technology to obtain 14 new, 32 known and 105 conserved miRNAs from chewing cane plants under low temperature stress. Studies have found that there are significant differences in the expression patterns of some endogenous miRNAs in chewing cane before and after low temperature stress, among which the expression differences of miR394 and miR825 families are more significant. These differentially expressed miRNAs tend to target the WRKY transcription factor family related to stress resistance, and may play an important regulatory role in the response of chewing cane to low temperature stress. Based on this, this study comprehensively screens important chewing cane miRNAs and their target genes that may respond to low temperature stress through literature retrieval and bioinformatics technology, and uses real-time quantitative PCR to verify the authenticity of the selected miRNAs and analyze their expression at the same time The characteristics lay a foundation for the in-depth study of the mechanism of miRNA in the process of low temperature stress and the process of cold resistance gene expression regulation, and provide a theoretical basis for the development of cold resistance varieties.

2 Materials

The test fruit cane is 'Guiguozhe No. 1', provided by the Sugarcane Research Institute of Guangxi Academy of Agricultural Sciences, and is planted in a greenhouse and potted.

3 Methods

3.1 Prediction and functional analysis of miRNA target genes in chewing cane in response to low temperature stress Combining with the results of the research in this laboratory, the miRNA of chewing cane in response to low temperature stress was counted, and the miRNA of the purpose of this study was selected from them. The mature sequence of miR394 obtained from miRBase21 database (http://www. mirbase. org/) and reported literature is 5'-UUUCUAGGAUU GAAGGGAGCU-3', and the mature sequence of miR825 is 5'-UCUCUUCGUUGUCUGU UCGAC-3'. According to miRNA sequence, predict its target gene in psRNATarget (A Plant Small RNA Target Analysis Server, http://plantgrn. noble. org/psRNATarget/), and search and analyze its function in Uni-protKB protein database and NCBI according to the name of each target gene.

3.2 Analysis of the expression characteristics of miRNA in chewing cane in response to low temperature stress After detoxification of the single bud stems of 'Guiguozhe No. 1' varieties, they are put into the sand tray for sand culture. When the fruit cane grows 2 to 3 leaves, the cane seedlings are removed from the sand, and the fruit cane seedlings with the same growth are selected. Planted in a 25 cm × 30 cm nutrient pot, planted 2 plants in each pot, grown for 50 d according to daily management, and grouped for treatment when the sugar cane is at the 5 – 6 leaf stage. Selected 10 pots of 'Guiguozhe No. 1' sugarcane seedlings with consistent growth and placed them in a low-temperature culture room at a temperature of 0 ℃, an illumination of 300 – 400 μmol/(m^2 · s), 12 h of light, a relative humidity of 65% to 75%. After 14 d of stress, the soil water content during the treatment was maintained at about 25%, and the normal growth of fruit cane seedlings in the greenhouse was used as the control. Samples were taken at 1^{st}, 3^{rd}, 7^{th}, 10^{th}, and 14^{th} d after the low temperature treatment, and all indicators were set to 3 replicates. Based on the miRNA mature sequence and the miRNAqRT-PCR primer design principle, the upstream primer sequence was designed (the downstream primer is the universal primer Uni-miRqPCRPrimer provided in the kit) and the upstream and downstream primer sequences of the internal reference gene Actin (Table 1).

Table 1 qRT-PCRprimers used in this study

Primer name	Sequence (5'→3')
Sof-miR159	TTTGGATTGAAGGGAGCTCTA
Sof-miR858	TCTCGTTGTCTGTTCGACCTT
Actin-F	ATTCTCCGTTTGGACCTTGCT
Actin-R	GCTCCGATGGTGATGACTTGT

We used RNAisoforSmallRNA kit (TaKaRa, 9573A) to extract SmallRNA of the test sample, and took 3 μL SmallRNA for 1% agarose gel electrophoresis detection. Used miRNAcDNA first-strand synthesis kit miRcutemiRNAFirst-StrandcDNA Synthesis Kit (TIANGEN, KR201) to carry out Poly(A) tailing and reverse transcription reaction on SmallRNA in the sample, and the resulting cDNA was stored at – 20 ℃. Real-time quantitative PCR was used to detect the relative expression of miRNA in different tissues at different times after low temperature stress, and the reaction solution was prepared according to the requirements of TIANGEN miRNA real-time fluorescent quantitative PCR kit (TIANGEN, FP401). The reaction system is 20 μL, including: 2 × miRcutemiRNAPremix 10 μL, cDNA template 1 μL, forward and reverse primers each 0. 4 μL (primer concentration is 10 μM), 8. 2 μL of ddH_2O. The reaction conditions were: 94 ℃ pre-denaturation for 2 min; 94 ℃ for 20 s, 60 ℃ for 34 s for 40 cycles. Using sugarcane Actin as an internal reference gene, the $2^{-\triangle\triangle Ct}$ method was used to analyze the relative expression of miRNA and draw a histogram[7].

