Comparative Biochemistry and Physiology, Part D 22 (2017) 78–89

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part D

journal homepage: www.elsevier.com/locate/cbpd

Transcriptome analysis and identification of induced genes in the response of *Harmonia axyridis* to cold hardiness

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ARTICLE INFO

Article history:
Received 17 June 2016
Received in revised form 18 January 2017
Accepted 27 January 2017
Available online 20 February 2017

Keywords:
Harmonia axyridis
Transcriptome
DGE
Induced gene
Cold hardiness
Cryopreservation

ABSTRACT

Harmonia axyridis is an important predatory lady beetle that is a natural enemy of agricultural and forestry pests. In this research, the cold hardiness induced genes and their expression changes in *H. axyridis* were screened and detected by the way of the transcriptome and qualitative real-time PCR under normal and low temperatures, using high-throughput transcriptome and digital gene-expression-tag technologies. We obtained a 10 Gb transcriptome and an 8 Mb gene expression tag pool using Illumina deep sequencing technology and RNA-Seq analysis (accession number SRX540102). Of the 46,980 non-redundant unigenes identified, 28,037 (59.7%) were matched to known genes in GenBank, 21,604 (46.0%) in Swiss-Prot, 19,482 (41.5%) in Kyoto Encyclopedia of Genes and Genomes and 13,193 (28.1%) in Gene Ontology databases. Seventy-five percent of the unigene sequences had top matches with gene sequences from *Tribolium castaneum*. Results indicated that 60 genes regulated the entire cold-acclimation response, and, of these, seven genes were always up-regulated and five genes always down-regulated. Further screening revealed that six cold-resistant genes, E3 ubiquitin-protein ligase, transketolase, trehalase, serine/arginine repetitive matrix protein 2, glycerol kinase and sugar transporter SWEET1-like, play key roles in the response. Expression from a number of the differentially expressed genes was confirmed with quantitative real-time PCR (HaCS_Trans). The paper attempted to identify cold-resistance response genes, and study the potential mechanism by which cold acclimation enhances the insect's cold endurance. Information on these cold-resistance response genes will improve the development of low-temperature storage technology of natural enemy insects for future use in biological control.

1. Introduction

Cold hardy insects in arctic and sub-arctic regions of North America, Scandinavia, Russia and China must survive the low temperatures of their habitats (Bale, 1996; Somme, 1999; Khani and Moharramipour, 2010). Cold acclimation is an important aspect of overwintering adaptation. The Asian lady beetle (*Harmonia axyridis*) is remarkably cold hardy and can overwinter as a diapausing adult (Koch et al., 2004). In nature, a large population of *H. axyridis* overwinters in Northeastern China. In the laboratory, short-time cold acclimation of these insects can significantly promote cold tolerance (Watanabe, 2002; Koch et al., 2004; Labrie et al., 2008; Berkvens et al., 2010).

H. axyridis is extensively distributed throughout Asia. As a ferocious predator of many pests, especially aphids (Koch, 2003), it is considered a natural enemy of several agricultural and forestry pests in Asia (Tang et al., 2014a; Shi et al., 2016). Its voracious appetite, reproductive capacity and strong resistance to cold have allowed it to become an invasive species: the first specimens were collected in North America and Canada in 1994 (Coderre et al., 1995; Brown and Miller, 1998), and the insect was observed in Belgium as early as 2001 (Adriaens et al., 2003). As an invasive species, *H. axyridis* has a detrimental effect on natural environments and indigenous species and has rapidly become dominant in many areas. Nonetheless, it is considered a beneficial organism and has been used to control pests, including aphids, in North America (Koch, 2003) and Canada (Coderre et al., 1995). It is believed that the use of *H. axyridis* in biological control can effectively reduce the harmful environmental impact of pesticide use.

However, the use of *H. axyridis* as a biological control presents the following limitations to large-scale application: cannibalism, the need for artificial feeding and the logistics of low-temperature storage (Watanabe, 2002; Shi et al., 2016). In particular, the beetles cannot be stored at low temperatures for periods that are longer than their natural overwintering conditions (Wu et al., 2016). Little is known about how these beetles endure the low winter temperatures and how the induced cold-hardiness genes function. Although the cold hardiness of this insect has been studied extensively, the molecular mechanisms and genetic regulators of cold adaptation are less well characterized.

Transcriptome and differential gene expression (DGE) analyses based on next-generation deep-sequencing technology provided

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http://dx.doi.org/10.1016/j.cbd.2017.01.004
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Table 1
Summary statistics of assembly of *Harmonia axyridis* transcriptome.

Parameters	HaRT_trans	HaCS_trans
Total raw reads	57,398,406	56,915,276
Total clean reads	53,875,178	53,590,364
Q20	97.90%	98.03%
GC percentage	43.09%	41.92%
Contig number	70,876	69,975
Contig N50 (bp)	916	1039
Unigene number	42,886	44,741
Unigene N50 (bp)	1005	1070

Table 2
Summary statistics of annotation of all unigenes.

Database	Unigene number	Percentage (%)
NR	28,037	59.7
NT	10,559	22.5
Swiss-prot	21,604	46.0
KEGG	19,482	41.5
COG	9921	21.1
GO	13,193	28.1
All unigenes	46,980	100.0

extensive data with enormous depth and coverage (Oppert et al., 2010; Husseneder et al., 2012). In addition, the application of RNA-Seq analysis, which allows the de novo assembly of short reads from RNA sequences without genome references, was also performed. As well as transcriptome and RNA-Seq analysis were used to find the new gene and its function in insect (Liu et al., 2015; Zhou et al., 2015; Cui et al., 2016; Niu et al., 2016), especially in discovering and studying of stress-induced genes (Dunning et al., 2013; Pan et al., 2015; Osachoff et al., 2016; Wang et al., 2016).

2. Materials and methods

2.1. Experimental samples and sample treatment

Beetles were maintained at 25 ± 1 °C with 70 ± 5% humidity. The population was established under the lab conditions of Hangzhou Normal University and raised for more than three generations. Insects were raised in rectangular plastic boxes (15 × 12 × 7 cm) at densities of 20 to 30 insects per cage and were fed with bean aphid (*Aphis medicaginis*) once per day. Folded paper was placed in the boxes to provide spawning substrates. After females spawned, eggs were removed to insect cages (aluminum alloy with 60 mesh gauze net system, 5 × 30 × 30 cm) for development and breeding.

Insects were exposed to normal conditions and different periods under low-temperature conditions, with all treatments replicated three. The populations of *H. axyridis* exposed to normal conditions (room temperature) were the RT group and termed HaRT_Trans or RT in transcriptome and DGE analysis, respectively. The population under different treatments of *H. axyridis* was exposed to the low temperature of 5 °C for 2 h, 12 d and 150 d – the corresponding treatments were termed "CS_A" and "CS_B", "DF_A" and "DF_B", "DFLT_A" and "DFLT_B" in DGE sequencing and analysis. At the same time, the library preparation for RNA under three treatment groups which 5 °C for 2 h, 12 d and 150 d, and every thirty *Harmonia axyridis* adults were isolated and mixed as "HaCS_Trans" sample for transcriptome sequencing and analysis.

