Comparative Biochemistry and Physiology, Part A 205 (2017) 48–57

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Transcription of four *Rhopalosiphum padi* (L.) heat shock protein genes and their responses to heat stress and insecticide exposure

Yuting Li [a], Qi Zhao [a], Xinle Duan [a], Chunman Song [b], Maohua Chen [a,*]

[a] College of Plant Protection, Northwest A&F University, State Key Laboratory of Crop Stress Biology for Arid Areas, Key Laboratory of Crop Pest Integrated Pest Management on the Loess Plateau of Ministry of Agriculture, Yangling 712100, China.
[b] Technology Center of China Tobacco Yunnan Industrial Co., Ltd., Kunming, Yunnan 650106, China.

ARTICLE INFO

Article history:
Received 30 July 2016
Received in revised form 14 December 2016
Accepted 20 December 2016
Available online 24 December 2016

Keywords:
Development
Heat shock protein
Heat stress
Insecticide
Rhopalosiphum padi

ABSTRACT

The bird cherry-oat aphid, *Rhopalosiphum padi* (L.), a worldwide destructive pest, is more heat tolerant than other wheat aphids, and it has developed resistance to different insecticides. Heat shock proteins (HSPs) play an important role in coping with environmental stresses. To investigate *Hsp* transcriptional responses to heat and insecticide stress, four full-length *Hsp* genes from *R. padi* (*RpHsp60*, *RpHsc70*, *RpHsp70-1*, and *RpHsp70-2*) were cloned. Four *RpHsps* were expressed during all *R. padi* developmental stages, but at varying levels. The mRNA levels of *RpHsps* were increased under thermal stress and reached maximal induction at a lower temperature (36 °C) in the alate morph than in the apterous morph (37 °C or 38 °C). *RpHsp* expressions under heat stress suggest that *RpHsp70-1* and *RpHsp70-2* are inducible in both apterous and alate morphs, *RpHsc70* is only heat-inducible in apterous morph, and *RpHsp60* exhibits poor sensitivity to heat stress. The pretreatment at 37 °C significantly increase both the survival rate and the *RpHsps* expression level of *R. padi* at subsequent lethal temperature. Under exposure to two sublethal concentrations (LC_{10} and LC_{30}) of beta-cypermethrin, both *RpHsp70-1* and *RpHsp70-2* expressions were induced and reached a maximum 24 h after exposure. In contrast, expression of *RpHsp60* was not induced by either sublethal concentration of beta-cypermethrin. Moreover, the responses of *RpHsp70-1* and *RpHsp70-2* to heat shock were more sensitive than those to beta-cypermethrin. These results suggest that induction of *RpHsp* expression is related to thermal tolerance, and that *RpHsp70-1* and *RpHsp70-2* are the primary genes involved in the response to both heat and pesticide stress.

1. Introduction

Heat shock proteins (HSPs) are a well-known group of proteins that help organisms respond to environmental stress, including heat stress and insecticide exposure (Kim et al., 2015; King and MacRae, 2015; Sørensen et al., 2003; Sun et al., 2014). Under stress conditions, they usually act as molecular chaperones, promoting correct folding and preventing aggregation of denatured proteins or newly synthesized polypeptides (Feder and Hofmann, 1999; Johnston et al., 1998; Sørensen et al., 2003). They are also involved in development and diapause in insects (Dean et al., 2016; Haass et al., 1990; King and MacRae, 2015; Okada et al., 2014). Based on molecular weight and amino acid sequence homology, HSPs can be classified into five families: HSP90, HSP70, HSP60, HSP40, and small HSPs (Feder and Hofmann, 1999; Sørensen et al., 2003). The HSP70 family is one of the most highly conserved and best studied and includes both inducible HSP70 and constitutive heat shock cognate 70 (HSC70) (Karlin and Brocchieri, 1998; Kregel, 2002). *Hsp60* is located mainly in the mitochondria of eukaryotic cells, but it is encoded by nuclear DNA and synthesized in the cytoplasm (Neupert, 1997).

Temperature is a major environmental factor affecting the survival, growth, development, population abundance, and geographic distribution of insects (Clarke, 2003; Luo et al., 2015). Due to the impact of global warming, the frequency and degree of high-temperature conditions are expected to rise substantially (Diffenbaugh et al., 2005; Easterling et al., 2000). Many behavioral and physiological strategies to avoid thermal stress and maintain thermotolerance have evolved in organisms. *Hsps* are best known for their functions in increasing thermotolerance and as protectors under thermal stress (Advani et al., 2016; Dahlgaard et al., 1998; Hoffmann et al., 2003; Lindquist, 1986; Lu et al., 2016; Sørensen et al., 2005).

In addition to ambient temperature, insecticides are another important stress factor for insects. Insecticides can affect many physical and biochemical processes in insects, including *Hsp* responses (Chen and Zhang, 2015; Gupta et al., 2005; Sun et al., 2014, 2016). *Hsps* may provide potential biomarkers for assessing insecticide risk in many insects (Chen and Zhang, 2015; Mukhopadhyay et al., 2002; Nazir et al.,

* Corresponding author at: College of Plant Protection, Northwest A&F University, Yangling 712100, China.
E-mail address: maohua.chen@nwsuaf.edu.cn (M. Chen).

http://dx.doi.org/10.1016/j.cbpa.2016.12.021
1095-6433/

2001) and may contribute to insecticide resistance (Yoshimi et al., 2002).

The bird cherry-oat aphid (*Rhopalosiphum padi* L.) is considered one of the most destructive insect pests worldwide (Duan et al., 2016; Hansen, 2000). *R. padi* has two wing morphs, alate (winged), with a high flight ability, and apterous (wingless), with a high reproductive capacity (Braendle et al., 2006). Previous studies found that apterous individuals are more heat resistant than alates in *R. padi* (Yang et al., 1995). Among the three main wheat aphids, *R. padi* is more tolerant to high temperature than *Schizaphis graminum* and *Sitobion avenae* (Ma and Ma, 2007). Frequent extreme high temperature events have led to *R. padi* becoming the dominant species in wheat fields in China (Ma et al., 2015). The application of insecticides is the main management strategy to control aphids, leading to resistance of *R. padi* to various insecticides (Zuo et al., 2016). However, little information is available on the molecular mechanisms underlying the differential thermotolerance of the two morphs. Whether *Hsp*s play significant roles in adaption to high temperatures and in the response to insecticides is unclear.

In the present study, we cloned and identified four full-length cDNAs of *Hsp* genes (*Hsp60*, *Hsc70*, *Hsp70-1*, and *Hsp70-2*) from *R. padi*. We observed transcriptional expression of these four *Hsp* genes during different developmental stages and compared the differences in mRNA expression levels of the four *Hsp* genes between apterous and alate morphs after heat shock. The thermotolerance and *Hsp* gene expression of *R. padi* under pretreatment with thermal stress were analyzed. The expression profiles of *Hsp*s in response to insecticide treatment were also investigated.

Table 1
Primers used for RT-PCR and quantitative PCR.

Gene	Primer name	Primer sequence (5′-3′)	Size (bp)	PCR type
Hsp60	60F1	AGTGCGAAAACTTATGCTTG	1098	PCR
	60R1	ATACCAGATGCTAACCTTGC		
	60F2	GCACCTGGATTTGGAGAT	879	PCR
	60R2	ACTTGCGATGTCAGCCTT		
	60GSP1	CTGTTGTTGTGCCATCACCAGCTTC	463	5′-RACE
	60GSP2	GCATCATTATTGACTACAGCGGAAGC	462	3′-RACE
	Rp60F	TTCTGTTTTGTGGCGTGG	1879	ORF
	Rp60R	CTTGCGATGTCAGCCTTT		
	60-qF	TTGGACAAGAAGGCAATGAAC	223	qPCR
	60-qR	AGATGCTAACCTCGCAAGACG		
Hsc70	70F1	CATCAATGAACCTACAGCCG	1064	PCR
	70R1	AGGTGGTGAAAGTTTGGGTC		
	70GSP1	GCTTGCTTGGGTAGACGAGGAAAGAGTA	1009	5′-RACE
	70GSP2	CAAGCGTAACACAACCATCCCCAC	857	3′-RACE
	70F	GCAGTGGTATCAACGCAG	2165	ORF
	70R	GTAGGAATGTGCTGATGAAT		
	70-qF	GTTCTACCCGTATCCCCAAGG	110	qPCR
	70-qR	TACAGCGGCACCGTAAGCA		
Hsp70-1	70-1F	GATTTGCCGATTTGCGTTA	1958	ORF
	70-1R	TCTTCATCATCAGAGCCGA		
	70-1qF	CCGACACCGAACGATTGA	174	qPCR
	70-1qR	TTTTAGGCTTCCCGCAGT		
Hsp70-2	70-2F	ATGTGTGGGTATCTGGCAAC	1862	ORF
	70-2R	CGACTTCTTCGACGGTAGGT		
	70-2qF	AACCTGTCCATCAATCCC	160	qPCR
	70-2qR	TTTTGGTCATCACTCCGC		

2. Materials and methods

2.1. Insects

R. padi samples were initially collected from wheat (*Triticum aestivum* L.) in Yangling, Shaanxi Province, China in 2012 and reared at 24 ± 1 °C and 70% relative humidity and under a photoperiod of 16:8 h (light: dark). A colony of *R. padi* was established on seedlings of the wheat cv. Xiaoyan 22 in a plastic cage to prevent infestations of other pests and the entry of natural enemies. Newly emerged adults were removed to plastic petri dishes containing wheat. Then, newly born nymphs (the first-instar), newly molted nymphs (the second-, third-, and fourth-instars), and 1-day old apterous aphids were used for developmental stage analysis. The aphids were frozen immediately in liquid nitrogen and stored at −70 °C until use. Each developmental stage included three replicates.

