Molecular cloning of heat shock protein 10 (Hsp10) and 60 (Hsp60) cDNAs from *Galeruca daurica* (Coleoptera: Chrysomelidae) and their expression analysis

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Abstract

Galeruca daurica (Joannis) is a new outbreak pest in the Inner Mongolia grasslands in northern China. Heat shock protein 10 and 60 (Hsp10 and Hsp60) genes of *G. daurica*, designated as *GdHsp10* and *GdHsp60*, were cloned by rapid amplification of cDNA ends techniques. Sequence analysis showed that *GdHsp10* and *GdHsp60* encoded polypeptides of 104 and 573 amino acids, respectively. Sequence alignment and phylogenetic analysis clearly revealed that the amino acids of *GdHsp10* and *GdHsp60* had high homology and were clustered with other Hsp10 and Hsp60 genes in insects which are highly relative with *G. daurica* based on morphologic taxonomy. The mRNA expression analysis by real-time PCR revealed that *GdHsp10* and *GdHsp60* were expressed at all development stages and in all tissues examined, but expressed highest in eggs and in adults' abdomen; both heat and cold stresses could induce mRNA expression of *GdHsp10* and *GdHsp60* in the 2nd instar larvae; the two Hsp genes were expressed from high to low with the extension of treatment time in *G. daurica* eggs exposed to freezing point. Overall, our study provides useful information to understand temperature stress responses of Hsp60 and Hsp10 in *G. daurica*, and provides a basis to further study functions of Hsp60/Hsp10 relative to thermotolerance and cold hardiness mechanism.

Keywords: Hsp10, Hsp60, cloning, mRNA expression, *Galeruca daurica*

Introduction

Heat shock proteins (Hsps), known as stress proteins and molecular chaperones, are highly conserved and produced in all organisms from lower prokaryotic organisms to higher mammals (Bose et al., 1996; Shi et al., 2016). In insects, Hsps include ATP-independent small Hsps and the larger ATP-dependent proteins, Hsp60, Hsp70, and Hsp90 (King & MacRae, 2015). They perform diverse biological functions during insect normal growth phase or physiological state (Li et al., 2009; Sasibhushan et al., 2012), and play essential roles in

transcription, transporting, protein synthesis, folding, assembling of degraded and misfolded proteins, cell signaling, and metabolism (Huang and Kang, 2007). They are synthesized constitutively and induced significantly when insects are exposed to undesirable stressors, including extreme temperatures (Kayukawa et al., 2005; Huang and Kang, 2007), crowding (Wang et al., 2007), anoxia (Michaud et al., 2011), desiccation (Tammariello et al., 1999), starvation (Wang et al., 2012), heavy metals, other toxic particles and chemical substances (Ge et al., 2013; Zhang et al., 2015).

Hsp60 is a homolog of phage growth E large in bacteria (Gupta, 1995; Xu et al., 2014), which is produced in the cytoplasm and mainly located in the mitochondria of eukaryotic cells (Neupert, 1997). In recent decades, extensive researches on multiple Hsp60 functions in diverse physiological processes have been conducted in various organisms, for example:

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involvement in apoptosis (Ghosh et al., 2008), germ cell differentiation (Prinsloo et al., 2009), tumorigenesis (Cappello et al., 2008), and so on. However, researches on insect Hsp60 have focused on response under multiple environmental stress and involvement in certain important physiological processes. Hsp60 can enhance cold hardiness and promote membrane stability by stabilizing microfilaments on insects, such as: *Delia antique* (Kayukawa and Ishikawa, 2009), *Eurosta solidaginis* (Zhang et al., 2011), and *Chilo suppressalis* (Cui et al., 2010).

Hsp10, known as phage growth E small (in *Escherichia coli*), is a co-chaperone for Hsp60 and exerts its biological functions in diverse conditions (Boettinger et al., 2015). Hsp60 and Hsp10 form a folding cage through their rings and Hsp60–Hsp10 complex may accelerate polypeptide folding, denatured protein refolding, and misfolded protein correcting (Corrao et al., 2010; Clare & Saibil, 2013; Hayer-Hartl et al., 2016). In insects, information about Hsp10 is far from sufficient. Establishing the molecular information of the Hsp10 and Hsp60 would be necessary for better understanding the tolerance mechanism associated with Hsp60–Hsp10 complex.

Galeruca daurica (Joannis) is a new pest occurring seriously in the Inner Mongolia grasslands in China in recent years, which has exclusively destructive effects on *Alliums* plants, the pest would lead to a devastating disaster in turf seeding growth on grassland, especially on barren pasture (Tan et al., 2016; Zhou et al., 2016). It was reported that extensive outbreaks of this pest beginning in 2009 have caused great losses to pasture in grasslands in northern China and levels of damage has increased year by year (Li et al., 2014). *G. daurica* occurs one generation each year, and overwinters as eggs in its natural habitats. Their overwintering eggs have strong cold hardiness with the lower half lethal temperature (LT_{50}) of $-33.08°C$ after 12 h exposure (Gao et al., 2015), and their 1st, 2nd, 3rd instars' larvae have also high cold hardiness with the LT_{50} of -8.5 to $-10.1°C$ after 2 h exposure (Li et al., 2014). *G. daurica* might survive through undergoing huge temperature fluctuation during larvae development process in spring, but how they response to drastic temperature changes and possible mechanism is unknown.

Although Hsps have been proposed to involve in cold hardiness and heat shock responses, the mechanisms underlying temperature stress in *G. daurica* are hardly known. Hence it is important to understand temperature–stress response and molecular regulation in this outbreak insect. Hsps, as called heat stress proteins, naturally became our focus. At present, there were no Hsp genes in *G. daurica* that have been studied. Here, we reported the full length of Hsp10 and Hsp60 cDNA sequences of *G. daurica*, their deduced amino acid sequences and gene characterizations. Furthermore, we examined the mRNA expression pattern of the two Hsps of *G. daurica* in different developmental stages, five adult tissues, larvae exposed to different temperatures, and eggs under cold stress. Our research should be valuable and provide the basis for further studies on Hsp10 and Hsp60 functions, and also allowed us to explore the roles of the two Hsps associated with thermotolerance and cold hardiness on this seriously occurring pest species.

Materials and methods

Experimental insects

G. daurica egg masses were collected from the Arxant village of Xilinhot City (44°62′N, 115°80′E), Inner Mongolia, China, in middle September in 2015, were brought back to the Research Center for Grassland Entomology (40°48′N, 111°42′E), Inner Mongolia Agricultural University (Hohhot, Inner Mongolia, China), and were maintained in an uninsulated container in outdoors. After 1 month, some of the egg masses were brought back and held in a growth chamber ($20 ± 1°C$, RH = $50 ± 10\%$, L16:D8). After 1 week, larvae hatched out and were fed on fresh garlic chives in a growth chamber under the same conditions; garlic chives were planted in our laboratory, which were without exposure to any chemicals and pesticides. The larval development durations were 8, 9, 15, and 7 days for 1st, 2nd, 3rd instars and pupal *G. daurica*, respectively, and then bugs enter the adult stage.

RNA isolation and cDNA cloning

Total RNA was isolated from test bugs (20–40 mg) using Takara Mini RNA Extraction Kit (Takara, Dalian, China) following the manufacturer's instructions. The RNA integrity was checked by analysis on a 1.0% (w/v) agarose gel; RNA concentrations were measured using a spectrophotometer. The first strand of cDNA was synthesized using PrimeScript First Strand cDNA Synthesis Kit (Takara, Dalian, China), following the manufacturer's protocol.

Based on the partial sequences annotated with *Hsp10* and *Hsp60* genes from our transcriptome analysis (unpublished), gene-specific primers were designed by Primer 5.0 (table 1). Cycling conditions for amplifying partial cDNA sequences of *GdHsp10* and *GdHsp60* were as follows: 5 min 94°C; 30 cycles of 20 s 94°C, 20 s 55°C, 30 s 72°C, and a final extension of 10 min at 72°C, using primers *GdHsp10*-f, *GdHsp10*-r, *GdHsp60*-f, and *GdHsp60*-r. Amplified fragments were eluted from agarose gels and cloned into the pMD™18-T vector (Takara) and then sequenced.

