



The Effect of Low Temperature Stress on the Leaves and MicroRNA Expression of Potato Seedlings

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In recent years, with the wanton destruction of the ecological environment by humans and the frequent occurrence of extreme bad weather, many places that should have been warm and blooming in spring have instead experienced the phenomenon of the “April blizzard,” which has seriously affected China’s crops, especially spring potato production in most areas. Potato cultivars, especially potato seedlings, are sensitive to frost, and low temperature frost has become one of the most important abiotic stresses affecting potato production. Potato cold tolerance is regulated by a complex gene network. Although some low temperature resistant microRNAs have been identified, little is known about the role of miRNAs in response to low temperature stress in potato. Therefore, the objective of this study is to clarify the influence of low temperature stress on the miRNA expression of potato by comparing the expression differences of miRNA in potato which was treated with different low temperatures. For the study, 307 known miRNAs belonging to 73 small RNA families and 211 novel miRNAs were obtained. When the temperature decreased, the number of both known and novel miRNA decreased, and the minimum temperature was -2°C . Most of the miRNAs respond to low temperature, drought, and disease stress; some conserved miRNAs were first found to respond to low temperature stress in potato, such as *stu-miR530*, *stu-miR156d*, and *stu-miR167b*. The Gene Ontology, Kyoto Encyclopedia of Genes, and Genomes pathway enrichment analysis of 442 different expression miRNAs target genes indicated that there existed diversified low temperature responsive pathways, but Abscisic Acid was found likely to play a central coordinating role in response to low temperature stress in many metabolism pathways. Quantitative real-time PCR assays indicated that the related targets were negatively regulated by the tested different expression miRNAs during low temperature stress. The results indicated that miRNAs may play an important coordination role in response to low temperature stress in many metabolic pathways by regulating abscisic acid and gibberellin, which provided insight into the roles of miRNAs during low temperature stress and would be helpful for alleviating low temperature stress and promoting low temperature resistant breeding in potatoes.

Keywords: abscisic acid, low temperature stress, miRNA, potato, qRT-PCR

INTRODUCTION

Potato (*Solanum tuberosum* L.) is from the *Solanaceae* family, which has a history of more than 7,000 years of cultivation. Potato cultivars are sensitive to frost, and low temperature is one of the important factors limiting the geographical distribution (Wang Pruski and Schofield, 2012; Xiao-ping, 2015). Research has shown that potato seedlings will stop growing when the temperature is lower than 7°C and will encounter chilling damage at -0.8°C, frost damage at -2°C, and death at -3°C (Fuyi and Mengyun, 1995). Low temperature freezing will damage the potato cell membrane system (Willick et al., 2018), and have a series of effects on the metabolism of malondialdehyde (Lin et al., 2017), ABA (Huang et al., 2017), proline (Phukan et al., 2016), sugar (Bustamante et al., 2016) and other substances, and lead to changes in molecular metabolism and subsequent physiological metabolism. These physiological responses include induction of transient increases in Ca²⁺ (Calcium ion) levels, alterations in membrane lipid composition, increases to antioxidant capacity, and accumulation of osmoprotectant (Fu et al., 2016).

MicroRNAs are a class of about 21–25 nucleotides in length, and are non-coding endogenous single stranded small molecule RNAs that, through post-transcriptional inhibition or degradation of target genes regulating gene expression, play important roles in plant stress, especially in cold regulation (Sun et al., 2015; Song et al., 2017). According to the target gene types, miRNAs that respond to low temperature stress are divided into three categories: the target genes that directly respond to external stimuli, those that indirectly respond to stress by regulating protein transcription factors that play a role in stimulation, and those cooperated by multiple miRNAs that can respond to a variety of stresses and target genes encoding hydrolases or oxidoreductases (Yang et al., 2017).

Currently, a lot of studies focus on the role of miRNAs in response to low temperature stress, such as in *oryza sativa* L. (Cui et al., 2015), *medicago sativa* L. (Shu et al., 2016) and *Prunus dulcis* Mill. (Karimi et al., 2016). At the same time, a lot of studies have been carried out using *Solanaceae* plants. For example, the expression of miR167, miR172, and miR393 were activated in tomato with cold treatment (Chen et al., 2015a,b; Koc et al., 2015). With low temperature stress, 84 differential expression (DE) miRNAs corresponding to 314 DE mRNAs were identified in *S. aculeatissimum* (Yang et al., 2017). Sp-miR396a-5p played critical roles in low temperature stresses through targeting *NtGRF7*-regulated expression of osmotic stress-responsive genes in *Nicotiana tabacum* (Chen L. et al., 2015).

More and more studies have shown that the second-generation high-throughput sequencing technology provides a new solution to revealing that miRNAs respond to adversity stress in plants. As the expression of miRNAs in different species has significant specificity, it is necessary to study the expression

of miRNAs in different plants in response to stress. At present, there are few studies on the response to miRNA in cultivated potato to low temperature stress. This study used Illumina solexa sequencing, qPCR validation, and some physiological analysis to study the effects of different low temperature treatments on the expression of miRNA in cultivated potatoes.

MATERIALS AND METHODS

Materials

The plant material used in the present research was *Solanum tuberosum* L. cv. Favorita. It 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. Then the test materials were divided into control group and treatment group. The control group was grown at 20°C for 4 h (S01). The treatment groups were grown at chilling temperature (0°C, S02), critical growth temperature (2°C, S04) and freezing temperature (-2°C, S05) for 4 h, respectively. The third fully expanded leaf was collected after the treatments, immediately frozen in liquid nitrogen, and stored at -80°C. The RNA samples were extracted from potato leaves and stored at -80°C. All of the RNA samples were subjected to small RNA-sequencing by Illumina solexa sequencing (Biomarker Technologies, Beijing, China) (Buschmann et al., 2016). The samples were also used for qRT-PCR analysis.

Methods

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 (Fisher, 2020). RNA purity was checked by using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured by using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed by using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system. The specific preparation process of the small RNA library is as follows: A total amount of 1.5 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ small RNA Sample Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. First of all, the 3' SR Adaptor was ligated. Then, the 3' SR Adaptor for Illumina, RNA, and Nuclease-Free Water were mixed in the mixture system after incubation for 2 mins at 70° in a preheated thermal cycler. The tube was then transferred to ice. Then, 3' Ligation Reaction Buffer (2×) and 3' Ligation Enzyme Mix ligate were added to the 3' SR Adaptor. It was then incubated for 1 h at 25°C in a thermal cycler. To prevent adaptor-dimer formation, the SR RT Primer hybridized to the excess of 3' SR Adaptor (that remained free after the 3' ligation reaction) and transformed the single-stranded DNA adaptor into a double-stranded DNA

Abbreviations: ABA, abscisic acid; Ca²⁺, calcium ion; miRNA, microRNA; DE, differential expression; DEGs, differential expression genes; qRT-PCR, quantitative real-time PCR; TFs, transcription factors; GO, gene ontology; BP, biological process; MF, molecular function; CC, cellular component; KEGG, Kyoto Encyclopedia of Genes and Genomes; ABE, ABRE binding factors; AAO3, abscisic-aldehyde oxidase; GA, gibberellin.

molecule. dsDNAs are not substrates for ligation mediated. After this, the 5' SR Adaptor was ligated. Then, reverse transcription synthetic first chain was performed. Finally, PCR amplification and Size Selection was carried out. PAGE gel was used for electrophoresis fragment screening purposes, rubber cutting recycling was carried out as the pieces get small RNA libraries, and then the pieces were sequenced with HiSeq 2500 and Hiseq x-ten platform.

Identification and Differential Expression Analysis of Known and Novel miRNAs

Using Bowtie tools soft, the Clean Reads were respectively blasted with Potato database, GtRNadb database, Rfam database and Rcpbase database sequence alignment, filter ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and other ncRNA (Non-coding RNA) and repeats. The remaining reads were used to detect known miRNA and novel miRNA by comparing with known miRNAs from miRBase 21. To identify potential novel miRNAs in potato, the rest of the unmapped small RNA sequences were searched by BLAST against potato genome (PGSC_DM_v4.03). Randfold tools soft was used for new miRNA secondary structure prediction (Rehmsmeier et al., 2004). Differential expression analysis was performed using the DESeq R package (1.10.1). DESeq provided statistical routines for determining differential expression of digital miRNA expression data using a model based on the negative binomial distribution. The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. The miRNAs 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 four samples was performed using the IDEG 6. *P*-value was adjusted using *q*-value. *q*-value < 0.005 and $|\log_2(\text{fold change})| \geq 1$ was set as the threshold for significantly differential expression.

MiRNA Target Prediction

TargetFinder software (<http://targetfinder.org/>) was employed to predict the target genes for all the conserved and novel miRNAs that expressed differentially in four samples under low temperature stress. We used the following rules to predict miRNA target genes:

1. The mismatch between the miRNA and the target gene shall not exceed 4;
2. There must not be more than 2 mismatches between adjacent sites in the miRNA/target gene complex;
3. In the miRNA/target gene complex, the 2–12 positions from the 5' end of the miRNA must not have any mismatches in adjacent positions;
4. MiRNA/target gene complex must not be mismatched at positions 10–11;
5. In the miRNA/target gene complex, there must be no more than 2.5 mismatches at positions 1–12 from the 5' end of the miRNA;
6. The minimum free energy (MFE) of the miRNA/target gene complex should not be less than 75% of the MFE when the miRNA is combined with

its best complement. The parameters in prediction were set as default from the webserver (Bo and Wang, 2005).