2.2. Molecular cloning of the four Hsp genes

Total RNA was extracted from 15 1-day-old apterous adults using TRIzol reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. The quality and concentration of the obtained RNA were determined using a biophotometer (Eppendorf BioPhotometer Plus, Eppendorf, Germany). First-strand cDNA was synthesized from 2 μg total RNA using oligo$(dT)_{15}$ primers and the Reverse Transcription System (Promega, WI, USA) following the manufacturer's instructions. Single-stranded cDNA for 3′-rapid amplification of cDNA ends (3′-RACE) and 5′-RACE experiments were synthesized from 1 μg RNA using the SMART RACE cDNA Amplification Kit (Clontech, CA, USA).

Primers designed using Primer Premier 5.0 (Premier Biosoft International, CA, USA) were used to amplify the partial segments and 5′- and 3′-termini of *Hsp* genes by PCR (Table 1). To ensure that the 5′ and 3′ fragments were derived from the same gene, specific primer sets flanking each ORF were designed and then used to amplify the entire ORF sequence. Primers amplifying the *Hsp70-1* and *Hsp70-2* ORFs were designed based on sequence data from the *R. padi* transcriptome (data not shown). PCR was performed in a solution containing 1 μL cDNA, 0.4 μM each primer, 100 μM each dNTP, 4 mM Mg^{2+}, 10 × PCR reaction buffer, 2 units Taq DNA polymerase (5 U/μL, Sangon Biotech Co., Ltd., Shanghai, China), and distilled water in a total volume of 25 μL. PCR conditions were set as follows: initial denaturation at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 1 min; final extension at 72 °C for 10 min. RACE PCR was performed according to the instructions included in the SMART RACE cDNA Amplification Kit (Clontech, CA, USA). PCR products were purified from 1% agarose gels using the Wizard PCR Preps kit (Promega, WI, USA). The purified fragment was cloned into the pGEM-T Easy vector (Promega) and transformed into *E. coli* DH5α-competent cells. Positive clones were selected using a blue-white screen and sequenced (Sangon Biotech Co., Ltd., Shanghai, China).

2.3. Sequences and phylogenetic analysis

Similarity searches for the nucleotide and amino acid sequences were conducted using the BLAST program from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nih.gov/BLAST/). The inferred amino acid sequences were analyzed using DNAMAN software (version 6.0, Lynnon Biosoft, Quebec, Canada). ORFs were identified using the assistance of the ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight and theoretical isoelectric point were calculated using the SWISS-PROT (ExPASy server) tool "Compute pI/Mw" (http://au.expasy.org/tools/pi_tool.html). The Maximum-Likelihood (ML) method in MEGA7 software (Kumar et al., 2016) was used to construct a phylogenetic tree based on known amino acid sequences of insect HSPs. Bootstrap analysis was carried out, and the confidence of each branch was estimated using 1000 replicates.

2.4. Treatment under heat and beta-cypermethrin stresses

Twenty-one-day-old adult aphids (apterous and alate adults) were placed on wheat leaves in a plastic petri dish with wet filter paper and heated in a dry bath incubator (Allsheng Instruments, Hangzhou, China). Adult aphids were heated at temperatures of 36, 37, 38, 39, and 40 °C for 1 h and at 36 °C for 1, 2, 3, 4, and 5 h, then allowed to recover at 24 °C for 1 h. Adult aphids reared at 24 °C were used as controls.

Apterous adult aphids were collected for thermotolerance assays. Control treatments were placed at 24 °C for 3 h. Apterous adult aphids for pretreatment treatments (PT) were placed in incubator at 37 °C for 1 h, then removed and placed at 24 °C for 2 h. Apterous adult aphids for heat shock treatments (HS) were placed at 24 °C incubator for 2 h then removed to 41 °C for 1 h. Apterous adult aphids for pretreatment plus heat shock treatments(PH) were firstly received 1 h pretreatment as above, followed by 1 h at 24 °C, then removed to 41 °C for 1 h as above. Following the PT, HS and PH treatments above, the aphids were transferred to 24 °C for 1 h recovery. The surviving *R. padi* after recovery were checked. The thermotolerance was scaled by survival rate in each treatment. Three independent biological replications were performed for each treatment. After treatment, all surviving aphids were flash-frozen in liquid nitrogen and stored at −70 °C until RNA exaction.

The leaf dipping method (Moores et al., 1996) was used as a bioassay for beta-cypermethrin (96% purity) (Yancheng Nongbo Bio-technology Co., Ltd., Jiangsu, China). Three replicates of 30–50 apterous aphids were used to evaluate each insecticide concentration, while 6–7 serial concentrations were used for the beta-cypermethrin test. Based on the initial test results, two beta-cypermethrin concentrations, 0.3987 and 0.9280 mg/L, were chosen for the following experiments, which correspond to the sublethal concentrations LC_{10} and LC_{30}, respectively. For the insecticide treatments, wheat leaves with apterous adult aphids were dipped in the beta-cypermethrin dilutions for 10 s. Then, these leaves were removed from the solution, and residual solution droplets on the leaves were adsorbed using clean, dry filter paper pieces. For the controls, wheat leaves with apterous adult aphids were dipped in distilled water. Aphids were then placed in a plastic petri dish and allowed to feed on wheat leaves for 12, 24, or 36 h. At the end of the test, all surviving aphids were quickly frozen in liquid nitrogen and stored at −70 °C until RNA extraction.

2.5. Quantitative real-time PCR (qPCR) analysis

A qPCR assay was performed to examine the *Hsp* gene mRNA levels. The primers used are shown in Table 1. Total RNA was isolated from individuals (4 mg) from each developmental stage and each treatment. qPCR was performed in a 20 μL total reaction volume containing 10 μL 2 × FastStart Essential DNA Green Master™ (Roche, Shanghai, China), 0.8 μL each gene-specific primer (10 μM) (Table 1), 1 μL cDNA template, and 6.4 μL RNase-free water in an iCycler iQ5 (Bio-Rad, CA, USA). β-actin was used as the reference gene (Kang et al., 2016). Thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and elongation at 72 °C for 20 s. The melting curve was obtained by raising the temperature from 65 °C to 95 °C for 10 s at 0.5 °C increments. All experiments were performed in both technical and biological triplicates. The relative expression levels were calculated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001).

2.6. Statistical analysis

All data are presented as mean ± standard error (S.E.) relative expression levels. The significance of the differences was determined using either *t*-test for comparison of two means or one-way analysis of variance followed by Tukey's test for multiple comparisons. The significance level was set at a value of $P < 0.05$. All statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cloning and characterization of RpHsps

The full-length coding sequences of four *Hsp* genes from *R. padi* were cloned. One showed high similarity with the known *Hsp60s* from other insects, whereas three were similar to members of the HSP70 family. These *Hsp* genes were named *RpHsp60*, *RpHsc70*, *RpHsp70-1*, and *RpHsp70-2*. The cDNA and protein characteristics of the four *Hsp* genes are shown in Table 2. The deduced amino acid sequence of *RpHsp60* contains an ATP-binding motif (KDGVITVKDGKTLEDELEV), a classical mitochondrial HSP60 family signature motif (AAVEEGIVPGGG), and a typical C-terminal GGM repeat motif (Fig. 1). Of the other three genes, *RpHsc70* was identified as *Hsc70* (Chen et al., 2006). The three deduced proteins contained HSP70 family signature sequences (Fig. 2). The ATP/GTP-binding site and non-organellar consensus motif were found in all deduced proteins. Meanwhile, the conserved C-terminal motif EEVD of cytoplasmic HSP70s was observed. Sequences in the N-terminus were more conserved than those in the C-terminus according to alignments of the three RpHSP70s (Fig. 2).

BLAST results from GenBank indicated high homology to *Hsp* genes from other insects. The deduced amino acid sequence of *RpHsp60* was 94% identical to the sequence of HSP60 from *Acyrthosiphon pisum* (XP_008178869), 94% identical to that from *Diuraphis noxia* (XP_015366602), and 93% identical to that from *Myzus persicae* (CAB58441). The similarity of RpHSP60 to other insect HSP60s was 73–81%. RpHSP70-1 showed 95%, 94%, and 93% identity with HSP70 from *A. pisum* (XP_001945786), *Aphis glycines* (AHG94986), and *D. noxia* (XP_015373896), respectively, while RpHSP70-2 showed 96%, 98%, and 93% similarity with HSP70 from *A. pisum* (XP_001945786), *A. glycines* (AHG94986), and *D. noxia* (XP_015373896), respectively. The similarity of the two inducible RpHSP70s to other insect HSP70s ranged from 77 to 86%. RpHSC70 displayed 99%, 99%, and 98% identity with HSC70 from *A. pisum* (XP_001951207), *D. noxia* (XP_015366535), and *A. glycines* (AFO70211), respectively. The similarity of RpHSC70 to other insect HSC70s was 85–94%.