Rapid amplification of the GdHsp10 and GdHsp60 cDNA ends

The 5′ and 3′ ends of cDNA were synthesized employing SMARTer® rapid amplification of cDNA ends (RACE) 5′/3′ Kit as specified by the manufacture (Clontech). PCR amplification was performed using 5′ and 3′ anchor primers (enclosed in 5′/3′ RACE kit) and a specific primer designed from the obtained fragment sequence. Thermal cycling was done using the following program for semi-nested PCR, the 5′GSP1 and 3′GSP1 primers were used to amplify the sequence in the first PCR run; 5′GSP2 and 3′GSP2 primers were for the second round PCR. Before the second run, 5 µl of the primary PCR product was diluted into 245 µl of the EDTA buffer, which was as the cDNA template in the secondary PCR. The thermal cycling was performed as follows: 25 cycles of 30 s 94°C, 30 s 68°C, 2 min 72°C. The expected-size fragments were purified, tailed with A, ligated into a vector and sequenced.

Sequence and phylogenetic analysis

The full cDNA sequences of *GdHsp10* and *GdHsp60* were analyzed for homology with the BLAST programs at the National Center for Biotechnology Information (NCBI). DNAMAN V6.0 (Lynnon Biosoft, Canada) was used to assemble gene sequences and predict molecular mass and theoretical isoelectric point (pI). The functional sites or domains in the amino acid sequences were predicted by the SMART program (http://smart.embl-heidelberg.de/). Multiple sequence alignments and phylogenetic-tree construction applying the

Table 1. PCR primers used in the experiment.

Primers	Sequences (5′−3′)	Sequence information
GdHsp10-f	GGCAACTACGAAAACGAAAGG	Cloning of *GdHsp10* partial sequence
GdHsp10-r	CCGCATTGTCTTCGAGGTTT	
GdHsp60-f	ACGCCAAAGATGTGAGGTTC	Cloning of *GdHsp60* partial sequence
GdHsp60-r	CAACCTGGCTCCAATGTTCT	
GdHsp10-5′GSP1	CTGGTATTCCGCATTGTCTTCG	5′RACE of *GdHsp10*
GdHsp10-5′GSP2	GGGGCGAAAGAACCATCCTGTAAT	
GdHsp60-5′GSP1	GGAAATAGTGGCGACTTGGCAGAT	5′RACE of *GdHsp60*
GdHsp60-5′GSP2	CTTCAGGAGTAGTGACGGGTTTG	
GdHsp10-3′GSP1	CTGTAGTCGCAGTCGGTCAA	3′RACE of *GdHsp10*
GdHsp10-3′GSP2	TCTTTCGCCCCACCTTCTGT	
GdHsp60-3′GSP1	CAAGGGGTTGATGTTCTAGC	3′RACE of *GdHsp60*
GdHsp60-3′GSP2	TTGAACAATCATGGGGCTCACC	
GdHsp10-F	TGGTATCAACGCAGAGTA	Cloning of *GdHsp10* full length sequence
GdHsp10-R	GGTTTGCCACAGGGTTTT	
GdHsp60-F	AGAAGTGTCGCGTCTCGT	Cloning of *GdHsp60* full length sequence
GdHsp60-R	GAGGCAATCACTGAGTTTAT	
q*GdHsp10*-F	GGCAACTACGAAAACGAAAGG	Quantitative real-time PCR-target gene
q*GdHsp10*-R	CCGCATTGTCTTCGAGGTTT	
q*GdHsp60*-F	GGAGAAGCCTTGACCACTCT	Quantitative real-time PCR-target gene
q*GdHsp60*-R	GATTTCGCCAGTTTGACCGA	
q*SDHA*-F	GGGAGACCACAATCTCCTCA	Quantitative real-time PCR-reference gene
q*SDHA*-R	AGCTGGTGCTCCTAAGTCCA	

bootstrapped neighbor-joining method were created using ClustalX and MEGA 5.0 software.

Expression of GdHsp10 and GdHsp60 in different development stages and tissues

To investigate the mRNA expression level of *GdHsp10 and GdHsp60* in different developmental phases, whole body samples were collected and pooled as follows: third day eggs, 1st, 2nd, 3rd instars' larvae, pupae, female and male adults, respectively, then all of the samples were kept in −80°C for RNA extraction.

For examining the tissue distribution of *GdHsp10* and *GdHsp60* transcripts in *G. daurica*, antenna, head, leg, thorax, wing, and abdomen were cut off with dissecting scissors from third day mixed sex adults. About 100 mg five body tissue samples, including the head (without antenna), thorax (without legs), abdomen (without wings), antenna, and legs (including foreleg, midleg, hindleg) were obtained and frozen in liquid nitrogen immediately, and stored at −80°C until RNA isolation and quantitative PCR (qPCR) determination.

Expression of GdHsp10 and GdHsp60 in response to heat and cold stresses

Different temperature treatments

Ten constant temperatures (−14, −10, −5, 0, 5, 10, 15, 20, 25, and 30°C) were chosen to treat the 2nd instar larvae for 1 h without any recovery and determine mRNA expression level of *GdHsp10* and *GdHsp60*, and the 20°C group was regarded as control. All of the samples were kept in −80°C until RNA extraction.

Low-temperature treatment for different times

Because expression variation of proteins to thermal stress not only influences the growth and development but also determine hatching of embryo (Punyavathi et al., 2017), the third day hatched eggs were set as sampling criteria. The tested eggs exposed to 0°C for 0 (control), 15, 30 min, 1, 1.5, 2, and 3 h without any recovery, were selected to test mRNA relative expression level. All samples were instantly frozen and stored at −80°C. Each of the processing was repeated at least four times.

Quantitative analysis of GdHsp10 and GdHsp60 mRNA expression

The relative transcription levels of *GdHsp10* and *GdHsp60* genes in different developmental periods, different adult tissues, larvae exposed to different temperatures, and eggs exposed to 0°C for a different time were examined using real-time qPCR. Total RNA isolation and first-strand cDNA synthesis were described above. The mRNA expression levels of *GdHsp10* and *GdHsp60* were examined using a SYBR Green® real-time PCR assay (SYBR PrimeScrip™ RT-PCR Kit II, Takara) with FTC-3000P Real-time PCR (Funglyn Biotech, Canada). The succinate dehydrogenase (SDHA) gene was confirmed stably expressed among different treatments in *G. daurica* (Tan et al., 2017), and was used as an internal control. Primers for qPCR were designed using online software Primer 3.0 (http://primer3.ut.ee/) and listed in table 1. The real-time PCR (final volume of 25 µl reactions) contained 1 µl cDNA template, 12 µl of SYBR Green Real-time PCR Master Mix (Takara, No. DRR420S), and 0.5 µl of each primer; sterile distilled water was added for a total of 25 µl. The experiment was performed as follows: 95°C for 5 min followed by 30 cycles of 95°C for 10 s, 52°C for 15 s, and 72°C for 20 s. The entire experiment was replicated four times with four determinations per sample. The amplification efficiency of each gene was estimated by using the equation: $E = [10^{(-1/\text{slope})}-1] \times 100$ (Pfaffl, 2001). Relative expression analysis of *GdHsp10* and *GdHsp60* mRNA was conducted according to the $2^{-\Delta\Delta Ct}$ value method (Livak & Schmittgen, 2001). All data were given as means ± SD; a statistical difference was determined by analysis of variance

