Profiling the lncRNA-miRNA-mRNA interaction network in the cold-resistant exercise period of grape (Vitis amurensis Rupr.)

Weifeng Ma
1579416408@qq.com
Gansu Agricultural University

Lijuan Ma
690245959@qq.com
Shantou Forestry Research Institute

Zonghuan Ma
1403334741@qq.com
Gansu Agricultural University

Wenfang Li
1304784689@qq.com
Gansu Agricultural University

Shixiong Lu
1697879025@qq.com
Gansu Agricultural University

Huimin Gou
1648885861@qq.com
Gansu Agricultural University

Juan Mao
maojuan@gsau.edu.cn
Gansu Agricultural University

Baihong Chen
bhch@gsau.edu.cn
Gansu Agricultural University

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Author name

Weifeng Ma[1], Lijuan Ma[2], Zonghuan Ma[1], Wenfang Li[1], Shixiong Lu[1], Huimin Gou[1], Juan Mao*[1] and Baihong Chen*[1]

Indication and e-mail address of the corresponding author

Juan Mao:Email: maojuan@gsau.edu.cn

Baihong Chen: Email: bhch@gsau.edu.cn

Abstract

Background Grape is a plant that is sensitive to low temperature and is vulnerable to low temperature damage. However, little is known about the roles of lncRNAs, miRNAs and mRNAs regulate the hypothermia response mechanism in *Vitis amurensis* Rupr.

Methods In this study, the expression and regulatory network of low-temperature response genes were studied in phloem of grape under different low temperature stress.

Results Here, we performed analyses related to RNA-seq and miRNA-seq on grape phloem tissues from five periods of cold resistance campaigns. Three RNA (lncRNAs, miRNAs and mRNAs) obtained by KEGG and GO analyses were used to identify starch and sucrose metabolic pathways associated with cold resistance, and specific changes in BP, CC, and MF were identified in four comparisons. The differentially expressed genes (DEGs) of these pathways were analysed by using Venn diagrams, thermograms and pathway maps respectively, to obtain their specific gene expression during cold exercise. The six DEGs were finally selected,

and they were used for qRT-PCR to verify the RNA-seq data. In addition, we found the regulatory networks of miRNAs and lncRNAs correspond to the six DEGs. This study will contribute to further experimental studies to elucidate the cold resistance mechanism of *Vitis amurensis* Rupr.

Conclusions The low temperature response genes of grape are mainly enriched in the metabolic pathways of starch and sucrose, and regulated by miRNA and lncrna, which will provide basic information for further understanding of the cold resistance mechanism of grape in the future.

Keywords Grape; lncRNA-miRNA-mRNA; Low temperature; Full transcriptome analysis; Starch and sucrose metabolic pathways

Background

Grapes (*Vitis amurensis* Rupr.), one of the most significant commercial fruit crops in the world, are utilized in a variety of food and beverage businesses, including wine production, raisins, juicing, and fresh cuisine [1]. Low temperature is the most important abiotic stress for normal growth and yield of grapes, and affects the area distribution of grapes [2, 3]. As a result, low temperature is a significant environmental issue that limits the ability of grapes to grow and develop and has an impact on grape output and quality. Enhancing ability of grapes to withstand low temperature and making research on how grapes react to low temperature are crucial for revealing the molecular mechanism of grape resistance to low temperature.

As deciduous perennials, vines need to be sheltered from freeze before each year’s freezing period so that the grapes can survive the winter [4]. Changing gene expression patterns is an efficient

and cost-effective strategy to respond to cold stress [5, 6]. Furthermore, numerous cold-responsive genes and the gene products are believed to contribute to cold tolerance at the transcriptional and biochemical levels, as evidenced by earlier research [7-9].

A class of endogenous tiny non-coding RNAs known as microRNAs (miRNAs) controls the expression of genes. [9]. Typically usually 19–24 nucleotides (nt) long, and they originate from stem-loop precursors that the DICER-LIKE 1 (DCL1) enzyme translates from endogenous miR genes [10, 11]. In most eukaryotes, the miRNAs control post-transcriptional gene expression either facilitating the cleavage of target messenger RNAs (mRNAs) or by suppressing the translation of target mRNAs. This regulation is significant for pathogen response, development regulation, and epigenetic modification [12-16]. Single-stranded RNA molecules, which imperfectly form secondary structures resembling hairpins locally, are the source of miRNAs [14, 17-19]. Dicer nuclease breaks down these 21 nucleotide molecules from a lengthy RNA precursor with a base pair reentry structure [20]. Base pairing allows the single-stranded version of miRNA to attach to the target RNA by forming a ribonucleoprotein complex with AGO [21, 22]. miRNAs are important post-transcriptional regulators of gene expression. Throughout their life cycle, plants face several abiotic stressors and hormonal cues, to which they might respond in a sequence-specific way. Numerous miRNAs have been found; for example, *miR156* is important in regulating the expression of its target gene, *SPL (PROMOTER BINDING-LIKE)*, which in turn affects plant growth and development [11].

