Identification of Cold-Resistant microRNAs in *Taxillus chinensis* (DC.) Danser Seeds

Empowering miRNA Research with MGI's Small RNA Library Preparation Technology and DNBSEQ Sequencing Platform

The research team led by Wei Shugen from the Guangxi Botanical Garden of Medicinal Plants utilized MGI's MGIEasy Small RNA Library Preparation Kit and DNBSEQ sequencing technology to conduct research on the issue of low-temperature stress sensitivity in *Taxillus chinensis* (DC.) Danser seeds. They analyzed and identified miRNAs that specifically respond to cold stress in *Taxillus chinensis* (DC.) Danser seeds[1]. The findings were published in the *BioMed Research International* journal in 2021 with the title "Identification of MicroRNAs in *Taxillus chinensis* (DC.) Danser Seeds under Cold Stress".

Recommended application: Agrigenomics

Recommended model: DNBSEQ-G400

- **Low initial RNA input**

  Allows the preparation of high-quality small RNA sequencing libraries from 10 ng - 1 μg of total RNA samples.

- **High sample type compatibility**

  Taxillus chinensis (DC.) Danser seeds can be used as plant samples for experiments. This library preparation technique is suitable for different types of samples of various species, while maintaining stable performance across all sample types.

- **Automatic operation compatible**

  This kit can be used on the MGI's automated sample processing systems, enabling an efficient and streamlined experimental workflow.

- **Efficient and high-quality sequencing data output**

  DNBSEQ sequencing technology exhibits many excellent features such as high accuracy, low repeat rate and low index hopping rate.

Background

Loranthus, known as *Taxillus chinensis* (DC.) Danser, is a parasitic plant and an ancient traditional Chinese medicine used to treat diseases such as rheumatism, high blood pressure, and stroke[2]. However, the loranthus plant has relatively low reproductive capability, largely due to the extreme sensitivity of its seeds to low temperature, causing them to rapidly lose their ability to survive and develop at temperature below 0°C. Cold stress is a significant factor affecting the geographical distribution, growing seasons, and yields of many plants[3]. Numerous studies have indicated that many MicroRNAs (miRNA) are related to cold stress response in plants, and play important roles in regulating the cold resistance of plants. Nevertheless, our understanding of the regulatory mechanisms involved in the response to cold stress in loranthus remains limited.

miRNAs are a class of endogenous small non-coding RNAs (18~24 nt). They function by cleaving messenger RNA (mRNA) or repressing translation following transcription, leading to the repression of protein expression[4]. In plants, the primary transcripts of miRNA are stabilized by DAWDLE and processed into miRNA: miRNA* duplexes by DCL1, HYL1, SE, and nuclear CBC in the D-bodies. Subsequently, the duplexes are methylated by HEN1 and transported to the cytoplasm[5]. Mature miRNA from the duplexes is incorporated into an AGO protein complex[6]. Currently, the miRBase database (v22.1) contains a total of 10,414 miRNA and miRNA* sequences for 82 different plant species, including rice, maize, Arabidopsis, grape, and others.

Research Description

Due to the lack of comprehensive information for the loranthus genome, research on miRNA sequences and their regulation in loranthus genome remains largely unknown. miRNA sequencing has been widely used to identify known and novel miRNAs in model and non-model plants, like okra. Previously, this team has reported the transcriptome data of loranthus seeds responding to moisture loss 7, which could be used for miRNA discovery in loranthus seeds. In this study, this research team initially assessed the viability of loranthus seeds stored at different temperatures for various durations. Subsequently, miRNA sequencing was performed to identify known and novel miRNAs in loranthus seeds, as well as miRNAs involved in the cold resistance process.

Materials and Methods

Sample preparation

100 samples of loranthus seeds were collected from the Guangxi Botanical Garden of Medicinal Plants. Seeds underwent different cold treatments, including different temperatures (-20°C, -5°C, -1°C, 0°C, 4°C, 10°C, 25°C) and different durations (1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 10 days, 20 days) and the viability was tested with the TTC staining method (1% 2,3,5-triphenyl tetrazolium chloride)[7]. Total RNA (100 mg, in triplicate) was extracted from seeds stored at 0°C for 0 hours (A0), 12 hours (A1), and 36 hours (A2) using TRIzol reagent, and the quantity and quality of total RNA were evaluated with Agilent Bioanalyzer 2100[8].

