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Effects of low temperature on mRNA and small RNA transcriptomes in *Solanum lycopersicoides* leaf revealed by RNA-Seq

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ABSTRACT

The plant low temperature tolerance mechanisms have been studied in the Arabidopsis, tomato, *Solanum commersonii*, *Solanum tuberosum*, *Chorispora bungeana*, and Chinese cabbage at the transcriptional level. Some genome-wide works to identify cold-regulated genes, but no comprehensive research of the *Solanum lycopersicoides* transcriptome under low temperature stress have been performed. *S. lycopersicoides* is more freeze-tolerant than the cultivated tomato. We analyzed the low temperature transcriptomes and small RNA fractions of *S. lycopersicoides* leaf tissue using an Illumina platform for high-throughput RNA sequencing (RNA-seq). There were 59,286 unigenes obtained using de novo assembly, and 2052 down-regulated and 2409 up-regulated unigenes were identified in response to chilling. The expression of six cold-regulated genes was confirmed by qPCR. Some biological processes were showed, by gene ontology term enrichment analysis of the cold-regulated genes, including 'response to stimulus', 'signaling', and 'cell killing' in the response of *S. lycopersicoides* to chilling. In addition, we identified a total of 952 novel miRNA candidates that may regulate relevant target genes. Our data indicated that certain miRNAs (e.g., sly-miR156a, sly-miR397, and unconservative_SL2.50ch00_21686) play roles in response to low temperature stress. Sequencing of mRNAs and miRNAs revealed new genes and allowed us to have new assumptions for a low temperature tolerance mechanism.

1. Introduction

Some plants increase their cold tolerance to deal with the low temperature. This phenomenon is considered as cold acclimation. In recent years, many cold-regulated genes have been identified in plants under cold stress. The CBF cold-responsive pathway was considered as the best well known cold tolerance pathway in plants [1]. There are three CBF/DREB1 family members, including CBF1, CBF2, and CBF3 (DREB1b, DREB1c, and DREB1a, respectively), encoding the DNA-binding proteins of AP2/ERF family [2]. Overexpression of CBF genes increase the cold tolerance of *Arabidopsis thaliana* [3], *Brassica napus* [4], poplar [5], and potato [6], but do not increase the cold tolerance in tomato [7].

The roles of cold regulated genes in plants cold acclimation show that different expression of genes are related to different abilities of plants cold adaption. In *A. thaliana* and *Chorispora bungeana*, many alterations in gene expression begin within minutes of transferring plants to low temperature [8–11].

In plants, 21 to 24 microRNAs (miRNAs) play a role as negative regulators in gene expression [12]. Research has indicated the key role of miRNAs in regulating organ development and biological

Abbreviations: ABA, Abscisic acid; CBF, C-repeat binding factor; DEG, Differentially expressed gene; EST, Expressed Sequence Tag; GO, Gene ontology; ICE1, inducer of CBF expression 1; miRNA, microRNA; JA, Jasmonic acid; MDA, Malondialdehyde; PCR, Polymerase chain reaction; PLC, phospholipase C; POD, Peroxidase; RPKM, Reads per kilobase of a gene per million reads; SNP, Single nucleotide polymorphism; SOD, superoxide dismutase; SSR, Simple Sequence Repeat; TF, Transcription factors; UV, Ultraviolet; ZAT10, Cys2/His2-type zinc-finger protein.

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processes [13]. miRNAs are also related with abiotic stress responses [14,15].

Solanum lycopersicoides (LA2408) is a perennial, alpine subnival plant which can grow at low temperature [16,17]. However, there are few reports about its cold tolerance mechanism, distinguishing *S. lycopersicoides* from other temperate and tropical plants. It is also unknown whether key regulators, for example miRNAs, play a role in *S. lycopersicoides* under cold stress. To understand the gene network underlying why *S. lycopersicoides* can acclimate to cold and survive freezing temperatures, we report the results of an RNA-Seq transcriptome and miRNA analysis of RNA populations obtained from the cold-treated leaves of plants. The results show that many changes in transcriptome happen in plants transfer from warm to cold.