The first miRNA was discovered to regulate development in *C. elegans* in 1993 and was designated as *Lin-4* [23]. *Ath-miRNA171*,

the first plant miRNA, was discovered in 2002 [24]. Recent research on miRNAs has demonstrated the significant function miRNAs play in fruits such as apple (*Malus domestica*) and grape (*Vitis vinifera*) to response to biotic/abiotic stress [25-27].

Among grapes, 110 miRNAs have been identified [28], including *vvi-miR156a /b/ c* against *Vv-SPL9*, which make function throughout plant growth [11], and *vvimiR061* targets *VvREV* and *VvHOX32*, which play a role in the gibberellin signaling pathway [29]. In addition, miRNAs may regulate certain transcription factors during copper stress, including *AP2*, *SBP*, *NAC*, *MYB* and *ARF* [30].

Although it is well established that long non-coding RNAs (lncRNAs) control a wide range of biological activities, it is unknown how the entire pool of grape lncRNAs interacts with cold stress. The role of plant non-coding RNAs, the major types of long non-coding RNAs (lncRNAs) in particular, have not been thoroughly investigated. lncRNA is defined as a non-coding RNA of more than 200 base pairs (bp) in length [31] and it can be divided into four types based on transcript length, including lncRNA, lincRNA (Long-intergenic noncoding RNA, large intervening noncoding RNA, long-intervening noncoding RNA), vlincRNA (Very long intergenic noncoding RNA), macroRNA and PALR (Promoter-associated long RNA) [32]. According to genome-wide analysis, lncRNAs are widely found in plants, including grape [33], *Arabidopsis* [34], rice [35, 36], maize [37], and cotton[38]. The formation of human cancer cells, abiotic and biological stress responses, plant photomorphogenesis, and numerous other biological processes be impacted by the action of lncRNA [39-41]. A previous study found that as a rival for *YUCCA7*, the lncRNA *TCONS_00021861* was demonstrated to suppress *miR528-3p*-mediated cleavage of *YUCCA7* in rice, thus increasing plant tolerance to drought stress [42]. Cotton *lncRNA973*

overexpression improves salt tolerance in *Arabidopsis* [43]. lncRNA *asHSFB2a* inhibited the expression of *HSFB2a* in *Arabidopsis*, affecting the reaction of plants to heat stress [44]. Similarly, *COLD INDUCED lncRNA 1* (*CIL1*), a novel lncRNA, was found to be a positive regulator of the plant response to cold stress [45]. *LNC_016398-MtCIR1* controls *CBF/DREB1* gene expression in *Medicago truncatula* in response to cold treatment [46]. In grape, lncRNA-mediated regulation of extrachromosomal genes, namely mitochondrial and chloroplast coding sequences, has been observed to be involved in processes such as key biological "photosynthesis" and "oxidative phosphorylation" [33].

Based on full transcriptome data of mRNAs, miRNAs and lncRNAs, we performed four comparisons over five different time periods in this study. The examination of the whole transcriptome data input revealed a high enrichment of mRNAs, miRNAs, and lncRNAs in the metabolic pathways of starch and sucrose. We carried out an investigation of the interaction networks between mRNA, lncRNA, and miRNA that wrere enriched in starch and sucrose metabolic pathways., and some mRNAs were selected for qRT-PCR verification.

Materials and methods

Plant materials and treatments

One-year old grapevine developed from cutting of Chinese wild *Vitis amurensis* was used in this study. Five different growth stages were selected, including growth stage (A stage, 28±2℃, Jul. 9, 2016), earlier low temperature stage (B stage, 5±2℃, Oct. 26, 2016), medium low temperature stage (C, 0±2℃, Nov. 21, 2016), later low temperature stage (D, -5±2℃, Dec. 28, 2016) and deep

dormancy stage (E, -10±2℃, Jan. 9, 2017), respectively. The samples were collected from the experiment nursery of Gansu Agricultural University (103°41′ E, 36°5′ N). The cultivation substrate includes nearly 30% vermiculite, nearly 40% humus and peat mixed in the proportion of 1:1, and nearly 30% perlite. We selected well-developed trees, cut branches from the ground 40 cm place and quickly brought them to the laboratory. With the help of garden shears, we cut 5-8 cm brachyplast from *Vitis amurensis* branch, and after that, we used the scalpel to remove the cortex and collected the phloem. Three samples were collected from each treatment mixed for transcriptome sequencing. After being gathered, the samples were frozen in liquid nitrogen and kept at -80°C.

