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Characterization and functional analysis of small heat shock protein gene HSP19.5 in *Bombyx mandarina*

Jiaqi Ding[a,†], Enxi Chen[a,†], Ruonan Li[a,†], Mingjun Lin[a], Heying Qian[a,b], Guodong Zhao[a,b,*]

[a] School of Biotechnology, Jiangsu University of Science and Technology, Jiangsu 212018 China
[b] Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Jiangsu 212100 China

*Corresponding author, e-mail: sdgdzhao@just.edu.cn
† These authors contributed equally to this work.

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ABSTRACT: Heat shock proteins (HSPs) act as molecular chaperones and are widely distributed in all kinds of organisms. In this study, the small heat shock protein HSP19.5 gene was cloned from *Bombyx mandarina* (*Bmm*). Its open reading frame (ORF) is 504 bp in length, encoding 167 amino acids with the HSP20 characteristic domain. The relative molecular weight of the encoding protein was 19.5 kD, and the isoelectric point (pI) is 6.54. Multiple comparisons and phylogenetic analyses showed that the insect small heat shock proteins (sHSPs) are highly conservative. *Bmm*HSP19.5 has the closest relationship with *Bm*HSP19.5 of *Bombyx mori*. The results of real-time quantitative PCR showed that the *BmmHSP19.5* was highly expressed in the testis and ovary of the 5th instar larvae. The expression level of *BmmHSP19.5* gene was increased in all tested tissues under short-term high/low temperature stress. *Bm*NPV can induce the expression of the *BmmHSP19.5* gene in hemolymph and midgut. The expression of *BmmHSP19.5* gene was up-regulated in the midgut of larvae under deltamethrin stress. Larval RNA interference of *BmmHSP19.5* caused 36.1% insect mortality and reduced the oviposition amount by 34.7%; while the whole cocoon weight, cocoon shell rate, and unfertilized rate showed no significant differences. The results suggested that the *BmmHSP19.5* gene might play an important role in the molecular mechanism of response to external stress and development in *B. mandarina*.

KEYWORDS: *Bombyx mandarina*, small heat shock protein gene, expression analysis, external stress, RNA interference

INTRODUCTION

Heat shock proteins (HSPs) are a kind of proteins that organisms preferentially synthesize in response to exogenous adverse stress and, hence, were named for their response to thermal stimulation. Based on their relative molecular mass and amino acid homology, HSPs can be divided into five families: HSP60, HSP70, HSP90, HSP110, and small heat shock protein [1–3]. Small heat shock proteins (sHSPs) are widely distributed among various organisms' ATP-independent molecular chaperones with a highly conserved α-crystallin (ACD) domain [4]. sHSPs participate in the molecular functions of protein synthesis, folding, assembly, and degradation, and as a result affecting the secretion and regulation of cells [5, 6].

sHSPs play an important role in the growth and development of insects and their adaptation to extreme environments. sHSPs involved in the adaptation of *Grapholita molesta* to high temperature [7]. *BmHSP20.1* took part in the immune response to *Bombyx mori* cytoplasmic polyhedrosis virus (*Bm*CPV) [8]. HSP19.8 of *Helicoverpa armigera* responded significantly to the induction of Cry1 Ac insecticidal proteins [9]. Eicosanoids could mediate the induction of Ap-sHSP20.8 in the fat body and midgut of *Antheraea pernyi* [10]. The expression of *sHSP* genes in the pupae of *Glyphodes pyloalis* parasitized by *Aulacocentrum confusum* was affected significantly [11]. In addition, *DmelHSP27* of *Drosophila melanogaster* was significantly increased after UV-A radiation [12].

Bombyx mandarina (*Lepidoptera*) mainly lives on mulberry and Chinese wolfberry trees, and is widely found in most parts of China. *B. mandarina* shares a common ancestor with *B. mori*. It is one of the major pests in summer and autumn mulberry fields. *B. mandarina* can indirectly transmit the disease to *B. mori* through feces and residue. The cross-infection of viruses such as *Bm*NPV and *Bm*CPV with silkworm will bring extreme damage to sericulture [13]. However, there have been few studies on the molecular mechanism of *B. mandarina* response to environmental stress. In the current work, the small heat shock protein gene *BmmHSP19.5* of *B. mandarina* was cloned by molecular biology; its sequence and protein characteristics were analyzed using bioinformatics methods. We studied the spatial expression patterns, transcriptional level changes under external stress and the role of *BmmHSP19.5* gene in growth and development of *B. mandarina* by RNAi technique. This study would provide a theoretical basis for exploring the mechanism of *B. mandarina* and other agricultural pests in responding to environmental stress in the future, including being used as reference for the formulation of scientific mulberry pest control strategies.