2. Materials and methods

2.1. Plant material and cold stress treatments

S. lycopersicoides (LA2408) was provided by Tomato Genetics Research Center (University of California, Davis, USA). *S. lycopersicoides* was treated after grown at 25 °C with 16 h light and 8 h dark cycles for 12 weeks. Cold treatments began at 12 pm at 4 °C under light and continued for 0 (control), 1 and 12 h to avoid changes from the circadian rhythm.

2.2. Physiological responses to cold stress

The malondialdehyde (MDA) content was assayed as described by Campos with slight modification [18]. Peroxidase (POD) activities were determined as described by Quiroga with slight modification [19].

2.3. Total RNA isolation and library preparation

The total RNA from leaf tissue was isolated using TRIzol reagent (Invitrogen) and digested with RQ1 DNase (Promega) to remove DNA. The quality and integrity of the total RNA were detected by a SmartSpec plus Spectrophotometer (Bio-Rad) and 1.5% agarose gel electrophoresis. Polyadenylated mRNAs were purified and concentrated with oligo(dT)-conjugated magnetic beads before being used for directional RNA-seq library preparation. Purified mRNAs were fragmented at 95 °C and performed by end repair and 5′ adaptor ligation, and followed by reverse transcription using randomized hexamer and RT primer with 3′ adaptor. Purified cDNAs were amplified, and 150–200 bp PCR products were quantified and purified. RNA-seq libraries were prepared and applied to the Illumina Genome Analyzer IIx system for 32 nt single-end sequencing by Biomarker Inc. (Beijing, China).

For small RNA library preparation, 3 μg total RNA was used for small RNA cDNA library preparation with the Balancer NGS Library Preparation Kit for small/microRNA (GnomeGen). The purified small RNA libraries were quantified with a Qubit Fluorometer (Invitrogen) and used for cluster generation and 18–30 nt single-end sequencing analysis using the Illumina Genome Analyzer IIx system.

2.4. De novo assembly

Reads were assembled separately from each *S. lycopersicoides* library using the Trinity method [20]. There were three software in Trinity, including Inchworm, Chrysalis and Butterfly, which were performed to process RNA-Seq reads sequentially. Firstly, the Inchworm program was used to assemble reads to contigs. Secondly, the Chrysalis program was used to cluster the minimal overlapping contigs. Thirdly, the Butterfly program was used to construct the transcripts. Finally, the multiple sequence alignment tool BLAST was used to cluster the transcripts by the similarity of right match length [21]. The coding sequences (CDS) of the unigenes were predicted by EMBOSS (http://emboss.sourceforge.net/) and the longest CDS was considered as the complete CDS of the unigenes.

2.5. Functional annotation and differential expression

A series of BLAST searches were used to annotate unigenes to find the most accurate difinition for each sequence [22]. The assembled unigenes were compared with sequences in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), the National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/), the Swiss-Prot protein database (http://web.expasy.org/docs/swiss-prot_guideline.html) and European Molecular Biology Laboratory European Bioinformatics Institute (EMBL-EBI) database (http://www.ebi.ac.uk/). GO annotations of unigenes were obtained by the Blast2GO program [23]. GO functional classification of unigenes was performed by the WEGO software. The expression level of genes were evaluated and normalized by RPKM (reads per kilobase of exon model per million mapped reads) [24]. Differentially expressed genes were identified by IDEG6 software [25], and the Benjamini–Hochberg false discovery rate (FDR <0.01) was used to correct the results of statistical tests. Significantly differentially expressed sequences were obtained by this method whose adjusted *P* value was <0.001 and there was at least a two-fold change in the RPKM values between two libraries.

2.6. Detection of SSR markers and SNPs

MISA software (http://pgrc.ipk-gatersleben.de/misa/) was used to identify the potential SSR markers in the unigenes. Potential SNPs were detected using soapsnp software [26].

