Gene Expression Profiles of Heat Shock Proteins 70 and 90 From Empoasca onukii (Hemiptera: Cicadellidae) in Response to Temperature Stress

Li Qiao,1,2 Jun X. Wu,1,3 Dao Z. Qin,1 Xiang C. Liu,2 Zhao C. Lu,2 Li Z. Lv,2 Zi L. Pan,2 Hao Chen,1 and Guang W. Li1

1Key Laboratory of Plant Protection Resources and Pest Management (Northwest A&F University), Ministry of Education; Key Laboratory of Applied Entomology, Northwest A&F University, Yangling, Shaanxi, China
2Xinyang City Academy of Agricultural Sciences, Xinyang, China
3Corresponding author, e-mail: junxw@nwsuaf.edu.cn

ABSTRACT. Empoasca onukii Matsuda is a worldwide pest that causes great economic loss in tea growing areas and is significantly affected by temperatures. Heat shock protein (Hsp) genes are important in insects’ response to temperature stress. In this study, two full-length Hsp genes, Eohsp90 and Eohsp70, were cloned from E. onukii using rapid amplification of complementary DNA ends. The open reading frames of Eohsp90 and Eohsp70 were 2,172 bp and 2,016 bp in length, respectively. Their deduced amino acid sequences of Eohsp90 and Eohsp70 showed high homology with other species. Subsequently, the transcriptional expression of Eohsp90 and Eohsp70 in E. onukii adults exposed to various temperatures (–5, 0, 10, 15, 20, 25, 30, 35, 41 and 44 °C) for 1 h, and at extreme temperatures (0 °C and 41 °C) for various time duration (0, 20, 40, 60, 80, 100, and 120 min) were investigated via real-time quantitative polymerase chain reaction. The relative expression levels of both Eohsp90 and Eohsp70 in E. onukii adults were upregulated as the temperature rises or falls over time, except in the –5 °C or 44 °C temperature groups. Moreover, the expression level in the temperature elevated groups was higher than that of the lower temperature groups. In addition, the Eohsp70 generally demonstrated a higher transcriptional level than Eohsp90, and both genes had a higher expression profile in female adults compared with the males. The expression profiles indicated that Eohsp90 and Eohsp70 may play important roles in E. onukii adult responses to ecologically relevant environmental temperature threat.

Key Words: Empoasca onukii Matsuda, heat shock protein, temperature stress, sex difference

When organisms are exposed to heat, cold, or some other stresses, they synthesize a small set of proteins called heat shock proteins (Hsps) (Parsell and Lindquist 1994, Sørensen et al. 2003, Arya et al. 2007), which participate in folding and unfolding, aggregation, degradation, and transport of proteins induced by the stress (Hartl 1996, Beissinger and Buchner 1998). The Hsps are a ubiquitous group of highly conserved proteins (Denlinger and Yocum 1998, Sørensen et al. 2003) that are organized into several families based on their molecular mass (kDa), with Hsp90 and Hsp70 proteins belonging to the two major families. Hsp90 is one of the most abundant proteins, accounting for 1–2% of all cell proteins under nonstressed conditions (Csermely et al. 1998). Hsp70 originates through the gene duplication process (Bettencourt and Feder 2001) and exhibits differential gene expression profiles under various stress conditions. Conserved structures of Hsp90 and Hsp70 proteins are characterized by the N-terminal ATPase domain and the C-terminal polypeptide-binding domain (Young et al. 2004). Consequently, the role of Hsp90 and Hsp70 as stress markers has been well recognized in many organisms.

