

Transcriptomic analysis of Asiatic lily in the process of vernalization via RNA-seq

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Abstract The new transcriptomes provided comprehensive sequence profiling data of transcriptomic variation during vernalization in Lily Asiatic Hybrids ‘Tiny ghost’. A number of 52,277,184 sequencing raw reads totaling 5.11 Gbp of the chilling treatment (4 °C) sample and 39,466,176 sequencing raw reads totaling 3.85 Gbp of room temperature control (25 °C) sample were assembled de novo into 68,718 unigenes with a mean length of 984 bp, and a total of 33,208 (45.6 %) unigenes were annotated by using public protein databases with a cut-off *E* value about 10^{-5} . There are 6,153 unigenes of which were assigned to specific metabolic pathways by the Kyoto encyclopedia of genes and genomes. Gene Ontology analysis of the annotated unigenes revealed that the majority of sequenced genes were associated with signal transduction mechanisms, posttranslational modification, protein turnover and chaperones. In addition, the genes expression levels were compared just after vernalization completion between the cold treatment and room temperature control. There are 68,116 unigenes were differentially expressed, and hierarchical clustering analysis arranged 7,301 significantly differentially-expressed unigenes into 56 groups. Six genes related to the vernalization were selected to confirm their expression levels by using quantitative real-time polymerase chain reaction. Furthermore, typical vernalization unigenes *VRN1* and *VRN2* were identified, and

also some vernalization-associated unigenes, such as *CBF*, *SOC*, *TaAGL*, *AP2*, *LEA*, *LIM* et al. were also annotated in the present study. As for *VRN1* and *VRN2*, their expressions were consistent with some previous related studies. Also, this was the first time the vernalization genes *VRN1* and *VRN2* were founded in lily. According to the results of the present studies, we predicted that they would play an important role during vernalization in Lily Asiatic Hybrids; these data provided the foundation for the future studies of metabolism during vernalization of Asiatic lily.

Keywords Lily Asiatic Hybrids · Transcriptome · RNA-Seq · Vernalization

Abbreviations

BLAST	Basic local alignment search tool
COG	Cluster of orthologous group Cluster
GO	Gene Ontology
KEGG	Kyoto encyclopedia of genes and genomes
NCBI	National Center for Biotechnology Information
qRT-PCR	Real-time quantitative reverse transcription polymerase chain reaction

Introduction

Vernalization is a critical developmental process that allows plants to flower by exposing to cold temperatures. Lily is one of the most important flowering crops and is second to the rose in the cut flower market worldwide [1]. Among the species in *Lilium*, Lily Asiatic Hybrids is loved by people for its richest variations in colors and beautiful shapes. But the molecular mechanisms regulating

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vernalization of these species remain unclear. As a powerful modern genetic research tool, transcriptome sequencing has been used to analyze plenty of species, e.g. *Gossypium hirsutum* [2], *Pyrus* (*Pyrus pyrifolia* ear group) [3], *Chrysanthemum lavandulifolium* [4], *Prunus mume* [5], *Cymbidium sinense* [6], but transcriptome and genomic information for lily is currently unavailable. Transcriptome and expression profiling data for this species would be needed as an important resource to identify genes and to better understand the biological mechanisms of vernalization in lily. Vernalization is the acquisition of a plant's ability to flower by exposing to the prolonged cold of winter. That is to say, plants grown in temperate climates which require vernalization must experience a period of low winter temperature to initiate or accelerate the flowering process [7]. After vernalization, plants have acquired the ability to flower, but they may require additional seasonal cues or weeks of growth before they would actually flower. Vernalization temperatures are usually between 5 and 10 °C. So far, much progress has been made on vernalization.

Genetic analysis of winter wheat found that *VRN1*, *VRN2*, *VRN3*, *VRN4* and *VRN5* were the major genes that control its vernalization of winter wheat [8–10]. They controlled wheat vernalization habits through combinations and mutual effect of different recessiveness and dominance. *VRN1* gene is a flowering promoting factor induced by low temperature in the hexaploid genome wheat, *VRN1* gene had three copies, the *VRNA1*, *VRNB1* and *VRND1*, located on chromosome 5A, 5B and 5D individually [11]. Further study showed that any one of three genes *VRNA1*, *VRNB1* and *VRND1* is dominant, it's growth characteristics of spring, they do not need cold-induced for vernalization, or shorter jarovization could induce blooming. If all three genes are recessive its growth characteristics of winter. In the process of growth, they would take a long time of low temperature jarovization to blossom.

VRN2 is a flowering inhibiting factor, and dominant for winter habit [12]. Any functional copy of *VRN2* is sufficient to inhibit flowering. And the product of *VRN2* is the repressor of *VRN1*. *VRN2* was cloned by Yang et al. [13] in *Arabidopsis*, and they found that *VRN2* was a single copy gene in *Arabidopsis* genome. Furthermore, scientist also found that the flowering inhibitor gene *FLC* in *Arabidopsis* and rice has a similar function to *VRN2*, both of whom inhibit flowering and are repressed by vernalization at the same time [9, 14, 15]. Wheat *VRT2* gene is a MADS-box transcription factor, with a similar vernalization response mode to *VRN2*. Danyluk being studied the molecular interaction relationship between *VRN1*, *VRN2* and *VRT2* genes and their products by transient expression analysis in *Nicotiana benthamiana* and found that *VRT2* was a transcription repressor of *VRN1*, *VRT2* bonding with CArG

domain in promoter region of *VRN1* to inhibit the expression of *VRN1*. Meanwhile, *VRN2* neither united with *VRN1* promoter region nor affected its activity directly. But it enhance the inhibitory effect of *VRT2*, indicating that there was a regulation complex joint affecting the expression of *VRN1* together [16]. In winter wheat, Timing expression showed that *VRT2*, *VRN2* and *VRN1* accumulate in apical meristems and tender leaves. And it also proved that *VRT2* and *VRN2* inhibit flowering, and *VRN1* promotes flowering. While in the short-day condition, the unvernalsized spring-habit wheat accumulate *VRT2* and postpone flowering, which indicated that *VRT2* was adjusted and controlled by light and low temperature separately. In a word, *VRT2* and *VRN2* inhibit the expression of *VRN1* together.

