Five small heat shock protein genes from *Chilo suppressalis*: characteristics of gene, genomic organization, structural analysis, and transcription profiles

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Abstract Small heat shock proteins (sHSPs) are the most diverse but also the most poorly known family of molecular chaperones, and they play essential roles in various biological processes. The striped stem borer, *Chilo suppressalis* (Insecta: Lepidoptera: Pyralidae), is one of the most serious pests of rice, causing extensive damage and yield loss. In this study, we isolated and characterized five members of the sHSPs family—*Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b*—from *C. suppressalis*. The cDNAs of these genes encoded proteins of 177, 187, 191, 191, and 191 amino acids with isoelectric points of 7.0, 5.6, 6.1, 6.3, and 6.3, respectively. While *Cshsp19.8*, *Cshsp21.5*, and *Cshsp21.7b* had no introns, *Cshsp21.4* and *Cshsp21.7a* contained one and two introns, respectively. Structural analysis indicated that all five *Cshsps* possessed conserved arginine and a V/IXI/V motif, which is related to hydrophobic characteristics of sHSPs. The five heat shock proteins can be classified into two main groups: an orthologous type (*Cshsp21.4* and *Cshsp21.7a*) and a species-specific type (*Cshsp19.8*, *Cshsp21.5*, and *Cshsp21.7b*). Real-time quantitative PCR analyses revealed that *Cshsp19.8*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b* all exhibited their highest expression levels within Malpighian tubules or the hindgut, while such levels were found in the head for *Cshsp21.4*. The expression of *Cshsps* at different developmental stages revealed that the mRNA levels of *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, and

Cshsp21.7b peaked in adults, whereas the highest level of *Cshsp21.7a* was observed in first instar larvae. *Cshsp19.8* and *Cshsp21.7b* were both upregulated dramatically by heat and cold, and *Cshsp21.5* could be induced by cold stress. Neither *Cshsp21.4* nor *Cshsp21.7a* responded to heat or cold. These results demonstrated that different *Cshsps* play distinctive roles in the regulation of the physiological activities in *C. suppressalis*.

Keywords sHSPs · *Chilo suppressalis* · Genome · Structure · Expression · Temperature

Introduction

Previous studies suggest that small heat shock proteins (sHSPs) are abundant and ubiquitous in almost all organisms (Kim et al. 1998; Aebermann and Waters 2008; Waters et al. 2008). Compared to other types of HSPs, sHSPs exhibit a greater variation in sequence, structure, size, and function (de Jong et al. 1998; Franck et al. 2004). They are a superfamily of proteins that contain an α -crystallin domain with molecular weights of 12–43 kDa, depending on the variable N- and C-terminal extensions (Stromer et al. 2004), and exist under physiological conditions as large oligomers of up to 50 subunits and 1.2 MDa in mass (Gusev et al. 2002; Horwitz 1992). Structurally, sHSPs remain poorly understood, mainly because members of this protein family are extremely dynamic and heterogeneous (Horwitz 2003; Haslbeck et al. 2005). Some studies have found sHSP chaperone activity to be involved with rearrangement of the sHSP oligomer (Giese and Vierling 2002; Morris et al. 2008), and surface-exposed hydrophobic sites of sHSP oligomer on the sHSPs bound to partially unfolded proteins (van Montfort et al. 2001). They are able to bind to denatured proteins and prevent irreversible protein aggregation during stresses, such as extreme temperatures, oxidation, and heavy