Insects are small-bodied poikilotherms that are relatively easily affected by heat or cold stress, which can quickly increase or decrease their body temperatures to lethal levels (Denlinger and Yocum 1998, Rinehart et al. 2007). Insects have evolved many behavioral and physiological strategies to avoid temperature impairments, and production of Hsp is one of them (Feder and Hofmann 1999, Sørensen et al. 2003). Inducible Hsps can be synthesized when temperatures increase to levels substantially above normal, and one of their best known functions is to increase heat tolerance of organisms to protect them from thermal injury and death (Gehring and Wehner 1995, Dahlgaard et al. 1998). It has been proposed recently that Hsps also contribute to the cold tolerance of insects (Chen et al. 2005, Rinehart et al. 2007). The green tea leafflower Empoasca onukii (E. onukii) Matsuda (Hemiptera: Cicadellidae), is a major pest in many Asian tea plantations (Feng et al. 2010, Wang et al. 2010, Jin et al. 2012). Both adults and nymphs actively move around to feed on fresh tender leaves causing characteristic symptoms referred to as “hopperburn” (Backus et al. 2005). The average tea loss is about 10–15% each year. This insect can also cause a dramatic decrease in tea quality and a loss of over 50% in some temperate years (Peng et al. 2010). In mid-south of China, E. onukii are active from mid-March to early December, and peak numbers occur in July and October. Populations sharply decline in late November with decreasing temperature. In the summer, this insect usually spends its life on deciduous trees and in the winter on conifers or evergreens adjacent to tea fields. It is now apparent that there is no physiological diapause in this pest and the optimal temperature range for survival, development, and reproduction of E. onukii is 20–25 °C (Yukio 2010). Therefore, E. onukii is frequently exposed to high or low temperature stress in nature. The temperature is normally above 20 °C during this period in China, occasionally with a temperature higher than 39 °C or lower than 0 °C (Cui et al. 2008). In previous studies, we have investigated the biological parameters of E. onukii under high- and low-temperature conditions and found that adults had the greatest thermal and cold tolerance among all developmental stages in natural environments, and with the higher or lower temperature conditions, there were more female than male adults in tea plantations (L.Q., unpublished data). This may be related to the expression of Hsp genes.

Comprehensive information about the changes in the level of Hsp genes from E. onukii in the response to temperature stress is not available yet. To explore changes of E. onukii to thermal and cold stress conditions at the molecular level, here in this article, we cloned two full-length genes encoding Hsp90 and Hsp70, respectively, and
analyzed their transcriptional expressions under various temperature stress scenarios and investigated their expression differences between females and males to elucidate the gene expression profiles.

Materials and Methods

Insects. *E. onukii* were originally collected from tea plantations in the Maan Mountains (113° 45′–115° 5′ E, 30° 23′–32° 27′ N, elevation 120 m; tea cultivar belongs to the Xinyang colony species), which is part of the Academy of Agricultural Sciences in Xinyang City, Henan Province, China. Adults and nymphs were reared on biennial tea trees in plastic flowerpots (15 cm in diameter) covered with 60 mesh filter gauze. The phytotron with *E. onukii* gauze. The phytotron with a relative humidity of 80 ± 5% and a photoperiod of 14:10 (L:D). The experimental adults were collected from the fifth generation reared in phytotron.

**Heat and Cold Treatments.** Five adults (1-day-old adults) were randomly selected for the experiment and placed in a 1.5-ml centrifuge tube blocked with cotton, and heated at 35°C for 1 h in a dry bath incubator (Allsheng Instruments, Hangzhou, China) to provide material for carrying out Hsp transcripts cloning. The temperature treatment levels chosen for these experiments were selected based on data from a prior pilot experiment. Before conducting real-time quantitative polymerase chain reaction (RT-qPCR), adults were exposed to various temperature treatments of −5, 0, 10, 15, 20, 25, 30, 35, 38, 41, and 44°C for 1 h and at two selected extreme temperatures of low 0°C and high 41°C for various time treatments (0, 20, 40, 60, 80, 100, and 120 min). After each stress temperature treatment, all adults were immediately snap-frozen in liquid nitrogen and stored at −80°C prior to processing. Five female and five male adults were included in each treatment. To observe the survival rates of *E. onukii* under various temperatures, 30 female and 30 male adults were exposed to above temperatures for 1 h. The survival rates were assessed after the treated insects were allowed to recover at 20 ± 1°C for 24 h. Adults set at 20 ± 1°C were regarded as the control group. Each treatment was repeated three times.