2.2. RNA isolation and library preparation for transcriptome analysis

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Three micrograms of total RNA was used as the input material for sequencing. All sample RNAs were detected with RIN values >8 using the 2100 Bioanalyzer (Agilent Technologies). The sequencing library was generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. mRNA was purified from total RNA using poly(T)-oligo-conjugated magnetic beads. Fragmentation was performed by heating the RNA in fragmentation buffer (Illumina) containing divalent cations. First-strand complementary DNA (cDNA) was performed using random oligonucleotides and SuperScript II reverse transcriptase (Invitrogen). Second-strand cDNA synthesis and RNA degradation were performed using DNA Polymerase I and RNase H. The overhangs were converted to blunt ends through the exonuclease activity of the polymerase, and the double-

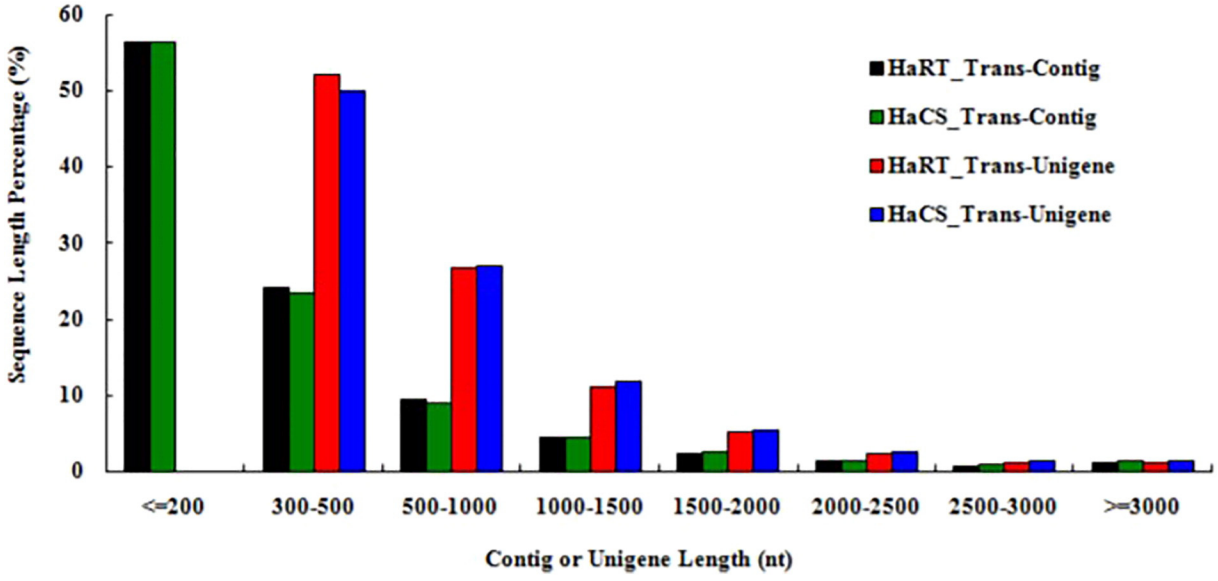

Fig. 1. Length distribution of *Harmonia axyridis* transcriptome unigenes and contigs. Sizes of all unigenes and contigs were calculated: the length (nt) is plotted on the x-axis and the number on the y-axis.

Table 3
Summary statistics of two parallel DGEs.

	Total reads	Mapping percentage	Perfect match	≤2 bp mismatch
The first sequencing				
RT	12,347,866	91.76%	67.40%	24.36%
CS (2 h)	12,121,879	91.85%	67.98%	23.88%
DF (12 d)	12,192,991	91.66%	66.38%	25.28%
DFLT (150 d)	12,109,521	90.37%	65.22%	25.15%
The second sequencing				
RT	11,512,895	92.12%	67.77%	24.35%
CS (2 h)	12,176,022	92.13%	68.02%	24.11%
DF (12 d)	11,393,954	90.79%	67.37%	23.42%
DFLT (150 d)	12,060,986	88.37%	61.45%	26.92%

stranded DNA fragments were purified. The 3′ ends of the DNA fragments were adenylated, and Illumina PE adapter oligonucleotides were ligated to both termini of the adenylated fragments for hybridization. To select adaptor-ligated cDNA fragments of approximately 200 bp in length, the library fragments were purified using the AMPure XP system (Beckman Coulter, Brea, CA, USA). The purified cDNA fragments were selectively enriched during 10 cycles of PCR using the Illumina PCR Primer Cocktail. Products were purified again, and quantified with the High-Sensitivity DNA Assay (Agilent Technologies, Santa Clara, CA, USA) performed on the Bioanalyzer 2100 system (Agilent). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina), according to the manufacturer's instructions.

2.3. Illumina sequencing, read assembly and annotation of transcriptome genes

The libraries were sequenced on an Illumina HiSeq 2000 platform, and 200 bp paired-end reads were generated. One transcriptome from the control group (HaRT_Trans) and another transcriptome (HaCS_Trans) from the three treatment groups (under low-temperature 2 h, 12 d, 150 d) which including ever thirty *Harmonia axyridis* adults, were obtained with a combined mixture total of $>5 \times 10^9 \times 2$ bp clean sequence reads.

Raw data in FASTQ format were processed using in-house Perl scripts. Clean data were obtained by filtering reads containing adapter or poly(N) sequences and low-quality reads. The Q20, GC-content, and level of sequence duplication of the clean data were calculated to ensure that all of the downstream analyses were based on high-quality data. The transcriptomes were assembled using the Trinity program (Grabherr et al., 2011) using a min_kmer_cov value of 2 and default settings for the other parameters. The resulting contigs were joined into scaffolds using paired-end joining and gap-filling methods. Paired-end reads were used again for gap filling of scaffolds to get sequences with the lowest number of Ns that could not be extended on either end.

Table 4
Summary cold hardiness-related genes.

ID	Transcript ID	Putative function	Description
1	CL2532.Contig2	DOPA decarboxylase	BAA95568.1
2	Unigene20527		
3	Unigene10292		
4	Unigene506	Cystathionine gamma-lyase	XP_309478.3
5	CL4373.Contig1		
6	CL4577.Contig1		
7	Unigene8416	Serine/arginine repetitive matrix protein 2	XP_966706.1
8	CL1295.Contig1	rRNA-processing protein CGR1	EFA07306.1
9	CL5063.Contig3	Odorant binding protein	EFA07430.1
10	Unigene14582		
11	Unigene1527	Chloride intracellular channel 6	ELU05672.1
12	Unigene5440	Endonuclease-reverse transcriptase	EHJ73704.1

Table 5
Primers used in QRT-PCR.

Gene	Primer name	Nucleotide sequences (5′-3′)
1	Uni506-qF	AAG CAC AAG ACC CAG AAC AAT G
	Uni506-qR	GGT GGT GAT GGC TCC TAA ACC
2	Uni8416-qF	TCT CAG AAG CGA CCT CAA GC
	Uni8416-qR	TGG CTA CCA TTT GGG ACT G
3	CL4373-qF	TTT GTG CGT AGC CGT TGG
	CL4373-qR	CTT TTG CCC TTG TCC GTG
4	CL1295-qF	GAG CGA CGA AAC TCC ACC AG
	CL1295-qR	TTG GCT GCT TGT TCC TCT GC
5	CL2532-qF	TGC GAG TGA AGC TAC GCT AG
	CL2532-qR	TCG GTT TGA TTC GTC TGT GG
6	Uni1527-qF	TGC AAC AAT CGC TCT GAC G
	Uni1527-qR	CTC CTA CTG GAC CCC ATA C
7	Uni8245-qF	ATG CGT CTC AGC AAC ACC AAC
	Uni8245-qR	CTG AAC ATT GCC TCT TGA AAC C
8	Uni5440-qF	GCA GGA ATT GAG CGA GAT G
	Uni5440-qR	TGC CGA TTC TTA TTG AGT GTC
9	Uni6041-qF	CAA GGT CAC CAA AGA TAC AGC C
	Uni6041-qR	CGT GAA GTT AGT ACC GTT GAA GTG
10	Uni10807-qF	TAC ATT ATG CTC GGG CTT GTC
	Uni10807-qR	AAT CCC TGG CTT GTT CCT TG
11	CL5063-qF	CAC TTT CGC CGA TGA CGA C
	CL5063-qR	CGG CGT ATC AAC GAC AAT G
12	Uni12773-qF	GAA AAG ACT GAG GGA CAA AGG C
	Uni12773-qR	TCC TCG TTC CAC CAA CCT AC
13	CL4426-qF	TGG AAT CCA CGC AGG TGT C
	CL4426-qR	AAG TTC GCT TTT CCA ATC TCG
14	CL1777-qF	CGA TTC AAC CAA CCC AAC G
	CL1777-qR	TCA TCC GAC AAA AGT CAA CCT C
15	18S-qF	ACG GACTTCGGTAGGACG
	18S-qR	CGCAGACAATCCCGAAA

The final sequences are referred to as unigenes. Following assembly, duplicate unigenes were removed from the two transcriptomes, and a new reference generated from the two transcriptomes for DGE analysis was obtained.

