

Identifications, Characteristics, and Expression Patterns of Small Heat Shock Protein Genes in a Major Mulberry Pest, *Glyphodes pyloalis* (Lepidoptera: Pyralidae)

Jie Chu,¹ De-lei Jiang,¹ Meng-wen Yan,¹ Yi-jiangcheng Li,¹ Jun Wang,^{1,2} Fu-an Wu,^{1,2} and Sheng Sheng^{1,2,3,*}

¹Jiangsu Key Laboratory of Sericultural Biology and Biotechnology, School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang 212018, PR China, ²Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture and Rural Affairs, Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, PR China, and ³Corresponding author, e-mail: parasitoids@163.com

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Abstract

Six candidate sHSP genes were identified from the *Glyphodes pyloalis* transcriptome. All sHSP genes included full-length open reading frames and shared high similarity with the sequences of other lepidopteran species. These sHSP genes encoded 175–191 amino acid residues, and the predicted proteins had a molecular weight from 19.5 to 21.8 kDa. All *GpsHSPs* were expressed at lower levels at larval stages. All *GpsHSPs* were expressed at higher levels at diapaused, prepupal, or pupal stages, suggesting that sHSPs may be involved in metamorphosis in *G. pyloalis*. In addition to the developmental stage, extreme temperatures can induce variations in the expression of sHSPs genes. All *GpsHSPs* were significantly upregulated in larvae following exposure to heat shock, except *GpHSP21.4* which downregulated at 4 h following exposure to the cold shock treatment. Furthermore, Starvation influenced the expression patterns of *GpsHSPs* as a function of the duration of food deprivation. Four *GpsHSPs* increased their expression with time of starvation until reaching to the peak level at 6 d of starvation. Finally, parasitism by the endoparasitoid *Aulacocentrum confusum* He et van Achterberg (Hymenoptera: Braconidae)-induced fluctuations in the expression of all *GpsHSPs*, and the expression varied with time after parasitization. Our results from this study strongly suggest functional differentiation within the sHSPs subfamily in *G. pyloalis*. The present study would provide further insight into the roles of sHSPs in *G. pyloalis* and novel avenues for promoting integrated management of this pest.

Key words: *Glyphodes pyloalis*, small heat shock protein, environmental stress, expression patterns, mulberry pest

Heat shock proteins (HSPs), known as stress proteins and highly conserved molecular chaperones, help organisms to response stress and protect organisms from damage to cell. (Kim et al. 1998, Bukau et al. 2006, Hartl et al. 2011). In insects, five major families of HSPs have been identified, including the ATP-independent small heat shock proteins (sHSPs) and the ATP-dependent HSP40, HSP60, HSP70, and HSP90 (Feder and Hofmann 1999, Huang et al. 2008, Gu et al. 2012). It has been known that HSPs have consequences on development, immunity, and other protective functions such as tolerance under high temperature, desiccation, anoxia/hypoxia, oxidative stress, and starvation (Sun and Macrae 2005, Sonoda et al. 2006, Nakamoto and Vigh 2007). The sHSP family is widely distributed across eukaryotes and exhibits diverse structures and functions in each compartment of the cell, mostly ranging in monomer size from 12 to 43 kDa (Huang et al. 2008, Gu et al. 2012). Compared with other families of HSPs, sHSPs are less conserved, even within a

species (Brocchieri et al. 2008, Johnson 2012). sHSPs can prevent irreversible denaturation of substrate proteins and protect them from damage caused by abiotic stressors such as heavy metals, antibiotics, and radiation (Nakamoto and Vigh 2007). sHSPs can also defend against pathological stressors, including virus infection, inflammation, and parasitization (Nakatsue et al. 1998, Roelofs et al. 2006). In addition, sHSPs play a significant role in mediating endogenous physiological processes such as hormone secretion, cell differentiation, and histogeny (King and Macrae 2015). Moreover, sHSPs have been found to be involved in many developmental processes, including insect diapause, metamorphosis, embryo formation, apoptosis, and autophagy (Haslbeck et al. 2019, Rodríguez et al. 2019, Sudnitsyna and Gusev 2019).

The first insect sHSP was identified in and isolated from *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Tissières et al. 1974). Thanks to the development of genomics, transcriptomics, and other

sequencing technologies, an increasing number of sHSPs have been reported in insects, including *Galeruca daurica* Joannis (Coleoptera: Chrysomelidae), *Antheraea pernyi* Hübner (Lepidoptera: Saturniidae), *Musca domestica* Linnaeus (Diptera: Muscidae), *Bactrocera dorsalis* Hendel (Diptera: Tephritidae), *Ericerus pela* Chavannes (Homoptera: Coccidae), *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) and *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae). (Li et al. 2009; Liu et al. 2014, 2018; Chen and Zhang 2015; Dou et al. 2017; Tan et al. 2017; Tian et al. 2018).

The expression of sHSPs vary depending on insect developmental stage or environmental conditions insects live in (Morrow and Tanguay 2012). For example, four of the six sHSPs in the tobacco armyworm *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) are expressed at varying levels across developmental stages (Shen et al. 2011). In *Apis cerana cerana* Fabricius (Hymenoptera: Apidae), *AccHSP22.6* was highly expressed at different developmental stages and was significantly upregulated by temperature (Zhang et al. 2014). In the parasitoid wasp, *Pteromalus puparum* Linnaeus (Hymenoptera: Pteromalidae), the expression level of *PpHSP20* increases significantly after 6 h of starvation but decreases after 24 h (Wang et al. 2012). Rinehart et al. (2007) demonstrated that inhibiting the expression of *SchHSP23* can effectively reduce cold tolerance in *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae).