GO Enrichment, KEGG Pathway and Network Analysis

Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the Goseq R packages based on Wallenius non-central hyper-geometric distribution, 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 (Kanehisa et al., 2008). Network analysis was conducted using the Cytoscape network platform (<http://www.cytoscape.org/>) (Lopes et al., 2010).

Validation of miRNA Expression by qRT-PCR

To examine the different expression of miRNAs in potato with low temperature, the miRNAs expression was detected by Poly (T) RT-PCR under different low temperatures. 0.5 mg total RNA extracted from potato leaves was used for the reverse transcription with miRNA mature sequence specific Poly (A) RT primers according to the Poly (T) RT-PCR protocol to produce miRNA fused Poly (T) cDNA. All the primers were listed in **Supplementary Table 1**.

Extraction and qRT-PCR Analysis for Target genes

Total RNAs of the samples was extracted by using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The DNase-treated RNA was reverse-transcribed by using M-MLV reverse transcriptase (Invitrogen). Primers for real-time fluorescent quantitative PCR (qRT-PCR) were designed by using Primer Express 3.0 software (**Supplementary Table 1**), and the *ef1- α* gene was used as an internal reference with primers synthesized by Sangon Biotech Co., Ltd (Shanghai, China).

Determination of Abscisic Acid and Gibberellin in Potato

Potato materials with different low temperature treatments were used for the determination of abscisic acid and gibberellin content. Assays were carried out by Anhui Guoke Testing Technology Co., Ltd. (Hefei). GA and ABA were extracted from potatoes using an isopropyl alcohol/water/hydrochloric acid extraction method, and analyzed by liquid chromatography-mass spectrometry (LC-MS/MS) (Izumi et al., 2009). Three biological repeats were made for each sample.

RESULTS

Effects of Different Low-Temperature Treatment of Potato Leaf

In order to compare the effects of different low temperature treatments for potatoes, the potatoes were processed under different temperature conditions. The results showed that with different low temperature treatments, the potato leaves' morphology changed significantly (**Figure 1**). The potato leaves remained normal when they were above 0°C, but when the temperature dropped to -2°C, the leaves showed serious

damage such as water-logging, wilting, or even death. Therefore, the potato was significantly affected by low temperature, and it was further used for exploring profiling of the low temperature responsive miRNAs by Illumina solexa sequencing in this experiment.

Small RNA Library Construction, Sequencing, and Data Analysis

In order to identify miRNAs involved in the process of low temperature stress response in the potato, the four sRNA libraries were generated from the four samples which were treated by different low temperature treatments. After deleting the low-quality sequences and adapter sequences from original data obtained by sequencing, the four samples' clean reads numbers were 24,497,210 (S01), 15,102,752 (S02), 21,559,435 (S04), and 19,464,286 (S05), respectively. All Q30 were greater than 98% (Table 1).

The sequenced small RNAs from the four libraries included ribosomal RNA (13.54–26.89%), small nucleolar RNA (0.02–0.04%), transfer RNA (0.76–0.82%), rpsb (0.87–1.14%), and unannotated (71.26–84.47%). By aligning unannotated sRNA sequences to reference rgenome, 7590356, 4269663, 7436784, and 5271029 reads were mapped (Supplementary Figure 5). To identify miRNAs in potato, all sRNA sequences were compared to known plant miRNAs in miRBase Release 21. 266, 259, 257, and 252 known miRNAs were identified respectively. By mapping all unique sRNA sequences to the potato genome and predicting the hairpin structures for their flanking sequences, 211, 209, 209, and 209 novel miRNAs candidates were identified (Supplementary Table 2). And interestingly, the highest levels of the numbers of the known and novel miRNAs occurred in the control group, and the lowest were at -2°C . This showed that with low temperature treatment, some miRNAs are in a suppressed state.

The majority of reads were in the range of 18–24 nt, in known miRNA, the majorities of them were 21 nt, followed by 24, 22, 20, and 23 nt. In novel miRNA, the majority of them were 24 nt, followed by 21, 22, 23, 20, and 18 nt (Figure 2A). It can be predicted that the pre-miRNA sequences minimum hairpin folding free energy of novel miRNAs was in the range of -137.1 to -18.0 kcal·mol $^{-1}$, and scores are in the range of 0.3–136,012.6 (Supplementary Table 3). The predicted novel miRNA precursor sequence can form a good stem loop structure, which also showed the high reliability prediction of novel miRNA. The candidate miRNAs stem-loop structures were shown in Figure 2B; Supplementary Figure 1.

The base groups in different sites have a certain preference in miRNA sequences, which help miRNA cutting, target gene identification, and other functions. Through the distribution and identification analysis of the different lengthed miRNA first point base, we are able to get all miRNA base distribution statistics. From Figure 2C, most first base of known miRNA and novel miRNA were A and U, and known miRNA had the most T nucleotide content and least C nucleotide, while novel miRNA had most of the A nucleotide content and least C nucleotide.

Cluster Analysis of miRNA in Four Samples

Cluster analysis of DE miRNAs was conducted and the results were shown in Figure 3; Supplementary Table 4. All the DE miRNAs were assigned to three main clades: 1. the miRNAs were down-regulated at 20°C and up-regulated during low temperature stress (2, 0, and -2°C); 2. The miRNAs were up-regulated at 20°C , but most showed a downward trend during low temperature stress (2, 0 and -2°C); and 3. the miRNAs were up-regulated at the 0°C (chilling temperature), but lower at other temperatures (20, 2, and -2°C), which suggested that these miRNAs might be involved in low temperature response.

The venn diagrams of the DE miRNAs among S01 vs. S02 and S01 vs. S04, S01 vs. S05 were shown in Figure 4A, the Venn diagrams of the DE miRNAs between S02 vs. S04 and S02 vs. S05, S02 vs. S04 and S04 vs. S05, S04 vs. S05 and S02 vs. S05, S05 vs. S01 and S05 vs. S02, S04 vs. S01 and S04 vs. S02, S02 vs. S01 and S02 vs. S05 were shown in Supplementary Figure 2. The data showed that there were 16 DE miRNAs in all groups; this indicated that 16 miRNAs were involved in the response of 2, 0, and -2°C low temperature treatment, there are 28, 16, and 10 miRNAs which should respond to low temperature stress of 0, 2, and -2°C respectively.

A total of 211 novel miRNAs were mapped to the potato Genome. TPM analysis showed that 50 novel miRNAs showed the highest expression level at 20°C , accounting for 23.7%. At 0°C , 39 novel miRNAs showed the highest expression level, accounting for 18.5%. At -2°C , 13 novel miRNAs showed the highest expression levels. And at 2°C , 109 novel miRNAs showed the highest expression levels, accounting for 51.7% (Figure 4B). Therefore, the highest expression levels occurred in 161 novel miRNAs, accounting for 76.3% at all the cold temperatures (2, 0, and -2°C).

Target Gene Prediction and Functional Analysis for DE miRNAs

To better understand the biological functions of the potato miRNAs in response to low temperature stress, the putative target genes of the miRNAs were respectively predicted in the potato. 1984 target genes were predicted (Supplementary Table 5). In the known miRNAs, abiotic stresses were related, e.g., *SRG1* for stu-miR156f-3p, ABC transcription factors for stu-miR172a-5p, and serine/threonine-protein phosphatase for stu-miR160a-3p and stu-miR4376-3p. On the other side, novel miRNAs were found where transcription factors (TFs) were related, e.g., cytochrome P450 for stu-novel-miR29658, bZIP transcription factor, ABC transporter and MYB transcription factors for stu-novel-miR43095, F-box protein for stu-novel-miR39617, and UDP-glucosyl transferase for stu-novel-miR12016. Meanwhile, a few miRNAs might regulate only one target, such as stu-miR171b-3p targeting scarecrow protein, stu-miR8020 targeting AFP3 protein, or stu-novel-miR29658 targeting cytochrome P450. Most of the miRNAs were found to regulate more than one target gene, for example, stu-miR172 family had 421 target genes, stu-novel-miR41822 had 211 target genes, stu-novel-miR27782 had 210 target genes, and stu-novel-miR2710 had 121 target genes. At the same time, a target could be regulated by multiple



FIGURE 1 | Morphological changes of leaves from potato treated during low-temperature stress. **(A,E)** The control group in which the plants were grown at 20°C for 4 h (S01); **(B,F)** The treatment groups in which plants were grown at chilling temperature (0°C, S02). **(C,G)** The treatment groups in which plants were grown at critical growth temperature (2°C, S04); **(D,H)** The treatment groups in which plants were grown at water freezing temperature (−2°C, S05).

TABLE 1 | Sequencing data statistics table.