3.2. Phylogenetic analysis of RpHSPs

A phylogenetic tree was constructed using MEGA7 based on HSP sequences from other insects to investigate the relationships among them. The amino acid sequence of *RpHsp60* is closest to those of other aphids (Fig. 3A). In addition, the distances between HSP60s were very close for taxa within the same insect order (Fig. 3A). The phylogenetic tree of HSP60 shows that it segregates in a similar manner to the different insect orders. Fig. 3B shows that the HSP70 amino acid sequences were divided into three clusters: HSP70 in the cytosol, HSP70 in the endoplasmic reticulum (ER), and HSP70 in the mitochondrion. The three HSP70 members from *R. padi* were clustered in the cytosol group. The cytosol cluster is divided into two branches, inducible HSP70 and HSC70. *RpHsp70-1* and *RpHsp70-2* were clustered in the inducible HSP70 branch, while *RpHsc70* was clustered in the HSP70 cognate branch (Fig. 3B).

3.3. Expression of RpHsps during different developmental stages

The mRNA expression levels of these four *Hsps* in *R. padi* during different developmental stages are shown in Fig. 4. The *RpHsps* were expressed during all developmental stages but at varying levels. *RpHsp60* transcripts did not differ significantly during the nymph stages ($F = 0.411, P = 0.749$) but were significantly higher than levels of adult

Table 2
Characteristics of four *R. padi Hsps*.

Gene name	GenBank accession no.	Full length (bp)	ORF (bp)	Deduced amino acids		
				Length (aa)	Molecular weight (kDa)	Isoelectric point (pI)
RpHsp60	KU311036	2184	1728	575	60.7	5.44
RpHsp70-1	KU311037	2378	1923	641	70.0	5.47
RpHsp70-2	KU311038	2233	1911	637	69.6	5.30
RpHsc70	KU311039	2343	1959	653	71.3	5.33

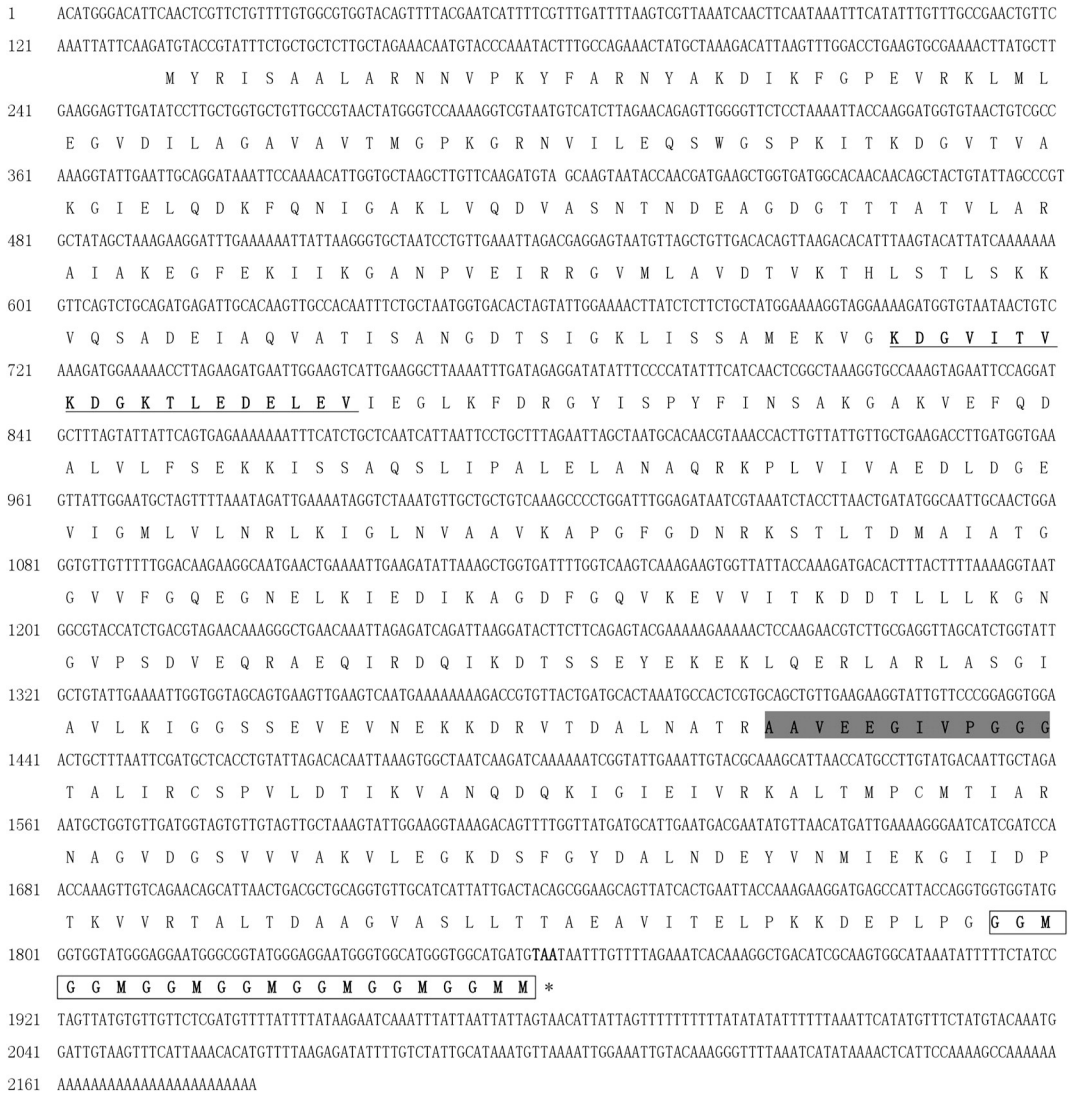

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *RpHsp60*. The ATP-binding motif is underlined. The classical mitochondrial HSP60 signature motif is shown in grey. The typical GGM repeat motif of the HSP60 family is shown within the box.

stage. The mRNA levels of *RpHsc70* and *RpHsp70-1* in the adult were higher than those in nymphs. *RpHsc70* transcript levels in 4th instar nymphs were higher than those in other instar nymphs, which did not significantly differ from levels during the adult stage. The relative *RpHsp70-1* mRNA levels of 3rd and 4th instar nymphs were lower than those of the other instars, and 2nd instar nymphs had the highest expression level of all nymph stages. Similarly, the *RpHsp70-2* mRNA level was the lowest in 4th instar nymphs among the developmental stages, and the expression level of *RpHsp70-2* was the highest in 2nd instar nymphs compared with other instar nymphs and adults.

3.4. Expression profiles of RpHsps in response to heat shock

The relative mRNA expression levels of the four *RpHsp*s were quantified by qPCR following exposure of apterous and alate adult aphids to heat shock for 1 h. Compared with the control, the transcription levels of these four *Hsp* genes were influenced by heat shock in both morphs. The expression of these four genes peaked at 37 °C or 38 °C in apterous aphids but at 36 °C in alate aphids. The expression of *RpHsp60* in apterous adults did not differ significantly between control (24 °C) and heat-shocked individuals (from 36 °C to 40 °C) ($F = 1.157$, $P = 0.384$) (Fig. 5). Moreover, *RpHsp60* transcripts in alate aphids were not significantly increased by the treatments, with the exception of the 36 °C treatment ($F = 3.235$, $P = 0.082$). *RpHsc70* expression levels in alate adults peaked at 36 °C and then declined as the temperature increased from 37 °C to 40 °C. In contrast, *RpHsc70* transcripts in apterous adults increased remarkably at 36 °C and achieved maximum levels at 38 °C. Additionally, expression of *RpHsc70* was lower in alate adults than in apterous adults under heat stress. The expression levels of *RpHsp70-1* and *RpHsp70-2* were similar under heat stress. In alate aphids, *RpHsp70-1* and *RpHsp70-2* expression decreased with increasing temperature, but in apterous aphids, an increase was seen at 37 or 38 °C. It is important to note that the degree of induction of the four *Hsp* mRNAs in response to heat stress differed significantly, with *RpHsp70-1* exhibiting the highest level and *RpHsc70* the lowest level of maximal mRNA induction (Fig. 5).

3.5. Expression of RpHsps at 36 °C at different time points

The expression patterns of *RpHsp60*, *RpHsc70*, *RpHsp70-1*, and *RpHsp70-2* after exposure to 36 °C for 1–5 h are presented in Fig. 6. No significant changes were detected in *RpHsp60* expression levels in response to 36 °C temperature from 1 to 5 h in apterous and alate adults (apterous: $F = 0.186$, $P = 0.936$; alate: $F = 0.612$, $P = 0.672$). The expression of *RpHsc70* in alate aphids remained constant after 36 °C heat shock for 1–5 h ($F = 1.837$, $P = 0.198$). A significant decrease in

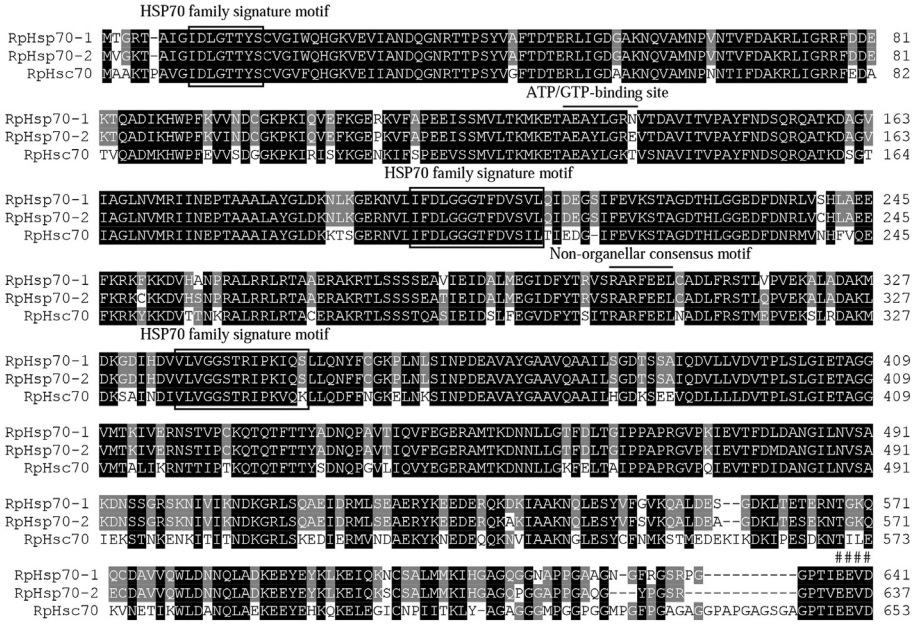

Fig. 2. Amino acid sequence alignment of three RpHSP70s from *R. padi*. The HSP70 family signature motifs are shown in the box. The ATP/GTP-binding site and non-organellar consensus motif are underscored above the sequences. The pound sign denotes the C-terminal motif EEVD.