RNA quantification and qualification

1.5% agarose gels were used to track RNA degradation and contamination, particularly DNA contamination. Thermo Fisher Scientific, Wilmington, DE's Nano Drop 2000 Spectrophotometer was used to quantify the concentration and purity of RNA [47]. With the Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, USA) RNA Nano 6000 Assay Kit, RNA integrity was evaluated [48].

Small RNA library construction

The RNA sample preparation process required a total of 2.5 ng of RNA per sample as input material. Following the manufacturer's instructions, sequencing libraries were created using the NEBNext Ultra small RNA Sample Library Prep Kit for Illumina (NEB, USA). Index codes were then applied to each sample to identify its sequences. In a nutshell, ligating the 3′SR Adaptor is the initial step. After mixing RNA, Nuclease-Free Water, and 3'SR Adaptor, the

mixture was heated to 70°C for two minutes and then put in the ice. Next, 3´ Ligation Enzyme and 3´ Ligation Reaction Buffer (2X) were added to create the combination and the heat cycler was set to 25℃ for an hour in order to attach the 3'SR Adaptor. After the 3' binding procedure, the excess 3'SR adaptors that are still free are hybridized with SR RT primers in order to stop dimer adaptor formation, which then transformed single stranded DNA (ssDNA) adaptors into double-stranded DNA (dsDNA) molecules (dsDNA is not a ligation-mediated substrate). The 5′SR Adaptor must be ligated in the second step. And the first chain was synthesized through reverse transcription. The last step includes PCR amplification and Size Selection. PAGE gel was used for electrophoresis and the fragment were sorted to form a small RNA library. Agilent Bioanalyzer 2100 system [48] was used to evaluate the library quality after PCR products were purified using the AMPure XP system (Wang et al., 2024).

lncRNA and mRNA library construction

The Ribo-Zero rRNA Removal Kit (Epicenter, Madison, WI, USA) was utilized to extract rRNA from the samples using 1.5 µg of RNA per sample. The NEBNextR UltraTM Directional RNA Library Prep Kit for IlluminaR (NEB, USA) was utilized to produce sequencing libraries in accordance with the manufacturer's instructions. Index codes were incorporated to assign sequences to individual samples. Divalent cations were used in NEBNext First Strand Synthesis Reaction Buffer (5X) at a high temperature to carry out the fragmentation process. Random hexamer primer and reverse transcriptase were used to create first strand cDNA. Next, RNase H and DNA Polymerase I were used to synthesise second-strand

cDNA molecules. Through the use of exonuclease and polymerase, the remaining overhangs were transformed into blunt ends. To get ready for hybridization, the 3' ends of DNA fragments were adenylated, and then the NEBNext Adaptor with a hairpin loop structure was ligated. AMPure XP Beads (Beckman Coulter, Beverly, USA) were used to purify the library fragments in order to choose pieces that were ideally 150–200 bp length [49]. Next, selector-ligated cDNA was treated with 3 µl USER Enzyme (NEB, USA) at 37°C for 15 minutes before to PCR. Then, Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index(X) primer were used to carry out PCR. Finally, the AMPure XP system [49] was used to purify the PCR products, and qPCR and the Agilent Bioanalyzer 2100 [48] were used to evaluate the library quality.

Clustering and sequencing

Following the manufacturer's instructions, the index-coded samples were clustered using a cBot Cluster Generation System and the TruSeq PE Cluster Kit v4-cBot-HS (Illumia) [50]. Following cluster creation, paired-end reads were produced and the library preparations were sequenced on an Illumina Hiseq 2500 platform [51].

Sequence analysis results of microRNA: mapping and differential expression

Initially, internal Perl scripts were used to process the raw data (raw readings) in the Fastq format. In this stage, low-quality reads, adapter-containing reads, and ploy-N-containing reads were eliminated from the raw data to provide clean data (clean reads). Next, sequences longer than 30 nt or less than 18 nt were removed from the readings in order to trim and clean them. Concurrently, the clean data's Q20, Q30, GC-content, and sequence duplication

level were determined. The clear, high-quality data served as the foundation for all downstream studies.

By using the Bowtie Tools software [52], Ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small nucleosomal RNA (snoRNA), transfer RNA (tRNA), other non-coding RNAs, and some repeats were filtered using clean reads that were sequenced against the GtRNAdb, Silva, Repbase, and Rfam databases, respectively. By comparing the remaining reads with known miRNAs from miRbase (https://www.mirbase.org/), it was possible to identify the known miRNA and the novel miRNA predicted. The prediction of new miRNA secondary structures was done using Randfold. [53]. For every sample, the levels of miRNA expression were estimated: 1. The precursor sequence was mapped back to the sRNAs.; 2. The mapping findings were used to determine the read count of each miRNA.