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metals (van Montfort et al. 2002; Haslbeck et al. 2005; Reineke 2005). In addition to stress response, sHSPs have been suggested to be involved in the apoptosis and autophagy, actin and intermediate filament dynamics, organization of the cytoskeleton, and membrane fluidity (Haslbeck 2002; Quinlan 2002; Sun and MacRae 2005; Tsvetkova et al. 2002). They are also therapeutic targets and biomarkers for many diseases (Arrigo et al. 2007). In insects, they are assumed to play an important role in the heat stress, metamorphosis, normal development, diapause, and immune responses (Gu et al. 2012; Hayward et al. 2005; Huang and Kang 2007; Jakob and Buchner 1994; Rinehart et al. 2007; Song et al. 2006), but their functional roles remain unclear. Different organisms have different numbers of sHSPs, ranging from only one up to 19 (Haslbeck et al. 2005). Insects are one of the most successful organisms, having evolved a strong ability to adapt to various habitats. Sixteen *shsps* in *Bombyx mori*, 11 in *Drosophila melanogaster*, 10 in *Apis mellifera*, and 7 in *Anopheles gambiae* have been identified (Li et al. 2009). However, studies of sHSPs in other insects are not as extensive and penetrating as in model insects.

Temperature is one of the most important factors determining the distribution and abundance of insects (Bale et al. 2002). When exposed to extreme temperatures, insects may respond in different ways, adopting behaviors to avoid or escape extreme temperatures or altering their physiology to better withstand them (Hoffmann and Parsons 1991). One important behavior is to synthesize a small set of proteins called heat shock proteins (HSPs) that participate in the refolding and relocalization of proteins damaged by such stresses (Sørensen et al. 2003).

The striped stem borer, *Chilo suppressalis* (Walker) (Insecta: Lepidoptera: Pyralidae), is one of the most economically damaging insect pests of rice in Asia, northern Africa, and southern Europe. In the district of Yangzhou (32.39°N, 119.42°E), Jiangsu Province, China, this insect now completes two full and a partial third generation each year, undergoing diapause in the larval stage (Lu et al. 2012). Moths of the overwintering generation lay egg masses on rice leaves and larvae, which undergo six instars and bore into rice leaf sheaths and stems to feed. Recently, increasing damage and yield loss has been caused by *C. suppressalis*. Three large and one small *hsp*s genes in *C. suppressalis* have been identified, and it has been suggested that they could be related to temperature stress (Cui et al. 2010a, b; Sonoda et al. 2006a, b). However, whether other sHSPs exist in *C. suppressalis* and what differences they display in genome aspect, structure, and function are as yet unknown.

Here, we identified five sHSP genes in *C. suppressalis* and described their genomic and structural characteristics. In addition, we compared the differential abundance of five *shsps* among tissues or organs and developmental stages. In

order to evaluate the potential relationships between regulation of the five *shsps* and temperature, we also studied *shsps* expression under various temperatures.

Materials and methods

Insects

The population of *C. suppressalis* used in this study was collected from a suburb of Yangzhou (32.39°N, 119.42°E). The rice stem borers were reared in an environmental chamber at 28±1 °C, a 16:8 (light/dark) photoperiod, and a relative humidity of 70±5 % as per Shang et al. (1979).

Cloning and RACEs

Total RNA was extracted using the SV Total RNA isolation system (Promega Z3100) combined with DNase digestion to eliminate DNA contamination. Total cDNA was synthesized by oligo(dT)₁₈ primer (TaKaRa). The full-length cDNAs of the small heat shock protein genes were determined using 5'- and 3'-RACE (SMART RACE, Clontech). The primers used are shown in Supplemental Data 1. The full length sequences of genes were confirmed by RACE 5' cDNA.

Characterization of the genomes

The genomic DNA of *C. suppressalis* was extracted by Axyprep™ multisource Genomic DNA Kit (Axygen, USA). According to the full-length cDNA of the five small heat shock protein genes, the pairs of specific primers (Supplemental Data 1) were designed to amplify *Csshsp*s genomic fragments. The products were purified using a gel extraction kit (Axygen, USA), cloned into PGEM-T Easy vector (Promega, USA), and transformed into competent *Escherichia coli* DH5α cells for sequencing.