Sample	Raw_reads	Containing “N” reads	Length<18	Length>30	Clean_reads	Q30 (%)
S01	28795169	421	851895	3445643	24497210	98.87
S02	16134299	258	285793	745496	15102752	98.91
S04	23396904	348	483758	1353363	21559435	98.79
S05	21226209	295	909250	852378	19464286	98.99

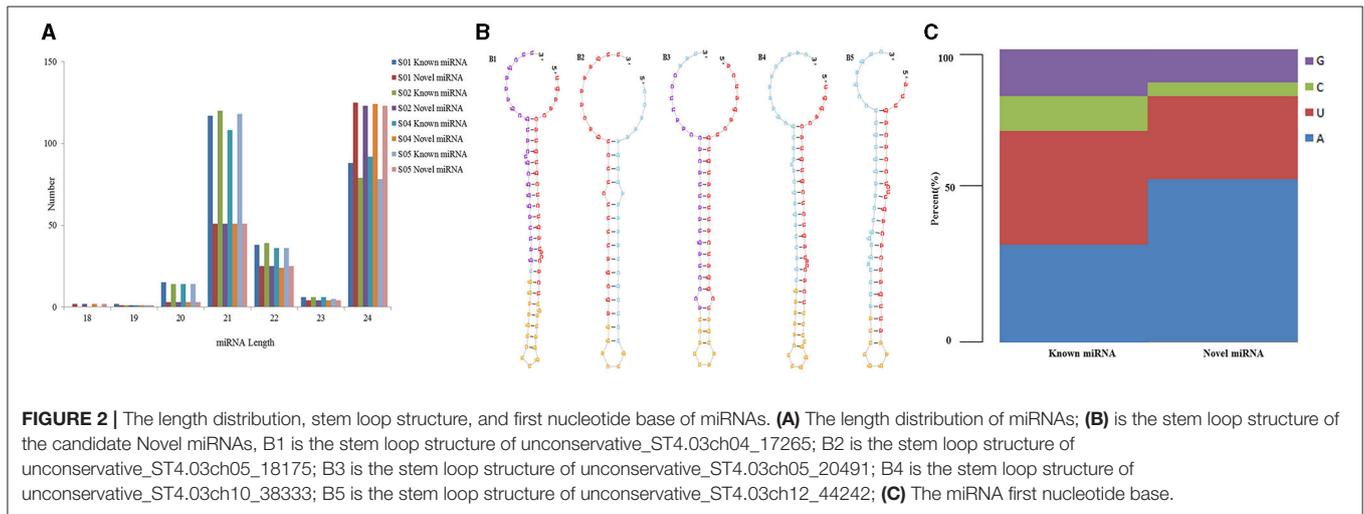
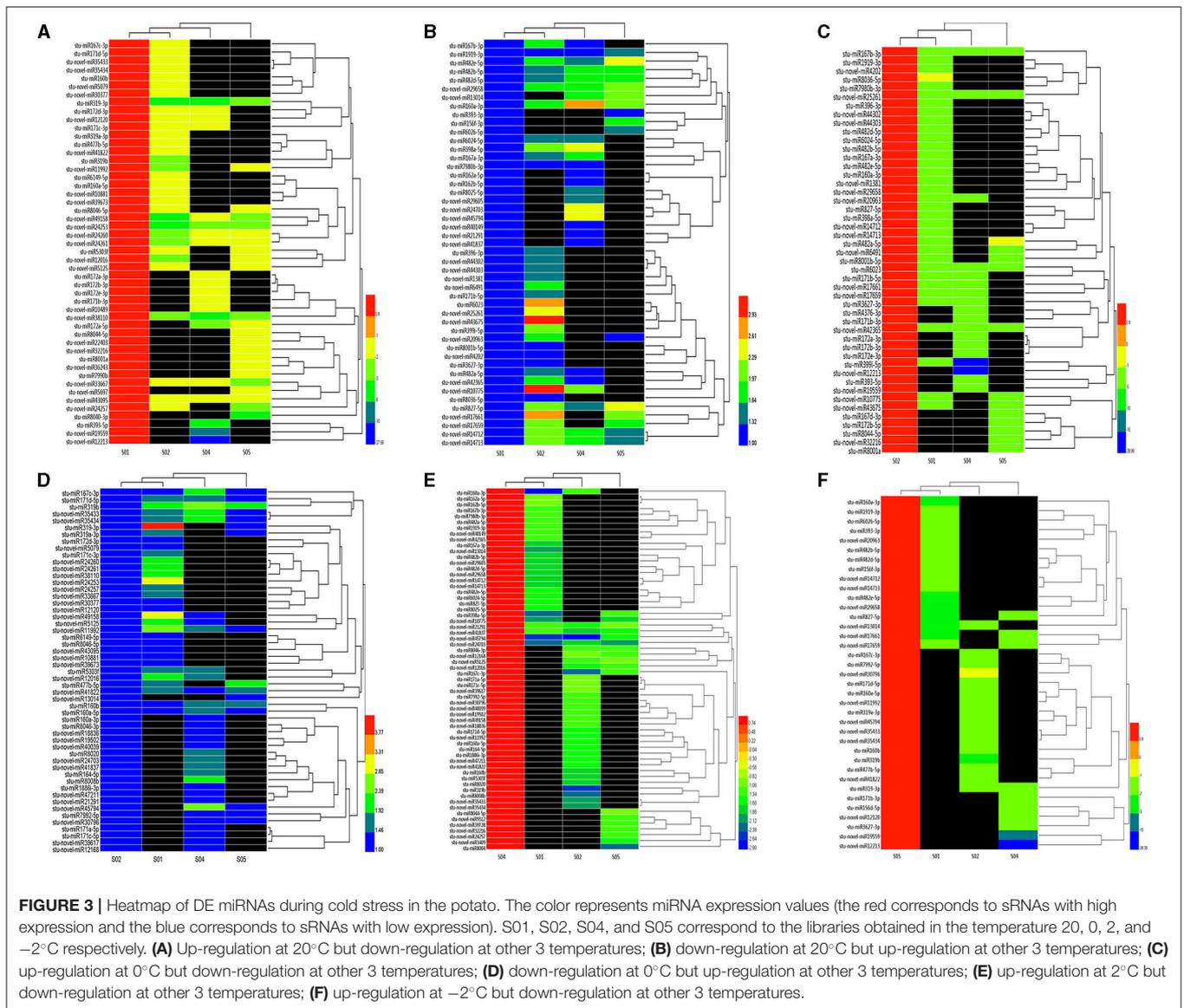


FIGURE 2 | The length distribution, stem loop structure, and first nucleotide base of miRNAs. **(A)** The length distribution of miRNAs; **(B)** is the stem loop structure of the candidate Novel miRNAs. B1 is the stem loop structure of unconservative_ST4.03ch04_17265; B2 is the stem loop structure of unconservative_ST4.03ch05_18175; B3 is the stem loop structure of unconservative_ST4.03ch05_20491; B4 is the stem loop structure of unconservative_ST4.03ch10_38333; B5 is the stem loop structure of unconservative_ST4.03ch12_44242; **(C)** The miRNA first nucleotide base.

miRNAs. For example, sucrose synthase genes were likely to be regulated by 112 miRNAs, while peroxidase were likely to be regulated by as many as 16 miRNAs each. Therefore, this result indicated that during low temperature stress in potato, miRNA regulation was complicated and involved forming a cold response complex network (**Supplementary Tables 5, 6**).

Moreover, the targets of the DE miRNAs for each group (S01 vs. S02, S01 vs. S04, S01 vs. S05, S02 vs. S04, S02 vs. S05, S04 vs.

S05) were respectively categorized by GO and KEGG pathway enrichment analysis. GO enrichment was applied on the DEGs (**Figure 5; Supplementary Table 7**). The BP category numbers was the most in all of the groups, and the second was MF category but except S02 vs. S05. The numbers of terms enriched in CC category were the least in the six groups. The most numbers of terms enriched in MF category was S01 vs. S04, in BP category was S02 vs. S04, and in CC category was S02 vs. S05. The GO



enrichment of different groups exhibited significant differences (Figures 5B,D,E).

KEGG pathway analysis was used for the DEGs (Supplementary Table 8), and the top 20 enriched pathways were shown as Table 2 with the details were shown in Supplementary Table 9. Interestingly, some pathways occurred only in one group. For example, inositol phosphate metabolism pathway and phosphatidylinositol signaling system pathway occurred only in S01 vs. S02, implying that the inositol phosphate might play specific roles in response to 0°C treated temperature in potato. Nitrogen metabolism pathway, purine metabolism pathway, and basal transcription factors pathway occurred only in S02 vs. S04, implying that the three pathways were essential for the critical growth to the 0°C treated temperature in the potato. The alanine, aspartate, and glutamate metabolism pathway and

terpenoid backbone biosynthesis pathway occurred only in S02 vs. S05, implying the two important roles in response to frozen stress. Among all the top 20 enriched pathways, there are four pathways that occurred in all groups. They are starch and sucrose metabolism, zeatin biosynthesis, biosynthesis of amino acids, and protein processing in endoplasmic reticulum, which suggested that these four pathways play an important role in response to low temperature stress in the potato, and soluble sugar and zeatin are also applied to alleviate low temperature stress.

