Phyton-International Journal of Experimental Botany
DOI:10.32604/phyton.2020.010322
Article

High-Throughput MicroRNA and mRNA Sequencing Reveals that MicroRNAs May Be Involved in Pectinesterase-Mediated Cold Resistance in Potato

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Received: 27 February 2020; Accepted: 28 April 2020

Abstract: Since potato cultivars are sensitive to low temperature, cold injury severely affects the geographical distribution and yield of potato. Although some miRNAs have been identified in response to cold stress in plants, there is no report about the role of miRNAs in the response to cold stress in potato. Here, via high throughput sequencing, we described the profiling of cold stress response to miRNA and mRNA in potato. Two small RNA and six mRNA libraries were constructed and sequenced. 296 known and 211 novel miRNAs were identified, in which 34 miRNAs in Cold Group (CG) had the higher expression quantity than which in Normal Group (NG) and 32 in CG had lower expression quantity than which in NG. 3068 differentially expressed genes were detected between NG and CG, in which 1400 genes were up-regulated and 1668 genes were down-regulated. The metabolism pathway of starch and sucrose (ko00500) is the common KEGG pathway in differentially expressed miRNA and mRNA. In this pathway, StuPME21575 and StuPME42971 are pectinesterase which mainly catalyzes the pectin-forming pectate, which are controlled by stu-miR6023 and stu-novel-miR42365. As the potato suffering cold stress, these two miRNAs expression levels became higher, but their target genes expression levels were just opposite and this result is the same with qRT-PCR.

Keywords: Potato; microRNA; RNA-Seq; low-temperature stress; pectinesterase

Abbreviations

PME	Pectin methyl esterase;
MFE	Minimum hairpin folding free energy;
DEGs	Differentially expressed genes;
GO	Gene ontology;
KEGG	Kyoto encyclopedia of genes and genomes.

1 Introduction

Potato (*Solanum tuberosum* L.) is the fourth food crop worldwide, which has a cultivation history of more than 7,000 years. Potato cultivars are sensitive to frost, low-temperature is one of the important factors limiting the geographical distribution and yield expansion [1,2]. Researches have shown that, potato seedlings will stop growing while the temperature is lower than 7°C, chilling damage at −0.8°C, frost damage at −2°C, death at −3°C [3]. Low-temperature will damage the pectin [4], ABA [5] and other physiological metabolism of potato.

MicroRNA (miRNA) is a class of about 21–25 nucleotides in length, non-coding endogenous single stranded small molecule RNA [6], through post-transcriptional inhibition or degradation of target genes regulating gene expression. It plays an important role in plant stress especially in cold regulation [7–9]. In rice, overexpression of miRNA319 could impact leaf morphogenesis and lead to enhanced cold tolerance [10], but overexpressed the OsmiR156k will lead to reduced tolerance to cold stress [11]. In *medicago sativa*, application of exogenous melatonin could down-regulate the expression of miR159-5p and novel-m0048-3p, this results in the up-regulation of their target genes involved in signal transduction and enhances cold stress resistance [12].

At the same time, a lot of studies have been carried out in *solanaceae* plants, such as Koc et al. found that in tomato cultivars, miR167, miR172 and miR393 were activated in the early time points of cold treatment [13]. With low temperature stress, there are 56 down-regulated and 28 up-regulated miRNAs corresponding to 220 up-regulated and 94 down-regulated mRNAs, respectively in *S. aculeatissimum* [14]. In transgenic tobacco, Sp-miR396a-5p plays critical roles in cold stresses through targeting *NtGRF7*-regulated expression of osmotic stress-responsive genes and pathogen infection via the regulatory networks of *NtGRF1* and *NtGRF3* [15].

Pectinesterase (PME) is the first key enzyme to catalyze pectin esterification [16]. It plays an important role in plant resistance to low-temperature stress. Such as, inhibition of PME can significantly increase the ability of wheat to resist low temperature stress [17]. However, few studies focus on the potato PME, especially how the potatoes use miRNA to regulate PME expression, thereby improving the ability of potatoes to resist low-temperature stress.

Therefore, in order to clarify the mechanism of cold resistance of potato, which is regulated by PME expression by using miRNAs, we used the transcriptome and small RNA sequencing to potato samples which were treated with low-temperature, and analyzed the relationship between mRNA and miRNA, which will help to clarify the responses of miRNAs and their target genes in potato at low temperature and the miRNA-regulated mechanism of cold response in potato plants.

2 Materials and Method

2.1 Materials

The plant materials used in the present research was *Solanum tuberosum* L. cv. Favorita, which was obtained from Anhui Academy of Agriculture Sciences. The potato tubers were placed into a controlled environment of 22 ± 2°C under 16 h light/8 h dark photoperiod (normal condition) for 35 days. The growth potential of the same plants was homogenized for 7 days in a 20°C degree artificial climate incubator. Environmental processing and other environmental parameters remained the same. The test materials were divided into control group and treatment group. The control group was that the plants grow at normal temperatures (20°C) for 4 hours. The treatment group was that the plants grow at low temperature environment (0°C) for 4 hours. The third fully expanded leaf was collected after treatment, immediately frozen in liquid nitrogen and stored at −80°C. RNA samples were extracted from potato leaf and stored at −80°C. All of RNA samples were subjected to transcriptome and small RNA sequencing by high throughput sequencing (Biomarker Technologies, Beijing, China). The samples also used for qRT-PCR analysis.

2.2 Method

2.2.1 RNA Isolation and Library Preparation

Total RNA was isolated from the above samples using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit®2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The RNA samples were sent to Biomarker (Beijing, China) to generate libraries for small RNA and mRNA sequencing. The small RNA and mRNA were reverse transcribed to cDNA, and then sequenced with HiSeq 2500 and Hiseq x-ten platform (Illumina, San Diego, CA, USA), respectively.

2.2.2 Identification and Differential Expression Analysis of Known and Novel miRNAs

Use Bowtie tools soft, the Clean Reads respectively with Potato database, GtRNAdb database, Rfam database and Repbase database sequence alignment, rRNA, tRNA, snRNA and other ncRNA were removed. The remaining reads were used to detect known miRNA by comparing with known miRNAs from miRBase 21. To identify potential novel miRNAs in potato, unmapped small RNA sequences were searched by BLAST against potato genome (PGSC_DM_v4.03). Randfold tools soft was used new miRNA secondary structure prediction. Differential expression analysis of two conditions was performed using the DESeq R package (1.10.1). The resulting p values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. miRNA with an adjusted $p < 0.05$ found by DESeq were assigned as differentially expressed.

Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of two samples was performed using the IDEG6. p-value was adjusted using q-value [18]. Q-value < 0.005 & |log2 (fold change)| ≥ 1 was set as the threshold for significantly differential expression.

2.2.3 Identification of Differentially Expressed Genes

Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality. The adaptor sequences and low-quality sequence reads were removed from the data sets. Raw sequences were transformed into clean reads after data processing. These clean reads were then mapped to the potato genome sequence. Only reads with a perfect match or one mismatch were further analyzed and annotated based on the reference genome. Tophat2 tools soft were used to map with reference genome. Quantification of gene expression levels were estimated by fragments per kilo base of transcript per million fragments mapped.

After calculating the fold change, differential expression analysis of two conditions/groups was performed using the DESeq R package (1.10.1). Genes with FDR-adjusted q-value ≤ 0.05 and |log2 (fold-change) | ≥ 1 were identified as differentially expressed genes.

2.2.4 miRNA Target Prediction

TargetFinder software (http://targetfinder.org/) was employed to predict the target genes for all the conserved and novel miRNA that expressed differentially in two samples under low temperature stress. The parameters in prediction were set as default from the webserver [19].

2.2.5 GO Enrichment, KEGG Pathway and Network Analysis

Gene Ontology (GO) enrichment analysis of the DEGs was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution [20], which can adjust for gene length bias in DEGs. Pathway analysis was based on the KEGG database. We used KOBAS software to test the statistical enrichment of differential expression genes in KEGG pathways [21]. Network analysis was conducted using the Cytoscape network platform (http://www.cytoscape.org/) [22].

2.2.6 Validation of miRNA and Gene Expression by qRT-PCR

To examine the expression of miRNAs in potato leaf which exposed to control and treatment were used. Expression of miRNAs was detected by Poly (T) RT-PCR. To produce miRNA fused Poly (T) cDNA, 0.5 mg total RNA was used for the reverse transcription with miRNA mature sequence specific Poly (A) RT primers according to the Poly (T) RT-PCR protocol. All the primers were listed in the Appendix A. The ABIStepOne PULS instrument was used for qRT-PCR experiments on cDNA samples from different samples. Experiments were repeated three times. Relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method [23]. Reactions contained the following: 10 µl of 2x*TransStar*® SybrGreen qPCR Master Mix, 2 µl of template cDNA, 0.4 µl of forward and Universal miRNA qPCR Primer, 0.4 µl of Passive Reference Dye (50x) and water to 20 µl. PCR amplification was carried out as follows: 50°C for 2 min, 95°C for 3 min, followed by 45 cycles of 95°C for 5 s, 60°C for 30 s.

2.2.7 Extraction and qRT-PCR Analysis for Target genes

In order to examine the expression of target genes in potato leaf, total RNAs of the samples were extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The DNase-treated RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). Primers for real-time fluorescent quantitative PCR (qRT-PCR) were designed using Primer Express 3.0 software (Appendix A), and the ef1-α gene was used as an internal reference with primers synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The ABIStepOne PULS instrument was used for qRT-PCR experiments on cDNA samples from two samples. Experiments were repeated three times. Relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method [23]. Reactions contained the following: 10 µl of 2xTransStar ® SybrGreen qPCR Master Mix, 2 µl of template cDNA, 0.4 µl of forward and Universal miRNA qPCR Primer, 0.4 µl of Passive Reference Dye (50x) and water to 20 µl. PCR amplification was carried out as follows: 50°C for 2 min, 95°C for 3 min, followed by 45 cycles of 95°C for 5 s, 60°C for 30 s.