Library construction and sequencing

1 µg of the extracted total RNA samples was used to construct small RNA libraries using the MGIEasy Small RNA Library Prep Kit from MGI. The process is briefly described as follows: total RNA underwent urea-PAGE gel electrophoresis and the small RNAs (18-30 bp) band was excised. The small RNAs were extracted and subsequently ligated with a 3' adenylated adapter. Then, the 3' adenylated adapter was annealed with an RT primer containing a barcode, ligated with a 5' adapter and underwent reverse transcription. When the first strand cDNA was ready, it was subjected to 15 cycles of PCR amplification and gel separation for 103-115 bp fragments, followed by purification and quantitation. The purified product was then circularized and quantitated using the Qubit ssDNA Assay Kit.

The library preparation process is relatively complex, especially when dealing with a large number of samples. It is time-consuming prone to errors and variations in sample volumes. The MGI's automated sample preparation system is highly recommended to complete library construction. This automated system eliminates the need for manual sample handling, improving the stability and overall efficiency of sample preparation.

For small RNA sequencing, DNA nanoballs (DNBs) were generated by rolling circle replication, and these DNBs were loaded onto sequencing chips for sequencing of single-end read of 50 bp (SE50) on the DNBSEQ platform.

Bioinformatic analysis The sequenced raw data was filtered with SOAPnuke. miRDeep2 was employed for predicting potential miRNA hairpin structures based on the previously published transcriptome of loranthus seeds. The predicted miRNA precursors were used as reference for miRNA expression analysis[9]. All processed data were compared with mature plant miRNA data from miRBase, and the Read with the highest expression, aligned to each miRNA family, was chosen as the miRNA sequence for loranthus plants. The published transcriptome data of loranthus seeds were utilized as a resource for miRNA target gene prediction. Three software tools, psRobot, TarHunterL, and TargetFinder, were used to predict differentially expressed miRNA target genes. Furthermore, Gene Ontology and KEGG pathway analyses were applied to annotate these transcriptomes. Lastly, qRT-PCR was employed to quantitatively verify the expression levels of miRNAs and their target genes.

Sample collection	Library preparation and sequencing	Bioinformatics analysis	Result analysis
100 samples of loranthus seeds collected from the Guangxi Botanical Garden of Medicinal Plants	 MGIEasy Small RNA Library Prep Kit  Genetic Sequencer DNBSEQ-G400	SOAPnuke miRDeep2 psRobot TarHunterL TargetFinder	· Analysis of differentially expressed microRNA (DEmiRs) · miRNA target prediction and pathway analysis

Results

miRNA identification and expression analysis

The miRNAs in loranthus seeds exhibit traditional miRNA structures, such as aly-miR390a and c51122_g1_i3_15022 (Figure 1a), and some miRNAs with siRNA-like structures (Figure 1b). These miRNAs with traditional structures in loranthus seeds display significant activation and suppression performance after 12 and 36 hours of cold treatment (Table 1). In this study, a total of 600 miRNAs were identified in loranthus seeds under cold stress for the first time. Among them, 242 differentially expressed miRNAs were identified, with 224, 229, and 223 miRNAs detected (TPM > 1) in A0 (control), A1 (0°C cold treatment for 12 hours), and A2 (0°C cold treatment for 36 hours), respectively (Figure 1c). 18, 17 and 16 miRNAs, exhibited expression levels exceeding 1000 TPM (Figure 1d) in A0, A1 and A2, respectively.