Moreover, the scatter diagrams of the top 20 KEGG pathways enriched of the putative target genes for DE miRNAs were shown as Supplementary Figure 3. Overall, the most enriched pathway evaluated by enrichment factor was in S01 vs. S04, they were photosynthesis, limonene and pinene degradation, riboflavin, and thiamine metabolism; the second most enriched

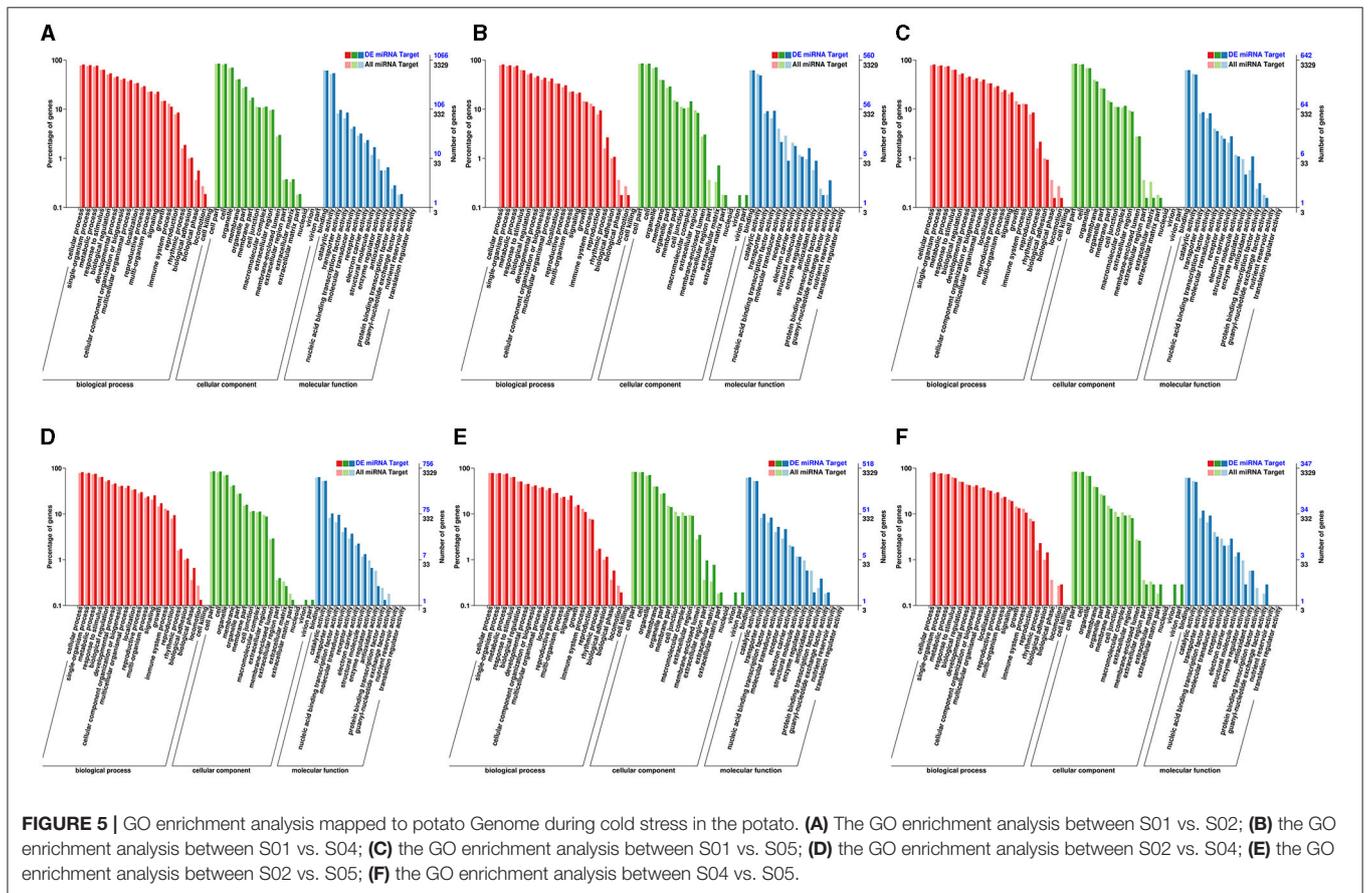
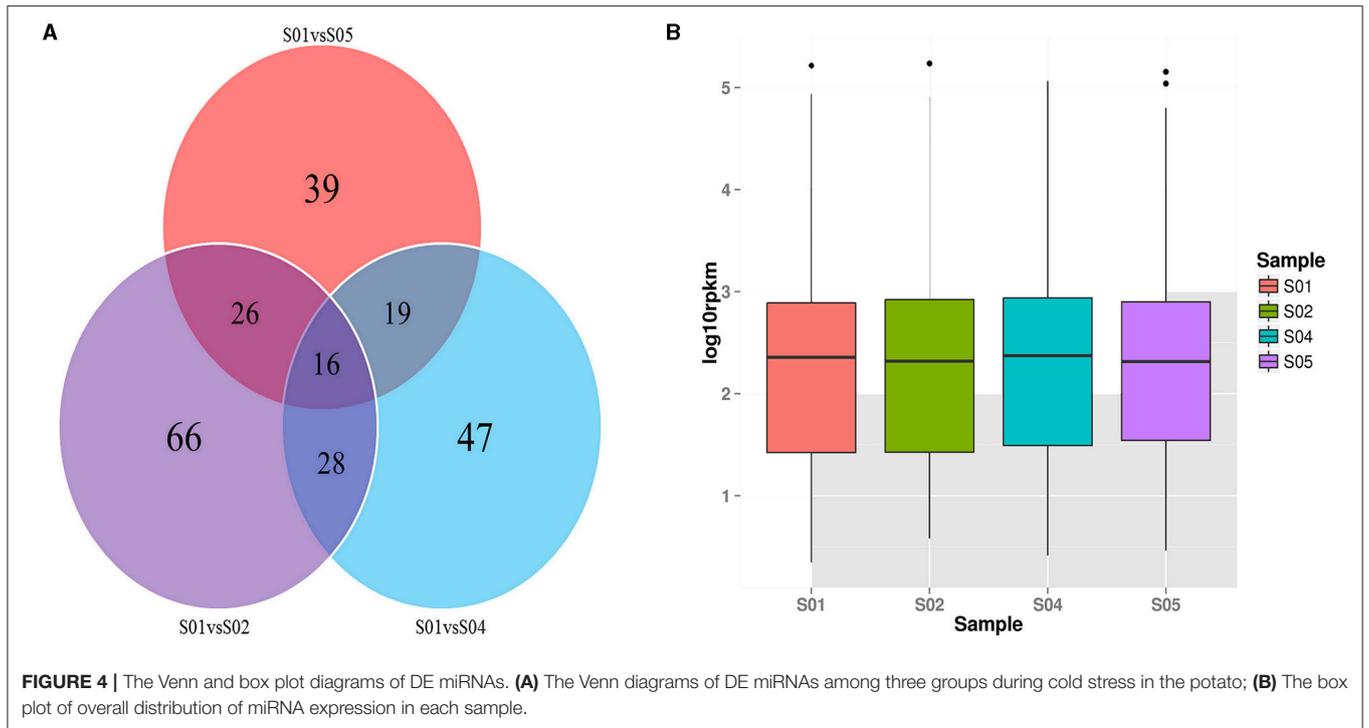


TABLE 2 | The top 20 KEGG pathways enriched by target genes of DE miRNA in 6 groups.

Pathway	Group	Pathway	Group	Pathway	Group
ABC transporters	▲●■	Glutathione metabolism	◆▲●▼	Starch and sucrose metabolism	★◆▲●■
Riboflavin metabolism	▲●■	Fatty acid metabolism	◆▼	Ubiquitin mediated proteolysis	◆▲■
Nitrogen metabolism	●	Linoleic acid metabolism	▲▼■	Protein processing in endoplasmic reticulum	★◆●▼■
Carbon metabolism	◆▼■	mRNA surveillance pathway	●▼	Cysteine and methionine metabolism	▲●▼■
RNA degradation	■	Homologous recombination	■	Limonene and pinene degradation	★◆
Photosynthesis	★◆▲	Oxidative phosphorylation	★▲●▼	Alanine, aspartate and glutamate metabolism	▼
Spliceosome	■	alpha-Linolenic acid metabolism	●▼	Ribosome biogenesis in eukaryotes	●▼
Ribosome	◆▼■	Inositol phosphate metabolism	★	SNARE interactions in vesicular transport	▲●
Peroxisome	★●▼	Biosynthesis of amino acids	★◆▲▼■	Stilbenoid, diarylheptanoid and gingerol biosynthesis	★◆■
Purine metabolism	●	Diterpenoid biosynthesis	★▼	Terpenoid backbone biosynthesis	▼
Thiamine metabolism	★◆▲	Carotenoid biosynthesis	★▲●■	Pantothenate and CoA biosynthesis	★▲●■
Endocytosis	▲▼	Basal transcription factors	●	Degradation of aromatic compounds	★◆▲●
Zeatin biosynthesis	★◆▲▼■	Plant-pathogen interaction	▲●■	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	★◆
Protein export	◆▲	Plant hormone signal transduction	◆●■	Amino sugar and nucleotide sugar metabolism	◆▼
Proteasome	★▲●	Circadian rhythm—plant	★◆▲▼	Phosphatidylinositol signaling system	★

★: S01 vs. S02; ◆: S01 vs. S04; ▲: S01 vs. S05; ●: S02 vs. S04; ▼: S02 vs. S05; ■: S04 vs. S05.

pathways were all in S04 vs. S05, they were carotenoid biosynthesis, pantothenate and CoA biosynthesis, stilbenoid, diarylheptanoid, and gingerol biosynthesis. We can find that S04 (2°C) was a tipping point in this experiment. Photosynthesis, carotenoid biosynthesis and stilbenoid, diarylheptanoid, and gingerol biosynthesis metabolic pathways might play important roles in response to chilling in the potato. In addition, the carotenoid biosynthesis pathway occurred four times with 3 times the highest *q*-value, which implied that carotenoid biosynthesis might play a special role in response to low temperature treatment in the potato.