3 Results

3.1 Analysis of MicroRNA Response to Low Temperature Treatment Based on High-Throughput Sequencing

To examine whether miRNAs are involved in low-temperature response in potato, we performed high throughput sequencing analysis of miRNA in potato leaves treated with cold stress for 0 h (Normal Group, NG) and 4 h (Cold Group, CG). A total of 28795169 (NG) and 16134299 (CG) raw reads were obtained. After the removal of low quality, containing 'N' reads, Length < 18 and Length > 30, a total of 24497210 and 15102752 clean reads were obtained. And then we obtained 17457940 and 12552035 unannotated sRNA sequences for NG and CG by removing rRNAs, tRNAs, snRNAs, and snoRNAs respectively. By aligning unannotated sRNA sequences to reference genome, 7590356 and 4269663 reads were mapped (Fig. 1). To identify miRNAs in potato, all sRNA sequences were compared to known plant miRNAs in miRBase Release 21. 266 and 259 known miRNAs with high sequence similarity to known plant miRNAs were identified respectively. By mapping all unique sRNA sequences to the potato genome and predicting the hairpin structures for their flanking sequences, 211 and 209 novel miRNAs candidates were identified (Tab. 1). The majority of reads were in the range of 20–24 nt, among which the most abundant sequences were 21 nt long in all libraries (Fig. 2). It can be predicted that the pre-

Table 1: Distribution of counts of sequ-seqs type in 2 libraries

Type	NG		CG	
Count	Count	Percent (%)	Count	Percent (%)
Total reads	28795169		16134299	
Clean reads	24497210	85.07%	15102752	93.61%
Containing 'N' reads	421	0.0015%	258	0.0015%
Smaller than 18nt	851895	2.96%	285793	1.77%
Bigger than 30nt	3445643	11.97%	745496	4.62%
>Q30	24220391	98.87%	14938132	98.91%
unannotated sRNA	17457940	71.26%	12552035	83.1%
Mapped_Reads	7590356		4269663	
Known miRNA	266		259	
Novel miRNA	211		209	

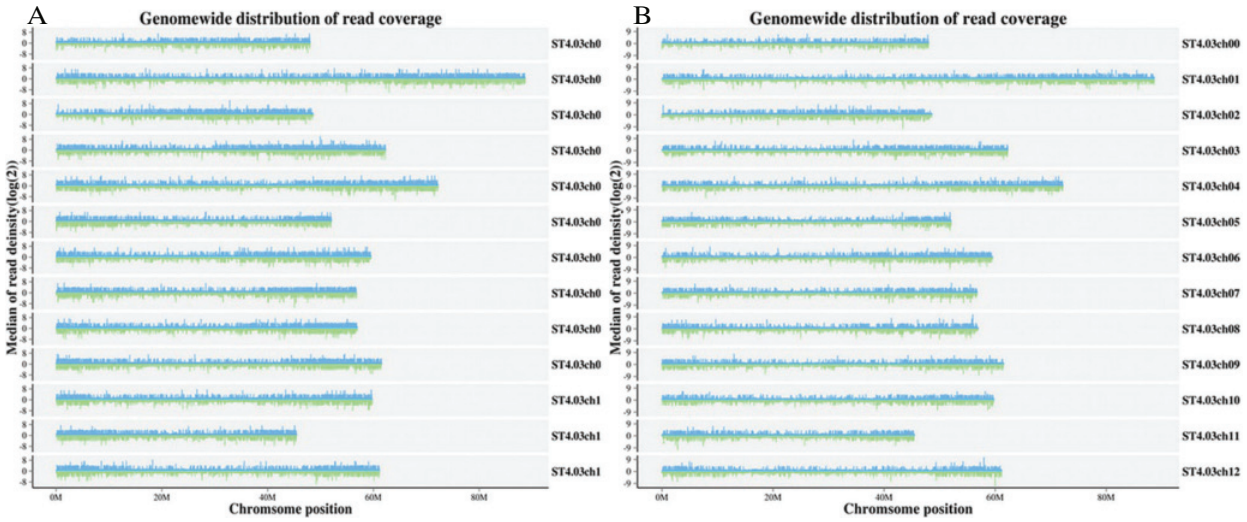

Figure 1: Distribution of sequence reads mapped to potato genome

Note: Abscissa is the chromosomal location; Ordinate is the logarithm of the base 2 with the depth of coverage. Sense, Antisense. A Represents the location and depth of coverage of NG on the potato genome; B Represents the location and depth of coverage of CG on the potato genome.

miRNA sequences minimum hairpin folding free energy (MFE) has the range of −137.1 kcal/moL ~ −18 kcal/mol and scores are in the range of 0.3 and 136012.6 which the maxium is 136012.6. All the information indicated was in high reliability prediction of novel microRNA. The predictive novel miRNA precursor sequence can form a good stem-loop structure, which also shows the high reliability prediction of novel microRNA. The candidate miRNAs stem-loop structures were shown in Fig. 3.

The base groups in different sites have a certain preference in miRNA sequences, we are able to get all miRNA base distribution statistics. We are able to get all microRNA base distribution statistics by the distribution and identification analysis of the different lengths microRNA first point base. From Fig. 4, most first base of known miRNA and novel miRNA are A and U, and known miRNA has relatively most

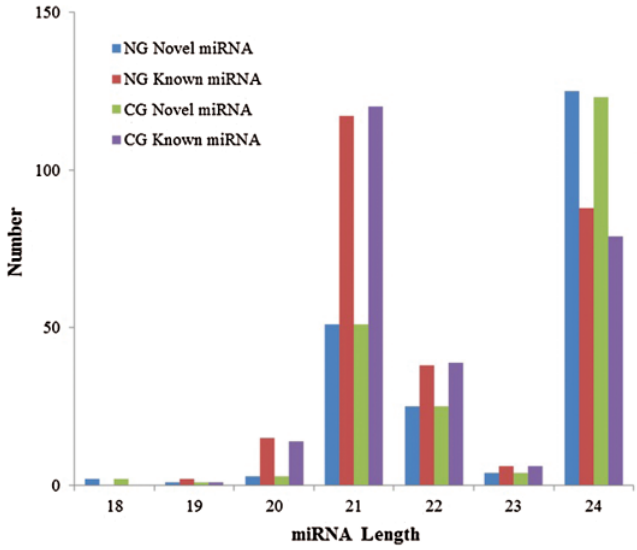

Figure 2: The length distribution of miRNAs

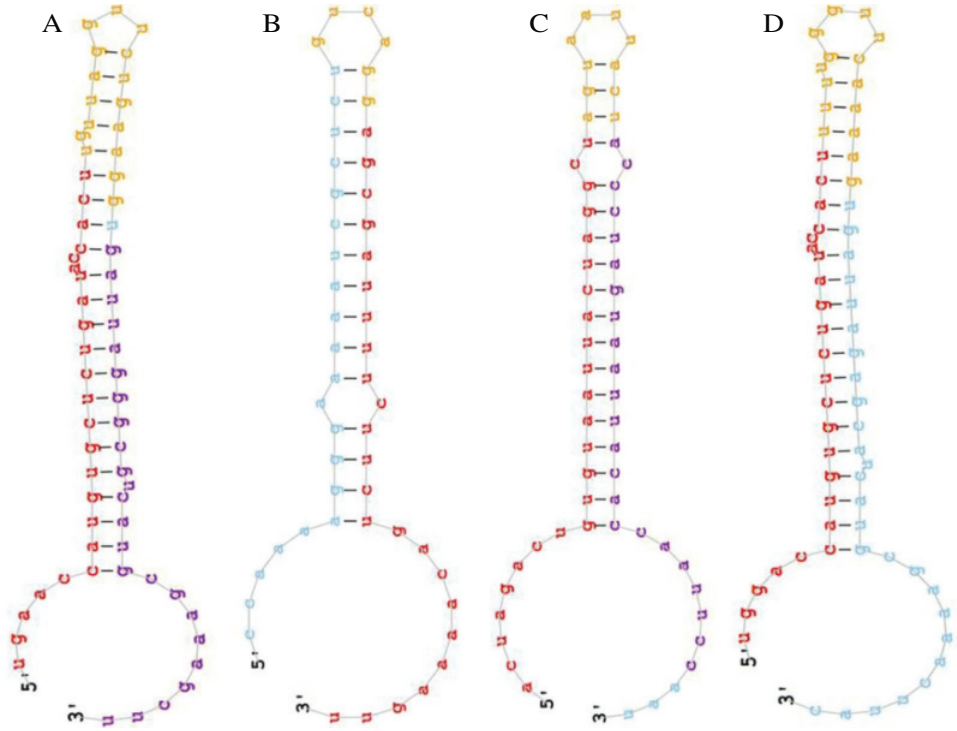

Figure 3: The stem loop structure of the candidate novel miRNAs

Note: A is the stem loop structure of unconservative_ST4.03ch04_17265; B is the stem loop structure of unconservative_ST4.03ch05_18175; C is the stem loop structure of unconservative_ST4.03ch05_20491; D is the stem loop structure of unconservative_ST4.03ch10_38333;

The hairpin structure of miRNA is composed of Mature, Star and arm sequence. Purple and blue sequences represent the star sequence of the hairpin structure; Red sequences represent the mature sequence of the hairpin structure; Yellow sequences represent the arm sequence of the hairpin structure.

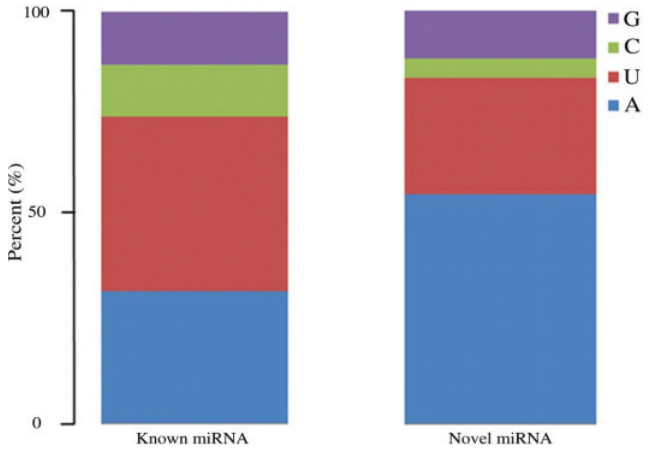

Figure 4: miRNA first nucleotide bias

U nucleotide content and least C nucleotide, while novel miRNA has most of A nucleotide content and least C nucleotide.

3.2 Cluster Analysis of microRNA in Two Samples

Putting the similar expression pattern of known and novel miRNA together (Fig. 5). stu-miR167c-3p, stu-miR171d-5p, stu-miR319-3p and other known miRNAs have the less expression quantity in CG than in NG; stu-miR3627-3p, stu-miR167d-3p and others in NG and CG have the same expression quantity basically; and stu-miR398a-5p, stu-miR399i-5p, stu-miR6023 and etc., have higher expression quantity in CG than in NG apparently.

In novel miRNA, stu-novel-miR49158, stu-novel-miR5125, stu-novel-miR11992 and other novel miRNAs expressed less in CG than in NG; stu-novel-miR48785, stu-novel-miR2258, etc., expressed consistently in both; and stu-novel-miR6491, stu-novel-miR10775, stu-novel-miR17661 and others expression quantity was significantly higher in CG than in NG; and the stu-novel-miR19890, stu-novel-miR19893 etc., are only expressed in NG, while no miRNA only expressed in CG.

3.3 The Variance Analysis of microRNA in Two Samples

By using the miRNA expressed statistics in NG and CG, we could check out if there are significant differences of the expression quantity. As we can see, there were 66 kinds of miRNA possessing significant expression differences in both samples (Fig. 6 and Appendix B). And 34 miRNAs had higher expression quantity in CG than in NG, where 19 kinds are known miRNA and 15 kinds are novel miRNA. 32 miRNAs had lower expressions in CG than in NG, where 13 kinds are known miRNA and 19 kinds are novel miRNA. stu-novel-miR43675 and stu-novel-miR10775 have been regulated to be the largest expression, which is 9.51 and 8 times higher than NG, but the expression of stu-miR319-3p and stu-novel-miR24253 are showing the most significant reduction while low-temperature was treated to potato. From the information above, we can conclude that low temperature treatment can significantly alter miRNAs expression in potato.

