Screening and expression analysis of genes related to cold tolerance in *Dendroctonus valens*

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Dendroctonus valens (Coleoptera: Scolytidae) is a major pest of the genus *Pinus*. It has strong cold tolerance, but the molecular mechanisms underlying its response to low temperatures are not clear. In this study, we sequenced the transcriptomes of adult and larval *D. valens* in May (-12.1℃) and January (18.7℃) using the Illumina HiSeq platform. We detected 4,996 and 8260 differentially expressed genes (DEGs) between sampling dates in larvae and adults, respectively, and 1189 common DEGs, including genes encoding protein phosphatase, very long-chain fatty acids, cytochrome P450, and putative leucine-rich repeat-containing proteins. In a GO enrichment analysis, 1,189 genes were assigned to 43 terms, with significant enrichment for cellulase activity, hydrolase activity, and carbohydrate metabolism. KEGG classification and enrichment analyses showed that the lysosomal and oxidative phosphorylation metabolic pathways involved the most DEGs. We also detected significant enrichment for pentose and glucuronate interconversions and lysosomal processes. We identified 112 candidate genes associated with cold tolerance, including genes with established roles in this trait (e.g., genes encoding trehalose transporter, fructose-1,6-bisphosphatase, and trehalase). Our comparative transcriptome analysis of adult and larval *D. valens* in different conditions provides basic data for the discovery of key genes and molecular mechanisms underlying cold tolerance.

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Abstract

Dendroctonus valens (Coleoptera: Scolytidae) is a major pest of the genus Pinus. It has strong cold tolerance, but the molecular mechanisms underlying its response to low temperatures are not clear. In this study, we sequenced the transcriptomes of adult and larval *D. valens* in May（-12.1℃） and January（18.7℃）using the Illumina HiSeq platform. We detected 4,996 and 8260 differentially expressed genes (DEGs) between sampling dates in larvae and adults, respectively, and 1189 common DEGs, including genes encoding protein phosphatase, very long-chain fatty acids, cytochrome P450, and putative leucine-rich repeat-containing proteins. In a GO enrichment analysis, 1,189 genes were assigned to 43 terms, with significant enrichment for cellulase activity, hydrolase activity, and carbohydrate metabolism. KEGG classification and enrichment analyses showed that the lysosomal and oxidative phosphorylation metabolic pathways involved the most DEGs. We also detected significant enrichment for pentose and glucuronate interconversions and lysosomal processes. We identified 112 candidate genes associated with cold tolerance, including genes with established roles in this trait (e.g., genes encoding trehalose transporter, fructose-1,6-bisphosphatase, and trehalase). Our comparative transcriptome analysis of adult and larval *D. valens* in different conditions provides basic data for the discovery of key genes and molecular mechanisms underlying cold tolerance.

Introduction

Dendroctonus valens mainly attacks the base of the trunk of *Pinus* tabulaeformis. Adults generally lay eggs in the phloem at the base of the trunk or 1.5 m below the base. After hatching, larvae consume decaying phloem and form a common tunnel. Adults and larvae eat the phloem, destroy the cambium, and cut off nutrient transport in swarms, thereby affecting tree growth or even causing death. This damage reduces the economic and landscape value of the tree *(Yan et al., 2005)*.

D. valens was introduced to Shanxi Province in 1998 and spread rapidly due to the abundant Pinus hosts and warm and dry climate *(Sun et al., 2013)*. The species was introduced to Hebei and Henan in 1999 *(Sun et al., 2004)*, Shaanxi and Qinghai in 2001, and Beijing in 2005, and its

distribution continued to expand northward. By 2017, it reached to Chaoyang of Liaoning and Chifeng of Inner Mongolia at approximately 41.5°N latitude. *D. valens* has evolved a unique cold tolerance mechanism.

In view of the expansion of the distribution of *D. valens*, extensive research has focused on biological characteristics *(Liu , Xu & Sun, 1938;Zhang et al., 2010; Lindeman & Yack, 2015; Liu et al., 2017)*, risk analyses *(Liu , Xu & Sun, 1938)*, biological control *(Storey , Storey & GuiJun , 2011; Yang, Wang & Zhang, 2014)*, symbiotic bacteria *(Wang et al., 2012; Raffa, Hanshew & Mason, 2015; Wang et al., 2017)*, and pheromones *(Chen et al., 2010; Xu, Liu & Sun, 2014; Kelsey & Westlind, 2018)*, but studies of the molecular mechanisms underlying cold tolerance in *D. valens* are lacking. Cold tolerance is an important indicator of the population density, diffusion, and distribution of insects and can explain adaptation to low temperatures in northern regions. Genes related to cold tolerance in insects have been identified in cold-sensitive *Drosophila melanogaster (Hoffmann, 2010)*. A number of candidate genes and proteins related to low-temperature responses (e.g., factors involved in metabolism, the epidermis, cytoskeleton, immune function, and signal transduction *(Vesala et al., 2012; Isobe, Takahashi & Tamura , 2013; Parker et al., 2015)*)have been reported, including HSPs *(Colinet et al., 2013; Enriquez & Colinet, 2019)* and Frost genes *(Colinet et al., 2013;MacMillan et al., 2016)*.

In this study, high-throughput transcriptome sequencing was used to explore key genes and metabolic pathways related to responses to low temperatures in *D. valens*. These results provide a foundation for further molecular and functional studies of this trait and provide a theoretical reference for the development of a prevention and control strategy.

Materials and Methods

Experimental insects

D. valens larvae and adults were collected from the field on January 19, 2019, and May 11, 2019, in Heilihe Town, Ningcheng County, Chifeng City, Inner Mongolia (Longitude: 118.43°; Latitude: 41.41°; Altitude: 1050 m). The average temperature was -12.1℃ and 18.7 ℃ on these months respectively. The samples were stored in liquid nitrogen, brought to the laboratory, and stored at -80°C until RNA extraction.

RNA extraction, cDNA library preparation, and Illumina sequencing assembly

Total RNA was extracted from *D. valens* collected in January and May using TRIzol reagent (Ambion, Austin, TX, USA) and the RNeasy Plus Mini Kit (No. 74134; Qiagen, Hilden, Germany) following the manufacturers instructions. Three biological repeats were evaluated per sample. The purity, concentration, and integrity of the total RNA samples were measured using the NanoDrop2000 (IMPLEN, Westlake Village, CA, USA), Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA) and 1.0% agarose gel electrophoresis. RNA samples of sufficient quality were sent to Shanghai Majorbio Bio-pharm Technology Co.,Ltd for cDNA library construction and Illumina sequencing. The Illumina platform converts sequenced image signals into text signals through CASAVA Base Calling and stores these raw data in fastq format. SeqPrep and Sickle were used to eliminate reads including sequencing adapter sequences, low-quality reads, sequences with a high N rate (where N indicates uncertain base information), and sequences that were less than 30bp. fastx_toolkit (version 0.0.14) was used to estimate the Q20, Q30, and GC contents. Clean reads were assembled using Trinity (version 2.5.0), and optimized filtering was performed using TransRate (version 1.0.3) and CD-HIT. A high-quality unigene library was obtained after evaluation using BUSCO (version 3.0.2) to obtain references for subsequent analyses.All of the raw sequence data were deposited in the NCBI Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/subs/) under BioProject accession No. PRJNA609406.

Functional annotation of genes

BLAST was used to obtain homologous sequences by searching against several public protein databases, including the National Center for Biotechnology Information (NCBI) non-redundant (Nr) protein database, the Swiss-Prot protein database, the Protein Family (Pfam) database, the Cluster of Orthologous Groups (COG) database, the Gene Ontology (GO) database*(Camacho et al., 2009)*, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database*(Kanehisa, 2000)*.

Differential gene expression analysis

Bowtie was used to compare newly obtained sequence reads with sequences in the databases. RSEM 1.2.31 was used to evaluate expression levels based on FPKM (fragments per kilobase of transcript per million fragments mapped) values. The Benjamini–Hochberg method was used to correct for multiple testing*(Benjamini & Yekutieli， 2001)*. Differentially expressed genes

(DEGs) were defined as unigenes with log2 (fold change) > 1 or log2 (fold change) < -1, with P < 0.01. These DEGs were evaluated by GO and KEGG functional classification and enrichment analyses.

