Molecular cloning and expression analysis of small heat shock protein gene *GdHsp20.6* under temperature stress in *Galeruca daurica* (Celeoptera: Chrysomelidae)

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Abstract: In order to clarify the function of small heat shock proteins (sHsps) of *Galeruca daurica* under tempersture stress, the complete open reading frame (ORF) sequence of *GdHsp20.6* (*Hsp20* gene in *G. daurica*) was cloned by PCR, and bioinformatics analysis was performed using on-line software. The gene expression was induced by prokaryotic expression technology, and the expressed protein was purified. The expression level of *GdHsp20.6* gene under temperature stress was analyzed using the real-time quantitative PCR (RT-qPCR). The results showed that the ORF sequence of *GdHsp20.6* gene was 543 bp in length, encoding 180 amino acids; the predicted molecular weight was 20.6 kD without a transmembrane region or signal peptide. The amino acid sequence of GdHsp20.6 had a highly conserved α-crystallin domain, a higher identity with those of the Hsp20s in other coleopteran insects and the highest identity with the Hsp20.99 from *Dastarcus helophoroides* (63%). *GdHsp20.6* gene was successfully expressed in *Escherichia coli* BL21 (DE3) cells, and then GdHsp20.6 protein was induced to express by isopropyl-β-d-thiogalactoside (IPTG). The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blot analysis showed that the size of the fusion protein was consistent with the predicted value, and the target protein with high purity was obtained. The results showed that the relative expression level of *GdHsp20.6* gene was significantly up-regulated by low (−10−5℃) and high (35−40℃) temperature treatments for one hour with recovery or no recovery at 25℃ for 30 min. Additionally, *GdHsp20.6* gene was also up-regulated when the *G. daurica* larvae were treated at 0℃ for 30−120 min. These results suggested that *GdHsp20.6* gene might play an important role in response to cold and hot stress in *G. daurica.*

Key words：*Galeruca daurica*; small heat shock protein; temperature stress; expression profiling; Western-blot analysis

温度胁迫下沙葱萤叶甲小热激蛋白基因*GdHsp20.6*的克隆及表达分析

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摘要：为明确小热激蛋白在沙葱萤叶甲 *Galeruca daurica* 应对温度胁迫中的作用，采用PCR方法克

基金项目：国家自然科学基金(31760517)

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收稿日期：2018-08-24

隆沙葱萤叶甲小热激蛋白基因*Hsp20*(*GdHsp20.6*)完整的开放阅读框(open reading frame,ORF)序列,应用在线软件对*GdHsp20.6*基因进行生物信息学分析,通过原核表达技术诱导表达及纯化其编码蛋白,并通过实时荧光定量PCR(real-time quantitative PCR,RT-qPCR)技术分析不同温度胁迫下*GdHsp20.6*基因的表达量。结果显示,*GdHsp20.6*基因ORF序列长度为543 bp,编码180个氨基酸,预测分子量为20.6 kD,无跨膜区和信号肽。GdHsp20.6氨基酸序列有高度保守的α-结构域。GdHsp20.6氨基酸序列与其它鞘翅目昆虫的Hsp20氨基酸序列有较高的一致性,其中与花绒寄甲*Dastarcus helophoroides*的Hsp20.99氨基酸序列的一致性最高,为63%。*GdHsp20.6*基因在大肠杆菌*Escherichia coli* BL21(DE3)细胞系中成功表达,经异丙基-β-D-硫代吡喃半乳糖苷(isopropyl-β-d-thiogalactoside,IPTG)诱导后GdHsp20.6蛋白成功表达,十二烷基硫酸钠–聚丙烯酰胺凝胶电泳(sodium dodecyl sulfate-polyacrylamide gel electrophoresis,SDS-PAGE)和Western-blot分析表明融合蛋白大小与预测大小一致,并纯化获得了纯度较高的目的蛋白GdHsp20.6。低温(−10~5℃)和高温(35~40℃)处理1 h以及处理后25℃恢复30 min均能诱导*GdHsp20.6*基因表达上调,并且0℃处理30~120 min也能诱导*GdHsp20.6*基因表达上调。表明*GdHsp20.6*基因在沙葱萤叶甲应对低温和高温胁迫中可能起着重要作用。