Before annotation, the unigenes of the two transcriptomes were pooled in a single database (all unigenes) by removing redundant unigenes, and this pool of 46,980 unigenes was used as a reference for DGE analysis (Table 2). Annotation of unigene sequences was conducted with a BLASTx comparison using sequences in the GenBank database. Non-redundant unigenes were used for BLASTx searches and annotation, using a cut-off E-value of 10^{-5} and protein databases, including nr (http://www.ncbi.nlm.nih.gov/), Swiss-Prot (http://www.ebi.ac.uk/uniprot/), KEGG (http://www.genome.jp/kegg/) and COG (http://www.ncbi.nlm.nih.gov/COG/). The first hit (the lowest E-value) for each sequence was used for the analysis. GO annotation was used to assign possible functions to the predicted genes. Functional annotation using gene ontology terms (GO; http://www.geneontology.org) was performed.

2.4. DGE library sequencing

Libraries were prepared with the data from a single replication of each treatment. The resulting eight libraries were independently deep-sequenced, as described above. Every sample obtained from 1×10^9 bp of clean sequence reads. The read counts were adjusted with edgeR software (Lambirth et al., 2015) using the default settings for the other parameters. Differential expression was identified by comparing data in the same group (samples A and B) and with another group (different treatment samples) using the DESeq R package and so on (Faherty et al., 2015). The data sets are available at the Sequence Read Archive (SRA) repository with accession number SRX540102.

2.5. Distribution of DGE tags on genes

The unigene expression level was calculated by using the RPKM method (Mortazavi et al., 2008). False discovery rate (FDR) is a method

to determine the threshold of *P*-value in multiple tests. During the analysis, "FDR ≤0.001 and the absolute value of log2Ratio ≥ 1" was used as the threshold to judge the significance of gene expression difference.

The *P*-values were adjusted using the Q value, with Q < 0.005 and |log2 (fold-change)| > 1 as the threshold for identifying significantly different levels of expression between the two inter-samples. The levels of gene expression were estimated based on RNA-Seq by expectation-maximization (Li and Dewey, 2011) for each sample. The clean data were mapped back onto the assembled transcriptome, and the read count for each gene was obtained from the mapping results. GO enrichment analysis of the differentially expressed genes was performed using the GOseq R software (Li et al., 2014) based on the Wallenius' noncentral hypergeometric distribution to adjust for gene length bias in the differentially expressed genes. We used the KOBAS software (Mao et al., 2005) to test the statistical enrichment of differentially expressed genes that were annotated based on KEGG pathways.

It is generally believed that similar gene expression patterns usually have similar function. Simultaneous hierarchy clustering analysis of genes and the experimental conditions were performed by Cluster software (de Hoon et al., 2004) based on the Euclidean distance formula for the matrix.

2.6. Confirmatory qRT-PCR

To confirm the DGE results, qualitative real-time PCR (qRT-PCR) was performed for 14 genes randomly selected from the 60 common differentially expressed genes in this study. Primers were designed using Primer 5.0 software. Expression levels for the selected genes were computed based on the stable expression level of the reference gene, 18s (Wu et al., 2016). The primers used for qRT-PCR are shown in Table 5. qRT-PCR was performed in 96-well plates on a Bio-Rad CFX96 Real-Time PCR Detection system using Premix Ex *Taq* (SYBR Green) reagents (Takara, Dalian). In 20 μl volumes, the qRT-PCR thermal profile consisted of 95 °C for 3 min, followed by 39 cycles of 95 °C for 10 s and 60 °C for 30 s. Dissociation curves were obtained from a thermal melting profile generated under a final PCR cycle of 95 °C for 30 s followed by a constant increase in temperature from 60 °C to 95 °C. Sample cycle threshold (C_T) values were standardized for each template based on the 18s control primer reaction, and the $2^{-\Delta\Delta CT}$ method was used to analyze relative changes in gene expression (Livak and Schmittgen, 2001). Three replicate reactions per sample were used to ensure statistical credibility.

3. Results

3.1. Transcriptome profile of Harmonia axyridis

The transcriptome of HaRT contained a total of 57,398,406 raw reads that, after filtering, yielded 53,875,178 clean reads (Table 1). The transcriptome of HaCS_Trans contained a total of 56,915,276 raw reads, with 53,590,364 clean reads after filtering. The average Q20 quality scores of the two transcriptomes were >97%, and the GC contents were >41%. Using clean reads, 70,876 and 69,975 contigs were assembled and 42,886 and 44,741 non-redundant unigenes were obtained

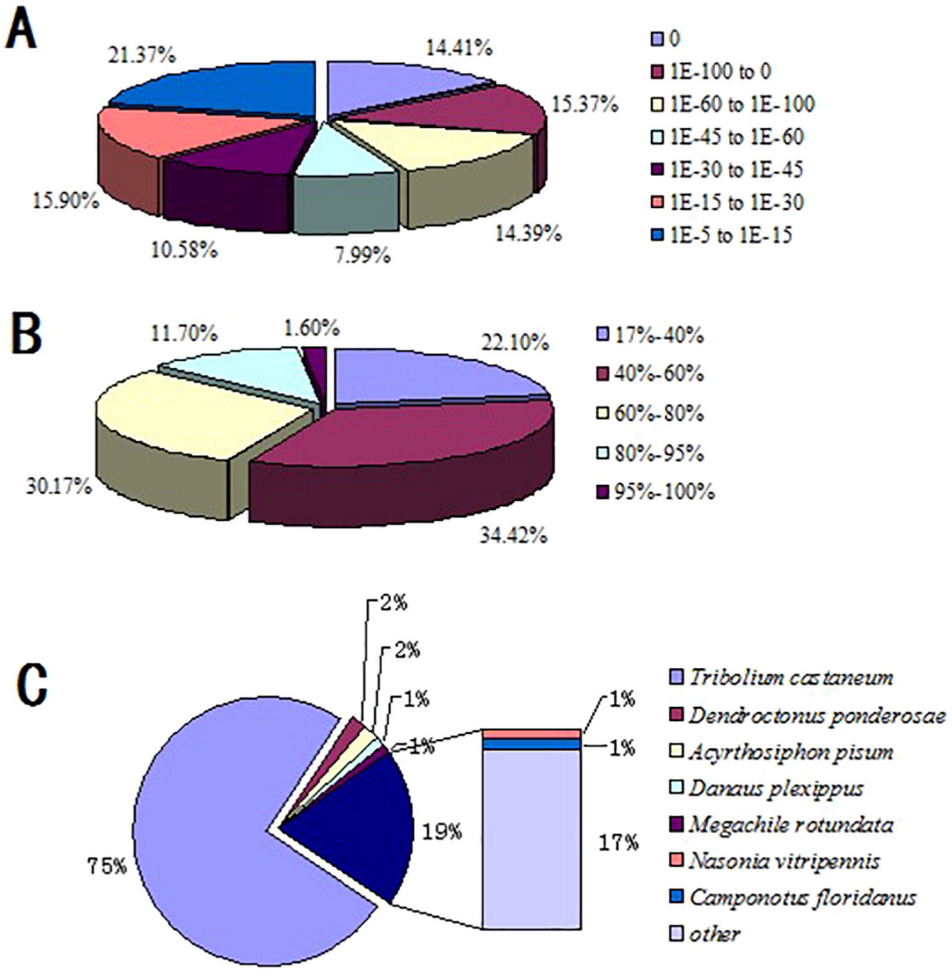

Fig. 2. Species distribution of BLASTx results. Unigenes were used in a BLASTx search (cutoff E value $<10^{-5}$) of the nr protein database, and charts were prepared for (A) E-value distribution, (B) similarity distribution and (C) species distribution.

for the HaRT_Trans and HaCS_Trans transcriptomes, respectively. Although the lengths of nearly half of the unigenes from the two transcriptomes were 300–500 bp (Fig. 1), the N50 contig lengths of the unigenes were 1005 bp and 1070 bp, respectively.