3.4 Cold Tolerance Candidate miRNAs and Target Genes

As previously mentioned, miRNAs promote the degradation of target mRNAs or the translation inhibition by binding the 3' untranslated region. This is the mode of action of plant miRNAs, and it depends on the degree of complementarity of the miRNA to its target site [24,25]. In this study, 0°C treatment with 4 hours potato was analyzed, we identified 66 differential expression miRNAs in NG *vs.*

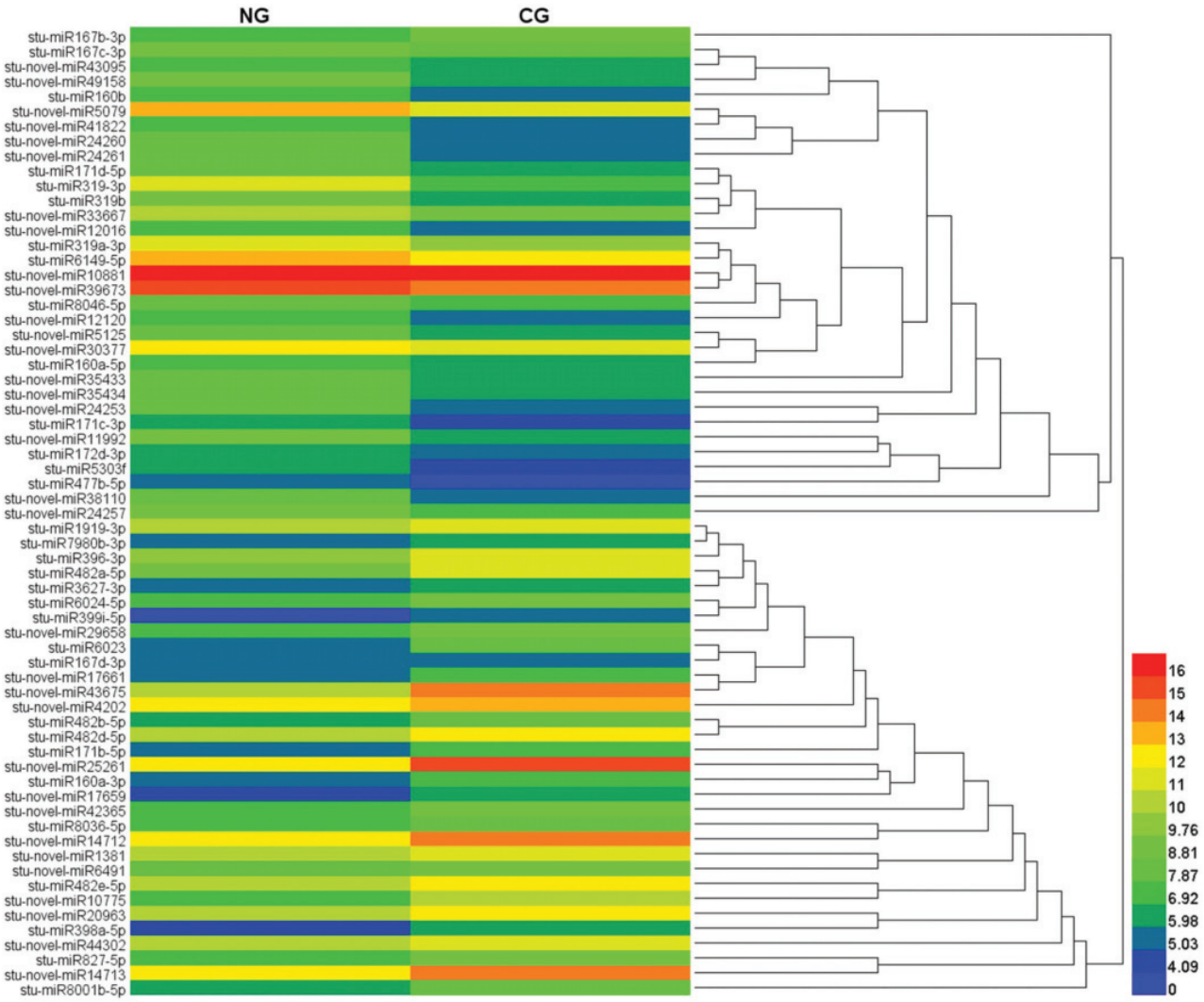

Figure 5: Cluster analysis of miRNA expression

Note: The figure shows the clustering diagram of differentially expressed miRNAs. The columns represent different samples, and the rows represent different miRNAs.

CG, corresponding to 1394 target genes. At the same time, we screened 8 significant differentially expressed miRNAs and mRNAs by association analysis. Among these, we focused on the analysis of stu-miR6023 and stu-novel-miR42365, which target genes pectinesterase (PME) are key regulators in catalyzing the pectin to form pectate (Appendix C).

The target genes of the differentially-expressed miRNAs were further submitted to GO analysis (Fig. 7). There are two significantly enriched functions among the differentially-expressed miRNAs target genes which cellular physiological processes and single-organism processes in the biological process. Cell part and cell were also significantly enriched in cellular component. Catalytic activity and binding combination of molecular function were significantly enriched in molecular function. We can see that, the GO term (q-value $\leq$ 0.05) mainly possess the biological process (68.52%) and molecular function (23.61%), and a small amount of cellular component (7.87%). That means the effect of low-temperature

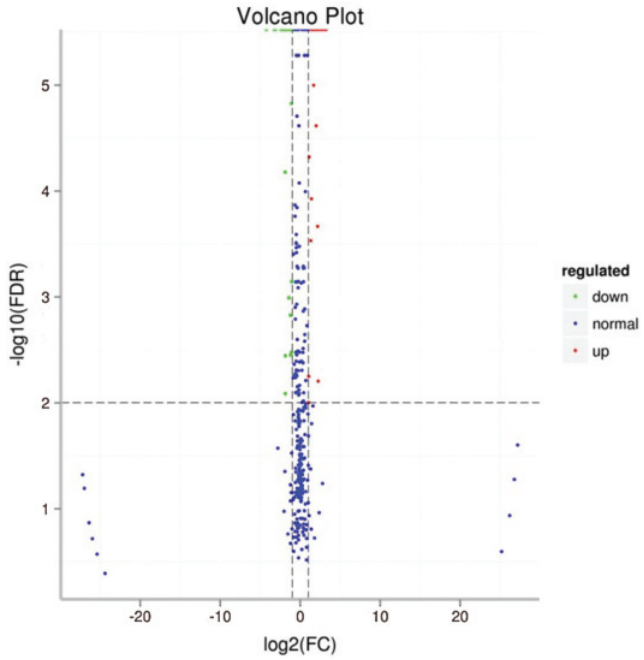

Figure 6: The volcano plot for differentially expressed miRNA

Note: Abscissa represents the microRNA expression multiple changes in the different samples, while ordinate represents the significant level of microRNA expression changes, and blue dots indicate no significant differences, red dots indicate up-regulated and green dots indicate down-regulated between NG and CG.

treatment is primarily by altering the miRNA expression quantity of target genes which has the biological process and molecular function, and is less related to potato cellular components.

KEGG pathway analysis is also used for the target gene candidates. From the KEGG pathway analysis of differentially expressed genes, we got 212 KEGG pathways enrichment in NG *vs.* CG (Fig. 8), which mainly include Environmental Information Processing (17), Metabolism (106), Organismal Systems (22), Genetic Information Processing (53) and Cellular processes (14). The significant enrichment of KEGG pathways mainly have 45 and they are Carotenoid biosynthesis (ko00906), Starch and sucrose metabolism (ko00500), Peroxisome (ko04146), Zeatin biosynthesis (ko00908), Photosynthesis (ko00195), Phenylpropanoid biosynthesis (ko00940), and so on.

3.5 Differential Expressed Genes and Pathways in Potato with Low-Temperature Treatment

To investigate low-temperature responsive genes as well as target genes of the miRNAs in potato, two RNA samples in triplicate were subjected for sequencing, including samples from the 0°C treatment for 0 h (NG) and 4 h (CG). In order to ensure the quality of the reads were high enough to ensure accuracy of subsequent analysis, we discarded the low-quality reads, and a total of 171,820,478 (total clean nucleotides 51,426,719,278 nt) clean reads were obtained in two samples. Besides, the Q30 percentage was over 93%. In order to further determine the accuracy of the sequencing data, we used STAR software to compare the clean reads with the published potato participating genomes to obtain their positional information on the reference genome (Fig. 9). There are 81.76% and 80.55% uniquely mapped reads in NG and CG, 5.73% and 6.00% reads mapped to multiple loci, 0.80% and 0.08% reads mapped to too many loci (Tab. 2).

Used the mRNA expressed statistics in NG and CG to check out if there are significant differences of the expression quantity between two samples, we used mRNA edgeR (R package) to identify differential