MATERIALS AND METHODS

Experimental insects and sample preparation

The larvae of *B. mandarina* used in this research were provided by Sericulture Research Institute of Chinese Academy of Agricultural Sciences and raised on mulberry leaves at 25 °C ± 1 °C and 60–70% relative humidity on a 12:12 light cycle under standard conditions. *Bm*NPV was provided by our laboratory, with a concentration of 1.1×10^7 PIBs/ml. Deltamethrin (25 g/l EC) is produced by Bayer Crop Science Co., Ltd. (Hangzhou, China).

RNA extraction and complementary DNA (cDNA) synthesis

Total RNAs were extracted from individual samples with the EASYspin Plus Tissue/Cell RNA Extraction Kit (Aidlab, Beijing, China). The absorbance ratio at 260–280 nm (A260/A280 ratio) was used to evaluate the purity of RNAs. Agarose gel electrophoresis was used to assess the integrity of RNA. First-strand cDNA for cloning and quantitative real-time qRT-PCR was generated from 1 µl of total RNAs per sample using HiScript® III RT SuperMix for qPCR (+gDNA wiper) Kit (Vazyme, Nanjing, China).

Cloning of *BmmHSP19.5* gene

The primers used for cloning were designed by SnapGene software and synthesized by Sango Biotech (Shanghai) Co., Ltd. according to the coding sequence of the homologous insect *B. mori HSP19.5* gene (GenBank accession number: GQ884144.1) obtained from the National Center for Biotechnology Information (NCBI) to amplify the *BmmHSP19.5* gene. Polymerase chain reaction (PCR) was performed by the following program: 94 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 64 °C for 1 min, and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. The PCR products were subjected to 1% agarose gel electrophoresis and examined. The PCR fragment of interest was purified using AxyPrep DNA Gel Extraction Kit (Corning, Wujiang, China) and sequenced by Sunya Biotech (Hangzhou) Co., Ltd.

Bioinformatics and phylogenetic analysis

Online analysis tools were used for bioinformatics prediction and analysis of *BmmHSP19.5* (Table S1). The sequence was aligned with Clustal W, and the phylogenetic tree was constructed by MEGA-X software using a neighbor-joining (NJ) method with a bootstrap test of 1,000 repetitions.

Quantitative real-time PCR (qRT-PCR) analysis

The primers used for qRT-PCR were designed by Primer Premier 6.0 software and synthesized by Sango Biotech (Shanghai) Co., Ltd. with the primers of the internal reference gene *Actin3* (Table S2). The qRT-PCR reactions were conducted using a Light Cycler 96 Real-time PCR System (Roche Molecular Systems, Inc., Basel, Switzerland) in 20 µl reaction volume containing 10 µl of ChamQ Universal SYBR qPCR Master Mix (Vazyme), 1 µl of cDNA as a template, 0.4 µl of gene-specific primers, and 8.2 µl of nuclease-free water. The procedure was: 95 °C for 30 s, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. All reactions were analyzed using melting curves from 60 °C to 95 °C. All experiments were performed in triplicate. The relative mRNA level was determined using the $2^{-\Delta\Delta CT}$ method.

Spatial expression patterns

For the tissue-specific experiments, the head, midgut, fat body, malpighian tubules, anterior silk gland, middle silk gland, posterior silk gland, hemolymph, epidermis, trachea, testis, and ovary tissues of 5th instar larvae were collected and stored at −80 °C until further analysis.

Temperature treatments

5th instar wild silkworm larvae ($n = 5$) were treated at a high temperature of 40 °C in a constant temperature incubator or a low temperature of 8 °C in the refrigerator for 1 h and 2 h. The midgut, fat body and silk gland tissues were immediately dissected on the ice, washed with PBS buffer and stored at −80 °C. The 25 °C treatment was used as a normal temperature control. Each treatment was repeated 3 times.