About 28,037 (59.7%) unigenes were matched to known genes in the GenBank, 21,604 (46.0%) in the Swiss-Prot, 19,482 (41.5%) in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and 13,193 (28.1%) in the Gene Ontology (GO) databases. A BLASTx search of protein sequences from the National Center for Biotechnology Information nr database using the unigenes and E-value distribution were performed (Fig. 2A and B), and the resulting species distribution revealed that 75% of the unigene sequences had top matches with gene sequences from

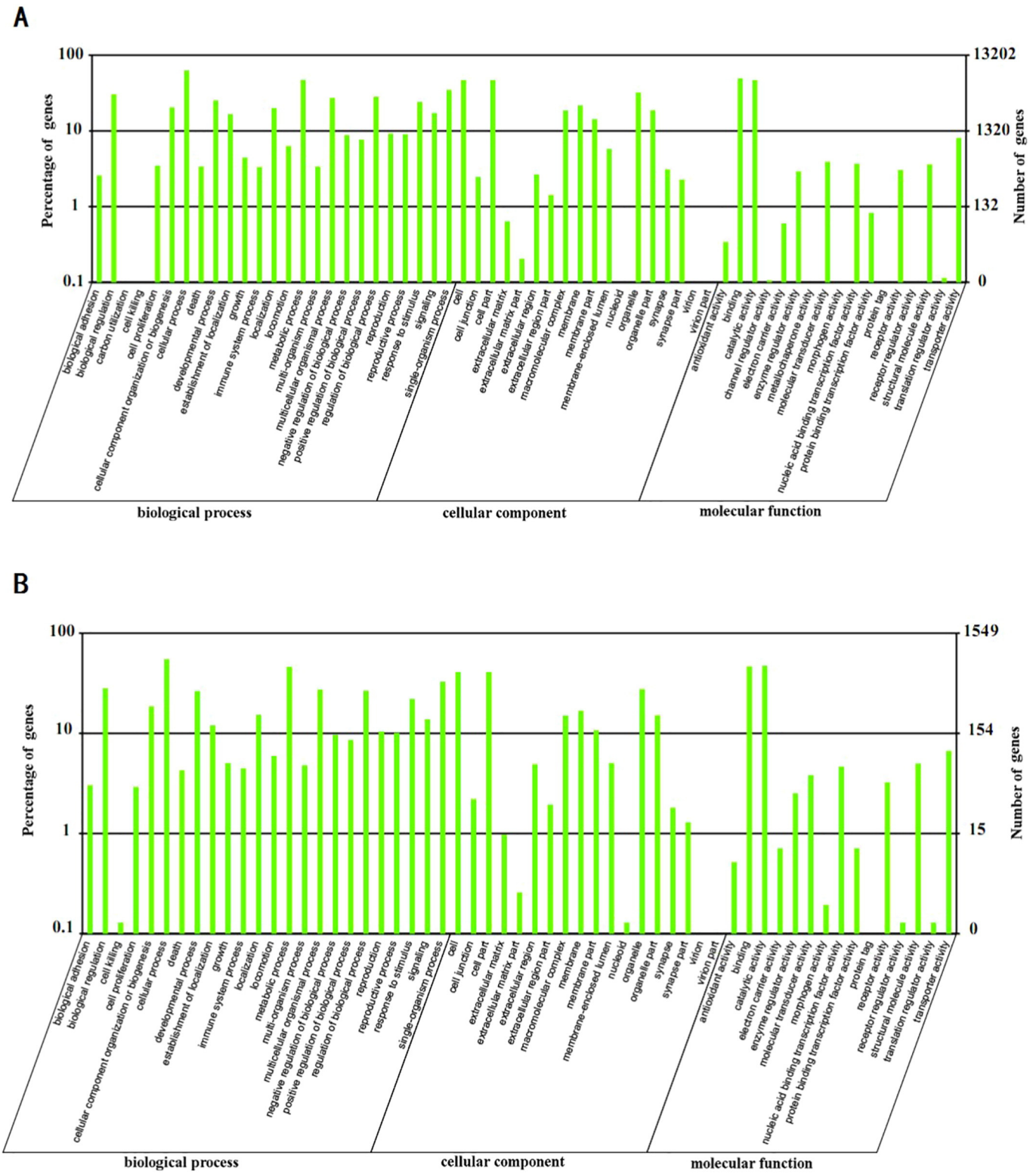

Fig. 3. Gene ontology classification of the unigenes. The unigenes were classified into the listed subcategories for *biological process*, *cellular component* and *molecular function* categories. The percentage and number of genes in each category are indicated on the left and right y-axes, respectively. Plots are based on annotation from (A) all unigenes in the two transcriptomes and (B) unigenes that were differentially expressed between the two transcriptomes.

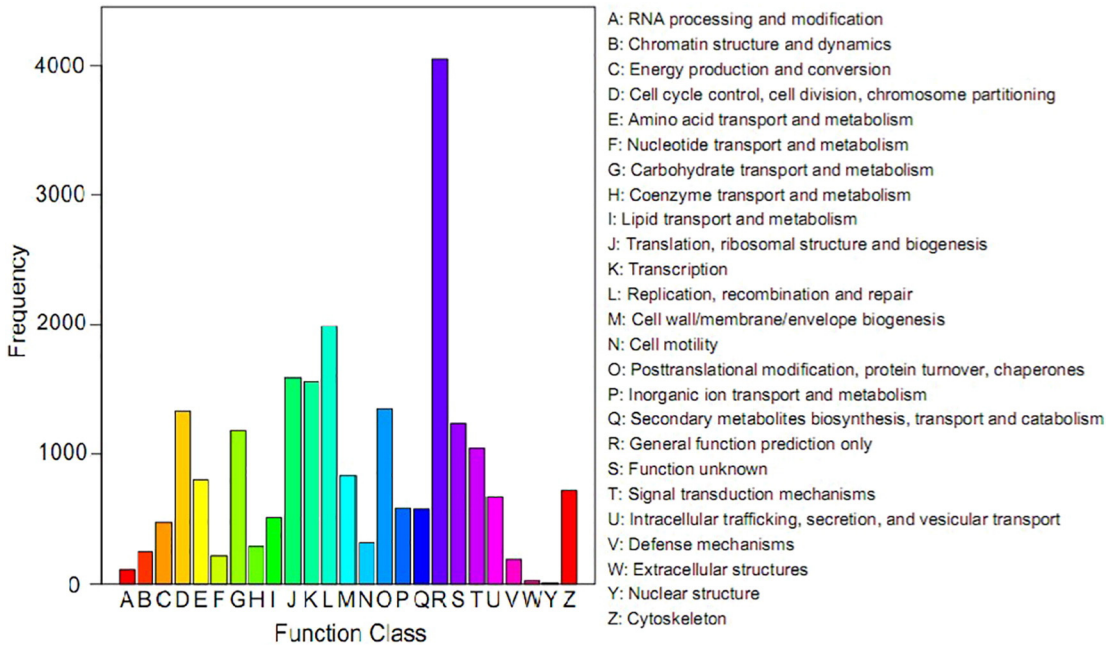

Fig. 4. Clusters of orthologous groups (COG) classification of the unigenes. Of 46,980 unigenes, 9921 sequences were grouped into 25 COG categories.