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  1  GAGATTCTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACATGGGGA
 61  TCTCAACGTGATCGTCAAGAAAACCACGTTTTGCAGATTTAAATACACAATTCATAAGAA
121  AATATAATA ATG TCTGCCTTCAAAAGATTGGTCCCCCTTTTCGACAGAATTCTAATAAAA
  1            M   S  A  F  K  R  L  V  P  L  F  D  R  I  L  I  K
181  AAATTCGAGGCAACTACGAAAACGAAAGGAGGTATCGTAATTCCAGAAAAAGCACAAGAT
 18  K  F  E  A  T  T  K  T  K  G  G  I  V  I  P  E  K  A  Q  D
241  AAAGTTTTACAAGGAACTGTAGTCGCAGTCGGTCAAGGATCTAGATTACAGGATGGTTCT
 38  K  V  L  Q  G  T  V  V  A  V  G  Q  G  S  R  L  Q  D  G  S
301  TTCGCCCCATTCGCCCCACCTTCTGTTAAAGTAGGTGATAAAGTACTTCTTCCCGAATAT
 58  F  A  P  F  A  P  P  S  V  K  V  G  D  K  V  L  L  P  E  Y
361  GGTGGAACGAAAGTAAACCTCGAAGACAATGCGGAATACCAGTTATTCAGAGAATCAGAT
 78  G  G  T  K  V  N  L  E  D  N  A  E  Y  Q  L  F  R  E  S  D
421  ATTTTAGCTAAAATTGATTCC TAA ACTTTACTCGAGTTGTAAATGTTGCATTTCCTTTTA
 98  I  L  A  K  I  D  S  *
481  AACTAAGTCCCAATAATAAGTCTGAACTATGTAGGTTTACATTTAAAATGATCTGATTCC
541  TGCAAAATTTCGGACGCTAAAAAACATTGCAACAAATTGTTTATTATATTATTGTTGTAA
601  ATGTTTTCTGGTAATATGTTTGTAGTCGATATCTTCATTGATTTAAGTTTGTTACAAAAA
661  TATATTTGTATTTAAAATTAAAAAAAAAAAAACCTATAGTGAAATCCCTAGGGGGGGGACC
721  CGCGGGGGGTGTAAAGGTAACCATTTATACTTTAATGTGCAGTACCTCGTCATGGGGGGG
781  CTGAAAAAAAACCCTGTGGCAAACCCCTTGGGAGGACCGGGG
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Fig. 1. Nucleotide sequence and deduced the amino acid sequence of *GdHsp10* cDNA clone. The deduced amino acid sequence is shown below the nucleotide sequence. The initiation and termination codons are marked boxes. The chaperonin hsp10/cpn10 signature is shown in gray and the predicted mobile loop is underlined.

followed by the Tukey's multiple comparison test using SPSS 16.0 for Windows.

Results

cDNA cloning, sequencing, and characterization of the GdHsp10 and GdHsp60 genes

After amplifying genes using two pairs of specific primers of *GdHsp10*-f, -r, and *GdHsp60*-f, -r, two fragment sequences with 206 and 215 bp were obtained and sequenced, respectively, which were identified as section fragments of *Hsp10 and Hsp60* genes by Blastn. For cloning the 5′ and 3′ ends of cDNA of Hsp10, RACE and semi-nested PCR were explored; two fragments of 525 and 402 bp were amplified by 3′ and 5′ RACE techniques, respectively. By sequence assembly of the above sequences using DNAMAN V6.0 (Lynnon Biosoft, Canada), a full length of *GdHsp10* cDNA from *G. daurica* was obtained, which is 822 bp, containing open reading frame (ORF) of 315 bp encoding a protein of 104 amino acid, a 129 bp 5′-UTR, and a 378 bp 3′-UTR (fig. 1). The predicted molecular mass of *GdHsp10* was 11.34 kD and the theoretical pI was 9.58. Chaperonin's hsp10/cpn10 signature (LVPLFDRILIKKFEATTKTKGGIVI) of the deduced amino acid sequence of *GdHsp10* cDNA is located at 7–31 (shown in gray). Furthermore, a mobile loop, which functioned as an interaction surface with the large subunit of the chaperonin complex, was found in the *GdHsp10* sequence (underlined).

The complete cDNA sequence of *GdHsp10* gene was deposited in GenBank under accession no. KY460464.

For cloning the 5′ and 3′ ends of cDNA of *Hsp60*, two fragments of 2017 and 658 bp were amplified by 3′ and 5′ RACE techniques, respectively. After assembling, the full length of *GdHsp60* cDNA with 2365 bp was obtained, including a 5′-UTR of 159 bp, a 3′-UTR of 484 bp, and an ORF of 1722 bp encoding 573 amino acids (fig. 2). The calculated molecular mass of GdHsp60 was 61.15 kD and the pI was 5.17. A classical mitochondrial Hsp60 signature shown in gray is shown at amino acid positions of 430–441, and the ATP-binding motif is underlined. In addition, the typical GGM repeat motif at the C-terminus is shown as waved lines. With the 3′-UTR, one putative polyadenylation signal was observed at positions 1919–1923. The nucleotide sequence of *GdHsp60* gene was deposited in GenBank under accession no. KY460463.

Homology and phylogenetic analysis of GdHsp10 and GdHsp60 genes

From the Blastn program, the nucleotide sequences of the *GdHsp10* and *GdHsp60* genes shared high homology with other known *Hsp10* and *Hsp60* genes, which indicated that the two cloned genes encode Hsp10 and Hsp60 proteins. The deduced amino acid sequences of *GdHsp10* revealed high similarity with Hsp10 from three insect species: *Apis florae* (67.3%), *Bombyx mori* (71.3%), and *Musca domestica* (59.4%), and three vertebrate species: *Penaeus monodon* (57.0%), *Danio*

