JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Characterization of Multiple Heat-Shock Protein Transcripts from *Cydia pomonella*: Their Response to Extreme Temperature and Insecticide Exposure

Xue-Qing Yang,[*,†] Ya-Lin Zhang,[§] Xiao-Qi Wang,[†] Hui Dong,[†] Ping Gao,[†] and Ling-Yi Jia[#]

[†]Key Laboratory of Economical and Applied Entomology of Liaoning Province, College of Plant Protection, Shenyang Agricultural University, Shenyang 110866, People's Republic of China

[§]Key Laboratory of Plant Protection Resources and Pest Management of Ministry of Education, College of Plant Protection, Northwest A&F University, Yangling 712100, Shaanxi, People's Republic of China

[#]Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China

Supporting Information

ABSTRACT: The economically important fruit pest *Cydia pomonella* (L.) exhibits a strong adaptability and stress tolerance to environmental stresses. Heat-shock proteins (HSPs) play key roles in insects in coping with environmental stresses. However, little is known about the spatiotemporal expression patterns of *HSPs* and their response to stresses in *C. pomonella*. In this study, a thermal treatment−recovery test was performed, and the expression profiles of a novel isolated *HSP*, named *CpHSP40*, and six *CpHSPs* were determined. Third-instar larvae were able to recover from cold shock (0 °C) and heat shock (40 °C). *Escherichia coli* BL21 (DE3) cells harboring recombinant pET-28a (+)-CpHSP40 plasmid showed significant temperature tolerance. *CpHSPs* were developmentally and tissue-specifically expressed. The responses of *CpHSPs* to 0 and 40 °C (with or without recovery) and insecticide exposure were varied. All of these indicated that the expression of *HSPs* plays a role in the development and in environmental adaptation in *C. pomonella*.

KEYWORDS: *heat-shock proteins, adaptation, development, stress, tolerance*

INTRODUCTION

Abiotic stresses such as high/low temperatures and insecticides, are important stressors, influencing the survival, development, seasonality, and evolutionary responses of insects and mites.[1−4] The codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) is one of the most serious pests in pome fruit orchards worldwide.[5] Fruit quality was seriously affected by *C. pomonella*, and the fruit damage by *C. pomonella* includes boring tunnels into the center of fruit to feed on seeds, contaminating fruits with their frass, and causing fruit abscission.[6] Like most pests, *C. pomonella* is constantly threatened by abiotic stresses, such as high/low temperatures[7] and insecticides in their habitat, and it exhibits a strong adaptability and stress tolerance to these stresses. Extreme low temperatures in winter and extreme high temperatures in summer are key stress factors that threaten the survival and development of codling moth.[7] *C. pomonella* is inevitably exposed to insecticides due to protection of pome fruits.[7,8] In northwestern China, apple orchards are intensively treated with insecticides, on average, >10 applications per year. They are thus a compelling interest to study the effects of extreme temperature and insecticides on the expression level of some stress tolerance related genes in *C. pomonella*.

Heat-shock proteins (HSPs) allow insects to adjust their tolerance levels, adapt to extreme environments, and remain active under a series of environmental stresses.[4,9] The HSP gene superfamily has been divided into several families, such as *HSP100*, *HSP90*, *HSP70*, *HSP60*, *HSP40*, and the small *HSPs* (*sHSPs*, with molecular masses ranging from 12 to 43 kDa) according to their sequence homology and molecular weight.[10] In insects, HSPs are suggested to be involved in diverse physiological processes including increasing tolerance to extreme temperatures to protect insects from thermal injury and death[11,12] and are also implicated in development and diapause in some species including *Drosophila melanogaster*,[13] *Liriomyza sativa*,[12] and *Sarcophaga crassipalpis*.[14,15] HSPs are found in a wide range of organisms, including both eukaryotes and prokaryotes; they usually act as molecular chaperones, promoting correct refolding and preventing aggregation of denatured proteins.[16] They also function in normal cellular processes, including protein trafficking, signal transduction, DNA replication, and protein synthesis.[17] In addition, *HSPs* are commonly used by environmental toxicologists as biochemical markers of exposure to various physical, chemical, and biological stresses, such as temperature change and insecticide toxicity.[18]

In the codling moth, previous research in overwintering strategies and biochemical mechanisms of cold tolerance has suggested that compounds such as alanine, fructose and some other sugars, polyols, and free amino acids are accumulated, whereas glycogen and glutamine are reduced during the overwintering stage.[7] However, few attempts have been made

Received: April 27, 2016
Revised: May 4, 2016
Accepted: May 9, 2016
Published: May 9, 2016

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

to isolate and characterize *HSP* genes in the codling moth. *CpHSP90* (GenBank no. JN624775) represents the first *HSP* gene isolated from the codling moth; a study focused on its gene expression response to heat shock suggests that this is a heat-inducible *HSP* gene.[19] Garczynski et al. characterized three *sHSPs* (*CpHSP19.8*, *CpHSP19.9*, and *CpHSP22.2*) from codling moth.[20] Apart from this, there are two *HSPs* deposited in GenBank, including *CpHSP70-1*(GenBank no. JN863694.1) and *CpHSP70-2* (GenBank no. JN863695.1). These recent studies on the identification and characterization of *CpHSPs* provide a primary base for further investigation of this important gene family. However, there is little research about the expression patterns of these *Hsps* in different tissues, across different development stages, and in response to stresses in *C. pomonella*.

In the present study, the *C. pomonella HSPs* were identified and compared with the equivalent information from published research. The main objectives of the present study were to (1) determine the capacity of third-instar larvae recovery from cold-shock (0 °C) and heat-shock (40 °C) treatments; (2) isolate the complete cDNA sequence of the *C. pomonella HSP40* (*CpHSP40*) and evaluate the temperature tolerance of recombinant CpHSP40; (3) investigate the spatiotemporal expression profiles of seven *CpHSPs* and the transcriptional responses of seven *CpHSPs* to cold and heat shock (with or without recovery) and insecticide exposure; (4) discuss the role of these *CpHSPs* in the development and environmental adaptation of *C. pomonella*.

MATERIALS AND METHODS

Insects and Chemicals. The *C. pomonella* larvae were originally collected from abandoned apple orchards in Wuwei Botanical Garden, Gansu Province, China. The larvae were reared on an artificial diet[21] in the laboratory. Pupae were sexed and placed with both sexes together in a 2:1 male/female ratio in a plastic case (20 × 20 × 20 cm) for adult emergence. Mated female moths laid eggs on filter paper, allowing us to obtain the next generation of neonates. Insects reared for eight generations without any treatments were used in this study. Environmental conditions maintained for culturing the insect were 25 ± 1 °C and 60 ± 5% relative humidity with a 16:8 h light/dark photoperiod.

Insecticides representing the major groups of neonicotinoids (imidacloprid and acetamiprid), carbamates (carbaryl and methomyl), and synthetic pyrethroids (cypermethrin and deltamethrin) with purities of >99% were obtained from Aladdin Reagent (Shanghai, China) and dissolved in acetone.

Sample Preparation. For developmental stage expression analyses, three replicates of 20 first- to second-instar larvae, 10 third-instar larvae, 5 fourth- to fifth-instar larvae, pupae, and adults were respectively collected and stored at −80 °C until use. Pupae and adults were divided to males and females, respectively. For tissue-specific expression analyses, six tissues including the head, cuticle, the silk gland, midgut, fat body, and Malpighian tubules were dissected from three groups of 30 fourth-instar larvae on ice. All sampled groups were flash-frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

Third-instar larvae were selected for abiotic stress treatments. For thermal treatments (40 and 0 °C), three replicates of 15 larvae were kept in 60 × 15 mm disposable Petri dishes, incubated in a dry bath, in respective groups, for 1, 2, and 4 h. After thermal treatment, all treated larvae were then allowed to recover at 25 °C for 1 h. As a control, larvae were kept at 25 °C. After that, larvae were checked for viability, and the percentages of larvae that recover from thermal treatments were determined. A larva was considered dead or unrecoverable from thermal treatments when it did not respond to stimulation with an ink brush. Three groups of five larvae recovered from thermal treatments, as well as larvae without recovery at 25 °C for 1 h after thermal treatments, or control, were flash-frozen in liquid nitrogen and stored at −80 °C for RNA extraction. For insecticide treatments, sublethal doses (approximately 10% of mortality rate) of 2.50, 1.10, 7.50, 6.00, 20.00, and 2.80 mg/L of these insecticide solutions (Table S1) were prepared, and 1 μL of each insecticide solution was applied on the surface of a 0.25 cm^3 artificial diet using an Eppendorf pipettor (Hamburg, Germany). To determine that the insects were exposed to the insecticides, each larva was starved for 12 h before treatment with insecticides. Acetone was used for the control. Three replicates of 10 larvae were placed on the treated artificial diet and reared under the conditions noted above. The larvae were collected after 36 h of exposure and were flash-frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

RNA Extraction and First-Strand cDNA Synthesis. Total RNA used for rapid amplification of cDNA (RACE) and cDNA cloning was extracted from five fourth-instar larvae using the RNAiso Plus Kit (Takara, Dalian, China). The extracted RNA was quantified by spectrophotometer analysis (Infinite M200 PRO, Tecan Group Ltd., Männedorf, Switzerland). The genomic DNA was then removed from RNA samples by digestion with DNase I (MBI, Fermentas). The cDNA template of conserved region amplification was synthesized using 1.0 μg of total RNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas) in a 20 μL reaction mixture following the manufacturer's instructions. The cDNA template of 3′RACE was generated from 1 μg of total RNA using the SMART RACE cDNA Amplification Kit with the 3′-RACE CDS Primer A (Clontech, Japan). For 5′RACE, the template was terminal deoxynucleotidyl transferase (TdT) tailed according to a previous study[22] using AUP1 (Table S2) as an adaptor. The product cDNAs were stored at −20 °C for future use.