4 Results and analysis

4.1 Prediction and functional analysis of miRNA target genes in chewing cane in response to low temperature stress The results of microarray hybridization in our laboratory have shown that multiple miRNAs in chewing cane can respond to low temperature stress, and their expression levels change to varying degrees at 3^{rd}, 7^{th}, 10^{th}, and 14^{th} d after stress. Based on literature search, it is found that miR394 and miR825 may play a cooperative regulatory role in the cold resistance signal pathway. Based on the above research results, we selected miR394 and miR825 as the main research objects of this experiment. According to the mature sequence of miRNA, it is predicted in the psRNATarget software that chewing cane miR394 has 16 target genes such as

TC146586, miR825 has 12 target genes such as *TC117317*, among which most members of WRKY type transcription factor family, and the target miRNA mainly passes complementary action targets the cutting of its target genes, which in turn affects gene expression. We searched and analyzed the function of each target gene in the Uni-protKB protein database and NCBI (refer to the function of each target gene in the model organism *Arabidopsis*), and the results show that it is mainly involved in the growth and development, metabolism, cell morphology and cold resistance of chewing cane process (Table 2).

4.2 Real-time quantitative PCR detection of chewing cane miRNA in response to low temperature stress qRT-PCR was used to detect the authenticity and expression of miR394 and miR825 in the samples of the treatment group and the control group after 1, 3, 7, 10, and 14 d of low temperature stress. The results showed that in stem and leaf tissues, the expression of miR394 was up-regulated within 7 d of low temperature stress, and decreased rapidly within 10 d. However, the expression of miR394 was down-regulated in root tissues after 7 d of low temperature stress, and gradually up-regulated within 10 d. In stem and leaf tissues, the expression of miR825 was down-regulated within 7 d of low temperature stress, and gradually up-regulated within 10 d. However, the expression of miR825 was up-regulated in root tissues after 3 d of low temperature stress, and gradually down-regulated within 7 d. On the whole, the expression level of miR394 in various tissues is higher than that of miR825 (Fig. 1). The above results indicate that miR394 and miR82 really exist in chewing cane, and their expression has temporal and spatial specificity and tissue specificity. Chewing cane miR394 and miR825 can respond to low temperature stress and have significant differential expression, which in turn induces changes in the expression of many downstream genes, including the differential expression of disease resistance genes regulated by them, which may play an important role in the resistance of chewing cane to low temperature stress.

Table 2 Predicted target genes of chewing cane miR394 and miR825

miRNA	Target gene	Target description
miR394	*TC146586*	Squamosa promoter binding protein (SBP)
	BQ534106	Nucleic acid binding protein
	CA162377	WRKY protein
	CA067690	Alcohol dehydrogenase
	CF577019	Putative senescence-associated protein
	CA072223	Beta-galactosidase precursor
	CA075255	Rf-1 gene for fertility restorer
	TC137037	Clathrin assembly protein, putative
	CA072223	sugar transporter
	CA120099	ABC transporter family protein
	CA101090	Putative early nodulin
	TC120009	Heat shock cognate 70 kDa protein
	CA203894	G-type lectin Screceptor-like protein kinase
	BU103176	Probable cytochrome P450 monooxygenase
	CA084820	Auxin response factor
	CF157375	Putative sorbitol transporter
MiR825	*TC117317*	cysteine proteinase inhibitor B
	CA072223	Membrane-associated zinc metalloprotease family
	CA231663	Nucleic acid binding protein
	CA084181	Kelch repeat-containing F-box-like
	TC126582	GRAS transcription factor family protein
	TC195758	blue copper protein
	CF213253	Putative protein kinase homolog
	CA228340	ABC1 family protein
	TC137024	CTL-like protein DDB, putative U-box superfamily protein
	CA154021	Putative senescence-associated protein
	CF150483	Phospholipase A-2-activating protein
	CF139748	Glucanases superfamily