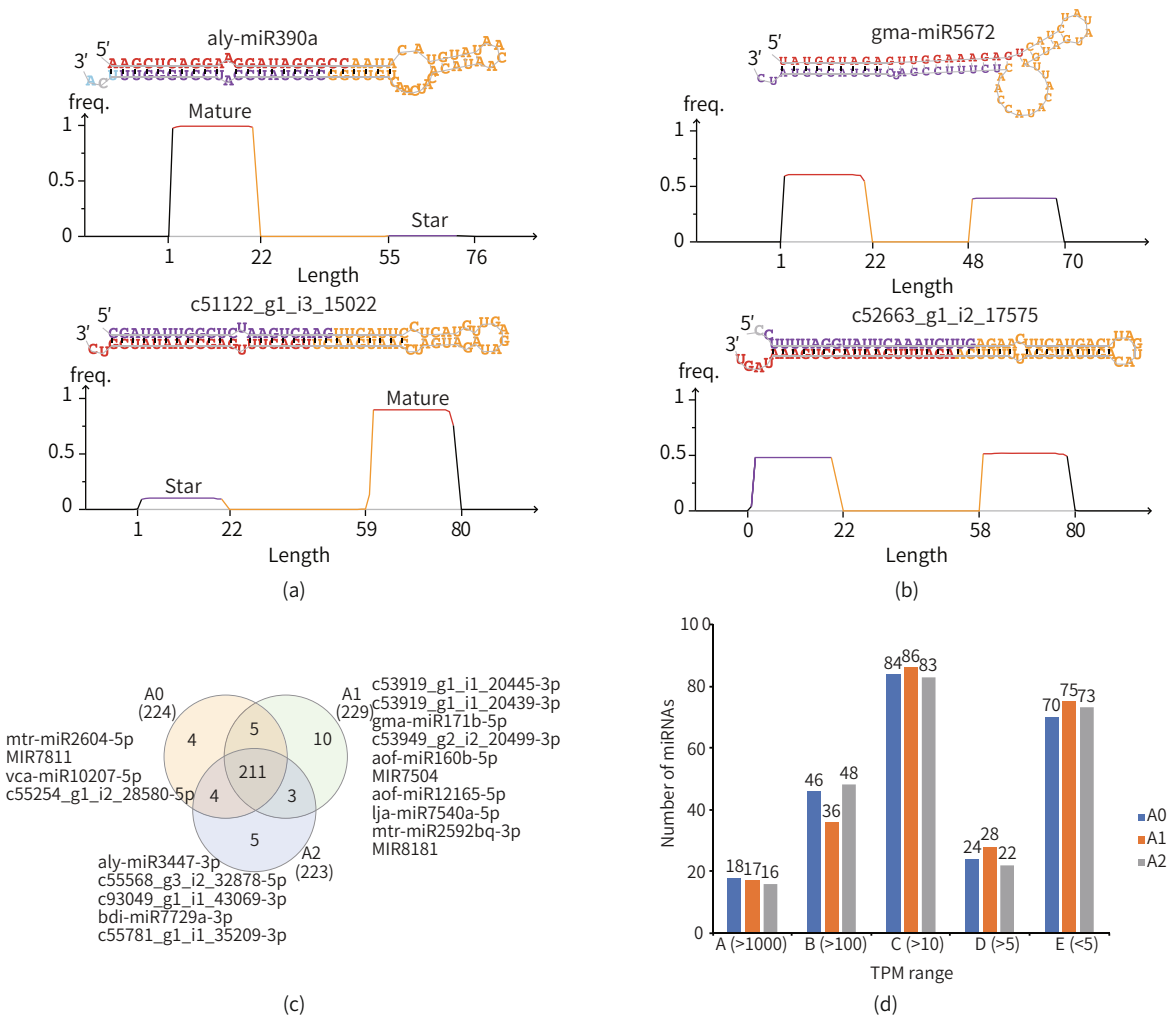

Figure 1. miRNAs isolated in loranthus seeds under cold stress. (a) Conventional structures of miRNAs isolated by miRDeep2 in loranthus seeds. (b) Possible siRNA-like structures of miRNAs in loranthus seeds. (c) Venn diagram showing the miRNAs isolated in the loranthus seeds at 0°C for 0 h (A0), 12 h (A1), and 36 h (A2). (d) Distribution of miRNA expression isolated in each sample.