Sample preparation

For the first experiment (focused on eight tissues within one life stage), randomly selected fifth instars were used. The larvae used in the experiments were all fifth instars of similar body size and were assigned randomly to each experimental group. Each experimental group contained ten larvae, and each experiment was repeated three times. Larvae were anesthetized on ice before dissection. Heads (HE), epidermis (EP), fat body (FB), foregut (FG), midgut (MG), hindgut (HG), Malpighian tubules (MT), and hemocytes (HC) were collected from larvae and rinsed with a 0.9 % sodium chloride solution. For the second experiment (focused on different life stages and sexes), relevant stages (egg masses; the first, second, third, fourth, fifth, and sixth

instar larvae; pupae [male and female]; and 1-day-old adults [male and female]) were randomly selected for the experiment. The samples were frozen immediately in liquid nitrogen and stored at -70°C until the experiment.

Temperature stress

Larvae were confined individually in glass tubes, and experimental groups of ten were exposed to a given temperature ($-11, -8, -6, -3, 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39$, and 42°C) for 2 h in a constant temperature subzero incubator (DC-3010, Jiangnan equipment). The larvae were recovered at $27\pm 1^{\circ}\text{C}$ for 2 h, after which surviving larvae were frozen in liquid nitrogen and stored at -70°C . The larvae set at $27\pm 1^{\circ}\text{C}$ were regarded as control. Each treatment included more than three surviving larvae.

Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted using the SV Total RNA isolation system (Promega Z3100), followed by DNase treatment to eliminate DNA contamination. The integrity of the RNA in all samples was verified by comparing the ribosomal RNA bands in ethidium bromide-stained gels. RNA sample purity was estimated using spectrophotometric measurements at 260 and 280 nm (Eppendorf BioPhotometer plus). Real-time PCR reactions were performed in a 20- μl total reaction volume comprised of 10 μl of $2\times$ SYBR[®] Premix EX Taq[™] (TaKaRa, Dalian, China) master mix, 0.8 μl of each gene-specific primer (Supplemental Data 1), 0.4 μl of Rox reference dye, and 2 μl of cDNA templates. Reactions were carried out on a CFX-96 real-time PCR system (Bio-Rad). The efficiencies of the target and reference genes were similar (Bustin et al. 2009; Nolan et al. 2006). The quantity of *Csshsp* mRNA was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method and normalized to the abundance of the 18S rRNA gene. Hemocytes, eggs, and larvae at $27\pm 1^{\circ}\text{C}$ were described respectively as a control (Schmittgen and Livak 2008; <http://www.ambion.com/techlib/basics/rtpcr/index.html>). Following qPCR, the homogeneity of the PCR products was confirmed by melting curve analysis, which was read each 5 s per 0.5°C increment from 65 to 95°C . Every treatment included three replicates, and each reaction was run in triplicate.

Bioinformatic analysis

The open reading frames (ORFs) were identified with the aid of the ORF Finder software (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The deduced amino acid sequences were aligned using ClustalX software. Sequence analysis tools of the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics, including Translate, Compute pI/MW, and

Blast, were used to analyze the deduced small heat shock protein sequences. Amino acid sequences were used to estimate phylogeny using neighbor-joining, minimum evolution, maximum likelihood, and maximum parsimony methods. Phylogenetic trees were constructed with 1,000 bootstrap replicates using MEGA version 5.0 (Tamura et al. 2011).

Computational molecular modeling

Homology models were generated using Protein Homology/analogy recognition engine V 2.0 (<http://www.sbg.bio.ic.ac.uk/~phyre2/html>) (Kelley and Sternberg 2009). The small heat shock protein sequences of *C. suppressalis* were aligned using Phyre2, and the best model of human αB -crystallin V* structure (PDB ID: 2YGD) was used for modeling analyses (Braun et al. 2011). The Chimera tool was used to visualize the 3D coordinates for the atoms of the model (Pettersen et al. 2004).

Statistical analysis

Homogeneity of variances among different groups was evaluated by Levene's test. The significance of differences between treatments was identified with either an LSD test (homogeneity of variances) or Dunnett's *C* test (nonhomogeneous) for multiple comparisons. The data were analyzed using SPSS16.0 software (Pallant 2005) and denoted as means $\pm$ SE (standard error).