VRN3 gene shows homologous with *FT* in *Arabidopsis* and *HvFT* in barley [10, 11, 17]. *VRN3* promotes flowering through the regulation of vernalization pathway and long-day pathway, and *VRN3* a dominant allele related to spring habit. Sequence analysis showed that *VRN3* in wheat is not mutated in the coding area, but the insertion of a reversal base to the promoter region can distinguish the recessiveness and show dominant allele genes. *VRN3* gene links *VRN1* and *VRN2* in the vernalization pathway and genes in the photoperiod pathway to control the flowering time together.

In 1972, the fourth vernalization gene *VRN4* was found by Pugsley in wheat (*Triticum aestivum* 'Gabo') [8], and the existence of *VRN4* was proved by Gonch [18]. *VRN5* was first proposed by Law and Wolfe [17], and was unified named *VRNB4* in 1998.

The vernalization gene *FaVRN1* was cloned from *Festuca arundinacea* by Wang et al. [19], and the vernalization gene *VRN2* was cloned and constructed expression vector in *Arabidopsis* by Zhang et al. [20]. Other vernalization genes, such as FLOWERING LOCUS C (*FLC*), *Arabidopsis* vernalization genes *FLC* belongs to MADS-box gene [21], which encodes a protein transcription factor's inhibitory effect on flowering, promoting some late flowering in *Arabidopsis* ecotypes and late flowering mutants flowering through negative regulation of transcription and protein expression levels.

When plants were exposed to low temperature, accompanied with vernalization, they will experience a serious of changes, such as feeling the low temperature, making a series of physiological and biochemical reaction changes, and adjusting genes' expression, which is the process that transcription factor CBF1 binds to the CRT/DRE motif to induce *COR* gene expression and then improve the cold resistance for the plants.

To elucidate these mechanisms, advances in the next generation high-throughput sequencing technologies are now being exploited not only to the analysis of static genomes, but also dynamic transcriptomes in an approach

termed RNA-seq [22–26]. Because of its higher sensitivity, base-pair resolution and the larger range of expression values can be detected [25–27]. In addition, RNA-Seq requires less prior knowledge of gene sequences. Through these powerful and rapidly evolving technologies, scientists have already made substantial contributions to understanding of genome expression and regulation in a number of plant species. The model species whose RNA-Seq analysis has been applied include *Arabidopsis* [28, 29], soybean [30, 31], rice [32], maize [33] and *Medicago truncatula* [34]. There are also some non-model plant species which lack a reference genome using RNA-Seq, such as *Eucalyptus grandis* [35], grape (*Vitis vinifera* L.) [36], California poppy (*Eschscholzia californica*) [29], avocado (*Persea americana*) [29], *Pachycladon ensyisii* [37], and *Artemisia annua* [29].

Materials and methods

Plant materials and RNA isolation

Plants of Asiatic lily ‘Tiny ghost’ were collected from Kunming Academy of Agricultural Sciences. The underground bulbs were harvested when the aboveground parts withered, and they were placed in planting boxes (50 × 60 cm), covered with 30 cm planting soil, 20 bulbs per box, a total of 20 boxes. Treatments as follows: 10 boxes were placed in cold storage at 4 °C (treatment), while the other 10 boxes were placed in the room temperature at 25 °C (control). The intermediate scales were sampled every 5 days and the content of soluble sugars were determined by anthrone method. When the soluble sugar content of the cold treatment group reaches peak, 40 stem tips of each group were sampled, and stored in the refrigerator at –80 °C for the isolation of RNA. Total RNA was extracted using the EASYspin Plus plant RNA extraction kit (Aidlab, China). The quantity and quality of the isolated total RNA was examined using spectrophotometry (BIO-RAD SmartSpec Plus, USA), and loaded on a denaturing agarose gel to check the concentration and integrity of the RNA (BIO-RAD, USA).

Library preparation for transcriptome analysis and sequencing

Poly-A-containing mRNAs were purified from the total RNA samples by using the OligoTex mRNA mini kit (Qiagen, China). Then the mRNA was fragmented into small pieces by using RNA fragmentation kit (Ambion, USA). With these short fragments as the templates, the first cDNA strand was synthesized by using random hexamer primers and reverse transcriptase (Invitrogen, China), and

the second-strand cDNA was synthesized by using DNA polymerase I and RNase H. The cDNA fragments were purified using the QiaQuick polymerase chain reaction (PCR) extraction kit (Qiagen, China) and resolved with EB buffer for end repair and poly (A) addition. The short fragments were then connected with sequencing adapters, and the products were subsequently purified and amplified via PCR to create the final cDNA libraries. The cDNA library was sequenced by using Illumina HiSeq™ 2000, and the sequencing-derived raw image data were transformed by base calling into sequence data by using Illumina Pipeline Software v1.6. The raw reads were cleaned by the trimming of adaptor sequences, empty reads and ambiguous nucleotides (‘N’ in the end of the reads). The reads obtained were then assembled by using the SOAPdenovo program [38]. SOAPdenovo first combined those reads of a certain length of overlap to form longer fragments without N, which were called contigs. The resulting contigs were joined into scaffolds by using the paired-end reads. The paired-end reads were used again for the gap filling of scaffolds to obtain sequences with the least Ns that could not be extended at either end. Such sequences were defined as unigenes. In the final step, Basic local alignment search tool X (BLASTX) alignments (*E* value 0.00001) between unigenes and protein databases, including UniProt, Kyoto encyclopedia of genes and genome (KEGG) and clusters of orthologous groups (COGs), were performed, and the best alignment results were used to decide the sequence direction of the unigenes. When a unigene happened to be unaligned with none of the above databases, ESTScan software was used to predict its coding regions and to decide its sequence direction [39].