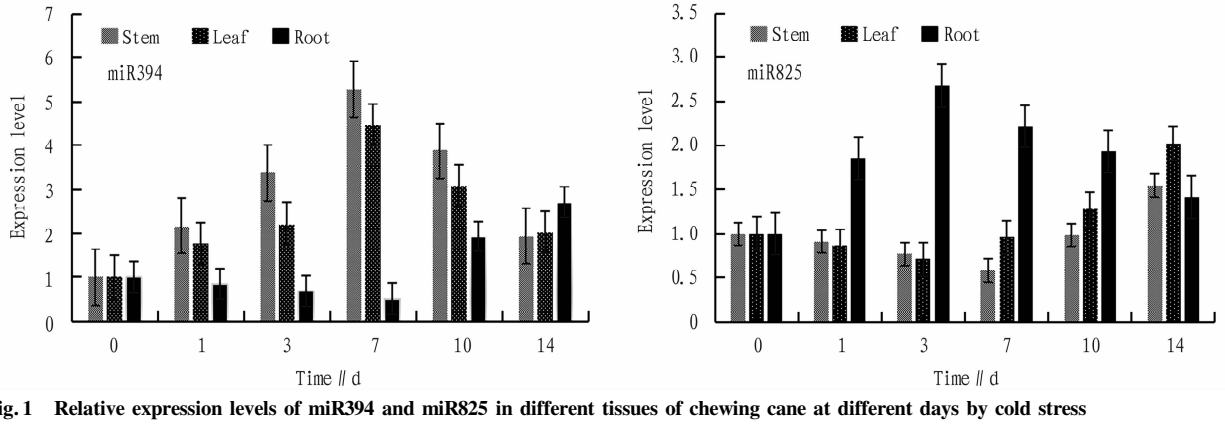

Fig. 1 Relative expression levels of miR394 and miR825 in different tissues of chewing cane at different days by cold stress

5 Discussion

As a kind of endogenous small molecule RNA, miRNA plays an important role in plant growth, development and stress response. The research on miRNA and its target genes is the basis of miRNA function research. With the rapid development of bioinformatics technology and molecular biology technology, more and more plant miRNAs and their functions have been discovered and confirmed by people. This study used bioinformatics technology to predict the target genes of chewing cane miR394 and miR825 and

(To page 66)

References

[1] ZHANG XT, WANG XS, JIA WF, *et al.* Research progress of plants under salt treatment[J]. Northern Horticulture, 2021(6): 137 - 143. (in Chinese).

[2] GUO DY, ZI PY, LEI G, *et al.* SnRK2s at the crossroads of growth and stress responses[J]. Trends in Plant Science, 2019, 24(8): 672 - 676.

[3] EHAB A, IBRAHIM G. Seed priming to alleviate salinity stress ingerminating seeds[J]. Journal of Plant Physiology, 2016(192): 38 - 46.

[4] ANNE C, JAN E L, MANUEL F, *et al.* Cytokinin action in response to abiotic and biotic stresses in plants[J]. Plant Cell & Environment, 2019, 42(3): 998 - 1018.

[5] HONG GL, KYOUNGHEE L, PIL JS. The Arabidopsis MYB 96 translation factor plays a role in seed dormancy[J]. Plant Molecular Biology, 2015, 87(4 - 5): 371 - 381.

[6] JAUME L, PERE V, MIQUEL P, *et al.* Post petalfall applications of gibberellins import fruit set on pear[J]. Scientific Horticulturae, 2019 (252): 149 - 155.

[7] ZHANG LL, LIN ZQ, MA DR, *et al.* Effects of exogenous gibberellin on seed germination and seedling growth of weedy rice under NaCl stress[J]. Seeds, 2014, 33(10): 5 - 8, 11. (in Chinese).

[8] LIU SC, CAO XM, MU JX, *et al.* Effects of exogenous hormones on tomato seed germination and seedling growth under salt stress[J]. Seeds, 2016, 35(12): 94 - 98. (in Chinese).

[9] LI L, ZHANG K, HE MC, *et al.* Effects of different salt concentrations on seed germination and seedling bud length of four tomato varieties[J]. Northern Horticulture, 2019(24): 1 - 6. (in Chinese).

[10] GARCIA IS, SOUZA A, BARBEDO CJ, *et al.* Changes in soluble carbohydrates during storage of Caesalpinia echinata LAM. (Brazilwood) seeds, an endangered legalized tree from the Brazilian Atlantic Forest [J]. Brazilian Journal of Biology, 2006, 66(2B): 739 - 745.