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1      CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACATGGGGTATCAAC
61     GCAGAGTACATGGGGATAGAAAGAAGTGTCGCGTCTCGTGTTGCCGGTCTGCAGAATTTT
121    AATAAAAAATTGTAATTAAATTCGAAATTATACTAAAAG ATG TATGTCTACCCAGCACT
1                                            M  Y  R  L  P  S  T
181    GTGCGCTCTTTGGCCCTCCGCAAAGCCCAACAATATAATCAAATTCAAAGATGGTACGCC
8       V  R  S  L  A  L  R  K  A  Q  Q  Y  N  Q  I  Q  R  W  Y  A
241    AAAGATGTGAGGTTCGGATCAGAGGTCAGAGCTCTCATGCTCCAGGGGGTTGATGTTCTA
28      K  D  V  R  F  G  S  E  V  R  A  L  M  L  Q  G  V  D  V  L
301    GCTGATGCAGTAGCTGTGACGATGGGACCAAAGGGCCGAAATGTAATTTTTGAACAATCA
48      A  D  A  V  A  V  T  M  G  P  K  G  R  N  V  I  F  E  Q  S
361    TGGGGCTCACCAAAAATTACCAAAGATGGAGTTACAGTTGCTAAAGGGGTAGAACTAAAA
68      W  G  S  P  K  I  T  K  D  G  V  T  V  A  K  G  V  E  L  K
421    GATAAATTCCAGAACATTGGAGCCAGGTTGGTGCAAGATGTTGCTAACAATACAAATGAA
88      D  K  F  Q  N  I  G  A  R  L  V  Q  D  V  A  N  N  T  N  E
481    GAGGCAGGTGATGGTACTACAACAGCTACGGTTTTGGCTCGTTCTATTGCCAAAGAAGGA
108     E  A  G  D  G  T  T  I  A  T  V  L  A  R  S  I  A  K  E  G
541    TTTGAGAATCTTGGAAAAGGAGCCAATCCAGTTGAAATTCGTAAAGGTATCATTATGGCC
128     F  E  N  L  G  K  G  A  N  P  V  E  I  R  K  G  I  I  M  A
601    GTTGAGGTAATCACAAAAGCTCTAAAGAACCTTTCCAAACCCGTTACTACTCCTGAAGAG
148     V  E  V  I  T  K  A  L  K  N  L  S  K  P  V  T  T  P  E  E
661    ATCTGCCAAGTCGCCACTATTTCCGCCAATGGTGACAGTTCAGTTGGAAATCTCATTGCT
168     I  C  Q  V  A  T  I  S  A  N  G  D  S  S  V  G  N  L  I  A
721    GATGCCATGAAGAGGGTTGGTAAAGAAGGTGTCATCACTGTTAAAGATGGTAAAACACTT
188     D  A  M  K  R  V  G  K  E  G  V  I  T  V  K  D  G  K  T  L
781    AACGACGAATTAGAAATCATCGAAGGAATGAAATTTGACAGAGGCTACATCTCACCTTAC
208     N  D  E  L  E  I  I  E  G  M  K  F  D  R  G  Y  I  S  P  Y
841    TTTGTCAACACAACTAAAGGTGCCAAAGTTGAATACCAAGATGCTCTAATTTTATTAAGC
228     F  V  N  T  T  K  G  A  K  V  E  Y  Q  D  A  L  I  L  L  S
901    GAAAAGAAATCTCATCTGTTCAAAGCATTGTACCTGCTTTGGAATTAGCAAATGCACAA
248     E  K  K  I  S  S  V  Q  S  I  V  P  A  L  E  L  A  N  A  Q
961    AGAAAACCACTTATAATCATCGCTGAGGATGTTGACGGAGAAGCCTTGACCACTCTTGTA
268     R  K  P  L  I  I  I  A  E  D  V  D  G  E  A  L  T  T  L  V
1021   GTCAATAGACTTCGCATTGGATTACAAATTGCTGCTGTCAAAGCTCCAGGATTTGGTGAC
288     V  N  R  L  R  I  G  L  Q  I  A  A  V  K  A  P  G  F  G  D
1081   AATAGAAAGGCTACCCTCCAAGATATTGCAATAGCCACTGGTGGTATTGTATTTGGAGAT
308     N  R  K  A  T  L  Q  D  I  A  I  A  T  G  G  I  V  F  G  D
1141   GACGCTAACATTGTCAAATTGGAAGATGTCAAATTATCCGATCTCGGTCAAACTGGCGAA
328     D  A  N  I  V  K  L  E  D  V  K  L  S  D  L  G  Q  T  G  E
1201   ATCGTAATAACCAAGGACGACACTCTAATTTTAAAAGGTAAAGGTAAAAAAGATGACATC
348     I  V  I  T  K  D  D  T  L  I  L  K  G  K  G  K  K  D  D  I
1261   GATAGAAGAGCAGAACAAATTAGAGATCAAATCGATACCACAACGTCCGAATATGAAAAA
368     D  R  R  A  E  Q  I  R  D  Q  I  D  T  T  T  S  E  Y  E  K
1321   GAAAAAACTACAAGAACGTCTCGCTAGATTGGCGTCCGGTGTTGCCTTATTGAAAGTAGGA
388     E  K  L  Q  E  R  L  A  R  L  A  S  G  V  A  L  L  K  V  G
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Fig. 2. Nucleotide sequence and deduced the amino acid sequence of *GdHsp60* cDNA clone. The deduced amino acid sequence is shown below the nucleotide sequence. The initiation and termination codons are marked boxes. The classical mitochondrial Hsp60 signature is shown in gray and the ATP-binding motif is underlined. The typical GGM repeat motif at the C-terminus is shown as waved lines.

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1381   GGTAGCAGCGAAGTGGAAGTAAATGAGAAAAAAGACAGAGTAACCGATGCTTTGAATGCA
408      G  S  S  E  V  E  V  N  E  K  K  D  R  V  T  D  A  L  N  A

1441   ACAAGGGCAGCTATTGAAGAAGGTATTGTTCCTGGAGGTGGCACAGCTCTTTTGAGATGT
428      T  R  A  A  I  E  E  G  I  V  P  G  G  G  T  A  L  L  R  C

1501   AGCAGCAGCTTAGATGAAATTAAACCTGCTAACAAAGACCAAGAAGTTGGTATTCAAATT
448      S  S  S  L  D  E  I  K  P  A  N  K  D  Q  E  V  G  I  Q  I

1561   GTAAAAAGGGCTCTTAAAGTACCTTGCATGACAATTGCTGCTAATGCAGGTGTTGATGGT
468      V  K  R  A  L  K  V  P  C  M  T  I  A  A  N  A  G  V  D  G

1621   GCAGCTGTAGTAGCCAAAGTTGAACAACATAGTGGAGACTATGGCTATGATGCTCTTAAC
488      A  A  V  V  A  K  V  E  Q  H  S  G  D  Y  G  Y  D  A  L  N

1681   AACGAATATGTAAATATGTTTGAACGAGGAATCATCGATCCTACAAAAGTTGTTAGAACT
508      N  E  Y  V  N  M  F  E  R  G  I  I  D  P  T  K  V  V  R  T

1741   GCACTTGTAGACGCTTCAGGAGTAGCTTCTCTCTTGACAACAGCTGAAGCCGTTATCACG
528      A  L  V  D  A  S  G  V  A  S  L  L  T  T  A  E  A  V  I  T

1801   GATATACCTAAAGAAGACGTTCCAGTTCCAACTGGTATGGGTGGAATGGGCGGTATGGGA
548      D  I  P  K  E  D  V  P  V  P  T  G  M  G  G  M  G  G  M  G