De novo assembly

Raw reads were assembled using SOAPdenovo (<http://soap.genomics.org.cn/soapdenovo.html>). The raw sequence data in fast a format were filtered to remove the following reads: (1) eliminate the low quality reads, they are less than 50 % proportion of the bases which their qualities are less than 20, and $Q = -10\log_{10}(\text{error_ratio})$, (2) eliminate the reads which containing adapter or the length is less than 20 bp, (3) eliminate the reads with fuzzy N-containing bases, (4) eliminate ribosome RNA, using the *Lilium*’s rRNA for the comparison template, and the match ones maybe the ribosome RNA reads, and (5) eliminate the 3’ bases which their qualities are less than 20, and the mistake rate of bases are less than 0.01. Only clean reads were used in the following analysis. The contigs are the longest assembled sequences containing no Ns. We mapped the reads back to contigs and combined paired-end information, linked the contigs into scaffolds. Scaffold length was estimated on the basis of the average segment

Table 1 Primers used in real-time quantitative PCR (RT-qPCR)

Unigene id	Protein descriptions	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Length (bp)
Contig554	LEA3 protein	CGTTACATCATCAACCTTCA	GAGAACACAGGCAACTTC	108
Contig4194	MADS box protein	CATCAACAAGCCTTCAGAA	TCTCCAACAGAATCATCTCT	257
Contig5976	AGL20-like MADS box transcription factor	CTTCACACAGCAGTTCT	GCTCTAGTATTCAAGGTACAGT	105
Contig9669	NAP2/ERF domain-containing transcription factor	CAGAGACTACAAAGTTTAGGG	GGAGTGATAAGACGGTGA	218
Contig82297	CBF-like transcription factor	CTCATTCTCTGCTTCTTCAC	TCAGTAGATCATCGGATAGTTC	230
Contig43966	LIM1	GCAAGCATCATCACATTCA	AGCAGTCCAAAATAAGAAGGT	271
Contig27291	Vernalization 2-1 protein	GTTCCATCAGAAATCTCAAGTC	GGTAGTTGAACAGGACAGT	212

Table 2 Summary of RNA-seq and de novo assembly of Asiatic lily ‘Tiny ghost’ unigenes

	Number	Median sizes	Mean sizes	N25 (bp)	N50 (bp)	N75 (bp)
Reads	91,743,360	92	92	92	92	92
Contig	122,064	535	618	1,812	860	447
Unigene	68,718	984	984	2,207	1,208	655

Reads = cold treatment sample reads + room temperature control sample reads

length of each pair of reads. By filling the gaps in scaffolds using paired-end reads, unknown bases were filled with Ns and unigene sequences were created. Unigene sequences were alignment to @blastdb using BLASTX (*E* value 0.00001). Sequence orientations were determined depending on the best hit in the database. If results from different databases clashed with each other, then the final choice was in line with @blastdb. The orientations and CDSs of sequences that had no hit in BLASTX were predicted using ESTScan [39]. The original transcript sequences (5′–3′) were supplied if their orientations could be determined. Other sequences were provided as assembler outputs functional annotation.

Unigene functional annotation provides information by BLASTX alignment (*E* value $<10^{-5}$) between unigenes and protein databases, such as nr, Swiss-Prot, KEGG and Gene Ontology (GO) The unigenes were retrieved from these databases along with their protein functional annotations as long as they showed high sequence similarities to proteins that found in this study. Using this process, protein function could be deduced by comparison with other similar genes. The possible functions of a fasciata unigenes could be predicted and classified by compared to the COG database.

Together with nr annotation, the BLAST2GO program was used to obtain GO annotations for a fasciata unigenes [40], and also was used for GO functional enrichment

analysis of certain genes by performing Fisher’s exact test with a robust false discovery rate (FDR) correction to obtain an adjusted *P* value between certain test gene groups and the whole annotation. After obtaining GO annotations for every fasciata unigene, GO functional classification for all the unigenes were undertaken by WEGO software, and also the distribution of gene function in the species at the macro level was investigated.

Analysis and mapping of DGE reads and differential gene expression

For gene expression analysis, in order to eliminates the influence of different gene lengths and sequencing discrepancies on the calculation of gene expression, the expression level of the unigenes was calculated and then normalized to RPKM (reads per kilobase of exon region per million) [22]. In this way, the calculated gene expressions can be directly compared among samples. The cut-off value for determining gene transcriptional activity was determined according to a 95 % confidence interval for all the RPKM values of each gene. Different expression genes were selected between treatment and control on condition of FDR <0.05 , and fold-change ≥ 2 .

Clustering of gene expression profiles

Hierarchical cluster analysis of the 75 gene expression patterns was performed with cluster [41] and Java Tree-view [42] software, and the hierarchical clustering were analyzed by the log₂Ratio for each gene.

qRT-PCR validation and expression analysis

Total RNAs were isolated from stem tip of the treatment and controlled as described above. First-strand cDNA

Fig. 1 Gene Ontology classification of assembled unigenes. *Note* the unigenes were assigned to **a** biological process, **b** cellular component and **c** molecular function