Gene expression validation by real-time quantitative PCR

To validate the reliability of RNA-Seq data, 15 common DEGs both larvae and adults were selected for an qRT-PCR assay, using TUB and PRS as reference genes for larvae and adults, respectively (data not published). Primers were designed using Primer 3Plus and synthesized by Ruibo Xingke Biotechnology Co., Ltd. (Beijing, China) (Table S1). The target fragment was amplified by conventional PCR and the optimal reaction conditions were explored. The SYBR Green dye method was used for qPCR, and the procedure was performed according to the instructions provided with the Fluorescence Quantitative Reaction Kit (Roche, Basel, Switzerland). The reaction system (12.5 μL) included SYBR® Premix Ex TaqII (6.25 μL), 0.5 μL of 10 μmol/L forward primer, 0.5 μL of 10 μmol/L reverse primer, cDNA (1 μL), and ddH_2O (4.25 μL), mixed well on ice. PCR conditions were as follows: 95°C for 3 min, 40 cycles at 95°C for 10 s, 60°C for 30 s, and 65°C to 95°C in increments of 0.5°C for 5 s to generate melting curves. Each reaction was performed with three biological and technical replicates. The experimental data were homogenized and analyzed by the $2^{-\Delta\Delta t}$ method *(Livak & Schmittgen, 2001)*. SPSS19.0 was used for statistical analyses, and GraphPad Prism was used to generate plots of real-time PCR results.

Results

Sequencing and assembly of *D. valens* transcriptomes

The Illumina sequencing platform was used to sequence 12 samples of *D. valens*. Over 6.1 Gb of clean data were obtained for each sample, with Q30 ≥ 93.21%, Q20 ≥ 97.82%, and a GC content of 42.98% (Table 1). Trinity was used to assemble all clean reads from scratch, and 110932 transcripts were obtained. After clustering and de-redundancy, 58956 unigenes were obtained. The average transcript length after assembly was 1163.73 bp, N50 was 2101 bp, and the BUSCO score was 96.3%(Table S2). Based on these parameters, the data quality and reliability were high, meeting the requirements for further analyses.

Functional annotation of genes

The assembled sequences were compared with sequence data in the Nr, Pfam, COG, Swiss-Prot, KEGG, and GO databases using BLAST ($e \leq 10\text{-}5$). Functional annotations were obtained for 28218 sequences, accounting for approximately 47.86% of all unigene sequences in the transcriptome (Fig. 1). Among these, 27,047 unigenes were annotated in the Nr database, accounting for the highest proportion (45.88%), followed by Pfam (33.84%), GO (32.22%), Swiss-Prot (33.32%), and KEGG (24.94%). The E-value distribution, identity distribution, and species distribution were used to further analyze homology between Illumina sequences and those in the Nr database. Based on the E-value distribution, 69.64% of the annotated unigenes (18836) had very high homology with proteins in the Nr database (E-value < 1e-30), and the other sequences had matches with E-values ranging from 1e-30 to 1e-5 (Fig. 2A). Further analysis showed that 86.99% of the sequences had similarities of >60% to those in the Nr database (Figure 2B). With respect to species, the annotated sequences had the highest degree of match to those of *Dendroctonus ponderosae*, with 12,848 sequences and a matching rate of 47.84% (Fig. 2C).

Differentially expressed gene screening results of *D. valens* under different temperature conditions

The FPKM method was used to calculate expression levels, and genes with p-adjusted < 0.01 and |log2 (fold-change)| > 1 were identified as DEGs. Among 58,956 unigenes in the transcriptome of *D. valens*, 4996 DEGs were found among the larvae sampled in January and May. Compared with larvae collected in May, 2937 (58.79%) of genes collected in January were up-regulated, 2059 (41.21%) were down-regulated, and 53,960 genes were not differentially expressed (Fig. 3A). There were 8,260 DEGs in adults, including 4726 (57.22%) up-regulated genes and 3534 (42.78%) down-regulated genes in January compared with May as well as 50,696 genes that were not differentially expressed (Fig. 3B).

As shown in a Venn diagram in Fig.4, 1189 genes were differentially expressed between January and May in both larvae and adults(Table S3), 7071 genes were differentially expressed only in adults, and 3807 genes were differentially expressed only in larvae. Among the common DEGs in adults and larvae, 175 lacked functional annotations in any database, 740 were down-regulated in adults and larvae, and 92 were up-regulated. In addition, 349 DEGs were up-regulated in larvae and down-regulated in adults, only 8 DEGs were down-regulated in larvae and up-regulated in adults. We speculated that the common DEGs in adults and larvae under different field temperatures might play an important role in the response to low temperatures; accordingly, we focused on these genes in further functional analyses.

Classification of common DEGs

The 1189 common DEGs were assigned to three GO domains: biological process (BP), cellular component (CC), and molecular function (MF), involving 43 total GO terms (Fig. 5). In the BP category, DEGs were assigned to 20 GO terms; the most frequent terms were metabolic process (234 genes), followed by cellular process (232 genes). In the CC domain, DEGs were assigned to 12 GO terms, and the most frequent terms were membrane and membrane part (257 and 245, respectively). In the MF category, DEGs were assigned to 11 GO terms, with catalytic activity accounting for the largest proportion of genes (359 genes). A KEGG analysis revealed that 1527 unigenes belonged to 282 pathways (Table 2). Two metabolic pathways contained more than 20 genes: lysosome (26) and oxidative phosphorylation (21). There were 28 metabolic pathways containing 10–20 genes, and the remaining pathways all had fewer than 10 genes. The pathways identified in this analysis were mainly involved in the processes of translation, endocrine system, transport catabolism, carbohydrate metabolism, signal transduction, nucleotide metabolism, and cell growth and death, indicating that signal transduction and substance transport activities may be important in *D. valens*.

Enrichment analysis of common DEGs

In an evaluation of the top 25 common DEGs (Table 3), 12 terms in BP, 8 terms in CC, and 5 terms in MF were significantly enriched. The entries carbohydrate metabolic process (GO: 0005975) and peptide metabolic process (GO: 0006518) were significantly enriched in BP, indicating that carbohydrate metabolism in *D. valens* might contribute to overwintering. In the CC category, the significantly enriched terms were organelle (GO: 0043226) and intracellular organelle (GO: 0043229). In the MF category, the two most significantly enriched GO terms were hydrolase activity, acting on glycosyl bonds (GO: 0016798) and hydrolase activity, hydrolyzing O-glycosyl compounds (GO: 0004553), further illustrating that the molecular activity of *D. valens* is strong under low temperatures. In these conditions, it is necessary to rapidly synthesize a large number of small molecules, such as sugars, as antifreeze protectants.

Among the common up-regulated DEGs between sampling dates in adults and larvae, the top three were iron ion binding (GO: 0005506), oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (GO: 0016705), monooxygenase activity (GO: 0004497). Among the common down-regulated DEGs, carbohydrate metabolic process (GO: 0005975), hydrolase activity, acting on glycosyl bonds (GO: 0016798), hydrolase activity, hydrolyzing O-glycosyl compounds (GO: 0004553), cellulase activity (GO: 0008810), and other terms were significantly enriched.

A KEGG analysis revealed that common DEGs could be classified into 282 pathways, including three significantly enriched pathways(Fig. S1). The most significantly enriched

pathway was pentose and glucuronate interconversions (map00040); this pathway involved 14 unigenes. The conversion of these substances is related to glycerol synthesis. Glycerin, an important small molecule, functions as an antifreeze substance in insects and thereby plays an important role in cold tolerance. The lysosome (map04142) and galactose metabolism (map00052) pathways, which belonged to transport and catabolic metabolism and carbohydrate metabolism pathways, respectively, were significantly enriched in common DEGs. It is possible that these processes contribute to overwintering in *D. valens*.

Among the common up-regulated DEGs between sampling dates in adults and larvae, the highly enriched terms included thyroid hormone synthesis (map04918), glycine, serine and threonine metabolism (map00260), antifolate resistance (map01523), and glutathione metabolism (map00480). The enrichment results for common down-regulated DEGs in adults and larvae were similar to common DEGs for adults and larvae.

Screening of genes related to cold tolerance among common DEGs

Based on previous studies of insect cold tolerance and the keywords obtained in manual searches of the annotation information for the common DEG set, we selected 112 candidate genes related to cold tolerance. In total, 44 genes encoded enzymes and proteins related to the degradation of cryoprotectants (Fig.6), including 21 genes involved in the synthesis and metabolism of low-molecular-weight polyols, 10 genes involved in trehalose synthesis, and 13 genes involved in low-molecular-weight amino acids.