Feeding *Bm*NPV

The *Bm*NPV virus solution with a concentration of 1.1×10^7 PIBs/ml was fed to the 5th instar larvae of the wild silkworm by a pipet gun, and the untreated larvae were selected as the control and then fed with normal mulberry leaves. At 12 h, 24 h, 48 h, and 72 h after treatment, the hemolymph tissues were treated on ice, rinsed with PBS buffer, and stored in a refrigerator at −80 °C. Five samples were taken from each treatment and three biological replicates were set.

Deltamethrin stress

The pesticide solution was diluted to 0.02 mg/l, and the mulberry leaves were soaked in the solution for 5 s. After taking out from the solution, the leaves were left air-dried and used to feed the well-developed third-day 5th instar larvae of *B. mandarina*. The larvae were fed with fresh mulberry leaves under the same conditions. At 12 h, 24 h, and 48 h after treatment, the midgut tissues were treated on ice for subsequent experiments. Larvae soaked in distilled water mulberry leaves were used as the control group. Samples of 5 larvae were taken from each treatment, and 3 replicates were set.

RNAi

According to the sequence obtained by sequencing, the siRNA design principle was commissioned by Sango Biotech (Shanghai) Co., Ltd.

The 5th instar wild silkworm larvae with consistent growth were selected and divided into an experimental group and a control group. The larvae were starved for 1 h before injection and placed on ice. The siRNA was injected from the 7–8 abdominal segment of the back of the larvae using microinjection techniques. Each was injected with 1 microgram. After 24 h, the testis and ovary of the experimental group and the control group were dissected and collected. Total RNA was extracted and reverse transcribed into cDNA. The expression of *BmmHSP19.5* after RNAi was detected by qRT-PCR.

The differences in the pupation, cocooning and mothing stages of silkworms in the control group and the experimental group were observed and recorded; and the survival rate, cocoon shell weight, cocoon shell rate, oviposition amount, and fertilization rate were investigated.

RESULTS

Cloning and characterization analysis of *BmmHSP19.5* gene from *B. mandarina*

BmmHSP19.5 gene was cloned from *B. mandarina*. Sequencing showed that the length of ORF was 504 bp, encoding a protein with 167 amino acids. The predicted molecular formula of the *BmmHSP19.5* was $C_{879}H_{1375}N_{245}O_{2551}S_3$, the total atomic number was 2757, the relative molecular weight was 19551.26 Da, the isoelectric point was 6.54, the instability coefficient was 58.83, and the total average hydrophilicity was −0.698. Protein signal peptide prediction revealed no signal peptide. There were 6 serine, 4 threonine and 3 tyrosine phosphorylation sites in *Bmm*HSP19.5 with a N-glycosylation site. An HSP20 domain (65–157 nucleotides) was detected in *Bmm*HSP19.5. Subcellular localization prediction results showed that the gene was located in the mitochondria The prediction of the secondary structure showed that the HSP19.5 contained 41.92% of random coil, 35.33% of α-helix, 14.97% of extended strand, and 7.78% of β-turn.

Multiple alignments and phylogenetic analysis of *Bmm*HSP19.5 from *B. mandarina*

The results of multiple sequence alignment showed that *Bmm*HSP19.5 had the closest relationship with *Bm*HSP19.5 of *Bombyx mori* with 98.20% amino acid sequence identity while the lowest relationship of 78.43% amino acid sequence identity was with *G.molesta* HSP19.6. The sequence identity with *Eogystia hippophaecolus* HSP20, *A. pernyi* HSP19.5, *Melitaea cinxia* HSP12.2, and *Mythimnia separata* HSP19.5 were 85.00%, 82.64%, 80.24%, and 79.75%, respectively. All amino acid sequences contained a typical α-crystallin domain (Fig. 1).

The phylogenetic tree constructed with the *Bmm*HSP19.5 protein and sHSPs from other insects by neighbor-joining method showed that the *Bmm*HSP19.5 was clustered into one branch with *B. mori* (Fig. 2).