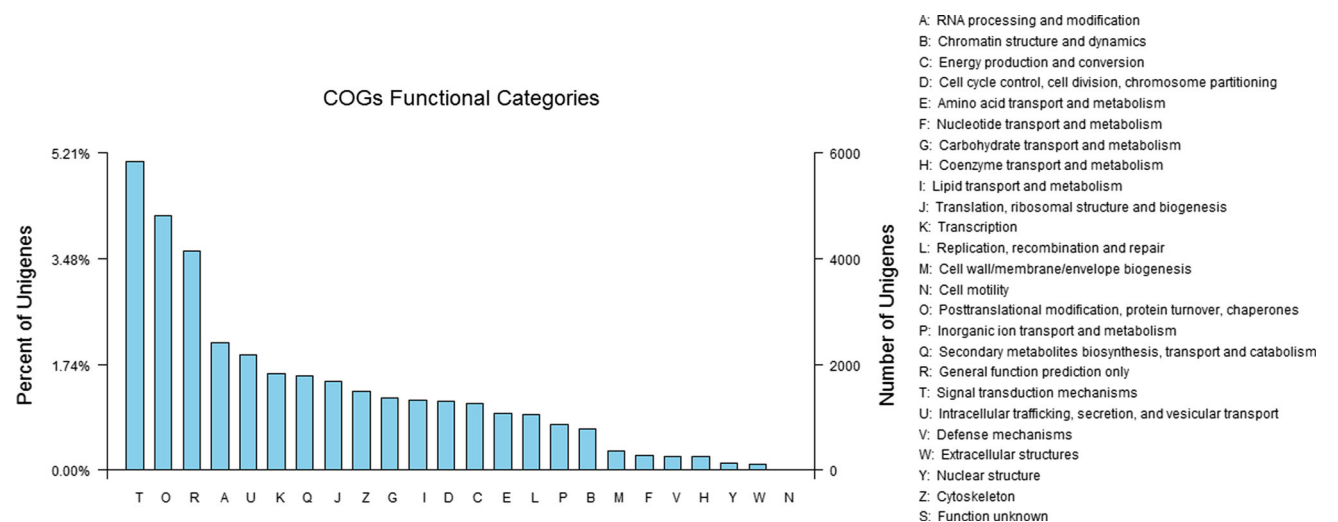
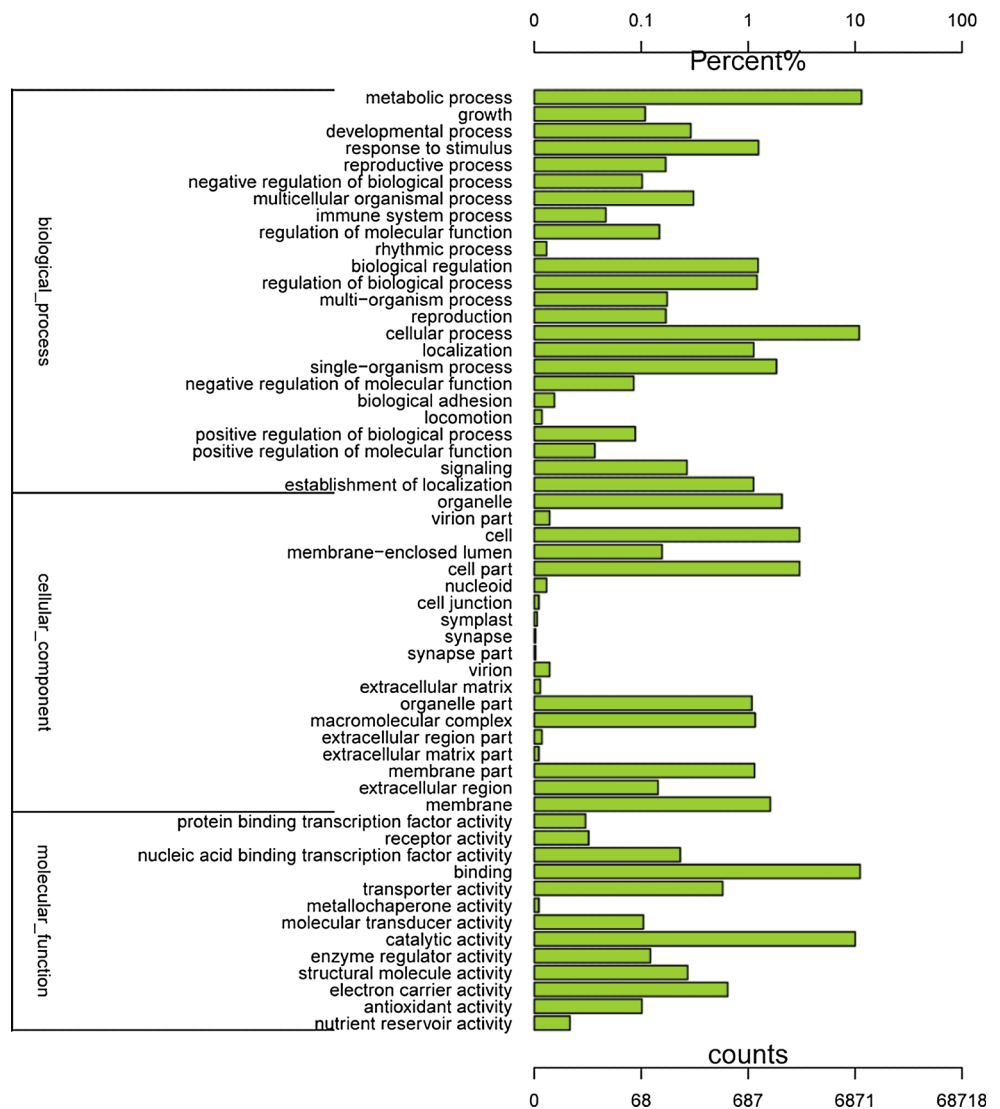


Fig. 2 COG functional classification of all unigene sequences. *Note* out of UniProt hits, 14,511 sequences were assigned to 25 COG classifications

synthesis was performed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions, using 1 µg of total RNA and oligo (dT) primers. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using a Rotor-Gene 3000 real-time PCR detection system (Qiagen) using SYBR[®] qPCR Mix (Toyobo, Tokyo, Japan) according to the manufacturer's protocol. The primers used in this study were designed by use of Beacon Designer (Premier, Palo Alto, CA, USA) and are listed in Table 1. Real time PCR reaction was carried out using the prepared cDNA (60 µg) with each set of primer and probe and iQTM SYBR[®] Green Supermix (Cat. number 170-8882, BIO-RAD, Hercules, CA, USA). PCR cycling was at 95 °C (10 s), 56 °C (30 s), and 72 °C (30 s). Three independent replicates were performed for each sample. The comparative CT method was used to determine the relative amount of *LaLEA3*, *LaAGL20*, *LaAP2*, *LaCBF*, *LaLIM1*, *LaSOC1* and *LaVRN2* in Asiatic lily 'Tiny ghost', with the expression of Actin used as an internal control.