Parasitism can be a stressor inducing sHSPs in hosts (Rinehart et al. 2002). For example, relative levels of the expression of sHSP genes in the host *Pieris rapae* Linnaeus (Lepidoptera: Pieridae) pupa are up-regulated in response to parasitism by *P. puparum* (Zhu et al. 2013). In contrast, *sHSP23* is downregulated in *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) on the fourth day after envenomation by *Bracon hebetor* Say (Hymenoptera: Braconidae) (Shim et al. 2008).

The mulberry pyralid caterpillar, *Glyphodes pyloalis* Walker is a destructive pest of mulberry (*Morus alba* L.) trees in China and other Asian countries (Khosravi and Sendi 2010, Poltavsky et al. 2017). It can cause heavy losses to the sericultural industry by decreasing the production of mulberry leaves, which is the exclusive natural food for the silkworm *B. mori*. The population of *G. pyloalis* usually reaches a high level from mid-summer through to mid-autumn when temperature gets to as high as 42°C in eastern and southern China. High temperature has consequences on the expression of multiple genes in *G. pyloalis* (Liu et al. 2017). Yet, it remains unknown whether sHSPs are involved in these gene responses to heat waves. *Aulacocentrum confusum* (Hymenoptera: Braconidae) is a preponderant parasitoid wasp of *G. pyloalis*, and generally parasitizes *G. pyloalis* larvae during their second to fifth instar with the parasitism rate reaching up to 58.2% under field condition (Yu et al. 2003). Whether sHSPs play any role in eliminating or reducing the parasitization risk in *G. pyloalis* is also unknown.

The present study aimed to identify sHSPs from transcriptome database, to analyze their sequence characteristics, and to examine gene expressions of sHSPs in *G. pyloalis* exposed to extreme temperatures, starvation, or parasitism. The information obtained from this study can improve the understanding of the significance of physiological function of sHSP in *G. pyloalis*.

Materials and Methods

Insects

The laboratory stock of *G. pyloalis* was collected from the mulberry field at the campus of Jiangsu University of Science and Technology in the city of Zhenjiang (119°44'N 32°20'E), Jiangsu Province,

China. The larvae were reared with fresh mulberry (variety 'Yu71-1') leaves in the insectary [26 ± 2°C, 60–80% relative humidity, and photoperiod of 14:10 (L: D) h]. Adult moths were provided as supplementary food with 10% honey solution while being kept with two males and one female in transparent glass jars (20 × 20 × 30 cm) for mating and oviposition. Fresh mulberry leaves were provided as the substrate for deposition of eggs.

The parasitoid *A. confusum* was collected from rearing parasitized *G. pyloalis* larvae attacking mulberry trees in the mulberry field. The parasitoid colony was established with *G. pyloalis* larvae as hosts in the insectary [26 ± 2°C, 60–80% relative humidity, and photoperiod of 14:10 (L: D) h]. A honey solution of 10% was supplied via cotton lines to adult parasitoids.

Sample Preparation

To determine the relative expression levels of the sHSPs at different life stages of *G. pyloalis*, samples of first-instar, third-instar, fifth-instar larvae, pupae, male, and female adult moths were collected and stored at –80°C until RNA extraction. To obtain diapaused larvae following Chen et al. (2015), the fifth-instar larvae were collected and reared at 16°C for 7–10 d. Once the larvae molted into the prepupal stage to enter into the diapause, they were collected and kept for half a month without pupation before the experiment.

In the thermal treatment, 3-d-old fourth-instar larvae were selected to undergo heat and cold shock. Three temperature levels, 40, 25, and 0°C, were imposed to the larvae (Yang et al. 2016, Liu et al. 2017). To evaluate the effect of exposure time on gene expression, two times of the exposure (1 and 4 h) were examined for their short- and long-term effects. In the starvation treatment, our preliminary trial showed that the fourth-instar larvae remained alive for 6–7 d without food while the first- to third-instar larvae were not able to survive this period of starvation; beyond this period, the fifth-instar larvae molted into the prepupae stage when food was absent. So, we applied food deprivation treatment to the fourth-instar larvae at three levels: 2, 4, and 6 d. To avoid cannibalism, these larvae were individually reared in Petri dishes (9 cm in diameter). The survived larvae were collected and stored at –80°C until further analysis. The control larvae were fed ad arbitrium with mulberry leaves.

The parasitization experiment was conducted following the methods described by Zheng et al. (2017). Briefly, five of the fourth-instar *G. pyloalis* larvae with similar body weights were exposed to one 6-d-old mated and naive female wasp in a glass tube (2 × 2 × 10 cm). Once the parasitization by the wasp was finished, the parasitized larvae were removed and individually placed in Petri dishes (9 cm in diameter). Healthy fourth-instar larvae were used as the control. These larvae at 0, 2, 4, or 6 d after parasitization were stored at –80°C until RNA extraction.

RNA Isolation and Reverse Transcription

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. RNase-Free DNase I was used to remove residual genomic DNA (Promega, Madison, WI, USA). The concentration of RNA was confirmed by the absorbance at 260 nm, and the purity was determined according to an OD260/280 ratio between 1.9 and 2.1 using a 2100 Bioanalyzer (Agilent Technologies). Agarose gel electrophoresis was used to ensure that the RNA was integrated. The PrimeScript RT reagent Kit (Takara, Dalian, China) was used to produce first-strand DNA, followed by reverse transcription following the manufacturer's recommendations, and the synthesized cDNA was stored at –20°C.