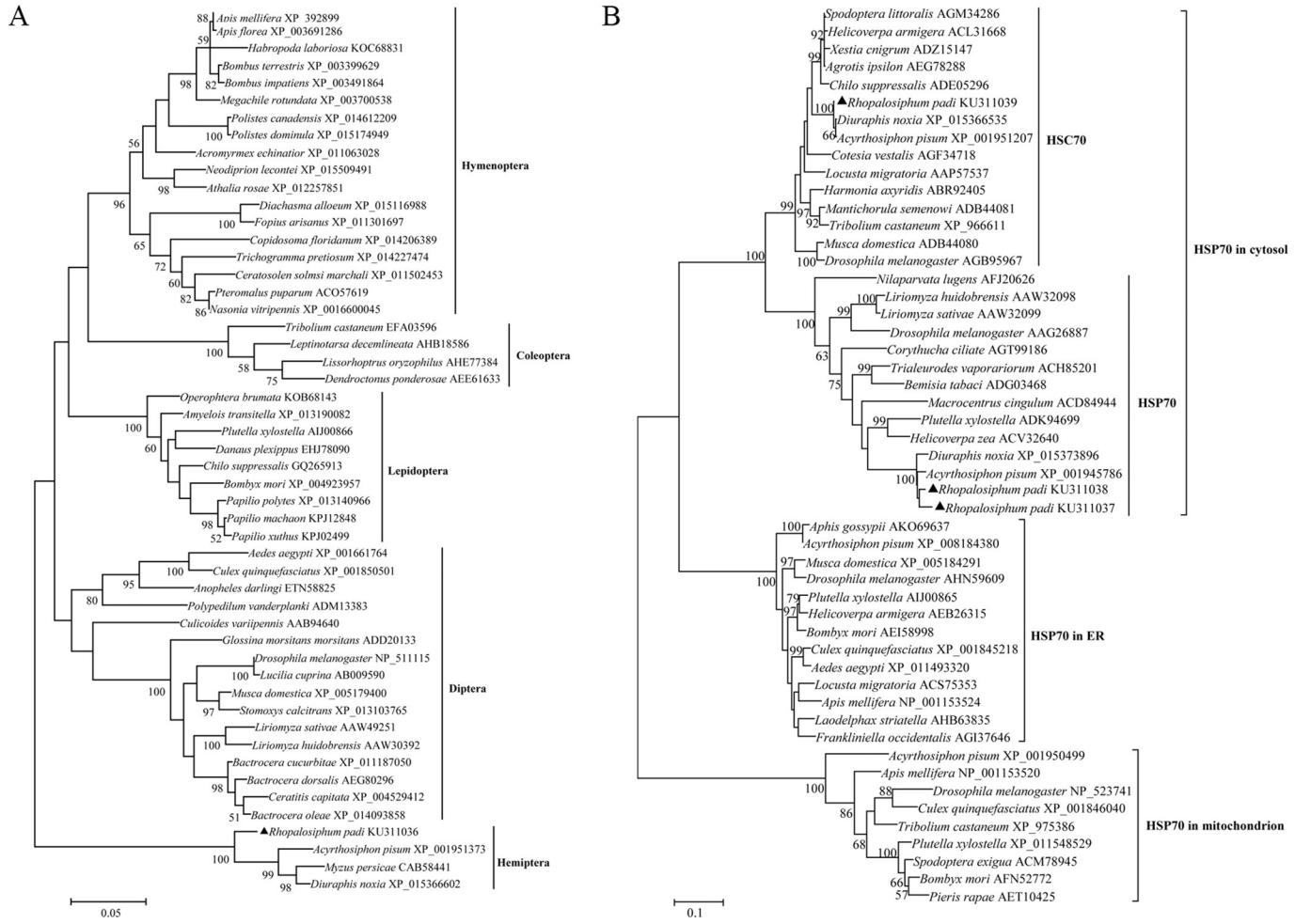

Fig. 3. Phylogenetic tree based on amino acid sequence alignment of HSP60s (A) and HSP70s (B) from insects. Sequences were downloaded from the GenBank protein database. Sequence labels are indicated by the species name and GenBank accession number. The triangle denotes the *R. padi* HSP sequence obtained.

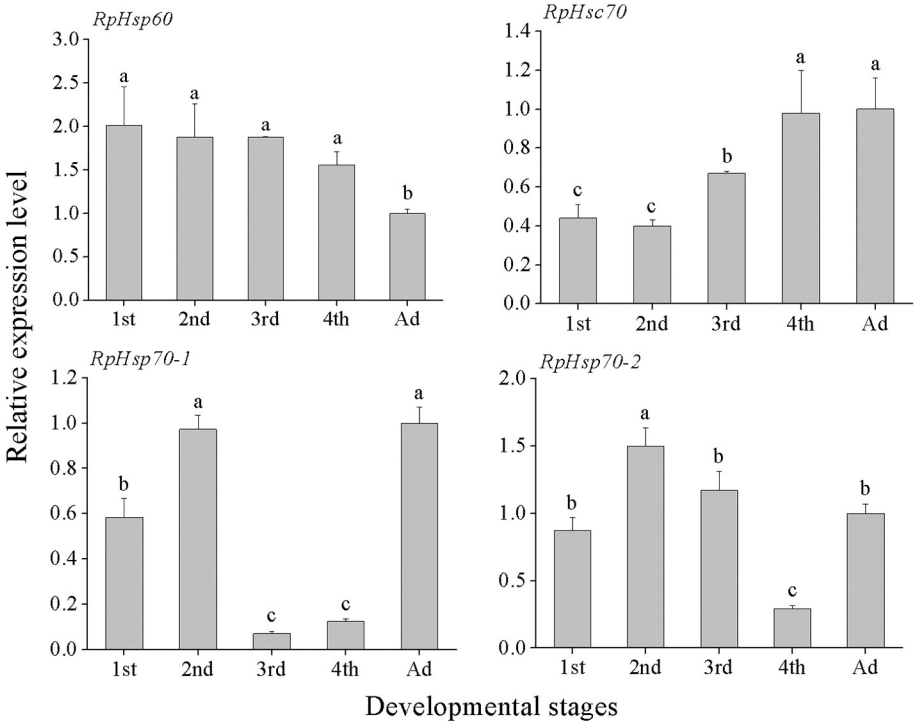

Fig. 4. Relative expression of *R. padi Hsps* during different developmental stages. Data are presented as means ± S.E. The mRNA level in the adult was arbitrarily set to 1.0. The different letters above the error bars indicate significant differences among stages (Tukey's test, $P < 0.05$). 1st: first instar nymphs; 2nd: second instar nymphs; 3rd: third instar nymphs; 4th: fourth instar nymphs; AD: apterous adults.

RpHsc70, *RpHsp70-1*, and *RpHsp70-2* expression was observed at 2 h compared with 1 h in apterous aphids. Moreover, the expression of *RpHsc70*, *RpHsp70-1*, and *RpHsp70-2* remained at relatively higher levels after 5 h in both apterous and alate aphids. Interestingly, alate aphids after stress expressed higher levels of *RpHsp70-1* and *RpHsp70-2* than those of apterous aphids.

3.6. Thermotolerance and expression of RpHsps

R. padi apterous adults showed significant plasticity in thermotolerance, indicating by the significant different survival rates of aphids among thermotolerance treatments ($F = 107.283$, $P = 0.0001$) (Fig. 7A). All apterous adults survived at Control and PT treatments.