Molecular Cloning of CpHSP40 cDNA by 3′ and 5′RACE. On the basis of the conserved amino acid sequences of the insects' HSP40 genes deposited in the NCBI database, degenerate primers used for conserved region amplification were designed (Table S2) using the CODEHOP Web tool.[23] PCR was performed in a C1000 Thermal Cycler (BioRad, USA) by nested PCR using HSP40-F1 and HSP40-R1 (Table S2) as the primer pair for the first-round reaction followed by the second-round amplification using primer pair HSP40-F2 and HSP40-R2 (Table S2). The PCR reactions for both the first and second rounds were as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and then a final extension at 72 °C for 7 min. The approximately 600 bp PCR product was gel purified using the Biospin Gel Extraction Kit (Bioer Technology Co., Ltd., Hangzhou, China). The purified DNA was cloned into the pMD-19 T vector (Takara) and was then transformed into *Escherichia coli* DH5α (Takara). A small colony was picked and suspended in a 25 μL total reaction volume containing 12.5 μL of 2×Taq MasterMix (CWBIO, Beijing, China), 1.0 μL each of M13 primers, and 10.5 μL of double-distilled water in a PCR tube. Thermal cycling conditions were 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 7 min. Three PCR screened positive clones were grown overnight in Luria−Bertani (LB), and plasmid DNA was extracted using an E.Z.N.A.Plasmid Mini Kit I (Omega, USA) according to the manufacturer's protocol, followed by sequencing at the Shanghai Sunny Biotech Co., Ltd., China.

The gene-specific primers (GSPs) and adaptor primers for 3′ and 5′RACE were subsequently synthesized (Table S2) on the basis of the obtained conserved region sequences. For 3′RACE, PCR was carried out by seminested PCR using the HSP40-3F1 (Table S2) combined with the universal primer 10×UPM (Table S2) for first-round PCR, and using HSP40-3F2 (Table S2) and 10×UPM for second-round PCR. The PCR conditions for both rounds of PCR reactions were 94 °C for 3 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 72 °C, with a final extension at 72 °C for 7 min. For 5′RACE, PCR was performed by nested PCR using HSP40-5R1 and AP-L (Table S2) as the primer pair for first-round PCR and primer HSP40-5R2 combined with primer AP-S (Table S2) for the second-round PCR. The PCR conditions for both rounds of PCR reactions were as

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 7 min. Only one 3′ and one 5′ cDNA end were obtained using those primers. The PCR products of 3′ and 5′RACE were respectively separated by electrophoresis on a 1% agarose gel, and the bands for 3′RACE (1000 bp) and 5′RACE (200 bp) were gel purified, TA cloned, and sequenced as described above. On the basis of the obtained sequence, the putative full length of *CpHSP40* sequence was spliced via searching the *C. pomonella* transcriptome (SRX371333) deposited in NCBI (http://www.ncbi.nlm.nih.gov/sra/SRX371333[accn]).

To amplify the full length of *CpHSP40*, specific primers CpHSP40-F and CpHSP40-R were synthesized (Table S2). The PCR was carried out using high-fidelity Ex *Taq* polymerase (Takara) under these conditions: 94 °C for 3 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 72 °C, and with a final extension at 72 °C for 7 min. The PCR product was TA cloned and sequenced as described above.

Sequence Alignment and Bioinformatic Analysis. The amino acid sequence of *CpHSP40* was deduced from the obtained ORF cDNA sequences, and the similarity analyses of the deduced amino acid sequence were performed using the BLAST program in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ExPASy Compute p*I*/M_w tool (http://web.expasy.org/compute_pi/) was used to predict the molecular weight and isoelectric points of CpHSP40. The deduced amino acid sequences of HSPs were aligned with the HSP members from other insect species using the ClustalW2 Web tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Motifs were predicted on the basis of scanning the Prosite database.[24] The deduced amino acid sequences of *CpHSPs* were used as queries to search for other Lepidopteran HSPs using the BLAST program in NCBI. Phylogenetic trees were constructed with MEGA 4.0[25] by the maximum parsimony method. The three-dimensional (3D) structure of CpHSP40 was predicted using homology modeling (automated mode) in the SWISS-MODEL (http://swissmodel.expasy.org/).

Expression of CpHSP40 in *E. coli*. To determine if the *CpHSP40* encodes an active CpHSP40 protein, the prokaryotic expression vector was constructed. The CpHSP40 ORF containing *Eco*RI and *Hin*dIII restriction endonucleases was cloned into the pMD-19 T vector. Sequence-verified plasmid was digested with *Eco*RI and *Hin*dIII and then cloned into *Eco*RI and *Hin*dIII sites predigested pET-28a (+) plasmid to obtain the recombinant vector, pET-28a-CpHSP40. The recombinant plasmid was sequenced to ensure that the inserted ORF sequence was correct and was subsequently transformed into *E. coli* BL21 (DE3). Ten clones were grown overnight at 37 °C in LB medium containing 50 μg/mL kanamycin, and plasmid DNA was extracted as described above, followed by digestion with *Eco*RI and *Hin*dIII and nucleotide sequencing. A positive colony was cultured in 10 mL of LB medium containing 50 μg/mL kanamycin at 37 °C and induced with a final concentration of 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 30 °C with shaking at 220 rpm when cell density reached 0.6. After incubation for 8 h, 1 mL of the culture medium was obtained, and cell pellets were harvested by centrifugation. The precipitation was washed twice by Tris-HCl buffer (50 mM, pH 8.0) and was resuspended in Tris-HCl buffer. The suspension was sonicated (Sonics Uibra Cell VCX130, Sonics) on ice for a total time of 5 min (10-s pulses, 10-s cooling). The lysate was centrifuged (12000*g*, 30 min, 4 °C), and the precipitate was resuspended with Tris-HCl buffer. The supernatant and suspension were added with loading buffer, respectively, and the mixtures were denatured at 95 °C for 10 min. The samples were then submitted to a 12% SDS-PAGE (Bio-Rad) to determine the expression of the recombinant CpHSP40 protein.

Thermotolerance and Cold-Tolerance Assay of *E. coli* Overexpressing CpHSP40. The thermotolerance and cold tolerance assay was performed as previously described.[26] In brief, the cells with the pET-28a-CpHSP40 plasmid were induced with 0.2 mM IPTG at 30 °C for 8 h. The cells were then incubated at 4 °C for 0, 12, 24, 36, and 48 h and at 50 °C for 0, 15, 30, 60, and 120 min, respectively. Subsequently, the cells were diluted to 5×10^6 cells/mL in cold LB buffer containing 50 μg/mL kanamycin, and 50 μL cultures were plated on LB agar plates. Colonies were counted after incubation at 37 °C overnight, and the cell survival rate (the ratios of viable cell numbers per plate in the treated samples and untreated samples) was calculated. Experiments were performed in triplicate.

Semiquantitative RT-PCR. The total RNA was isolated from different developmental stages and various tissues of fourth-instar larvae using the RNAiso Plus Kit (Takara, Dalian, China) according to the manufacturer's instructions. The quality and concentration of extracted RNA were determined by agarose gel electrophoresis and spectrophotometer analysis (Infinite M200 PRO, Tecan Group Ltd., Männedorf, Switzerland). The first-strand cDNA was then synthesized from 1.0 μg of total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) in a 20 μL reaction mixture as described by the manufacturer. Meanwhile, the potential genomic DNA contamination was also eliminated by this kit. Transcript levels were checked using semiquantitative RT-PCR using *C. pomonella* β-actin gene (*CpActin*) (155 bp, GenBank no. KC832921) as the internal control.[27] Primer pairs (Table S2) were designed to amplify 200−330 bp long regions of *HSP* genes from *C. pomonella HSP* genes, including *CpHSP19.8* (GenBank no. HQ219475.1), *CpHSP19.9* (GenBank no. HQ219476.1), *CpHSP22.2* (GenBank no. HQ219477.1), *CpHSP40* (GenBank no. KU748778), *CpHSP70-1*(GenBank no. JN863694.1), *CpHSP70-2* (GenBank no. JN863695.1), and *CpHSP90* (GenBank no. JN624775.1). The semi-qPCR reactions were performed on a C1000 Thermal Cycler (BioRad, Hercules, CA, USA) using a 2×Taq MasterMix (CWBIO, Beijing, China). A 20 μL reaction mixture was assembled for each gene including the following: 1 μL of 5-fold diluted cDNA template, 0.5 μL each of gene-specific primers (10 μLM), 10 μL of 2×Taq MasterMix, and 8 μL of double-distilled water. PCR was performed under the following cycling program: 95 °C for 3 min, 24−40 cycles (based on a preliminary experiment) of 95 °C for 30 s, 55−60 °C (annealing temperatures of each primer pairs were optimized by gradient PCR before use) for 30 s, and 72 °C for 30 s. A no-template control (NTC) and a no reverse transcriptase control (NRC) reaction were included as a check for contamination and nonspecific amplification. To determine the plateau of PCR amplification, nine reaction tubes were prepared, and the Thermal Cycler was programmed to pause every two cycles—beginning at 24 cycles and ending at 40 cycles—to remove each reaction tube for agarose analysis. Each PCR was conducted in triplicate. Resultant PCR products were TA cloned and sequenced as described above to verify that the PCRs were specific and produced the correct amplicons. PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. To obtain relative consistency of gel backgrounds, the same photographic settings for each gel were used during photographic exposure. The intensities of the resultant PCR products were quantified by Quantity One software on the BioRad Gel Doc 2000 system.