Through the annotation of the predicted target genes of DE miRNAs (Supplementary Table 6) and the enrichment analysis of GO and KEGG pathways, it could be seen that the functions of the candidates of targets for DE miRNAs in response to low temperature stress were as follows:

The prediction of potato DE miRNAs target genes under low temperature stress indicated that there were multiple low temperature response pathways; these indicated that miRNAs played a key role under low temperature stress by regulating photosynthesis, fatty acid metabolism, carotenoid biosynthesis, and plant hormone signal transduction pathway. We also found that transcription factors, secondary metabolism, and signaling were targeted by so many DE miRNAs; this indicated that transcription factors participated in the function of low temperature stress signal transduction by regulating related functional genes. By increasing the content of peroxidase, sesquiterpenes, aromatic compounds, and carotenoids to degrade peroxides to reduce low temperature-induced oxidative stress, antioxidants improved potato resistance to low temperature stress.

The Target Candidates Belonged to Plant Hormone Signal Transduction Genes in the Potato

Many target candidates belonged to plant hormone signal transduction genes, and some of them occurred at high frequencies (Supplementary Table 10; Supplementary Figure 4). In tryptophan metabolism, the expression of AUX1, TIR1, AUXIAA, and SAUR were affected with the low temperature stress. The AUX1 was targeted by stu-miR167b-3p, which was up-regulated in S01 vs. S02 and S01 vs. S04 and down-regulated in S02 vs. S04 and S02 vs. S05. The AUXIAA was targeted by stu-miR827-5p and stu-miR398a-5p, which were up-regulated in S01 vs. S02, S01 vs. S04 and S01 vs. S05 and down-regulated in S04 vs. S05. The SAUR was targeted by stu-novel-miR41822, which was up-regulated in S02 vs. S05 and down-regulated in S01 vs. S02 and S01 vs. S04; it was both up- and down-regulated in S02 vs. S04.

In carotenoid biosynthesis, just ABF corresponding miRNA was changed with low temperature stress. ABF might be down-regulated in S02 vs. S04, and up-regulated in S01 vs. S02, S01 vs. S04, S01 vs. S05, and S04 vs. S05. This means that, when the temperature was lower than 0°C, the freezing damage began and the expression of ABF began to increase, which led to the stomatal closure. However, by observing the difference between chilling (S02 vs. S04) and freezing (S01 vs. S02, S01 vs. S04, S01 vs. S05 and S04 vs. S05) treatment, we find that the ABF expression was significantly increased at chilling stress (2°C), it means that, at the begin of chilling injury, the plant began to increase the ABF expression level to promote the stomatal closure, thereby reducing the plant’s heat loss and improving the ability of plants to resist low temperature stress.

Target Candidates of DE miRNAs Involved in Carotenoid Biosynthesis Pathways in the Potato

In the scatter diagrams of the top 20 enriched pathways, the carotenoid biosynthesis pathway occurred in the four groups (S01 vs. S02, S01 vs. S05, S02 vs. S04, and S04 vs. S05). The KEGG figures indicated that synthetizes related to the carotenoids were differentially expressed in the pathway, and the synthetic major metabolites included prephytoene-PP, α -carotene, lutein, β -carotenoid, abscisic aldehyde, abscisate, and so on. In the potato, different low temperature treatments had significantly different effects on carotenoid metabolism. From **Supplementary Table 11**, we can see the carotenoid pathway was linked to ABA biosynthesis pathway. ABA belongs to carotenoids 15 carbon steroids; it was reported that ABA is an important signal factor under low temperature stress. Under low temperature stress, the binding ability of abscisic acid and its receptor protein is enhanced, which could sense and transmit signals, thereby playing an important regulatory role in the regulation of plant material balance and stress resistance during low temperature stress. In a word, carotenoids, especially ABA, likely played an important role in the response to low temperature stress in the potato.

Most of the target candidates for DE miRNAs coded the upstream and downstream enzymes in the pathway, e.g., crtB (15-cis-phytoene synthase) targeted stu-miR5303 and stu-novel-miR6491, crtZ (beta-carotene 3-hydroxylase) targeted by stu-novel-miR5125; AAO3 (abscisic-aldehyde oxidase) also targeted by stu-novel-miR5125, and all the miRNAs of the 3 targets expressed differentially during low temperature stress. In addition, crtB might be down-regulated in S01 vs. S02, but up-regulated in S02 vs. S05. CrtZ might be up-regulated in S01 vs. S02, S01 vs. S05, and S04 vs. S05, and down-regulated in S02 vs. S04. AAO3 might be down-regulated in S02 vs. S04, and up-regulated in S01 vs. S02, S01 vs. S05 and S04 vs. S05 (**Supplementary Table 11**). With low temperature treatment, the crtZ expression might be increased, suggesting that the pathway forming zeaxanthin, adonixanthin, and astaxanthin from α -carotene, β -carotene, echinenone, and canthaxanthin should be promoted under the chilling temperature, which further implied that the four kinds of carotenoids might play a key role in cold acclimation in the potato.

Target Candidates of DE miRNAs Involved in the Circadian Rhythm in the Potato

The circadian rhythm is a regular cycle established by the various physiological functions of the organism to adapt to the circadian changes of the external environment. In the potato, the circadian rhythm-plant pathway shared the top 20 pathways in S01 vs. S02, S01 vs. S04, S01 vs. S05, and S02 vs. S05 (**Table 2; Figure 6; Supplementary Table 12**). The targets of DE miRNAs included the genes coding PRR5, FKF1, SPA1, COP1, CRY, and PHYA. PHYA is phytochrome A and was targeted by stu-miR171d-5p. SPA1 is phytochrome A inhibitor; it is involved in the regulation of COP1 protein by cryptochromes. Cryptochrome (CRY) is a kind of flavin-binding protein. In the potato, SPA1

was targeted by stu-novel-miR25261 and CRY2 was targeted by stu-miR827-5p. FKF1 is a member of the F-box protein family (Lee et al., 2017), this gene was targeted by stu-novel-miR43095. COP1 protein is a core inhibitor of photo morphogenesis; it was targeted by stu-miR167a-3p. In the potato, PPR5 was targeted by stu-novel-miR33667, stu-miR171b-5p, and stu-miR8040-3p.

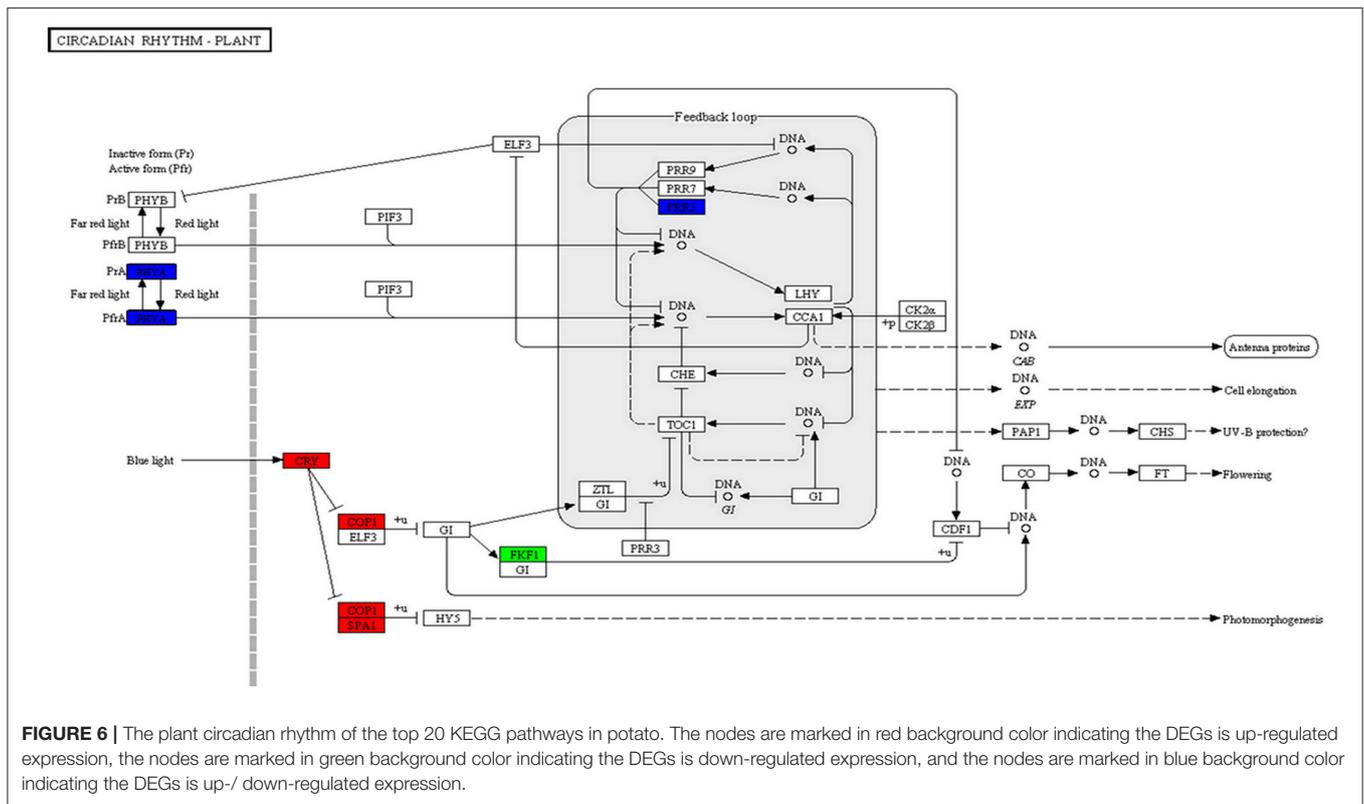
In a word, the target genes PRR5, FKF1, SPA1, COP1, CRY, and PHYA in the circadian rhythm-plant pathway were the key components responsive to low temperature stress in the potato, and it was inferred that during low temperature stress the CRY and FKF1 regulated the biological clock and further activated various cold acclimation pathways; at the same time, the COP1/SPA1 compound negatively regulated HY5 to alleviate growth stress of plants for cold acclimation by the regulation of sulfur and N assimilation by HY5. Furthermore, stu-miR171 and stu-miR171d-5p regulated PPR5 and PHYA to control the biological clock. Therefore, miR171 family was the key miRNA for regulating the biological clock during low temperature stress in the potato.