关键词：沙葱萤叶甲；小热激蛋白；温度胁迫；表达谱；Western-blot分析

As ectothermic organisms, insects do not regulate their body temperature, and the effects of temperature on the activity, distribution and abundance of insects likely exceed those of any other abiotic factor. Insects respond to extreme changes of temperature by activating expression of heat shock proteins (Hsps), a set of evolutionary conserved proteins (King & MacRae, 2015). Hsps in insects contain four main families: Hsp90, Hsp70, Hsp60, and small Hsps (sHsps) (King & MacRae, 2015). sHsps are a superfamily of stress protein with a molecular weight range from 12 to 43 kD, and characterized by the presence of a highly conserved α-crystallin domain comprised of several β-strands (Basha et al., 2012). sHsps exhibit a larger variation in sequence, size, structure, and function compared with other families of Hsps (Franck et al., 2004). They are the first line of cell defense, avoiding irreversible denaturation of substrate proteins, especially when cells are stressed (Basha et al., 2012). sHsps presumably play important roles in insect adaptation to environmental stresses, including heat, cold, drought, starvation, anoxia, infection, and ultraviolet light (Basha et al., 2012; King & MacRae, 2015; Quan et al., 2018). sHsps can be up-regulated or down-regulated by environmental stresses when insect tolerance to stresses is enhanced (Zhao & Jones, 2012; King & MacRae, 2015). Most of insect sHsps have evolved independently in different insect orders (Huang et al., 2008; Li et al., 2009; Zhang et al., 2015), implying that species-specific sHsps may play important roles in their adaptability to diverse environments. Therefore, it is very useful to identify and study the species-specific sHsps to clarify the mechanisms by which insects survive in severe environments (Quan et al., 2018). Since sHsps were firstly described in *Drosophila melanogaster* (Southgate et al., 1983), a large number of sHsps have been identified in other insect species, including *Bombyx mori* (Li et al., 2009), *Chilo suppressalis* (Pan et al., 2018), *Choristoneura fumiferana* (Quan et al., 2018), *Tribolium castaneum* (Mahroof et al., 2005), *Lissorhoptrus oryzophilus* (Yuan et al., 2014), *Harmonia axyridis* (Wang et al., 2017), *Apis cerana* (Liu et al., 2012), *Lucilia cuprina* (Singh & Tiwari, 2016) and *Chironomus riparius* (Martín-Folgar et al., 2015). However, the knowledge about the functions of insect sHsps is much limited (Bakthisaran et al., 2015; Pan et al., 2018).

Galeruca daurica is a new insect pest in the Inner Mongolian Autonomous Region, which feeds only on the species of *Allium* plants (Hao et al., 2015). Since its abrupt outbreak in the Xilinhaot grassland of Inner Mongolia Autonomous Region, China in 2009, its distribution and damage area have continually enlarged, causing great losses to pasture in the Inner Mongolia Autonomous Region, and it has been one of

the most important insect pests in Inner Mongolia Autonomous Region in recent years (Li et al., 2014; Zhou et al., 2016). The overwintering eggs of *G. daurica* begin to hatch as early as early April when local temperature fluctuates greatly, and the lowest temperature sometimes falls to −10℃ (Li et al., 2015). In such a cold condition, eggs and larvae could survive, which showed that they had strong cold hardness (Li et al., 2014; 2015; Gao et al., 2015). Nevertheless, its cold hardness mechanisms were unknown. Three *Hsps* genes (*Hsp10*, *Hsp60* and *Hsp70*) had been cloned from the *G. daurica* larvae, and their expression profiles had been analyzed under temperature stress and in various development stages (Tan et al., 2017a; 2018). In this study, a new *sHsp* gene in *G. daurica* was cloned, a phylogenetic tree was constructed, its expression profiles were analyzed under different temperature stress, and Western-blot analysis was conducted in order to further investigate the functions of Hsps in *G. daurica.*

1 Materials and Methods

1.1 Materials

Insects: The overwintering eggs of *G. daurica* were collected from the Xilinhot grassland (44°62′N, 115°80′E) of Inner Mongolia Autonomous Region in 2016, and then were maintained in a climatic cabinet that the temperature was 25±1℃, relative humidity was (70±5)%, photoperiod was 14 L∶10 D. The larvae of *G. daurica* were reared with *Allium mongolium*.

LB (Luria-Bertani) liquid cultures: 1% yeast extract, 0.5% NaCl, 1% tryptone.

Reagents: MiniBEST Universal RNA Extraction Kit, PrimeScript First Strand cDNA Synthesis Kit, MiniBEST Agarose Gel DNA Extraction Kit, pMD-19T vector, made by Takara Biomedical Technology (Dalian) Co., Ltd, China; Go*Taq*®qPCR Master Mix, made by Promega Corporation, USA; yeast extract, trypton, made by Oxoid Ltd., UK; anti-6×His tag antibody-ChIP grade, goat anti-rabbit IgG H & L, made by Abcam, UK; antibody dilution buffer, BeyoECL star A and B, made by Beyotime Biotechnology, China; TIANprep Mini Plasmid Kit, pET-28a (+) plasmid, *Escherichia coli* BL21 (DE3), 2 × SDS-PAGE sample loading buffer, isopropyl-β-D-thiogalactoside (IPTG), DL-dithiothreitol (DDT), made by Tiagen Bioche (Beijing) Co., Ltd., China; imidazole, Tris-HCl, NaCl, Levamisole, PVDF membranes, skimmed milk, made by Xilong Scientific, China; *Bam*H I and *Sal* I, T4 DNA ligase, made by New England BioLabs Ltd., UK; Ni-NTA agarose, made by Qiagen Bioinformatics, Germany.