Results

Annotation of predicted proteins

The output of sequenced data was from two experimental samples of cold treatment and room temperature control. 52,277,184 sequencing raw reads a total number of 5.11 Gbp from cold treatment sample and 39,466,176 sequencing raw reads totaling 3.85 Gbp of room temperature control sample. Total 91,743,360 sequence data had a mean length of 96 bp. These clean reads were assembled and produced to contigs, scaffolds and unigene sequences (Table 2). Finally, unigene data from the cold treatment and room temperature control were summarized into an all-unigene consisting of 68,718 sequences with a mean size of 984 bp, which included all non-redundant unigene sequences from both the cold treatment and room temperature control plant samples.

CDS prediction

All of the unigenes were compared with the sequences in public databases, including the National Center for Biotechnology Information (NCBI) COGs database, the UniProt protein database, and the KEGG database, using the BLASTX algorithm with an *E* value threshold of 10^{-5} . A total of 33,208 unigenes had significant hits (*E* value 10^{-5}) to the sequences in the UniProt protein database. The results also showed that 66.78 % of the unigenes over 500 bp in length had BLAST matches, whereas only 21.87 % of the unigenes were shorter than 300 bp. This

Table 3 Categorization of *Lilium* Asiatic unigenes to KEGG biochemical pathways

KEGG categories	Number of unigenes
Metabolic pathways	2,191
Biosynthesis of secondary metabolites	1,210
Microbial metabolism in diverse environments	513
Ribosome	324
Protein processing in endoplasmic reticulum	311
Ribosome biogenesis in eukaryotes	310
Biosynthesis of amino acids	307
Spliceosome	301
Pyrimidine metabolism	277
RNA transport	239
Citrate cycle (TCA cycle)	229
Epstein–Barr virus infection	224
Starch and sucrose metabolism	216
Plant hormone signal transduction	211
Oxidative phosphorylation	198
Calcium signaling pathway	194
Alcoholism	192
Plant–pathogen interaction	187
Purine metabolism	185
Cell cycle	176
Endocytosis	168
Viral carcinogenesis	168
Glycolysis/gluconeogenesis	167
RNA degradation	165
Ubiquitin mediated proteolysis	163
mRNA surveillance pathway	158
Homologous recombination	156
HTLV-I infection	152
Amino sugar and nucleotide sugar metabolism	146
Oocyte meiosis	146
Hippo signaling pathway	142
Phenylpropanoid biosynthesis	136
Glutathione metabolism	134
Progesterone-mediated oocyte maturation	129
Pyruvate metabolism	126
Cell cycle: yeast	123
Phagosome	123
Insulin signaling pathway	121
Carbon fixation in photosynthetic organisms	110
PI3K-Akt signaling pathway	110
Legionellosis	109
Peroxisome	106
Influenza A	106
Estrogen signaling pathway	104
Methane metabolism	103
Herpes simplex infection	100
Others	8,399

absence of homology could be caused by several factors. There is limited information about the genomes or transcriptomes of Asiatic lily and its related species. Some unigenes were too short to allow statistically meaningful matches.

GO is an international standardized gene functional classification system that provides a standardized vocabulary that is used to assign function to uncharacterized sequences. BLAST2GO program [40]. A tool that associates GO terms with sequences based on several pieces of annotation evidence, was used to classify gene function in our dataset. Putative functions were assigned to 28,954 unique sequences involved in the categories of biological process (11,931), cellular component (4,751) and molecular function (12,272; Fig. 1). As for the molecular function, the most two common types of genes were localized to the binding and catalytic activity, and the functions of the identified genes cover various electron carrier activity, transporter activity and structural molecule activity. The sequences encoded a broad set of transcripts represented

within the biological process category. Among these, the single-organism process, response to stimulus, biological regulation, regulation of biological process and localization were better represented.

Unigenes are aligned to the COG database, a database where orthologous gene products are classified, to predict and classify possible functions. A total of 14,511 sequences were assigned to the COG classifications (Fig. 2). Among the 25 COG categories, the cluster for signal transduction mechanisms (5,838; 15.27 %) represented the largest group, followed by posttranslational modification, protein turnover, chaperones (4,813; 12.59 %), general function prediction only (4,141; 10.83 %), RNA processing and modification (2,416; 6.31 %), intracellular trafficking, secretion, and vesicular transport (2,177; 5.60 %), transcription (1,689; 4.79 %) and secondary metabolites biosynthesis, transport and catabolism (1,598; 4.64 %).

The assembled unigenes were assigned to the biochemical pathways described in KEGG based on their EC numbers. The KEGG database contains a systematic analysis of inner-cell metabolic pathways and functions of gene products. Pathway-based analyses help to understand the biological function of genes further. A total of 19,865 unigenes demonstrated sequence similarities to the genes in the KEGG database, and 3,825 unigenes were assigned to five KEGG biochemical pathways, including metabolic pathways (2,191 unigenes), biosynthesis of secondary metabolites (1,210), microbial metabolism in diverse environments (513), ribosome (324), and pyrimidine metabolism (311; Table 3). These results highlight the immense capacity of Illumina sequencing to discover genes in metabolic pathways.

Difference in genes related to vernalization of lily

The difference in levels of differentially expressed genes between cold treatment and room temperature control was calculated according to RPKM value of the obtained