Tribolium castaneum (Fig. 2C). Also the meaning of the 19% blue pie indicated *Nasonia vitripennis* (1%), *Camponotus floridanus* (1%) and other (17%) (Fig. 2C).

Based on sequence similarity, 13,193 unigenes were assigned to the *biological process*, *cellular component*, or *molecular function* categories, which were further divided into 60 subgroups (Fig. 3). Clusters of orthologous groups (COG) analysis revealed that 9921 (21.1%) of the unigenes were assigned at least one of the 25 different COG annotation types (Fig. 4).

3.2. H. axyridis DGE profile at low temperature

Eight DGEs were obtained from a $1 \times 10^9 \times 8$ bp database (Table 3), and above 88.37% of the differential expression gene tags were mapped to the reference, which is acceptable for DGE analysis (>61.45% is considered a perfect match). A search of differentially expressed genes in which unigenes with ≥2 bp mismatch were removed, resulted in a change from 23.42% to 26.92%. Two RNA-Seq results, including the first and the second sequencing, showed >3000 up- or down-regulated genes when insects were exposed to 5 °C for 12 d (DF) and 150 d (DFLT) (Fig. 5).

Venn diagrams for the two databases revealed unique and common DGE patterns. Up- and down-regulated genes from each DGE database were analyzed statistically and differentially expressed genes were analyzed as potential cold-hardiness-related genes. This gene analysis was based on 76 up-regulated genes and 18 down-regulated genes from the CS (2 h at 5 °C) treatment group: DF (12 d at 5 °C), 2515 up- and 3787 down-regulated genes; DFLT (150 d at 5 °C), 3137 up- and 3649 down-regulated genes (Fig. 6). Fig. 7 shows the unique and common patterns of differential expression for the three treatments. Cold-

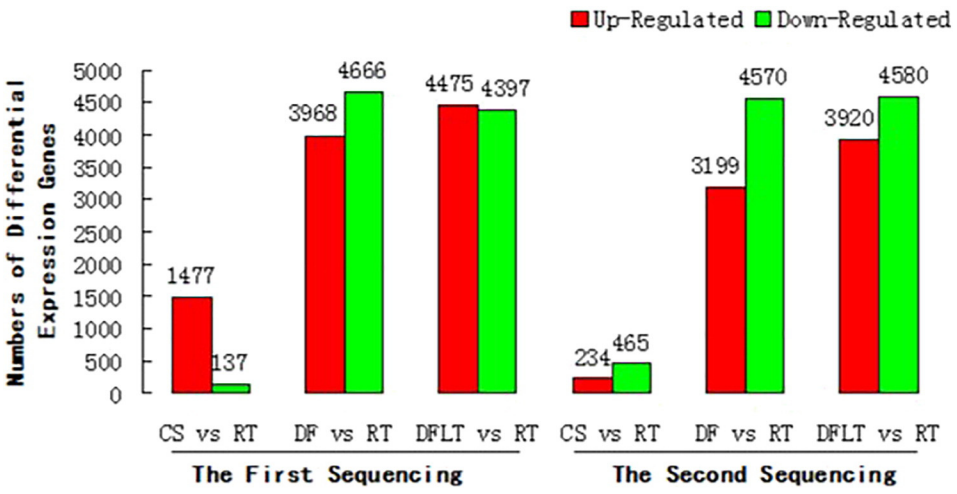

Fig. 5. Total number of differentially expressed genes. The differential expression of unigenes in the two DGE databases is plotted according to regulation for each treatment, that is CS, DF and DFLT post-treatment. Red: up-regulated; green: down-regulated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

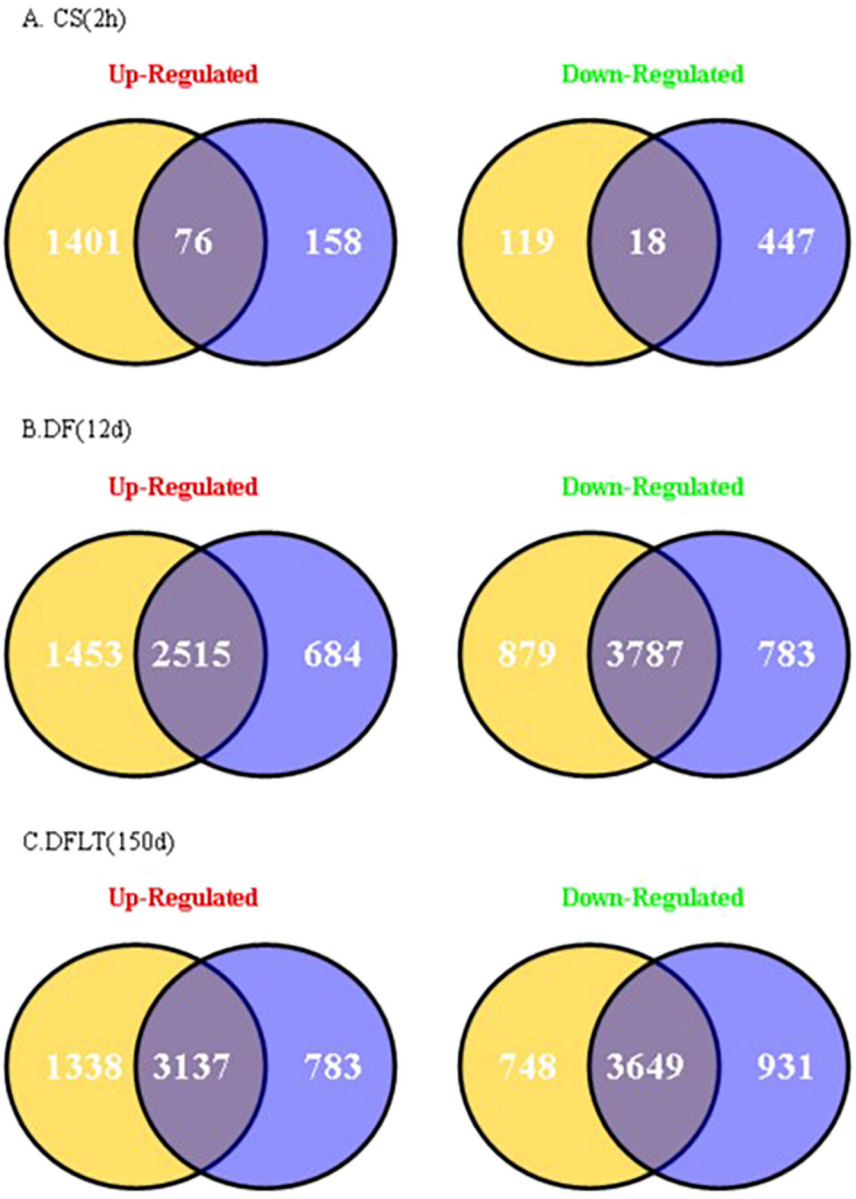

Fig. 6. Number of differentially expressed genes. Comparison of the number of differentially regulated unigenes from the two DGE databases for each of the treatments. Yellow: the first DGE-seq database only; dark purple: the second DGE-seq database only; light purple: both databases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hardiness-related genes were predicted based on the common genes (five up-regulated, seven down-regulated and 60 common differentially expressed genes).