Sequence Identification and Bioinformatics Analysis

cDNA sequences encoding sHSPs were retrieved from a previously constructed *G. pyloalis* transcriptome dataset (GenBank SRR9959773) using the TBLASTN algorithm (E -value $< 1 \times 10^{-5}$), and all candidate genes were manually checked using BLASTX (E -value $< 1 \times 10^{-5}$) against the GenBank nonredundant (nr) protein database at the National Center for Biotechnology Information (NCBI). Annotated sHSP protein sequences from other insect species (including *Apis mellifera ligustica* Linnaeus [Hymenoptera: Apidae], *P. rapae*, *Sesamia inferens* Walker [Lepidoptera: Noctuidae], *Cnaphalocrocis medinalis* Guenée [Lepidoptera: Pyralidae], *B. mori*, *Lymantria dispar* Linnaeus [Lepidoptera: Lymantriidae], and *Pyrausta nubilalis* Hubert [Lepidoptera: Pyralidae]) were used as queries. The open reading frames (ORFs) of the putative sHSPs were predicted using ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). The homologous sequences of the sHSPs were identified in the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Motif scans (https://myhits.isb-sib.ch/cgi-bin/motif_scan) by choosing 'PROSITE profiles' as the mot_source were used to find all known motifs in the present sequences (Hulo et al. 2006). Then, we predicted the molecular weight and isoelectric point of the proteins with ExPASy (https://web.expasy.org/compute_pi/) by choosing 'Average' as the Resolution. Sequence alignments and homology analysis were performed using ClustalX 2.1 multiple alignment with default gap penalty parameters of gap opening 10 and extension 0.2. The neighbor-joining (NJ) tree is constructed based on the principle of minimum evolution and suggested to be high accuracy, fast computation and has been widely and successfully used in phylogenetic analysis, especially in sHSPs (Gkouvitass et al. 2008, Kriehuber et al. 2010, Dou et al. 2017). Therefore, a tree was constructed using MEGA 7.0 (Tamura et al. 2007) with a p-distance model and a pairwise deletion of gaps. The bootstrap support of tree branches was assessed by resampling amino acid positions from 1,000 replicates. The secondary structure predictions were performed with the online PHD software accessed via the NPS@Web server (<http://npsa-pbil.ibcp.fr>; Combet et al. 2000) by choosing 'PHD' as the Method and

with the output width as 70. We named the sHSPs according to their molecular weight.

Real-Time Quantitative PCR

RT-qPCR was conducted in QuantStudio 6 Flex (Thermo Fisher Scientific) in a total reaction volume of 20 μ l, including 10 μ l of 2 \times iQTM SYBR Green I (Takara-Bio, Dalian, China), each gene-specific primer (Supp Table 1 [online only]) at 10 μ M and the cDNA template. A cDNA dilution series (1, 1/3, 1/9, 1/27, and 1/81) with the sample cDNA was used to construct the standard curve and calculate the efficiency of amplification. In addition, a dissociation curve analysis (60–95°C at the end of each RT-qPCR run) was used to verify amplification of a single product. The qPCR conditions were as follows: 95°C for 5 min and 40 cycles of 95°C for 15 s and 60°C for 31 s. Each sample was run in triplicate with three biological replicates, and the relative gene expression levels among the different samples were measured via the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The *G. pyloalis* GAPDH gene (GenBank MK243490) was used as the housekeeping gene to normalize target gene expression since it had been evaluated as the most stable reference gene for the temporal and spatial distribution of *G. pyloalis* in our previous studies. Differences in the expression levels of each target gene among the treatments were compared by one-way ANOVA followed by the least significant difference (LSD) test using R 3.2.2 software (R Development Core Team 2015).

Results

Identification and Characterization of Six *GpsHSPs*

Six sHSP genes were identified from the *G. pyloalis* transcriptome and named according to their molecular weights (i.e., *GpHSP19.5*, *20*, *20.2*, *21.4*, *21.6*, and *21.8*). The ORFs ranged from 525 to 573 bp and encoded 175 to 191 amino acids (Table 1). Their isoelectric points and predicted molecular weights are shown in Table 1. The deduced amino acid sequences of *GpsHSPs* contained a typical

Table 1. Characteristics of sHSP mRNAs in *Glyphodes Pyloalis*

Gene	GenBank accession number	Sequence length (bp)	Protein length (aa)	ORF	Molecular weight (kDa)	Isoelectric point	BLASTX best hit				
							Species	Description	Accession number	E -value	Identity(%)
<i>GpHSP19.5</i>	MK225525	528	175	complete	19.5	6.30	<i>Bombyx mori</i>	heat shock protein 22.6	FJ602788.1	7E-41	78
<i>GpHSP20</i>	MK225526	534	177	complete	20.0	6.53	<i>Mamestra brassicae</i>	heat shock protein 19.7	AB251898.1	3E-104	80
<i>GpHSP20.2</i>	MK225527	537	178	complete	20.2	5.88	<i>Grapholita molesta</i>	small heat shock protein 22.5	KP843904.1	3E-10	83
<i>GpHSP21.4</i>	MK225528	564	187	complete	21.4	5.79	<i>Chilo suppressalis</i>	heat shock protein 21.4	JX491642.1	0	83
<i>GpHSP21.6</i>	MK225529	576	191	complete	21.6	7.03	<i>Helicoverpa armigera</i>	heat shock protein 20.8	KX845562.1	2E-100	80
<i>GpHSP21.8</i>	MK225530	576	191	complete	21.8	6.33	<i>Maruca vitrata</i>	small heat shock protein 22.0	KY565573.1	2E-157	85