Instruments: NanoPhotometer® P330 spectrophotometer, made by Implen, Germany; T100™ thermal cycler, PowerPac™ HV power supply, made by Bio-Rad, USA; FTC-3000P real-time quantitative thermal cycler, made by Funglyn Biotech Inc., Canada; affinity chromatography column, made by Sangon Biotech (Shanghai) Co., Ltd, China; LRH-100CB cooling incubator, Shanghai Yiheng Instruments Co., Ltd., China; BG-verMIDI standard vertical gel tank, made by Baygene Biotech Co., Ltd., China.

1.2 Methods

1.2.1 RNA extraction and *GdHsp20.6* cloning

Total RNA was isolated from 15 three-day-old 2nd-instar larvae of *G. daurica* using Universal RNA Extraction Kit following the manufacturer's instructions. The RNA integrity was checked using the 1.0% agarose gel and the RNA concentrations were measured using the spectrophotometer. The first strand of cDNA was synthesized using PrimeScript First Strand cDNA Synthesis Kit following the manufacturer's protocol.

Based on the sequence annotated with the *GdHsp20.6* gene from the larval transcriptome data of *G. daurica* (GenBank accession: SRP150886), and blasted with complete *sHsps* published in other insects, the result showed that *GdHsp20.6* had a complete open reading frame (ORF). The gene-specific primers were designed using Primer Premier 5.0 software. The primers of cDNA cloning were *Hsp20.6*-F (5′-ATGGCATTTCTTCCTATT-3′) and *Hsp20.6*-R (5′-TTACTCCATTGGTTTCTC-3′), all of which were synthesized by Beijing Liuhe Huada Gene Technology Company. The PCR amplification was performed as follow: 3 min at 94℃; 30 cycles of 30 s at 94℃, 30 s at

58℃, 1 min at 72℃, and extension at 72℃ for 7 min by T100™ thermal cycler. The PCR products were eluted from the 1% agarose gel, extracted using Agarose Gel DNA Extraction Kit, transferred into pMD-19T vector following the manufacturer's protocol, and finally sequenced by Beijing Liuhe Huada Gene Technology Company.

1.2.2 Sequence and phylogenetic analysis of *GdHsp20.6*

ORF of *GdHsp20.6* gene was identified on the ORF finder website (https://www.ncbi.nlm.nih.gov/orffinder/), and then compared with other Hsp20 amino acid sequences for verification by BLASTx soft available at the NCBI website (http://www.ncbi.nlm.gov/BLAST/). The theoretical isoelectric point (pI), molecular weight, and amino acid composition of GdHsp20.6 amino acid sequence were predicted on the websites (http://web.expasy.org/protparam/ and http://web.expasy.org/protscale/). Predictions of transmembrane region, secondary structure, phosphorylation sites, and signal peptide of GdHsp20.6 amino acid sequence were conducted on the websites of http://www.cbs.dtu.dk/services/TMHMM-2.0/, http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi, http://www.cbs.dtu.dk/services/ NetPhos-3.1/, and http://www.cbs.dtu.dk/services/SignalP/, respectively. Furthermore, protein subcellular localization was predicted on the PSORT software website of https://wolfpsort.hgc.jp/. Multiple sequence alignment was performed using the Clustal 2 software. To study the evolutionary relationships among different sHsps from various insects, a phylogenetic tree was constructed by the neighbor-joining method and performed using Mega 6.0 software.

1.2.3 Prokaryotic expression, protein purification and Western-blot analysis of GdHsp20.6

The PCR product of *GdHsp20.6* was digested with *Bam*H I and *Sal* I, and was ligated into the pET-28a (+) plasmid digested with the same enzymes to get the pET-28a (+)/GdHsp20.6 recombinant plasmid by T4 DNA ligase. The pET-28a (+)/GdHsp20.6 recombinant plasmid was transferred into *E. coli* BL21 (DE3). Recombinant plasmids were extracted by TIANprep Mini Plasmid Kit and insertion was correct or not was confirmed using restriction enzyme digestion and sequencing. *E. coli* BL21 (DE3) that contained the correct insertion of recombinant plasmids was cultured overnight at 37℃, and then added into LB liquid cultures (1∶100) and cultured for an additional two hours. IPTG was added into this bacterial culture with a final concentration of 1 mmol/L for four hours to induce recombinant GdHsp20.6 protein expression. The bacterial culture was centrifuged at 8 000 r/min for two min, and the precipitation was collected and mixed with 50 μL 2×SDS-PAGE sample loading buffer, 40 μL nuclease-free water and 1 mol/L 1×DTT 10 μL, and finally transferred on ice for five min immediately after boiled for ten min. The recombinant GdHsp20.6 protein samples were analyzed using 12% SDS-PAGE.