Real-Time Quantitative PCR (RT-qPCR). Total RNA was isolated from larvae exposed to temperatures and insecticides using the RNAiso Plus Kit (Takara) following the manual instructions. The quality and concentration of extracted RNA were determined, and cDNA was synthesized from 1.0 μg of total RNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara) to remove any residual genomic DNA contaminatio, and stored at −20 °C. The transcripts of different *CpHSPs* after larvae exposure to temperatures and insecticides were examined using RT-qPCR using the same internal control and gene-specific primers for *CpHSPs* (Table S2) described above. Real-time reactions were conducted on a BioRad iCycler iQ5 in a 20 μL total reaction volume containing 10 μL of 2×UltraSYBR Mixture (CWBIO, Beijing, China), 0.5 μL each of gene-specific primers (10 μM) and 5-fold diluted cDNA templates, and 8.5 μL of double-distilled water. Relative standard curves of *CpHSP* genes and the endogenous control were constructed using linear gradient (10-fold) cDNA as template and were included within each RT-qPCR. Thermal cycling conditions were 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 55−60 °C (depending on primer pairs) for 1 min, followed by a dissociation analysis to confirm the homogeneity of

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

Table 1. Percentages (Mean ± SD, %) of Larvae Recovery from Cold and Heat Treatments[a]

	treatment time			
temperature (°C)	0 h	1 h	2 h	4 h
40	100 ± 0	91.11 ± 10.18	80.00 ± 13.33	66.67 ± 17.64
0	100 ± 0	93.33 ± 6.67	88.89 ± 10.18	75.56 ± 10.18

[a]Three replicates of 15 third-instar larvae were exposed to 0 and 40 °C for 0, 1, 2, and 4 h and then recovered at 25 °C for 1 h. The surviving number of larvae was recorded, and the percentages of larvae that recover from thermal treatments were determined.

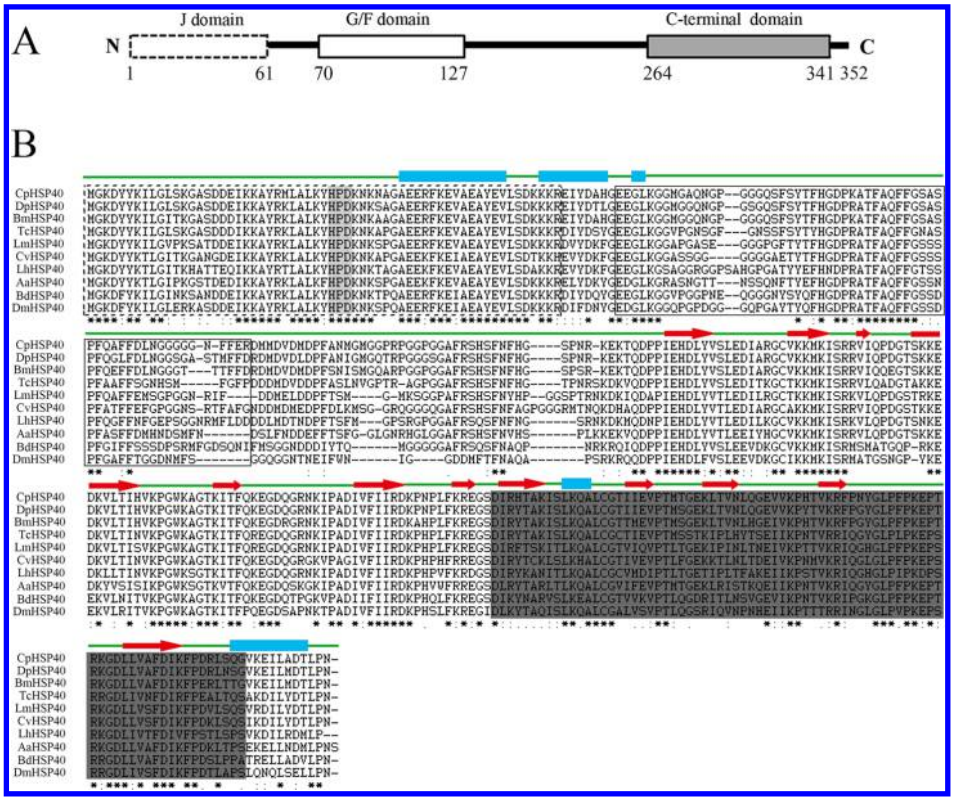

Figure 1. Schematic diagram of the protein domains of CpHSP40 (A). The conserved J-domain (1−36), G/F domain (45−102), and C-terminal substrate binding domain (239−316) are shown. (B) Structure-based sequence alignments of HSP40 from *C. pomonella* and other insects. The selected sequences are HSP40 from *B. mori* (GenBank no. ACT34039.1), *T. castaneum* (GenBank no. EFA11191.1), *L. migratoria* (GenBank no. ABC84495.1), *Cotesia vestalis* (GenBank no. AGF34716.1), *L.nbs hesperus* (GenBank no. AFX84558.1), *A. aegypti* (GenBank no. XP 001658074.1), *B. dorsalis* (GenBank no. AEJ88465.1), and *D. melanogaster* (GenBank no. AAP31279.1) by ClustalW2. The J-domain is dash boxed. The G/F domain is solid boxed. The C-terminal substrate binding domain is in gray. The predicted α-helices and β-sheets of CpHSP40 are marked on the top of sequences using blue boxes and red arrows, respectively. Asterisks (*) indicate identical residues among the sequences; colons (:) indicate residues with conserved substitutions; dots (.) represent residues with weakly conserved residues in the sequence alignments.

the PCR products. The reaction was repeated three times for each gene. Each replicate was performed with an independent RNA sample preparation and consisted of three technical replicates. Negative controls were carried out as described above. Samples were normalized using *CpActin* (GenBank no. KC832921) *Ct* values. The relative mRNA expression was represented as fold over the expression levels of *CpActin*. Fold changes were calculated according to the $2^{-\Delta\Delta CT}$ method,[28] and the expression level of control samples was set at 1.

Statistical Analysis. Statistics were performed using SPSS 12.0 software (IBM, IBM Inc., Chicago, IL, USA). Student's *t* test (*, $P \leqslant 0.05$; **, $P \leqslant 0.01$; ***, $P \leqslant 0.001$) was used to compare the relative expression level of *CpHSPs* under cold and heat stresses, as well as the transcript levels of *CpHSPs* after larvae exposure to insecticides. The results are represented as the mean of triplicates ± standard deviation (SD). The results were plotted using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

RESULTS

Larvae Recovery from Thermal Treatments. After exposure of third-instar larvae to 40 °C for 0, 1, 2, and 4 h, followed by a recovery at 25 °C for 1 h, larvae recoveries from heat-shock (40 °C) treatment were (mean ± SD) 100 ± 0, 91.11 ± 10.18, 80.00 ± 13.33, and 66.67 ± 17.64%, respectively (Table 1). Similarly, 100 ± 0, 93.33 ± 6.67, 88.89 ± 10.18, and 75.56 ± 10.18% of third-instar larvae recovered from 0 °C treatment for 0, 1, 2, and 4 h, respectively (Table 1). The results suggest that the percentage of larvae recovery from thermal treatment decreased as the treatment time increased from 1 to 4 h.

Identification and Characterization of CpHSPs Transcripts. A 553 bp conserved region of *CpHSP40* was obtained using the degenerate primers designed on the basis of the conserved regions of the HSP40 gene (Table S2). After 3′ and 5′RACE, a 3′cDNA fragment of 995 bp and a 5′cDNA fragment of 169 bp were obtained, respectively. A full-length

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

cDNA of 1655 bp (GenBank no. KU748778) composed of a 56 bp 5′-untranslated region (5′-UTR), an open reading frame (ORF) of 1059 bp, and a 540 bp 3′-UTR was subsequently amplified by RT-PCR. The ORF encodes a protein of 352 amino acids with a calculated molecular mass of 38.93 kDa and theoretical isoelectric point of 9.22. Putative polyadenylation signal (AATAAA) was present at 513 nucleotides downstream from the termination codon (Figure S1).