Prior to performing the differential gene expression analysis, each sequenced library's two treatments were subjected to a differential expression analysis using IDEG6 for samples lacking biological duplicates. The criterion for significantly differential expression was established at qvalue<0.05 & |log2 (foldchange) |⩾ 2 [54].

Sequence analysis results of lncRNA and mRNA: mapping and differential expression

Initially, internal Perl scripts were used to process the raw data (raw readings) in the Fastq format. In this stage, low-quality reads, adapter-containing reads, and ploy-N-containing reads were eliminated from the raw data to provide clean data (clean reads). Next, sequences longer than 30 nt or less than 18 nt were removed

from the readings in order to trim and clean them. Concurrently, the clean data's Q20, Q30, GC-content, and sequence duplication level were determined. The clear, high-quality data served as the foundation for all downstream studies.

Based on the sequences mapped to the reference genome, the transcriptome was constructed using StringTie [55]. The collected transcripts were annotated using the gffcompare software. To find potential lncRNAs, the unidentified transcripts were screened. The transcriptome was assembled by using the StringTie based on the reads mapped to the reference genome. The gffcompare program was used to annotate the assembled transcripts. The unknown transcripts were used to screen for putative lncRNAs. In order to separate potential protein-coding RNAs from non-protein-coding RNA candidates in the unidentified transcripts, four computational techniques—CPC/CNCI/Pfam/CPAT—were combined. Potential RNAs that code for proteins were eliminated based on a cutoff point for exon number and minimum length. As lncRNA candidates, transcripts longer than 200 nt and including more than two exons were chosen. These transcripts were then screened using CPC/CNCI/Pfam/CPAT, which can differentiate between genes that code for proteins and those that do not. Additionally, the various kinds of lncRNAs—lincRNA, intronic lncRNA, anti-sense lncRNA, and sense lncRNA—were chosen through the application of cuffcompare.

The FPKMs of the coding genes and lncRNAs in each sample were determined using StringTie (1.3.1) [56]. The FPKMs of transcripts in each gene group were added up to calculate the gene FPKMs. Based on the length of the fragment and the number of reads mapped to it, FPKM stands for fragment per kilo-base of exon per million fragments mapped.

Before performing a differential gene expression analysis on samples without biological replicates, the edgeR computer package corrected the read counts for each sequenced library using a single scaling normalization factor. Two samples were subjected to differential expression analysis using the EBseq (2010) R program. The posterior probability of being DE, or PPDE, was used to modify the resulting false discovery rate, or FDR. The criterion for significantly differential expression was chosen at FDR < 0.05 & |log2 (Fold Change) | ⩾ 2.

Functional analysis of DEGs

To determine which DEGs were significantly enriched in GO keywords or metabolic pathways, functional enrichment analysis using KEGG and GO was carried out. Utilizing the Wallenius non-central hyper-geometric distribution based on GO seq R tools, GO enrichment analysis of the DEGs was carried out [57]. Specifically, large-scale molecular datasets produced by genome sequencing and other high-throughput experimental technologies are the main source of information for KEGG, a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism, and the ecosystem (http://www.genome.jp/kegg/) [58]. We tested the statistical enrichment of differential expression genes in KEGG pathways using the KOBAS [59] software.

Quantitative real-time PCR validation of RNA-Seq data.

In order to verify the expression of mRNA grape phloem samples in five stages of A, B, C, D, and E, the RNA returned by the sequencing company was reversely transcribed into cDNA by using TaKaRa (China Da lian) PrimeScriptTMRT Reagent Kit (Perfect

Real Time). The cDNA was then adopted for q-PCR experiments with the help of a Bio-Rad iCycler iQ real-time quantitative PCR instrument and SYBR Primer Ex TaqTM Ⅱ reagent. Sangon Biotech designed and synthesized the primers for qRT-PCR. The first cDNA strand of miRNA was synthesized by tail addition method, and the 3 'primer was synthesized by Accurate Biotechnology (Hunan) Co., Ltd. *VvGAPDH* (NCBI reference sequence ID: XM_0022663109) was used as an internal control gene for mRNA and lncRNA expression standardization. And *VvU6* was used as an internal control gene for miRNA expression standardization. The primers used are shown in the following Supplementary table1.

Statistical analysis

Excel 2010 was used to analyze the transcriptome data and qRT-PCR results. Differences among means were evaluated by the Least-Significant Difference (LSD) with the Statistical Program for Social Science 19 (SPSS, Chicago, IL, USA). Graphs were generated with Origin 9.0.