Results

Isolation, cloning, sequencing, and structure of five *shsps* from *C. suppressalis* larvae

In this study, five small heat shock protein genes—*Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b*—were obtained from *C. suppressalis* (GenBank accession nos. JX491641, JX491642, KC710018, JX491640, and KC710019, respectively). They possessed the ORFs of 177, 187, 191, 191, and 191 amino acids with an isoelectric point of 7.0, 5.6, 6.1, 6.3, and 6.3, respectively. All five *shsps* contained a conserved α -crystallin domain, a typical characteristic of *shsps* (Fig. 1a). *Cshsp21.5* and *Cshsp21.7b* had the highest sequence similarity, while the other three *shsps* shared only low levels of similarity (Fig. 1b). To investigate the structural characteristics of these five small heat shock proteins from *C. suppressalis*, we generated their homology models with Phyre using human αB -crystallin V* structure (PDB ID: 2YGD) as a template (Braun et al. 2011). The structures of all five *Csshsp* models exhibited low homology, compared to that of αB -crystallin (Fig. 2). Alignment analyses demonstrated that CsHSP19.8,

Fig. 1 Alignment of the deduced amino acid sequences of five *shsps* from *Chilo suppressalis*. **a** The horizontal line indicates the conserved α -crystallin domain bordered by variable amino- and carboxy-terminal regions. (–) no amino acid residue; (*) identical residues; (:) strong positive residues; (.) weaker positive residues. **b** Sequence similarities of *C. suppressalis*

Fig. 2 Structure analyses of five small heat shock proteins from *C. suppressalis*. **a** Homology modeling analyses of the CsHSP19.8 (yellow), CsHSP21.4 (blue), CsHSP21.5 (cyan), CsHSP21.7a (purple), and CsHSP21.7b (green) with human α B-crystallin V* structure (PDB ID: 2YGD) (white) as template. **b–e** The α -helix shown in yellow and β -strand shown in orange. The conserved Arg is shown in blue sphere and the V/IXI/V motif is shown in cyan stick and ball. **b** CsHSP19.8, **c** CsHSP21.4, **d** CsHSP21.5, **e** CsHSP21.7a, and **f** CsHSP21.7b

CsHSP21.4, CsHSP21.5, CsHSP21.7a, and CsHSP21.7b possessed 37, 25, 36, 41, and 35 % identity, respectively. All five CshHSPs also consisted of α -helices and β -strands. For example, CsHSP21.5 only included seven β -strands, which differed from the other CshHSPs, which possessed nine β -strands (Fig. 2). CsHSP19.8, CsHSP21.5, and CsHSP21.7b contained five α -helices, while CsHSP21.4 contained six and CsHSP21.7a contained four. The conserved Arg was found in the $\beta 6 + \beta 7$ of all five CshHSPs (Arg115, Arg143, Arg119, Arg121, and Arg123, respectively). The carboxy-terminal extensions of all five CshHSPs contained a V/IXI/V motif, and CsHSP19.8, CsHSP21.5, CsHSP21.7a, and CsHSP21.7b contained a V/P/I motif (Val156/Pro157/Ile158, Val160/Pro161/Ile162, Val161/Pro162/Ile163, and Val164/Pro165/Ile166). Meanwhile, CsHSP21.4 contained an I/P/I motif (Ile182/Pro183/Ile184) (Fig. 2).

Phylogenetic analysis

The deduced amino acid sequences of the five *shsps* displayed a high degree of homology with those of other insects reported so far (Table 1). We used CLUSTALX and MEGA 5.0 phylogenetic analysis to compare *Csshsp*s with other *shsps* and crystallins through the neighbor-joining, minimum evolution, maximum likelihood, and maximum parsimony methods, and four phylogenetic trees exhibited a consistent trend. As shown in Fig. 3, the tree was separated into three clusters. *Cshsp19.8*, *Cshsp21.5*, and *Cshsp21.7b*, all with high sequence similarity to each other, fell into a well-supported cluster. Meanwhile, *Cshsp21.4* belonged to the group of the lepidopteran *hsp21.4*, and *Cshsp21.7a* and *l(2)efl* proteins were placed together (Fig. 3). For *Cshsp21.5*, the highest amino acid identity of 75 % was observed with the α -crystallin cognate protein gene of *Plodia interpunctella*; *Cshsp21.4* had 98 % identity with the *hsp21.4* of *Spodoptera litura*.