Expression levels of the *BmmHSP19.5* gene in different tissues

The *BmmHSP19.5* gene was expressed in all the 12 tissues of 5th instar of *B. mandarina*, and the expression levels of the gene were highest in testis and lowest in hemolymph (Fig. 3).

Expression levels of the *BmmHSP19.5* gene in response to temperature stress

After short-term high (40 °C) and low (8 °C) temperature stress treatments, the relative expression levels of the *BmmHSP19.5* gene in all three tissues of the 5th instar larvae of wild silkworm were higher than the control (25 °C). In the midgut, both high and low temperatures could induce the expression of *BmmHSP19.5*, and the induction effect of low temperature was significant. In the fat body, the expression of *BmmHSP19.5* was up-regulated significantly at both 1 h and 2 h after high and low-temperature treatments. The expression pattern of the silk gland was similar to that of the midgut. Both high and low stresses could introduce the expression of *BmmHSP19.5* (Fig. 4).

Expression levels of the *BmmHSP19.5* gene in response to BmNPV

The results showed that the expression of *BmmHSP19.5* in hemolymph increased significantly at 24 h, 48 h, 72 h, and 96 h after feeding the 5th instar larvae with *Bm*NPV and reached the peak at 72 h after treatment. For the midgut, *Bm*NPV could also increase the expression levels of *BmmHSP19.5* and showed very significant response after 72 h and 96 h of treatment (Fig. 5).

Expression levels of the *BmmHSP19.5* gene in response to deltamethrin stress

Expression levels of *BmmHSP19.5* in midgut under deltamethrin stress were shown in Fig. 6. The levels were up-regulated significantly and increased with the prolongation of treatment time.

RNAi phenotypes of *BmmHSP19.5*

To investigate the effect of *BmmHSP19.5* on the growth and the development in *B. mandarina*, RNAi technology was used to interfere the transcription of the *BmmHSP19.5* gene, and the transcription levels of the gene in the testis and the ovary of wild silkworms were detected by real-time quantitative PCR. Fig. 7 shows that the transcription levels in the testis and the ovary compared with the control group decreased by 36.5% and 66.5%, respectively, indicating that the interference effect of *BmmHSP19.5* gene was significant.

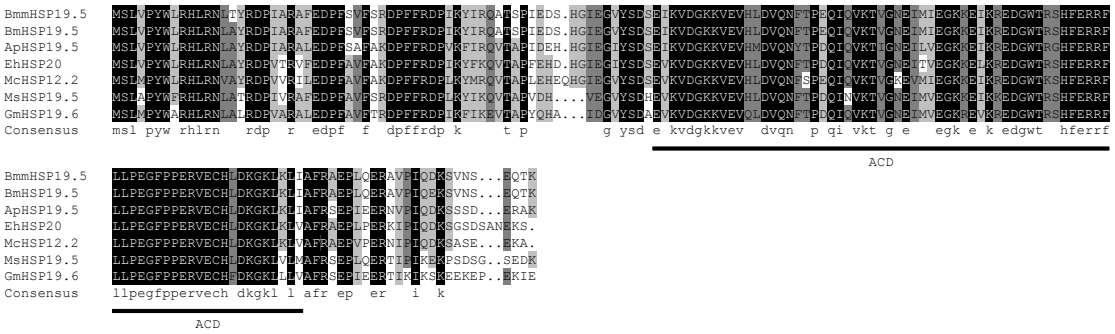

Fig. 1 Multiple sequence alignments of *BmmHSP*19.5 in *B. mandarina* and sHSPs of other species. Origin species of sHSPs and their GenBank accession numbers: BmmHSP19.5, *B. mandarina*, OP830474; BmHSP19.5, *B. mori*, 001164470.2; ApHSP19.5, *A. pernyi*, APX61064.1; EhHSP20, *E. hippophaecolus*, AYA93247.1; McHSP12.2, *M. cinxia*, XP_045454427.1; MsHSP19.5, *M. separata*, ATN45242.1; GmHSP19.6, *G. molesta*, AKS40076.1. The single underline represents the Alpha-crystallin domain (ACD).

Table 1 Effects of siRNA of *BmmHSP19.5* on phenotypic traits of *B. mandarina BmmHSP19.5*.