**RNA Extraction and First Strand cDNA Synthesis.** Total RNA was extracted using RNAsiso Plus (TaKaRa, Dalian, China) and treated with *Dnase I* (MBI Fermentas, Hanover, MD) to remove any contaminating DNA (per the manufacturer’s instructions). The mRNA quality was determined by using a spectrophotometer (Nanodrop2000c, Thermo Fisher Scientific, West Palm Beach, FL) and gel electrophoresis,respectively. To generate the first strand of complementary DNA (cDNA), 0.5 μg of total RNA was used as template, and oligo dT primers and the PrimeScript RT reagent kit (TaKaRa, Dalian, China) were used for reverse transcription and cDNA synthesis.

**Cloning of Hsp Fragments.** To amplify partial cDNA fragments of *Hsp90* and *Hsp70*, degenerate primers (Table 1) were designed based on sequences from other insect species. PCR reactions were performed as follows: 3 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 50°C for *Hsp90*, or 30 s at 48°C for *Hsp70* and 72°C for 75 s; 10 min at 72°C. The PCR products obtained were separated on a 1.5% agarose gel. After being purified by a universal DNA purification kit, bands with expected sizes (Tiangen Biotech, Beijing, China) were cut and then cloned into a pGEM-T vector (Promega, Madison, WI) and transformed into DH5α competent cells (Transgen Biotech, Beijing, China). Positive products were determined based on blue–white screening and then cultured on liquid Luxol blue (LB) culture medium. Plasmids were extracted by using a plasmid mini kit (Tiangen Biotech) and sequenced (AuGCT DNA-SYN Biotechnology, Beijing, China).

Based on the sequence information of partial fragments, 5′ and 3′ regions of the corresponding cDNA were obtained by 5′ and 3′ RACE (rapid amplification of cDNA ends) using designed gene-specific primers (Table 1), and the 5′-full RACE kit and 3′-full RACE core set (ver. 2.0, TaKaRa). The nested PCR reaction for 3′ RACE was 3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 50°C for *Hsp90* or 30 s at 58°C for *Hsp70* and 72°C for 1 min; and 10 min at 72°C. The nested PCR reaction for 5′ RACE was 3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, with a final extension phase at 72°C for 10 min for *Hsp90* and *Hsp70*, followed by sample storage at −20°C. Distinct bands with expected sizes were recovered from the gel, and then cloned and sequenced as above. Finally, based on the preliminary sequence of the obtained cDNA, full sequences validation of *Hsp90* and *Hsp70* were implemented using designed gene-specific primers (Table 1). The reaction for full sequences validation was 3 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55°C and 72°C for 75 s; 10 min at 72°C, respectively.

**Gene Sequence Analysis.** Analogs of the obtained cDNA sequences were searched using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Partial sequences were assembled using CAP3 sequence assembly program (http://pbil.univ-lyon1.fr/cap3.php). The sequence alignment and identity analysis were carried out using the DNAMAN software package (Lynnon Corporation, Pointe-Claire, Quebec, Canada). Open reading frames (ORF) were identified with the aid of the ORF Finder software (http://www.ncbi.nlm.nih.gov/orf/orf.html). The molecular weights of predicted proteins were calculated by the SWISS-PROT (ExPaSy server) program “Compute pi/Mw” (http://au.expasy.org/tools/pi_tool.html).

**Real-Time qPCR.** RT-qPCR reactions were performed in 25 μl total reaction volume including 12.5 μl of 2× SYBR Green Mix (Hangzhou Bloor Technology Co., Ltd, China), 1 μl of gene-specific primers (Table 1) each, 2 μl of cDNA template, and 8.5 μl of ddH2O. Samples without cDNA template were used as negative controls. Reactions were carried out on the iQ5 (Bio-Rad, Hercules, CA) according to the operation manual. PCR conditions were initial step at 95°C for 3 min, followed by 40 cycles of 95°C 10 s, 55°C 15 s and 72°C 30 s, then 15 s at 95°C, 30 s at 55°C, and 15 s at 55°C (gradient rising to 95°C, at an interval of 0.5°C) to generate the melting curve. All reactions were carried out in three biological and technical replicates.