2.7. q-PCR analysis

To validate the transcript abundance of genes measured by RNA-Seq, we performed q-PCR using Power SYBR Green Mastermix in an Applied Biosystems 7500 Real-Time PCR System. The RNAs from *S. lycopersicoides* used in RNA-seq were reverse transcriped into cDNAs. Twelve primers are listed in S5 Table. The ACTIN gene was used as a reference in these experiments. Three technical replicates were used for q-PCR. The single amplicons were confirmed by melting curve analysis and gel electrophoresis of the final product. The CT value of each gene was normalized to the CT value of the reference gene to determine the relative fold changes for each sample, and it was calculated using the ΔΔCT method as described [27].

2.8. Identification of miRNA in S. lycopersicoides

The reads generated after Solexa sequencing were analyzed on the FASTX-toolkit website (http://hannonlab.cshl.edu/fastx_toolkit/). After basic analysis (filtering out low quality reads, trimming the adaptors and removing overrepresented sequences and noise), clean reads and unique reads (reads with non-redundancy) were obtained. The SOAP tool package (http://soap.genomics.org.cn/index.html) [26] was used to BLAST clean reads against Repbase (http://www.girinst.org/) and Rfam (ftp://ftp.sanger.ac.uk/pub/databases/Rfam). The rRNA, tRNA, snRNA, and snoRNA were annotated by aligning them to the Rfam database [28]. The repeat sequences were annotated by aligning them to the Repbase

database. The remaining non-annotated sequences were performed a BLAST process to the miRNA database (http://mirbase.org/) to identify known miRNAs [29]. The novel miRNAs were identified by Mireap (http://sourceforge.net/projects/mireap) was used to. The secondary structures of putative pre-miRNAs were checked using Mfold (http://mfold.rit.albany.edu/?q=mfold/) [30]. The equation: MFEI = AMFE/(G + C)% was used to calculate the minimum free energy index (MFEI). The equation: (MFE/length of RNA sequence) × 100 was used to calculate the adjusted MFE (AMFE) [31]. Novel miRNAs in *S. lycopersicoides* were used as query sequences for a target gene search against *S. lycopersicoides* transcriptome data using Targetfinder (http://carringtonlab.org/resources/targetfinder). Hits with <4 mismatches were chosen as candidate targets. Functional annotations of target genes were performed by sequence comparison with the NCBI database (http://www.ncbi.nlm.nih.gov/).

3. Results and discussion

3.1. Phenotypic and physiological responses to cold stress

S. lycopersicoides (LA2408) leaf tissue was chosen to study cold responses. The degree of cold stress was identified by the malondialdehyde (MDA) content and peroxidase (POD) activity exchange in the leaves. *S. lycopersicoides* exhibited less severe wilting after cold treatment at 4 °C (Additional file 10: Figure S1A–C). The cold stress showed significantly increased MDA content and POD activity of these plants (Additional file 10: Figure S1D–E).

3.2. S. lycopersicoides transcriptome analyses

The low temperature transcriptomes of *S. lycopersicoides* (LA2408) were analyzed by RNA-seq using the Illumina Genome Analyzer II. After grow at 25 °C for 12 weeks, plants were removed to 4 °C for 0, 1, and 12 h, and the total RNA from leaf was extracted and analyzed. More than 71 million reads were produced with approximately 24 million reads from each sample (Table 1). The reads were assembled into 127,889 transcripts and 59,286 non-redundant unigenes by Trinity method with default parameters, and the unigenes were longer than 200 bp [20]. The assembly results were displayed in Additional file 1. In total, 3839 sequences containing 4921 SSRs were identified from 14,747 unigenes (Additional file 2), and 1,472,709 SNPs between S1 and S0 and 1,330,795 SNPs between S12 and S0 (Additional file 3).

To identify *S. lycopersicoides* miRNAs that were involved in the regulation of the cold stresses, three miRNA libraries were constructed from the leaves of *S. lycopersicoides* that were or were not treated with cold. The three miRNA libraries were sequenced using high-throughput RNA-seq and yielded approximate 19 million raw reads in each sample. We excluded the poor-quality reads and those whose leagth was smaller than 18 nucleotides in further analysis. Finally, we obtained approximate 15.3 million non-redundant reads (18–30 nucleotides) in each sample (Table 2).