Treatment	Survival rate (%)	Average cocoon weight (g)	Average cocoon shell rate (%)	Average oviposition amount	Average fertilization rate (%)
Control	92.4	0.572	22.9	409 ± 17	97.57 ± 2.28
siRNA	63.9**	0.536	23.2	267 ± 12**	97.98 ± 1.82

The results are shown as mean ± S.E. (*t*-test). Asterisks denote significant differences between the treated and the control groups, as indicated by * $p < 0.05$, and ** $p < 0.01$.

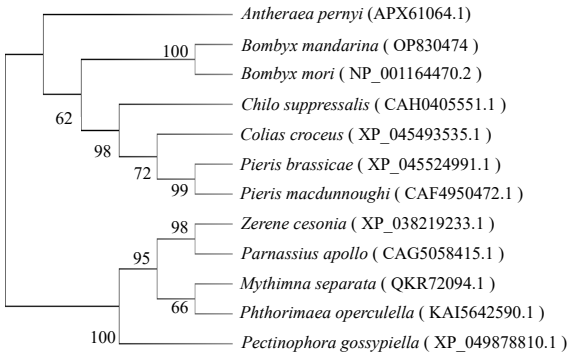

Fig. 2 Phylogenetic tree of sHSP proteins of *B. mandarina* and other species based on the amino acid sequence constructed by the neighbor-joining method (1,000 repetitions). The phylogenetic tree was generated using the MEGA-X software by the neighbor-joining method. Numbers on the branches are the bootstrap value obtained from 1,000 replicates.

Injection of siRNA of *BmmHSP19.5* caused a total mortality of 36.1% during larval stage, and the oviposition amount was reduced by 34.7%. We found no marked impact on the average cocoon weight, average cocoon shell rate, and average fertilization rate after the siRNA injection (Table 1).

DISCUSSION

In this study, the *BmmHSP19.5*-encoding gene was cloned from *B. mandarina*. Sequence analysis showed that the gene had a typical α-crystallin domain. Its secondary structure contained 7.78% of β-turn, which contributed to the antiparallel structure of *BmmHSP19.5* protein in turn to form a dimer [14]. *BmmHSP19.5* contains 13 phosphorylation active sites, and it could easily resist external stress by phosphorylation modification [15, 16]. The conserved domain prediction showed that the encoded protein had a typical α-crystallin domain. The results of amino acid homology and phylogenetic analysis showed the highest homology with *Bm*HSP19.5 of *B. mandarina* and closely related to *B. mori*, which suggested that sHSPs proteins were relatively conserved during the evolution.

Significant differences were found in the relative expression of *BmmHSP19.5* gene in different tissues (Fig. 3). The results were consistent with the relative expression of *sHSP* gene from *Spodoptera frugiperda*, indicating that the expression of *sHSP* genes in *B. mandarina* had tissue specificity [17]. The expression levels of *BmmHSP19.5* in the testis and the ovary were higher than in other tissues, which could imply that sHSPs proteins of *B. mandarina* might be involved in the development of sexual glands [18]. However, the proteins' specific biological function in sexual glands remains to be further studied and explored.