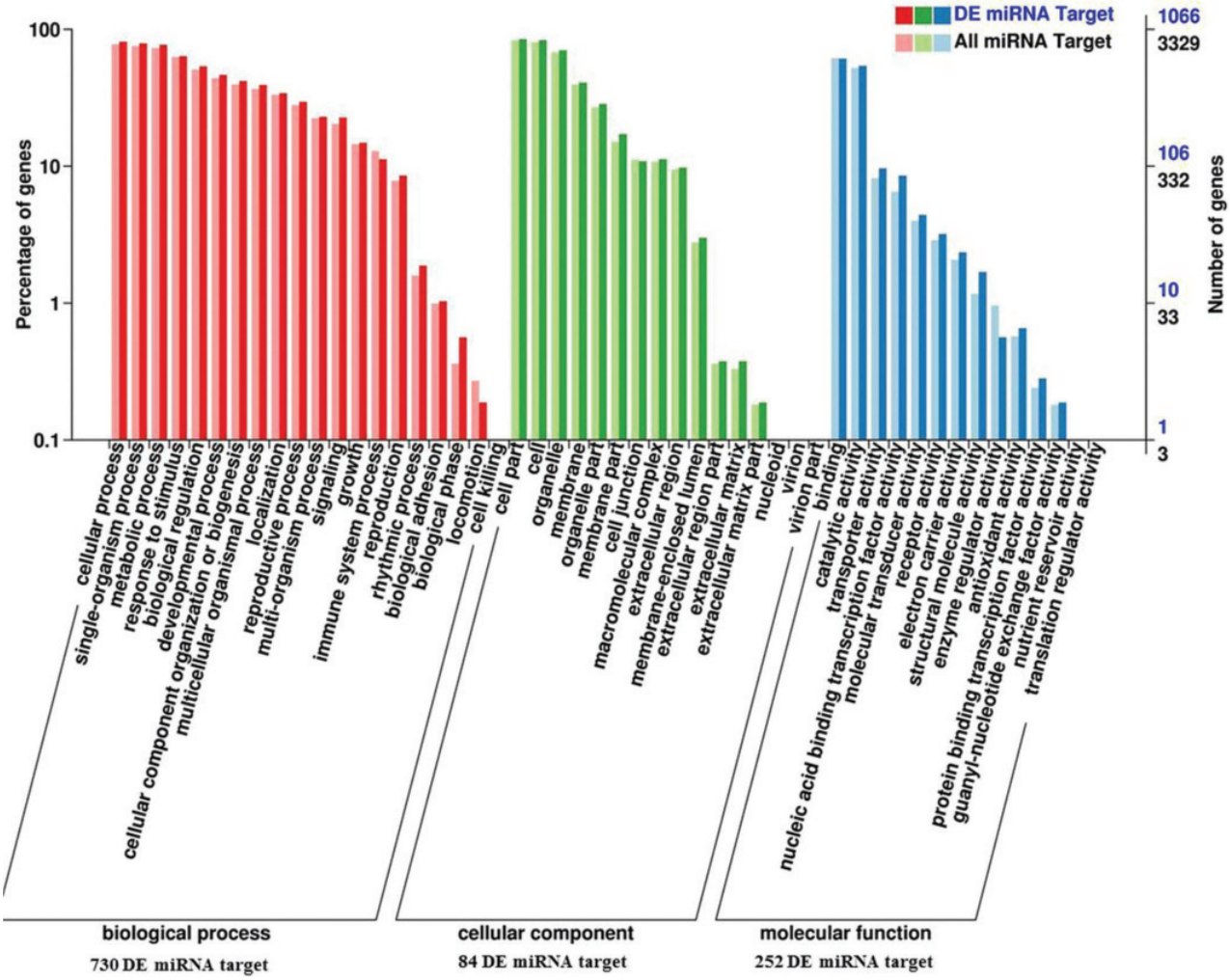

Figure 7: GO annotation class of differentially expressed genes

Note: This is the Gene Ontology (GO) enrichment analysis of the DEGs which were targeted by differentially-expressed miRNAs.

expression, and draw differential expression trend. After gene annotation and expression analysis, a total of 55189 genes were detected in the RNA samples of NG and CG. 3068 DEGs were detected between NG and CG among them ($|\log2 \text{ fold-change}| \geq 1$ and q-value ≤ 0.05), with 1400 up-regulated and 1668 down-regulated (Fig. 10).

3.6 Transcriptome GO and KEGG Analysis

Gene ontology (GO) enrichment was applied on the DEGs with low temperature treatment. 752 significantly enriched GO terms were identified in NG *vs.* CG. There are 62, 519 and 171 significantly enriched GO terms in cellular component, biological process and molecular function, respectively (Appendix D). Moreover, with low-temperature treatment, the biological processes have the highest proportion (69.02%), and the cellular component have the lowest proportion (8.25%). It means that, the effect of low-temperature on potato leaf transcript is primarily altering the expression of biological processes and molecular regulation functions genes, and less involved in cellular components.

KEGG pathway analysis is also used for the DEGs between NG and CG, we got 105 enriched KEGG pathways, they are mainly include cellular processes (21), environmental information processing (63),

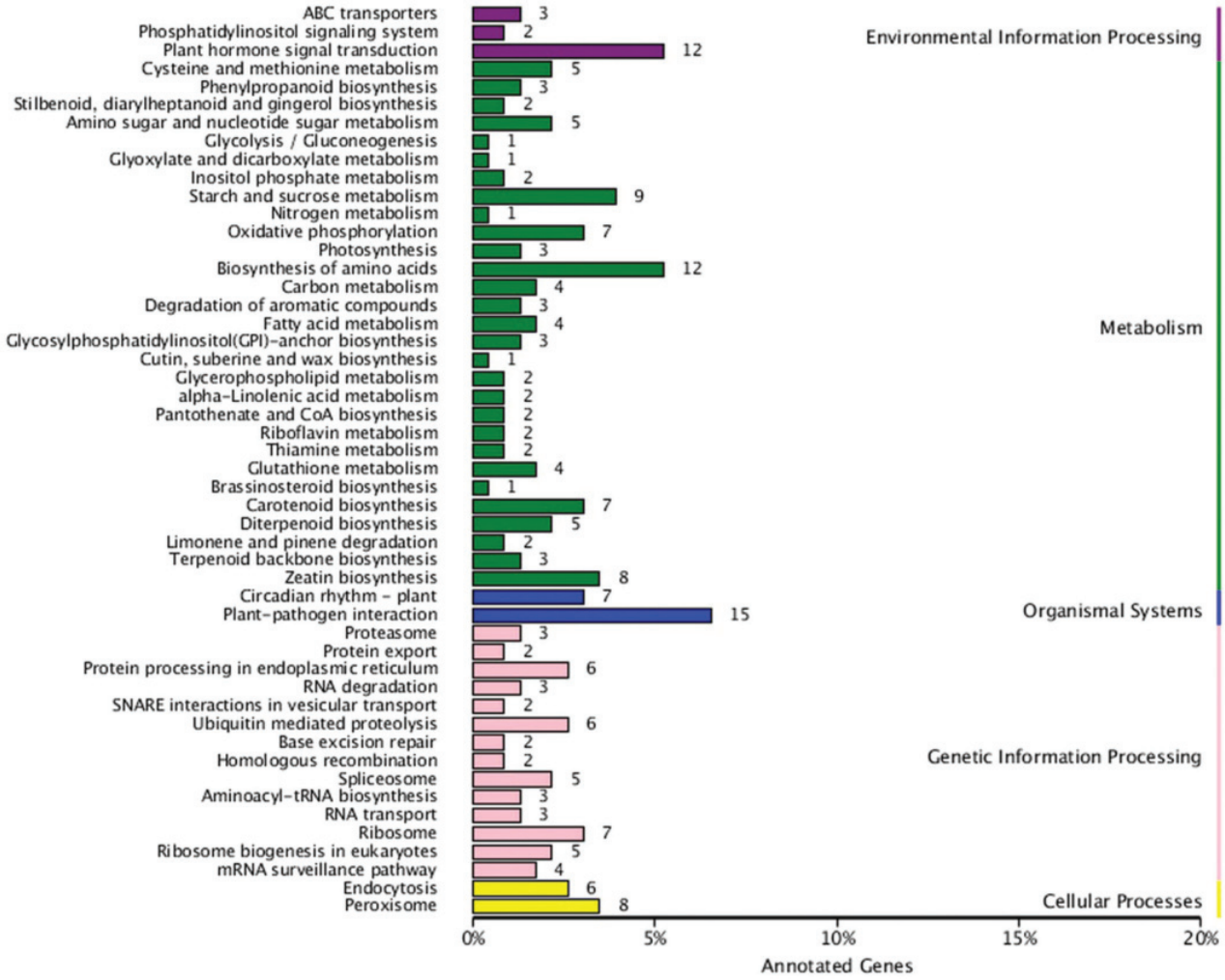

Figure 8: KEGG annotation class of different expressed genes

Note: This is the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the DEGs which were targeted by differentially-expressed miRNAs.

genetic information processing (62), metabolism (382) and organismal systems (43) (Fig. 11). When the parameter p-value ≤ 0.05, the significant enrichment of KEGG pathways mainly have 15, they are phenylpropanoid biosynthesis (ko00940), starch and sucrose metabolism (ko00500), plant hormone signal transduction (ko04075), plant-pathogen interaction (ko04626), phenylalanine metabolism (ko00360), flavonoid biosynthesis (ko00941), DNA replication (ko03030) and so on (Appendix E).

3.7 Analysis of Common Differential Expression KEGG Pathways between Small RNA and Transcriptome Sequencing

The common differentially expressed KEGG pathway between transcriptome and small RNA sequencing in NG *vs.* CG is starch and sucrose metabolism (ko00500). In this pathway, the expression levels of stu-miR6023 and stu-novel-miR42365 in CG were significantly higher than in NG (Fig. 12). Through target genes prediction of these miRNAs, 52 target genes were obtained. PGSC0003DMG400021575 and PGSC0003DMG400042971 encode for pectinesterase (StuPME21575 and StuPME42971), which were involved in catalyzing the pectin to form pectate. In transcriptome

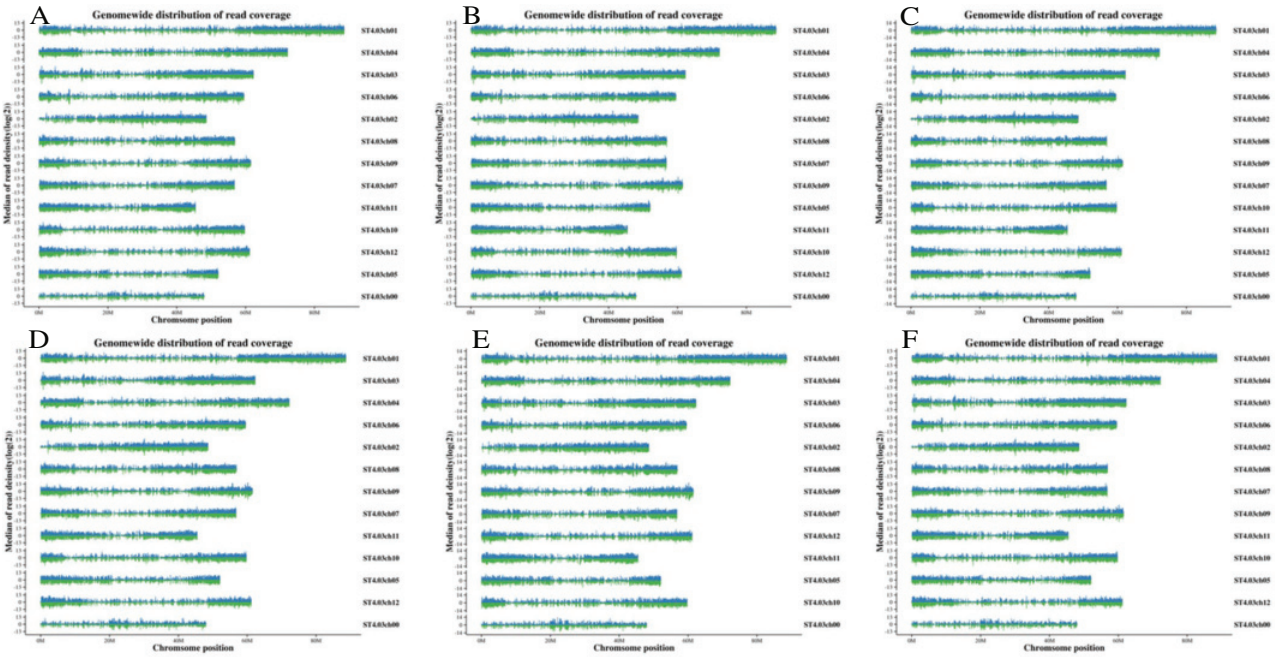

Figure 9: Distribution of the sequence reads mapped to potato genome

Note: A, B and C are three repetitions of NG; D, E and F are three repetitions of CG; Abscissa is the chromosomal location; Ordinate is the logarithm of the base 2 with the depth of coverage. — Sense, — Antisense.