Table 1
Statistics of the sequences for control and cold stress libraries from the *Solanum lycopersicoides* leaf.

Samples	Raw data (G)	rRNA percent	Use data (G)	Use GC
S0	4.78	4.7%	4.78	43.49%
S1	4.67	4.58%	4.67	43.47%
S12	5.01	4.94%	5.01	43.59%

S0, S1, and S12 stand for *S. lycopersicoides* cold treatment for 0 h, 1 h, and 12 h, respectively.

Table 2
Statistics of the sequences for control and cold stress microRNA libraries from the *Solanum lycopersicoides* leaf.

Samples	Total reads	"N" reads	Length <18	Length >30	Clean reads
S0	19,505,585	778	1,225,080	2,937,299	15,342,428
S1	18,369,746	687	883,572	2,204,641	15,280,846
S12	19,212,919	810	1,780,988	2,026,161	15,404,960

S0, S1, and S12 stand for *S. lycopersicoides* cold treatment for 0 h, 1 h, and 12 h, respectively.

3.3. Differential expression under cold stress

To study the impact of cold stress on gene expression in *S. lycopersicoides*, the transcript abundance of each gene was estimated by RPKM (Additional file 4). As a comparison of low temperature transcriptomes in *S. lycopersicoides*, a heat map was generated to present the transcript abundance for all differentially expressed genes (DEGs) under cold stress at 0, 1, and 12 h (Additional file 10: Figure S2-4). The results showed that a series of changes in *S. lycopersicoides* transcriptome happen when plants are moved from warm to cold temperature.

We used a threshold of a minimum 2-fold change in abundance between any two time points to define DEGs in the following analysis (Additional file 4). In *S. lycopersicoides*, transcripts for 617 and 1928 ESTs increased at 1 and 12 h, respectively, and 136 ESTs increased at both time points tested. 1066 and 1171 ESTs decreased at 1 and 12 h, respectively, and 185 ESTs decreased at both time points tested (Fig. 1). In summary, the expression profiling of DEGs showed that gene expression was different in response to cold stress in *S. lycopersicoides*.

To verify the correctly of the RNA-Seq data, some increased expressed genes, decreased expressed genes, and non-differentially expressed genes were choose for qRT-PCR under cold stress. There was a relativity between RNA-Seq and qRT-PCR (Additional file 5). The results indicated that the expression patterns of these genes in qRT-PCR were similar with that in RNA-Seq (Additional file 10: Figure S5).

3.4. GO term enrichment analysis

To clarify the obviously changed biological processes under cold stress in *S. lycopersicoides*, the up-regulated and down-regulated genes in RNA-seq were employed to the GO term enrichment analysis. The results showed that some stress-related GO terms were dramatically enriched among the up-regulated or down-regulated genes of *S. lycopersicoides*, including 'response to stimulus', 'signaling', and 'cell killing'. (Fig. 2; Additional file 6).

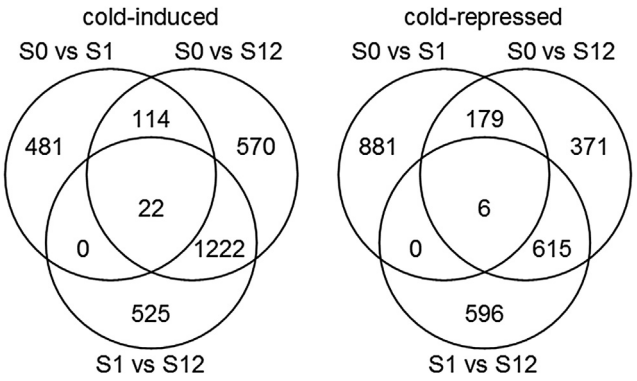

Fig. 1. 2-fold cold-induced or 2-fold cold-repressed ESTs in *Solanum lycopersicoides*. The results were from 0, 1, and 12 h of cold stress at 4 °C.