(From page 54)

analyzed their functions. The results showed that the target genes of miRNA were mostly WRKY transcription factors. This result is consistent with previous research results[8 - 10], which mainly use complementary effects to target and cut its target genes, and then participate in the growth and development and disease resistance process of sugar cane. For example, miR394 targets its target gene WRKY18-like, and functional analysis results show that transcription factors *WRKY18* and *WRKY17* jointly promote the biosynthesis of glucosinolate. Glucosinolates and their hydrolysates play an important role in the defense of Arabidopsis, rice and other plant[11 - 12]. The target gene of miR825 is WRKY-related proteinHv1-like gene, which responds to external stimuli in barley and participates in the biosynthesis of flavonoids. Flavonoids play a role in plant growth, development, flowering, fruiting, cold and drought resistance, *etc.* Plays an important role[13]. miR394 and miR825 both carry their target genes and exist alone and are not related to each other, indicating that the two miRNAs may play a regulatory role in different signaling pathways, or there may be different mechanisms of action. In order to clarify the expression characteristics of miR394 and miR825 in chewing cane subjected to low temperature stress, we used the qRT-PCR method to detect the relative expression of the target miRNA in different tissues of chewing cane and at different treatment times. It was found that both miR394 and miR825 could be Low temperature stress responds, and there are significant differences.

In this study, we analyzed the expression characteristics of miRNA in chewing cane related to low temperature stress and predicted the function of its target genes, laying a foundation for the study of chewing cane cold resistance mechanism and providing a new idea. However, scientific issues such as how cane miRNA regulates its target genes as a consequence of low temperature stress, and how the predicted cold resistance-related target genes play their roles, need to be further explored.

References

[1] LEE RC, FEINBAUM RL, AMBROS V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14 [J]. Cell, 1993, 75(5): 843 - 854.

[2] REINHART BJ., WEINSTEIN EG., RHOADES MW, *et al.* MicroRNAs in plants[J]. Genes and Development, 2002, 16(13): 1616 - 1626.

[3] LIU C, WANG B, LI Z, *et al.* TsNAC1 is a key transcription factor in abiotic stress resistance and growth[J]. Plant Physiology, 2017(1): 742 - 756.

[4] KHRAIWSH B, ARIF MA, SEUMEL GI, *et al.* Transcriptional control of gene expression by microRNAs[J]. Cell, 2010, 140(1): 111 - 122.

[5] WU L, ZHOU H, ZHANG Q, *et al.* DNA methylation mediated by a microRNA pathway[J]. Molecular Cell, 2010, 38(3): 465 - 475.

[6] SUN X, FAN G, SU L, *et al.* Identification of cold-inducible micro RNAs in grapevine[J]. Front Plant Science, 2015(6): 595.

[7] LIVAK KJ, SCHMITTGEN TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle Ct}$ method[J]. Methods, 2001(25): 402 - 408.

[8] MAO W, LI Z, XIA X, *et al.* A combined approach of high-throughput sequencing and degradome analysis reveals tissue specific expression of microRNAs and their targets in cucumber[J]. PLoS ONE, 2012, 7(3): e33040.

[9] HU JH, SUN LL, ZHU ZX, *et al.* Characterization of conserved microRNAs from five different cucurbit species using computational and experimental analysis[J]. Biochimie, 2014(102): 137 - 144.

[10] LI CH, LI YS, BAI LQ, *et al.* Grafting-responsive miRNAs in cucumber and pumpkin seedlings identified by high-throughput sequencing at whole genome level[J]. Physiologia Plantarum, 2014(151): 406 - 422.

[11] HIRAI MY, SUGIYAMA K, SAWADA YJ, *et al.* Omics-based identification of *Arabidopsis* Myb transcription factors regulating aliphatic glucosinolate biosynthesis[J]. PNAS, 2007, 104(15): 6478 - 6483.

[12] CHEN YH, YANG XY, HE K, *et al.* The MYB transcription factor superfamily of *Arabidopsis*: Expression analysis and phylogenetic comparison with the rice MYB family[J]. Plant Molecular Biology, 2006, 60 (1): 107 - 124.

[13] ZHANG RX. Suppression and comparison of Brassica napus TT8 gene family by RNAi and CRES-T[D]. Chongqing: Chongqing: Southwest University, 2011. (in Chinese).