Successfully expressed recombinant GdHsp20.6 in *E. coli* BL21 (DE3) was purified following the Qiagen protein purification protocol. The precipitation of positive expression production was collected by centrifugation, and the cell lysate of the target protein was obtained by ultrasonication with 20 mmol/L imidazole, and the supernatant was collected by centrifugation (7 800 r/min) for one hour at 4℃. The supernatant was added into the equipment composed of Ni-NTA agarose and affinity chromatography column, gently mixed, and stored at 4℃ for one hour. The unbound protein was washed successively with 20 mmol/L imidazole for three times and 250 mmol/L imidazole for eight times to get purified recombinant GdHsp20.6. Next, all of the ingredients were collected for SDS-PAGE analysis and verification.

Purified proteins were electrophoretically transferred onto PVDF membranes at 80 V for 75 min. The membrane was blocked in TBST (0.02 mol Tris-HCl, 0.50 mol NaCl, 0.05% Tween 20, 0.01 mol levamisole, pH 7.5) containing 5% skimmed milk at room temperature for one hour, and then rinsed the membrane by TBST. Subsequently, the membrane was incubated in the primary antibody dilution buffer containing the primary antibody (anti-6×His tag antibody-ChIP grade) with 1∶10 000 at 4℃ for 12 h. The membrane was cleaned and put it into the secondary antibody (goat anti-rabbit IgG H & L), which was diluted

1∶20 000 in the secondary antibody dilution buffer at room temperature for one hour. Finally, the mixed BeyoECL star A and B (1∶1) was dripped to the membrane to check.

1.2.4 Temperature treatments of *G. daurica* larvae

Recovery treatment: three-old-day 2nd-instar larvae of *G. daurica* were selected and were exposed for one hour at −10, −5, 0, 5, 10, 15, 20, 25, 30, 35 and 40℃, respectively, in a cooling incubator. They were recovered at 25℃ for 30 min, and then were snap-frozen in liquid nitrogen before being stored at −80℃. No recovery treatment: similar to recovery treatment, but no samples were recovered at 25℃ for 30 min. Different time treatment: three-old-day 2nd-instar larvae of *G. daurica* were treated, respectively, at 0, 10, 15, 20, 30, 45, 60, 90, 120, 150 and 180 min at 0℃, then frozen in liquid nitrogen and stored at −80℃. Each sample included three independent biological replicates, and each biological replicate included fifteen 2nd-instar larvae of *G. daurica*.

1.2.5 RT-qPCR analysis of *GdHsp20.6* expression

The relative expression levels of *GdHsp20.6* in the 2nd-instar larvae of *G. daurica* with recovery treatment, no recovery treatment and different time treatment were measured using RT-qPCR. Total RNA isolation of each sample and first-strand cDNA synthesis were the same with 1.2.1. The expression levels of *GdHsp20.6* under different treatments were examined using real-time quantitative thermal cycler with BRYT Green® dye as the fluorescence reporter for each elongation cycle. The succinate dehydrogenase *SDHA* gene (GenBank accession: KU240575) was used as a reference gene (Tan et al., 2017b), primers of reference gene were SDHA-F (5′-GGGAGACCACAATCTCCTCA-3′) and SDHA-R (5′-AGCTGGTGCTCCTAAGTCCA-3′). Primers for RT-qPCR were *Hsp20.6*-qF (5′-AAGAAATCACCGTCAG-3′) and *Hsp20.6*-qR (5′-CAGTTTATCCCCCAC-3′), all primers were designed using Primer Premier 5.0 software. The volume of RT-qPCR reaction system was 20 μL and included 2 μL cDNA template, 10 μL Go*Taq*®qPCR Master Mix, 0.4 μL *Hsp20.6*-qF primer, 0.4 μL *Hsp20.6*-qR primer; sterile distilled water was added until reaction system reached 20 μL. RT-qPCR reaction conditions as follow: 95℃ for 60 s; 40 cycles of 95℃ for 15 s, 56℃ for 30 s, and 72℃ for 30 s. The relative expression levels of *GdHsp20.6* were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

1.3 Statistical analysis

Statistical analysis of test data was performed using SPSS 16.0 software. Difference significance analysis of *GdHsp20.6* expression levels in different treatments were compared by one-way ANOVA followed by a Tukey's test for multiple comparisons, and paired samples *t* test was used to compare the difference between recovery treatment and no recovery treatment.

2 Results

2.1 *GdHsp20.6* gene cloning and sequence analysis

The sequence obtained from positive clone shared 100% identity with the corresponding sequence from the transcriptome. Based on the predicted molecular mass with 20.6 kD of its encoding protein, the identified *Hsp20* gene in *G. daurica* was named as *GdHsp20.6* (GenBank accession: KY437812) with a complete ORF. The complete ORF was 543 bp in length, encoding 180 amino acids with an isoelectric point of 6.39 but without signal peptide and transmembrane region, and the PSORT software predicted that GdHsp20.6 in *G. daurica* existed in nuclear.