Transcription factors (TFs) play a key role in the regulation of genes expression under abiotic and biotic stresses in plants. RNA-seq results showed that analysis many TFs were regulated under cold stress in *S. lycopersicoides*, and some members of C-repeat binding factor (CBF) were up-regulated under cold stress at 1 h or 12 h, and some were down-regulated after 12 h (Fig. 3; Additional file 4). Two additional transient-term cold-regulated genes were found which encode transcription factors, homolog to Cys2/His2-type zinc-finger protein (ZAT10) [32] and a zinc finger protein that involved in high light and cold stress (ZAT12) [33].

Sucrose accumulates during cold acclimation in Arabidopsis [34]. A gene encoding Suc synthase (c58591.graph_c0) showed increased transcript abundance in *S. lycopersicoides* under 12 h of

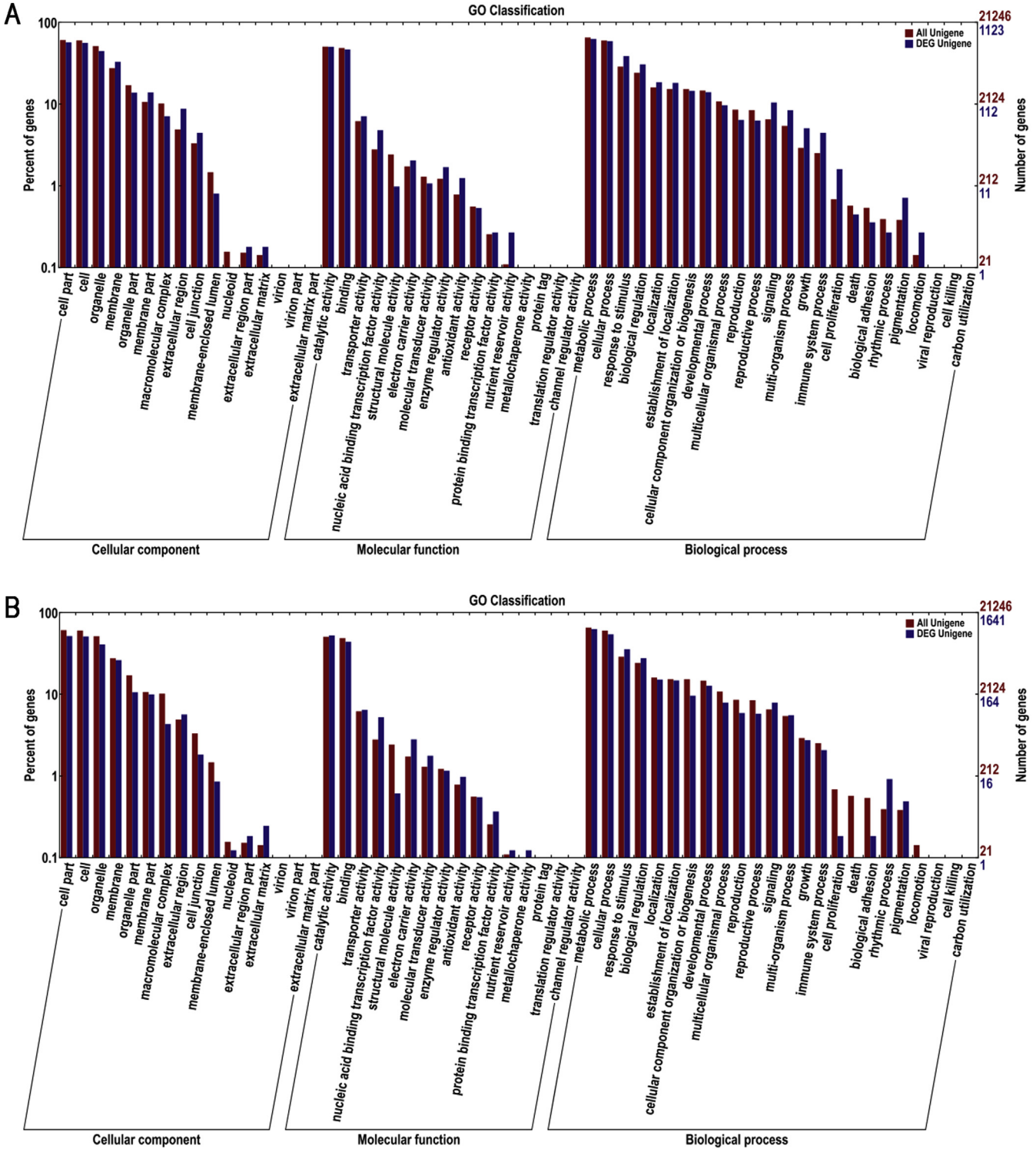

Fig. 2. Functional categories of the GO terms of all *Solanum lycopersicoides* unigenes between the following samples: S1 versus S0 (A) and S12 versus S0 (B). S0, S1, and S12 represent *S. lycopersicoides* cold treatment for 0 h, 1 h, and 12 h, respectively.

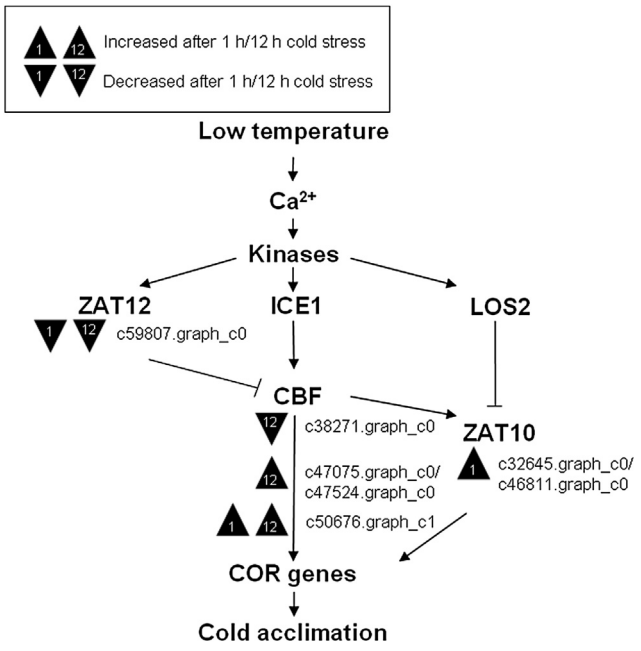

Fig. 3. Diagram of transcriptional network under cold stress in plants. Solid arrows indicate activation, whereas lines ending with a bar show inhibition. Abbreviations: CBF, C-repeat binding factor; ICE1, inducer of CBF expression 1; LOS2, low expression of osmotically responsive genes 2; ZAT10, a Cys2/His2-type zinc-finger protein; ZAT12, a zinc finger protein involved in high light and cold stress.

cold stress (Additional file 10: Figure S6; Additional file 4; Additional file 7).

The photosynthesis-related genes were suppressed significantly in *A. thaliana* under cold stress [35]. In *S. lycopersicoides*, the suppression of the photosynthetic included electron transport (Additional file 10: Figure S7A). On the contrary, many genes involved in PSI and PSII were strongly induced in *S. lycopersicoides* under cold stress, such as c51355.graph_c0, and c55618.graph_c0 (Additional file 10: Figure S7B; Additional file 4; Additional file 7).

In addition to the biological process mentioned above, which were involved in the cold tolerance of plants, the biological processes 'phosphorylation' (Additional file 10: Figure S8), 'peroxisome' (Additional file 10: Figure S9), 'hormones' (Additional file 10: Figure S10), 'natural killer cell' (Additional file 10: Figure S11), 'glycerolipid metabolism', 'RNA degradation', 'inositol phosphate metabolism', 'spliceosome', 'ABC transporters', 'Vitamin B6 metabolism', etc. were found in *S. lycopersicoides* response to chilling stress. Other examples of cold stress-associated biological process are provided in Additional file 7.