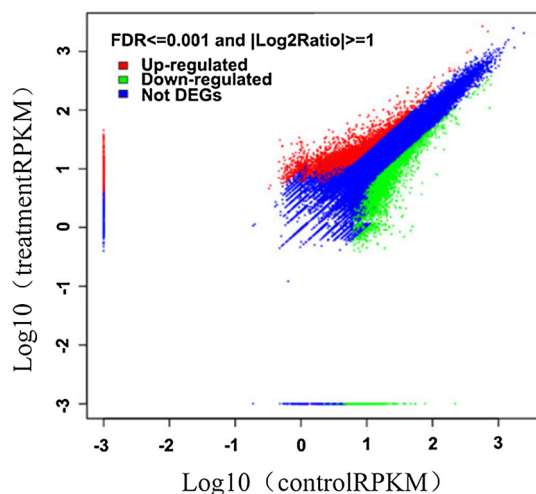


Fig. 3 Differential expression level comparison of treatment with control

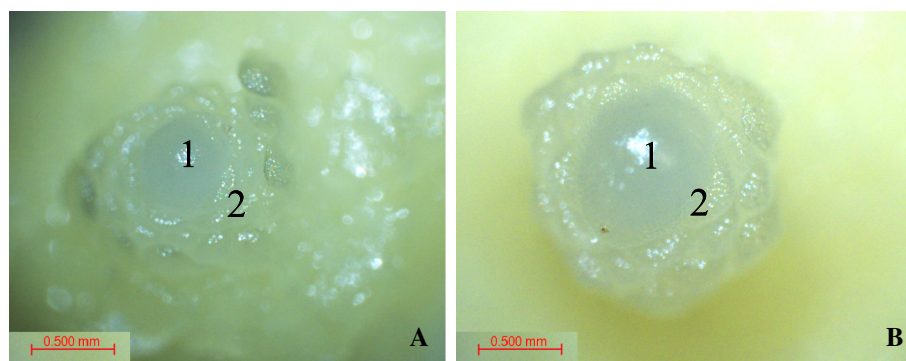


Fig. 4 Lily growing point of cold treatment. *Note a* Before vernalization (cold storage for 0 days). *b* Just completed vernalization (cold storage for 27 days). (1) Shoot tips, and (2) inner scales

Table 4 VRN1 GO functional annotation

GO	Terms	GO_namespace	Unigene_num
GO:0008152	Metabolic process	biological_process	10,623
GO:0005488	Binding	molecular_function	9,853
GO:0016817	Hydrolase activity, acting on acid anhydrides	molecular_function	1,182
GO:0001882	Nucleoside binding	molecular_function	2,438
GO:0035639	Purine ribonucleoside triphosphate binding	molecular_function	2,299
GO:0001883	Purine nucleoside binding	molecular_function	2,425
GO:0016817	Hydrolase activity, acting on acid anhydrides	molecular_function	1,182
GO:0016818	Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	molecular_function	1,168
GO:0019148	D-Cysteine desulfhydrase activity	molecular_function	1
GO:0032549	Ribonucleoside binding	molecular_function	2,437
GO:0032553	Ribonucleotide binding	molecular_function	2,449
GO:0017076	Purine nucleotide binding	molecular_function	2,426
GO:0005524	ATP binding	molecular_function	2,068
GO:0016462	Pyrophosphatase activity	molecular_function	1,160
GO:0030554	Adenyl nucleotide binding	molecular_function	2,183
GO:0032555	Purine ribonucleotide binding	molecular_function	2,425
GO:0032550	Purine ribonucleoside binding	molecular_function	2,425
GO:0016818	Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	molecular_function	1,168
GO:0032553	Ribonucleotide binding	molecular_function	2,449
GO:0017076	Purine nucleotide binding	molecular_function	2,426
GO:0005524	ATP binding	molecular_function	2,068
GO:0016462	Pyrophosphatase activity	molecular_function	1,160
GO:0030554	Adenyl nucleotide binding	molecular_function	2,183
GO:0017111	Nucleoside-triphosphatase activity	molecular_function	1,147
GO:0032555	Purine ribonucleotide binding	molecular_function	2,425
GO:0032559	Adenyl ribonucleotide binding	molecular_function	2,183
GO:0005524	ATP binding	molecular_function	2,068
GO:0017111	Nucleoside-triphosphatase activity	molecular_function	1,147
GO:0032559	Adenyl ribonucleotide binding	molecular_function	2,183
GO:0005524	ATP binding	molecular_function	2,068

that the architecture formation of lily may be related to hormone metabolic regulation. By comparison, the cold treatment and room temperature control showed significant differences in vernalization. Classical genetic studies showed that the vernalization of lily was a quality trait controlled by the combined effect of some vernalization genes. These differences are controlled by genes. In differentially expressed genes, we found the vernalization gene *VRN1* (Contig63927) and *VRN2* (Contig27291), *VRN1* and *VRN2* gene sequence showing homology with *Oryza sativa* subsp. and *Malus domestica*, respectively.

In GO functional annotation, *VRN1* gets 29 clusters, among which GO:0008152 attributed to metabolic process of biological processes, another 28 clusters were attributable to molecular function (Table 4).

In COG annotation, the function attributed to post-translational modification, protein turnover, chaperones. The predicted functions is AAA ATP enzyme. The best five results (cut off 10^{-5} *E* values) are shown in Table 5.

To further verify the expression profiles of genes in our Illumina sequencing analyses, six DEGs were selected randomly by qRT-PCR using the same samples originally used for RNA-Seq, including late embryogenesis abundant gene (*LaLEA3*), CBF-like transcription factor (*LaCBF1*), MADS-box transcription factor AGL6a (*LaTaAGL*), vernalization gene (*VRN2*), transcription factors regulate the expression of target gene (*LaLIM1*) and ethylene-responsive element binding protein (*LaAP2*). The results presented in Fig. 6 show that the expression levels of four genes were higher in treatment than those in control, including late embryogenesis

Table 5 The COG annotation of VRN1

Unigene	Unigene length	Descriptions	Subject length	E value
Contig63927	892	gnlCDDI35952 KOG0733, KOG0733, KOG0733, nuclear AAA ATPase (VCP subfamily) [posttranslational modification, protein turnover, chaperones]	802	8.00E−21
Contig63927	892	gnlCDDI35949 KOG0730, KOG0730, KOG0730, AAA+-type ATPase [posttranslational modification, protein turnover, chaperones]	693	3.00E−14
Contig63927	892	gnlCDDI35950 KOG0731, KOG0731, KOG0731, AAA+-type ATPase containing the peptidase M41 domain [posttranslational modification, protein turnover, chaperones]	774	3.00E−12
Contig63927	892	gnlCDDI35956 KOG0737, KOG0737, KOG0737, AAA+-type ATPase [posttranslational modification, protein turnover, chaperones]	386	1.00E−11
Contig63927	892	gnlCDDI35870 KOG0651, KOG0651, KOG0651, 26S proteasome regulatory complex, ATPase RPT4 [posttranslational modification, protein turnover, chaperones]	388	2.00E−10

abundant gene (*LaLEA3*), MADS-box transcription factor AGL (*LaTaAGL*), vernalization gene (*LaVRN2*) and transcription factors regulating the expression of target gene (*LaLIM1*), CBF-like transcription factor (*LaCBF1*) and ethylene-responsive element binding proteins (*LaAP2*) expressed much higher in control than that in treatment.