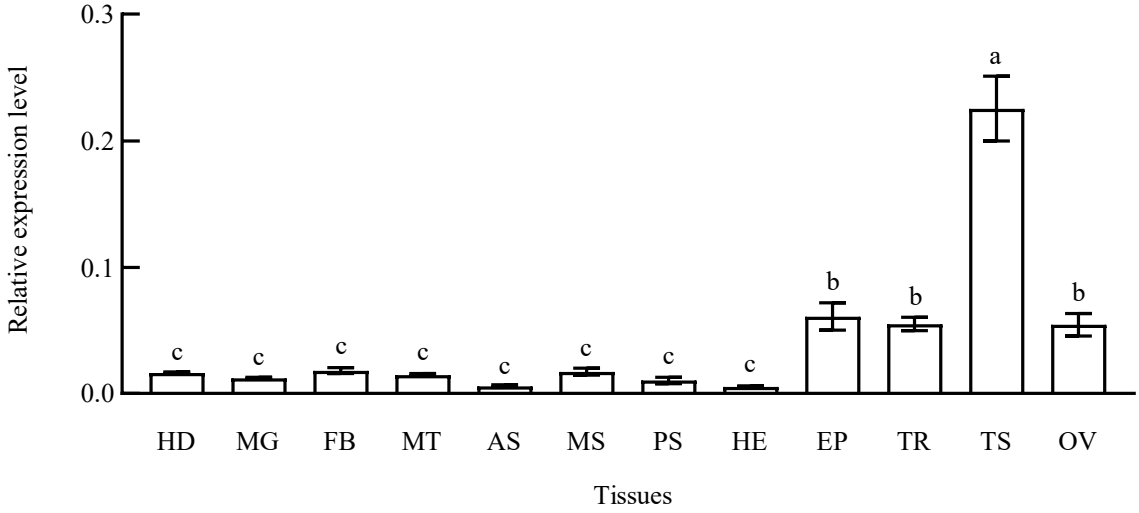

Fig. 3 Relative expression levels of *BmmHSP19.5* in different tissues of the 5th instar larvae of *B. mandarina*. The results are shown as the means ± S.E. Abscissa represents different tissues and ordinate represents relative expression levels. HD, Head; MG, Midgut; FB, Fat body; MT, Malpighian tubules; AS, Anterior silk gland; MS, Middle silk gland; PS, Posterior silk gland; HE, Hemolymph; EP, Epidermis; TR, Trachea; TS, Testis; OV, Ovary.

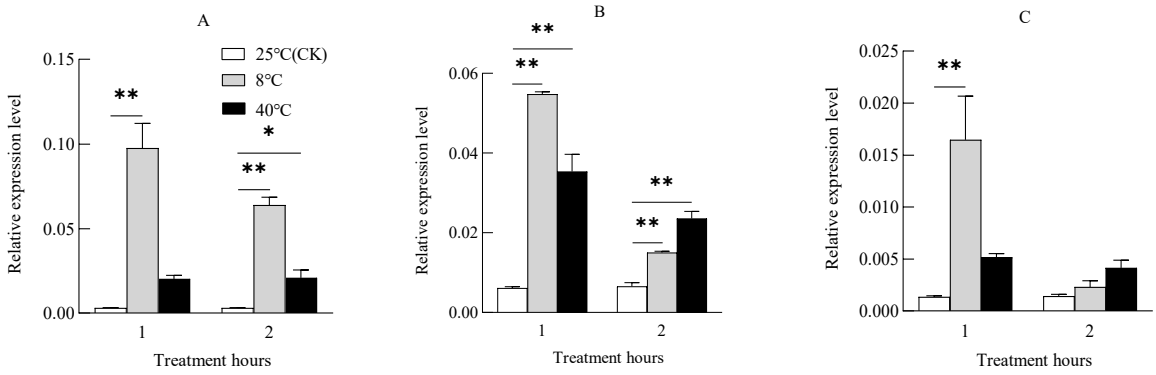

Fig. 4 Relative expression levels of *BmmHSP19.5* in different tissues of 5th instar larvae of *B. mandarina* after treated with high and low temperature stresses. A, Midgut; B, Fat body; C, Silk gland. Abscissa represents different treatment hours; ordinate represents relative expression levels. The results are shown as the mean ± S.E. (*t*-test). Asterisks denote significant differences between the treated and the control groups, as indicated by * $p < 0.05$, and ** $p < 0.01$. The same for Fig. 5, Fig. 6 and Fig. 7.

With the global climate changes, the temperature of natural conditions could become more and more extreme [19]. In 2020, the Chinese government introduced carbon neutrality and emission peak to the construction of ecological civilization in order to solve the problem. Climate warming has a huge impact on the plant-pest-natural enemy interaction system [20]. As ectotherms, insects are very sensitive to changes in temperature. Not only the response ability of insects to temperature stress affects the cellular structure, neogenesis metabolism, physiological process, growth and development rate [21], it also determines the distribution and the density of insect population [22]. Corresponding measures must be taken during the whole growth and development stages of insects for these extreme temperatures, and the regulation of heat shock protein is an important means [23]. This study mainly focused on the effects of high and low temperature stresses on the expression levels of *BmmHSP19.5* gene in the 5th instar larvae of *B. mandarina* (Fig. 4). The results showed that the sHSP protein of *B. mandarina* could actively respond to temperature stress in all tissues [24, 25]. Although both low and high temperature stresses could induce *BmmHSP19.5*, the