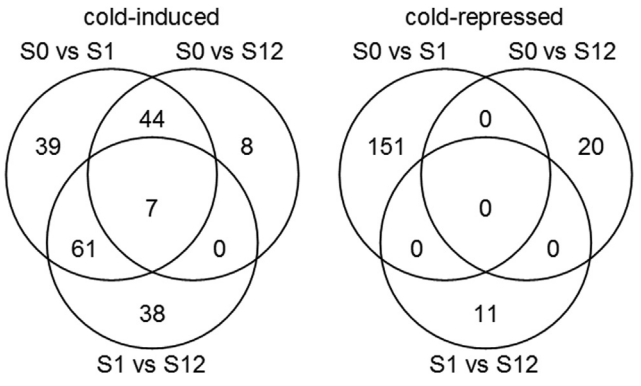

Fig. 4. 2-fold cold-induced or 2-fold cold-repressed miRNAs in *S. lycopersicoides*. The results were from 0, 1, and 12 h of cold stress at 4 °C.

3.5. Impact of cold stress on miRNAs in *S. lycopersicoides*

To identify novel microRNAs (miRNAs) in *S. lycopersicoides*, we analyzed miRNAs using the miRDeep2 tool. The sequences of non-coding RNAs (rRNAs, tRNAs, and small nuclear RNAs) were abandoned using BLASTn process to search the Rfam database (http://rfam.xfam.org/). The remaining sequences were categorized as either other types of endogenous small RNAs or miRNA candidates and used to predicte the fold-back structure. We compared all unique miRNAs with the miRBase database (version 21.0). In this analysis, 952 novel miRNA candidates were obtained matching with published miRNAs (mismatch ≤ 1) (Additional file 8)

The transcript abundance of each miRNA was estimated by RPKM. The RPKM of the miRNAs varied from 0 to 214,075 (sly-miR167a, sample S12), showing that the expression patterns of miRNAs changed greatly in *S. lycopersicoides* (Additional file 8). A heat map generated to present the transcript abundance for all differentially expressed miRNAs under cold stress at 0, 1, and 12 h is shown (Additional file 10: Figure S12). In *S. lycopersicoides*, transcripts for 151 and 59 miRNAs increased at 1 and 12 h, respectively, and 51 miRNAs increased at both of the tested time points; transcripts for 151 and 20 miRNAs decreased at 1 and 12 h, respectively, and none of the miRNAs decreased at both tested time points (Fig. 4). The expression of some miRNAs in *S. lycopersicoides* under cold stress at 1 h had the opposite pattern compared to the plant under cold stress at 12 h. For example, unconservative_SL2.50ch09_1295135 and unconservative_SL2.50ch10_1310967 were up-regulated under cold stress for 1 h in *S. lycopersicoides*, whereas they were down-regulated under cold stress for 12 h (Additional file 8).

We used Targetfinder to identify the predicted targets for the miRNAs. For the miRNAs that were annotated as described above, we identified a total of 6107 mRNA targets (Additional file 9). To further analysis the role of the miRNAs under cold stress, the target unigenes for miRNAs, which were related to the cold stress, were identified based on RNA-seq data (Additional file 9). For example, one of the target gene was the high affinity nitrate transporter 2.5 (NRT2.5, AT1G12940). NRT2.5 is repressed in *S. lycopersicoides* after 12 h of cold stress based on RNA-seq results (Additional file 4). The miRNA that is predicted to target NRT2.5 is sly-miR156a. Our sequencing data showed that sly-miR156a is up-regulated in *S. lycopersicoides* after 12 h of cold stress (Additional file 8). The repression of NRT2.5 in response to 12 h of cold stress correlates with sly-miR156a induction by cold, suggesting that the expression levels of NRT2.5 were post-transcriptional regulated by miRNA under cold stress. Therefore, sly-miR156a/NRT2.5 represent an abiotic stimulus module that could be important for the cold response in the *S. lycopersicoides* leaf. Other examples of cold stress-associated modules of miRNAs are provided in Additional file 9.

For comprehensive annotation, all putative targets in each library were analyzed by GO terms by Blast2GO program. An analysis of GO enrichment for the mRNA targets revealed that mRNA target functions were enriched in different biological processes (Additional file 9). The putative target transcripts of miRNAs were related to the metabolic process (1745 terms), cellular process (1628 terms), response to stimulus (816 terms), and biological regulation (713 terms).

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.029.

Transparency document

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