1861   GGCATGGGAGGAATGATGTAAACGTAATTCTTCCCATACATGAACTGTTAAACAGTTAAA
568      G  M  G  G  M  M  M  *

1921   TAAACTCAGTGATTGCCTCGCAAAGGCAAAGCAAATGACGTCTGTCAAAAAAGTTCCATT
1981   TTCGTCTTTGACAGACACGAGTGTTTTTTTTAGTTAACATTTAATTATTTTGCTACCAGA
2041   ATTCGTGTGTAGTGTTATTATATGTAACACGAATTGAAAATTTGTGTGAATGAGAAATTG
2101   TAAGTTTCGCATATGGCGGTCGAAGGTGGTTTTTTCGTAGATGGTACTTTGAGTGTTGTT
2161   TGGGGAATCCTCTTTGGTCGCCGAACTGATCTGCATTTAAATCCTAAAGAATGTTTGAAT
2221   ATAGTTTTAATGACTCTCAAATGTACCTATATTGTTATAAATATATTGTTATGTAACTTT
2281   CTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACCACTGCTTGCC
2341   CTATAGTGAGTCGTATTAGAATCGA
```

Fig. 2. (*continued*)

rerio (59.0%), and *Homo sapiens* (59.0%). Multiple sequence alignment of *GdHsp60* with other known Hsp60 amino acid sequences of seven insects revealed that it was highly conserved. *GdHsp60* showed the highest identity with *Tribolium castaneum* (90.4%), as well as *Nasonia vitripennis* (81.3%), *Apis mellifera* (80.2%), *B. mori* (79.2%), *Plutella xylostella* (79.2%), *M. domestica* (78.7%), and *Myzus persicae* (73.8%).

Twenty-two different Hsp10 sequences and 23 Hsp60 sequences of invertebrate and vertebrate species were downloaded from NCBI to conduct phylogenetic analyses. For Hsp10, *G. daurica* was clustered together with four Lepidoptera insects, and this clade was closed to the other clade with several Diptera and Hymenoptera species. Besides, other invertebrate and vertebrate species were well grouped based on their taxonomic status (fig. 3). *GdHsp60* was most closely related to sequences from Coleoptera species with high bootstrap support, and they fell into distinct clades with sequences from mammals and Osteichthyes (fig. 4). The phylogenetic trees indicated that the evolution of Hsp60 was almost in accord with the evolution of species.

Expression of GdHsp10 and GdHsp60 in different developmental stages and tissues

The qPCR was used to determine mRNA expression levels of *GdHsp10 and GdHsp60* in the whole life cycle of *G. daurica*; *GdHsp10* and *GdHsp60* were expressed at all developmental stages tested. For *GdHsp10*, it was revealed that the mRNA expression levels were significantly high in the eggs and 1st instar larvae with 4.43 and 4.19 times, respectively. Furthermore, the expression levels of *G. daurica* in 2nd, 3rd instars' larvae, pupae, and female adults were 2.82, 2.02, 2.77, and 1.25 times of that in the male adults, in which the relative expression level was set as fiducial value (1.00) ($F_{6,27} = 2.66$, $P = 0.06$) (fig. 5a). The *GdHsp60* was highly expressed in the eggs, and the relative expression level was 2.68 times of that in the male adults. Besides, the expression levels of *G. daurica* in the 1st and 3rd instar larvae were 1.60 and 2.10 times of that in the male adults. However, the *GdHsp60* was lowly expressed in the 2nd instar larvae, pupae, and female adults, and their relative expression levels were 0.80, 0.82, and 0.53
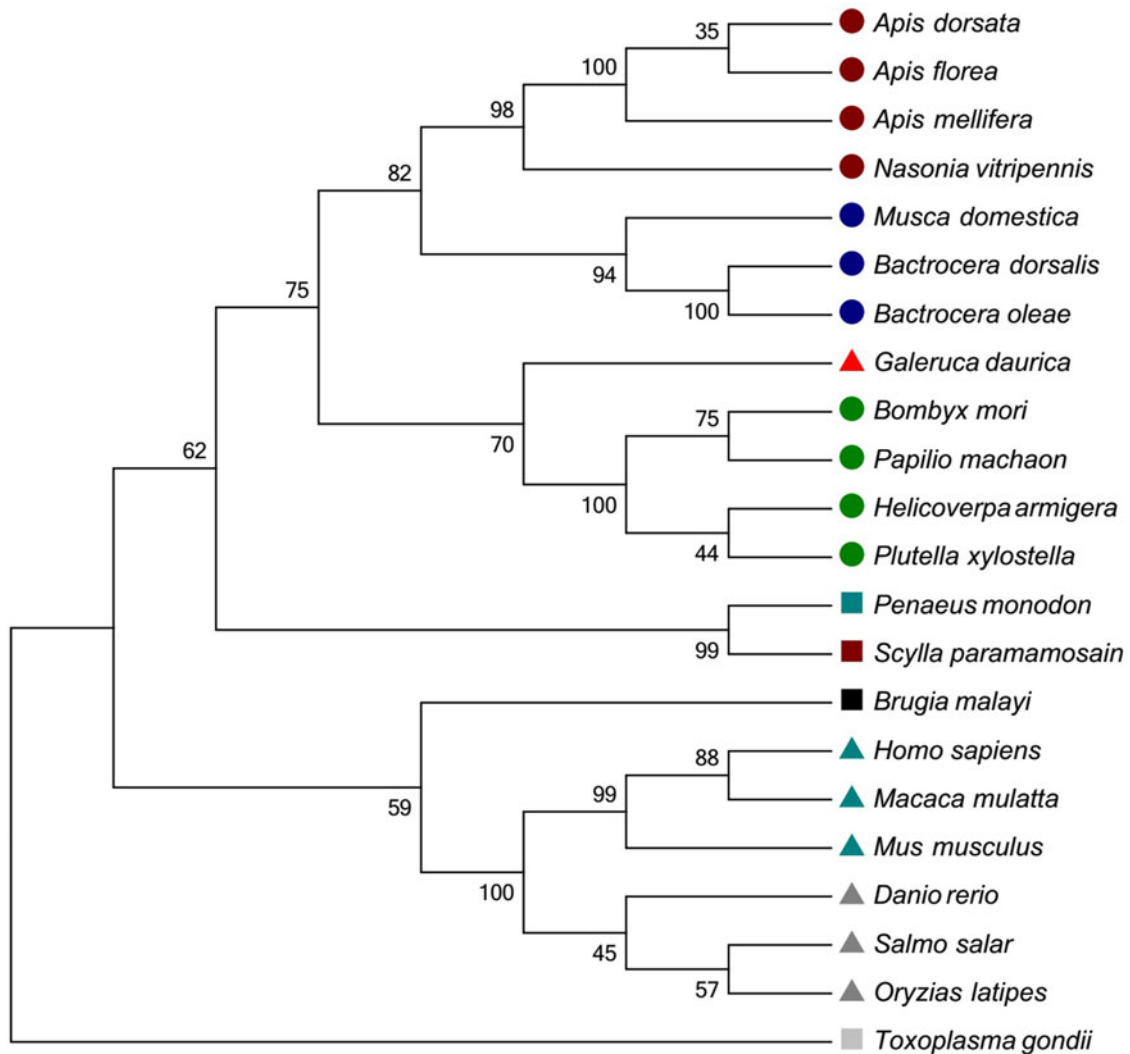
Fig. 3. Phylogenetic trees of GdHsp10. The deduced amino acid sequences were aligned with other known species Hsp10 by the ClustalW program. The tree was constructed using MEGA 5.0 software with the neighbor-joining method. GenBank accession numbers encoding Hsp60 are as follows: *Apis dorsata* (XM_006622720.1); *Apis florae* (XM_01245356.1); *Apis mellifera* (XM_624907.4); *Nasonia vitripennis* (XM_00159992.4); *Bombyx mori* (XM_004932889.2); *Papilio machaon* (XM_014506630.1); *Helicoverpa armigera* (KC689791.1); *Plutella xylostella* (XM_011556418.1); *Bactrocera dorsalis* (XM_011201618.2); *Musca domestica* (XM_005175857.3); *Bactrocera oleae* (XM_014241027.1); *Homo sapiens* (NM_002157.2); *Macaca mulatta* (XM_001100531); *Mus musculus* (NM_008303.4); *Salmo salar* (NM_001139672.1); *Danio rerio* (NM_131526.1); *Oryzias latipes* (NM_001104762.1); *Penaeus monodon* (KT001212.1); *Scylla paramamosain* (AGI74966.1); *Toxoplasma gondii* (AY644772.1); *Brugia malayi* (XM_001902716.1); *Galeruca daurica* (KY460464). ●: Hymenoptera; ●: Diptera; ●: Lepidoptera; ▲: Coleoptera (*G. daurica*); ▲: Primates; ▲: Osteichthyes; ■: Coccidae; ■: Malacostraca; ■: Crustacea; ■: Nematoda.

times of that in the male adults ($F_{6,27} = 27.42$, $P < 0.05$) (fig. 5b).