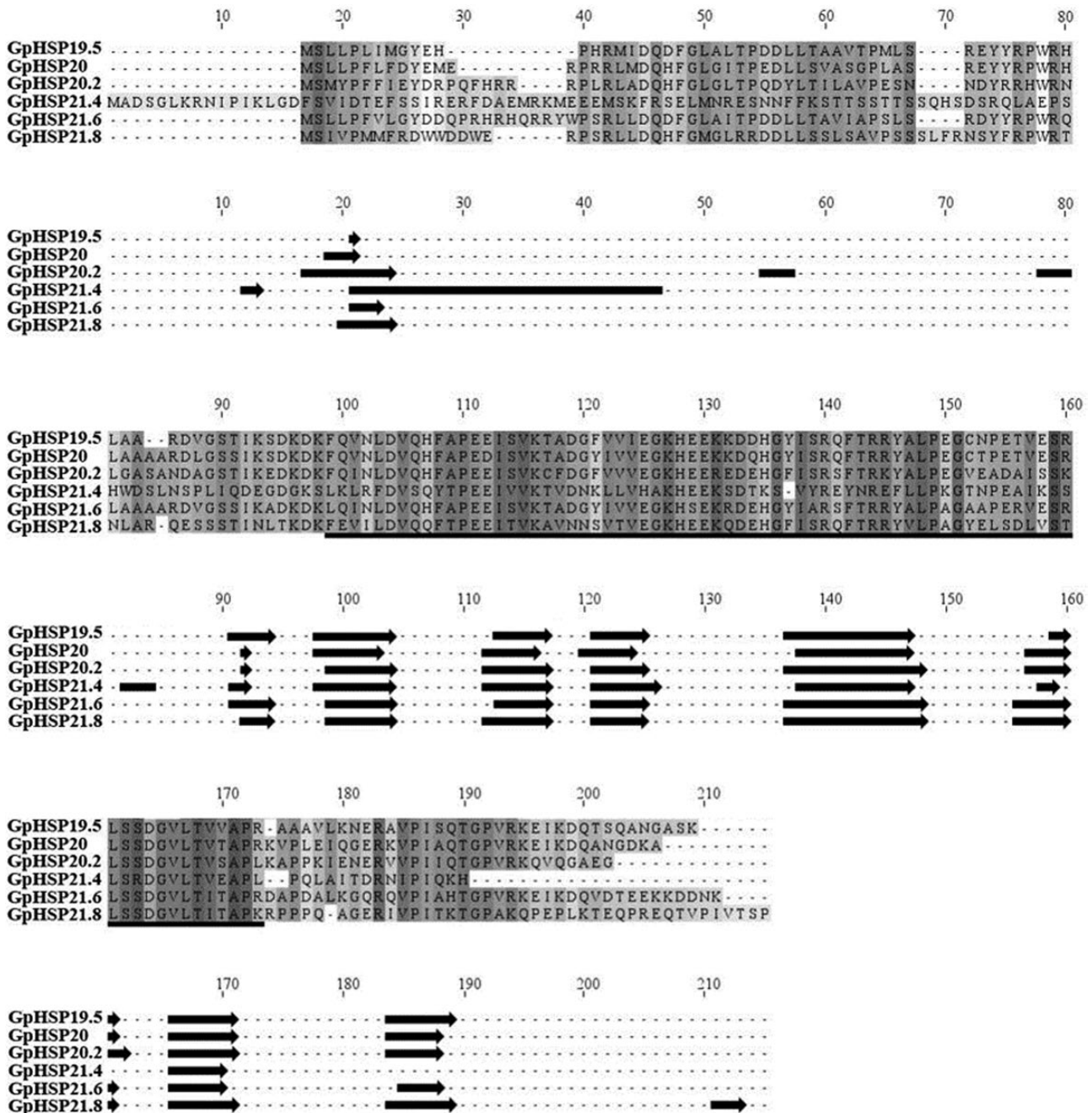


Fig. 1. Alignment of the deduced amino acid sequences of six *GpsHSPs* from *Glyphodes pyloalis*. Secondary structure elements are predicted for individual sequences. The conserved α -crystalline domain is underlined and is illustrated with cylinders (α -helices) and arrows (β -strands) below the alignment.

α -crystalline domain consisting of six β -strands (Fig. 1). Unlike the conserved α -crystalline domain located in the C-terminal, their N-terminal is highly variable (Fig. 1).

Phylogenetic Analysis of GpsHSPs

Results of the phylogenetic tree showed that insect sHSPs were divided into different clades (Fig. 2). Six *G. pyloalis* sHSPs were clustered into distinct branches, suggesting functional differentiation of GpsHSPs. GpHSP20 was grouped into a subbranch along with other insect sHSPs, which had similar molecular weight. By contrast, although GpHSP21.4, 21.6 and 21.8 had the similar

molecular weight, they were divided into separate branches. GpHSP21.4 was clustered with HSP21.4 in *S. inferens*, *B. mori*, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), *S. litura*, and *D. plexippus*, suggesting that these sHSPs may be orthologs and undertake conserved functions. GpHSP21.4, GpHSP21.6, and GpHSP21.8 were grouped into a subbranch with other insect sHSPs but did not follow the similar molecular weight. GpHSP19.5 was assembled into a subbranch with *C. suppressalis* HSP24.3 and GpHSP20.2 fell into the subbranch with *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae) HSP19.9, *D. plexippus* HSP15.1, and *S. litura* HSP20.7 (Fig. 2).