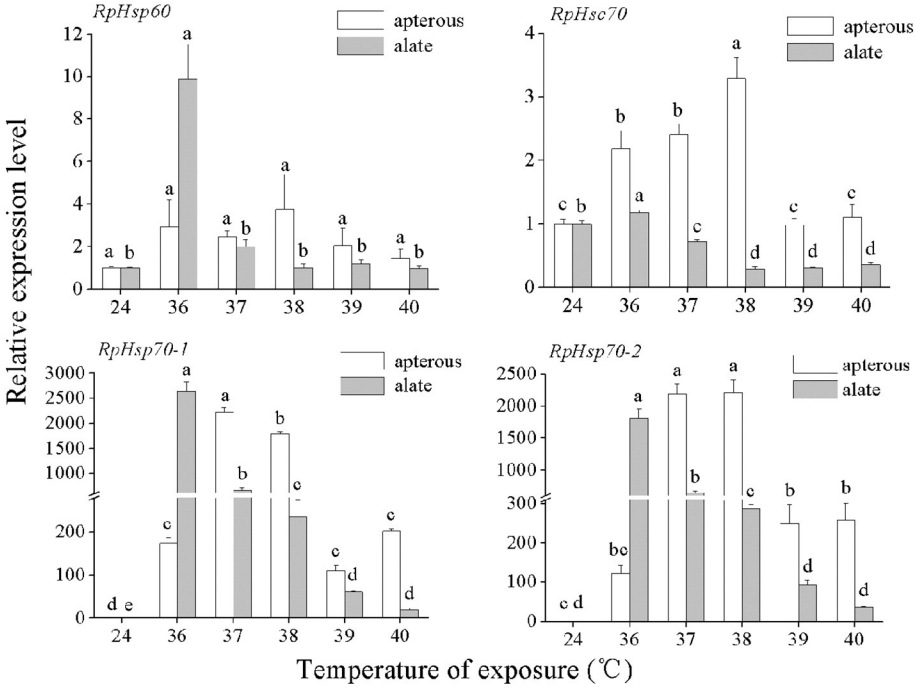

Fig. 5. Relative expression of *R. padi Hsps* under thermal stress for 1 h. Expression levels were first normalized to the abundance of the reference gene, β-actin, and then to the 24 °C control. Data are presented as means ± S.E. Different letters above the error bars indicate significant differences among treatment temperatures by Tukey's test ($P < 0.05$).

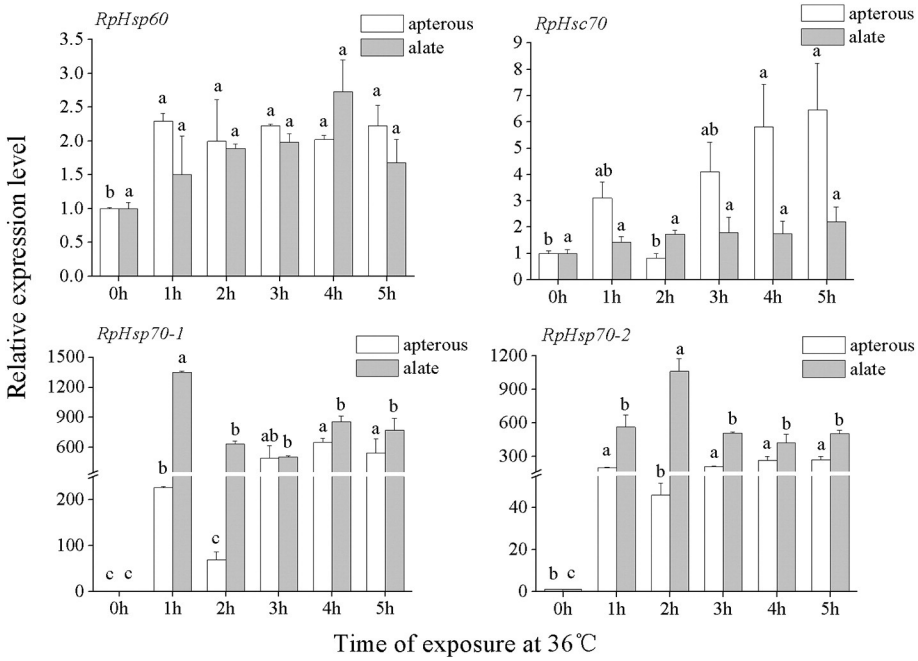

Fig. 6. Relative expression of *R. padi Hsps* under different time durations at 36 °C. Expression levels were first normalized to the abundance of the reference gene, β-actin, and then to the 24 °C control. Data are presented as means ± S.E. The different letters above the error bars indicate significant differences among treatment times by Tukey's test ($P < 0.05$).

The survival rate after 41 °C heat shock (HS) was 5.48%, whereas the pretreatment at 37 °C increased the survival rate of 41 °C heat shock treatment to 22.90%. Compared to the control, the expressions of four *RpHsps* in the two types of pretreatments (PT and PH) were significantly increased compared to the control, whereas the expressions of four *RpHsps* in HS treatments were not significantly increased (Fig. 7B, C).

3.7. Expression of RpHsps after exposure to beta-cypermethrin

The mRNA levels of the four *RpHsp* genes (*RpHsp60*, *RpHsc70*, *RpHsp70-1*, and *RpHsp70-2*) were influenced by exposure to sublethal concentrations (0.3987 and 0.9280 mg/L) of beta-cypermethrin (Fig. 8). After exposing apterous adult aphids to the two sublethal concentrations, the expression of *RpHsp60* was significantly lower than that of the control (Fig. 8). After exposure to 0.3987 mg/L and 0.9280 mg/L beta-cypermethrin, the expression level of *RpHsc70* peaked at 24 h (1.44-fold for 0.3987 mg/L; 0.84-fold for 0.9280 mg/L) and did not significantly differ among the treatment time points (0.3987 mg/L: $F = 2.045$, $P = 0.275$; 0.9280 mg/L: $F = 1.641$, $P = 0.330$). The mRNA levels of *RpHsp70-1* and *RpHsp70-2* were significantly higher at 24 h than at 12 h or 36 h for both concentrations of beta-cypermethrin. Furthermore, the transcription levels of *RpHsp70-1* and *RpHsp70-2* were higher after exposure to 0.9280 mg/L than 0.3987 mg/L beta-cypermethrin (Fig. 8). In the case of beta-cypermethrin stress, maximal induction of *RpHsp70-1* and *RpHsp70-2* mRNA synthesis was more than two orders of magnitude lower compared with heat stress (Figs. 5 and 8).

4. Discussion

In this study, four *Hsp* genes from *R. padi*, *Hsp60*, *Hsc70*, *Hsp70-1*, and *Hsp70-2*, were cloned and identified for the first time. As expected, the conserved sequences and characteristic motifs of HSP60 and HSP70 family members were observed in the deduced amino sequences. The typical C-terminal GGM repeat motif and signature "AAVEEGIVPGGG" of RpHSP60 indicate that *RpHsp60* belongs to the mitochondrial HSP60 family (Huang and Kang, 2007). The highly conserved ATP-binding motif of HSP60 may indicate that a similar mechanism exists in the substrate-refolding process among HSP60s coupled to ATP hydrolysis

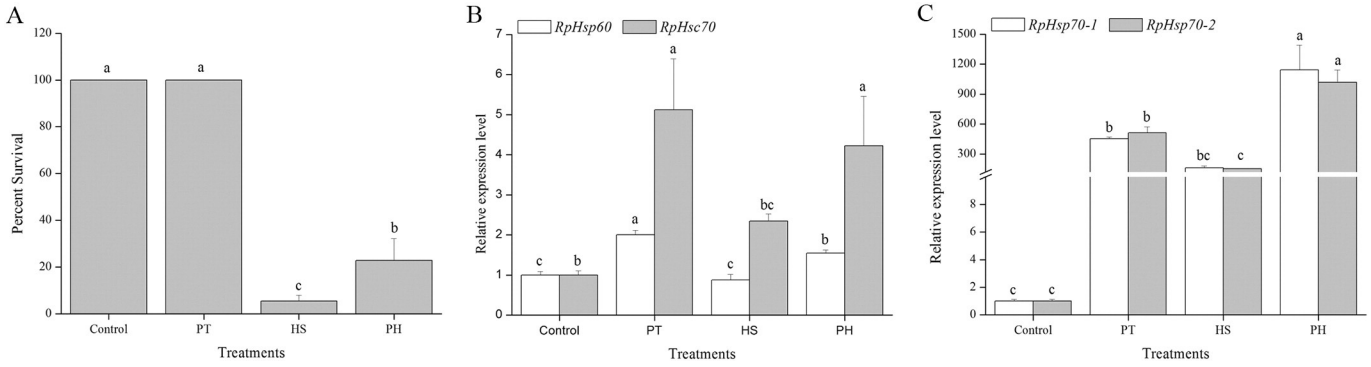

Fig. 7. Survival rate of *R. padi* apterous adults (A) and relative expression of *R. padi Hsps* (B, C) under different treatments. Data are presented as means ± S.E. The different letters above the error bars indicate significant differences among treatments by Tukey's test ($P < 0.05$). PT, apterous aphids were placed in incubator at 37 °C for 1 h, then removed and placed at 24 °C for 2 h. HS, apterous aphids were placed at 24 °C incubator for 2 h, then removed to 41 °C for 1 h. PH, apterous aphids were firstly received 1 h pretreatment as above, followed by 1 h at 24 °C, then removed to 41 °C for 1 h. All aphids were transferred to 24 °C for 1 h recovery after PT, HS and PH treatments. Expression levels were first normalized to the abundance of the reference gene, β-actin, and then to the control.