In addition, one gene encoding the cytoprotective protein *HSP70*, 11 genes encoding cytoskeletal proteins, 1 gene with cuticular functions, 1 gene involved in antioxidant defense, 15 genes related to signal transduction, 6 genes related to apoptosis, and 31 genes associated with other processes related to cold tolerance were annotated (Table 4).

Expression level verification

We selected 15 candidate genes with potential roles in cold tolerance, including 12 annotated genes described in Table 4 and Fig. 6, and three genes with high differential expression in the common DEG set but without functional annotation. Fifteen genes encoding staphylococcal nuclease domain-containing protein 1 (TRINITY_DN22243_c0_g1), ATP synthase subunit alpha (TRINITY_DN18757_c0_g2), protein phosphatase 1A (TRINITY_DN26187_c0_g1), fructose-1,6-bisphosphatase 1 (TRINITY_DN21780_c1_g1) glyceraldehyde-3-phosphate dehydrogenase 2 (TRINITY_DN22610_c0_g2), putative glutamate synthase (TRINITY_DN27207_c0_g1), fatty acid synthase (TRINITY_DN27539_c3_g1), cathepsin L1 (TRINITY_DN18927_c0_g1), probable low-specificity L-threonine aldolase 2

(TRINITY_DN22377_c1_g1),serine/threonine-protein kinase (TRINITY_DN26090_c0_g1), fatty acid desaturase (TRINITY_DN19691_c0_g1), E3 ubiquitin-protein ligase (TRINITY_DN25425_c0_g4) and three genes annotated to unknown or uncharacterized proteins (TRINITY_DN27537_c0_g1, TRINITY_DN20229_c0_g1, and TRINITY_DN22439_c0_g5) were evaluated by qPCR (Fig. 7 and Fig. 8).

Based on the qPCR results, the expression differences in the 15 DEGs were consistent with those detected by RNA-seq, indicating that the sequencing data were accurate and reliable and can be used as a reference for subsequent research. Slight differences in fold change values may be due to operational errors and the different principles of the analysis methods for transcriptome sequencing and qPCR.

Discussion

Transcriptome sequencing quality and analysis of differentially expressed genes

Based on searches against the Nr protein database of NCBI, 27,047 genes (45.88%) were homologous to known sequences, indicating that the transcriptome includes a large number of new genes with unknown functions. These genes may be related to the unique biological characteristics and environmental adaptability of the species. Of the 1189 common DEGs between sampling dates in both larvae and adults, only 92 were up-regulated in both adults and larvae and 740 (62.24%) were down-regulated, similar to the results of transcriptome analyses of *Ericerus pela* and *Microdera punctipennis*, although the frequency of upregulated genes is low, these results suggest that some physiological activities remain active or are initiated *(Yu et al., 2016; Tusong et al., 2017)*.

The 92 up-regulated genes in both adult and larvae encoded several proteins that may be related to cold tolerance, including protein phosphatase, very long-chain fatty acids, E3 SUMO-protein ligase, cytochrome P450, and putative leucine-rich repeat-containing protein. Studies show that under low-temperature and freeze-thaw conditions, protein phosphorylation and dephosphorylation are important regulatory mechanisms related to many metabolic functions. Protein kinases and phosphatases can regulate the activity of many transcription factors and participate in the differential expression of genes involved in cold and freeze resistance*(Pfister & Storey, 2006)*. Overwintering insects need to neutralize or prevent the production of harmful metabolites. To remove toxic and harmful metabolites, cytotoxic P450 enzymes in *Belgica antarctica* are up-regulated during recovery from dehydration*(Benoit et al., 2009)*. We speculate that cytochrome P450 plays a similar role in cold tolerance. In addition, fatty acid proteins and leucine-rich repeat-containing proteins contribute to the synthesis and metabolism of low-

molecular-weight cryoprotectants, and other genes may be important for the maintenance of dormancy and related physiological processes.

A large number of DEGs were related to the synthesis and metabolism of small cryoprotectants. Freeze-resistant insects accumulate low-molecular-weight cryoprotectants, such as polyols (e.g., glycerol), sugars (e.g., trehalose), or amino acids (e.g., proline), to confer freezing tolerance. Elevated proline concentrations in the body can improve the survival of cold-sensitive *Drosophila melanogaster* larvae at low temperatures, and trehalose, an antifreeze protein, plays a role in cold tolerance in *Harmonia axyridis* and New Zealand alpine insects *(Imek et al., 2012; Wharton, 2011; Toxopeus et al., 2017)*. In addition, low-molecular-weight cryoprotectants are related to anabolism genes, cytoskeleton protein genes, and repair-related genes*(Toxopeus & Sinclair, 2018)*, in addition to many unannotated genes. Although the specific mechanism of action of these genes in insects is unclear, they may also play an important role in cold resistance. Cold tolerance in insects is a complex process, involving interactions among multiple genes *(Toxopeus & Sinclair, 2018)*.

We identified 1189 unigenes with differential expression in both adults and larvae, accounting for only 14.38% of the DGEs in adults and 23.8% of the DGEs in larvae. Most of the DGEs were not expressed in adults and larvae but were involved in the synthesis and expression of adaptation-related genes at low temperatures, such as some heat shock proteins, the sugar transporter SWEET1, and calcium channel proteins. For cell survival, heat shock proteins are important for survival during the winter. Many insects alleviate stress by up-regulating HSP *(Storey & Storey, 2011; King & Macrae, 2015; Toxopeus, Koštál & Sinclairet, 2019; Li, Wang & Jiang, 2019)*, but only a subset of HSP genes are up-regulated, consistent with previous results*(Zhang et al., 2011)*. In this study, HSP genes were up-regulated in both adult and larvae, including *hsp20*, *hsp70*, and *hsp90*. The differentially co-expressed gene set only included hsp70. Although the specific function of *hsp70* in survival at low temperatures in insects is not clear, it improves the response to stress by re-folding damaged proteins and re-dissolving insoluble proteins *(Craig et al.,1985)* as well as marking irreparable proteins for degradation *(Terlecky et al., 1992)*. The sugar transporter SWEET1 is a cold tolerance gene based on a transcriptome analysis of *H. axyridis* at normal and low temperatures*(Tang et al., 2017)*. In larvae, calcium channel proteins are significantly up-regulated compared to levels in adults, and calcium signaling pathways are also important in cold stress signaling*(Reddy et al., 2011; Denlinger et al., 2013; Wang et al., 2013)*.

Many studies show that cold tolerance in insects is directly related to antifreeze proteins (AFP) and the inhibition of ice recrystallization *(Duman, 2015)*. However, AFPs were not included in our significant DEG sets. A previous study showed that cold tolerance depends on the structure of AFP rather than the amount of protein *(Koštál et al., 2011)*. It is possible that the relationship between transcript and protein levels is complex, and transcriptome and proteome analyses often show weak correlations between transcript and protein level *(Cui, 2017)*. It is also possible that the final transcriptome lacks AFP sequences as a result of unassembled reads or short contigs *(Tang et al., 2017)*.

DEGs involved in cold tolerance are expected to be up-regulated under cold stress; however, most DEGs in our study were down-regulated. It is possible that genes and proteins related to low temperature adaptation were upregulated at the beginning of the winter, with the saturation of expression levels in late January. Therefore, protein expression levels should be a focus of future research.

Cold tolerance-associated GO enrichment analysis

The terms iron binding, oxidoreductase activity, and monooxygenase activity were significantly enriched in the up-regulated gene set in both adults and larvae. DEGs in *Galeruca daurica* under cold stress were also significantly enriched for these terms. In addition, the term oxidoreductase activity was also enriched in *Nilaparvata lugens* and *Sogatella furcifera* at low temperatures*(Huang et al., 2017; Zhou et al., 2019)*. Enrichment for iron ion binding suggests that the base of the trunk and subsurface roots, were the overwintering of *D. valens* occurs, is a low-oxygen environment, where molecular damage is caused by reactive oxygen species (ROS), requiring the production of ferritin or transferrin to remove ROS from cells. In insects, transferrin normally inhibits stress-induced apoptosis *(Lee et al., 2006;Chen et al., 2019)*. Overwintering *Escherichia coli* aggregates ferritin to remove ROS and neutralizes glutathione *(Storey & Storey 2010)*. We hypothesized that cold exposure would increase oxidative stress in insects and induce antioxidant defense systems to resist oxidative stress.