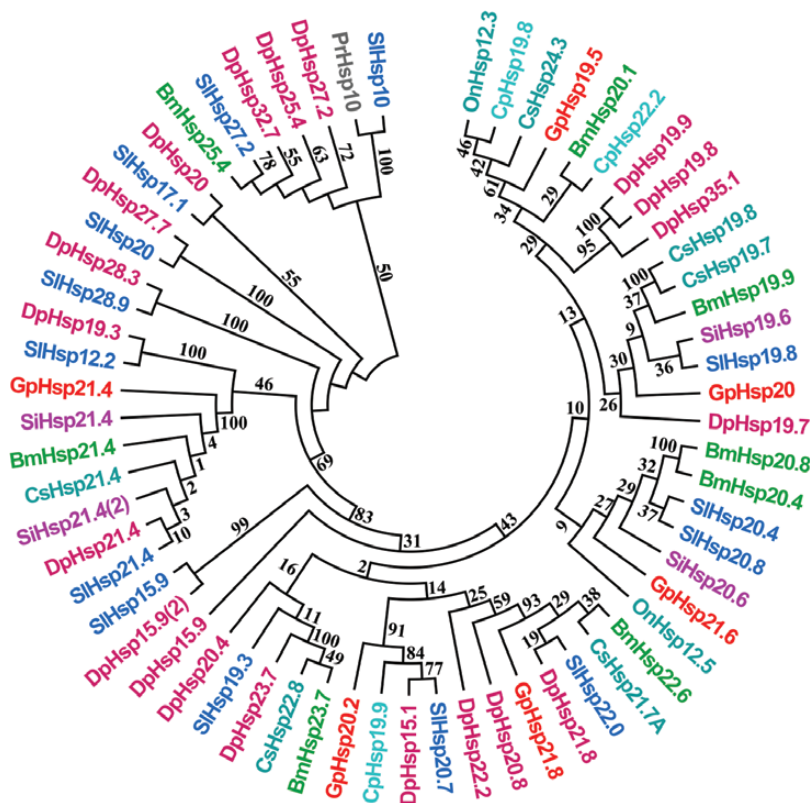


Fig. 2. Neighbor-joining phylogenetic tree of sHSP amino acid sequences from different insect species. The *Glyphodes pyloalis* sHSPs are labeled in red words. The full Latin names of all the species and the accession numbers of the genes from GenBank are presented with the corresponding abbreviations and listed in [Supp Table 2 \(online only\)](#). (Bm: *Bombyx mori*; Cp: *Cydia pomonella*; Cs: *Chilo suppressalis*; Dp: *Danaus plexippus*; On: *Ostrinia nubilalis*; Pr: *Pieris rapae*; Si: *Sesamia inferens*; Sl: *Spodoptera litura*)

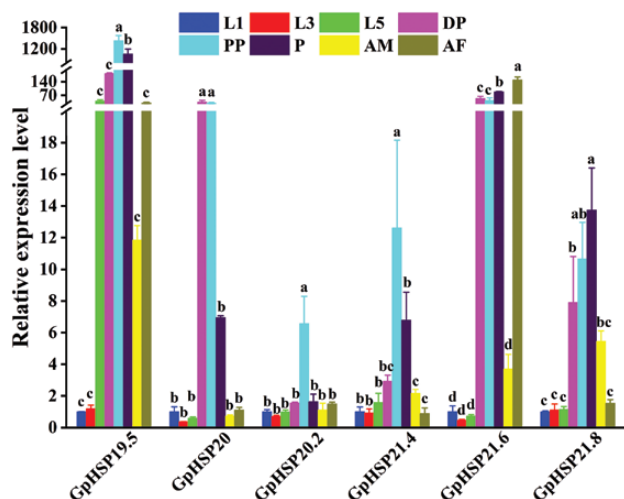


Fig. 3. Relative mRNA expression levels of six *GpsHSPs* at different developmental stages. first-instar larvae (L1), third-instar larvae (L3), fifth-instar larvae (L5), diapause larvae (DP), prepupae (PP), male adults (AM), and female adults (AF) of *Glyphodes pyloalis*. The relative expression levels of the *GpsHSPs* were determined by comparison with the first instar larvae. The different letters above the bars indicate significant differences ($P < 0.05$).

Expression of *GpsHSPs* at Different Developmental Stages

We used RT-qPCR to verify the expression levels of six *GpsHSPs* at different developmental stages (first-, third-, fifth-instar larvae,

diapused larvae, prepupae, pupae, female, and male adults). The results showed that all six sHSPs were expressed at relatively lower levels in first-, third-, and fifth-instar larvae than in other stages. *GpHSP19.5*, *GpHSP20.2*, and *GpHSP21.4* reached their highest expression levels in the prepupae stage compared with the other stages. Furthermore, *GpHSP20* showed the highest expression levels in the prepupae and diapause stages. Remarkably, *GpHSP21.6* was highly transcribed in female adults but was expressed at extremely low levels in male adults. Meanwhile, it was expressed at high levels in pupae, diapause larvae and prepupae. *GpHSP21.8* was expressed at the highest level at the pupal stage (Fig. 3).

Expression of *GpsHSPs* at Different Temperatures

The temperature treatment significantly affected the expression of six *GpsHSPs*. The expression level of the sHSPs under the cold shock treatment did not significantly differ from that in the control, but *GpHSP21.4* expression decreased after 4 h of the cold shock treatment. The expression levels of *GpHSP19.5*, 20, and 21.6 were highest after 1 h of heat treatment. Alternatively, the expression levels of *GpHSP20.2*, 21.4, and 21.8 were highest after 4 h of heat shock (Fig. 4).