miRNA	A1 vs. A0			A2 vs. A0			A2 vs. A1		
	log2FC	FDR	Regulation	log2FC	FDR	Regulation	log2FC	FDR	Regulation
miR1886	1.183	0.013	Up	1.041	0.039	Up	-0.156	0.831	NC
c55345_g4_i1_29371-3p	1.242	0.018	Up	1.010	0.081	Up	-0.237	0.764	NC
miR5067	1.062	0.032	Up	1.284	0.010	Up	0.262	0.710	NC
miR477	1.966	0.001	Up	1.552	0.026	Up	-0.373	0.646	NC
miR398_2	2.115	0.000	Up	3.057	0.000	Up	0.991	0.054	NC
miR398	1.439	0.002	Up	2.203	0.000	Up	0.815	0.097	NC
c52663_g1_i2_17575-3p	1.141	0.014	Up	2.087	0.000	Up	0.837	0.087	NC
miR5179	1.792	0.001	Up	1.717	0.003	Up	-0.014	1.000	NC
miR408	1.681	0.001	Up	3.507	0.000	Up	1.890	0.000	UP
c52663_g1_i7_17584-3p	1.232	0.013	Up	1.737	0.000	Up	0.443	0.455	NC
ath-miR779.2-3p	-1.202	0.013	Down	-1.902	0.000	Down	-0.625	0.263	NC
ath-miR2934b-5p	-5.742	0.001	Down	-5.738	0.001	Down	0.000	1.000	NC
miR156r	-1.064	0.030	Down	-1.286	0.009	Down	0.030	1.000	NC
ath-miR859-3p	-6.434	0.000	Down	-2.375	0.006	Down	4.134	0.113	NC
miR2865	-6.318	0.000	Down	-6.324	0.000	Down	0.000	1.000	NC

Table 1. Activation and suppression of miRNAs in the loranthus seeds after cold treatment for 12 h and 36 h.

Cold-Responsive miRNAs in Loranthus Seeds

103 differentially expressed miRNAs that response to cold stress were identified in loranthus seeds (Figure 2a~d). Among them, miR408 was upregulated during the cold treatment, while some differentially expressed miRNAs were specific to the A1 group and likely functioned in the early stages of cold stress response, such as miR390a, miR160b, miR171b, and miR167c. In contrast, miR408 played a continuous role throughout the cold treatment (Figure 2e~h).