2.2 GdHsp20.6 amino acid sequence characterization

Multiple sequences alignment between GdHsp20.6 and other four coleopteran species' Hsp20 were performed (Fig. 1). The predicted GdHsp20.6 amino acid sequence exhibited the highest identity with Hsp20.99 amino acid sequence of *Dastarcus helophoroides* (63%), followed by 57% identity with Hsp21 amino acid sequence of *Gastrophysa atrocyanea*, 53% identity with Hsp21 amino acid sequence of *Agasicles hygrophila*, and 41% identity with Hsp21 amino acid sequence of *Lissorhoptrus oryzophilus*. Multiple sequences alignment in secondary structures showed that GdHsp20.6 had a conserved α-crystalline family domain comprised of about 100 amino acid residues, and another highly conserved domain of the

metazoan α-crystalline domain comprised of about 80 amino acids, consisted of eight β-strands from β2 to β9, N-terminal and C-terminal for 65 and 35 amino acids, respectively.

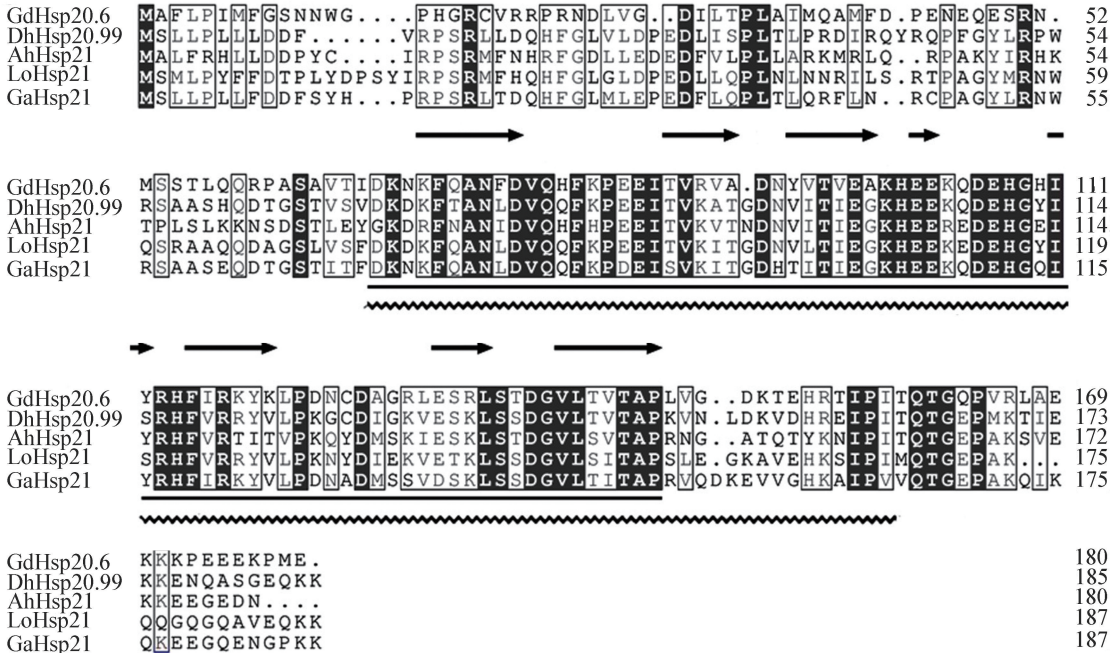

Fig. 1 Alignment of Hsp20 family amino acid sequences of five coleopteran species and GdHsp20.6 second structure characters

The shaded dark background and wireframe shows 100% and ≥60% identity in amino acid sequences, respectively. The wavy line indicates a conserved α-crystalline family domain, and the underlined section shows another highly conserved domain of the metazoan α-crystalline domain. Arrow shows β-strands. GdHsp20.6: sHsp of *Galeruca daurica*; LoHsp21: sHsp of *Lissorhoptrus oryzophilus*; DhHsp20.99: sHsp *of Dastarcus helophoroides*; AhHsp21: sHsp *of Agasicles hygrophila*; GaHsp21: sHsp of *Gastrophysa atrocyanea*.

2.3 GdHsp20.6 amino acid phylogenetic analysis

A phylogenetic tree of the sHsps was constructed on the basis of amino acid sequences from different insects' sHsps to investigate the evolutionary relationships among the sHsps of different insects. The result showed that all five sHsps from Coleoptera were clustered into the same clade while all eight sHsps from Lepidoptera were grouped into another clade. However, two sHsps from Hemiptera were firstly grouped into one subclade, then clustered into one subclade with one sHsp from Diptera, and finally grouped into one clade with two sHsps from Diptera (Fig. 2). The tree reflected the evolution relationship of species to some extent.