Amino acid sequence alignment indicated that CpHSP40 shared the highest identity with the DnaJ-5 (HSP40) from *Danaus plexippus* (92%, EHJ65096.1). It also shares a high degree of amino acid identity (>60%) with the HSP40 members from *Bombyx mori* (90%, ACT34039.1), *Locusta migratoria* (76%, ABC84495.1), *Tribolium castaneum* (74%, EFA11191.1), *Aedes aegypti* (69%, XP_001658074.1), *Cotesia vestalis* (72%, AGF34716.1), *Lygus hesperus* (67%, AFX84558.1), *Bactrocera dorsalis* (63%, AEJ88465.1), and *D. melanogaster* (64%, AAP31279.1) (Figure 1A). To investigate the structural characteristics of CpHSP40, the 3D structure model of CpHSP40 was predicted using SWISS-MODEL. We selected the *Thermus thermophilus* Chaperone protein DnaJ 2 (PDB ID 4j80.1A, resolution = 2.9 Å) as template; it shares a 32.06% sequence similarity with CpHSP40 (Figure S2). The DnaJ 2 was a homodimer protein, and each monomer contains 5 α-helices and 12 β-sheets, and the loops are linked (Figure 1B). The second structure of this chaperones is composed of an N-terminal conserved domain (J-domain, residues 1−61), a glycine/phenylanine domain (G/F, residues 70−127), and a C-terminal substrate binding domain (residues 264−341). The J-domain contains a highly conserved tripeptide of histidine, proline, and aspartic acid (the HPD motif, residues 32−34).

To determine the relationship of CpHSP40 with the DnaJ-like members from other species deposited in the GenBank database, a phylogenetic tree was constructed (Figure S3A). The result showed that the HSP40s of *C. pomonella*, *D. plexippus*, and *B. mori* form a small cluster, which is almost in line with the amino acid similarity. The phylogenetic trees of HSP70 and HSP90 were also constructed to examine their genetic relationships of *C. pomonella* to other insect species (Figure S3B,C). This analysis showed that the CpHSP70-1, CpHSP70-2, and *Grapholita molesta* (GenBank no. AFU06382.1) form a small cluster due to their belonging to the same family, Tortricidae. The phylogenetic tree of HSP90 revealed that CpHSP90 is located on the same cluster with the HSP90 from the tortricid insect, *G. molesta* (GenBank no. AFV09397.1), with 99% bootstrap support, and they share 98% amino acid identity, indicating CpHSP90 shares the closest relationship with *G. molesta* HSP90. Most Lepidopterous HSP90s clustered together in the tree (Figure S3C). The sequence and phylogenetic analysis of sHSPs (CpHSP19.8, CpHSP19.9, and CpHSP22.2) were previously reported.[20]

Expression and Characterization of Recombinant CpHSP40 in *E. coli*. A high level of soluble N-terminal fusion His-tag-CpHSP40 recombinant protein was expressed with IPTG induction for 3 h at 37 °C. The expected band was detected between 35 and 45 kDa, which is the sum of the predicted M_w of CpHSP40 (38.93 kDa) and the M_w of pET-28a (+) tag (approximately 4 kDa) (Figure S4).

To provide direct evidence that CpHSP40 protected cells from extreme temperature injury in vivo, we further examined the effect of heat and cold stresses on the survival rate of *E. coli* BL21 (DE3) cells transformed with a vector expressing CpHSP40. As shown in Figure 2, recombinant cells showed

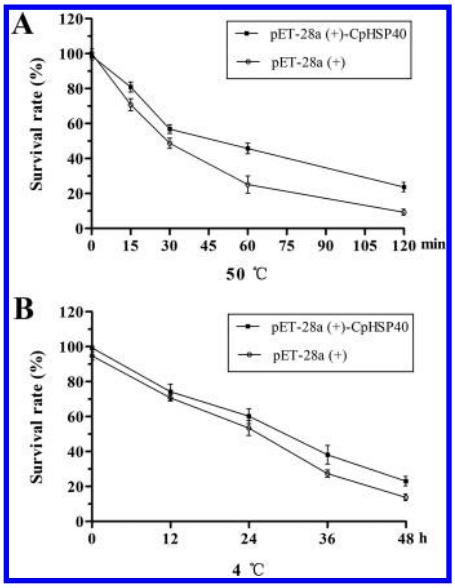

Figure 2. Effect of heat (A) and cold (B) stresses on the survival rate of *E. coli* BL21 (DE3) cells harboring pET-28a (+)-CpHSP40 (■) and pET-28a (+) vector only (○).

higher tolerance to heat (50 °C) and cold (4 °C) stresses than cells harboring the pET-28a (+) vector. After 120 min of thermal (50 °C) exposure, the survival rate of *E. coli* BL21 (DE3) cells containing pET-28a (+) dropped to 9.26%, whereas the cells harboring the pET-28a (+)-CpHSP40 remained at a relatively higher survival rate (23.69%) (Figure 2A). After incubation at 4 °C for 48 h, the survival rate of *E. coli* BL21 (DE3) cells containing pET-28a (+)-CpHSP40 was 23.07%; however, the value was 13.77% for cells harboring only the pET-28a (+) vector (Figure 2B). These results indicate that recombinant CpHSP40 exhibited significant temperature tolerance. Whether CpHSP40 functions as DnaJ in *E. coli* or not needs to be further investigated.

Developmental Expression Profiles of Seven *CpHSP* Transcripts. To determine the expression patterns of *CpHSPs* at various developmental stages, including first- to fifth-instar larvae, male and female pupae, and male and female adults, a semiquantitative RT-PCR was conducted. The results showed that the seven *CpHSP* transcripts were detectable in all developmental stages (Figure 3). The transcriptional levels of *CpHSP22.2* were similar during the larval, pupal, and adult stages. The expression levels of *CpHSP19.8*, *19.9*, *40*, *70-1*, *70-2*, and *90* increased as larval instars progressed from the first to fifth. The expression profiles of these seven transcripts were subsequently performed by semiquantitative RT-PCR to investigate their expression levels between sexes in the pupal stage and at adulthood. At the pupal stage, the expressions of *CpHSP19.9*, *22.2*, *40*, and *90* were similar between the male and female. Interestingly, *CpHSP19.8*, *70-1*, and *70-2* exhibited higher expression levels in females than in males (Figure 3A).

Tissue-Specific Expression Profiles of Seven *CpHSP* Transcripts. Semiquantitative RT-PCR showed that all seven *CpHSP* transcripts were expressed in all tested tissues, and all transcripts were found to have a higher expression level in the head than in other tissues (Figure 3B). Significantly lower expression levels of *CpHSP19.9*, *40*, and *70-1* were detected in the midgut than in other tissues, whereas *CpHSP90* has a remarkably lower expression in fat body than in other tissues (Figure 3B). It is noteworthy that the expressions of

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

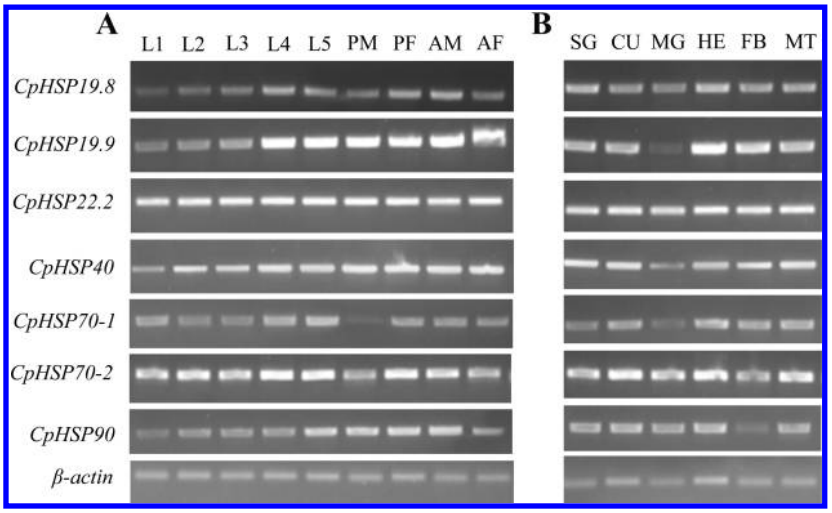

Figure 3. Relative expression level of *CpHSPs* at developmental stages (A) and tissues (B) as determined by semiquantitative RT-PCR. The numbers of cycles for *CpHSP19.8*, *19.9*, *22.2*, *40*, *70-1*, *70-2*, *90* and *CpActin* were 26, 26, 32, 26, 32, 28, 22, and 23, respectively. L1−L5, first−fifth-instar larvae; PM, pupa (male); PF, pupa (female); AM, adult (male); AF, adult (female); SG, silk gland; CU, cuticle; MG, midgut; HE, head; FB, fat body; MT, Malpighian tubules.