Results

Illumine sequencing

To reveal the mechanism of the response to low-temperature stress in *Vitis amurensis* Rupr., the whole transcriptome sequencing was performed on phloems of different low-temperature periods and the transcripts were compared. Low-quality data were removed and quality analysis can be found in Supplementary table 2. The total reads of mRNA with lncRNA for the five samples of A, B, C, D and E with different low temperature exercise periods were 127.6, 132.4,

110.7, 108.7, 108.8. And the Total reads of miRNA for the five samples were 5.3, 11.5, 2.7, 9.5, 9.8 million. The Total reads of mRNA with lncRNA were much higher than those of miRNA. The Mapped Reads of mRNA and lncRNA in 5 samples were 56.17% at the lowest and 75.79% at the highest. And the Mapped Reads of miRNA were all above 34%, with the lowest being 34.16% and the highest being 43.24%. Uniq Mapped Reads of each sample mRNA and lncRNA were above 50%, and the highest was sample B, which reached 74.07%. The Multiple Mapped Reads of each sample were low, ranging from 1.34% to 1.80%. In addition, the Reads Map to '+' of each sample was higher than that to '-', the Reads Map to '+' was higher than 20%, and the Reads Map to '-' was higher than 12%. the GC content ranged from 46.44% to 52.55 and the Q30 was above 95% in all samples. It can be seen that the transcripts are of good quality and can be used for subsequent analysis.

Global gene analysis

Gene expression analysis of each comparison group revealed that more than half of the gene expression was up-regulated in each low-temperature treated sample compared to that in the growth period, with C VS A, D VS A and E VS A up-regulating almost three times more genes than down-regulating genes (Figure 1A). In addition, with the increase of low-temperature, the number of differential genes in D period was the highest (5,175), with 3,686 up-regulated genes and 1,498 down-regulated genes. It can be seen that most genes were up-regulated in expression after the low-temperature treatment in response to low temperature stress. Different from mRNA, with the deepening of low temperature stress, the up-regulated miRNA of B VS A was 1.67 times that of down-regulated gene, and the up-regulated genes of C VS A, D VS

A and E VS A were 0.78 times, 0.69 times and 0.975 times that of down-regulated gene, respectively, indicating a decrease in the number of up-regulated miRNAs. The change trend of mRNA gene expression was opposite (Figure 1B). The number of differential lncRNAs decreased sharply in C VS A, and the number of up-regulated lncRNAs was lower than that of down-regulated lncRNAs. After the intensification of low temperature stress, the number of differential lncRNA gradually increased, and the number of up-regulated lncRNA was higher than that of down-regulated lncRNA in D VS A and E VS A, respectively (Figure 1C).

The assembled data were analyzed against major databases by using BLAST software to obtain annotation information for all genes. A total of 20,260 genes were annotated in the given protein database search. The NR database had the most annotated genes (20,258), the GO and Swiss-Prot databases also had more than 10,000 annotated genes (17,243 and 15,186, respectively), while the KEGG database had the least annotated genes (6,062) (Figure 1D). Similar to mRNA, miRNA and lncRNA had the most annotations in the RN database, with 7,567 and 15,066, respectively. The numbers of their genes annotated in GO database ranked the second, 7,267 and 13,397, respectively, while the numbers of their genes annotated in KEGG database were the least, 2,897 and 5,516, respectively (Figure 1E, F).

Differential gene enrichment analysis

Different databases were used to compare and comment the enhanced DEGs. Alpha-Linolenic acid metabolism, flavonoid biosynthesis, circadian rhythm plant, starch and sucrose metabolism, and alpha-Linolenic acid metabolism were the primary areas in which DEGs were abundant in the KEGG database (Figure

2A). Proteins inferred from the identified RNA sequences matched DEGs identified in the KOG database, grouped into 24 functional classes, mostly annotated in the areas of chaperones, protein turnover, signal transduction processes, carbohydrate transport and metabolism, posttranslational modification, and the formation, transport, and catabolism of secondary metabolites (Figure 2B). The DEGs were classified into 55 functional groups by the GO database, which were divided into 3 subclasses, including: biological processes, cell components, and molecular functions (Figure 2C). In the COG database, DEGs were annotated and grouped into 23 categories, of which Cell motility was the least annotated (Figure 2D).

Venn analysis of B_VS_A, C_VS_A, D_VS_A and E_VS_A revealed that 2,378 genes co-occured in the four treatment groups, showing that all 2,378 genes were differentially expressed with increasing levels of stress. Subsequent analyses will be carried out on these 2,378 genes (Figure 2E).

GO enrichment analysis was performed on 2,378 genes in B_VS_A, C_VS_A, D_VS_A and E_VS_A. It was found that Biological process, cellular component and molecular function contained 19, 14 and 14 functional groups, respectively (Figure 3A). In addition, the frequency of up-regulated genes in biological processes, cell components and molecular functions reached 17,930, the frequency of down-regulated genes was 5,650, and the frequency of up-regulated genes was three times that of down-regulated genes. Further analysis showed that GO:0016530 and GO:0045735 were both up-regulated genes in molecular function.