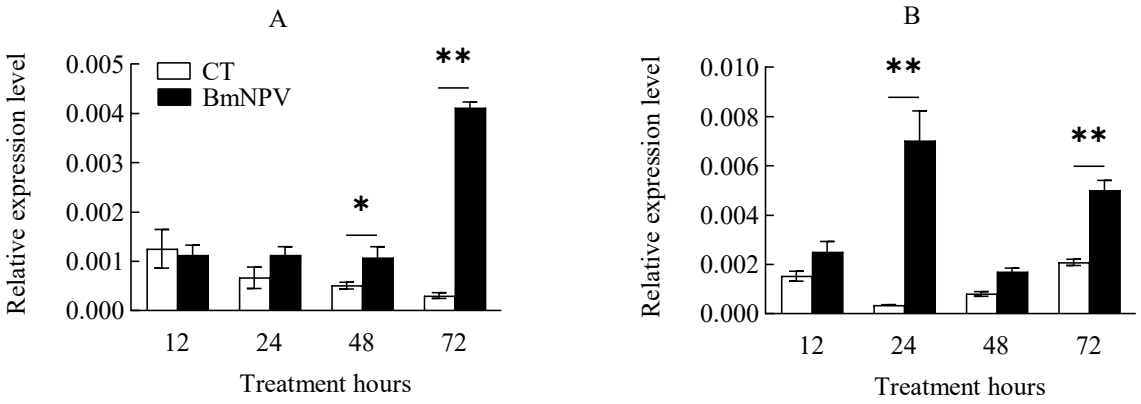

Fig. 5 Relative expression levels of *BmmHSP19.5* in the hemolymph (A) and the midgut (B) of 5th instar larvae of *B. mandarina* at different time after feeding with *Bm*NPV (1.1×10^7 PIBs/ml). CT, Control group without *Bm*NPV feeding; *Bm*NPV, *Bombyx mori* nucleopolyhedro viruses.

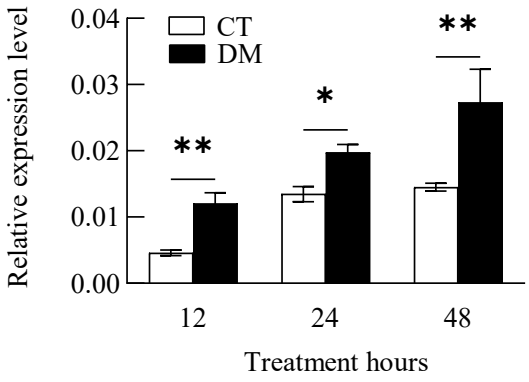

Fig. 6 Relative expression levels of *BmmHSP19.5* in the midgut of 5th instar larvae of *B. mandarina* at different time after treated with deltamethrin (0.02 mg/l). CT, Control group fed with mulberry leaves soaked in distilled water; DM, Deltamethrin.

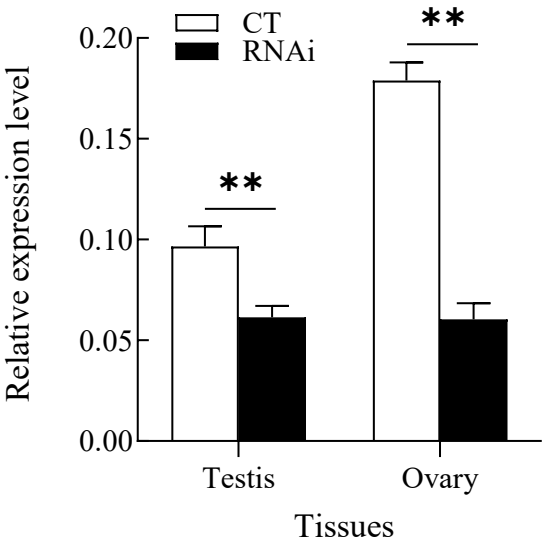

Fig. 7 Expression levels of *BmmHSP19.5* gene in the testis and the ovary of *B. mandarina* after treated with RNAi.

expression level of *BmmHSP19.5* gene under the high temperature was higher than the low temperature, which was the same as the results of sHSPs genes in *Liriomyza trifolii* and *Chilo suppressalis* [26, 27].