Expression of *GpsHSPs* in Response to Starvation Stress

Starvation had a great impact on the expression levels of all six sHSPs, and this effect was dependent on the duration of food scarcity. The expression levels of *GpHSP19.5*, 20, 20.2, and 21.6 were highest until the sixth day of deprivation. In contrast, *GpHSP21.8* was expressed most abundantly until the fourth

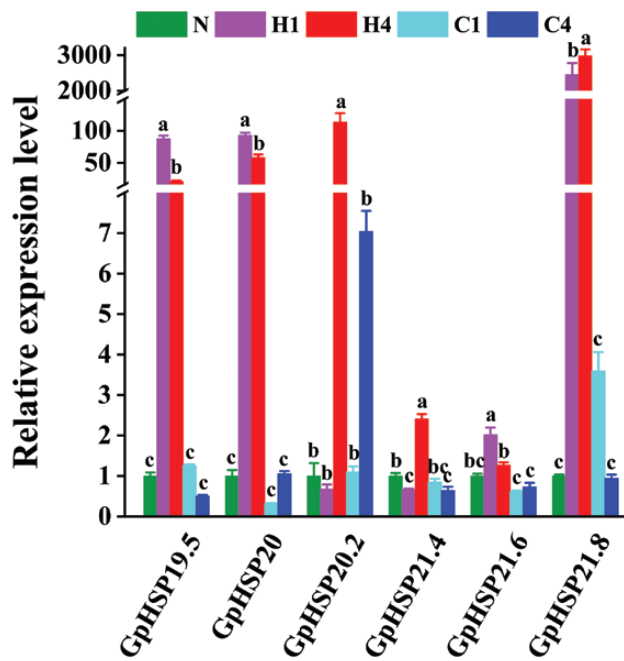


Fig. 4. Relative mRNA expression levels of six *GpsHSPs* at different temperatures. N: normal temperature control (25°C); H1: heat shock (40°C) for 1 h, H4: heat shock (40°C) for 4 h, C1: cold shock (0°C) for 1 h, C4: cold shock (0°C) for 4 h. Different lowercase letters indicate significant differences ($P < 0.05$).

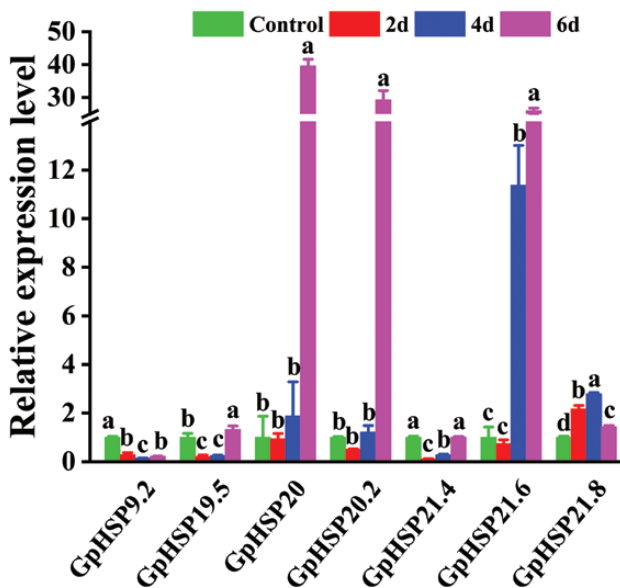


Fig. 5. Expression of *GpsHSPs* after different durations of starvation. Different lowercase letters indicate significant differences ($P < 0.05$).

day of starvation, while its expression level on the sixth day was lower than that on the second and fourth days. The expression level of *GpHSP21.4* was comparable between the sixth day and the control but lower on the second and fourth day (Fig. 5).

Expression of *GpsHSPs* in Response to Parasitization Treatment

RT-qPCR validations were conducted to examine the mRNA transcription levels of six *GpsHSPs* in *G. pyloalis* larvae parasitized by

A. confusum. Parasitization significantly changed the expression levels of all candidate sHSPs in *G. pyloalis* larvae, and these differences exhibited temporal variability. When at parasitism (within one day after parasitization), *GpHSP20.2* and *21.4* were expressed at lower levels in parasitized hosts than in healthy cohorts. The expression levels of *GpHSP19.5*, *21.6*, and *21.8* were higher in the parasitoid-exposed groups than in unparasitized hosts, whereas *GpHSP21.4* presented the opposite pattern 2 d after parasitization. After 4 d of development, the expression levels of *GpHSP19.5* and *20.0* were higher in parasitized *G. pyloalis* larvae than in the control groups. The expression levels of *GpHSP19.5*, *GpHSP20.0*, and *GpHSP20.2* were downregulated 6 d after parasitization compared with the healthy cohorts. In addition, *GpHSP21.6* and *GpHSP21.8* were expressed at higher levels in parasitized host larvae at this time point (Fig. 6).

Discussion

In the present study, a total of six sHSPs were identified from the *G. pyloalis* larval transcriptome dataset. In *Grapholita molesta* Busck (Lepidoptera: Tortricidae), fourteen new members from the sHSP families were identified by transcriptome sequencing (Zhang et al. 2015). The largest number of sHSP genes among insects has been reported in the silkworm *B. mori* until now. In contrast, in *M. domestica*, only four *MdsHSPs* have been identified (Tian et al. 2018). The variable numbers of sHSPs among insect species may result from gene duplication and a lack of data on these proteins. A full understanding of sHSP evolution could be achieved via genome-wide analysis in the future (Dou et al. 2017). Furthermore, apart from the conserved core domain existing in the members of the sHSP superfamily, which consists of the characteristic α -crystalline domain and its flanking sequences, variations in the N- and C-terminal regions contribute to the variability of the interspersed sequences of the protein and result in functional and structural differentiation of sHSPs (Kriehuber et al. 2010). In the present study, all *GpsHSPs* were predicted to contain the characteristic α -crystalline domains consisting of six β -strands. The further construction of a phylogenetic tree showed that all six *GpsHSPs* fell within homologous clades in terms of similar molecular weights, suggesting that the evolution of sHSPs is somewhat complex.