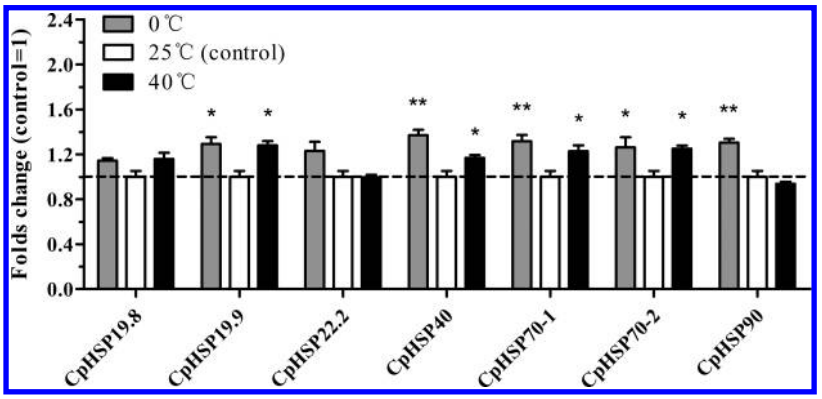

Figure 4. Relative expression level of *CpHSPs* in response to 1 h of cold- and heat-shock (1 h recovery). The relative mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ method, and the *y* axis represents the level of change. The expression level of each *CpHSP* transcript was first normalized to the quantity of *CpActin* and then divided by the abundance of the corresponding *CpHSP* at 25 °C. The normalized value (fold difference) was then applied to establish the relative expression analysis. The expression level of samples treated at 25 °C (control) was set at 1. The error bar represents the standard deviation calculated from three replicates. Asterisks above represent statistically significant differences by Student's *t* test (∗, $P \leqslant 0.05$; ∗∗, $P \leqslant 0.01$).

CpHSP22.2 were not significantly different among tissues (Figure 3B).

Expression of Multiple *CpHSPs* in Response to Temperatures. To examine the effect of temperature on *CpHSPs* expression, the transcription of multiple *CpHSPs* was examined. After exposure of third-instar larvae at 0 or 40 °C for 1 h, followed by recovery at 25 °C for 1 h, the transcripts of seven *CpHSPs* were not significantly different between treatment and control or were slightly up-regulated (Figure 4). Maintenance of third-instar larvae for 1 h at 0 °C did not change the expressions of *CpHSP19.8* and *CpHSP22.2*, but slightly up-regulated the expression levels of *CpHSP19.9* (df = 1,4, $F = 21.469$, $p = 0.1$), *CpHSP40* (df = 1,4, $F = 47.523$, $p = 0.002$), *CpHSP70-1* (df = 1,4, $F = 25.336$, $p = 0.007$), *CpHSP70-2* (df = 1,4, $F = 8.051$, $p = 0.047$), and *CpHSP90* (df = 1,4, $F = 16.981$, $p = 0.015$) for 1.29-, 1.37-, 1.32-, 1.26-, and 1.30-fold, respectively (Figure 4).

In contrast, the transcription levels of *CpHSP19.9* (df = 1,4, $F = 47.746$, $p = 0.02$), *CpHSP40* (df = 1,4, $F = 21.247$, $p = 0.01$), *CpHSP70-1* (df = 1,4, $F = 8.051$, $p = 0.047$), and *CpHSP70-2* (df = 1,4, $F = 16.981$, $p = 0.015$) were 1.28-, 1.17-, 1.26-, and 1.30-fold up-regulated, respectively, after heat-shock treatment at 40 °C for 1 h, but there was no up-regulation of *CpHSP19.8*, *CpHSP22.2*, and *CpHSP90* expression determined after heat stress (Figure 4).

The expression levels of *CpHSP19.9*, *CpHSP40*, *CpHSP70-1*, and *CpHsp90* were up-regulated to various degrees after 1 h of cold- or heat-shock treatments (0 and 40 °C), followed by a recovery. To determine if the expression levels of these *CpHSPs* after 1, 2, and 4 h of heat shock without recovery at 25 °C differ with recovery at 25 °C after heat shock, the transcription of these *CpHSPs* was examined. The *Ct* values of *CpActin* and *CpHSP19.9*, *CpHSP40*, *CpHSP70-1*, and *CpHsp90* for NRC as well as cold- and heat-shock treatments are shown in Table S3. *CpHSP19.9*, *CpHSP40*, *CpHSP70-1*, and *CpHsp90* were all up-regulated (Figure 5). The abundance of *CpHSP19.9* was most remarkably induced among the four *CpHSPs* determined. After 1, 2, and 4 h of cold shock (0 °C), the expression level of *CpHSP19.8* was highly increased by 24.86-, 52.99-, and 57.90-fold, respectively (Figure 5A). *CpHSP19.8* was 18.96-. 40.65-,

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

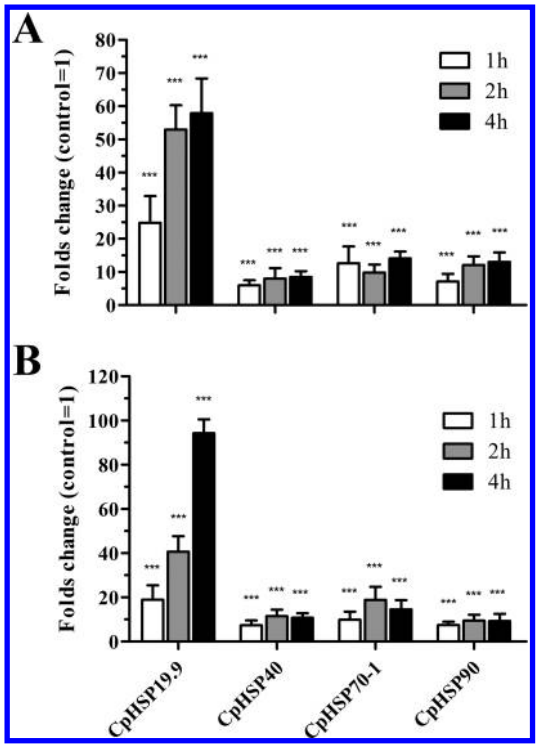

Figure 5. Relative expression level of *CpHSPs* in response to 1, 2, and 4 h of cold shock (A) and heat shock (B) (no recovery). The relative mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ method, and the *y* axis represents the level of change. The expression level of each *CpHSP* transcript was first normalized to the quantity of *CpActin* and then divided by the abundance of the corresponding *CpHSP* at 25 °C. The normalized value (fold difference) was then applied to establish the relative expression analysis. The expression level of samples treated at 25 °C (control) was set at 1. The error bar represents the standard deviation calculated from three replicates. Asterisks above represent statistically significant differences by Student's *t* test (***, $P \leqslant 0.001$).

and 94.27-fold overexpressed after heat-shock treatment at 40 °C for 1, 2, and 4 h, respectively (Figure 5B). After cold-shock treatment for 1, 2, and 4 h, the expressions of *CpHSP40* (5.99-, 8.00-, and 8.52-fold), *CpHSP70-1* (12.62-, 9.84-, and 14.10-fold), and *CpHsp90* (7.12-, 12.08-, and 13.01-fold) were up-regulated to different degrees (Figure 5A). After exposure of third-instar larvae at 40 °C for 1, 2, and 4 h, the abundances of *CpHSP40* (7.33-, 11.44-, and 10.74-fold), *CpHSP70-1* (9.82-, 18.83-, and 14.55-fold), and *CpHsp90* (7.46-, 9.40-, and 9.27-fold) were increased bto different degrees (Figure 5B).

Expression of Multiple *CpHSPs* in Response to Insecticides. After exposure of third-instar larvae to imidacloprid, acetamiprid, methomyl, carbaryl, cypermethrin, and deltamethrin for 36 h, the mRNA levels of these seven *CpHSPs* were determined by RT-qPCR. The expression of *CpHSP19.8* was slightly suppressed by imidacloprid (0.45-fold), acetamiprid (0.74-fold), and methomyl (0.67-fold) (Figure 6). In contrast, the abundance of *CpHSP19.9* was 0.36-fold down-regulated by imidacloprid and was increased by 1.37-, 1.97-, 3.52-, 3.40-, and 1.79-fold when exposed to acetamiprid, methomyl, carbaryl, cypermethrin, and deltamethrin, respectively (Figure 6). Acetamiprid has little effect on *CpHSP22.2* transcript levels, with only 1.82-fold of transcripts enhanced (Figure 6). The *CpHSP70-2* showed 2.32-, 1.67-, 1.92-, and 1.30-fold up-regulation by acetamiprid, methomyl, carbaryl, and cypermethrin, compared with blank control (Figure 6). No significant differences in *CpHSP40*, *CpHSP70-1*, and *CpHSP90* expression were observed after exposure to these six insecticides for 36 h (Figure 6).

DISCUSSION

Insects have evolved complex defense systems for adapting to various environmental stresses, such as extreme temperatures and insecticides. Stress response proteins, such as HSP70 and HSP90, are well-known stress proteins conferred in response to various environmental stresses. In this study, third-instar larvae of *C. pomonella* were employed to check their capacity of recovery from cold-shock (0 °C) and heat-shock (40 °C) treatments, and the expression profiles of a novel isolated *CpHSP40*, as well as six *CpHSPs*, were determined.