Meanwhile, KEGG enrichment of 2,378 common DEGs showed that the DEGs were mainly in Pentose and glucuronate interconversions, alpha-Linolenic acid metabolism, Flavone and

flavonol biosynthesis, Starch and sucrose metabolism pathway, Flavonoid biosynthesis and Circadian rhythm-plant (Figure 3B). After that, the target genes of differentially expressed miRNAs and lncRNAs were analyzed by KEGG classification (Figure 4A, B). And it was found that their target genes were classified into five categories: Metabolism and Organismal Systems, Genetic Information Processing, Environmental Information Processing, and Cellular Processes. The distinction is that in the context of environmental information processing, ABC transporters, phosphatidylinositol signaling system, and plant hormone signal transduction were the target genes of miRNAs. Conversely, the annotation of lncRNA target genes was limited to the Plant hormone signal transduction pathway. In Genetic Information Processing, miRNAs were not annotated to the SNARE interactions in vesicular transport pathway, and lncRNAs are not annotated to the Aminoacyl-tRNA biosynthesis pathway. In Metabolism, the target gene annotations of miRNAs and lncRNAs varied greatly, but both of them were annotated in the Carbon metabolism and Starch and sucrose metabolism pathways. Therefore, subsequent analyses focus on the starch and sucrose metabolic pathways.

mRNA, miRNA and lncRNA length analysis

As shown in Figure 5A, the length of mRNAs was above 200nt, with the number of mRNAs of 200 nt in length being 1,358, which is less than that of mRNAs of 3,000 nt in length; the number of mRNAs of 400 nt in length was the highest, with 103,766, and among those with the length of over 400 nt, the longer, the fewer; the total number of mRNAs that are over 3,000 nt was 26,315, which is lower than the number of lengths of mRNAs of 600 nt in length. The length of lncRNA was over 400 nt, and the number of lncRNA

fragments that are 400 nt long was the highest, which was 4,669. As the length of lncRNA fragments increased, the number of lncRNAs longer than 400 nt decreased; there are 129 lncRNAs totaling 3,200 nt in length (Figure 5B).

The lengths of the miRNAs varied from 18 to 24 nt, with 104 with the longest length of 21 nt being the most common. And there were 75 miRNAs with a 24 nt length, making it the second most common kind. There are the fewest amount of miRNAs, with 4 with lengths of 18 and 19 nt (Figure 5C).

Correlation analysis of mRNA with miRNA and lncRNA based on FPKM value

Correlation analysis was performed on the FPKM values with 41 mRNAs, 5 miRNAs and 17 lncRNAs. As shown in Figure 6, *unconservative_4_28837*, *unconservative_4_28838*, *unconservative_13_34980*, and *unconservative_13_34981* were positively correlated with 29 mRNAs with similar correlation patterns. While *vvi_miR3624_5p* was negatively correlated with 33 mRNAs. In addition, *MSTRG.108081.3*, *MS TRG.152515.1*, *MSTRG.10557.2*, *MSTRG.19181.1*, *MSTRG.19130.1*, *MSTRG.19148.5*, and *MSTRG.20793.5* were positively correlated with most of the genes in the sucrose and starch metabolic pathways.

Analysis of mRNA interactions with miRNA and lncRNA

To explore the expression of mRNAs in the starch and sucrose metabolic pathways and the interactions network relationship between mRNA and miRNAs and lncRNAs, we drew a heatmap of miRNA expression based on the FPKM values and visualized the interaction network among mRNA, miRNA and lncRNA.

In Figure 7A, heatmaps were drawn for the FPKM values of 41 genes enriched in the starch and sucrose metabolism pathways. *TPP* (Trehalose 6-phosphate phosphatase), *CWINV* (Cell wall invertase), and *TPS* (alpha,alpha-trehalose-phosphate synthase) were up-regulated in D and down-regulated in B, C, and E, *INVA* (Invertase) was up-regulated in E and *4-α-GT* (4-alpha-glucanotransferase) is upregulated in C, D, and E, compared with that in A.