Terms that were significantly enriched in the down-regulated gene set were carbohydrate metabolism processes, hydrolase activity, cellulase activity, and so on. Under stress, metabolic processes are down-regulated in many organisms under stress, and the down-regulation of hydrolase and cellulase activity may be the result of metabolic down-regulation. The downregulation of carbohydrate metabolism suggests that the relationship between energy utilization and temperature is altered in insects during the winter by changes in thermal

sensitivity and the inhibition of metabolic rates; these insects adapt to low temperature by changing energy metabolism*(Storey & Storey 1990; Sinclair, 2015)*.

Cold tolerance-associated KEGG enrichment analysis

In the up-regulated gene set, a KEGG enrichment analysis showed enrichment for thyroid hormone synthesis, glycine, serine and threonine metabolism, antifolate resistance, glutathione metabolism, and so on, indicating that metabolic regulation in response to low temperatures is very important. Three significantly enriched pathways (pentose and glucuronate interconversions and galactose metabolism related to carbohydrate metabolism, and lysosome related to transport catabolism) were detected in the common DEG set. The lysosomal pathway is similarly enriched in the cold-accumulated *D. melanogaster* transcriptome and metabolome *(MacMillan et al., 2016)*. The three most significant enrichment pathways were similar to those for the co-downregulated gene set in adults and larvae of *D. valens* , indicated that overwintering *D. valens* may produce or consume less energy, consistent with the general response of winter insects to low temperatures by inhibiting the metabolic rate *(Sinclair, 2015)*.

In conclusion, we used RNA-Seq technology to analyze the *D. valens* transcriptome at different periods in the field. We identified many common genes and pathways with potentially important roles in overwintering in the species at -18.7°C, improving our understanding of the molecular basis for survival in low temperatures. This study provides an overview of candidate genes associated with cold tolerance in insects, and further validation and functional analyses are needed. Our data will facilitate further molecular studies of cold tolerance in *D. valens* and provide new insights into insect adaptation to harsh environments.

Conclusions

In a comparative transcriptome analysis of *D. valens* in January and May, we detected 4996 and 8260 DEGs in larvae and adults, respectively, including 1189 genes that differentially expressed at both stages. During the overwintering process, one strategy for survival in low temperatures is the synthesis of low-molecular-weight antifreeze substances, anti-oxidative stress factors, molecular chaperones, and signal transduction factors. The DEGs were enriched for the GO terms iron ion binding, oxidoreductase activity, cellulase activity, hydrolase activity, and carbohydrate metabolism and for the lysosomal and oxidative phosphorylation metabolic pathways. We screened 112 genes that may be related to cold tolerance, some of which are associated with cold tolerance based on previous studies. The results provided basic data for the

subsequent discovery of key genes for cold tolerance in *D. valens* and the discovery of molecular mechanisms underlying this trait.

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Competing Interests

The authors declare no conflict of interest.

Author Contributions

- Dongfang Zhao conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote original draft preparation, prepared figures and/or tables, reviewed drafts of the paper.
- Chunchun Zheng performed the experiments, contributed reagents/materials/analysis tools.
- Fengming Shi performed the experiments, contributed reagents/materials/analysis tools.
- Yabei Xu conceived and designed the experiments.
- Jing Tao and Shixiang Zong conceived and designed the experiments, reviewed drafts of the

paper, approved the final draft.

DNA Availability

The following information was supplied regarding data availability:

The National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (BioProject accession number: PRJNA609406).

Supplemental Information

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Table 1(on next page)

Statistical summary of sequencing data for 12 cDNA samples from *D. valens.*

In sample names, CL indicates larvae collected in January, NL indicates larvae collected in May, CA indicates adults collected in January, and NA indicates adults collected in May.

Table 1 :

Statistical summary of sequencing data for 12 cDNA samples from *D. valens*.

Sample	Raw Bases	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
CL_1	7625015592	7435581614	0.0258	97.81	93.21	42.31
CL_2	6606424254	6408262061	0.0258	97.82	93.23	42.14
CL_3	7213969734	7003588404	0.0253	98.01	93.71	43.54
NL_1	6855725858	6669592971	0.0247	98.16	94.46	49.37
NL_2	6275942634	6141265236	0.0245	98.22	94.58	46.91
NL_3	6658060516	6433890287	0.0251	97.96	94.05	50.28
CA_1	7973857302	7780530188	0.0235	98.61	95.64	42.34
CA_2	7687548920	7512555521	0.0237	98.55	95.48	42.12
CA_3	7478884134	7284498797	0.0234	98.67	95.72	39.96
NA_1	8155209510	7979742248	0.0235	98.63	95.62	38.6
NA_2	8403092318	8234779825	0.0233	98.71	95.86	40.07
NA_3	7578902910	7436520894	0.0236	98.62	95.59	38.13

In sample names, CL indicates larvae collected in January, NL indicates larvae collected in May, CA indicates adults collected in January, and NA indicates adults collected in May.

Table 2(on next page)

KEGG pathway analysis of common DEGs between sampling dates in *D. valens* larvae and adults.

1 **Table 2:**

2 **KEGG pathway analysis of common DEGs between sampling dates in *D. valens* larvae and adults**

3

Pathway ID	Secondary Category	Description	Count
map04142	Transport and catabolism	Lysosome	26
map00190	Energy metabolism	Oxidative phosphorylation	21
map04024	Signal transduction	cAMP signaling pathway	18
map04140	Transport and catabolism	Autophagy - animal	18
map04530	Cellular community – eukaryotes	Tight junction	17
map04141	Folding, sorting and degradation	Protein processing in endoplasmic reticulum	16
map04210	Cell growth and death	Apoptosis	15
map00040	Carbohydrate metabolism	Pentose and glucuronate interconversions	14
map04010	Signal transduction	MAPK signaling pathway	13
map04260	Circulatory system	Cardiac muscle contraction	13
map04510	Cellular community - eukaryotes	Focal adhesion	13
map00230	Nucleotide metabolism	Purine metabolism	12
map04145	Transport and catabolism	Phagosome	12
map04261	Circulatory system	Adrenergic signaling in cardiomyocytes	12
map04723	Nervous system	Retrograde endocannabinoid signaling	12
map00020	Carbohydrate metabolism	Citrate cycle (TCA cycle)	11
map04390	Signal transduction	Hippo signaling pathway	11
map00520	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism	11
map04015	Signal transduction	Rap1 signaling pathway	11

map04921	Endocrine system	Oxytocin signaling pathway	11
map04919	Endocrine system	Thyroid hormone signaling pathway	11
map04910	Endocrine system	Insulin signaling pathway	11
map04810	Cell motility	Regulation of actin cytoskeleton	11
map00052	Carbohydrate metabolism	Galactose metabolism	10
map04020	Signal transduction	Calcium signaling pathway	10
map03013	Translation	RNA transport	10
map04013	Signal transduction	MAPK signaling pathway - fly	10
map04151	Signal transduction	PI3K-Akt signaling pathway	10
map04146	Transport and catabolism	Peroxisome	10
map04022	Signal transduction	cGMP-PKG signaling pathway	10

4

Table 3(on next page)

GO enrichment of common DEGs between sampling dates in *D. valens* larvae and adults.

1 **Table 3:**

2 **GO enrichment of common DEGs between sampling dates in *D. valens* larvae and adults**

GO category	Term type	GO code	Number	P-value
carbohydrate metabolic process	BP	GO:0005975	67	3.10E-07
peptide metabolic process	BP	GO:0006518	3	8.55E-06
peptide biosynthetic process	BP	GO:0043043	3	2.34E-05
cellular amide metabolic process	BP	GO:0043603	6	2.00E-04
external encapsulating structure organization	BP	GO:0045229	8	3.00E-04
peptide metabolic process	BP	GO:0006518	3	8.55E-06
translation	BP	GO:0006412	3	2.53E-05
amide biosynthetic process	BP	GO:0043604	4	7.55E-05
cell wall organization or biogenesis	BP	GO:0071554	8	3.50E-04
cell wall organization	BP	GO:0071555	8	3.50E-04
positive regulation of biological process	BP	GO:0048518	3	7.89E-04
cellular nitrogen compound metabolic process	BP	GO:0034641	68	1.18E-03
organelle	CC	GO:0043226	58	1.95E-06
intracellular ribonucleoprotein complex	CC	GO:0030529	8	2.34E-05
ribonucleoprotein complex	CC	GO:1990904	8	2.34E-05
cell part	CC	GO:0044464	186	2.34E-05
ribosome	CC	GO:0005840	3	2.00E-04
non-membrane-bounded organelle	CC	GO:0043228	9	1.46E-05
intracellular non-membrane-bounded organelle	CC	GO:0043232	9	1.46E-05

macromolecular complex	CC	GO:0032991	62	3.67E-04
cellulase activity	MF	GO:0008810	7	3.00E-04
nucleic acid binding	MF	GO:0003676	88	3.00E-04
hydrolase activity, acting on glycosyl bonds	MF	GO:0016798	49	3.10E-07
hydrolase activity, hydrolyzing O-glycosyl compounds	MF	GO:0004553	45	3.64E-07
structural constituent of ribosome	MF	GO:0003735	4	1.00E-04

3

4

Table 4(on next page)

Common differentially expressed gene set may be related to cold tolerance.