Target Candidates of DE miRNAs Involved in the Diterpenoid Biosynthesis Pathway in the Potato

The KEGG analysis indicated that the most enriched pathway evaluated by the rich factor in diterpenoid biosynthesis pathway was gibberellin synthetic (**Supplementary Table 13**). During low temperature stress, the diterpenoid biosynthesis pathway had the most significant difference in the treatment of 0°C (S01 vs. S02, S02 vs. S04 and S02 vs. S05). A total of 5 key synthetase genes' expression changed in the pathway: ent-copalyl diphosphate synthase (CPS), ent-kaurene synthase (KS), ent-kaurene oxidase (KO), gibberellin-44 dioxygenase, and gibberellin 3beta-dioxygenase. CPS was targeted by stu-miR171d-5p, which was down-regulated in S01 vs. S02 and up-regulated in both S02 vs. S04 and S02 vs. S05. KS was targeted by stu-miR477b-5p, which was down-regulated in S01 vs. S02 and up-regulated in S02 vs. S05. KO was targeted by stu-miR167d-3p, and not changed in S01 vs. S02 but down-regulated both in S02 vs. S04 and S02 vs. S05. Gibberellin-44 dioxygenase was targeted by stu-novel-miR6491, it was up-regulated in S01 vs. S02 and down-regulated in S02 vs. S05. Gibberellin 3beta-dioxygenase (GA_3OX_1) was targeted by stu-novel-miR10881; it was just down-regulated in S01 vs. S02. It can be seen that the freezing temperature treatment (0°C) and other temperature treatment expressions were significantly different. In S01 vs. S02, the content of GA_9 and GA_{20} might be significant decreased, but the GA_4 content was increased. In S02 vs. S04 and S02 vs. S05, the GA_4 content might be significantly decreased and GA_9 and GA_{20} content increased. This may have something to do with the different biological functions they have.

Differentially Expressed miRNAs and Their Target Genes qRT-PCR Validation

We verified the above results using qRT-PCR, and determined the expression patterns of three miRNAs and their target genes under different low temperature stresses. The results were shown



in **Figure 7; Supplementary Table 14**. The expression of *stu-novel-miR5125* in different low temperatures was significantly changed, it began to decrease, and the lowest expression level was at 2°C (**Figures 7A,B**). The target genes of *stu-novel-miR5125* were *StuABF8011* and *StuAAO36826*, the *StuABF8011* expression trend was up and down and reached a maximum at 2°C; *StuAAO36826* expression trend was also up and down and reached a maximum at 0°C. It means that the expressions of these two genes were significantly negatively regulated by *stu-novel-miR5125*.

The expression level of *stu-miR827-5p* was up-down-up and the maximum was at -2°C (**Figure 7C**). But the expression level of its corresponding target gene, *StuCRY217172*, was also increased significantly. The expression level of *stu-novel-miR10881* was down and up and the minimum was at 0°C; the corresponding expression change of target gene *GA3ox123158* was increased first and then decreased and the maximum was at 0°C. This is exactly the opposite of its corresponding miRNA expression (**Figure 7D**).

The expression patterns of the three target genes, namely *StuABF8011*, *StuAAO3682*, and *GA3ox123158*, were opposite to those of the related miRNAs, which implied the three targets were negatively regulated by the two miRNAs (**Figures 7A,B,D**). The expression pattern of *stu-miR827-5p* was the same with its target gene *StuCRY217172*; we speculated that this might be due to the simultaneous regulation of *StuCRY217172* by multiple miRNAs.

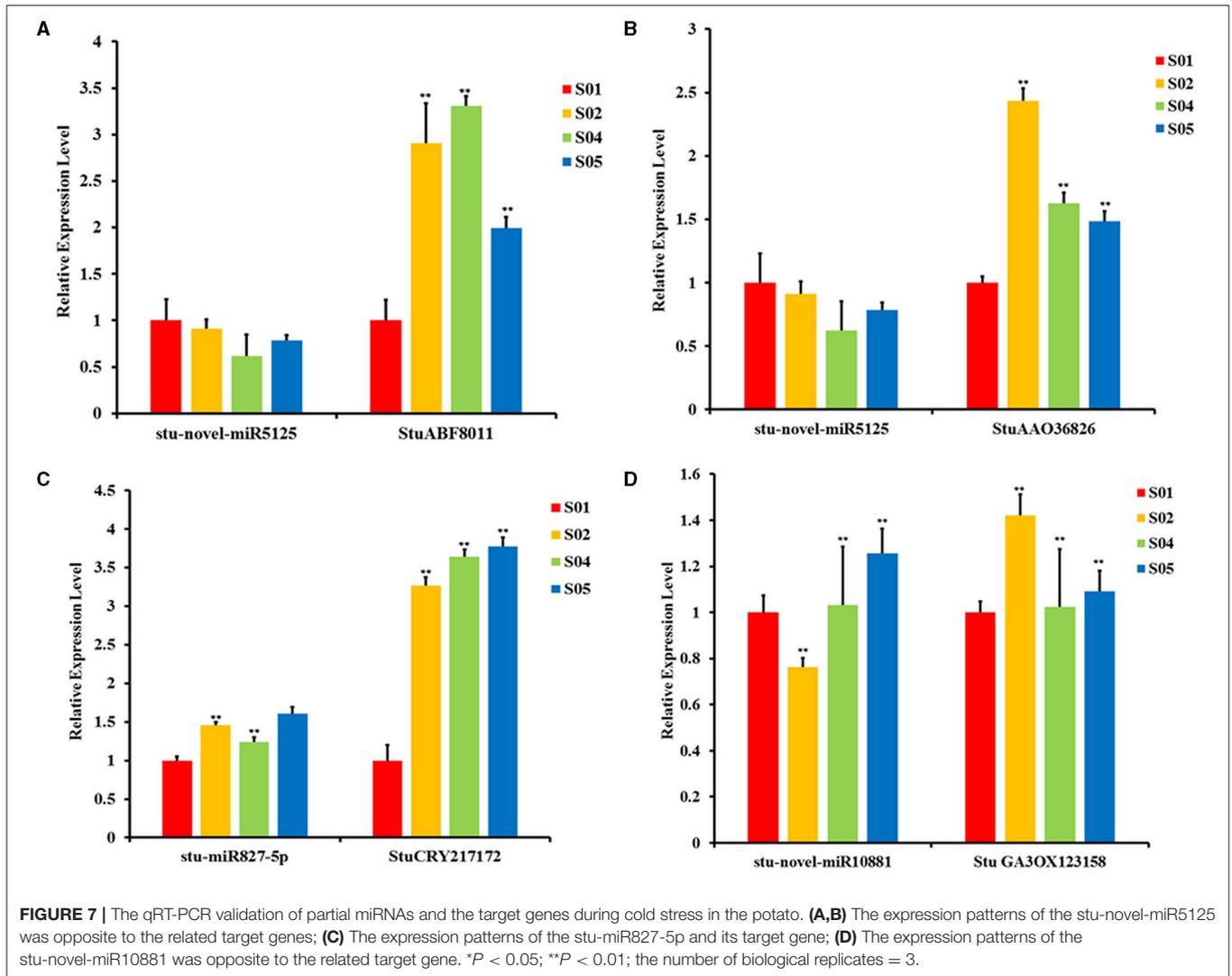
Furthermore, we found that *stu-novel-miR5125* simultaneously regulated plant hormone signal transduction and

carotenoid biosynthesis. During low temperature stress in the potato, *StuABF8011* and *StuAAO36826* were targeted by *stu-novel-miR5125*. In plant hormone signal transduction pathway of potato, *StuABF8011* could activate ABA-dependent SnRK₂, thereby activating downstream ABA signals. In Carotenoid biosynthesis pathway, *StuAAO36826* mainly catalyzed abscisic aldehyde to form abscisic acid. With low temperature treatment, the expression of these two genes had increased significantly, it showed that low temperature treatment significantly induced the ABA synthesis and promoted the strengthening of ABA-dependent signal transduction. We can also conclude that in the potato with low temperature treatment, *stu-novel-miR5125* significantly regulated abscisic acid expression levels in two metabolic pathways, and this also verified the accuracy of the target gene prediction.