Table 2: Summary for the *Solanum tuberosum L.* transcriptome

Sample	NG	CG
ReadSum	29505863.7	27767629
BaseSum	8831379198	8310860561
N percentage (%)	0.00	0.00
GC percentage (%)	43.49	43.00
Q30 (%)	93.50	93.42
Uniquely mapped reads (%)	81.76	80.55
reads mapped to multiple loci (%)	5.73	6.00
reads mapped to too many loci (%)	0.08	0.08

sequencing, the pectinesterases are significantly lower in NG than in CG (Fig. 13). Therefore, we speculated that while the potatoes were subjected to low temperature stress, the expression of stu-miR6023 and stu-novel-miR42365 were increased, they inhibited the expression of pectinesterase in starch and sucrose metabolism and changed the pectin content in potato leaves, thereby improving the ability of potato to resist low temperature stress.

3.8 Differentially Expressed microRNAs and its Target Genes qRT-PCR Validation

In order to verify the above results, we used qRT-PCR to determine the expression of stu-miR6023, stu-novel-miR42365 and their target genes *StuPME21575* and *StuPME42971* in NG and CG. From Fig. 14, it can be found that the expression of stu-miR6023 in potato with different time's low-temperature treatment

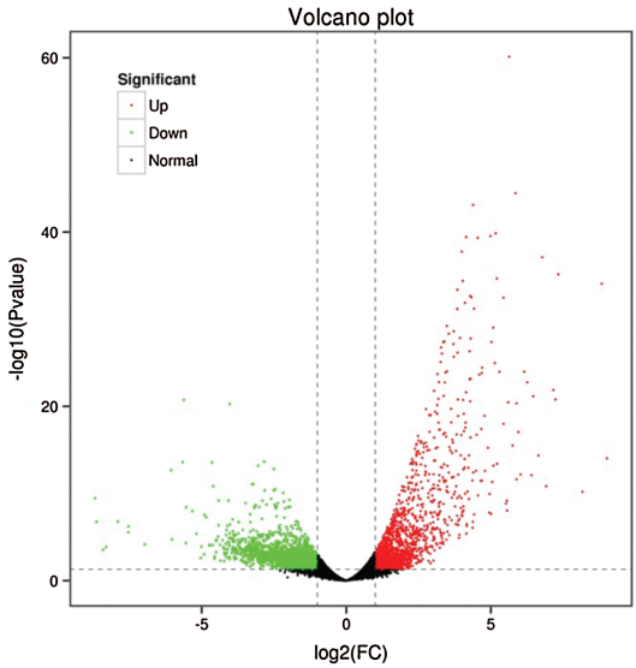

Figure 10: The MA plot for differentially expressed mRNA

Note: Abscissa represents the mRNA expression multiple changes in the different samples, while ordinate represents the significant level of mRNA expression changes, and black dots indicate no significant differences in the mRNA, red dots represent up-regulated differentially expressed genes, green dots represent down-regulated differentially expressed genes.

was significantly changed. The expression trend of stu-miR6023 is increasing, and reaches the maximum at 12 h. The expression quantity trend of stu-novel-miR42365 is the same with stu-miR6023. This result suggested that low-temperature treatment had a significant impact on miRNA expression in potato. The expression trend of *StuPME21575* is initial increase, subsequent decrease, and the maximum at 4 h. The expression trend of *StuPME42971* is initial decrease and then increase, the expression peak at 12 h.

We can see that when the stu-miR6023 reaching a maximum expression at 12 h, *StuPME21575* expression decreased nearly half. The expression trend of *StuPME42971* is exactly opposite to stu-novel-miR42365. We concluded that with low-temperature treatment in potato, stu-miR6023 and stu-novel-miR42365 significantly regulate the expression levels of pectinesterases (StuPME21575 and StuPME42971) in starch and sucrose metabolism pathways, and this also verifies the accuracy of the target gene prediction.

3.9 Pathway Analysis of MicroRNA Regulation in Potato Resistance to Low Temperature Stress

According to the previous results in this study, we hypothesized that potato respond low-temperature stress through the following steps (Fig. 15): (1) low-temperature affects the expression quantity of stu-miR6023 and stu-novel-miR42365; (2) the expression changes of stu-miR6023 and stu-novel-miR42365 result in the corresponding regulation of target gene (StuPME21575, StuPME42791, StuPG6906, StuPG6944, StuPG39920, StuSPS27936, StuSS2895, StuSS6672 and StuSBE9981) expression level changes, especially pectinesterase *StuPME21575* and *StuPME42791* expressions have significant difference in different low-temperature treatment; (3) *StuPME21575* and *StuPME42791*, through expression changing, lead to catalyzing pectin to form pectate change and pectin content change; and (4) the change of pectin content lead to the change of potato cold resistance. To our knowledge, these results

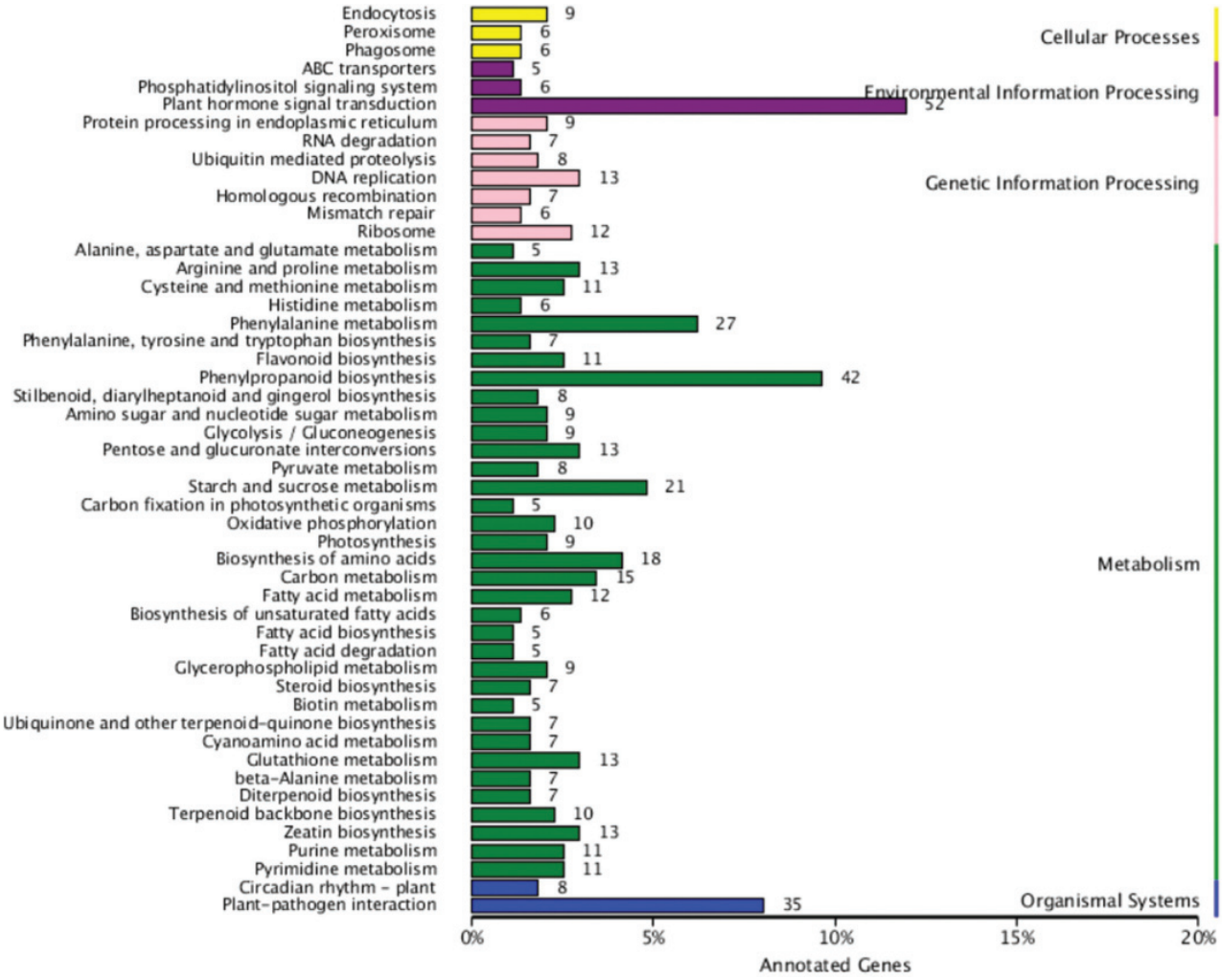

Figure 11: KEGG annotation class of differentially expressed genes

provide the first evidence for a potential regulatory role of miRNAs in Pectinesterase-Mediated cold resistance in potato.

4 Discussion

Plant mobilizes many defense mechanisms to cope with the environmental challenge, which involves molecular, biochemical and physiological changes [26]. In this research, we employ high-throughput sequencing of miRNA and mRNA to address the molecular differences between two samples with different treatment.

4.1 Effect of Frost Damage on Potato

Potato is one of the four major staple crops in the world. However, potato cultivars are sensitive to frost, low-temperature is an important factor limiting the geographical distribution and yield expansion [1,2,27]. Favorita is a precocious and high-quality potato cultivar first introduced from Netherlands, which is widely cultivated in China. It is yellow flesh, smooth skin, good taste and high yield, but it is frost sensitive and highly susceptible to frost damage, the cold spell in later spring will have a serious impact on it. Therefore, research on its ability to resist cold is urgent.