The expression levels of targeted *Hsp90* and *Hsp70* genes were quantified according to geNorm directions (Vandesompele et al. 2002).

| Primer name | Primer sequence from 5′ to 3′ |
|-------------|------------------------------|
| **Hsp90**   | CATGACGGAAACCTTGAAGTGAATG   |
| **Hsp70**   | TCAGGTCTTGCAGGTTTCATATDAT    |
| **3′ RACE Hsp90 outer** | CGATACATGAAATGGAATAATGCGNA YAYCACA |
| **3′ RACE Hsp90 inner** | GCTGTCAGGGGCGNCCRTANGC |
| **3′ RACE Hsp70 outer** | TACGGTCGCCACATGATGATTT   |
| **3′ RACE Hsp70 inner** | CGGATGATGATCCAGAAAGG   |
| **5′ RACE Hsp90 outer** | TGCAGATCCATCATGCAGACTCA   |
| **5′ RACE Hsp90 inner** | GGATACGCAACGGTCACTGACTG |
| **5′ RACE Hsp70 outer** | TACGGTCGCCACATGATGATTT   |
| **5′ RACE Hsp70 inner** | CGGATGATGATCCAGAAAGG   |
| **Hsp90 full validation** | TACGGTCGCCACATGATGATTT |
| **Hsp70 full validation** | CGGATGATGATCCAGAAAGG   |
| **RT-PCR Hsp90** | TTATCGGCTATCCTATCAA   |
| **RT-PCR Hsp70** | TTATCGGCTATCCTATCAA   |
| **β-actin** | GGAAGGAGCGTGACAGCTC    |
| **Hsp90 full validation** | GACGGATCAGCGTACCTGACTGATCAGTCGAT |
| **Hsp70 full validation** | GACGGATCAGCGTACCTGACTGATCAGTCGAT |
| **RT-PCR Hsp90** | CGGACGGAAACCTTGAAGTGAATG   |
| **RT-PCR Hsp70** | GCAGGTCCTTACATGCAATAT |

| Table 1. Primers used in transcript cloning and real-time quantitative PCR |
The expression of each gene was determined from the Ct (cycles threshold) value that corresponded to a number of cycles required for the PCR amplification to reach a fixed threshold in the exponential phase (Walker 2002). The real-time dissociation curve was used to determine the presence of a unique PCR product. Following the normalization strategy outlined in Vandesompele et al. (2002), we tested the stability of both β-actin and α-tubulin as reference genes (GenBank accession number: KJ476139). β-actin was selected and used as the final reference gene, because its expression was stable and had less change than α-tubulin under different temperature treatments based on analyses with Normfinder and Genorm. For all reactions, melting analysis confirmed that only a single amplicon was generated.

**Statistical Analysis.** Transcript levels under different temperature treatments were determined by qPCR and the result analysis using 2^ΔΔCt method (Livak and Schmittgen 2001). β-actin of *E. onukii* was used as a reference gene (Table 1) and data collected at the treatment of 20°C or 0 min was used as the calibrator. All test and calibration samples were normalized to the ΔCt value (ΔCt (test) = Ct(target test) − Ct(reference test)) and then to the ΔΔCt value (ΔΔCt = ΔCt (target) − ΔCt (calibrator)). The final relative expression level was calculated using the formula of F = 2^−ΔΔCt.

Significant differences among treatments were analyzed using a one-way analysis of variance to compare means of three samples and followed by a Student–Newman–Keuls test for multiple comparisons (means for groups in homogeneous subsets) using SPSS 16.0 software (SPSS Inc., Chicago, IL). The significance level was 0.05. Values throughout the manuscript are means ± standard deviation.