Discussion

The advantages of high throughput sequencing technology in the study of lily vernalization

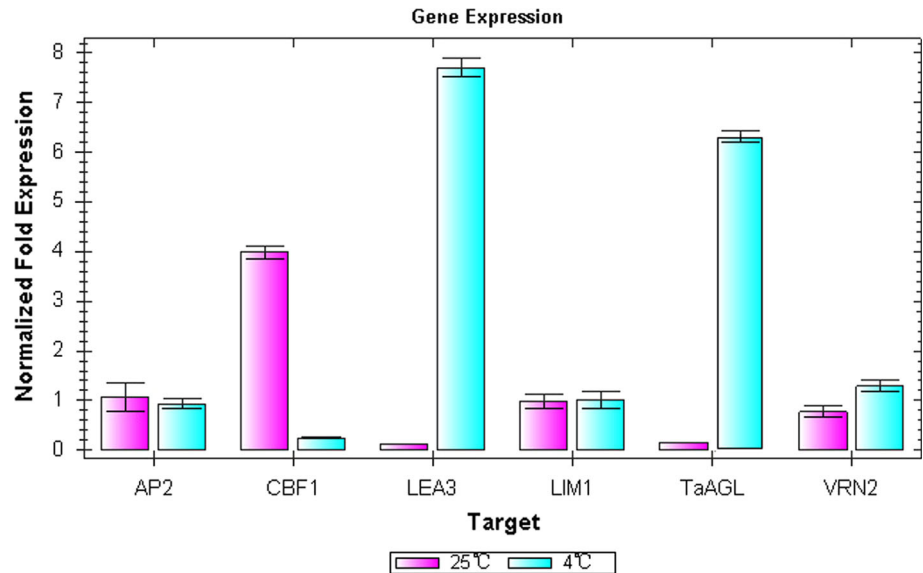
Compared with the chip technology, RNA-seq does not need reference genome information, but also has a higher accuracy. In addition, with the rapid development of NGS technology, the cost of sequencing is rapidly declining, and the data analysis technology has been constantly improved. These characteristics are especially attractive for the study of non-model species transcriptomics [43, 44]. Illumina/

Solexa is the most extensive sequencing platform for NGS application, and has been widely used in the plant transcriptome research. In this research, a total number of 91.74 million 90 bp paired-end clean reads were achieved, 68,718 unigenes were generated and compared with the sequences in UniProt databases. 625,149,167 and 14,511 unigenes had significant similarity to existing sequences in GO, KEGG and COG databases, respectively. A bit of cold-induced up-regulated and down-regulated genes were found, which revealed the flower complicated regulation network during lily vernalization at transcriptome levels. According to Fig. 7, the results suggest that this library is a good source for studying the useful characters in the lily breeding.

Basal metabolism plays an important role in the lily vernalization process

In early twentieth century, scientists proved that the nutritional status of plants could influence plant flowering

Fig. 6 Expression levels of highly abundant *LaAP2*, *LaCBF1*, *LaLEA3*, *LaLIM1*, *LaTaAGL* and *LaVRN2* genes in treatment and control of Asiatic lily ‘Tiny ghost’. Note total of RNA was using the same samples originally used for RNA-Seq. Real-time quantitative RT-PCR was performed to assay the gene expression of *LaAP2*, *LaCBF1*, *LaLEA3*, *LaLIM1*, *LaTaAGL* and *LaVRN2* as indicated. The resulting mean values are relative to the expression of Actin. Legend 25 °C on behalf of the control group, and Legend 4 °C on behalf of the treatment group



through a lot of experiments. The results of this study showed that the gene expression in nitrogen metabolism, starch and carbohydrate metabolism were significantly up-regulated during vernalization.

As shown in Table 6, Compared to the treatment sample, *VRN1* express higher in control, on the contrary, *VRN2* expresses higher in treatment group. According to the results and the previous studies on *VRN1* and *VRN2*, they probably play an important role in vernalization of lily. However, considering the complexity and quantity of lily genome, it is not clear how these genes affect the architecture formation of lily vernalization.

VRN2 is a homeodomain protein of *PRC2* complex subunit, is needed to sustain *FLC* gene silencing and the expression after vernalization in epigenomics [45, 46]. There are four pathways controlling lily flowering, floral repression pathway, autonomous promotion pathway, photoperiodic promotion pathway and vernalization promotion pathway. SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (*SOC1*) can integrate these blossom signaling pathways to regulate flowering time [47, 48], which is presents in both monocotyledonous and dicotyledonous plants, and they all could encode the MADS-box transcription factor [49–54]. At present, some *SOC1* homologous genes have been cloned, such as *GMGAL1* of soybean (*Glycine max*) [55], *CsCLI* and *CsCL2* of orange (*Citrus sinensis*) [56], *UNS* of petunia (*Petunia hybrid*) [57], *NAC1*, widely found in higher plants. They were characterized by their abilities to regulate development of plants and response to abiotic-stresses, respectively. These genes had been implicated in a plant defense mechanism against environmental stresses such as high temperature and low temperature or drought stress and various pathogens. *NAC*, found in strawberry and peach, they were induced by abscisic acid or salicylic acid, which mediates

plant responses to abiotic stresses [58, 59]. However, their biological function in plants is still unclear.

Genetic regulatory pathway during lily vernalization

Lily is a flower of summer dormancy. It can not be planted annually in natural conditions, and would not germination or rendered blind flower without being broken the dormancy. The dormancy of lily is not compulsive structural dormancy, but endogenous physiological dormancy caused by inhibitor, It needs the stimulation of low temperature, to complete flower bud differentiation to blossom and yield fruit, which limits the ornamental and market application to a certain extent. In this research, genes relate to four pathway controlling the timing of flowering, including floral repression pathway, autonomous promotion pathway, photoperiodic promotion pathway and vernalization promotion pathway [59] were found, such as *VRN1*, *VRN2*, *LFY*, *SEP*, *FT*, *AP2*, *AG*, *SOC1*.