To clarify the interactions of mRNAs, miRNAs and lncRNAs, the interaction network was visualized by Cytoscape (Figure 7B, Supplementary table 3). *VIT_17s0053g00700* was found to be regulated by two miRNAs, *unconservative_13_34981* and *unconservative_13_34980*. The expression of *TPP* and *4-α-GT* were regulated by *unconservative_4_28837* and *unconservative_4_28838*, and the expression of *4-α-GT* was also regulated by *vvi-miR3624-5p*. In addition, the expression of *VIT_02s0154g00090* was regulated by three lncRNA, which were *MSTRG.108081.6*, *MSTRG.108081.3* and *MSTRG.108081.1*, And the expression of *VIT_11s0016g03020* was regulated by *MSTRG.19181.1*, *MSTRG.19130.1* and *MSTRG.19148.5*. The expression of *VIT_07s0005g01030* was regulated by *MSTRG.152678.1* and *MSTRG.152676.1*, the expression of *BG* (beta-glucosidase) was regulated by *MSTRG.10557.2* and *MSTRG.10557.1*, and the expression of *VIT 03s0063g01510* was regulated by *MSRG.11516.1000101016*. After that, based on the interaction genes that regulate low-temperature stress and have different correlation types with miRNA and lncRNA, 5 mRNA interacting with 3 miRNA, 2 miRNA, 2 lncRNA and 1 lncRNA and 1 mRNA without interaction with miRNA and lncRNA were selected for in-depth analysis (Figure 7A). Meanwhile, miRNAs

(*unconservative_4_28838*, *vvi-miR3624-5p*, *unconservative_13_34980*) and lncRNAs (*MSTRG.115204.7*, *MSTRG.115190.2*, *MSTRG.171251.2*, *MSTRG.10557.1*, *MSTRG.10557.2*) that interacted with candidate mRNAs and had inconsistent expression patterns were selected for further analysis (Figure 7C and D).

Transcriptome data qRT-PCR validation

In order to verify the results of RNA-seq, we performed qRT-PCR verification, and selected 6 differentially expressed mRNA in the starch and sucrose metabolism pathways. Additionally, five lncRNAs and three miRNAs that interact with the six genes mentioned above but have different expression patterns were chosen for qRT-PCR. For every gene, three biological replicates were carried out, and the relationship between the RNA-seq and qRT-PCR data was examined. The results are shown in Figure 8 and 9.

Both *BG* and *vvi-miR3624-5p* showed differences in transcriptome sequence data at stage A, *unconservative_4_28837* showed differences in transcriptome sequencing data at stage E, and *MSTRG.10557.2* showed differences in transcriptome sequencing data at stage B. The remaining genes showed consistent expression trends in RNA-seq and RT-qPCR at all stages, indicating there was consistency between transcript abundance determined by RNA-Seq and RT-qPCR data.

Discussion

From the sequencing quality of the whole transcriptome data (as shown in Supplementary table 2), the sequencing quality is better and can be used for later data analysis. Among all types of RNA

distributions, mRNA was the largest, followed by lncRNA and miRNA was the smallest. From the overall distribution of three expression levels, it is concluded that the five samples have a high degree of coincidence, and the peaks are basically the same. We identified the length distributions of lncRNAs, mRNAs and miRNAs, and found that the length of lncRNA and mRNA is over 200 nt. As for length is mainly concentrated at 21nt, followed by 24nt. Due to the specificity of Dicer and DCL enzymes, the length of mature miRNAs is mainly concentrated in the range of 20 nt to 24 nt. Among animals, the length of miRNA is mainly 22 nt, while the length of 21 nt or 24 nt is dominated by plant miRNA [60].

By effectively carrying out expansion analysis on a specific pathway with the help of KEGG enrichment analysis, *DEGs* can be identified throughout the pathway, and upstream and downstream relationship nodes can be obtained [61]. Numerous DEGs were considerably enriched in the production of flavones and flavonols, as well as in the metabolism of starch and sucrose, according to KEGG expression analysis. Plants with a circadian rhythm and flavonoid production were identified in four pathways. Both miRNAs and lncRNAs have target genes that are abundant in the starch and sucrose metabolic pathways, according to the KEGG classification of those genes. It can be seen that genes related to starch and sucrose metabolism pathway significantly respond to low temperature stimulation under low temperature exercise. GO Database established by GO Organization (Gene Ontology Consortium) is a structured standard biological annotation system, built in 2000. The purpose is to establish a standard vocabulary system of product knowledge and its genes, which is applicable in various species [62]. In this paper, GO analysis of DEGs showed that a large amount of DEGs was enriched in the molecular functional

module, and a small amount of DEGs was distributed in the cellular component module. The results showed that the molecular function played a significant role under low temperature stimulation.

The primary glucose-related pathways found in KEGG are those linked to the metabolism of starch and sucrose, fructose and mannose, galactose, glycosaminoglycan degradation of pentose and glucuronic acid interconversion, and glycans. Although the target gene annotation pathways of lncRNA and miRNA were different, they were both annotated in the starch and sucrose metabolic pathways. Coincidentally, mRNA is also significantly enriched in the starch and sucrose metabolic pathways.