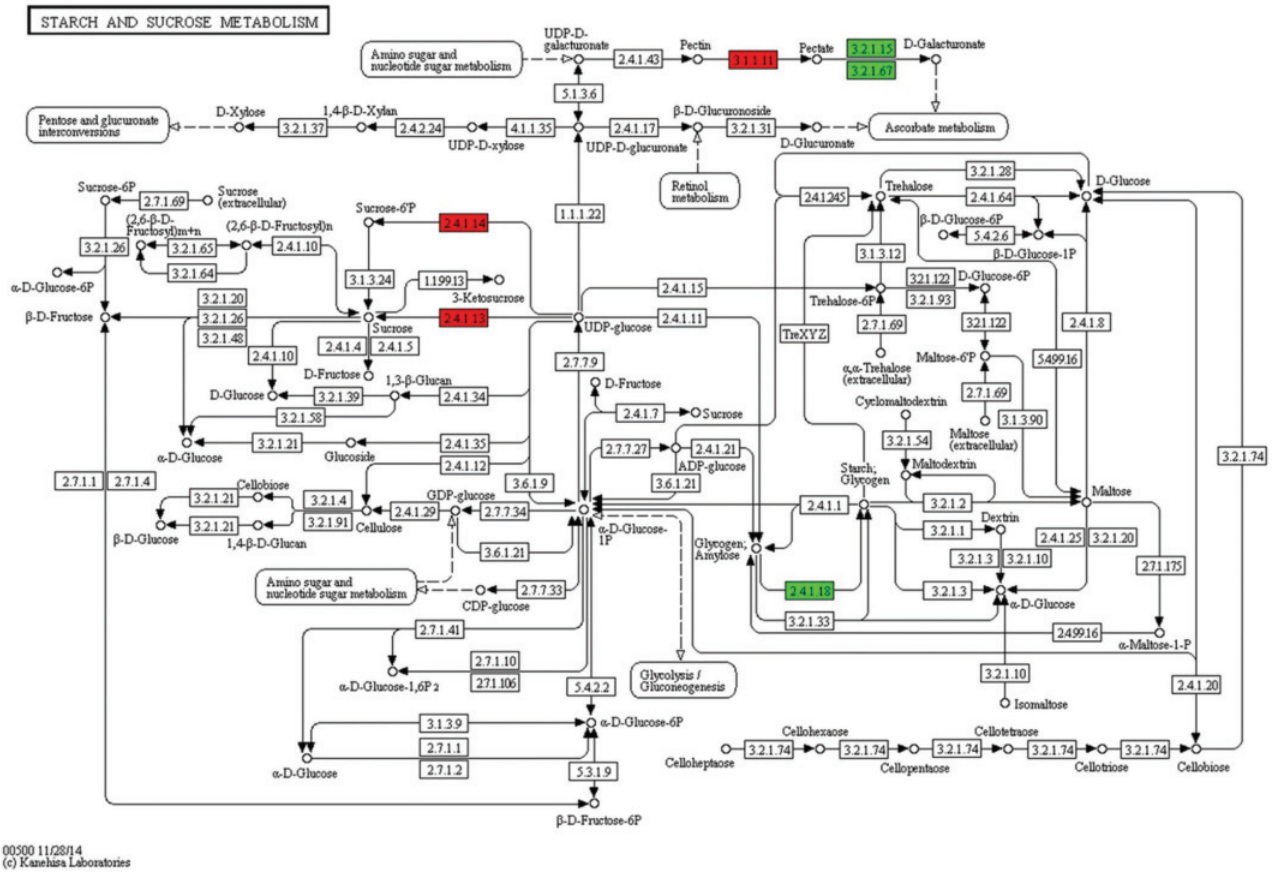

Figure 12: The starch and sucrose metabolism of small RNA sequencing in potato

Note: The enzymes marked with red boxes are related to target genes of up-regulation miRNA; the enzymes marked with green boxes are related to target genes of down-regulation miRNA; the enzymes marked with blue boxes are related to target genes of up and down-regulation miRNA; the numbers in the box represent the enzyme number; 3.1.1.11 is pectinesterase.

4.2 Known and Novel miRNAs and Their Differential Expressions in Potato

In this study, we employed deep sequencing technologies to investigate the miRNA expression profile upon cold stress in Favorita, which shows the effect of low-temperature treatment on miRNA expression in Potato.

In the two small RNA libraries sequenced, a total of 253 conserved miRNAs belonging to 74 families and 106 novel miRNAs have been identified. Similar to previous reports from pepper and tomato, different conserved miRNA had different expression levels, with reads ranged from a few to thousands [28–30]. However, most novel miRNAs had a high expression level, with reads more than 100, which was different from previous results from tomato and arabidopsis [28,31].

According to the data analysis, 32 known and 34 novel miRNAs were characterized as differentially expressed miRNAs. Among them, 34 miRNAs had a higher expression quantity in CG than NG, where 19 kinds are known and 15 kinds are novel miRNAs. 32 miRNAs had the lower expressions in CG than NG, where 13 kinds are known and 19 kinds are novel miRNAs. When low-temperature treatment to potato (CG), stu-novel-miR43675 and stu-novel-miR10775 have been regulated to be the largest expression, which is 9.51 and 8 times higher than NG, but the expression of stu-miR319-3p and stu-novel-miR24253 are showing the most significant reduction (Appendix B). From the above-mentioned information, we can conclude that low-temperature treatment can significantly alter miRNA expression in potato, this kind of results have also been observed in previous studies on sugarcane and cotton [32,33].

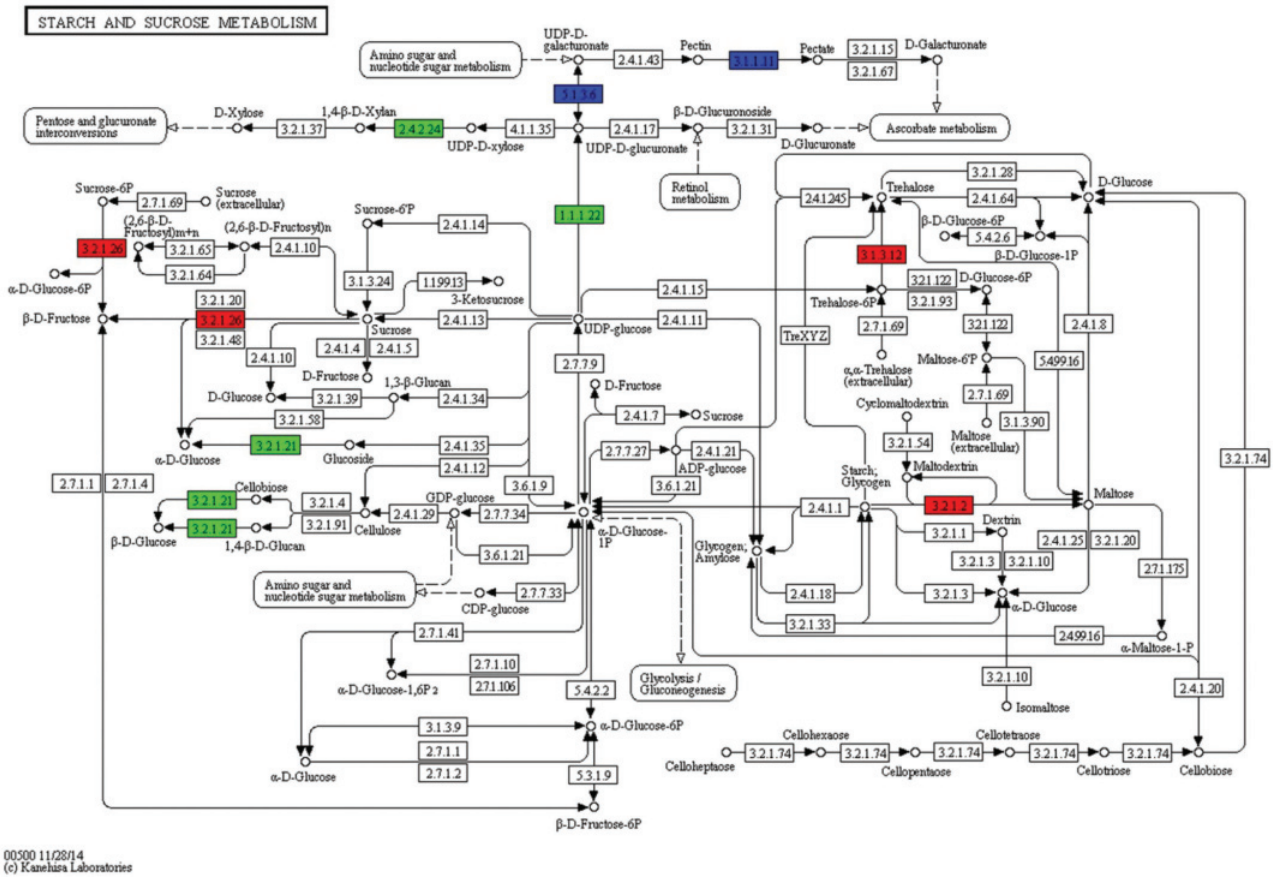

Figure 13: The starch and sucrose metabolism of transcriptome sequencing in potato

Note: The nodes are marked in red background color indicating the DEGs is up-regulated expression, the nodes are marked in green background color indicating the DEGs is down-regulated expression, and the nodes are marked in blue background color indicating the DEGs is up-/ down-regulated expression; 3.1.1.11 is pectinesterase.

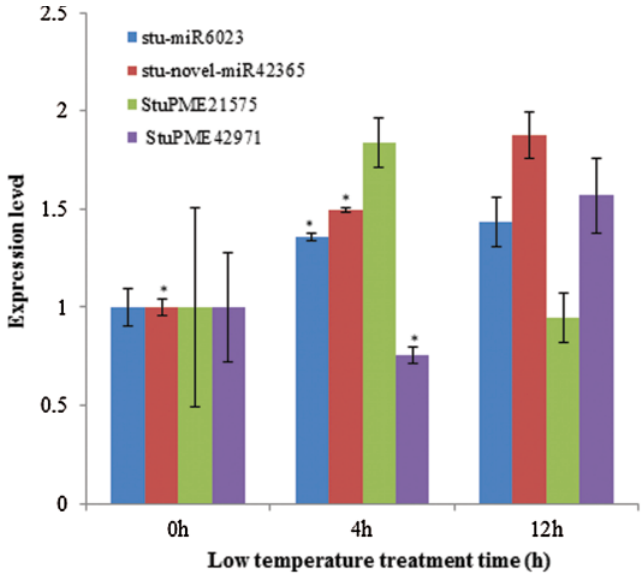

Figure 14: Expression pattern of miRNAs and target genes

Note: Duncan's multiple range test, the * represents $p < 0.05$, the number of biological replicates = 3.

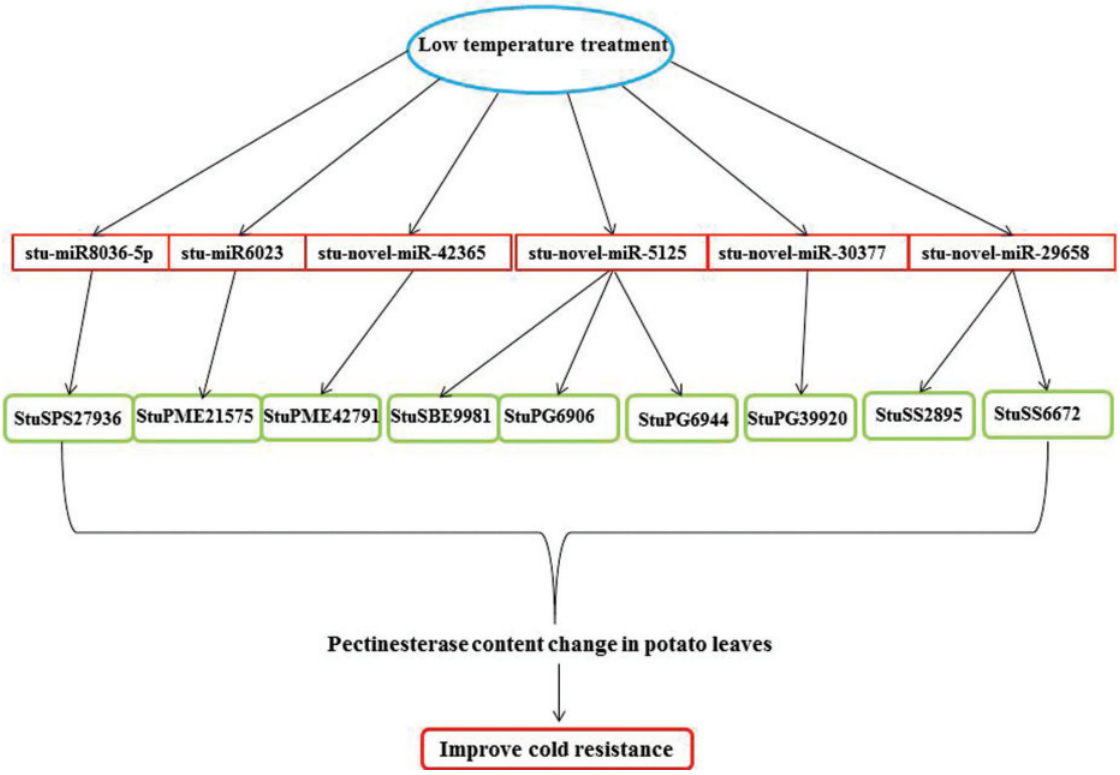

Figure 15: Model depicting the regulatory pathways of miRNAs in Pectinesterase-Mediated cold stress resistance in potato

Note: Under low temperature stress, the expression of stu-miR6023 and stu-novel-miR42365 were promoted. The up-regulation of miRNAs result in the down-regulation of their target genes involved in starch and sucrose metabolism (StuPME21575, StuPME42791, StuPG6906, StuPG6944, StuPG39920, StuSPS27936, StuSS2895, StuSS6672 and StuSBE9981), and changing the pectinesterase activity, thereby changing the ability of potato to resist low temperature stress.