Effects of Low-Temperature Treatment on Abscisic Acid and Gibberellin Content in Potato Plants

In order to clarify the effect of low temperature treatment on the content of ABA and gibberellin in potatoes, we measured the ABA and gibberellin in potato leaves which were under 20°C, 2°C, 0°C, and -2°C treatment for 4 h (**Figure 8**).

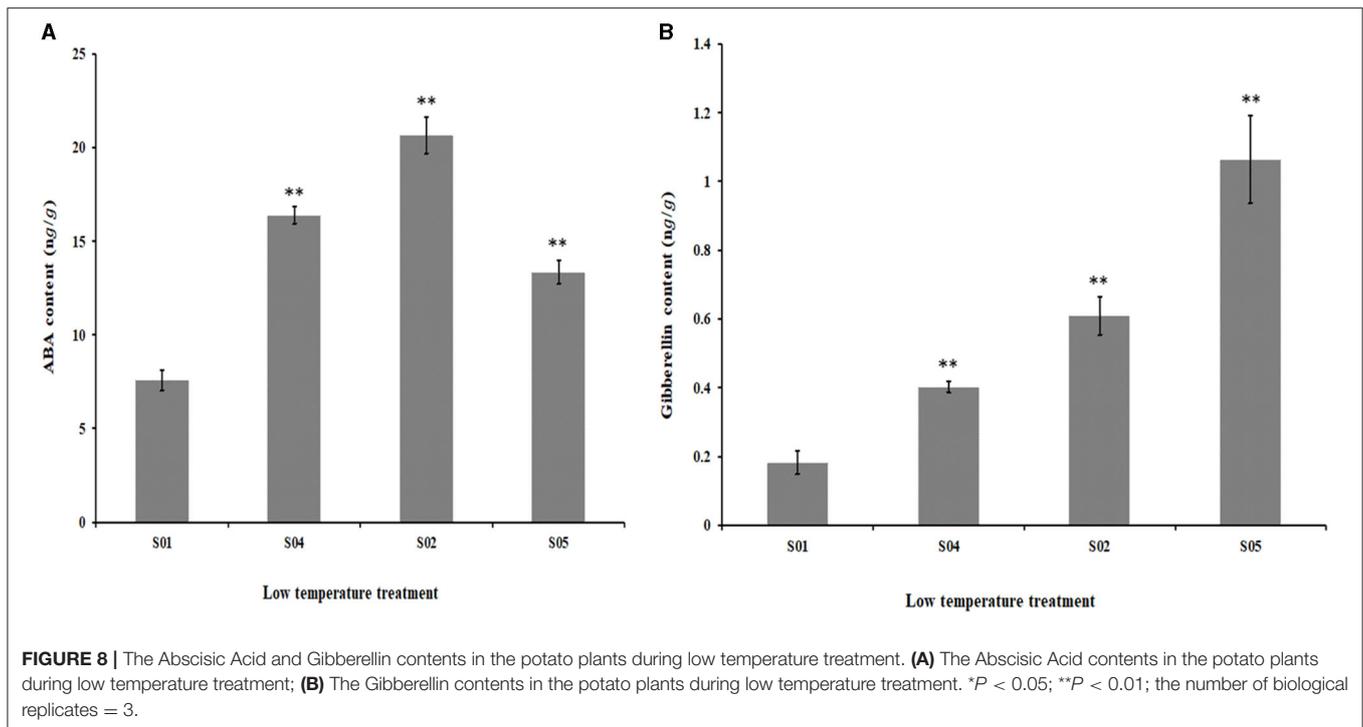
ABA is the first messenger of resistance gene expression in plants and plays an important role in regulating the balance of substances in plants and inducing stress resistance (Wang et al., 2019). With the low temperature treatment, the content



of ABA in potato leaves showed a trend of increasing and then decreasing, and the expression reached a maximum at 0°C and then began to decrease. It can be seen that the change trend of ABA content was consistent with the change trend of *StuAAO36826* expression, but was opposite to *stu-novel-miR5125*. The expression level of *StuAAO36826* was highest when treated at 0°C, and the ABA content also reached the highest at this time, which indicated that when potato was subjected to low temperature stress at 0°C, the expression of *stu-novel-miR5125* was reduced to promote the expression of its corresponding target gene *StuAAO36826*, and then increased ABA content, which strengthened the role of ABA's first messenger and promoted a series of physiological reactions such as the closure of stomata and increase of the content of proline and soluble sugar, thereby improving the ability of plants to resist low temperature stress. It can also be seen that the ABA content in potato leaves increased first, but decreased at -2°C. This may be due to the long-term low temperature inhibiting or destroying the structure and function of the enzymes related to ABA

synthesis, and its protective effect on potato low temperature injury was reduced.

Gibberellin is a type of diterpene acid, which plays an important role in plant growth and adversity adaptability. It promotes the accumulation of protein and non-structural carbohydrates by participating in the transmission of light signals, and induces the expression of cold-resistant calcium-dependent protein kinase genes to improve the cold-resistant ability of plants (Zhou et al., 2014). External application of gibberellin can reduce the electrolyte leakage rate of plants under low temperature conditions, enhance the stability of plant cell membranes, improve the water retention capacity of leaves, restore plant tissue damage caused by low temperature treatment, and offset the inhibitory effect of adverse environmental conditions on plant growth (Sun et al., 2020). With low temperature treatment, the gibberellin content of potato leaves had a rising trend, and reached the maximum at -2°C. It can be seen that there was a certain difference between the changing trends of gibberellin and *GA3ox123158*. The highest



expression of *GA₃ox₁23158* was at 0°C, while the expression of gibberellin reached its maximum at -2°C. This may be due to the fact that when the temperature dropped below 0°C, the low temperature had severely restricted the enzyme activity associated with *GA₃ox₁23158* expression, resulting in a decrease in its expression. At the same time, although the expression of gibberellin synthesis-related enzymes decreased, the activity of gibberellin degradation-related enzymes also decreased, leading to an increase in its accumulation in the plants, thus enhancing its role as a signal transducer and promoting the plants to initiate a series of physiological and biochemical responses corresponding to low temperature stress and improving the plants' cold resistance (Liu Y. et al., 2017; Niharika et al., 2020).

DISCUSSION

Low temperature freezing damage is an uncontrollable climatic factor; when it occurs during the potato growing season, especially the potato seedling stage, it will seriously affect the growth of the potato. Low temperature has become one of the most important factors restricting the distribution and development of the potato industry (Kou et al., 2018). The cultivated potato Favorita is an early-maturing and high-quality potato variety that is widely planted in China. However, it is sensitive to low temperature frost, and it is extremely vulnerable to early spring and early autumn frost in the second season of potato cropping. Therefore, it is of great significance to carry out research on potato cold resistance.

Known and Novel miRNAs and Their Differential Expression in Potato

Increasing evidence demonstrated that miRNAs play important roles in plant response to abiotic stress (Shukla et al., 2018; RGd et al., 2019; Yan et al., 2020). In this study, 307 known miRNAs which belonged to 73 small RNA families and 211 novel miRNAs had been identified in S01, S02, S04, and S05. It was similar to previous reports on tomato and pepper. The expression levels ranged from a few to thousands and different conserved miRNAs had different expression levels (Cao et al., 2014; Din et al., 2016; Liu M. et al., 2017; Liao et al., 2021). However, a lot of novel miRNAs in potato had a higher expression level, with reads of more than 100, which was different from the previous studies in tomato and Arabidopsis (Fahlgren et al., 2007).

32, 24, 17, 26, 17, and 10 known and 34, 23, 22, 25, 13, and 18 novel miRNAs were characterized as differentially expressed miRNAs in potato. There were 16 common differentially expressed miRNAs in cryogenic treatment, they were stu-miR160a-3p, stu-miR319-3p, stu-novel-miR24253, stu-novel-miR43095 and so on, which suggested that the 16 miRNAs might be involved in low temperature treated responses in the potato. stu-miR319-3p had a significant reduction in S01 vs. S02 and S01 vs. S04, which was down-regulated 2^{4.2} and 2^{4.1} times. stu-novel-miR24253 had a significant reduction in S01 vs. S05, it was down-regulated 2^{3.0} times. From the above studies, it can be seen that low temperature stress significantly changed the expression of miRNAs in potatoes. This result is consistent with previous studies on potatoes and other species (Zhou et al., 2019; Yan et al., 2020). In this study, we identified many miRNAs that respond to low temperature stress. At the same time, we also

identified many miRNAs involved in drought stress, pest defense, salt tolerance, and secondary metabolism, such as *stu-miR167b-3p*, *stu-miR171b-3p*, and *stu-miR827-5p* (Wang et al., 2016; Qiao et al., 2017; Sarkar et al., 2017; Shin et al., 2017). At the same time, this study also discovered some known miRNAs that only have the function of responding to low temperature stress in potatoes, such as *stu-miR530*, *stu-miR156d*, and *stu-miR167b*; these conserved miRNAs were likely to focus on regulating the cold resistance of the potato, which is consistent with the research results of significant differences in miRNA expression of different species (Juan Liu et al., 2017).