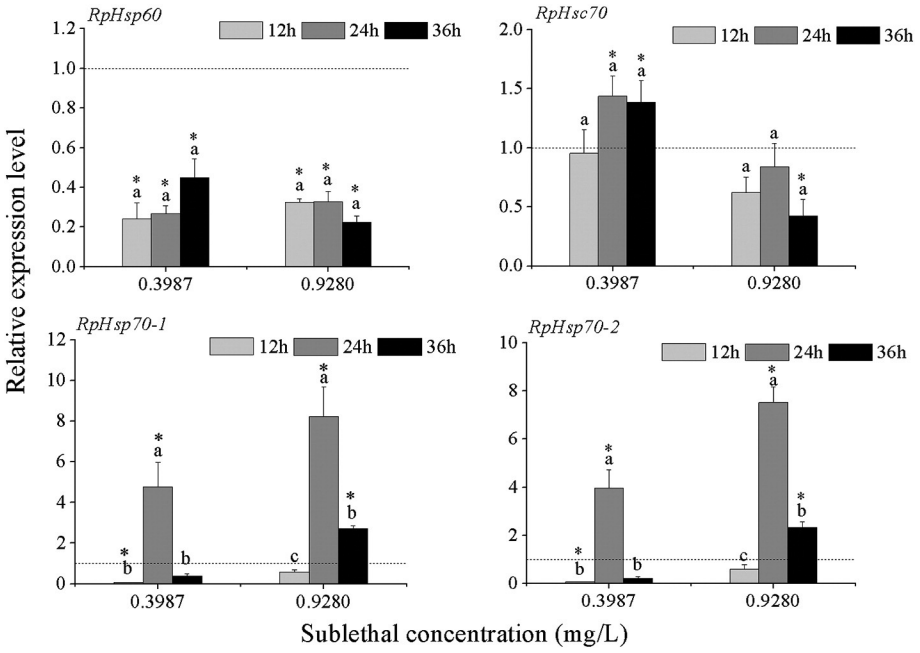

Fig. 8. The mRNA expression levels of four *RpHsps* in response to beta-cypermethrin in *R. padi*. Expression levels were first normalized to the abundance of the reference gene, β-actin, and then to the control. Data are presented as means ± S.E. The different letters on the error bars indicate significant differences between the indicated hours post-exposure at the 0.05 level (Tukey's test). The asterisk above the bars indicates a significant difference between the treatment and corresponding control (*t*-test, $P < 0.05$).

(Wong et al., 2004). Our results underscore that members of the HSP70 family are highly conserved, and their C-terminal regions are often more divergent than their N-terminal regions. The variation in the C-terminal sequences may determine the functional specificity of individual HSPs (Luo et al., 2015). The conserved "EEVD" motif, located at the C-terminus, confirmed that the three *R. padi* HSP70s are cytosolic homologs and may bind to other co-chaperones (Daugaard et al., 2010). Furthermore, some HSP70s have GGMP repeats at the C-terminus, whereas other HSP70 family members lack such structural elements (Chen et al., 2014; Huang and Kang, 2007; Luo et al., 2015; Moribe et al., 2010; Wang and Kang, 2005). In our study, only RpHSC70 was found to possess the GGMP tetrapeptide. The similarities between *RpHsps* and their counterparts in other insect species indicate that *RpHsps* play crucial roles in the Hsp chaperone system in response to environmental stresses.

The phylogenetic tree suggests that HSP60s cluster according to the taxonomy of the different insect orders, and RpHSP60 is closest to those obtained from other aphids. Therefore, HSP60 is highly conserved and an informative phylogenetic marker at the ordinal taxonomic level within insects (Abdallah et al., 2000; Wang et al., 2014). The phylogenetic tree of HSP70s is divided into three clades according to localization, implying that the specific functions of the different HSP70 members diverged before the speciation of these insects (Luo et al., 2015). Furthermore, the cytosol group was clustered into two branches, inducible HSP70 and cognate HSP70, indicating a different evolutionary mode between them (Luo et al., 2015).

Hsps are involved in the development of many insect species (Haass et al., 1990; King and MacRae, 2015; Okada et al., 2014), and species-specific differences in Hsp transcription have been observed frequently (Craig et al., 1983; Huang et al., 2009; Mahroof et al., 2005; Sharma et al., 2007; Wang et al., 2014). *Hsp60* levels increased gradually in L. *huidobrensis* during development (Huang et al., 2009) and were significantly elevated in adult females of *Chilo suppressalis* (Lu et al., 2014) and *Neoseiulus cucumeris* (Chen et al., 2015). Meanwhile, *Hsp70* is highly variably expressed during different developmental stages, such as larvae, eggs, and adults (Chen et al., 2015; Jiang et al., 2012; Shu et al., 2011). Expression of *Rphsp60* was significantly higher in nymphs than in apterous adults, implying that *RpHsp60* may be related to nymph development. The expression of *RpHsc70* was similar to that of homologs in *Drosophila melanogaster* and *Plutella xylostella*, with higher expression in adults than in larvae (Craig et al., 1983; Sonoda et al., 2006). The expression of *RpHsp70-1* was high in young nymphs (1st and 2nd instar nymphs) and adults, which is consistent with results from the red flour beetle *Tribolium castaneum* (Mahroof et al., 2005). *RpHsp70-2* gene expression in 4th instar nymphs was lower than that in other instar nymphs and adults. The different expression patterns of *RpHsps* suggest that they could play different roles during various developmental stages.

Temperature is a major environmental factor determining the population abundance and geographic distribution of insects (Lu et al., 2016). As a result of global warming, insects are expected to encounter higher temperatures in the future (Diffenbaugh et al., 2005; Easterling et al., 2000). The induction of Hsps may provide protection against this environmental stress, and the levels of inducible *Hsps* are associated with thermotolerance (Lu et al., 2016). In the present study, the expression levels of four *R. padi Hsp* genes were increased under heat stress and after different exposure times at 36 °C in apterous and alate aphids. The expression of *Hsp60* appears to be species-specific in response to heat stress in insects, as it is inducible by thermal stress in some insects (Cui et al., 2010; Huang and Kang, 2007; Sharma et al., 2006), but not in others (Chen et al., 2015; Sørensen et al., 2005; Wong et al., 2004). Our data indicate that *RpHsp60* expression was significantly increased only in alate adults at 36 °C for 1 h, suggesting that *RpHsp60* exhibits poor sensitivity to heat stress. *RpHsc70* expression was heat-inducible only in apterous adults under heat shock. In addition, the relative expression of *RpHsp70-1* was approximately three orders of magnitude higher than that of *RpHsc70* under heat stress. For example, the maximum expression of *RpHsp70-1* in alate aphids was 2645.50-fold, while that of *Rphsc70* was only 1.18-fold. Moreover, *RpHsp70-1* and *RpHsp70-2* had different expression profiles compared with *Rphsc70*. This phenomenon suggests a different response mechanism of *Hsc70* to heat stress (Luo et al., 2015; Yocum, 2001). Among the HSP superfamily, *Hsp70* was more responsive to heat stress than were the other Hsps in insects (Chen et al., 2014; Huang and Kang, 2007; Wang et al., 2014; Zhang and Denlinger, 2010). This indicates that HSP70 plays a predominant role

in thermotolerance in insects. Under different exposure times at 36 °C, the expression of *RpHsps* varied with time, but it was maintained for 5 h. Similar response patterns were reported in *Musca domestica* (Tang et al., 2012), *Grapholita molesta* (Chen et al., 2014), and *Neoseiulus cucumeris* (Chen et al., 2015). In addition, a reduction in *RpHsp* expression was found at 39 °C and 40 °C, indicating that *Hsps* can respond to high temperature but not beyond a certain tolerance (Cui et al., 2010; Huang and Kang, 2007; Kristensen et al., 2002). These data imply that *RpHsps* are involved in the response to thermal stress.

Heat stress-induced *Hsp* expression varies in different morphs and sexes of insects (Chen et al., 2014; Lu et al., 2016). Aphids exhibit wing dimorphism, producing both wingless and winged morphs, which differ strongly in morphology, physiology, behavior, life history, and capacity to cope with environmental stress (Braendle et al., 2006). Huang and Kang (2007) demonstrated that the temperatures for maximal induction (T_{max}) of *Hsps* can represent differences in temperature tolerance. Our results showed that *RpHsp* T_{max} in apterous aphids was commonly 1–2 °C higher than that in alate aphids under heat stress, confirming that the apterous morph is more heat tolerant than alate morph (Yang et al., 1995). Winged aphids benefit from their mobility, which allows them to avoid severe heat that may activate the expression of *Hsps*. This may result in different responses and fitnesses in wingless and winged aphids, regulating physiological homeostasis through differential expression patterns of *Hsps* under heat stress. Insect thermotolerance showed plasticity and can be remarkably improved by pretreatment with thermal stress (Huang et al., 2007; Shen et al., 2014). Our results showed that pretreatment (1 h exposure to 37 °C) significantly improved survival of *R. padi* adults. In addition, Hsps play a role in mediating thermal tolerance, and Hsp expression is related to thermotolerance (Advani et al., 2016). In this study, pretreatment at 37 °C remarkably increased the expressions of four Hsp genes at subsequent lethal temperature. Our results were in agreement with the previous studies in other insect species (Bettencourt et al., 2008; Shen et al., 2014). The results that pretreatment under thermal stress significantly increase both the survival rate and the *RpHsps* expression level at subsequent lethal temperature suggest that the increasing expression of heat shock proteins highly relates to the thermotolerance.