After exposure of third-instar larvae at 40 °C for 1, 2 and 4 h, followed by a recovery at 25 °C for 1 h, >66.67% of individuals returned to normal viability. It worth noting that the *C. pomonella* inhabited the inside of pome fruit to feed on pulp or seeds during the larval stage except for the newly hatched stages; thus, the temperature inside the fruit and that of the external environment might be quite different, and the external environmental temperature is much higher than that inside fruit. Therefore, the thermal response of third-instar larvae investigated in this study does not represent the real consequences of *C. pomonella* in response to low or high temperature in the field. Garczynski et al. incubated various stages of codling moth, including eggs, third- and fifth-instar larvae, pupae, and adults, at 42 °C for 4 h and then the viability was checked; however, the survival data were not given. Previous research had demonstrated that fifth-instar is the most heat-tolerant stage.[29] Thus, whether other larval stages possess high heat tolerance needs to be further determined.

Compared to the previous HSPs[19,20] identified from published studies, we identified one additional gene. The *CpHSP40* gene encoding HSP40 protein was cloned from codling moths using the RACE technique. The deduced amino acid sequence of CpHSP40 showed high (56−75%) similarity to HSP40 sequences known from other insect species. Structural analysis showed that CpHSP40 contains an N-terminal conserved domain (J-domain, residues 1−36), a G/F domain (residues 45−102), and a C-terminal substrate binding domain (residues 239−316). The J-domain, ubiquitous in DnaJ/HSP40 proteins, functions to target HSP70 to their substrates by catalyzing ATP hydrolysis by HSP70.[30] Furthermore, the J-domain in CpHSP40 contains a highly conserved HPD.[31]

A previous study demonstrated that HSPs are developmentally regulated in some insects. The expression of *HSP70 III* from the red flour beetle, *T. castaneum* (*TcHSP70 III*), varied among developmental stages. The abundance of *TcHSP70 III* decreased from young larvae to pupae and then increased at the adult stage, suggesting that *TcHSP70 III* is developmentally regulated.[32] In the leafminer, *L. sativa*, the expression levels of three sHSPs (*LsHSP19.5*, *LsHSP20.8*, and *LSHSP21.7*) reached a peak in the pupal stage, and the HSP60 homologue, *LsTCP1α*, *LsTCP1ζ*, and HSP90 increased with the developmental process, suggesting that these HSPs are involved in the development of *L. sativa*.[12] The developmental regulation of HSPs has also been reported in other insect species, including *D. melanogaster*,[32] *S. crassipalpis*,[33] *Manduca sexta*,[34] and *Chironomus* spp.[35] In the present study, the expression of *CpHSP22.2* was constant during all developmental stages. This

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

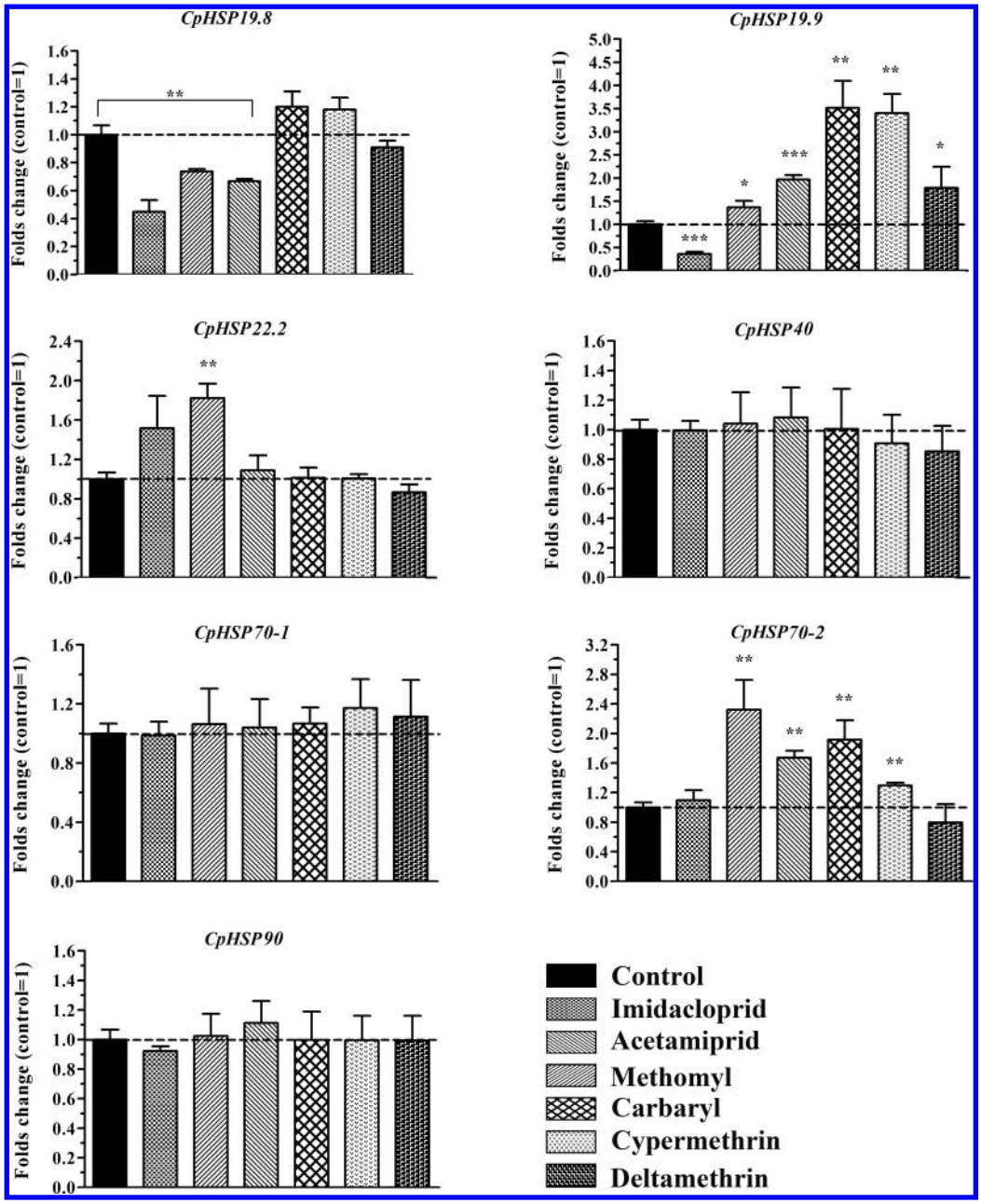

Figure 6. Relative expression level of *CpHSPs* exposure to six insecticides. The relative mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ method, and the *y* axis represents the level of change. The expression level of samples exposed to acetone (control) was set at 1. The error bar represents the standard deviation calculated from three replicates. Asterisks above represent statistically significant differences by Student's *t* test (∗, $P \leqslant 0.05$; ∗∗, $P \leqslant 0.01$; ∗∗∗,$P \leqslant 0.001$).

result is not in line with previous work,[20] but agrees well with *TcHSP70 II* genes from *T. castaneum*.[36] Moreover, we found that the transcript abundance of other *CpHSPs*, *CpHSP19.8*, *19.9*, *40*, *70-2*, and *90* increased as larval instars progressed from first to fifth, and the *CpHSP70-1* and *CpHSP70-2* reached their peak at the fifth instar. This result is in line with previous characterization of *CpHSP19.8* and *CpHSP19.9* from *C. pomonella*.[20] The expression level of *CpHSP70-1* was slightly decreased in the second and third instar larvae, but increased in the fourth- and fifth-instar larvae and reached its peak at the fifth-instar larval stage. This expression pattern is similar to that of *CvHSP70* from the endoparasitoid wasp, *C. vestalis*.[10] Apart from *CpHSP19.9*, *22.2*, and *40*, *CpHSP19.8*, *70-1*, *70-2*, and *90* were found to have a higher expression level in the female pupae and male adult than in the male pupae and female adult. The gender-specific expression profiles of these transcripts in codling moth suggest that males and females perform different functions during the development of pupae and adulthood and also imply differing roles of these *HSPs* in response to environmental stresses during sex-specific physiological processes, such as the production of hormones or pheromones. As a result, better stress tolerance compared to the opposite gender was exhibited during these two stages. The expressions of *CpHSP19.8*, *19.9*, *40*, *70−1*, *70−2*, and *90* mRNAs varied among developmental stages, suggesting their involvement in codling moth development, and might play important roles in this pest's defense against stresses during life stages. The regular expression patterns of *CpHSP22.2* among development stages

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

indicate that it is involved in a basic physiological function of the codling moth.

To better understand the function of *CpHSP* transcripts biologically and physiologically, the tissue expression profiles of these transcripts from codling moth were determined. Although these transcripts were expressed in the tested tissues (Figure 3), the abundance of these transcripts varied among tissues. The transcripts of *CpHSP19.8*, *19.9*, *70-1*, *70-2*, and *90* in the head were higher than in other tissues, suggesting that these transcripts are involved in either olfaction or neuro/developmental processing. *CpHSP22.2* was consistently expressed in all tested tissues. If the amounts of *CpHSP19.8*, *19.9*, *40*, and *70-1* transcripts are higher in fat body than midgut, these may participate in development and metamorphosis, rather than in defense against xenobiotics such as insecticides or plant toxins produced by plants during feeding. This tissue-specific expression profile was consistent with *HSP70* and *90* from *Spodoptera litura*,[37] but was not in line with *HSP70* and *90* from *S. crassipalpis*,[38] *Lucilia sericata*,[39] and *Helicoverpa zea*,[40] whereas the transcripts of *HSP70* and *90* in midgut were higher than in fat body in these species. Concerning *CpHSP70-2* and *90*, their expression levels in fat body were lower than those in midgut, which is consistent with previous studies.[38−40] The relatively high expressions of *CpHSP19.8*, *19.9*, *40*, *70-2*, and *90* in silk gland and cuticle suggest that these *HSPs* play a key role in protection of the codling moth from mechanical injuries.