ABA and GA3ox1 Are Involved in miRNA Response to Low Temperature Stress

In plants, ABA is a semiterpenoid derived from carotenoid which is ubiquitous and potentially plays an important role in the adaptation of these plants to different stresses (Arif et al., 2018). ABF belongs to A subfamily of bZip transcription factors, they specifically exist in plants (Yoshida et al., 2015). They can recognize and regulate the expression of ABA-responsive genes and enhance the ability of plant resistance to environmental stresses. They are in a critical position in ABA transduction pathway and their functions involve signal transduction for freezing and drought (Hwang et al., 2019). ABF transcription factors recognize and bind to ABRE element of stress-responsive genes' promoter and improve plants low temperature resistance by regulating stress-related gene expression (Nakashima et al., 2014). Therefore, ABF transcription factors act as a transmission switch in abiotic signal transduction process, playing an important role in plant adversity resistance. For example, over expression of *TaEXPB7-B* in *Arabidopsis* promoted the stomatal closure, thereby increasing the tolerance and survival of plants under conditions of low temperature stress (Feng et al., 2019). In wild-type *Arabidopsis thaliana*, they demonstrated that ABFs mediate rapid ABA induction of group A PP2C genes, thus playing a role in the negative feedback regulation of ABA signaling (Wang et al., 2019). In our study, with low temperature treatment, *stu-novel-miR43095* and *stu-novel-miR5125* expression changed significantly, which indicated that they were affected by low temperature stress. Further analysis showed 1 out of the 284 target genes of them was ABF gene; it was targeted by *stu-novel-miR43095* and *stu-novel-miR5125*. qRT-PCR analysis showed that the expression of *StuABF8011* increased with low temperature treatment, this was exactly the opposite of its corresponding miRNAs expression, indicating that its expression was negatively regulated by *stu-novel-miR5125*.

AAO₃ is an acetaldehyde dehydrogenase that catalyzes the final step of ABA biosynthesis. In *Arabidopsis* mutants, the AAO3 gene deletion caused ABA deficiency in plants (Seo et al., 2000). It was found that the early senescing phenotype of *CSAPOX* lines under dark condition is possibly caused by the increased AAO3, and thus increased ABA expression; this is the same as Yang et al. (So et al., 2020). In our study, *StuAAO36826* was the target gene of *stu-novel-miR5125*; qRT-PCR analysis showed that it was negatively regulated by *stu-novel-miR5125*. Therefore, we speculated that with low temperature stress, the

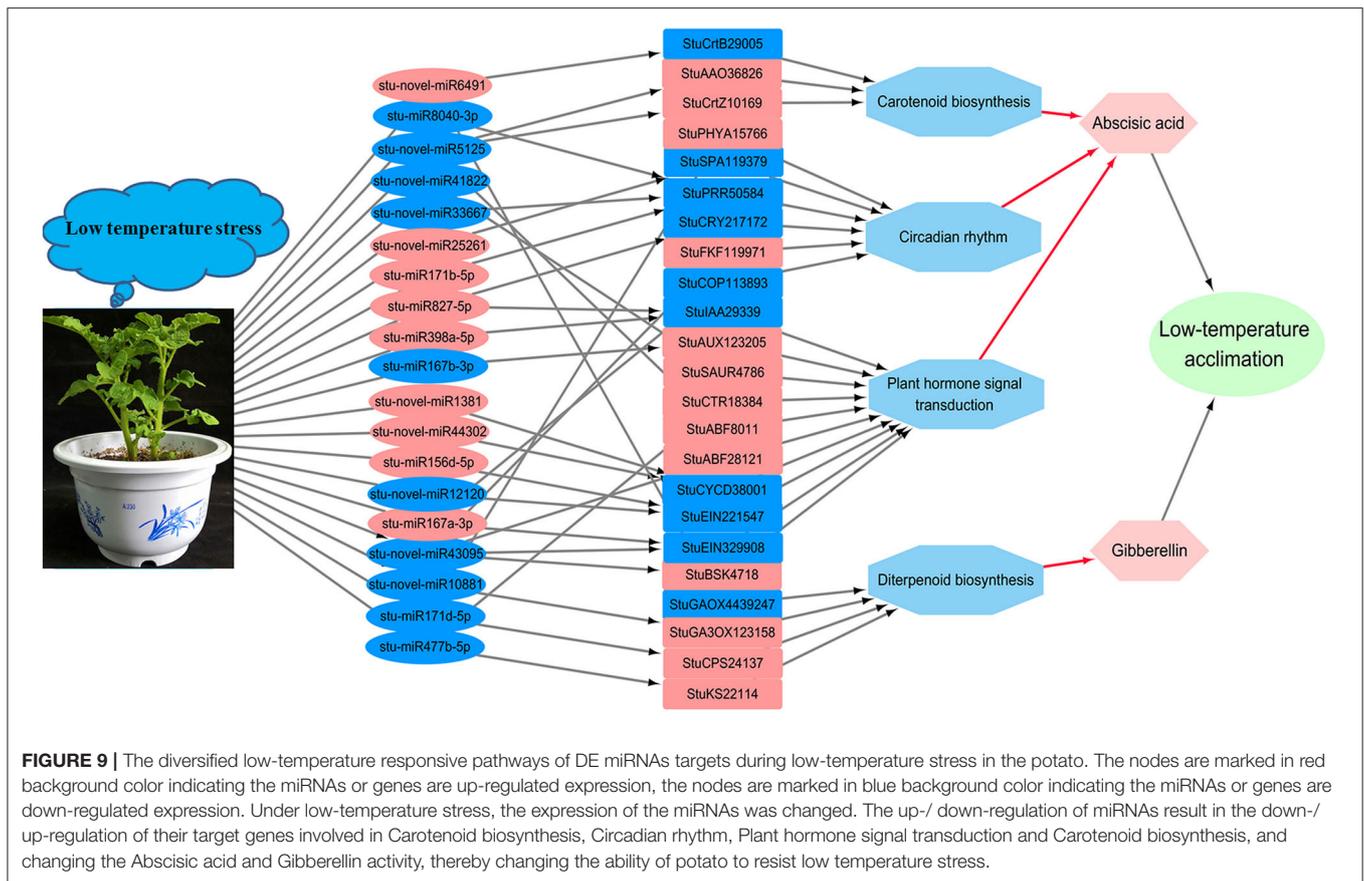
expression of *stu-novel-miR5125* was suppressed. The down-regulation of *stu-novel-miR5125* resulted in the up-regulation of *StuABF8011* and *StuAAO36826* which were involved in ABA metabolism and promoted the strengthening of ABA-dependent signal transduction, thereby improving the ability of potato to resist low temperature stress. This result is consistent with Feng and Wingler (Feng et al., 2019; Wingler et al., 2020).

CRY is the blue-light receptor which mediates light regulation in the growth and development of plants. Inhibition of CRYs in *Arabidopsis thaliana* can promote leaf stomatal closure, thereby reducing plant energy loss and improving plant resistance to low temperature stress. In rape, it was found that over expression of CRY inhibited ABA activity, indicating that CRY was involved in the ABA signal transduction pathway (Pooja, 2014). This is mainly because CRY2 inhibits ABA expression by combining with ABI4, an important transcription factor of ABA signal transduction pathway. When plants are stressed, CRY2 expression will be reduced to promote ABA expression, thus improving plant response to stress (Zhou et al., 2017; D'Amico-Damiao and Carvalho, 2018). In our study, the expression of *StuCRY217172* was increased; this was the same with its corresponding miRNAs. We speculated that this may be due to the fact that *StuCRY217172* received the regulation of multiple miRNAs at the same time and other miRNAs expression level decreased with low temperature treatment.

Gibberellin 3beta-dioxygenase (*GA3ox1*) is a key enzyme involved in gibberellin synthesis, it directly acts on the formation of active GAs (Geshnizjani et al., 2018). It participates in the process of regulating plant growth and stress resistance by controlling GA activity. For example, while corn was subjected to low temperature stress, *GA3ox* reduced the low temperature damage to corn by promoting the synthesis of active GA1 (Yang et al., 2016). In this paper, *GA3ox1* significantly received negative regulation from *stu-novel-miR10881*.

CONCLUSIONS

In this study, the profiling of the low temperature responsive miRNAs by Illumina solexa sequencing was performed during low temperature stress. According to the previous results, we hypothesized that the potato responds to low-temperature stress through the following steps (Figure 9): (1) The low temperature affected the expression quantity of miRNAs; (2) The expression change of miRNAs resulted in the corresponding regulation of target genes expression level changing, especially the genes in carotenoid biosynthesis, circadian rhythm, and plant hormone signal transduction; (3) Through the expression changing of the genes which related to abscisic acid synthesis in the above three metabolic pathways, and related to gibberellin synthesis in diterpenoid biosynthesis, the content of abscisic acid and gibberellin in potato leaves changed; and (4) The change in abscisic acid and gibberellin content caused the change in the potato's ability to respond to low temperature stress. These



results provided the ABA and gibberellin was likely to play a central coordinating role in response to low temperature stress in many metabolism pathways in potato. The profiling of miRNAs by Illumina solexa sequencing in response to low temperature stress in potato, which provided insight into the roles of miRNAs during low temperature stress, and would be helpful to promote the selection of low temperature resistant potato variety.

DATA AVAILABILITY STATEMENT

The sequencing data of this study are available in the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) (accession number: PRJNA641179). The other supporting data are included as **Supplementary Material**.

AUTHOR CONTRIBUTIONS

CY, HL, and NZ designed the experiments and drafted the manuscript. CY, NZ, FW, BX, and LZ analyzed the data. CY, NZ, YF, and YS carried out the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.727081/full#supplementary-material>

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