The insect *Hsp* response to insecticide pressure has received increasing attention (Gupta et al., 2007; Sharma et al., 2008; Tungjitwitayakul et al., 2016; Wang et al., 2014). Currently, *R. padi* has developed resistance to various types of insecticides, including beta-cypermethrin (Lu and Gao, 2009; Zuo et al., 2016), which is now widely used for aphid management in China. In the current study, while *RpHsc70* expression increased only at the lower sublethal concentration of beta-cypermethrin (0.3987 mg/L), the relative expression of *RpHsp70-1* and *RpHsp70-2* increased dramatically after 24 h of exposure to 0.3987 mg/L beta-cypermethrin and after 24 h and 36 h of exposure to 0.9280 mg/L beta-cypermethrin, demonstrating time-dependent induction. However, the expression of *RpHsp60* was decreased by both sublethal concentrations. Down-regulation of *Hsp60* was reported in *Myzus persicae* exposed to imidacloprid (Ayyanath et al., 2014). Increased expression of *Hsp70* was seen in response to chlorfenapyr, cypermethrin, avermectin, dichlorvos, and insecticide mixtures (Doganlar and Doganlar, 2015; Gupta et al., 2005; Mukhopadhyay et al., 2002; Sonoda and Tsumuki, 2007). However, *Hsp70* expression was not induced by protiofos, permethrin, chlorfluazuron, methomyl, or thiocyclam in *Mamestra brassicae* (Sonoda and Tsumuki, 2007) or by dimethoate in *Leptinotarsa decemlineata* (Brom et al., 2015). These results suggest that different *Hsp* genes exhibit insecticide-specific responses depending on the duration and severity of the stress.

Acknowledgment

This work was funded by the National Natural Science Foundation of China (Grants Nos. 31471766, 31272036), the Doctoral Program Foundation of Institutions of Higher Education of China (20110204110001), and the National Key Technology R&D Program of the Ministry of Science and Technology of China (2012BAK11B03).

References

Abdallah, M.A., Pollenz, R.S., Nunamaker, R.A., Murphy, K.E., 2000. Identification and characterization of a cDNA clone encoding the heat shock protein (Hsp60) from the biting midge, *Culicoides variipennis sonorensis* Wirth and Jones. Biochem. Genet. 38, 154–162.

Advani, N.K., Kenkel, C.D., Davies, S.W., Parmesan, C., Singer, M.C., Matz, M.V., 2016. Variation in heat shock protein expression at the latitudinal range limits of a widely-distributed species, the Glanville fritillary butterfly (*Melitaea cinxia*). Physiol. Entomol. 41, 241–248.

Ayyanath, M.M., Cutler, G.C., Scott-Dupree, C.D., Prithiviraj, B., Kandasamy, S., Prithiviraj, K., 2014. Gene expression during imidacloprid-induced hormesis in green peach aphid. Dose-Response 12, 480–497.

Bettencourt, B.R., Hogan, C.C., Nimali, M., Drohan, B.W., 2008. Inducible and constitutive heat shock gene expression responds to modification of Hsp70 copy number in *Drosophila melanogaster* but does not compensate for loss of thermotolerance in Hsp70 null flies. BMC Biol. 6, 1.

Braendle, C., Davis, G.K., Brisson, J.A., Stern, D.L., 2006. Wing dimorphism in aphids. Heredity 97, 192–199.

Brom, K.R., Dolezych, B., Tarnawska, M., Brzozowska, K., Nakonieczny, M., 2015. Expression of the Hsp40, Hsp70 and Hsp90 proteins in Colorado potato beetle (*Leptinotarsa decemlineata* Say) after the dimethoate treatment. J. Entomol. Res. Soc. 17, 39–49.

Chen, X.N., Zhang, Y.L., 2015. Identification of multiple small heat-shock protein genes in *Plutella xylostella* (L.) and their expression profiles in response to abiotic stresses. Cell Stress Chaperones 20, 23–35.

Chen, B., Kayukawa, T., Monteiro, A., Ishikawa, Y., 2006. Cloning and characterization of the HSP70 Gene, and its expression in response to diapauses and thermal stress in the onion maggot, *Delia antiqua*. J. Biochem. Mol. Biol. 39, 749–758.

Chen, H., Xu, X.L., Li, Y.P., Wu, J.X., 2014. Characterization of heat shock protein 90, 70 and their transcriptional expression patterns on high temperature in adult of *Grapholita molesta* (Busck). Insect Sci. 21, 439–448.

Chen, W., Li, D.S., Zhang, M., Zhao, Y.L., Wu, W.J., Zhang, G.R., 2015. Cloning and differential expression of five heat shock protein genes associated with thermal stress and development in the polyphagous predatory mite *Neoseiulus cucumeris* (Acari: Phytoseiidae). Exp. Appl. Acarol. 67, 65–85.

Clarke, A., 2003. Costs and consequences of evolutionary temperature adaptation. Trends Ecol. Evol. 18, 573–581.

Craig, E.A., Ingolia, T.D., Manseau, L.J., 1983. Expression of *Drosophila* heat-shock cognate genes during heat shock and development. Dev. Biol. 99, 418–426.

Cui, Y.D., Du, Y.Z., Lu, M.X., Qiang, C.K., 2010. Cloning of the heat shock protein 60 gene from the stem borer, *Chilo suppressalis*, and analysis of expression characteristics under heat stress. J. Insect Sci. 10, 100.

Dahlgaard, J., Loeschcke, V., Michalak, P., Justesen, J., 1998. Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. Funct. Ecol. 12, 786–793.

Daugaard, M., Rohde, M., Jäättelä, M., 2010. The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions. FEBS Lett. 581, 3702–3710.

Dean, C.A., Teets, N.M., Koštál, V., Šimek, P., Denlinger, D.L., 2016. Enhanced stress responses and metabolic adjustments linked to diapause and onset of migration in the large milkweed bug *Oncopeltus fasciatus*. Physiol. Entomol. 41, 152–161.

Diffenbaugh, N.S., Pal, J.S., Trapp, R.J., Giorgi, F., 2005. Fine-scale processes regulate the response of extreme events to global climate change. Proc. Natl. Acad. Sci. U. S. A. 102, 15774–15778.

Doganlar, O., Doganlar, B.Z., 2015. Responses of antioxidant enzymes and heat shock proteins in drosophila to treatment with a pesticide mixture. Arch. Biol. Sci. 67, 869–876.

Duan, X.L., Peng, X., Qiao, X.F., Chen, M.H., 2016. Life cycle and population genetics of bird cherry-oat aphids *Rhopalosiphum padi* in China: an important pest on wheat crops. J. Pest Sci. http://dx.doi.org/10.1007/s10340-016-0752-9.

Easterling, D.R., Meehl, G.A., Parmesan, C., Changnon, S.A., Karl, T.R., Mearns, L.O., 2000. Climate extremes: observations, modeling, and impacts. Science 289, 2068–2074.

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61, 243–282.

Gupta, S.C., Siddique, H.R., Saxena, D.K., Kar Chowdhuri, D., 2005. Hazardous effect of organophosphate compound, dichlorvos in transgenic *Drosophila melanogaster* (*Hsp70-lacZ*): induction of *Hsp70*, *anti*-oxidant enzymes and inhibition of acetylcholinesterase. Biochim. Biophys. Acta Gen. Subj. 1725, 81–92.

Gupta, S.C., Siddique, H.R., Mathur, N., Vishwakarma, A.L., Mishra, R.K., Saxena, D.K., Chowdhuri, D.K., 2007. Induction of *hsp70*, alteration in oxidative stress markers and apoptosid against dichlorvos exposure in transgenic *Drosophila melanogaster*: modulation by reactive oxygen species. Biochim. Biophys. Acta Gen. Subj. 1770, 1382–1394.

Haass, C., Klein, U., Kloetzel, P.M., 1990. Developmental expression of *Drosophila melanogaster* small heat-shock proteins. J. Cell Sci. 96, 413–418.

Hansen, L.M., 2000. Establishing control threshold for bird cherry-oat aphid (*Rhopalosiphum padi* L.) in spring barley (*Hordeum vulgare* L.) by aphid-days. Crop Prot. 19, 191–194.

Hoffmann, A.A., Sørensen, J.G., Loeschcke, V., 2003. Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. J. Therm. Biol. 28, 175–216.