In order to investigate the tissue distribution of *GdHsp10* and *GdHsp60*, the relative expression levels of their mRNA in five adult body tissues (head, thorax, abdomen, antenna, and leg) were examined by qPCR in the third day adults. Both of the two Hsp genes were expressed omnipresently in five tissues examined in *G. daurica* adults, but were abundantly expressed in the abdomen, while the head and antenna showed the lowest expression (fig. 5c, d). The relative expression levels of *GdHsp10* and *GdHsp60* showed a significant difference ($P < 0.05$) among different tissues ($F_{4,19} = 209.09$, $P < 0.05$; $F_{4,19} = 518.81$, $P < 0.05$).

Expression of GdHsp10 and GdHsp60 in response to heat and cold stresses

The expression patterns of *GdHsp10* and *GdHsp60* were tested using qPCR after different temperature treatments for 1 h on the 2nd instar larvae. Both of the *GdHsp10* and *GdHsp60* genes had a significant response to heat and cold stresses. For cold treating, the *GdHsp10* expression was significantly induced to increase more than triple times in the −5 and 0°C treatments, and relative expression levels in the −14, −10, 5, 10, and 15°C treatments were 1.81, 2.75, 1.93, 2.10, and 2.11 times of that in the 20°C treatment, respectively. For heating, the 25 and 30°C heat treating larvae showed overexpressed levels (2.46 and
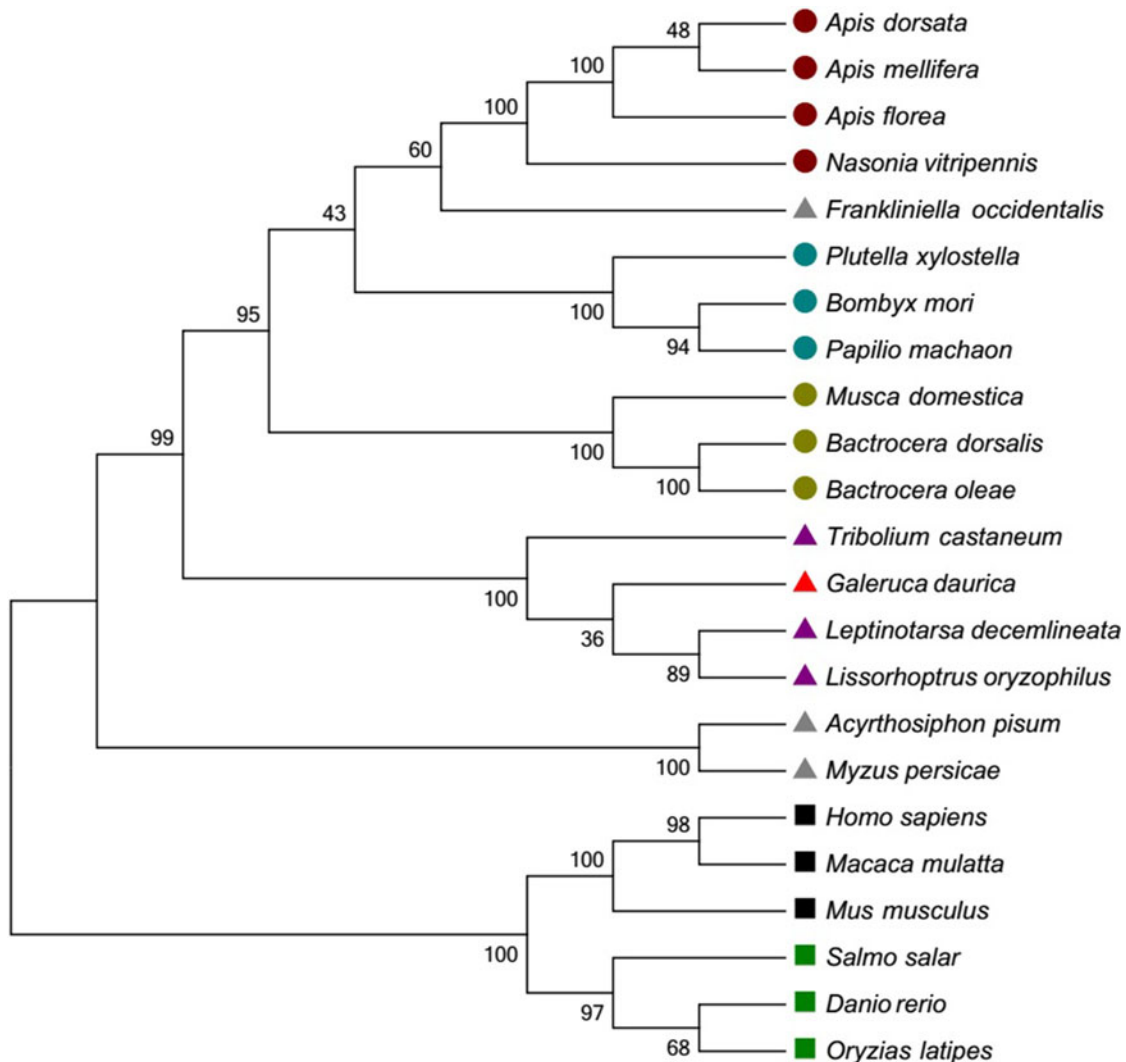
Fig. 4. Phylogenetic trees of GdHsp60. The deduced amino acid sequences were aligned with other known species Hsp60 by the ClustalW program. The tree was generated by MEGA 6.0 using the neighbor-joining (NJ) method. GenBank accession numbers encoding Hsp60 are as follows: *Apis dorsata* (XM_006622721.1); *Apis florae* (XM_003691238.2); *Apis mellifera* (XM_3392899.6); *Nasonia vitripennis* (XM_003427885.3); *Bombyx mori* (XM_004923900.2); *Papilio machaon* (XM_014505853.1); *Plutella xylostella* (KM215269.1); *Bactrocera dorsalis* (NM_001317412.1); *Musca domestica* (XM_005179343.3); *Bactrocera oleae* (XM_014238382.1); *Tribolium castaneum* (XM_966537.4); *Leptinotarsa decemlineata* (KC556801.1); *Galeruca daurica* (KY460463); *Lissorhoptrus oryzophilus* (KC620435.1); *Frankliniella occidentalis* (JX967580.1); *Myzus persicae* (AJ250348.1); *Acyrthosiphon pisum* (XM_008180647.2); *Homo sapiens* (ABB01006.1); *Macaca mulatta* (NM_034607.3); *Mus musculus* (AF137008); *Salmo salar* (ACN1137.01); *Danio rerio* (BC068415.1); *Oryzias latipes* (XM_004086882.2). ●: Hymenoptera; ●: Lepidoptera; ●: Diptera; ▲: Coleoptera; ■: Hemiptera; ▲: Primates; ■: Osteichthyes.

1.96 times than in the 20°C group) ($F_{9,39} = 8.33$, $P < 0.05$) (fig. 6a). A similar pattern appeared in the expression of *GdHsp60* gene under different temperature exposures. As fig. 6b showed, the expression of *GdHsp60* gene under cold temperature gradient was obviously higher than in the 20°C treatment, their expression levels were 3.66, 5.81, 4.38, 3.19, 4.13, 4.78, and 3.29 times for 15, 10, 5, 0, −5, −10, and −14°C. Moreover, the 25 and 30°C heat treating larvae showed overproduced *GdHsp60* transcripts (3.38 and 3.36 times than in the 20°C group) ($F_{9,39} = 65.10$, $P < 0.05$) (fig. 6b). The mRNA expression levels of *GdHsp10* and *GdHsp60* at 0°C for different times in the third day eggs were examined by qPCR. It was clearly exhibited that *GdHsp10* and *GdHsp60* genes were upregulated when *G. daurica*

eggs were exposed at 0°C, and a clear time-dependent expression pattern of the two genes was observed. The *GdHsp10* mRNA expression reached peak (5.19-fold higher than that of the control) after 1.5 h and then decreased ($F_{6,27} = 33.21$, $P < 0.05$) (fig. 6c), and the *GdHsp60* transcript increased to a maximum level (2.82-fold higher than that of the control) after 30 min and declined gradually ($F_{6,27} = 14.21$, $P < 0.05$) (fig. 6d).

Discussion

As a new and severely outbreaking insect species in grasslands in northern China, *G. daurica* has an adaptive strategy to escape extremely low-temperature condition in eggs and
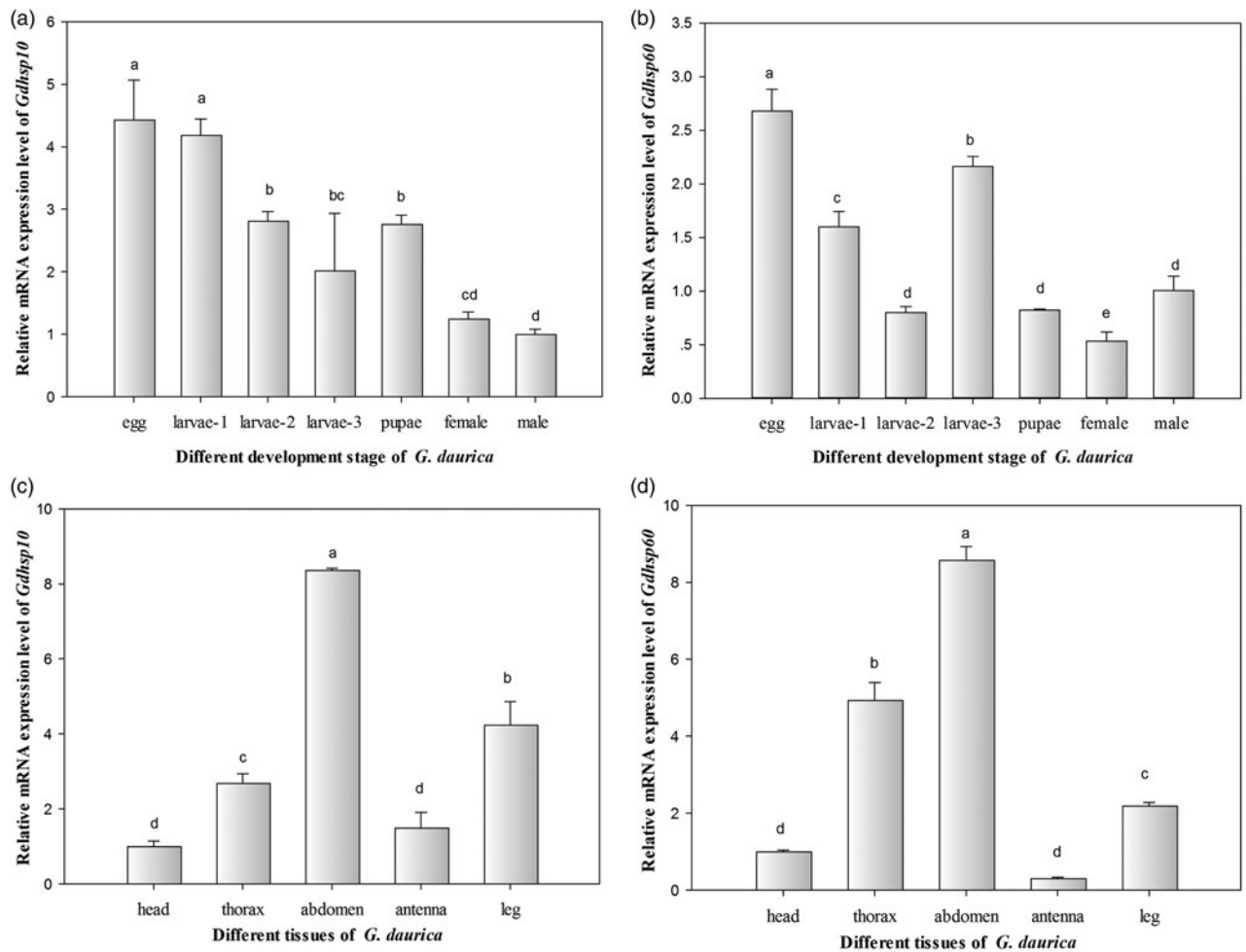
Fig. 5. Relative mRNA expression levels of *GdHsp10* and *GdHsp60* gene in different development stages and tissues. (a) *Gdhsp10*, (b) *GdHsp60* in different development stages; (c) *Gdhsp10*, (d) *Gdhsp60* in different tissues of adults. The bar colored light brown in each figure was considered as control. Different lowercase letters indicate significant differences (*P*<0.05). Each bar represents the mean ± SD (N = 4).