To protect organisms from lethal thermal and other stresses, *HSPs* are overexpressed[36] and HSP proteins are accumulated.[41] In insects, altered expression profiles of *HSPs* during temperature stress have been reported in many species. Originally reported from *D. melanogaster* in response to heat shock, *HSP70* and *HSP68* were up-regulated.[42] In *Lucillia cuprina*, the expression level of *HSP70* was significantly increased by heat shock at all developmental stages.[43] The transcription profiles of *HSP70* and *HSP90* of the oriental fruit moth, *G. molesta* from the same family, Tortricidae, were significantly induced by high-temperature treatment, with transcripts up-regulated 100-fold.[44] Apart from *HSP70* and *HSP90*, thermal-inducible *sHSPs* involved in protecting organisms against low-temperature stress were also reported in *S. crassipalpis*[33] and *Lymantria dispar*.[45] Intriguingly, the instantaneous (1 h) cold-shock (0 °C) and heat-shock (40 °C), followed by recovery at 25 °C for 1 h, did not significantly influence the normal physiological functions of third-instar *C. pomonella* larvae and only slightly induced expression of seven *HSP* transcripts observed from third-instar larvae of codling moth by heat or cold shock. These results align with *HSP83* from *L. cuprina*[43] and *HSP40* and *HSP70* from the kuruma shrimp, *Marsupenaeus japonicas*,[46] and *PaHSC70* from *Pyrrhocoris apterus*,[47] but not with previous characterization of *CpHSP19.8*, *CpHSP22.2*, and *CpHSP19.9* from *C. pomonella*.[20] The possible reason for such a large difference may be due to different sources and treatment methodologies used. However, given a longer cold- and heat-shock treatment time (2 and 4 h) without recovery at 25 °C, the abundances of *CpHSP19.9*, *CpHSP40*, *CpHSP70-1*, and *CpHsp90* were all significantly up-regulated. The expression level of *CpHSP19.9* was 94.27-fold increased after exposure of third-instar larvae for 4 h at 40 °C, and this value is much smaller than the result of Garczynski et al.,[20] who treated the same stage of *C. pomonella* larvae for 4 h at 42 °C. In this study, the transcript levels of seven *CpHSPs* in response to temperatures were investigated only in third-instar larvae. Thus, whether these *HSPs* are up-regulated or down-regulated in other stages of *C. pomonella* and how the level of heat-shock proteins is expressed after cold and heat shock should be further analyzed.

HSPs from other sources, such as *TcHSP90* from *Tetranychus cinnaharinus*[2] and *AlHSP90* from *Apolygus lucorum*,[4] were involved in the insect defense against temperature and insecticide stresses. However, in our study, weak changes in mRNA levels of the tested *CpHSPs* were observed in insecticide-exposed *C. pomonella* larvae, suggesting that this pest has a strong tolerance to insecticides, and such strong environmental adaptability contributes to its persisting as a serious pest in orchards. Notably, third-instar larvae rarely if ever come in contact with insecticides in the orchard as codling moth larvae are internal fruit feeders except for neonate larvae. Our current study did not provide complete life stage data for the codling moth, especially for the neonate larvae, which is the main stage contact with insecticides in the orchards; the result suggests that ingestion is an important means to determine the effects of a toxicant on the transcripts of *HSPs*.

Extreme temperatures in winter and summer pose a major challenge for the codling moth, and this pest might have evolved high levels of heat and cold tolerance during their overwintering and oversummering. Diapause is a critical biological process in insects, which involves many life-history parameters crucial for survival and reproductive fitness at both the individual and population levels.[48] Diapause is an evolutionary developmental pathway regulated by complex patterns of gene expression that result in the sequestration of nutrient reserves, suppression of metabolism, a halt or slowing of development, a decline of water content, and the acquisition of increased tolerance to environmental stresses such as low temperatures.[49] In insects, research on the linkage between diapause and *HSPs* expression has been described for many species. *HSP23* and *HSP70* are highly up-regulated during the overwintering pupal diapause of *S. crassipalpis*.[15,49] Down-regulation of *HSP83* is associated with photoperiodic diapause in *Drosophila montana*.[48] Similar down-regulation of the *HzHSP90* transcript from the corn earworm, *H. zea*, was reported during diapauses.[40] To our knowledge, there are at least 3 *HSC70s*, 14 *HSP70s*, 5 *HSP40s*, 2 *HSP90s*, 1 *HSC90*, 1 *HSP105*, 1 *HSP68*, and 14 *sHSPs* in addition to *sHSP19.8*, *19.9*, and *22.2*. This raises questions in the codling moth: why are there so many *HSPs*? Are these or additional *HSPs* involved in the diapause of codling moth? Do they each have a specific function? Future work is required to answer these questions.

To date, this is the most comprehensive study on identification, characterization, and expression profile of the HSPs in *C. pomonella*. Different expression patterns of the genes reveal that the *C. pomonella* HSPs are evolutionarily and functionally diversified and may be involved in the evolution of adaptive capacity in response to environmental stresses. Thus, further functional studies focused on these and more *CpHSPs* may facilitate a better understanding of the mechanisms involved in environmental adaptation of *C. pomonella* and its extreme temperature and insecticide tolerances.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b01914.

Tables S1−S3 and Figures S1−S4 (PDF)

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

AUTHOR INFORMATION

Corresponding Author

*(X.-Q.Y.) Phone: +86 024 88487148. Fax: +86 024 88487148. E-mail: sling233@hotmail.com.

Funding

This study was financially supported by the Scientific Research Foundation of Talent Introduction of Shenyang Agricultural University (20153011), the Open Project Program of Key Laboratory of Plant Protection Resources and Pest Management of Ministry of Education, the National Natural Science Foundation of China (Grant 31501666), and the General Project of Education Department of Liaoning Province (L5015489).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank John Richard Schrock from Emporia State University (Emporia, KS, USA) for proofreading the manuscript.

REFERENCES

(1) Bale, J. Insects and low temperatures: from molecular biology to distributions and abundance. *Philos. Trans. R. Soc., B* **2002**, *357*, 849−862.

(2) Feng, H.; Wang, L.; Liu, Y.; He, L.; Li, M.; Lu, W.; Xue, C. Molecular characterization and expression of a heat shock protein gene (HSP90) from the carmine spider mite, *Tetranychus cinnabarinus* (Boisduval). *J. Insect Sci.* **2010**, *10*, 112.

(3) Chidawanyika, F.; Terblanche, J. S. Rapid thermal responses and thermal tolerance in adult codling moth *Cydia pomonella* (Lepidoptera: Tortricidae). *J. Insect Physiol.* **2011**, *57*, 108−117.

(4) Sun, Y.; Sheng, Y.; Bai, L.; Zhang, Y.; Xiao, Y.; Xiao, L.; Tan, Y.; Shen, Y. Characterizing heat shock protein 90 gene of *Apolygus lucorum* (Meyer-Dür) and its expression in response to different temperature and pesticide stresses. *Cell Stress Chaperones* **2014**, *19*, 725−739.

(5) Rodríguez, M. A.; Marques, T.; Bosch, D.; Avilla, H. Assessment of insecticide resisitance in eggs and neonate larvae of *Cydia pomonella* (Lepidoptera: Tortricidae). *Pestic. Biochem. Physiol.* **2011**, *100*, 151−159.

(6) Yang, X. Q.; Zhang, Y. L. Investigation of insecticide-resistance status of *Cydia pomonella* in Chinese populations. *Bull. Entomol. Res.* **2015**, *105*, 316−325.

(7) Rozsypal, J.; Koštál, V.; Zahradníčkovč, H.; Ŝimek, P. Overwintering strategy and mechanisms of cold tolerance in the codling moth (*Cydia pomonella*). *PLoS One* **2013**, *8*, e61745.

(8) Fuentes-Contreras, E.; Reyes, M.; Barros, W.; Sauphanor, B. Evaluation of azinphos-methyl resistance and activity of detoxifying enzymes in codling moth (Lepidoptera: Tortricidae) from central Chile. *J. Econ. Entomol.* **2007**, *100*, 551−556.

(9) Vesala, L.; Salminen, T.; Laiho, A.; Hoikkala, A.; Kankare, M. Cold tolerance and cold-induced modulation of gene expression in two *Drosophila virilis* group species with different distributions. *Insect Mol. Biol.* **2012**, *21*, 107−118.

(10) Shi, M.; Wang, Y. N.; Zhu, N.; Chen, X. X. Four heat shock protein genes of the endoparasitoid wasp, *Cotesia vestalis*, and their transcriptional profiles in relation to developmental stages and temperature. *PLoS One* **2013**, *8*, e59721.

(11) Gehring, W. J.; Wehner, R. Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2994−2998.

(12) Huang, L. H.; Wang, C. Z.; Kang, L. Cloning and expression of five heat shock protein genes in relation to cold hardening and development in the leafminer. *J. Insect Physiol.* **2009**, *55*, 279−285.