2.4 Prokaryotic expression, protein purification and Western-blotting of GdHsp20.6

The recombinant protein pET-28a (+)/GdHsp20.6 was isolated from the protein samples, which were induced using IPTG from *E. coli* BL21 (DE3) with pET-28a (+) for four hours. Close to the protein maker 25 kD, there was a recombinant protein similar to the predicted result at the solid triangle showed the position of label 1, including the target protein about 20.6 kD and some of the proteins on the vector detected about 3.6 kD and label 2−4 as the control without recombinant GdHsp20.6, using 1.5% SDS-PAGE analysis (Fig. 3-A). Through testing the supernate and sediment collected by centrifuging of the induced sample, the result revealed that the recombinant GdHsp20.6 was present as a soluble protein in label 6. Label 7 showed that the recombinant GdHsp20.6 was purified for later research (Fig.3-A), and further checked by Western-blot analysis with His-antibody (Fig.3-B), which confirmed the successful expression of GdHsp20.6.

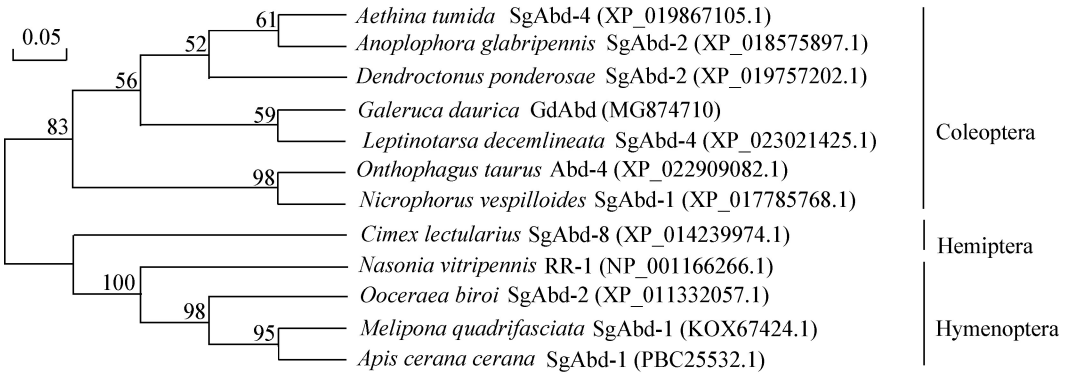

Fig. 2 Phylogenetic tree of sHsp20 amino acid sequences from different insect species based on neighbor joining method

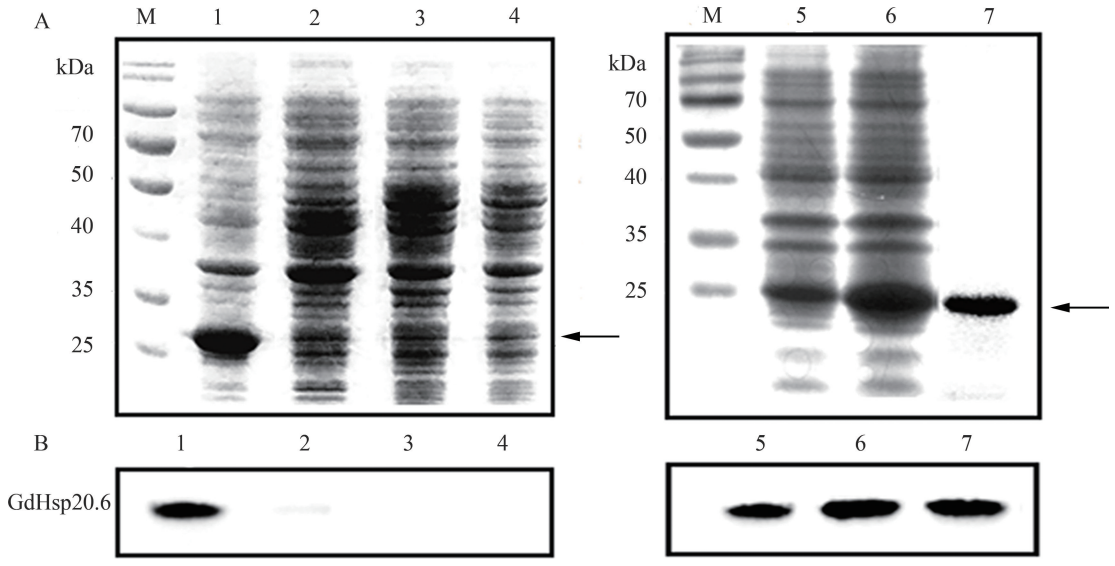

Fig. 3 SDS-PAGE (A) **and Western-blot** (B) **analysis of GdHsp20.6**

M: Protein maker; 1: recombinant protein pET-28a (+)/GdHsp20.6 induced for four hours with 1 mmol/L IPTG; 2: recombinant protein pET-28a (+)/GdHsp20.6 without induced; 3: pET-28a (+) induced for four hours with 1 mmol/L IPTG; 4: pET-28a (+) without induced; 5: sediment of recombinant protein pET-28a (+)/GdHsp20.6 induced with 1 mmol/L IPTG for four hours in 37℃; 6: supernatant of recombinant protein pET-28a (+)/GdHsp20.6 induced with 1 mmol/L IPTG for four hours at 37℃; 7: purified protein of recombinant protein pET-28a (+)/GdHsp20.6 induced with 1 mmol/L IPTG for four hours at 37℃. Arrows showed the position of the target protein.