Genomic structure of *C. suppressalis*

The genomic DNA sequences of the five *Csshsp*s were identified (GenBank accession nos. KC210020, KC210021, KC210022, KC210023, and KC210024, respectively). The positions and sizes of the introns were noted by aligning the cDNA sequences of the five *Csshsp*s with the genomic DNA sequences. The nucleotide sequences at the splice junctions are consistent with the canonical GT-AG rule. We found that *Cshsp19.8*, *Cshsp21.5*, and *Cshsp21.7b* had no introns, similar to the homology sequences of the other insects (Figs. 3 and 4), while *Cshsp21.4* and *Cshsp21.7a* had one and two introns, respectively (Fig. 4). Although *Cshsp21.4* and *Bmhsp21.4* (*B. mori*) had the high identity mentioned above, they had different numbers and sizes of introns. *Cshsp21.4* had one intron with 746 bp, while *Bmhsp21.4* had two introns with 720 and 5531 bp. And while we found only one intron

position in the alignment of the orthologous *Cshsp21.7a* sequences, the position of *Bmhsp1* is at 242–3219 and that for *Dpl(2)efl* of *D. melanogaster* was at 176–239. However, position of the two *Cshsp21.7a* introns was at 177–243 and 314–786, respectively (Figs. 3 and 4).

Tissue (organ) distribution of mRNA expression in *C. suppressalis* larvae

Real-time PCR was used to study the tissue (organ)-specific expressions of *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b* in *C. suppressalis* larvae. All five small heat shock protein genes were commonly expressed in all tissues or organs though they exhibited different expression patterns. For example, the lowest mRNA levels of *Csshsp*s were observed consistently in the hemocytes. The highest expression level of *Cshsp21.7a* was in the heads, which was as high as 5,382.51-fold that of the level in the hemocytes. Also, the epidermis, Malpighian tubules, and hindgut also exhibited abundant levels. Three *Csshsp*s (*Cshsp19.8*, *Cshsp21.5*, and *Cshsp21.7b*) all exhibited the highest expression levels in the Malpighian tubules or hindguts. The mRNA level of *Cshsp19.8* in the midguts was significantly higher than that of the foreguts ($F_{7, 16} = 9.956$, $P < 0.001$). Meanwhile, *Cshsp21.4* was expressed more highly in the heads than in the other tissues (organs) ($F_{7, 16} = 28.227$, $P < 0.001$) (Fig. 5).

Expression of different *shsps* in the developmental stages of *C. suppressalis*

We investigated the mRNA level of five *shsps* through the developmental stage of *C. suppressalis*, including eggs, larvae (first, second, third, fourth, fifth, and sixth instar larvae), pupae (male and female), and adults (male and female). We found that *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b* all showed irregular expression variations throughout the developmental stages ($F_{10, 18} = 29.595$, $P < 0.001$; $F_{10, 21} = 3.487$, $P = 0.008$; $F_{10, 17} = 10.730$, $P < 0.001$; $F_{10, 19} = 17.790$, $P < 0.001$; $F_{10, 20} = 5.239$, $P = 0.001$). We observed the highest expression levels for *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, and *Cshsp21.7b* in adults and in the first instar larvae for *Cshsp21.7a*. Moreover, the first instar larvae contained the lowest levels of *Cshsp19.8*, *Cshsp21.5*, and *Cshsp21.7b*. Interestingly, the expression levels of five *Csshsp*s all showed significant differences between male and female adults, but between male and female pupae, only *Cshsp19.8* had a significant difference ($P < 0.050$) (Fig. 6).