1 **Table 4:**

2 **Common differentially expressed gene set may be related to cold tolerance**

Gene description	DEG number	Larval expression (number)	Adult expression (number)
Molecular chaperone			
HSP70	1	up (1)	down (1)
Cytoskeleton proteins and regulators			
tubulin	2	down (1); up (1)	down (2)
actin	5	down (4); up (1)	down (5)
myosin	4	down (3); up (1)	Down (4)
integrin	2	down (2)	down (2)
Cuticle protein			
cathepsin	1	down (1)	down (1)
antioxidants			
thioredoxin	1	up (1)	down (1)
Signaling genes			
guanine nucleotide-binding protein	3	down (1); up (1)	down (3)
rho guanine nucleotide exchange factor	4	down (3); up (1)	down (4)
calcium channel protein	8	down (2); up (6)	down (8)
Genes associated with cell cycle and division			
inhibitor of apoptosis	1	down (1)	down (1)
serine/threonine-protein kinase	5	down (4); up (1)	down (5)
Other genes			

ATP synthase subunit alpha	1	down (1)	down (1)
endonuclease-reverse transcriptase	3	down (2); up (1)	down (3)
E3 ubiquitin-protein ligase	7	down (5); up (2)	down (7)
zinc finger protein	8	down (6); up (2)	down (8)
NADH dehydrogenase	4	down (3); up (1)	down (4)
elongation factor	1	down (1)	down (1)
ribosomal protein	6	down (1); up (1)	down (6)
staphylococcal nuclease domain-containing protein 1	1	down (1)	down (1)

3

4

Figure 1

Statistical summary of the functional annotations of unigenes in public databases.

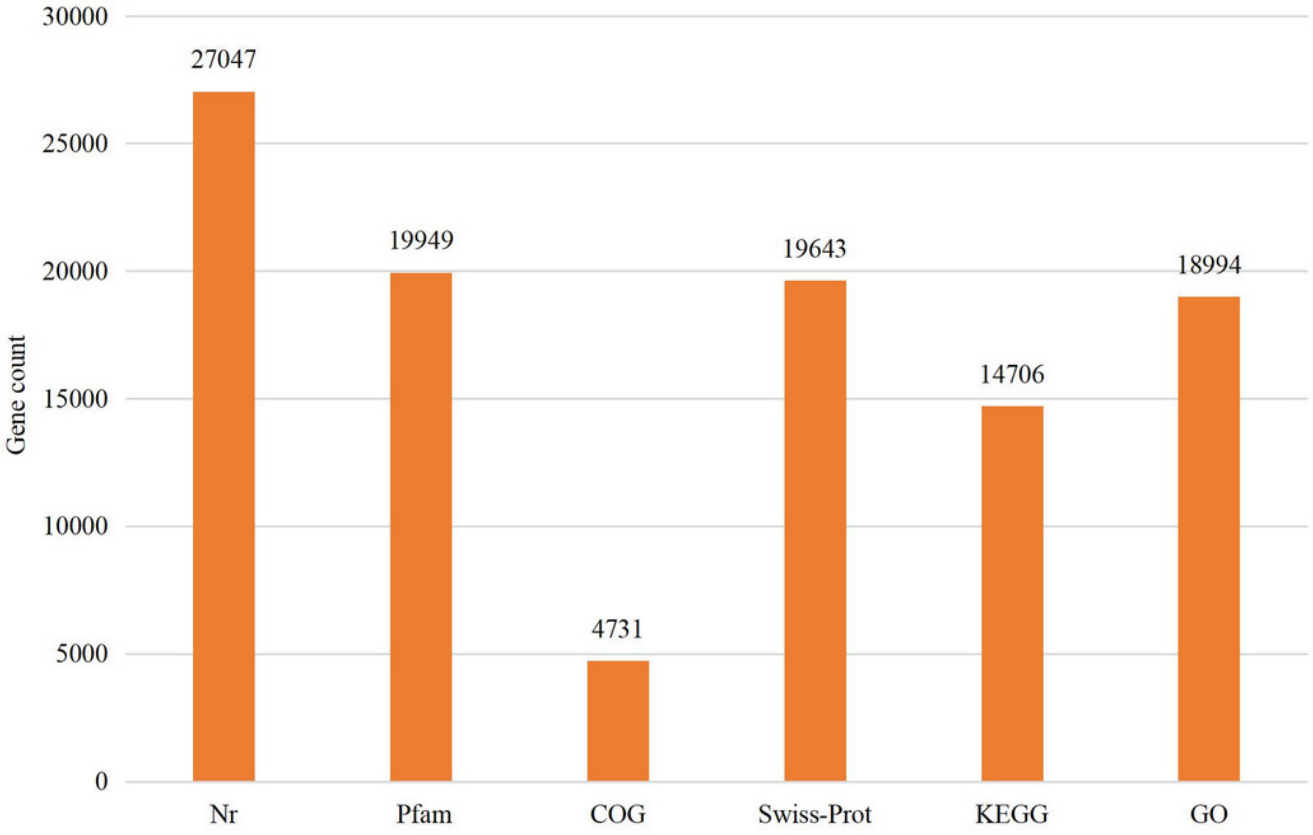


Figure 2

Pie charts showing distributions of BLAST matches for the *D. valens* unigenes with respect to (A) e-values (B) similarity, and (C) species.

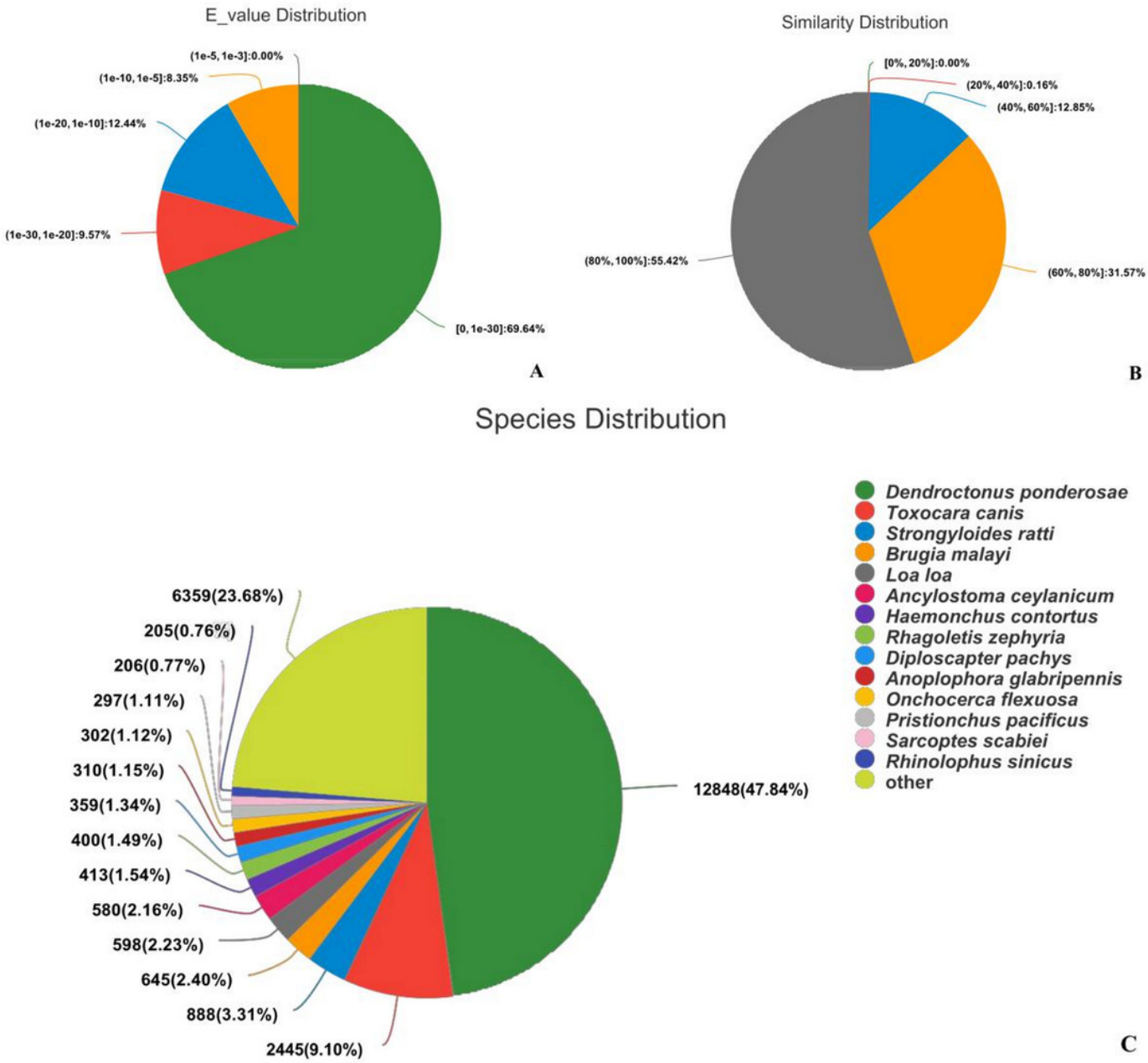


Figure 3

Distribution of DEGs between January and May in different sample types.