It has been shown that the expression of insect HSPs varies between developmental stages (Wang et al. 2017, Yang et al. 2019). The expression levels of *PxHSP19.7* in *P. xylostella* (Sonoda et al. 2006) and *LsHSP19.5*, *LsHSP20.8*, and *LsHSP21.7* in *Liriomyza sativa* Blanchard (Diptera: Agromyzidae) (Huang et al. 2009) is low at both larval and adult stages but highest at the pupal stage. Yet, *SIHSP20.4* and *SIHSP20.8* are expressed at a similar level across life stages in *S. litura* (Shen et al. 2011). In the present study, all sHSPs were expressed at higher levels in diapaused, prepupal, or pupal stages but lower at larval or adult stages. A previous study showed that the expression of HSP genes in *S. crassipalpis* increased upon entry into diapause (Yocum et al. 1998). In *Choristoneura fumiferana* Linnaeus (Lepidoptera: Tortricidae), most of the sHSP genes are involved in the diapause process, and they may play multiple and vital roles in different phases of this physiological process (Quan et al. 2018). Remarkably, the upregulation of sHSPs in diapaused insects is often explained in terms of increased cold tolerance and suppression of the development (Rinehart et al. 2007, King and Macrae 2015, Quan et al. 2018). So, we assume that the expression of sHSPs in diapaused larvae may be associated with overwintering in *G. pyloalis*. Alternatively, it may be correlated with metamorphosis as all *GpsHSPs* were highly expressed at prepupal stage when

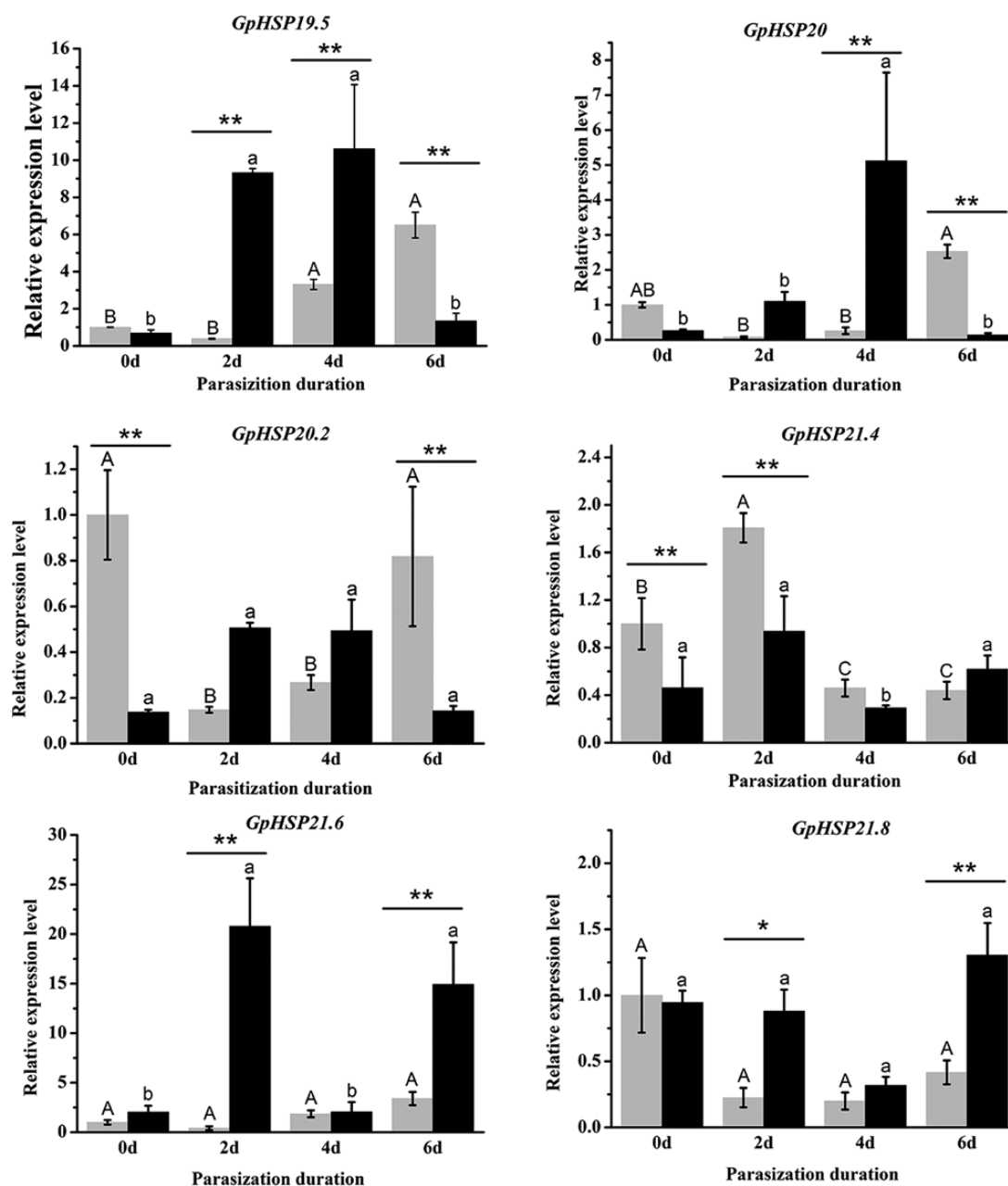


Fig. 6. Expression of *GpsHSPs* after parasitization by endoparasitoid, *A. confusum* for different periods. The gray bar represents the healthy groups and the black bar represents the parasitization groups. Different upper and lower case letters indicate significant differences between healthy and parasitization groups within the different days ($P < 0.05$). Single asterisks ($P < 0.05$) and double asterisks ($P < 0.01$) indicate significant differences between the healthy and parasitization groups within the same day.

the larvae terminated feeding and prepared for metamorphosis. It has been showed that sHSPs, as important chaperones, are markedly up-regulated during larval-pupal transformation, suggesting their essential roles in metamorphosis (Huang et al. 2009).