Expression of five *shsps* from *C. suppressalis* in response to temperatures

The relative mRNA levels of the five *shsps* were observed at temperature gradients from -11 to 42 °C. Different *Csshsp*s

Table 1 sHsps sequences collected from the database from which the phylogenetic tree was constructed

No.	Species	Name	Accession number
1	<i>Chilo suppressalis</i>	CsHsp19.8	JX491641
2		CsHsp21.4	JX491642
3		CsHsp21.5	KC710018
4		CsHsp21.7a	JX491640
5		CsHsp21.7b	KC710019
6	<i>Bombyx mori</i>	BmHsp19.9	Q5R1P7
7		BmHsp20.1	Q5R1P6
8		BmHsp20.4	Q9GSB6
9		BmHsp20.8a	Q9GN07
10		BmHsp21.4	Q5R1P5
11		BmHsp22.6	B9VTS8
12		BmHsp23.7	Q5R1P4
13	<i>Danaus plexippus</i>	BmHsp1	F1C929
14		DpHsp19.7	G6CMJ5
15		DpHsp19.8	G6DDW4
16		DpHsp20.4	G6DR93
17		DpHsp21.4	G6D9T7
18		DpHsp23.7	G6D7N2
19		DpHsp1	G6DC87
20	<i>Spodoptera litura</i>	SlHsp19.7	E2F3A1
21		SlHsp20.4	E2F399
22		SlHsp20.8	E2F397
23		SlHsp21.4	E2F396
24		SlHsp22.0	I3QQD5
25	<i>Lonomia obliqua</i>	LoHsp1	Q5MGP0
26		LoHsp2	Q5MGL1
27		LoHsp3	Q5MGN8
28	<i>Choristoneura fumiferana</i>	CfHsp19.8	Q1AMF5
29		CfHsp19.9	Q1AMF6
30		CfHsp21.5	Q1AMF7
31	<i>Cydia pomonella</i>	CpHsp19.8	F1C928
32		CpHsp19.9	F1C929
33		CpHsp22.2	F1C930
34	<i>Papilio polytes</i>	PpHsp20	I4DM98
35		Ppl(2)efl	I4DLZ6
36	<i>Sesamia nonagrioides</i>	SnHsp19.5	B2ZHW3
37		SnHsp20.8	Q2LCS7
38	<i>Mamestra brassicae</i>	MbHsp19.7	Q0KKB1
39		MbHsp20.7	Q0KKB2
40	<i>Heliconius erato</i>	HeHsp21.4	A7KCX9
41	<i>Plodia interpunctella</i>	α -Crystallin cognate protein	O18634
42	<i>Papilio xuthus</i>	Pxl(2)efl	I4DIM9
43	<i>Drosophila melanogaster</i>	DpHspB8	Q9VWG1
44		Dpl(2)efl	P82147
45	<i>Liriomyza sativae</i>	LsHsp21.7	Q1PCB6
46	<i>Liriomyza huidobrensis</i>	LhHsp20	Q1PCB7
47	<i>Homo sapiens</i>	HSPB1	NM_001540
48		α -Crystallin	NM_000394
49		β -Crystallin	NM_001885

names are listed in Table 1

151.72- and 34.39-fold, respectively, after 2 h at 42 °C.

Cshsp21.4 and *Cshsp21.7a* did not respond to the heat and

Fig. 4 Schematic representation of five insect *shsps* genomes. The species names and accession numbers of genomic DNA sequences were listed in Supplemental Data 3. Light gray and black rectangles are used to highlight the exons and introns, respectively

cold (Fig. 7b, d). While *Cshsp21.5* could be induced by cold stress and the mRNA level increased by 10.75-fold after 2 h at -11°C , it did not respond to heat (Fig. 7c).

Discussion

Previous research has suggested that small heat shock proteins participate in diverse physiological processes, one of the best known of which is to increase the heat and cold tolerance of an organism (Liu et al. 2012; Sun and MacRae 2005). In addition to stress response, sHSPs are produced

during normal developmental and diapause stages in insects (Feder and Hofmann 1999; Gkouvitsas et al. 2008; Hayward et al. 2004; Kokolakis et al. 2009; Shirk et al. 1998).