Huang, L.H., Kang, L., 2007. Cloning and interspecific altered expression of heat shock protein genes in two leafminer species in response to thermal stress. Insect Mol. Biol. 16, 491–500.
Huang, L.H., Chen, B., Kang, L., 2007. Impact of mild temperature hardening on thermotolerance, fecundity, and Hsp gene expression in *Liriomyza huidobrensis*. J. Insect Physiol. 53, 1199–1205.
Huang, L.H., Wang, C.Z., Kang, L., 2009. Cloning and expression of five heat shock protein genes in relation to cold hardening and development in the leafminer, *Liriomyza sativa*. J. Insect Physiol. 55, 279–285.
Jiang, X.F., Zhai, H.F., Wang, L., Luo, L.Z., Sappington, T.W., Zhang, L., 2012. Cloning of the heat shock protein 90 and 70 genes from the beet armyworm, *Spodoptera exigua*, and expression characteristics in relation to thermal stress and development. Cell Stress Chaperones 17, 67–80.
Johnston, J.A., Ward, C.L., Kopito, R.R., 1998. Aggresomes: a cellular response to misfolded proteins. J. Cell Biol. 143, 1883–1898.
Kang, X.L., Zhang, M., Wang, K., Qiao, X.F., Chen, M.H., 2016. Molecular cloning, expression pattern of multidrug resistance associated protein 1 (MRP1, ABCC1) gene, and the synergistic effects of verapamil on toxicity of two insecticides in the bird cherry-oat aphid. Arch. Insect Biochem. Physiol. 92, 65–84.
Karlin, S., Brocchieri, L., 1998. Heat shock protein 70 family: multiple sequence comparisons, function, and evolution. J. Mol. Evol. 47, 565–577.
Kim, H., Yu, Y.S., Lee, K.Y., 2015. Differential induction of heat shock protein genes to the combined treatments of heat with diatomaceous earth, phosphine or carbon dioxide on *Plodia interpunctella*. Entomol. Res. 45, 332–338.
King, A.M., MacRae, T.H., 2015. Insect heat shock proteins during stress and diapause. Annu. Rev. Entomol. 60, 59–75.
Kregel, K.C., 2002. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J. Appl. Physiol. 92, 2177–2186.
Kristensen, T.N., Dahlgaard, J., Loeschcke, V., 2002. Inbreeding affects Hsp70 expression in two species of *Drosophila* even at benign temperatures. Evol. Ecol. Res. 4, 1209–1216.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Boil. Evol., msw054
Lindquist, S., 1986. The heat-shock response. Annu. Rev. Biochem. 55 (1), 1151–1191.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.
Lu, Y., Gao, X.W., 2009. Multiple mechanisms responsible for differential susceptibilities of *Sitobion avenae* (Fabricius) and *Rhopalosiphum padi* (Linnaeus) to pirimicarb. Bull. Entomol. Res. 99, 611–617.
Lu, M.X., Liu, Z.X., Cui, Y.D., Du, Y.Z., 2014. Expression patterns of three heat shock proteins in *Chilo suppressalis* (Lepidoptera: Pyralidae). Ann. Entomol. Soc. Am. 107, 667–673.
Lu, K., Chen, X., Liu, W.T., Zhou, Q., 2016. Identification of a heat shock protein 90 gene involved in resistance to temperature stress in two wing-morphs of *Nilaparvata lugens* (Stål). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 197, 1–8.
Luo, S.Q., Ahola, V., Shu, C., Xu, C.R., Wang, R.J., 2015. Heat shock protein 70 gene family in the *Glanville fritillary* butterfly and their response to thermal stress. Gene 556, 132–141.
Ma, G., Ma, C.S., 2007. Upper critical temperatures for behaviors of three species of cereal aphids in leaf temperature gradients. Acta Ecol. Sin. 27, 2449–2459.
Ma, G., Rudolf, V.H., Ma, C.S., 2015. Extreme temperature events alter demographic rates, relative fitness, and community structure. Glob. Chang. Biol. 21, 1794–1808.
Mahroof, R., Zhu, K.Y., Neven, L., Subramanyam, B., Bai, J., 2005. Expression patterns of three heat shock protein 70 genes among developmental stages of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 141, 247–256.
Moores, G.D., Gao, X.W., Denholm, I., Devonshire, A.L., 1996. Characterisation of insensitive acetylcholinesterase in insecticide-resistant cotton aphids, *Aphis gossypii* Glover (Homoptera: Aphididae). Pestic. Biochem. Physiol. 56, 102–110.
Moribe, Y., Oka, K., Niimi, T., Yamashita, O., Yaginuma, T., 2010. Expression of heat shock protein 70a mRNA in *Bombyx mori* diapause eggs. J. Insect Physiol. 56, 1246–1252.
Mukhopadhyay, I., Nazir, A., Saxena, D.K., Chowdhuri, D.K., 2002. Toxicity of cypermethrin: hsp70 as a biomarker of response in transgenic *Drosophila*. Biomarkers 7, 501–510.
Nazir, A., Mukhopadhyay, I., Saxena, D.K., Kar Chowdhuri, D., 2001. Chlorpyrifos induced *hsp70* expression and effect on reproductive performance in transgenic *Drosophila melanogaster* (*hsp70-lacZ*) Bg^9. Arch. Environ. Contam. Toxicol. 41, 443–449.
Neupert, W., 1997. Protein import into mitochondria. Annu. Rev. Biochem. 66, 863–917.
Okada, Y., Teramura, K., Takahashi, K.H., 2014. Heat shock proteins mediate trade-offs between early-life reproduction and late survival in *Drosophila melanogaster*. Physiol. Entomol. 39, 304–312.
Sharma, S., Reddy, P.V.J., Rohilla, M.S., Tiwari, P.K., 2006. Expression of HSP60 homologue in sheep blowfly *Lucilia cuprina* during development and heat stress. J. Therm. Biol. 31, 546–555.
Sharma, S., Rohilla, M.S., Tiwari, P.K., 2007. Developmental and hyperthermia-induced expression of the heat shock proteins HSP60 and HSP70 in tissues of the housefly *Musca domestica*: an *in vitro* study. Genet. Mol. Biol. 30, 159–168.
Sharma, S., Rohilla, M.S., Reddy, P.V., Tiwari, P.K., 2008. In vitro induction of 60-kDa and 70-kDa heat shock proteins by endosulphan and monocrotophos in sheep blowfly *Lucilia cuprina*. Arch. Environ. Contam. Toxicol. 55, 57–69.
Shen, Y., Gong, Y.J., Gu, J., Huang, L.H., Feng, Q.L., 2014. Physiological effect of mild thermal stress and its induction of gene expression in the common cutworm, *Spodoptera litura*. J. Insect Physiol. 61, 34–41.
Shu, Y.H., Du, Y., Wang, J.W., 2011. Molecular characterization and expression patterns of *Spodoptera litura* heat shock protein 70/90, and their response to zinc stress. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 158, 102–110.
Sonoda, S., Tsumuki, H., 2007. Induction of heat shock protein genes by chlorfenapyr in cultured cells of the cabbage armyworm, *Mamestra brassicae*. Pestic. Biochem. Physiol. 89, 185–189.
Sonoda, S., Ashfaq, M., Tsumuki, H., 2006. Cloning and nucleotide sequencing of three heat shock protein genes (*hsp90*, *hsc70* and *hsp19.5*) from the diamondback moth, *Plutella xylostella* (L.) and their expression in relation to developmental stage and temperature. Arch. Insect Biochem. Physiol. 62, 80–90.
Sørensen, J.G., Kristensen, T.N., Loeschcke, V., 2003. The evolutionary and ecological role of heat shock proteins. Ecol. Lett. 6, 1025–1037.
Sørensen, J.G., Norry, F., Scannapeico, A.C., Loeschcke, V., 2005. Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the new world. J. Evol. Biol. 18, 829–837.
Sun, Y., Sheng, Y., Bai, L.X., Zhang, Y.J., Xiao, Y.F., Xiao, L.B., Tan, Y.A., Shen, Y.M., 2014. 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 19, 725–739.
Sun, Y., Zhao, J., Sheng, Y., Xiao, Y.F., Zhang, Y.J., Bai, L.X., Tan, Y., Xiao, L.B., Xu, G.C., 2016. Identification of heat shock cognate protein 70 gene (*Alhsc70*) of *Apolygus lucorum* and its expression in response to different temperature and pesticide stresses. Insect Sci. 23, 37–49.
Tang, T., Wu, C., Li, J.Q., Ren, G.D., Huang, D.W., Liu, F.S., 2012. Stress-induced HSP70 from *Musca domestica* plays a functionally significant role in the immune system. J. Insect Physiol. 58, 1226–1234.
Tungjitwitayakul, J., Tatun, N., Vajarasathira, B., Sakurai, S., 2016. Effects of ultraviolet-C and microwave irradiation on the expression of heat shock protein genes in the maize weevil (Coleoptera: Curculionidae). Eur. J. Entomol. 113, 135–142.
Wang, X.H., Kang, L., 2005. Differences in egg thermotolerance between tropical and temperate populations of the migratory locust *Locusta migratoria* (Orthoptera: Acridiidae). J. Insect Physiol. 51, 1277–1285.
Wang, H.H., Reitz, S.R., Wang, L.X., Wang, S.Y., Xue, L.I., Lei, Z.R., 2014. The mRNA expression profiles of five heat shock protein genes from *Frankliniella occidentalis* at different stages and their responses to temperatures and insecticides. J. Integr. Agric. 13, 2196–2210.
Wong, C.S., Mak, C.H., Ko, R.C., 2004. Cloning and characterization of the mitochondrial heat-shock protein 60 gene of *Trichinella spiralis*. Parasitol. Res. 93, 461–467.
Yang, Y.Z., Dai, Z.Y., Shen, W.H., Zhao, T.F., 1995. A comparasive study of some biological traits between alatae and apterous of bird cherry-oat aphid. J. Jiangsu Agric.Coll. 16, 31–34.
Yocum, G.D., 2001. Differential expression of two *HSP70* transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. J. Insect Physiol. 47, 1139–1145.
Yoshimi, T., Minowa, K., Karouna-Renier, N.K., Watanabe, C., Sugaya, Y., Miura, T., 2002. Activation of a stress-induced gene by insecticides in the midge, *Chironomus yoshimatsui*. J. Biochem. Mol. Toxicol. 16, 10–17.
Zhang, Q.R., Denlinger, D.L., 2010. 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. 56, 138–150.
Zuo, Y.Y., Wang, K., Zhang, M., Peng, X., Piñero, J.C., Chen, M.H., 2016. Regional susceptibilities of *Rhopalosiphum padi* (Hemiptera: Aphididae) to ten insecticides. Fla. Entomol. 99, 269–275.