Overall, we have identified a whole bunch of low-temperature responsive miRNAs, many of them are also involved in secondary metabolism, drought tolerance, disease defense and salt tolerance, such as stu-miR827-5p, stu-miR482e-5p, stu-miR1919-3p and stu-miR160a-5p [34–37]. At the same time, some conserved miRNAs were firstly identified in our study to be cold-responsive in potato, such as stu-miR6023, stu-miR5303f and stu-miR160b; these conserved miRNAs are likely to focus on regulating the cold resistance of potato.

4.3 RNA-seq and expression profiles of potato under cold stress

Transcriptomic approach has been widely used to characterize expression profiles under various stress conditions in plants, and the quantification of mRNAs is generally based on RNA-Seq analysis [38,39]. In this study, we utilized deep sequencing technology to compare the mRNA and miRNA expression between NG and CG.

With low-temperature stress, 3068 DEGs were detected between NG and CG, with 1400 up-regulated and 1668 down-regulated. GO and KEGG pathway analysis revealed that the stress response genes were involved in physiological, metabolism and biochemical processes, which is normal as the plant has to adapt to environmental stress by adjusting to the basic cellular metabolism [40]. The enrichment of biological process GO term of response to abiotic stimulus, additional GO terms related to stress were enriched in our study, such as response to cold, sucrose metabolic process and starch biosynthetic

process. DEGs were significantly enriched in GO term 'sucrose metabolic process' and 'starch biosynthetic process' in NG *vs.* CG, indicating that these genes could be essential for the cold response of potato. Pathway analysis on DEGs revealed various stress-related pathways, including pathways of plant hormone signal transduction, phenylpropanoid biosynthesis, flavonoid biosynthesis, starch and sucrose metabolism and circadian rhythm-plant. Starch and sucrose metabolism is one of the most important metabolic pathways in plants [41]. A total of 21 DEGs were detected in starch and sucrose metabolism, indicating that starch and sucrose are essential in the low-temperature response of potato.

4.4 Target Genes of miRNA in Potato

In this study, we have analyzed low-temperature stress treatment potato and 66 differential expression miRNAs were identified between NG and CG, corresponding to 1394 target genes. Many of these expressions were related to plant stress tolerance, including genes encoding transcription factors, cold-tolerance related proteinase, protein kinase and phosphatase, proteinase in signal transduction pathways, and enzymes in various metabolic pathways. GO analysis has shown the enriched biological process GO terms of defense response, response to stress, and response to stimulus.

Pectinesterases (PMEs) are key regulators in catalyzing the pectin to form pectate [42,43]. In rice, overexpression miRNA3980a/b-3p has the effect in reducing expression of PME genes, and further affects the downstream genes polygalacturonase and galacturan 1, 4-alpha-galacturonidase that function in starch and sucrose metabolism pathway [44]. In our study, low-temperature treatment significantly increased stu-miR6023 and stu-novel-miR42365 expression. Further analysis showed 2 out of the 52 target genes of them were PME genes (PGSC0003DMG400021575 and PGSC0003DMG400042971). Moreover, *StuPME21575* and *StuPME42791* were targeted by stu-miR6023 and stu-novel-miR42365; and transcriptome analysis showed that their expression decreased with low temperature treatment, indicating that their expression is negatively regulated by stu-miR6023 and stu-novel-miR42365.

Therefore, we concluded that the expression of stu-miR6023 and stu-novel-miR42365 were promoted by the low-temperature treatment. In addition, the up-regulation of miRNAs resulted in the down-regulation of their target genes involved in starch and sucrose metabolism, and changed the content of pectin in leaves, thereby changed the ability of potato to resist low-temperature stress.

5 Conclusions

In this study, 507 miRNAs and 3068 mRNAs involved in potato cold response were identified by high-throughput sequencing. Bioinformatics analysis showed that the starch and sucrose metabolism was the common KEGG pathway between miRNA and transcriptome, StuPME21575 and StuPME42971 are pectin methyl esterase which were regulated by stu-miR6023 and stu-novel-miR42365. Sequencing showed that the expression of stu-miR6023 and stu-novel-miR42365 was up-regulation, *StuPME21575* and *StuPME42971* was down-regulation with low-temperature. This result was the same with qRT-PCR. This is the first discovery that potato responds to low-temperature stress by regulating pectinesterase via miRNA, which will provide new insights into the complex regulatory network of plant adaption to low-temperature stress.

Authorship and Contribution: Chongchong Yan, Huajun Liao designed the experiments and drafted the manuscript. Nan Zhang, Qianqian Wang and Xuexiang Ren analyzed the data. Chongchong Yan, Xiaojing Pu, Jiajia Wang, Zhihong Xu and Bingjie Xue carried out the experiments. All authors read and approved the final manuscript.

Funding Statement: This work was supported by grants from the Earmarked Fund for Modern Agro-Industry Technology Research System of China (CARS-09-ES10), National Key Research and Development Project 'Integrated Research and Demonstration of Potato Fertilizer and Pesticide Reduction Technology (2018YFD0200800)', Anhui Academy of Agricultural Sciences Dean's Youth Fund

(17B0306), and Anhui Academy of Agricultural Sciences 'New Dr.' scientific research project, Anhui Science and Technology Major Project 'Breeding of New Strawberry Varieties in Facilities and Integration and Demonstration of Cultivation Techniques for Improving Quality and Efficiency' (201903a06020004), Potato Taro Breeding and Cultivation Team Project (2020YL029). All the authors read and approved the manuscript.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

Availability of Data and Materials: The sequencing data of this study are available in the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) (accession number: PRJNA587793). The other supporting data are included as Appendix.

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Appendix

Appendix A: Primers for qRT-PCR

Homolog	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
stu-miR6023	ACACTCCAGCTGGGTTCCATGAA AGTGTAGATCCAAAA	CTCAACTGGTGTCGTGGAGTCGGC AATTCAGTTGAGATCCAAAA
stu-novel-miR42365	ACACTCCAGCTGGGTATAGTTGC AGCAG	CTCAACTGGTGTCGTGGAGTCGGC AATTCAGTTGAGACTTGCAG
St 18s RNA	TTAGAGGAAGGAGAAGTCGTAACAA	CTCAACTGGTGTCGTGGAGTCGGC AATTCAGTTGAGTTGTTACGA
StuPME21575	TCTTCATGGCATTGGATCTAAC	TTAGCGGATAAAGTTGGGTGT
StuPME42971	TGGCGACACTGCTCAAATC	ACACACAATATAACACAATCACCG
ef1-α	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA
stu-novel-miR42365	ACACTCCAGCTGGGTATAGTTGC AGCAG	CTCAACTGGTGTCGTGGAGTCGGC AATTCAGTTGAGACTTGCAG