A crucial determinant of insect abundance and distribution is temperature, which at extremes can cause adaptive induction of HSP gene expression required for survival (King and Macrae 2015, Dou et al. 2017). A number of lepidopteran pests have been investigated to elucidate the relationship between temperature extremes and the expression level of sHSPs (Sun and Macrae 2005, Nakamoto and Vigh 2007, Haslbeck et al. 2019). sHSPs play important roles in insects upon exposure to thermal stresses (Gehring and Wehner 1995, Huang et al. 2009, Shen et al. 2011). In *Harmonia axyridis* Pallas

(Coleoptera: Coccinellidae), the expression level of *HaHSP16.25* and *HaHSP21.00* increased initially and then decreased under the short-term cooling treatment, while the expression level of *HaHSP10.87* and *HaHSP21.56* increased during the entire process (Wang et al. 2017). In *S. inferens*, two sHSPs were significantly upregulated by a heat treatment, and three sHSPs were dramatically upregulated under low-temperature stresses (Sun et al. 2014). In *C. pomonella*, the relative expression level of sHSPs varied with different heat shock durations (Garczynski et al. 2011). In the present study, all sHSPs were highly expressed following exposure to heat stress as an increasing function of the duration of heat stress. The sHSPs in *G. pyralis* reached their expression peaks after exposure to a short heat shock (1 h) but were notably down-regulated after

exposure to a long heat shock (4 h), possibly because these HSPs responded more sensitively at the initial heat shock stage. In contrast, the higher expression level of *GpsHSPs* after exposure to a long heat shock may come as a result of an accumulation of expressed sHSPs with extension of heat shock (Gkouvitsas et al. 2008). Compared with heat shock associated with sHSPs, cold shock with them has received less attention until now. Recently, Tian et al. (2018) revealed that in *M. domestica*, one sHSP was expressed at a high level while another sHSP at a low level, following exposure to a cold treatment. In the present study, none of the *GpsHSPs* was expressed at a higher level following exposure to the cold shock than to normal temperature, yet even one sHSP was expressed at lower level under long cold treatment. Our previous study showed that one *HSP70* in *G. pyloalis* was upregulated following 4-h exposure to a cold shock treatment (unpublished data). Therefore, it is assumed that the expression patterns of sHSPs associated with cold tolerance in insects are complicated or that sHSPs are less active as other HSP classes under such stresses. More studies are required to unravel the mechanism behind the expression patterns of sHSPs in response to temperature extremes in *G. pyloalis*.

Starvation is a fatal stress in the wild and can interfere or destroy energy homeostasis in insects. In *D. melanogaster*, the function of *DmHSP27* is associated with starvation stress (Hao et al. 2007). In starving *M. domestica*, the expression of *MdHSP27* was downregulated as compared with the control (Tian et al. 2018). In a recent study, Xie et al. (2019) found that all adults of *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) died within 18 d of starvation stress after the knockdown of *TcHSP18.3*, the longevity being much shorter than the control. These findings suggested that sHSPs can be sensitively responsive to starvation in insects. In the present study, four *GpsHSPs* were expressed at the highest level after 6 d following food deprivation, implying that these sHSPs could be activated specifically in response to long-term starvation. In contrast, two *GpsHSPs* showed a lower level of expression than the control at 2 or 4 d following the food deprivation, indicating that a shorter period of food starvation may suppress the expression of sHSPs. These sHSPs gene performances in *G. pyloalis* was similar to *PpHSP20* in *P. puparum* (Wang et al. 2012). A possible explanation may lie that *G. pyloalis* cells can absorb nutrition through autophagy, which occurs in the comprehensive reorganization of cellular activities aimed at surviving low nutrient levels (Scott et al. 2004).

Parasitism causes severe stress on the host. Parasitoids inject at parasitization PDVs, venom or VLPs into their host bodies while depositing their eggs to guarantee a suitable environment for their offspring (Moreau and Asgari 2015). Our results of RT-qPCR showed that the expression level of the six *GpsHSPs* were significantly influenced by parasitism, and these effects depended on the developmental duration after parasitization. Similarly, in *P. rapae*, the *PrHSP20* transcript level in pupae was higher at 12, 24, and 48 h after parasitization than in the healthy pupae (Zhu et al. 2013). Parasitism increases the relative expression level of sHSPs in *P. interpunctella*, indicating that sHSPs may be immunoregulatory agents that play important roles in the response to xenobiotic invasion (Shim et al. 2008). In contrast, *GpHSP20.2* and *GpHSP21.4* were downregulated immediately upon parasitization. A previous review showed that parasitism-associated factors can effectively regulate host development to create an environment favoring the development of parasitoids offspring (Beckage and Gelman 2004). Therefore, we assume that these *GpsHSPs* are inhibited following parasitism, especially during the initial stages.

Conclusion

In this study, we identified and characterized six sHSP genes in *G. pyloalis*. The structure of these sHSPs was predicted to contain conserved structural and functional domains and to incorporate typically variable C-terminal extensions. We determined the homology of *GpsHSPs* to sHSPs in other lepidopteran insect. Subsequently, we found that the six sHSPs exhibited unique expression patterns at different developmental stages and in response to various types of stresses. The information gained from the present study could provide a better understanding of the roles sHSPs play in *G. pyloalis* and thus helps in the integrative management of this pest.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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