Distribution of DEGs between January and May in different sample types. (A) Distribution of DEGs in larvae. (B) Distribution of DEGs in adults. Volcano plot displays the relationship between the fold change and P-adjusted. Each point in the figure represents a specific unigene. Red dots represent significantly up-regulated unigenes, green dots represent significantly down-regulated unigenes, and gray dots represent unigenes that are not differentially expressed.

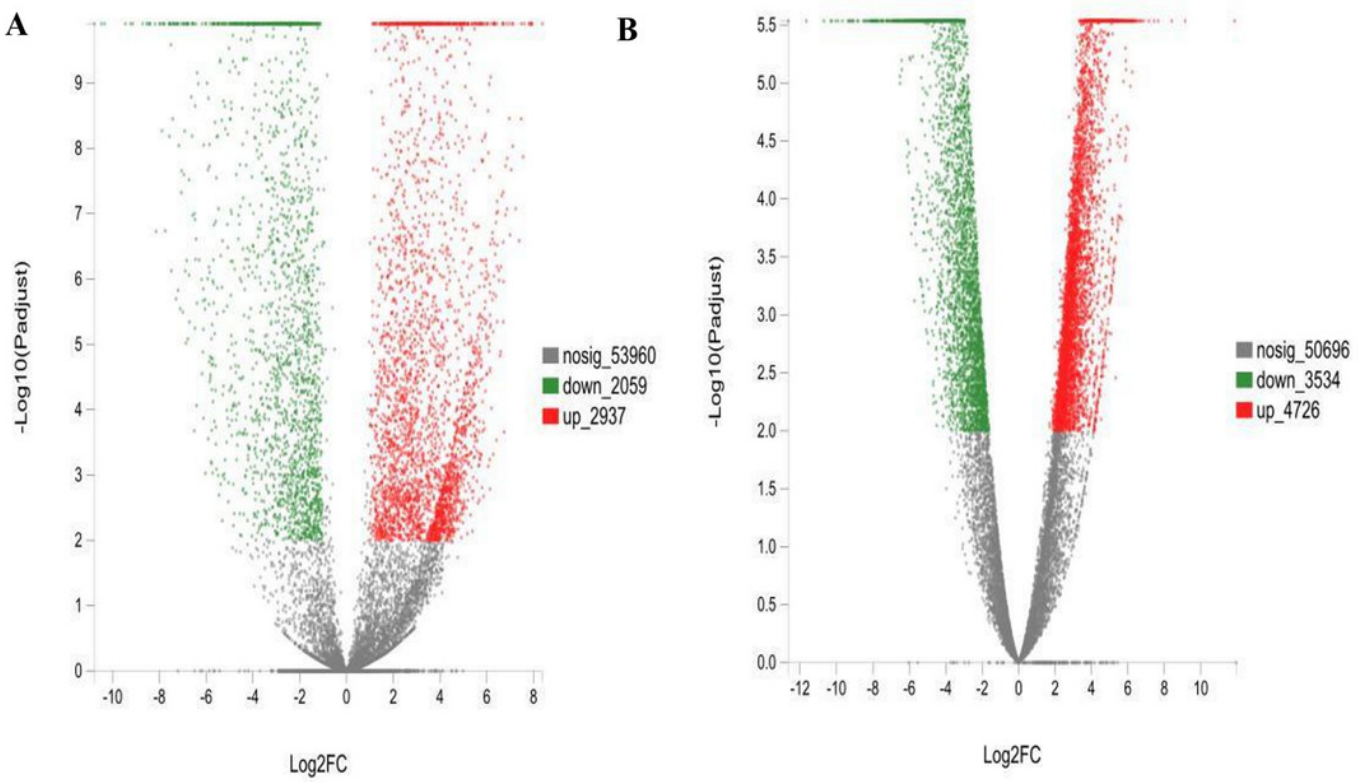


Figure 4

Venn diagram showing the number of commonly up- and down-regulated genes between the two different stages in *D. valens*.

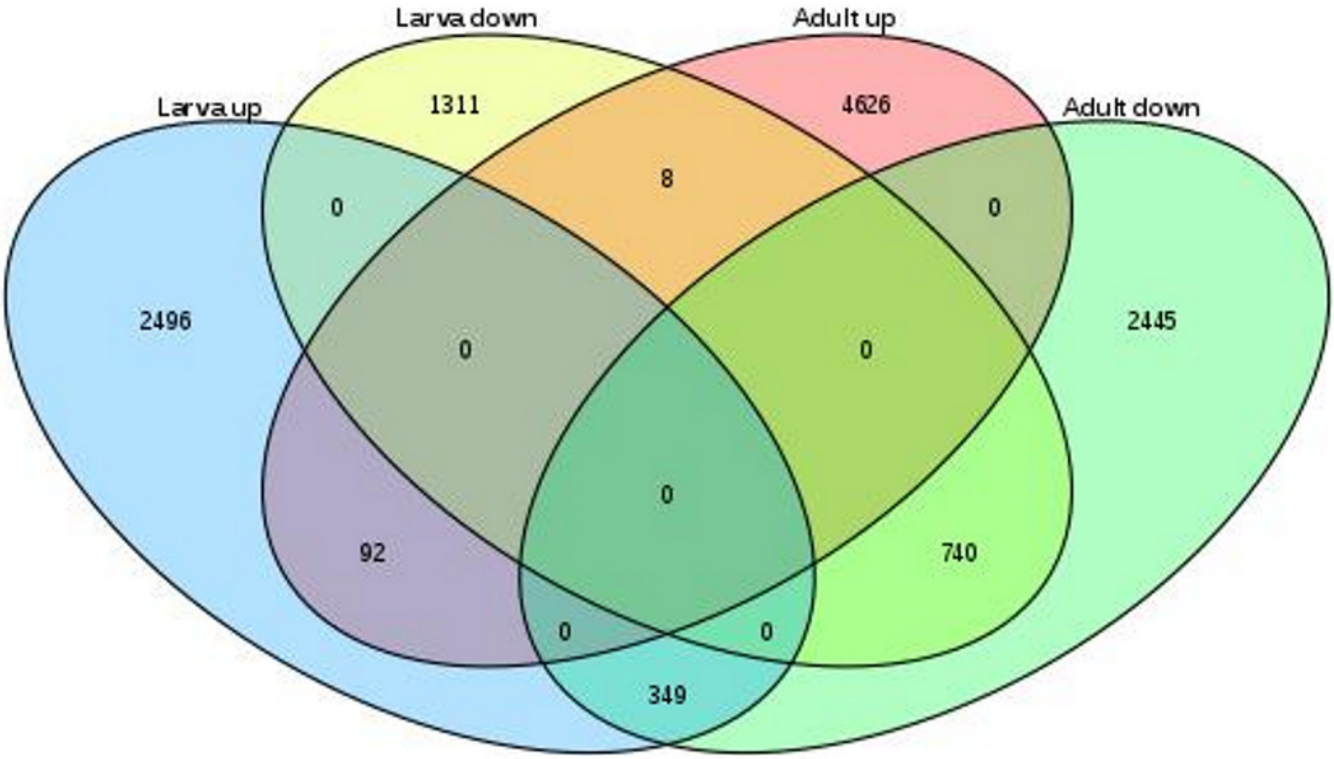


Figure 5

GO term assignments for common DEGs between collection times in *D. valens* larvae and adults.