**Results**

**Cloning and Characterization of Hsps.** Partial sequences of Hsp90 and Hsp70 obtained from *E. onukii* were 378 bp and 1,070 bp, respectively. Based on BLAST searches, a high homology to the Hsps of other insects was discovered, and we further performed RACE and obtained their full-length sequences and designated them as Eohsp90 (GenBank accession number: KF730250) and Eohsp70 (GenBank accession number: KF730249). Their complete nucleotide and deduced amino acid sequences are shown in Figs. 1 and 2, respectively.

The ORF of EoHsp90 was 2,172 bp, encoding 723 amino acids, with a predicted molecular mass of 83.34 kDa, and the theoretical isoelectric point of 4.96. All five conserved amino acid motifs characteristic of the Hsp90 protein family (Gupta 1995) were discovered in *E. onukii* EoHsp90 including a C-terminal MEEVD motif found in the C-terminus (Fig. 1). The ORF of Eohsp70 was 2,016 bp, encoding 671 amino acids with predicted molecular mass of 73.36 kDa and a pI of 5.65. Within the predicted E. onukii EoHsp70 protein sequence, three EoHsp70 protein family signatures (Sonoda et al. 2006) were identified: IDLGTTYS, IFDLAGGGTFDVSIL, and VVLGGSTRIPIKO, including the cytosolic EEVD signal at the C-terminus (Fig. 2). The termination codon (TAA) occurred at nucleotide 2,169 in Eohsp90 and 2,013 in Eohsp70. The possible polyadenylation signal (AAUAAA) was present at 164 and 31 nucleotides downstream from the termination codon in Eohsp90 and Eohsp70, respectively.

The deduced amino acid sequences of Eohsp90 and 70 Hsp genes and a range of highly homologous Hsp genes from other Hemiptera insect species (selected from BLAST analysis) were used for the construction of alignments (Figs. 3 and 4). These sequences proved to be highly conservative. The Eohsp90 of our insect displayed 86.50%, 86.33%, 87.97%, 91.38%, and 91.26% identity with that of Trialeurodes vaporariorum (FJ457626), Bemisia tabaci (HM367080), Lygus hesperus (JX627809), Laodelphax striatellus (KF660250), and Nilapavarta lugens (GU723300), respectively. The Eohsp70 displayed 81.82%, 79.64%, 74.07%, 76.30%, and 81.22% identity compared with B. tabaci (HM367079), Corythucha ciliata (KF018929), Eiricurus pela (KC161300), N. lugens (JQ782193), and T. vaporariorum (EU934244), respectively. For the identified Hsp90 amino acid sequence, the greatest similarity was found to be with other Hemiptera insect species.

**Survival Rates After Temperature Treatments.** As shown in Fig. 5, after different temperature treatments for 1 h, the survival rates of *E. onukii* adults were at the similar level at 5–35°C, both males and females. The survival rates of males were gradual declining starting at 38°C, were significantly different from other treatments, but females undifferentiated at 38°C. As the temperature rose (>38°C) or fell (<5°C), the survival rates declined significantly. A large number of *E. onukii* were unable to survive above the 44°C and under ~5°C treatment points, and the survival rates of females were higher than males (females: 48% under 44°C and 52% under ~5°C; males: 20% under 44°C and 39% under ~5°C).

**Expression of Hsps at Heat and Cold Shock Temperature Stress.**

The expressions of Eohsp90 and Eohsp70 were induced by both high (25°C to 44°C) or low (~5°C to 15°C) temperatures. The higher (25°C to 44°C) or the lower (~5°C to 15°C) the temperature, the higher the expression level of both Eohsp90 and Eohsp70, except for the ~5°C and 44°C temperature points (Fig. 6). Moreover, the expression level of Eohsp90 and Eohsp70 at the high temperature points was higher than that of the low temperature points, and the expression level of female was higher than that of the male as the temperature was raised or lowered beyond the normal physiological range (15–25°C). The expression of Eohsp90 and Eohsp70 remained constant between 15°C and 25°C but significantly differed when the temperature rose above 25°C or dropped below 15°C.