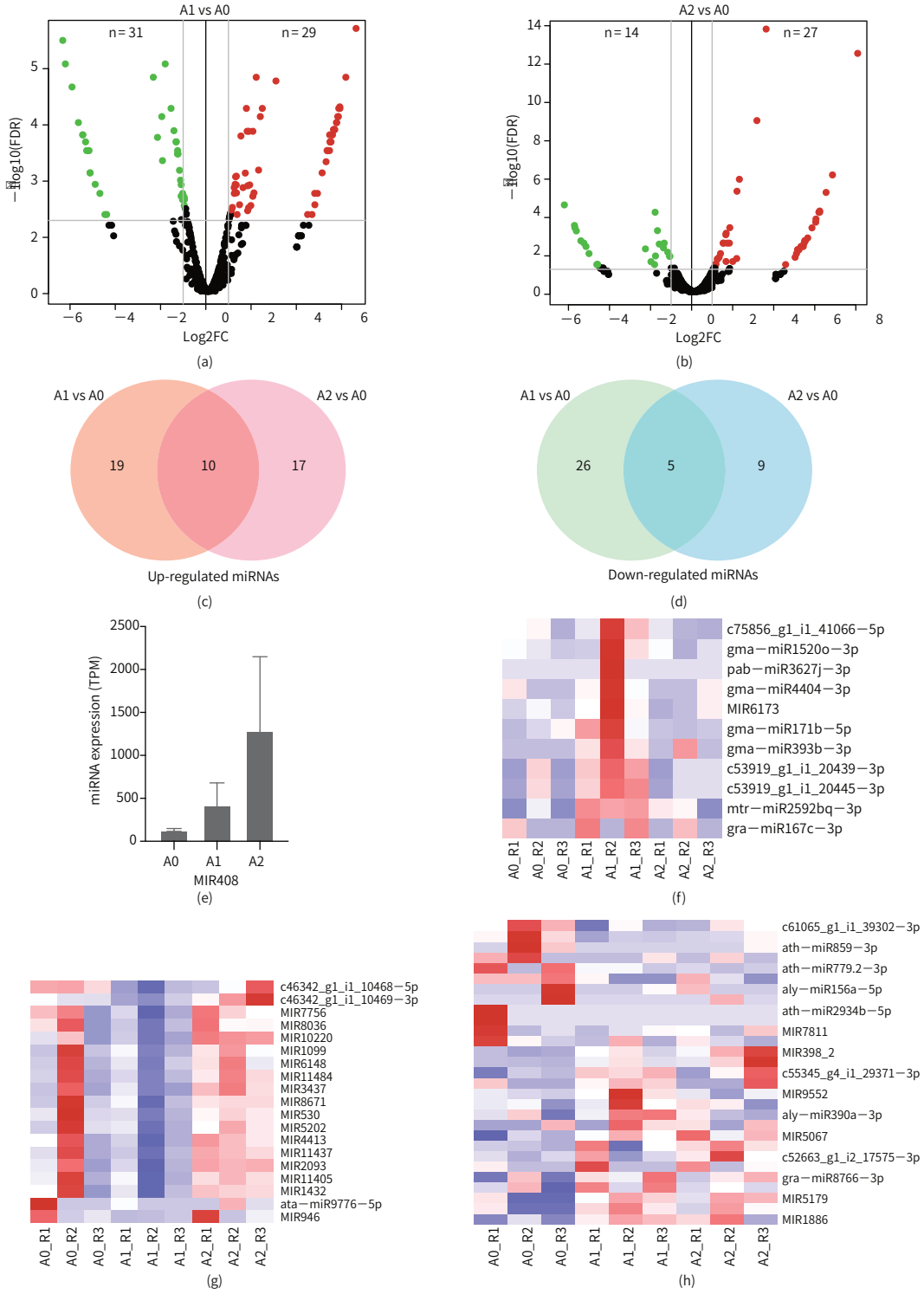

Figure 2. In cold stress situation, the differentially expressed miRNAs was isolated in the loranthus seeds.

Target gene prediction, pathway and regulatory network analysis

Based on the published transcriptome of loranthus seeds, the target genes of differentially expressed miRNAs were predicted using psRobot, TarHunterL, and TargetFinder software. This resulted in 479 miRNAs and 5,610 target genes, along with the regulatory relationships of these target genes under different cold treatment conditions (Table 2). The target gene prediction results suggest that some known cold-responsive genes in the loranthus seeds may be regulated by DEmiRNAs, such as ICE1, UBC, and several transcription factors (such as WRKY and TCP). This indicates that these differentially expressed miRNAs may function by regulating key regulatory genes in the cold stress response. Subsequently, the research team performed KEGG pathway analysis for the target genes of DEmiRs identified in each comparison. For most regulatory pathways, the number of target genes regulated by DEmiRs in A1 compared to A0 and A2 compared to A1 was similar (Figure 3). However, some pathways may be regulated by DEmiRs found only in A2.

Type	Type	Number of miRNAs	Number of targets
Loranthus	ALL	479	5,610
	Total	53	501
A1 vs. A0	Upregulated	24	316
	Downregulated	29	185
	Total	36	216
A2 vs. A0	Upregulated	24	180
	Downregulated	12	39
	Total	55	624
A2 vs. A1	Upregulated	37	331
	Downregulated	19	293