2.5 Expression profiles of *GdHsp20.6* under different temperature stress

When temperature was lower than the control of 25℃, there was an increasing trend in the relative expression level of *GdHsp20.6* in the *G. daurica* larvae with stress temperature decreasing from 20℃ to −10℃, but significantly higher only at −5℃ and −10℃ with a maximum of 7.65-fold compared with the control at 25℃ (P<0.05, Fig. 4-A). On the contrary, when temperature was higher than 25℃, the relative expression level of *GdHsp20.6* increased with stress temperature increasing from 30℃ to 40℃, and reached the maximums of 33.43-fold for recovery and 20.61-fold for no recovery, respectively (Fig. 4-B). Moreover, there was no significant difference between recovery and no recovery at most treatment temperatures except for 10, 15, 20, 30 and 35℃; the relative expression level of *GdHsp20.6* decreased significantly after recovery for 30 min at 25℃ from stresses at 10, 15, 20 and 30℃, whereas increased at 35℃ (Fig. 4).

2.6 Time-dependant expression profile of *GdHsp20.6* under cold stress

The stress duration at 0℃ had significant effects on the relative expression level of *GdHsp20.6* (F=

10.28, P<0.001). The relative expression level of *GdHsp20.6* increased gradually over time, and reached a maximum of 7.59-fold in 60 min but declined abruptly near to the control (0 min) level after 150 min (Fig. 5).

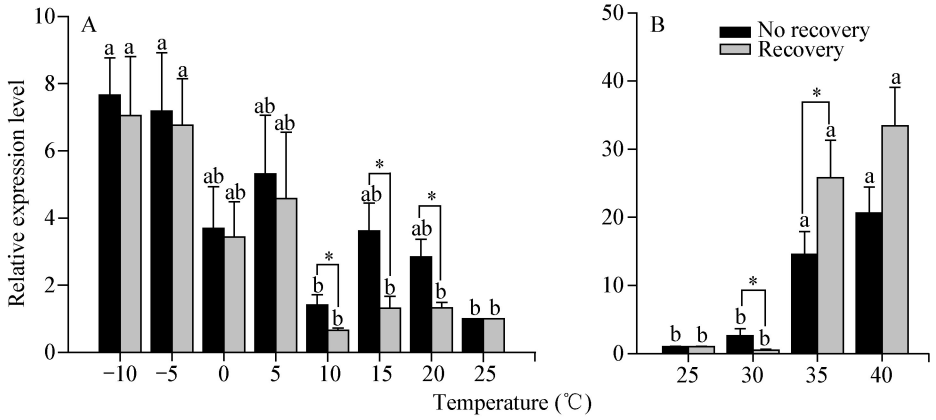

Fig. 4 Relative expression level of *GdHsp20.6* under different temperature stresses

Data are mean±SE. Different letters on the same color bars indicate significant difference at P<0.05 level by Tukey test. * indicates significant difference between recovery and no recovery at same temperature at P<0.05 level by t test.

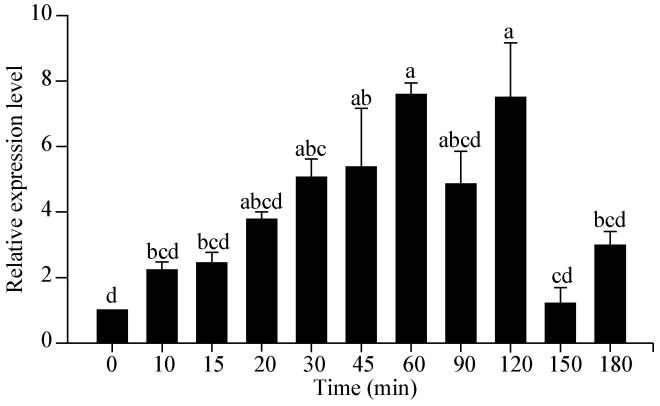

Fig. 5 Relative expression level of *GdHsp20.6* at 0℃ for 0−180 min

Data are mean±SE. Different letters on the bars indicate significant difference at P<0.05 level by Tukey test.

3 Discussion

A cDNA sequence in *G. daurica*, named *GdHsp20.6*, was cloned in this study. The amino acid alignment of GdHsp20.6 with other coleopteran species showed a mutual and highly conserved domain structure, namely α-crystalline domain, which belongs to all of Hsp20 family (Sun & MacRae, 2005), including eight β-strands, similar to GmHsp18.9, 19.6, 19.8a/b, 19.9, 20.4, 21.3, 21.4, 21.7, 22.1, 22.5, and 24.8 in *Grapholita molesta* (Zhang et al., 2015). The identity of GdHsp20.6 with DhHsp20.99, GaHsp21, AhHsp21, and LoHsp21 gradually decreased from 63% to 41% because of various and flexible carboxy-terminal and amino-terminal (Zhang et al., 2015), including two flanks of α-crystalline domain, which played important roles in their functions and evolution (Kriehuber et al., 2010). Moreover, the amino/N-terminal of GdHsp20.6 contains two α-behaves helices, as an intrinsically disordered protein that was very prone to proteolysis, and was not primarily to maintain the dodecamer (Franck, 2004; Rutsdottir et al., 2017).