In this study, five new members of the *shsps* family have been identified from the rice stem borer, *C. suppressalis*: *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b*. The cDNA sequences contained open reading frames that encoded 177, 187, 191, 191, and 191 amino acids for the genes, respectively. The predicted amino acid sequences for *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b* shared considerable sequence similarity with *shsps* from other insects and α -crystallin proteins from

Fig. 5 Relative mRNA expression levels of the *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b* genes in different tissues (organs) of *Chilo suppressalis*. Abbreviations: HG hindgut, MG midgut, FG foregut, HC hemocytes, MT Malpighian tubules, EP epidermis, FB fat body, HE head

vertebrate lenses. The *Csshsp*s share low levels of similarity among themselves, and the neighbor-joining dendrogram placed the predicted proteins of the five *Csshsp*s in different clusters. In general, *shsp*s from different insect species were

Fig. 6 Relative mRNA expression levels of *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b* in different developmental stages of *Chilo suppressalis*. Abbreviations: E eggs, L1 first instar larvae, L2 second instar larvae, L3 third instar larvae, L4 fourth instar larvae, L5 fifth instar larvae, L6 sixth instar larvae, P pupae, A adults

phylogenetically closer than those from the same insect. In addition, *Cshsp21.4* belonged to the group of the lepidopteran *hsp21.4*, an orthologous cluster that includes one sHSP from each insect, whereas most insect sHSPs are species specific (Li et al. 2009). This may imply that *Cshsp21.4* might have experienced a specific evolutionary process. The five *Csshsp*s all have a conserved structural organization of α -helices and β -strands (Kim et al. 1998; van Montfort et al. 2001). The highly conserved arginine was found to correlate to surface-exposed hydrophobic sites of sHSPs (Clark et al. 2011; Jehle et al. 2011). The five *Csshsp*s contained Arg115, Arg143, Arg119, Arg121, and Arg123, respectively, in the $\beta 6 + \beta 7$. In the carboxy-terminal extensions, every *Csshsp* had a V/IXI/V motif, which was one of several features that determined the assembly of sHSP dimers into a variety of oligomeric structures (Basha et al. 2012). Two of the five *Csshsp*s contained introns, and their number and size differ from their homology sequences of insects. The negative correlation between intron size and gene expression level was suggested in some studies, and the shorter or no intron genes had highly expressed level (Comeron 2004). The shorter or no intron genes also could be sensitive to various environmental stresses. According to the information of chromosome location and intron number, the sHSP genes were roughly divided into two types: orthologous and species specific (Li et al. 2009). Thus, we considered five small heat shock protein genes from *C. suppressalis* and classified them into two groups: orthologous type that contained introns (*Cshsp21.4* and *Cshsp21.7a*) and species-specific type with no intron (*Cshsp19.8*, *Cshsp21.5*, and *Cshsp21.7b*).

The role of small heat shock proteins in the tissues or organs is not well understood. One possibility is that sHSP genes expressed in special tissues or organs may play an important role in maintaining the normal organism functioning (Gu et al. 2012). In this study, five small *Csshsp*s (*Cshsp19.8*, *Cshsp21.5*, *Cshsp21.4*, *Cshsp21.7a*, and *Cshsp21.7b*) all exhibited abundant levels within the Malpighian tubules or hindgut. Similar results were found in *S. litura* and *Apis cerana cerana* (Liu et al. 2012; Shen et al. 2011). All five *Csshsp*s in *C. suppressalis* demonstrated tissue (organ)-specific expressions. Hindgut and Malpighian tubules reabsorb water, salts, and other substances before excretion by the insect, and it remains unclear why *shsp*s should be highly expressed in either. One explanation is that *shsp*s protect these tissues or organs from toxic injury. However, *Cshsp21.4* exhibited the highest mRNA level in the head, similar to the high expression levels of Bm19.1 and Bm22.6 in the head (Li et al. 2009). Thus, it is evident that different *shsp*s play different roles in insect biological processes.

sHSPs also play important roles in insect development. Previous studies have indicated that they were involved in the regulation of development in insects (Concha et al.

