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

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**A B S T R A C T**

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 alate morph, and *RpHsp60* exhibits poor sensitivity to heat stress. The pretreatment at 37 °C significantly increase both the survival rate and the *RpHsp* expression level of *R. padi* at subsequent lethal temperature. Under exposure to two sublethal concentrations (LC10 and LC30) 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.

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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 thermostolerance have evolved in organisms. Hsps are best known for their functions in increasing thermostolerance 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., 2016).
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 Mo, 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 Hsps 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 Hsps in response to insecticide treatment were also investigated.

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 Mg2+, 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 2 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.nlm.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.

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**Table 1**

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Apteronous adult aphids were collected for thermotolerance assays. Control treatments were placed at 24 °C for 3 h. Apteronous 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. Apteronous adult aphids for heat shock treatments (HS) were placed at 24 °C incubator for 2 h then removed to 41 °C for 1 h. Apteronous 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 extraction.

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 apteronous 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 LC50 and LC90, respectively. For the insecticide treatments, wheat leaves with apteronous 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 apteronous 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^−ΔΔCT 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 (KDGVTVDKDGKTELELEV), a classical mitochondrial HSP60 family signature motif (AAVEEGVPGGG), 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 EEDV of cytoplasmic HSP70s was observed. Sequences in the N-terminus were more conserved than in 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 *Acrystosiphon pisum* (XP_008178869), 94% identical to that from *Duaphis noxia* (XP_015366602), and 93% identical to that from *Myzus persicae* (CAH58441). The similarity of *RpHsp60* to other insect HSP60s was 73–81%. *RpHsp70-1* showed 95%, 94%, and 93% identity with HSP70 from *A. pism* (XP_001945786), *Aphis glycines* (AHG94986), and *D. noxia* (XP_015373896), respectively, while *RpHsp70-2* showed 96%, 98%, and 93% similarity with HSP70 from *A. pism* (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. pism* (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 *RpHsp70s*

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. *R. padi* was clustered in the HSP70 containing group. The cytosol cluster is divided into two branches, inducible HSP70 branch, while *RpHsp60* was 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 *Hsp* genes in *R. padi* during different developmental stages are shown in Fig. 4. The *RpHsps* were expressed during all developmental stages but at varying levels. *RpHsps* transcripts did not differ significantly during the nymph stages (*F* = 0.411, *P* = 0.749) but were significantly higher than levels of adult
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 Hsp genes were quantified by qPCR following exposure of apterous and alate adults 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 adults but at 36 °C in alate adults. 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 adults were not significantly increased by the treatments, with the exception of 36 °C treatment ($F = 3.235, P = 0.082$). RpHsp70 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.
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.
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 fourRpHsps 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 “AA VEEGIVPGGG” 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.
(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 (Luo 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 apterous 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 apterous 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 RpHsp56 showed a positive correlation with time, whereas 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 Hsp expression is related to thermotolerance extremes: bringing together quantitative and molecular approaches. J. Therm. Biol. 28, 175–182.


Dillenbaugh, N.S., Pal, J.S., Trapp, R.J., Giorgi, F., 2005. Fine-scale processes regulate the 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. padii 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 0.9280 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 protoxos, 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 response involving the duration and severity of the stress. Acknowledgment

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