Temperature was one of the most important factors in determining the distribution and abundance of insects (Bale et al., 2002). sHsps had been generally thought to play an important role to improve insect tolerance to extreme temperature (Sun & MacRae, 2005; King & MacRae, 2015). In the present study, *GdHsp20.6* was up-regulated by cold and hot stresses. In response to cold shock, the mRNA level of *GdH-*

sp20.6 reached the highest after exposure to −5℃ and −10℃. The other researchers also obtained similar results, such as *Hsp21.56* in *H. axyridis* (Wang et al., 2017), *Hsp20.8* in *Liriomyza sativae* (Huang et al., 2009), and *Cshsp21.5*, *Cshp22.9b* and *Cshsp24.3* mRNA in *C. suppressalis* (Lu et al., 2014; Pan et al., 2018). With respect to heat shock, the highest expression level of *GdHsp20.6* were found at 35℃ and 40℃, and this phenomenon was similar to *Cshsp22.9b* and *Cshsp24.3* in *C. suppressalis* (Pan et al., 2018), *Bmhsp27.4* in *B. mori* (Wang et al., 2014) and *Hsp27* in *L. cuprina* after heat shock (Singh & Tiwari, 2016). However, *GdHsp20.6* was not significantly up-regulated by mildly cold (0−20℃) or warm (30℃) temperatures, which was similar to the results reported for some *sHsp*s in *L. sativa* (Huang et al., 2009), *L. cuprina* (Concha et al., 2012) and *C. suppressalis* (Lu et al., 2014; Pan et al., 2018). There were also some *sHsps* insensitive to cold and hot shock, such as *Cshsp21.4* and *Cshsp21.7a* in *C. suppressalis* (Lu et al., 2014), *CrHsp21* and *CrHsp22* in *C. riparius* (Martín-Folgar et al., 2015). On the other hand, the expression level of *Gdhsp20.6* increased after recovery at 25℃ from exposure to 35℃ and 40℃. An increasement of the mRNA expression after recovery from heat shock had also been reported in other insects, including *B. mori* (Sakano et al., 2006), *Ceratitis capitata* (Kokolakis et al., 2009) and *A. cerana* (Lu et al., 2014). Interestingly, this results indicated that *GdHsp20.6* was much more sensitive to heat than cold stress, suggesting that this protein might play a greater role in repairing the injury caused by heat shock than by cold shock.

To date, a large number of studies had shown the *Hsp* up-regulation in response to cold and hot stress, which led to imprudent general interpretation of its role in the repair of cold injury. However, so far, direct functional research in *D. melanogaster* and other insects brought either limited or no support for such interpretation (Štětina et al., 2015), and there were only a few examples supporting such interpretation (Rinehart et al., 2007; Koštál & Tollarová - Borovanská, 2009; Colinet et al., 2010). In the present study, both acute cold (−10℃ and −5℃) and hot (35℃ and 40℃) shock induced the up-regulation expression of *GdHsp20.6* in the *G. daurica* larvae. RNAi technology had been widely used in functions of genes. After *dsAccsHsp22.6* was injected in adults of *Apis cerana cerana*, the adults had a lower survival rate than control groups at two extremely temperatures (Zhang et al., 2014), which gave an insight in next research and guidance in controlling this pest. Nevertheless, it was necessary to further study if the up-regulation of *GdHsp20.6* led to increase the product of GdHsp20.6 protein, and then, promote the thermotolerance in the *G. daurica* larvae because an increasement of sHSPs at the transcript level was not always correlated with higher protein levels under thermal stress (Zhao & Jones, 2012).

The *GdHsp20.6* expression level increased significantly after exposure for 30 min at 0℃, and a peak value was observed at 60 min, which suggested that *GdHsp20.6* might be quickly and strongly induced to protect the denatured proteins in host cells under cold stress (Georgopoulos & Welch, 1993). The expression level of *GdHsp20.6* declined suddenly after 150 min. This might be a tradeoff strategy of survival for *G. daurica* because it cannot afford the excessive energy consumption required for the synthesis of Hsps (Krebs & Feder, 1997). The similar results were also obtained in other *Hsps*. For example, when *Apis cerana cerana* adults were exposed at a similar low temperature condition for five hours, the most abundance of *AccsHsp22.6* occurred in two hours of cold exposure, then slumped after this time point (Zhang et al., 2014). Moreover, *Hsp70* and *Hsc70* in *Sitodiplosis mosellana* (Cheng et al. 2016), and *Hsp16.25* in *H. axyridis* (Wang et al., 2017) existed a similar phenomenon.

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