The cluster analysis was based on the intersection and unionsection of differentially expressed genes from an inter-comparison among three low-temperature treatments and two repeats per treatment, that is

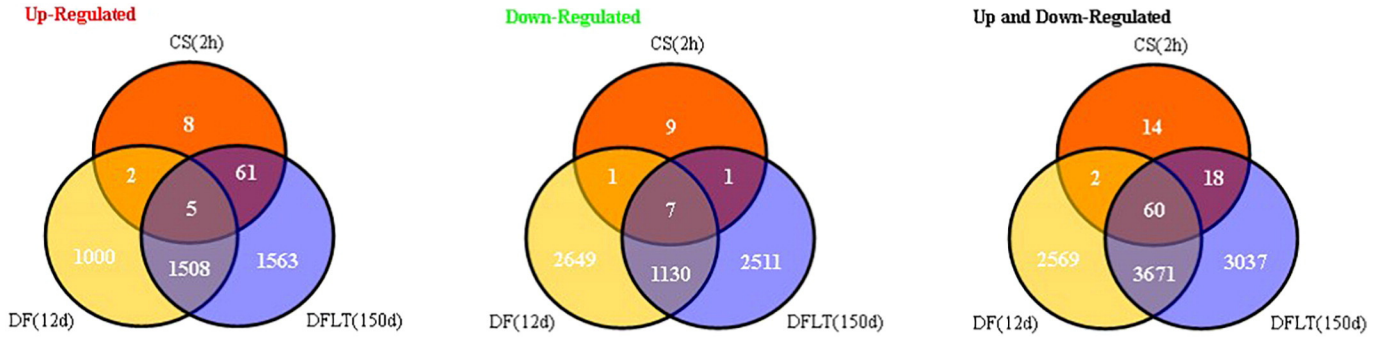

Fig. 7. Venn diagrams comparing DEG results from four conditions. The Venn diagrams represent unique and shared changes in the expression levels of the differentially expressed genes for the RT, CS, DF and DFLT treatment groups.

CS_A, DF_A, DFLT_A, CS_B, DF_B and DFLT_B (Figs. 8 and 9). It is generally believed that similar gene expression patterns usually have similar function (Calla et al., 2014; David et al., 2014).

3.3. Predicted cold-hardiness-related genes

Twelve genes (five up-regulated and seven down-regulated) were predicted to be related to cold hardiness. Of these, seven genes were detected from the 60 common differentially expressed genes and assigned putative functions possibly related to cold hardiness (Table 4): E3 ubiquitin-protein ligase, transketolase, trehalase, serine/arginine repetitive matrix protein 2, glycerol kinase and sugar transporter SWEET1-like.

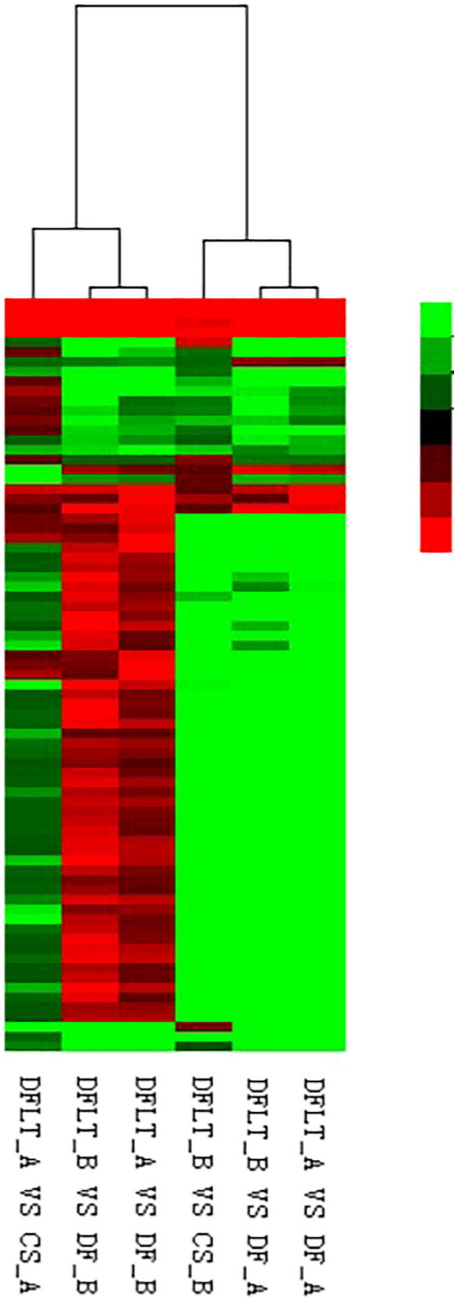

Fig. 8. The cluster analysis of the intersection of differentially expressed genes. Each column represents an experimental condition, and each row represents a gene. The ratio of change for the differentially expressed genes is color coded: red indicates high expression and green indicates low expression. The cluster analysis is based on the intersection of differentially expressed genes from inter-comparison from CS_A, DF_A, DFLT_A, CS_B, DF_B, DFLT_B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Qualitative real-time PCR

Most of the qRT-PCR results agreed with changes in the RPKM (average reads per million reads) of each sample, but Unigene506, CL4577.Contig1 and Unigene1527 fold changes calculation for NGS DGE data can't prove the congruence with qRT-PCR data (Fig. 10). As well as fold changes calculated for DGEs cannot be obtained from qRT-PCR data (Xu et al., 2015).

4. Discussion

4.1. Transcriptome sequencing quality and DGE analysis

The 10 Gb transcriptome and 8 Mb gene expression tag pool obtained with next-generation sequencing technology can potentially be used to enrich the *H. axyridis* gene database and provide a valuable resource for effective control of this pest. Nearly half of the unigenes from the two transcriptomes had a length of 300–500 bp (Fig. 1), presumably due to missing *H. axyridis* genome sequences and the complexity of the insect's genetics. The N50 of the *H. axyridis* transcriptome reference data (46,980 unigenes) in our study were 1005 bp and 1070 bp for the two databases. While with de novo assembly, the raw reads of *Propylea japonica* were further assembled into 33,647 unigenes using the methods in the Velvet and Oases programs (Tang et al., 2014b). Our results are slightly larger than reported from other studies, just like *Cryptolaemus montrouzieri* transcriptome reference data for N50 was 933 bp (Zhang et al., 2012). The *Nilaparvata lugens* transcriptome reference data for N50 was 933 bp (Xue et al., 2010) and, for the Asian honey bee (*Apis cerana cerana*) was 998 bp (Wang et al., 2012). In comparison, the quality of sequencing and assembly in our study was relatively high. These results indicate that the average length and sequence quality of the unigenes were acceptable for performing BLAST search and gene annotation procedures.

The percentage of genes similar to known annotated genes is not particularly high, and is likely due to the relatively low number of insect genes in public databases. The similarity between *H. axyridis*, *C. montrouzieri* and *T. castaneum* transcriptomes reflects the relatively close phylogenetic relationship within the order (Coleoptera) (Zhang et al., 2012). Now, some species of Coleoptera have completed the transcriptome sequencing, including *C. montrouzier* (Zhang et al., 2012), *T. castaneum* (Oppert et al., 2010), one firefly (Viviani et al., 2013), *Dastarcus helophoroides* (Wang et al., 2014) and *Tenebrio molitor* (Liu et al., 2015).

DGE analysis revealed that the number of differentially expressed genes in the CS group was lower than for the other two groups, suggesting the CS condition (2 h exposure at 5 °C) was not sufficient to stimulate most cold-resistance genes in *H. axyridis* (Fig. 5). As well as some up-regulated genes, including prolyl 4-hydroxylase subunit alpha-1 (P4HA1), staphylococcal nuclease domain-containing protein 1 (snd1) and cuticular protein analogous to peritrophins 3-D2 (Cpap3-d2) was candidate cold responsive genes in *Micrarchus* nov. sp. 2 (Dunning et al., 2013).