tolerate great daily temperature fluctuation in larvae to guarantee adequate population density, they mainly reply on their physiological stress responses. This survival strategy represents the first layer of self-defense in decreasing potentially negative effects of adverse conditions (Rosic et al., 2014). Gao et al. (2015) reported that the half-lethal time (LT50) at −30°C was 33.33 days in overwintering eggs of *G. daurica*, and Zhou et al. (2016) discovered that exposure to low temperatures was required to terminate egg diapause; furthermore, the longer chilling would shorten post-diapause time and accelerate egg development. So far, more efforts have been concentrated on studying the effects of temperature stress and treating time on the physiology of *G. daurica*, while little is known about the mechanism of responses and adaptability to temperature variation at molecular levels. Hsp is associated with cryoprotection of cells and tissues from thermal stress (Denlinger, 2002), and has been studied in some organisms subjected to thermal stress from ecological and physiological aspects (Feder & Hofmann, 1999).

In the present study, the two genes encoding *GdHsp10* and *GdHsp60* were cloned and characterized from *G. daurica*. The full-length cDNAs are 822 and 2365 bp with ORF of 315 and 1722 bp for *GdHsp10* and *GdHsp60*, respectively, encoding 104 and 573 amino acids with predicted molecular weights of 11.34 and 61.15 kD. A mobile loop structure and hsp10/cpn10 signature was detected in *GdHsp10* cDNA, a highly conserved mitochondrial Hsp60 signature, the ATP-binding sites, and four Gly-Gly-Met repeat motifs at the C-terminus were found in the *GdHsp60* sequence, in accordance with the structures of Hsp10/Hsp60 in marine organisms' studies (Xu et al., 1997; 2014). A dome-shaped heptameric ring of Hsp10 subunit and Hsp60 cylinder with ATPase form special structure called chaperon folding cage (Saibil & Ranson, 2002); the ATP hydrolysis triggers conformational changes of cage structure, producing a protected environment favoring protein folding (Clare & Saibil, 2013); meanwhile, the mobile loop in Hsp10 and an apical domain in Hsp60 cylinder bind and function as folding protein (Hayer et al., 2016). However, how Hsp60/Hsp10 complex co-works in cells, what proteins they can protect, and how temperature variation regulates Hsp60/Hsp10 expression and triggers protein protection mechanism are hardly known in insects; therefore, these
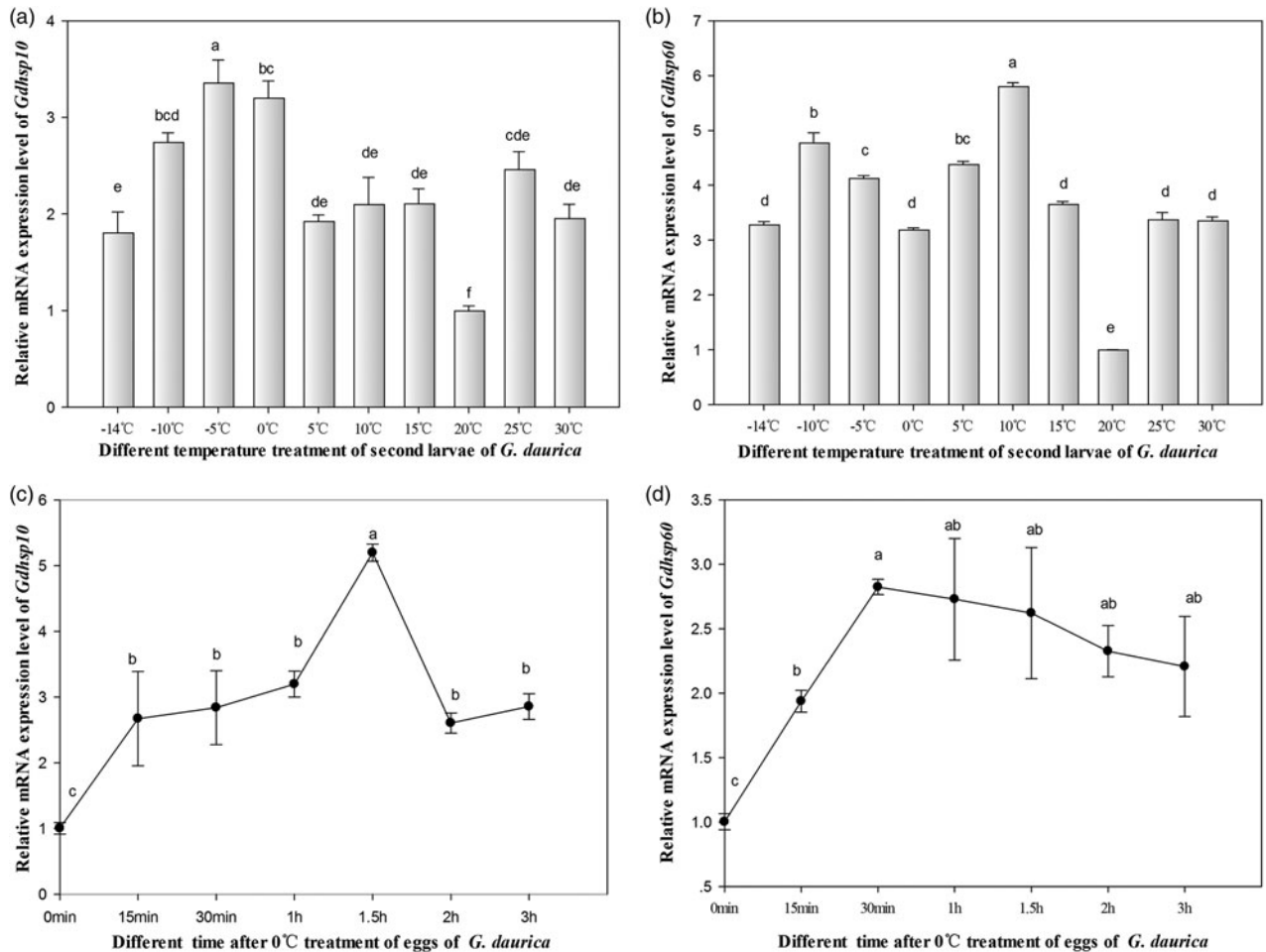
Fig. 6. Relative mRNA expression levels of *GdHsp10* and *GdHsp60* genes in response to thermal stress. (a) *Gdhsp10* and (b) *GdHsp60* in different temperature treatments of the 2nd instar larvae; (c) *Gdhsp10* and (d) *GdHsp60* in eggs under 0°C low-temperature exposure for different times. The bar colored light brown in each figure was considered as control. Different lowercase letters indicate significant differences ($P < 0.05$). Each bar represents the mean ± SD ($N = 4$).

researches are our main focus and interest on *G. daurica* in the future.

Homologous alignment analysis demonstrated that both *GdHsp10* and *GdHsp60* had high similarity with other *Hsp10* and *Hsp60* from other insects which have the closest relationship with *G. daurica* based on morphologic taxonomy. Phylogenetic trees showed that the evolution of *Hsp10* and *Hsp60* was almost in accord with the evolution of species. These analyses further suggested that *GdHsp10* and *GdHsp60* belonged to *Hsp10* and *Hsp60* families.

Some Hsps played essential roles in regulating growth, development, and cellular differentiation in insects, with tissue and time specificity (Michaud et al., 1997; Sharmas et al., 2007). *GdHsp10* and *GdHsp60* were proven to be expressed during the whole life cycle, but expressed highest in embryonic development stage (fig. 5a, b). As tissue distribution of mRNA expression analysis showed, *GdHsp10* and *GdHsp60* were expressed in five tissues tested in adults, and to be abundant in the abdomen of *G. daurica* (fig. 5c, d). It was reported that Hsps exist in high concentration in cells and fulfill a crucial role as a defense mechanism by sustaining protein

homeostasis during cellular stress through overproduction (Kastle & Grune, 2012; Bozaykut et al., 2014). *G. daurica* overwinters as eggs in bitter cold northern China; it was suggested that high expression of *Hsp10* and *Hsp60* in eggs might be helpful for tolerating extreme low temperatures, transmitting signals for cell proliferation, protein denaturation, programed death to protect them from severe cold injury, which might be one important cold resistance mechanism. More experiments on function and structure of *GdHsp10*/*GdHsp60* are required to prove our speculation in further research.