Appendix B: NG *vs.* CG. DEG final

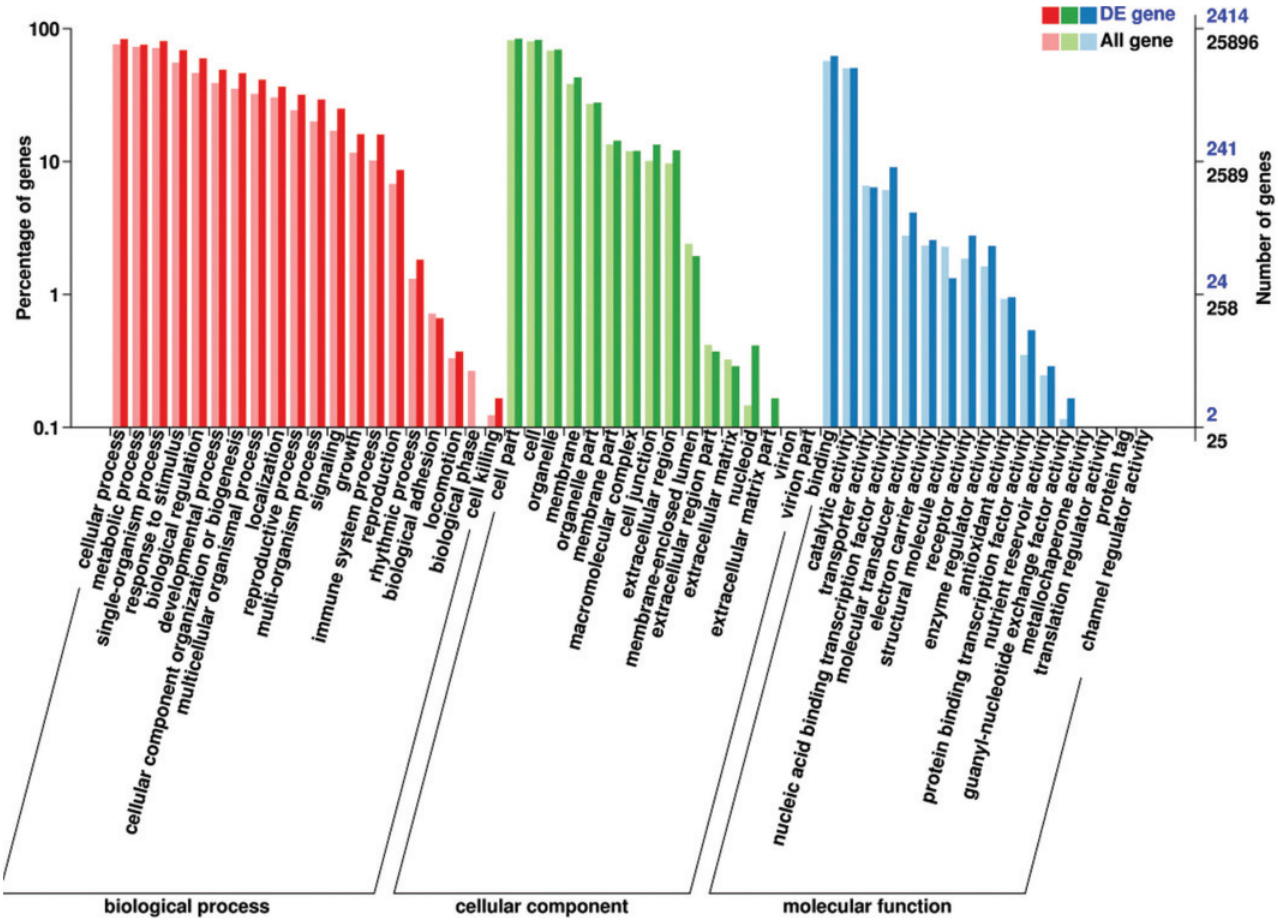
ID	NG	CG	*p*-value	FDR	log2FC	regulated
stu-miR167b-3p	149.971	576.2589	0	0	1.942034	up
stu-miR167c-3p	672.664	282.405	0	0	−1.25212	down
stu-miR171d-5p	295.5311	106.856	0	0	−1.46764	down
stu-miR1919-3p	1515.148	3285.821	0	0	1.116795	up
stu-miR319-3p	2439.234	129.7537	0	0	−4.23258	down
stu-miR319a-3p	3182.473	893.0105	0	0	−1.8334	down
stu-miR319b	458.7348	91.5908	0	0	−2.32439	down
stu-miR396-3p	990.2497	2793.52	0	0	1.49622	up
stu-miR482a-5p	707.9513	2087.507	0	0	1.560059	up
stu-miR482b-5p	108.0673	286.2213	0	0	1.405201	up
stu-miR482d-5p	1636.448	4739.825	0	0	1.534266	up
stu-miR482e-5p	1746.721	5560.326	0	0	1.67052	up
stu-miR6023	39.6982	263.3236	0	0	2.729691	up
stu-miR6024-5p	222.751	637.3194	0	0	1.516585	up
stu-miR6149-5p	16296.11	7640.201	0	0	−1.09285	down
stu-miR8036-5p	216.1347	435.0564	0	0	1.009272	up
stu-miR827-5p	130.1219	633.5032	0	0	2.283488	up
unconservative_ST4.03ch00_49158	540.3367	64.8768	0	0	−3.05808	down
unconservative_ST4.03ch01_1381	1310.041	3946.038	0	0	1.590793	up
unconservative_ST4.03ch01_4202	5553.338	12017.48	0	0	1.113707	up
unconservative_ST4.03ch01_5079	8191.063	3396.493	0	0	−1.27	down
unconservative_ST4.03ch01_5125	302.1475	76.3257	0	0	−1.98501	down
unconservative_ST4.03ch02_6491	242.6001	828.1336	0	0	1.771283	up
unconservative_ST4.03ch03_10775	189.6692	1518.881	0	0	3.001452	up
unconservative_ST4.03ch03_10881	163865.4	80077.09	0	0	−1.03305	down
unconservative_ST4.03ch03_11992	593.2676	110.6722	0	0	−2.42239	down
unconservative_ST4.03ch03_12016	156.5874	34.3466	0	0	−2.18873	down
unconservative_ST4.03ch04_14712	6823.68	28473.3	0	0	2.060988	up
unconservative_ST4.03ch04_14713	6823.68	28473.3	0	0	2.060988	up
unconservative_ST4.03ch05_17661	33.0818	217.5282	0	0	2.717093	up
unconservative_ST4.03ch06_20963	1140.221	4411.624	0	0	1.951997	up
unconservative_ST4.03ch06_24253	363.9002	38.1628	0	0	−3.2533	down
unconservative_ST4.03ch06_24257	535.9258	190.8142	0	0	−1.48986	down
unconservative_ST4.03ch06_24260	244.8056	57.2443	0	0	−2.09643	down
unconservative_ST4.03ch06_24261	244.8056	57.2443	0	0	−2.09643	down

Appendix B (continued).

ID	NG	CG	*p*-value	FDR	log2FC	regulated
unconservative_ST4.03ch07_25261	6190.715	36613.43	0	0	2.564195	up
unconservative_ST4.03ch08_29658	143.3546	503.7495	0	0	1.813118	up
unconservative_ST4.03ch08_30377	5573.187	2167.649	0	0	−1.36237	down
unconservative_ST4.03ch09_33667	1380.615	461.7704	0	0	−1.58006	down
unconservative_ST4.03ch09_35433	324.202	114.4885	0	0	−1.50169	down
unconservative_ST4.03ch09_35434	324.202	114.4885	0	0	−1.50169	down
unconservative_ST4.03ch10_38110	275.682	61.0605	0	0	−2.17469	down
unconservative_ST4.03ch10_39673	48242.14	23355.66	0	0	−1.04652	down
unconservative_ST4.03ch11_42365	196.2856	759.4405	0	0	1.951983	up
unconservative_ST4.03ch12_43675	1759.954	16791.65	0	0	3.254135	up
unconservative_ST4.03ch12_44302	1457.806	4045.261	0	0	1.472434	up
unconservative_ST4.03ch12_44303	1457.806	4045.261	0	0	1.472434	up
stu-miR160a-3p	48.52	156.4676	0.000002	1.00E-05	1.689212	up
stu-miR8046-5p	308.7638	145.0188	0.000003	1.49E-05	−1.09026	down
unconservative_ST4.03ch05_17659	28.6709	114.4885	0.000005	2.42E-05	1.997544	up
stu-miR8001b-5p	110.2728	240.4259	0.00001	4.77E-05	1.124515	up
unconservative_ST4.03ch11_41822	125.711	34.3466	0.000014	6.62E-05	−1.87187	down
stu-miR167a-3p	57.3419	152.6514	0.000026	0.000119	1.412579	up
stu-miR398a-5p	17.6436	80.142	0.000049	0.000216	2.183414	up
stu-miR171b-5p	59.5473	148.8351	0.000068	0.000294	1.321607	up
unconservative_ST4.03ch11_43095	185.2583	87.7745	0.000193	0.000718	−1.07766	down
unconservative_ST4.03ch03_12120	121.3001	45.7954	0.000282	0.001019	−1.40531	down
stu-miR160b	134.5328	57.2443	0.000426	0.001487	−1.23275	down
stu-miR160a-5p	136.7383	64.8768	0.001018	0.003334	−1.07564	down
stu-miR172d-3p	114.6837	49.6117	0.001112	0.003539	−1.20891	down
stu-miR171c-3p	68.3691	19.0814	0.001139	0.003591	−1.84118	down
stu-miR5303f	68.3691	19.0814	0.001139	0.003591	−1.84118	down
stu-miR3627-3p	52.9309	110.6722	0.001946	0.005627	1.064111	up
stu-miR399i-5p	8.8218	41.9791	0.00222	0.006241	2.250526	up
stu-miR477b-5p	55.1364	15.2651	0.003025	0.008185	−1.85277	down
stu-miR7980b-3p	46.3146	95.4071	0.003786	0.009926	1.04263	up

Appendix C: stu-miR6023 and stu-novel-miR42365 and their target genes

miRNA	Target
stu-miR6023	PGSC0003DMG401004019;PGSC0003DMG400002885; PGSC0003DMG400021575;PGSC0003DMG402021491; PGSC0003DMG400002495;PGSC0003DMG400005542; PGSC0003DMG400019834;PGSC0003DMG401005729; PGSC0003DMG400011546;PGSC0003DMG400030394; PGSC0003DMG400039819;PGSC0003DMG400011170; PGSC0003DMG401021049;PGSC0003DMG400016424; PGSC0003DMG400005226;PGSC0003DMG400036994; PGSC0003DMG400044667;PGSC0003DMG400015824; PGSC0003DMG402005729;PGSC0003DMG400008584; PGSC0003DMG400036266;PGSC0003DMG400011270; PGSC0003DMG400005648;PGSC0003DMG400015212; PGSC0003DMG403005729;PGSC0003DMG401025899; PGSC0003DMG400005773;PGSC0003DMG400007090; PGSC0003DMG400004725;PGSC0003DMG400017599; PGSC0003DMG400021067;PGSC0003DMG400020420; PGSC0003DMG402021049
stu-novel-miR42365	PGSC0003DMG400002710;PGSC0003DMG400044563; PGSC0003DMG400009926;PGSC0003DMG400005850; PGSC0003DMG400004006;PGSC0003DMG400028474; PGSC0003DMG400042971;PGSC0003DMG402006305; PGSC0003DMG400002818;PGSC0003DMG400025620; PGSC0003DMG400027431;PGSC0003DMG400025336; PGSC0003DMG400021134;PGSC0003DMG400010180; PGSC0003DMG401000517;PGSC0003DMG400016219; PGSC0003DMG400025310;PGSC0003DMG400007237; PGSC0003DMG400030625



Appendix D: GO annotation class of different expressed genes

Appendix E: Significantly enriched KEGG pathway

Kegg_pathway	ko_id	Cluter_frequency	Genome_frequency	*p*-value	Corrected_ *p*-value
Phenylpropanoid biosynthesis	ko00940	42 out of 435 9.6551724137931%	255 out of 6100 4.18032786885246%	1.77E-07	1.86E-05
Plant hormone signal transduction	ko04075	52 out of 435 11.9540229885057%	392 out of 6100 6.4262950819672%	6.26E-06	0.000657
Plant-pathogen interaction	ko04626	35 out of 435 8.04597701149425%	236 out of 6100 3.86885245901639%	2.19E-05	0.002295
Phenylalanine metabolism	ko00360	27 out of 435 6.20689655172414%	191 out of 6100 3.13114754098361%	0.000427	0.044806
Flavonoid biosynthesis	ko00941	11 out of 435 2.52873563218391%	57 out of 6100 0.934426229508197%	0.001968	0.206603
Starch and sucrose metabolism	ko00500	21 out of 435 4.82758620689655%	255 out of 6100 4.18032786885246%	0.002751	0.313543
DNA replication	ko03030	13 out of 435 2.98850574712644%	81 out of 6100 1.32786885245902%	0.004431	0.46524
Stilbenoid, diarylheptanoid and gingerol biosynthesis	ko00945	8 out of 435 1.83908045977011%	40 out of 6100 0.655737704918033%	0.006344	0.666081
Steroid biosynthesis	ko00100	7 out of 435 1.60919540229885%	35 out of 6100 0.573770491803279%	0.010464	1
Biotin metabolism	ko00780	5 out of 435 1.14942528735632%	20 out of 6100 0.327868852459016%	0.011408	1
Ubiquinone and other terpenoid-quinone biosynthesis	ko00130	7 out of 435 1.60919540229885%	36 out of 6100 0.590163934426229%	0.012215	1
Arginine and proline metabolism	ko00330	13 out of 435 2.98850574712644%	98 out of 6100 1.60655737704918%	0.021118	1
Circadian rhythm - plant	ko04712	8 out of 435 1.83908045977011%	50 out of 6100 0.819672131147541%	0.023774	1
Riboflavin metabolism	ko00740	3 out of 435 0.689655172413793%	10 out of 6100 0.163934426229508%	0.029632	1
Terpenoid backbone biosynthesis	ko00900	10 out of 435 2.29885057471264%	77 out of 6100 1.26229508196721%	0.045632	1