The abscissa indicates the secondary GO classification. The left ordinate indicates the percentage of unigenes included in the secondary classification. The right ordinate indicates the number of unigenes in the secondary classification and the three colors represent the three major branches of GO (i.e., BP, CC, and MF).

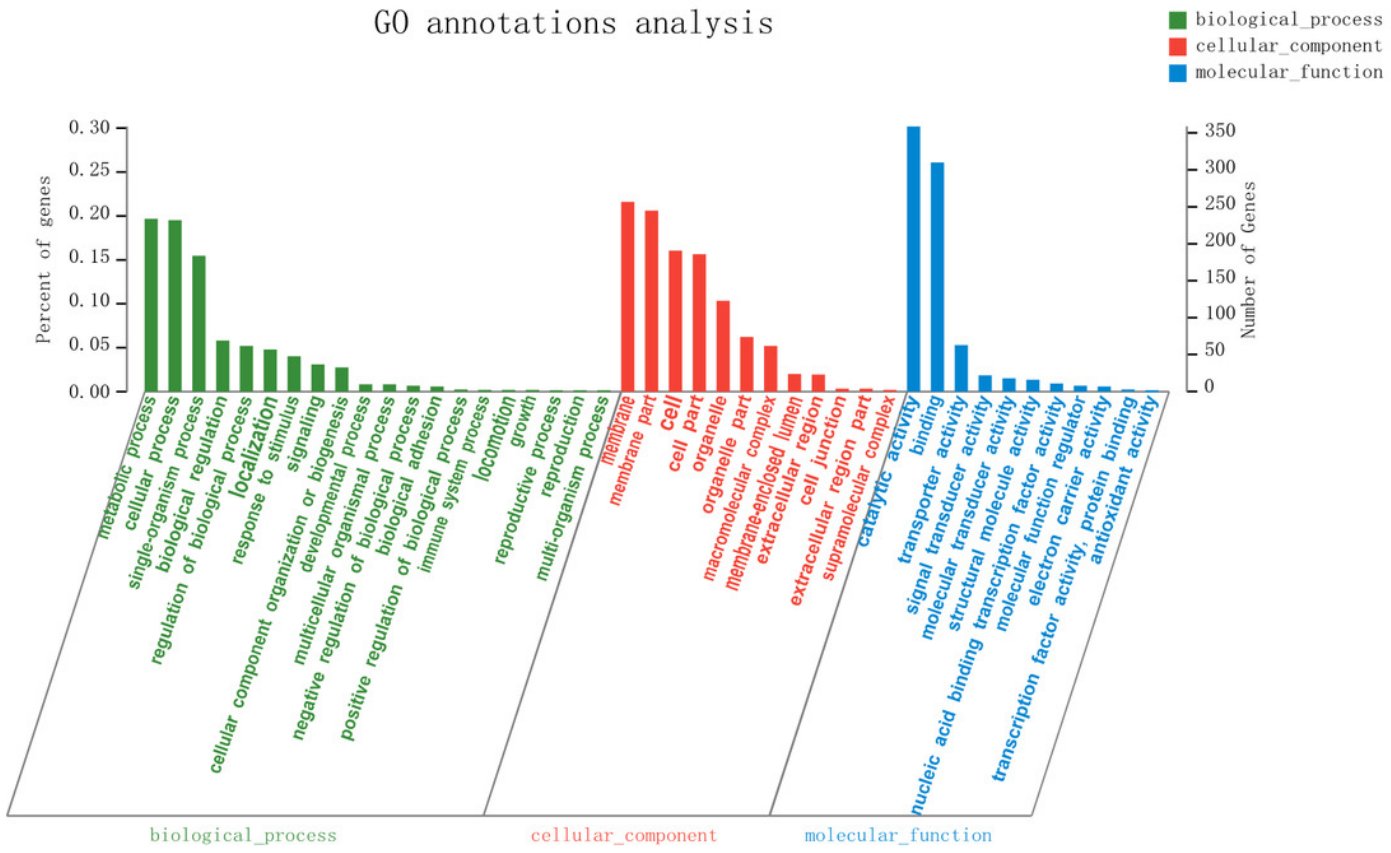


Figure 6

Heatmap of normalized FPKM values for DEGs related to cryoprotectant activity.

Heatmap of normalized FPKM values for DEGs related to cryoprotectant activity. The Z-score represents the number of standard deviations from the mean. Red indicates up-regulated expression, whereas green indicates down-regulated expression. FPKM: fragments per kilobase of transcript per million fragments mapped; CL: Larva in January; NL: Larva in May; CA: Adult in January; NA: Adult in May.

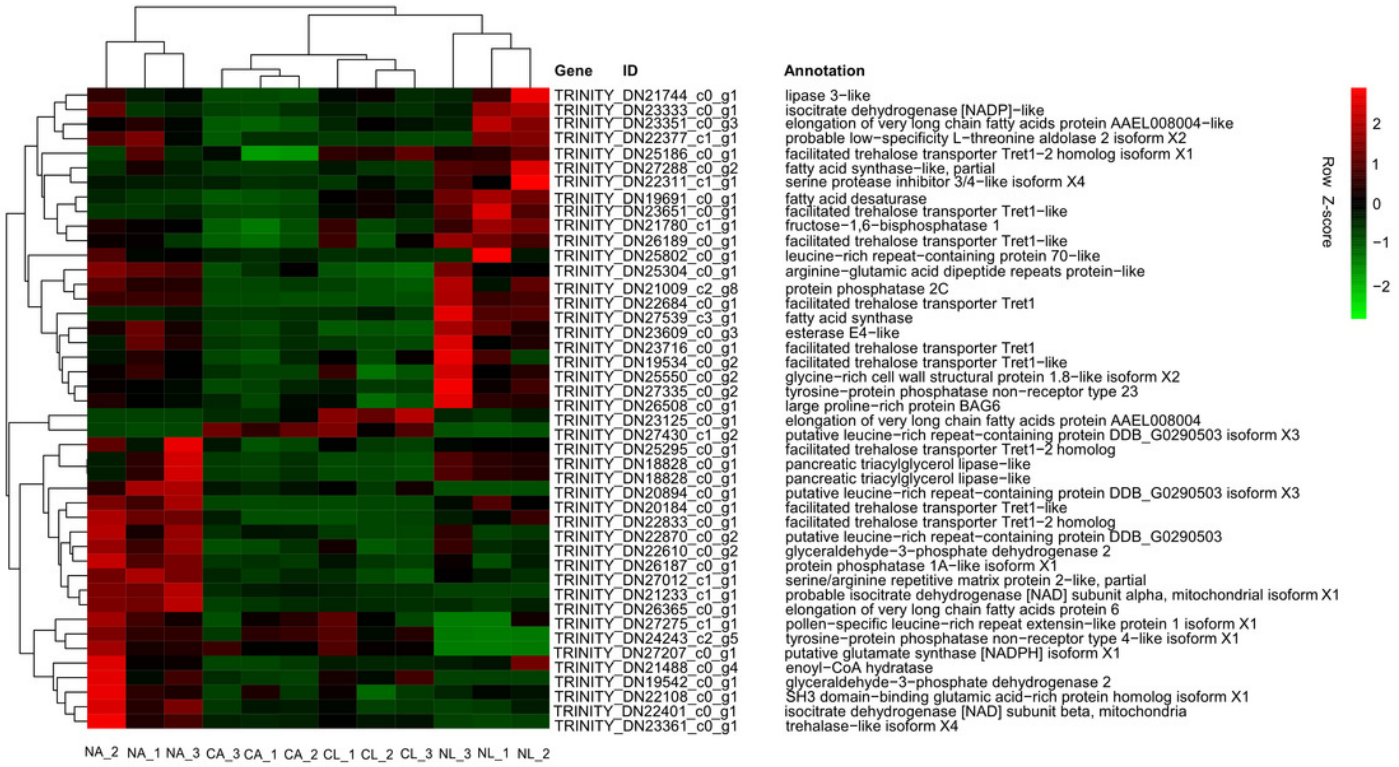


Figure 7

Validation expression patterns in *D. valens* adults by qPCR.

The abscissa indicates different collection dates. The left ordinate represents the qPCR-based expression levels and the right ordinate represents the RNA-seq-based expression levels.