Table 2. Number of targets for differentially expressed miRNAs

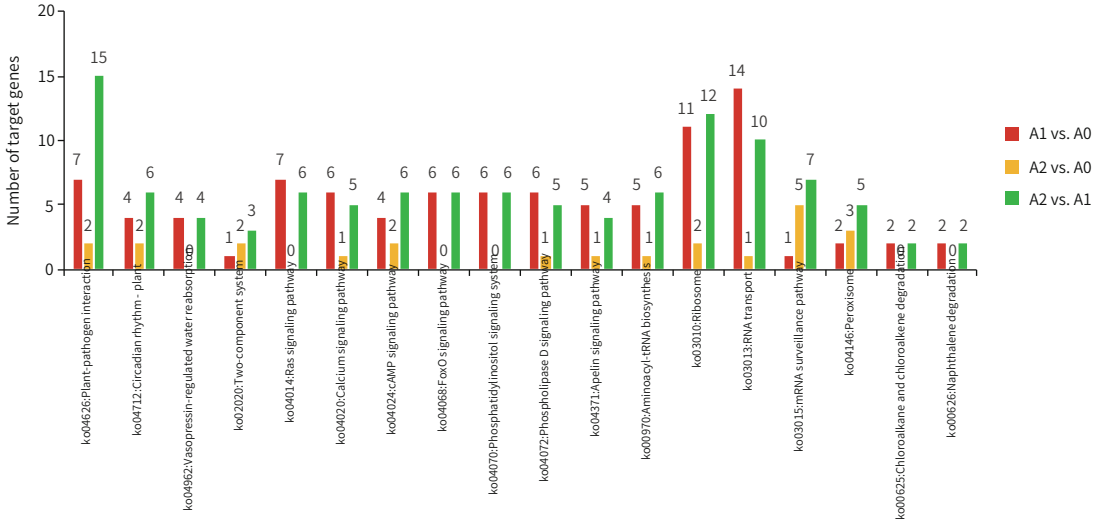

Figure 3. KEGG pathway analysis of DEmiRs

Key miRNAs and the regulatory networks

Through Gene Ontology (GO) and KEGG annotation of the miRNA target genes, functional analysis revealed that four DEmiRNAs (miR4228, miR8036, aly-miR390a-3p, zam-miR164d-5p) were involved in cold stress regulation. Three (gma-miR1520o-3p, c53051_g1_i1_18088-5p, sly-miR10539-3p) were involved in abiotic stimulus response regulation. Some of the other identified DEmiRNAs were involved in regulating processes such as seed embryo development, germination, dormancy process, and mucilage metabolism during seed coat development. KEGG pathway analysis revealed that the target genes of DEmiRs were also involved in pathways such as "Plant-Pathogen Interaction" (Table 3).

Function	Target	Annotation	miRNA	A1_vs._A0	A2_vs._A0	A2_vs._A1
Response to cold	c43520_g1_i1	Soluble inorganic pyrophosphatase 1, chloroplastic-like	miR4228	NC	NC	Up
	c48194_g1_i4	Phospholipase D p1-like	miR8036	Down	NC	Up
	c47711_g1_i2	Thylakoid lumenal 15 kDa protein 1, chloroplastic	aly-miR390a-3p	Up	NC	NC
	c47711_g1_i4	Hypothetical protein GLYMA_11G222100				
	c47711_g1_i3	Hypothetical protein VITISV_013914				
	c47711_g1_i1	Hypothetical protein GLYMA_11G222100				
Response to abiotic stimulus	c51250_g1_i1	Gibberellin receptor GID1B	zma-miR164d-5p	NC	NC	Down
	c29605_g1_i1	Uncharacterized protein LOC100247992	gma-miR1520o-3p	Up	NC	Down
	c34743_g1_i3	RNA pseudouridine synthase 1-like	c53051_g1_i1_18088-5p	NC	Down	Down
	c44975_g1_i2	Uncharacterized protein LOC105644596	sly-miR10539-3p	Up	NC	Down
Embryo development ending in seed dormancy	c46765_g2_i1	ruBisCO large subunit-binding protein subunit alpha	th-miR3434-5p	NC	Up	Up
	c52633_g2_i3	ATP-dependent zinc metalloprotease FtsH	MIR5998	NC	NC	Up
Seed germination Seed dormancy process	c51250_g1_i1	Gibberellin receptor GID1B	zma-miR164d-5p	NC	NC	Down
Mucilage extrusion from seed coat Mucilage metabolic process involved in seed coat development	c49078_g1_i1	Subtilisin-like protease SBT1.7	gma-miR1520o-3p	Up	NC	Down
Seed coat development	c50468_g1_i1	Subtilisin-like protease SBT1.7				

Table 3. Key miRNAs and their targets involved in cold stress response and seed germination

Conclusions

This research is the first study of miRNAs in loranthus plants and has identified 600 miRNAs, including 103 differentially expressed miRNAs, in loranthus seeds under cold stress. These miRNAs were involved in pathways related to "cold response", "abiotic stress response" and "seed development/germination". It provides valuable resources for future studies on loranthus plants. The findings can enhance our understanding of miRNA regulation in plant responses to cold stress, and can also offer insights for better reproductive planning for loranthus plants.

Utilizing the MGIEasy Small RNA library preparation technology and the DNBSEQ sequencing platform from MGI, this study accomplished the sequencing and identification of small RNA in loranthus seeds. This application case demonstrates the capability of relevant small RNA library preparation and sequencing technologies of MGI for fast and accurate miRNA identification.

DNBSEQ-G400 Genetic Sequencer

References

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Recommended Ordering Information

Category	Product	Cat. NO.
Instruments	Genetic Sequencer DNBSEQ-G400RS	900-000170-00
	MGISP-100RS Automated Sample Preparation System	900-000206-00
	MGISP-960RS Automated Sample Preparation System	900-000146-00
Software	MegaBOLT Bioinformatics analysis accelerator	900-000555-00
Library Prep	MGIEasy Small RNA Library Prep Kit（24 RXN)	940-000196-00
Sequencing Reagents	DNBSEQ-G400RS High-throughput Sequencing Set (FCL SE50)	1000016941

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