4.2. Insect cold hardiness strategy

Insects are ectotherms and can take strategy for freeze avoider or freezer tolerant, correspondingly, growth, development, behavior and evolution are affected by temperature (Leather, 1995). Insects living in cold climates and long winters have evolved a number of strategies (Lee, 1989) enhancing cold hardiness (Régnière and Bentz, 2007). For example, some insects respond to low temperatures by controlling the expression of genes for substances that promote cold tolerance, including antifreeze proteins (AFPs) (Graham et al., 1997), heat-shock

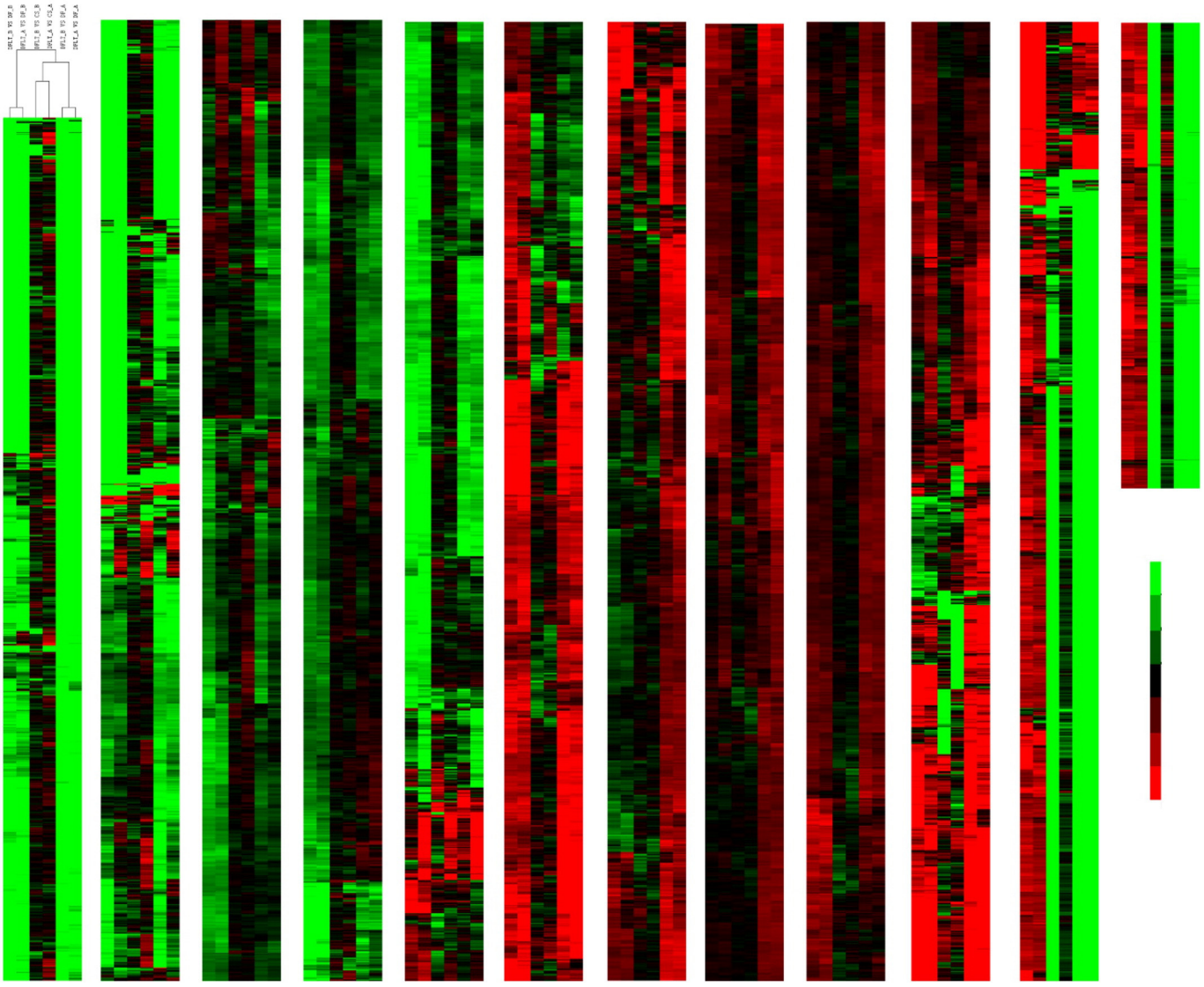

Fig. 9. Cluster analysis of the unionsection of differentially expressed genes. Cluster analysis based on the unionsection of differentially expressed genes from an inter-comparison between CS_A, DF_A, DFLT_A, CS_B, DF_B, DFLT_B. The 12 sections are continuous. The columns are DFLT_B VS DF_B, DFLT_A VS DF_B, DFLT_B VS CS_B, DFLT_A VS CS_A, DFLT_B VS DF_A, DFLT_A VS DF_A.

proteins (HSPs) and trehalose (Elbein et al., 2003). The AFP family of proteins, first identified in *Notothenenііd* (DeVries and Wohlschlag, 1969), protects from freezing or sub-freezing conditions by creating a difference between the melting point and freezing point, a process known as thermal hysteresis (Yue and Zhang, 2009). AFPs inhibit the growth of ice crystals and reduce the freezing point of a solution with non-colligative properties (Holden and Storey, 1994). AFP activity is 3–4 times higher in insects than it is in fish (Yue and Zhang, 2009) and has been identified in the following insect species: *T. molitor* (AF160494) (Graham et al., 1997), *Blaps kashgarensis* (EU368860), *Rhagium inquisitor* (HQ540314) (Kristiansen et al., 2011), *Dorcus curvidens* (AB264322), *Dendroctonus ponderosae* (BT128482) (Keeling et al., 2012), *Anatolica polita* (GU358704), *Dendroides canadensis* (U79781), *Microdera dzhungarica punctipennis* (AY821793) and *Choristoneura fumiferana* (AY004228) (Tyshenko et al., 2005).

4.3. Antifreeze proteins and cold-resistant

Although AFPs are known to have an important role in cold resistance, our analysis did not identify any AFP genes. It is reported that AFP cold-resistant activity depends on the structure of the AFPs and not the quantity of the proteins (Koštál et al., 2011). There are two reasons for not detecting any existing AFPs. The first is that the non-assembled reads and/or shorter contigs were not used for the final transcriptome analysis for any AFP-like sequences. The second is that the AFPs may have been expressed at very low levels – even under the cold treatment conditions of this experiment. Alternatively, this could also imply that neither approach used here was valid or that AFPs might indeed not play any role in cold hardiness in *H. axyridis*.

4.4. Heat-shock proteins and cold hardiness

HSPs, first identified in *Drosophila* (Ritossa, 1964), are important molecular chaperones involved in protein folding, protein degradation and transportation of cellular material. These proteins act by regulating target cell activity, rather than participating in target protein synthesis, increase tolerance to environment stresses (Craig et al., 1983) and are widely distributed in microbes, animals and plants. The HSPs superfamily has been classified into the following categories according to the relative molecular weight as well as the structure and function: HSP40, HSP60, HSP70, HSP90, HSPl00 and low-molecular-weight HSPs. Twenty-five HSPs have been identified in *T. castaneum* (Mahroof et al., 2005), and many other insects with multiple HSP genes have been reported (Wang et al., 2007). In *Liriomyza sativae*, cold shock