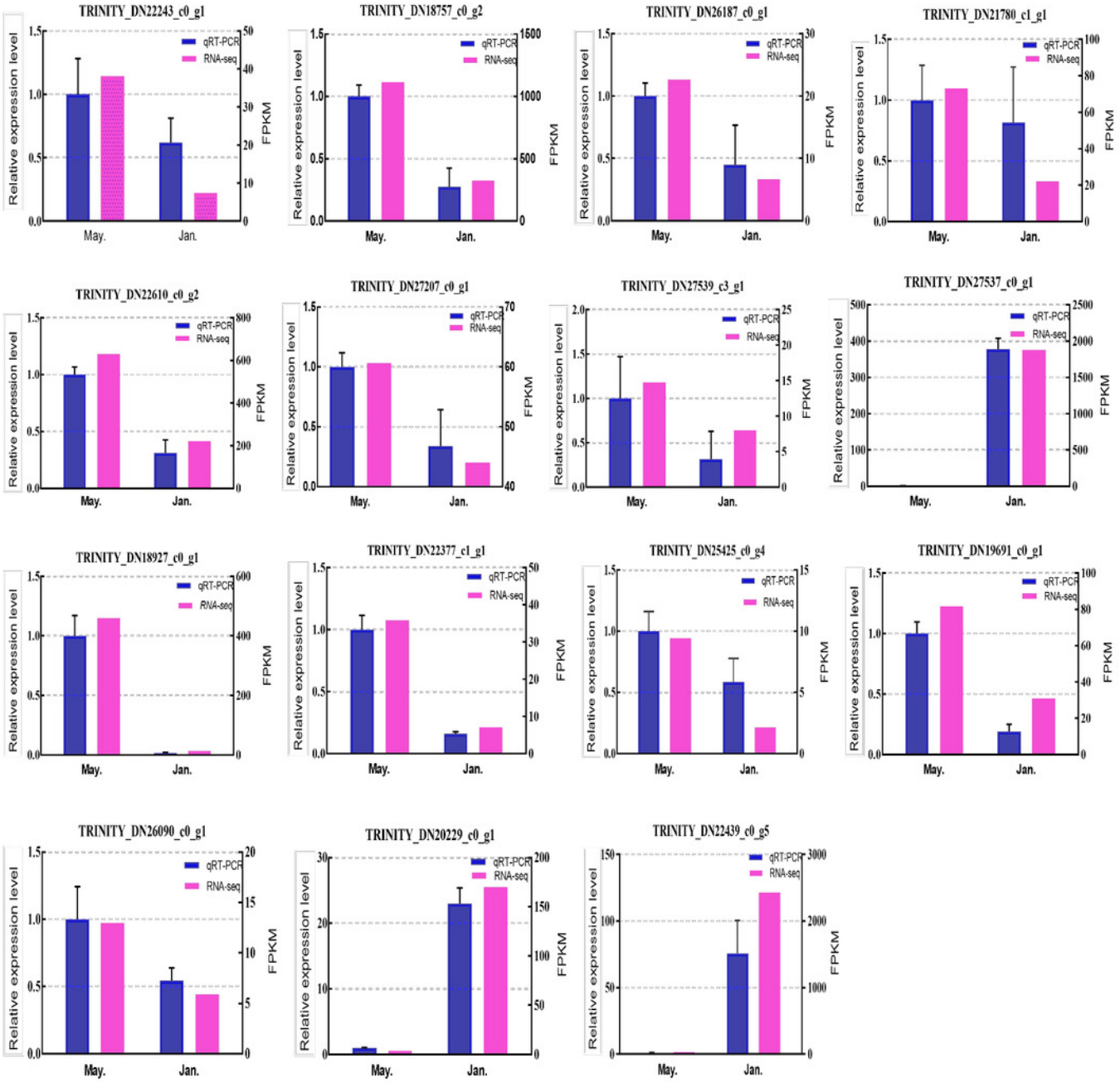
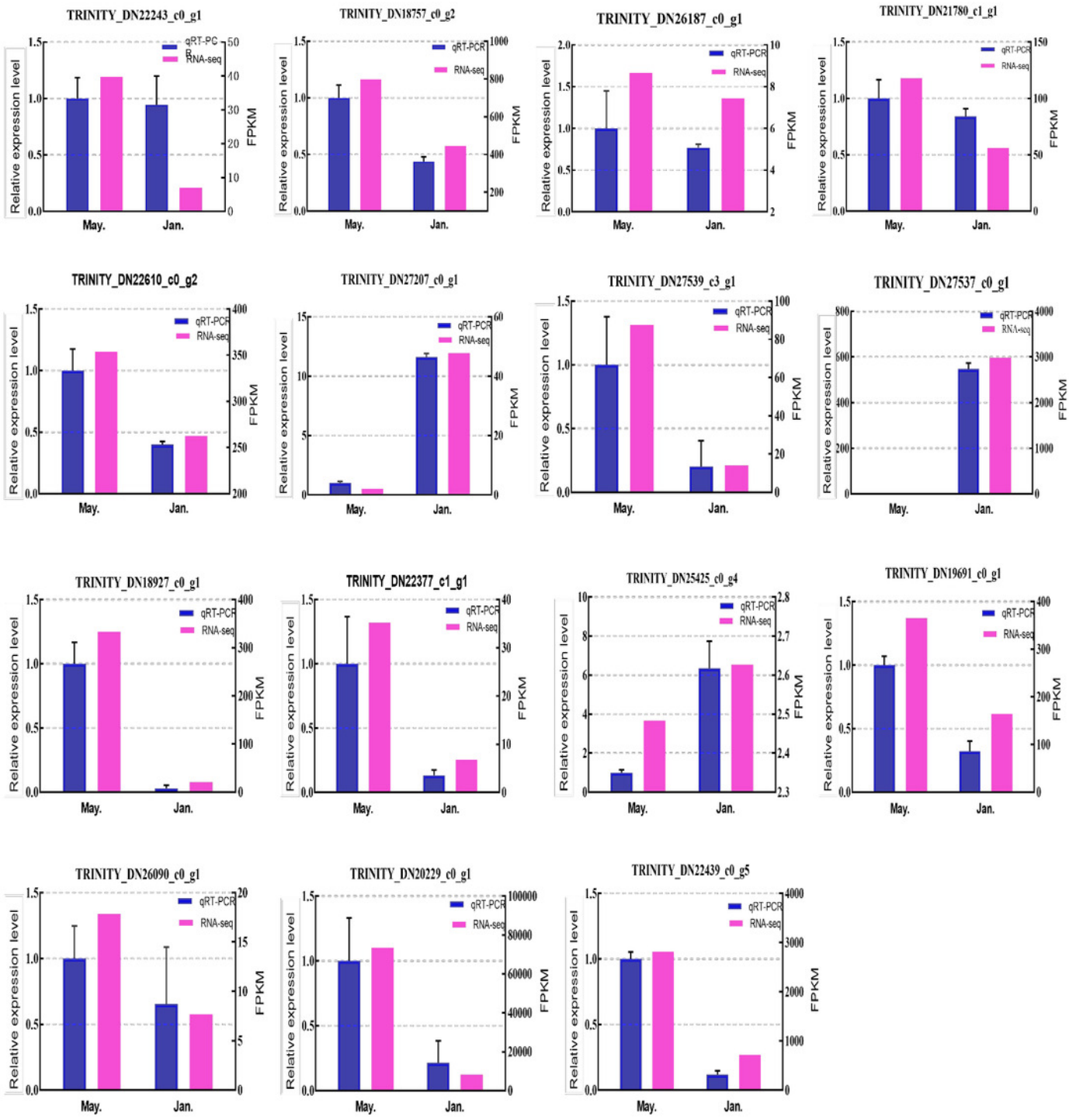


Figure 8

Validation of expression patterns in *D. valens* larvae by qPCR.

The abscissa indicates different collection dates. The left ordinate represents the qPCR measurements, and the right ordinate represents the RNA-seq measurements.


TRINITY_DN22243_c0_g1
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TRINITY_DN26187_c0_g1
TRINITY_DN21780_c1_g1
TRINITY_DN22610_c0_g2
TRINITY_DN27207_c0_g1
TRINITY_DN27539_c3_g1
TRINITY_DN27537_c0_g1
TRINITY_DN18927_c0_g1
TRINITY_DN22377_c1_g1
TRINITY_DN25425_c0_g4
TRINITY_DN19691_c0_g1
TRINITY_DN26090_c0_g1
TRINITY_DN20229_c0_g1
TRINITY_DN22439_c0_g5
qRT-PCR
RNA-seq
Relative expression level
FPKM
May.
Jan.