According to the results of this research and other related reports, a gene regulation network of lily vernalization to flowering could be preliminary as shown in Fig. 7. There are four main pathways controlling lily flowering: floral repression pathway, autonomous promotion pathway, photoperiodic promotion pathway and vernalization promotion pathway. The first two pathways regulated the internal states; the other two pathways transmitted signals from external environment. In this four pathways, vernalization promotion pathway makes the largest difference on lily. Vernalization process is regulated by a series of genes. *FLC* was found as one of the key genes in the process of flowing, *FLC* interacts with *CArG* boxes of *FT*, *SOC1* and *LFY* to inhibit their activity, and inhibits flowering induction [60]. *FLC* is mainly expressed in root and stem tip. Another vernalization related gene is *FRI*, which plays an important

Fig. 7 Schematic diagram of floral induction pathways on *Arabidopsis*. Genes in red type were found by Illumina sequencing. (Color figure online)

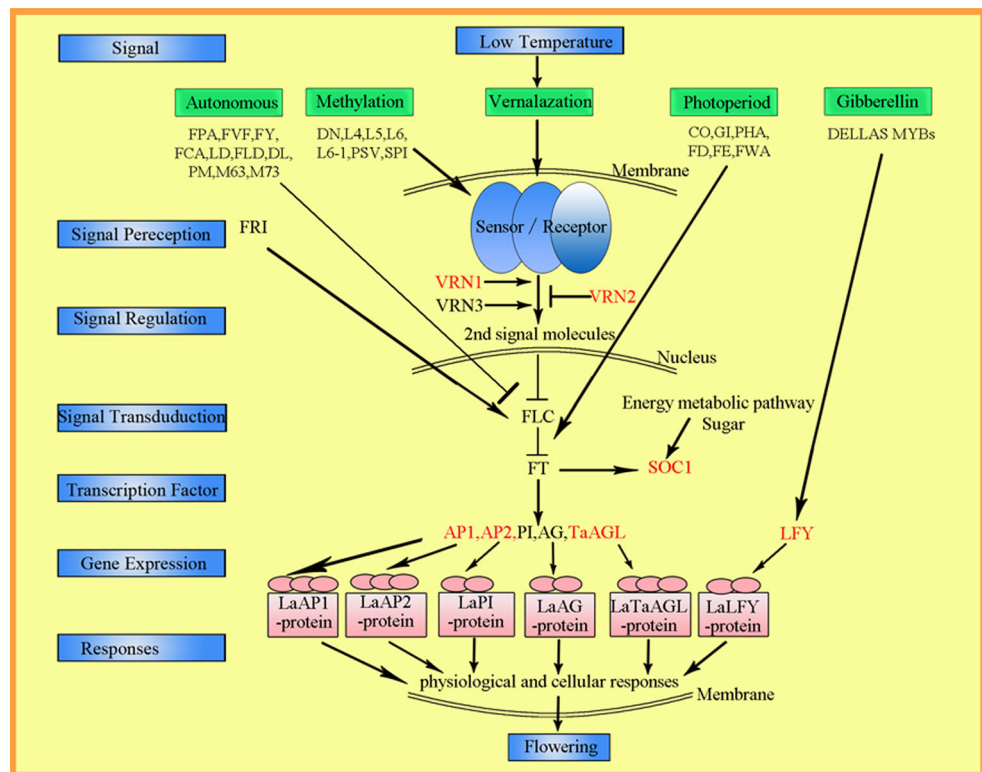


Table 6 The description and expression of VRN1 and VRN2 in *Lilium* Asiatic ‘Tiny ghost’

Unigene	Length	Description:Subject_length	Control_RPKM	Treatment_RPKM
Contig63927	892	trIQ5VRN1IQ5VRN1_ORYSJ:Os06g0109400 protein OS=Oryza sativa subsp. japonica GN=OSJNBa0004I20.8 PE=3 SV=1	2.628306	0.076471
Contig27291	415	trIB5B0L7IB5B0L7_MALDO:Vernalization 2-1 protein OS=Malus domestica GN=VRN2-1 PE=2 SV=1	27.28487	72.60081

role in *Arabidopsis thaliana* vernalization through the up-regulation of the expression of *FLC* gene. The activation of *FRI* and *FRI*-like gene on *FLC* is more significant, which can overcome the plant itself inhibitory effect on *FLC* [61]. Besides, two genes *VRN1* and *VRN2*, related to vernalization could keep the inhibitory action of *FLC*. Methylation in general was closely related to vernalization, which might influence flowering time by regulating the expression of *FWA*. In our study, *LaVRN1*, *LaVRN2*, *LaSOC1*, *LaLFY*, *LaAPI*, *LaAPX*, *LaTaAGL* genes related to vernalization were found. The interactions and the function in vernalization pathway are shown in Fig. 7.

Conclusion

In this study, genomics and bio-informatics approaches were used to identify vernalization-related genes that are expressed specifically in reproductive organs of Asiatic

lily. The RNA-Seq library revealed a great number of vernalization related genes in the constructed ESTs (Table 3). This is the first time that the vernalization gene *VRN1* and *VRN2* has been found in lily. According to the results of previous studies, we predict that they play an important role during vernalization in Lily Asiatic Hybrids, and the expression analysis proves that *VRN1* gene is a flowering promoting factor, and *VRN2* is flowering inhibiting factor. The results are consistent with previous studies. In Fig. 8, the *FLC* gene was not detected in this study, we speculated that there are two reasons, one is that no significant differences in expression, and the other is that maybe lily does not pass through this pathway, that is to say, there is another pathway to flower of lily which different from the model plant *Arabidopsis*. More research must be conducted. Whatever, these data provided a basis for future studies of metabolism in the process of vernalization. Furthermore, the highly expressed vernalization genes *LoVRN2* was further identified by qRT-PCR and the

vernalization regulatory network in *Arabidopsis* was outlined, thus it provided a scientific basis for further investigation of the molecular mechanisms for the underlying vernalization development of Asiatic lily.

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