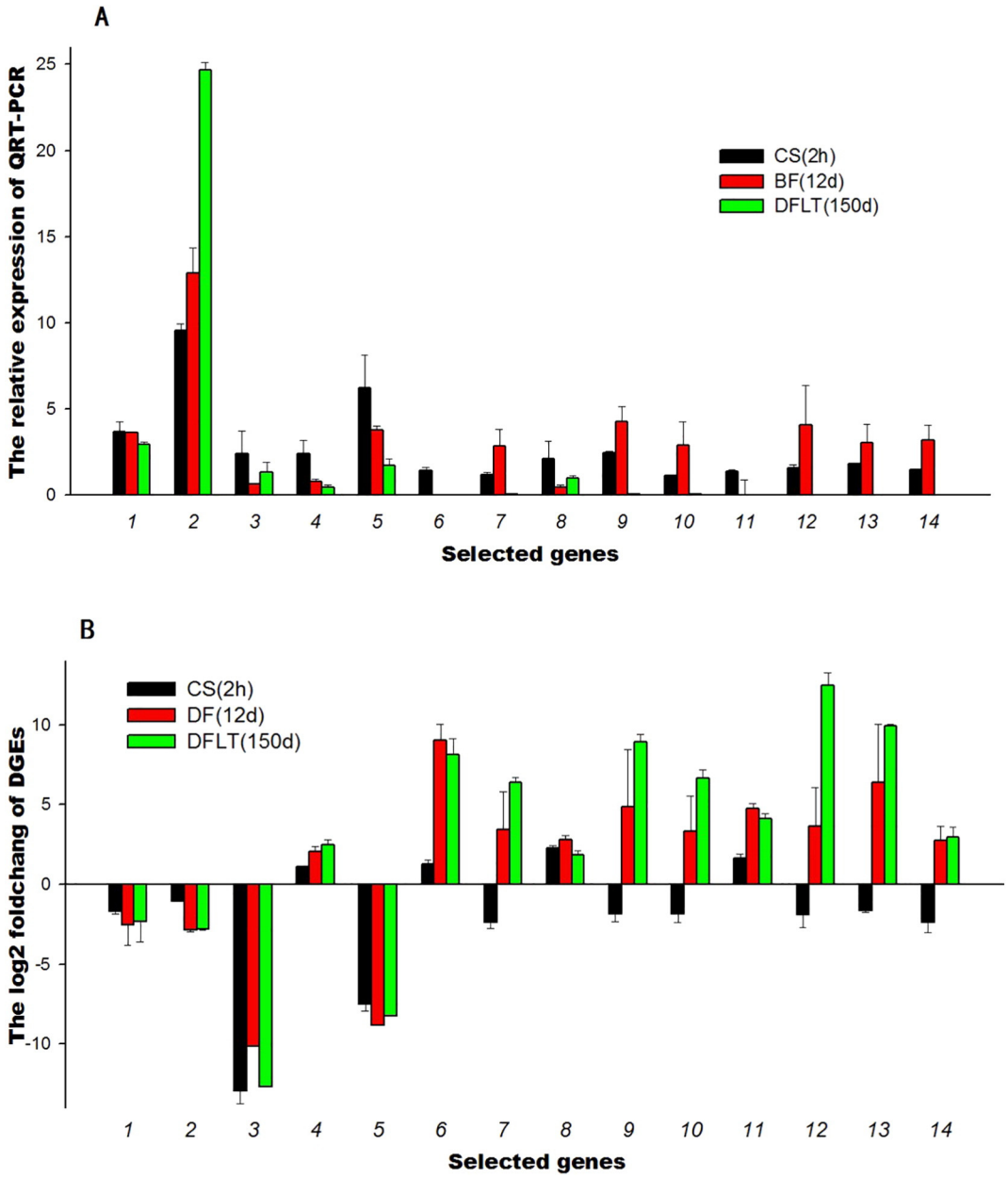

Fig. 10. Confirmation by qualitative real-time PCR. Of the 60 differentially expressed genes, 14 were randomly selected to confirm the stability and accuracy of DGE analysis by qRT-PCR. Result from (A) qRT-PCR and (B) DGE for each of the four treatments.

induces the expression of HSP90, HSP70, HSP40 and small HSPs (Huang and Kang, 2007). A high level of similarity has been observed from HSP sequences from different species: HSP70 and HSP84 protein sequences from *Drosophila* and *Saccharomyces cerevisiae* are 72% and 63% identical, respectively (Kantengwa and Polla, 1991). One HSP21.4 gene was identified in our study among 60 common differentially expressed genes (Fig. 7), and also this HSP21.4 was expressed highly when *H. axyridis* adults stored in cold condition for 10 to 30 d (Wu et al., 2016). The E3 ubiquitin-protein ligase and serine/arginine repetitive matrix protein 2 genes were identified in our study (Table 4). The latter may be an intrinsic bioremediation gene conferring cold resistance to beetles.

4.5. Trehalose metabolism and cold hardiness

Trehalose is important component of insect hemolymph, present at the larva, pupa and adult stages (Becker et al., 1996; Elbein et al., 2003). Trehalose stores energy for growth, development and activity and has been shown to protect against adverse environments (Thompson, 2003). As a metabolic product of stress, trehalose content is modified with changes in the environmental conditions (Van Laere, 1989). *Escherichia coli* grown in a high-salt environment accumulated trehalose to mediate osmotic pressure (Falkenberg and Landfald, 1986). In addition, high trehalose concentration accumulated in *S. cerevisiae* challenged with heat, cold, insufficient nutrition, osmotic stress, antioxidants and other noxious stimulation (Argüelles, 2000; Elbein et al., 2003; Bonnett et al., 2012), and trehalose has been shown to be a molecular signal controlling the glycolysis process in yeast (Argüelles, 2000). As frost resistance and intracellular trehalose content are closely related in yeast, it is believed that trehalose protects against frost damage.

Also in insects, certain energy-intensive movements, such as flight, require sufficient levels of trehalose, suggesting that trehalose can be used as a glucose reserve to provide energy. In addition to this physiological function, trehalose also plays an important role in low-temperature storage. One study of the cold-resistance mechanism of cotton

bollworm revealed that trehalose content is significantly different in diapause and the non-diapause pupae, and that trehalose levels fell sharply after injecting with diapause hormone to break the diapause (Xu et al., 2009). This mechanism has also been described for *Delia antiqua*, which have summer and winter diapause (Nomura and Ishikawa, 2001).

The biosynthetic pathway of trehalose differs in different organisms, and at least five types of trehalose synthesis have been identified: trehalose synthase (trehalose-6-phosphate synthase, TPS)/trehalose-6-phosphate lipase (trehalose-6-phosphate phosphatase, TPP) pathway, TS pathway, TreY/TreZ (maltooligosyl trehalose synthase/maltooligosyl trehalose trehalohydrolase) pathway, TreP (trehalose phosphorylase) approach and TreT (trehalose glycosyltransferring synthase) approach (Thompson, 2003; Avonce et al., 2006; Tang et al., 2010). In the TPS/TPP pathway, TPS catalyzes glucose to generate 6-phosphate trehalose and UDP, and then TPP catalyzes 6-phosphate trehalose to generate trehalose (Strom and Kaasen, 1993). This reaction was originally described for yeast, but has been identified in many other organisms, including insects (Tang et al., 2014a).

Cold endurance indicates that insects can strengthen cold hardiness after cold acclimation. Much research has been conducted in recent years on cold endurance in insects and the related regulation of cold-resistance and cold-acclimation genes, including HSPs, AFPs and other relative genes. In our study, not only the common differentially expressed genes in three treatments, but also common in two of three treatments, or unique in one of the treatments, can also be related to cold hardiness. Therefore, the rest of 60 common differentially expressed genes (but not commonly up- or down regulated) are also possibly related to cold hardiness (Table 4). This research contributes by identifying additional induced cold-hardiness genes and elucidating the mechanism of cold endurance and lays a solid foundation for the low-temperature storage of *H. axyridis* and other natural enemies.

Acknowledgements

This work was supported by National Basic Research Program of China (Grant No. 2013CB127600), National Natural Science Foundation of China (Grant Nos. 31071731 and 31371996), the Beijing Nova Program (Grant No. Z121105002512039), College Students in Zhejiang Province Science and Technology Innovation Plan (Grant No. 2015R423043) and the Program for Excellent Young Teachers in Hangzhou Normal University (Grant No. JTAS 2011-01-031).

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