From mid-April to early May, *G. daurica* in larval stage can emerge overnight and rapidly cause destructive losses on young grasses in the vast pasture. If the population density can be reduced below the economic threshold before 3rd instar larvae outbreak, the losses would be relieved. For low age larvae, *G. daurica* experienced daily temperature oscillation even more than 20°C in the field; the potential mechanism of tolerating severe temperature variation is our main concern. Hsps are commonly used as gene markers to study temperature-induced cell stress and explore potential protective mechanism when the organism is exposed to thermal stress (*Chow*

et al., 2009; Olsen et al., 2013). From our results, both heat and cold stresses can induce mRNA expression of *GdHsp10* and *GdHsp60* after 1 h exposure (fig. 6a, b). The similar results were reported in other studies (Fang et al., 1997; *Chow et al., 2009*; Meng et al., 2014; Xu et al., 2014; Rinehart et al., 2007). *Hsp10* and *Hsp60* in the sea cucumber were slightly upregulated when temperature increased to 2–4°C and sharply overexpressed after exposed to 6°C rising (Xu et al., 2014); HSP60 expression in the reef-building coral increased after exposed to moderate, severe cold and heat in short time of 6 h (Seveso *et al.*, 2016). These results suggested that *Hsp10* and *Hsp60* were rapidly induced to protect the threatened proteins in organisms under elevated and depressed temperatures, in line with the role of Hsps as rapid and temporally dynamic cellular machinery associated with cellular defense (Georgopoulos and Welch 1993; Feder & Hofmann, 1999). However, some researchers considered that *Hsp10* and *Hsp60* expression trends and modulation magnitudes have close relationship with the intensity of the thermal stress and exposure time, either hot or cold (Rodriguez-Lanetty et al., 2009; Meyer et al., 2011; Seveso *et al.*, 2016). Further studies are required to verify the conclusion on *G. daurica*.

For 0°C exposure of *G. daurica* eggs, we also tracked the change of expression of *GdHsp10* and *GdHsp60* over time; it was indicated that the transcriptional expression levels of the two Hsp genes have a peculiar time-dependent response and both of them showed similar patterns of mRNA expression (fig. 6c, d). The expression of *GdHsp10* and *GdHsp60* reached a peak at 1.5 h and 30 min, respectively, then decreased slowly. The expression of *Hsp10* and *Hsp60* in other organisms was strongly induced under thermal stress and decreased gradually (Georgopoulos and Welch, 1993; Xu et al., 2014). Researchers explained that upregulation of *Hsp10* or *Hsp60* might be due to cellar defenses triggered to counteract stresses in short time (Georgopoulos and Welch, 1993; King & MacRae 2015); but for subsequent stabilization or gradual downexpression of the two genes, more explanations mainly focus on energy balance strategy and metabolic disorder. The former considers that organisms cannot afford the excess energy consumption required for the synthesis of Hsps after long exposure to threatened stressors (Krebs & Feder, 1997, 1998). The later reflects strong repression of the metabolic activities, such as restricted enzymatic activities, cell division, development, growth (Sokolova & Portner, 2001; Roth et al., 2012). However, some researchers put forward a viewpoint that the phenomena were associated with host mitochondria injury, causing electron transport and ATP produce degradation, because Hsp60s are ATP-dependent (Dunn et al., 2012). In other studies, it was found that Hsp60 expression returned to the basal value, and they speculated that it might be due to a rapid physiological acclimation of the organism's ability (Seveso *et al.*, 2016). At the cellular level, the cellular stress responses could transitorily increase the stress tolerance threshold to remain homeostatic (Kultz, 2005).

In summary, the *GdHsp10* and *GdHsp60* cDNA sequences were cloned and characterized from *G. daurica* for the first time. We found that both the Hsp genes were induced by heat and cold stresses in the 2nd instar larvae, and their transcriptional levels promptly increased with an apparent time-dependent response after eggs were exposed to freezing point. This information provides a foundation to research the biological function of the Hsp10/Hsp60 complex, tolerating temperature upheaval mechanism and cold resistance

mechanism on *G. daurica*, meanwhile, confirms the practicability of Hsps as molecular markers to test biological responses to heat and cold stresses.

Acknowledgements

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