The starch and sucrose metabolic pathways were selected, and the differentially expressed mRNAs were analysed by heatmap, and the interactions between mRNAs, miRNAs and lncRNAs were mapped. Six differentially expressed mRNAs in the pathway that were positively regulated by miRNAs and lncRNAs were used for further analysis. There were only seven miRNAs that regulate mRNAs, most of which were unknown miRNAs, and the only known miRNA was *vvi-miR3624-5p*. Previous studies have found that *vvi-miR3624* can be induced by cold stress, and its expression tends to increase when treated at low temperatures [63]. The study confirmed that *Metal Ion Binding Protein* mRNA is the target of *miR3624-3p* [25, 63]. Nevertheless, the lack of reports in other species suggests that miR3624 is unique to Vitis. Moreover, the regulatory link between miRNA and mRNA is evident: several miRNAs can control a single mRNA, and one miRNA can control several mRNAs. Then, from the correlation analysis of 42 key miRNAs to lncRNA, and mRNA, it can be seen that miRNA is closely related to lncRNA, and mRNA. The evidence suggests that non-coding RNA, especially miRNA, is a key regulator of cold stress

in plants [64, 65].

Certain lncRNAs function as decoys, mimicking the target DNA or RNA, to control proteins or microRNAs (miRNAs). For example, *Arabidopsis* microRNA targets mimic *IPS1* lncRNA and bait *ASCO* lncRNA [66, 67]. This demonstrates the competitive endogenous RNA (ceRNA) idea, which has gained widespread acceptance and substantial support [68, 69]. Given that the quantity of each individual miRNA is restricted, the ceRNA theory postulates that mRNA, lncRNA, pseudogenes, and other miRNA sponges share a similar miRNA binding site [69].

Conclusion

We identified the mRNA and target genes of miRNA and lncRNA were significantly enriched in sucrose and starch metabolic pathways. 6 mRNAs of sucrose and starch metabolic pathways were regulated by 7 miRNAs and 17 lncRNAs. Analysis showed that qRT-PCR results of most genes were consistent with the sequencing results. These results indicated that grape low-temperature-responsive genes were mainly enriched in starch and sucrose metabolic pathways and were regulated by miRNAs and lncRNAs (Figure 10), which will provide basic information for further understanding of the cold-resistance mechanism in grape in the future.

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Author contributions

BH C, J M, WF M and LJ M contributed to the conception of the study; WF M and SX L performed the experiment; WF L and HM G

contributed to analysis and collection data; WF M performed the data analyses and wrote the manuscript; YM L, ZH M, WF L, and HM G contributed to modify paper grammar; BH C and JM helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/, reference number [PRJNA1027130].

Declarations

Ethics approval and consent to participate

This manuscript is an original paper and has not been published in other
journals. The authors agreed to keep the copyright rule.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] College of Horticulture, Gansu Agricultural University, Lanzhou, Gansu Provice 730070, China

[2] Shantou Forestry Research Institute, Shantou , Guangdong Provice 515041, China

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Figure legends

Figure 1 The number of DEGs was compared with the annotation library. Number of mRNAs, miRNAs and lncRNAs up- and down-regulated at different low-temperature comparisons, (A)mRNA, (B) miRNA, (C) lncRNA. Basic Local Alignment Search Tool (BLAST) was used to compare nucleic acid sequence to protein sequence library (BLASTx) against specific platforms, (D)mRNA, (E) miRNA, (F) lncRNA.

Figure 2 Annotation of differentially expressed genes (DEGs) in different databases and differential venn plots. (A) KEGG database enrichment plot. (B) KOG database enrichment plot. (C) GO database enrichment plot. (D) COG database enrichment plot. (D) Venn diagram.

Figure 3 Annotation of differential DEGs in different databases. (A) GO database enrichment diagram. (B) KEGG database enrichment map.

Figure 4 miRNA and lncRNA target gene KEGG annotation. (A) miRNA target gene KEGG annotation. (B) lncRNA target gene KEGG annotation.

Figure 5 The lengths of the three RNAs. (A) mRNA length. (B) lncRNA length. (C) miRNA length.

Figure 6 Correlation analysis of mRNA, miRNA and lncRNA based on FPKM values.

Figure 7 Heat map of expression of starch and sucrose metabolic pathway genes and regulatory network of related genes with miRNAs and lncRNAs. (A, C, D) Differential expression levels are based on the fragments per kilobase of transcript per million fragments (FPKM) values. The FPKM values of genes were transformed by log2. (B) Network diagram of mRNA interactions with miRNA and lncRNA.

Figure 8 qRT-PCR verifies transcriptome data of mRNA.

Figure 9 qRT-PCR verifies transcriptome data of miRNA and lncRNA.

Figure 10 Hypothetical model of mRNAs, miRNAs and lncRNAs regulating low-temperature response through starch and sucrose metabolic pathways in '*Vitis amurensis* Rupr.'.

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

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