Fig. 7 Relative expression levels of small heat shock protein genes in *Chilo suppressalis* under temperature treatments. **a** Relative expression levels of *Cshsp19.8* gene. **b** Relative expression levels of *Cshsp21.4*

gene. **c** Relative expression levels of *Cshsp21.5* gene. **d** Relative expression levels of *Cshsp21.7a* gene. **e** Relative expression levels of *Cshsp21.7b* gene

2012; Shen et al. 2011; Takahashi et al. 2010). For instance, *l(2)efl*, a type of small *hsp*, reached its maximum value in the third larval instar of *D. melanogaster* (Kurzik-Dumke and Lohmann 1995). In *Lucilia cuprina*, the *hsp24* gene was expressed at its lowest level in the third instar larvae (Concha et al. 2012). In this study, the highest expression level of *Cshsp21.7a* was observed in the first instar larvae,

while those for *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, and *Cshsp21.7b* were observed in adults, which corresponded with the expression of *hsp19.7*, *hsp20*, and *hsp20.7* in *S. litura* and *hsp19.7* and *hsp19.8* in *Cydia pomonella* (Garczynski et al. 2011; Shen et al. 2011). However, we found the adult phase of *C. suppressalis* possessed the lowest thermotolerance in our previous study. Therefore,

we speculated that *Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, and *Cshsp21.7b* also correlated with the reproduction of *C. suppressalis*. Conversely, the *hsp20.4* of *S. litura* and three *shsps* of *Liriomyza sativa* had their lowest expression levels in adults (Huang et al. 2009; Shen et al. 2011). However, the *hsp21.4* of *S. litura* exhibited a constitutive expression pattern during all developmental stages (Shen et al. 2011). In general, small heat shock proteins of different insects could have evolved respectively specific roles in the development. The expression levels of five small heat shock protein genes from *C. suppressalis* exhibited differences based on sex, and a similar phenomenon was observed in *B. mori* and *A. mellifera* (Aamodt 2008; Li et al. 2009).

Recent reports have stressed the fact that regulation and expression levels of HSPs contribute to the thermotolerance of organisms (Sørensen et al. 2003; Queitsch et al. 2002). All five *Csshsp*s (*Cshsp19.8*, *Cshsp21.4*, *Cshsp21.5*, *Cshsp21.7a*, and *Cshsp21.7b*) differed in their expression profiles. *Cshsp19.8* and *Cshsp21.7b* were both upregulated dramatically by heat and cold, with an intensive response to heat, which is similar to *hsp20.4* and *hsp20.8* from *S. litura*, which were also significantly upregulated by both heat and cold treatments. Five *shsps* from *B. mori* and three *shsps* from *C. pomonella* demonstrated increased expression to heat shock stress (Garczynski et al. 2011; Li et al. 2009; Sakano et al. 2006). However, none of the five *Csshsp*s were induced by only mild cold or heat, a phenomenon shared by the three *shsps* of *L. sativa* and two *shsps* of *L. cuprina* (Concha et al. 2012; Huang et al. 2009). However, *hsp19.7* and *hsp20.7* of *S. litura* responded only to heat treatment, not to cold stress (Shen et al. 2011). In contrast to *hsp19.7* and *hsp20.7*, our study found that *Cshsp21.5* only responded to cold stress. Meanwhile, neither *Cshsp21.4* nor *Cshsp21.7a* responded to heat or cold, similar to *hsp20* and *hsp21.4* of *S. litura* and *hsp21.4* of *B. mori*, all of which were insensitive to thermal stress (Li et al. 2009; Shen et al. 2011). Because overexpression of HSP may cause some deleterious effects in organisms, it is very important to understand the balance between benefits and costs (Krebs and Feder 1998; Sørensen et al. 2003). In order to balance, *C. suppressalis* could regulate different *shsps* under different temperature stresses.

The more studies there are on the levels of such proteins, the better we will understand the roles of these genes in insect behavior and development. Future investigations of *shsps* will help reveal the underlying physiological mechanisms of *C. suppressalis*, allowing for improved management of this important pest.

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