(13) Joanisse, D.; Michaud, S.; Inaguma, Y.; Tanguay, R. Small heat shock proteins of *Drosophila*: developmental expression and functions. *J. Biosci.* **1998**, *23*, 369−376.

(14) Flannagan, R. D.; Tammariello, S. P.; Joplin, K. H.; Cikra-Ireland, R. A.; Yocum, G. D.; Denlinger, D. L. Diapause-specific gene expression in pupae of the flesh fly *Sarcophaga crassipalpis*. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5616−5620.

(15) Rinehart, J. P.; Li, A.; Yocum, G. D.; Robich, R. M.; Hayward, S. A.; Denlinger, D. L. Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 11130−11137.

(16) Johnston, J. A.; Ward, C. L.; Kopito, R. R. Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* **1998**, *143*, 1883−1898.

(17) Hartl, F. U. Molecular chaperones in cellular protein folding. *Nature* **1996**, *381*, 571−580.

(18) Jing, J.; Liu, H.; Chen, H.; Hu, S.; Xiao, K.; Ma, X. Acute effect of copper and cadmium exposure on the expression of heat shock protein 70 in the Cyprinidae fish *Tanichthys albonubes*. *Chemosphere* **2013**, *91*, 1113−1122.

(19) Shen, J. R.; Li, M. F.; Chen, N. Z.; Wang, J. J.; Wan, F. H. Cloning and analysis of the expression characteristics under heat stress of Hsp90 gene in the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). *Acta Entomol. Sinica* **2011**, *54*, 1236−1248.

(20) Garczynski, S. F.; Unruh, T. R.; Guédot, C.; Neven, L. G. Characterization of three transcripts encoding small heat shock proteins expressed in the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). *Insect Sci.* **2011**, *18*, 473−483.

(21) Landolt, P. J.; Suckling, D. M.; Judd, G. J. R. Positive interaction of a feeding attractant and a host kairomone for trapping the codling moth, *Cydia pomonella* (L.). *J. Chem. Ecol.* **2007**, *33*, 2236−2244.

(22) Luo, C.; He, X. H.; Chen, H.; Wei, Y. L.; Li, M. H. A high-efficient method of RACE technique for obtaining the gene 5′ end. *Plant Physiol. J.* **2011**, *47*, 409−414.

(23) Rose, R. L.; Goh, D.; Thompson, D. M.; Verma, K. D.; Heckel, D. G.; Gahan, L. J.; Roe, R. M.; Hodgson, E. Cytochrome P450 (CYP)9A1 in *Heliothis virescens*: the first member of a new CYP family. *Insect Biochem. Mol. Biol.* **1997**, *27*, 605−615.

(24) Hulo, N.; Bairoch, A.; Bulliard, V.; Cerutti, L.; Castro, E. D.; Langendijk-Genevaux, P. S.; Pagni, M.; Sigrist, C. J. A. The PROSITE database. *Nucleic Acids Res.* **2006**, *34*, D227−D230.

(25) Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596−1599.

(26) Crack, J. A.; Mansour, M.; Sun, Y.; MacRae, T. H. Functional analysis of a small heat shock/alpha-crystallin protein from *Artemia franciscana*. *Eur. J. Biochem.* **2002**, *269*, 933−942.

(27) Yang, X. Q.; Zhang, Y. L. Molecular cloning, sequence analysis and mRNA expression stability detection of *β-actin* gene in codling moth *Cydia pomonella* (L.). *J. Northwest A&F Univ. (Nat. Sci. Ed.)* **2014**, *42*, 135−140 (in Chinese).

(28) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **2001**, *25*, 402−408.

(29) Yin, X.; Wang, S.; Tang, J.; Hansen, J. D. Thermal resistance of fifth-instar *Cydia pomonella* (L). (Lepidoptera: Tortricidae) as affected by pretreatment conditioning. *J. Stored Prod. Res.* **2006**, *42*, 75−85.

(30) Laufen, T.; Mayer, M. P.; Beisel, C.; Klostermeier, D.; Mogk, A.; Reinstein, J.; Bukau, B. Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 5452−5457.

(31) Qian, Y. Q.; Patel, D.; Hartl, F. U.; McColl, D. J. Nuclear magnetic resonance solution structure of the human *Hsp40* (HDJ-1) J-domain. *J. Mol. Biol.* **1996**, *260*, 224−235.

(32) Hass, C.; Klein, U.; Kloetzel, P. M. Developmental expression of *Drosophila melanogaster* small heat-shock proteins. *J. Cell Sci.* **1990**, *96*, 413−418.

(33) Joplin, K. H.; Denlinger, D. L. Developmental and tissue specific control of the heat shock induced 70 kDa related proteins in the flesh fly. *J. Insect Physiol.* **1990**, *36*, 239−249.

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298

(34) Rybczynski, R.; Gilbert, L. I. cDNA cloning and expression of a hormone-regulated heat shock protein (Hsc70) from the prothoracic gland of *Manduca sexta*. *Insect Biochem. Mol. Biol.* **2000**, *30*, 579−589.
(35) Karouna-Renier, N. K.; Zehr, J. P. Short-term exposures to chroni-cally toxic copper concentrations induce HSP70 proteins in themidge larvae (*Chironomus tentans*). *Sci. Total Environ.* **2003**, *312*, 267−272.
(36) Mahroof, R.; Zhu, K. Y.; Neven, L.; Subramanyam, B.; Bai, J. Expression patterns of three heat shock protein 70 genes among developmental stages of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2005**, *141*, 247−256.
(37) Shu, Y.; Du, Y.; Wang, J. Molecular characterization and expression patterns of *Spodoptera litura* heat shock protein 70/90, and their response to zinc stress. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2011**, *158*, 102−110.
(38) Denlinger, D. L.; Rinehart, J. P.; Yocum, G. D. Stress proteins: a role in insect diapause. In *Insect Timing: Circadian Rhythmicity to Seasonality*; Denlinger, D. L., Rinehart, J. P., Yocum, G., Eds.; Elsevier: New York, 2001; pp 155−171.
(39) Tachibana, S. I.; Numata, H.; Goto, S. G. Gene expression of heat-shock proteins (*Hsp23*, *Hsp70* and *Hsp90*) during and after larval diapause in the blow fly *Lucilia sericata*. *J. Insect Physiol.* **2005**, *51*, 641−647.
(40) Zhang, Q.; Denlinger, D. L. Molecular characterization of heat shock protein 90, 70 and 70 cognate cDNAs and their expression patterns during thermal stress and pupal diapause in the corn earworm. *J. Insect Physiol.* **2010**, *56*, 138−150.
(41) Yin, X.; Wang, S.; Tang, J.; Hansen, J. D.; Lurie, S. Thermal conditioning of fifth-instar *Cydia pomonella* (Lepidoptera: Tortricidae) affects HSP70 accumulation and insect mortality. *Physiol. Entomol.* **2006**, *31*, 241−247.
(42) Artavanis-Tsakonas, S.; Schedl, P.; Mirault, M. E.; Moran, L.; Lis, J. Genes for the 70,000 Da heat shock protein in two cloned *Drosophila melanogaster* DNA segments. *Cell* **1979**, *17*, 9−18.
(43) Concha, C.; Edman, R.; Belikoff, E.; Schiemann, A.; Carey, B.; Scott, M. Organization and expression of the Australian sheep blowfly (*Lucilia cuprina*) hsp23, hsp24, hsp70 and hsp83 genes. *Insect Mol. Biol.* **2012**, *21*, 169−180.
(44) Chen, H.; Xu, X. L.; Li, Y. P.; Wu, J. X. Characterization of heat shock protein 90, 70 and their transcriptional expression patterns on high temperatures in adults of *Grapholita molesta* (Busck). *Insect Sci.* **2014**, *21*, 439−448.
(45) Yocum, G. D.; Joplin, K. H.; Denlinger, D. L. Expression of heat shock proteins inresponse to high and lowtemperature extremes in diapausing pharate larvae of the gypsy moth. *Arch. Insect Biochem. Physiol.* **1991**, *18*, 239−249.
(46) Danwattananusorn, T.; Fagutao, F. F.; Shitara, A.; Kondo, H.; Aoki, T.; Hirono, I. Molecular characterization and expression analysis of heat shock proteins 40, 70 and 90 from kuruma shrimp *Marsupenaeus japonicus*. *Fish. Sci.* **2011**, *77*, 929−937.
(47) Koštál, V.; Tollarová-Borovanská, M. The 70 kDa heat shock protein assists during the repair of chilling injury in the insect, *Pyrrhocoris apterus*. *PLoS One* **2009**, *4*, e4546.
(48) Kankare, M.; Salminen, T.; Laiho, A.; Vesala, L.; Hoikkala, A. Changes in gene expression linked with adult reproductive diapause in a northern malt fly species: a candidate gene microarray study. *BMC Ecol.* **2010**, *10*, 3.
(49) Rinehart, J. P.; Yocum, G. D.; Denlinger, D. L. Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect Biochem. Mol. Biol.* **2000**, *30*, 515−521.

DOI: 10.1021/acs.jafc.6b01914
J. Agric. Food Chem. 2016, 64, 4288−4298