There have been many studies on the changes of gene expression levels after feeding baculovirus of insects. The transcription level of *Ap-sHSP25.4* in *A. pernyi* increased 87 times after NPV injection [28]. Liu also found that *Ap-sHSP21* was involved in the immune response of *A. pernyi* to NPV [29]. The results of our study showed that the expression of *BmmHSP19.5* in the hemolymph of wild silkworm was up-regulated after *Bm*NPV infection, and reached the highest value at 72 h. It was, hence, speculated that the virus required time for the proliferation; and, after reaching a certain number, the induction effect of the gene could be reflected. At 24 h and 48 h after treatment, the expression of *BmmHSP19.5* in *B. mandarina* increased sharply, which could be due to the fact that the virus was fed orally, so the response in the midgut was more rapid. Subsequently, its expression showed high expression at 72 h and 96 h, which was the same as that of *BmHSP19.5* observed in silkworm and *in vitro Bm*E cells [30]. Studies have shown that *Bm*CPV could also induce the expression of *BmHSP23.7* in the midgut [31]. All these results suggested that *sHSP* genes might play an important role in the resistance of insects to infestation by pathogenic microorganisms.

Both organophosphorus compounds and oxime compounds can induce the expression of sHSP proteins [32]. The expression level of *AsHSP21.7* gene in *Anopheles sinensis* increased under pyrethroid stress [33]. The results of this study showed that deltamethrin could induce the expression of *BmmHSP19.5* gene in the midgut, which was also consistent with the results observed by Lertkiatmongkol et al in *Aedes aegypti* [34].

There has been a long history of using RNAi technology in the studies of gene function of insects [35, 36]. In this experiment, substantial mortality was observed in the pupation period when the expression level of *BmmHSP19.5* was significantly down-regulated, resulting in the knockdown of *BmmHSP19.5* and consequently causing the larvae to become weaker and eventually die because of the interfere with ecdysis. The mortality was reflected in the survival rate, which was aligned with the results of *HSP21.8b* in *Tribolium castaneum* [37]. The silencing of sHSPs in *Agrilus planipennis* [38] and *Dendroctonus frontalis* [39] could also lead to high mortality. Previous studies demonstrated that heat shock protein genes affected the fecundity of *T. castaneum* [40] and *Agasicles hygrophila* [41]. Suppression of *BmmHSP19.5* had an impact on fecundity, causing significant declines of oviposition amount [42]. However, no significant differences were found on the cocoon shell weight, cocoon shell rate and fertilization rate. The results showed that *Bmm*HSP19.5 had an important effect on the growth and the development of wild silkworms.

The results of this study only indirectly indicate that *Bmm*HSP19.5 is involved in the development and the response of wild silkworms to adverse exogenous stress, but its regulatory mechanism remains to be further studied.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.085.

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Appendix A. Supplementary data

Table S1 Bioinformatics online analysis tools.

Name	Website
NCBI BlastP	https://blast.ncbi.nlm.nih.gov/Blast.cgi
ProtParam tool	https://web.expasy.org/protparam/
SOPMA	https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html
ProtScale	https://web.expasy.org/protscale/
NetPhos 3.1 server	https://services.healthtech.dtu.dk/service.php?NetPhos-3.1
NetNGlyc-1.0 server	https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0
SignalP-6.0 Server	https://services.healthtech.dtu.dk/service.php?SignalP-6.0
ScanProsite tool	https://prosite.expasy.org/scanprosite/
ProtComp 9.0	http://linux1.softberry.com/all.htm

Table S2 Primers used in this study.

Application of primers	Gene	Forward primer ($5'-3'$)	Reverse primer ($3'-5'$)
Cloning	*BmmHSP19.5*	CTAGCTAGCATGTCGCTTGTACCGTA	CGGGGTACCTCACTTTGTTTGCTCACT
qRT-PCR	*BmmHSP19.5*	TCAGGCAAGCCACCTCACCAA	TCCTCGTCCAGCCGTCTTCTCT
	Actin3	CGGCTACTCGTTCACTACC	CCGTCGGGAAGTTCGTAAG