In heat treatments, the expression level of Eohsp90 increased dramatically at 30°C compared with 20°C in both males and females (Fig. 6A). The maximal expression value was found to be approximately 4.53-fold higher in males and approximately 4.87-fold higher in females when the temperature of the *E. onukii* increased from 30°C up to 41°C. In cold treatment, the expression was increased dramatically at the 0°C temperature point. The maximal expression value was found to be approximately 3.05-fold in males and approximately 4.16-fold in females when the temperature of the *E. onukii* decreased from 10°C down to 0°C.

In heat treatments, the expression level of Eohsp70 (Fig. 6B) increased dramatically at 30°C compared with that of 20°C and reached a peak at 41°C. The maximal expression value was found to be approximately 8.72-fold in males and approximately 7.90-fold in females when the temperature of the *E. onukii* increased from 30°C up to 41°C. In cold treatment, the expression was also increased dramatically at the 0°C temperature point. The maximal expression value was increased substantially to approximately 5.93-fold in males and approximately 5.10-fold in females when the temperature of the *E. onukii* decreased from 10°C down to 0°C. The expression of Eohsp90 or Eohsp70 was lower (~5°C and 44°C) than that at 35–41°C and 0–5°C but were still elevated relative to those under 10–30°C temperature points.

**Expression of Hsps at Different Time Duration.**

The expression of Eohsp90 and Eohsp70 of females was higher than that of males during both heat and cold treatments at all checked time points except that at 0 min, and furthermore, Eohsp70 expression was higher than the expression of Eohsp90. Within the 20-min duration, the Eohsp90 and Eohsp70 had similar expression profiles (Fig. 7). In male and female adults, the increase in expression of Eohsp90 and Eohsp70 was highly significant after 40 min heat treatment compared with that at 0 min. At 41°C, the expression of Eohsp90 increased maximum with the increase of treatment time for about 120 min; however, there was no significant difference of the expression levels between the 100 and the 120 min treatments (Fig. 7A). Eohsp70 expression was the highest at 80 min and leveled off to 100 min, after that there was a significant decrease at 120 min treatment (Fig. 7B).

The increase of expression level of Eohsp90 in both males and females was clearly visible after 20 min of cold exposure and continued to increase for up to 40 min in males or 60 min in females at which time,
Fig. 1. Nucleotide and deduced amino acid sequences of Hsp90 from E. onukii. Five highly conserved motifs of the Hsp90 family are underlined. The cytoplasm Hsp90 C-terminal region (MEEVD) is emphasized with double underlines. A possible consensus signal sequence for polyadenylation is indicated with a box. The asterisk indicates the translational termination codon. Bold numbers indicate amino acid residue positions and nonbold numbers indicate nucleotide residue positions.
Fig. 2. Nucleotide and deduced amino acid sequences of Hsp70 from *E. onukii*. Three highly conserved motifs of Hsp70 family are underlined. The cytoplasm Hsp70 C-terminal region (V/IEEVD) is emphasized with double underlines. A possible consensus signal sequence for polyadenylation is indicated with a box. The asterisk indicates the translational termination codon. Bold numbers indicate amino acid residue positions and nonbold numbers indicate nucleotide residue positions.
Fig. 3. Alignment of the amino acid sequences of Eohsp90 with other five insects. The species and the GenBank accession numbers are as follows: TV, *T. vaporariorum* (FJ457626); BT, *B. tabaci* (HM367080); LH, *Lygus hesperus* (JX627809); EO, *E. onukii*; LS, *Laodelphax striatella* (KF660250); NL, *N. lugens* (GU723300).
Fig. 4. Alignment of the amino acid sequences of Ehs70 with other five insects. The species and the GenBank accession numbers are as follows: BT, B. tabaci (HM367079); CC, Corythucha ciliata (KFO18929); EO, E. onukii; EP, Ericerus pela (KC161300); NL, N. lugens (JQ782193); TV, T. vaporariorum (EU934244).
Fig. 5. Survival rates of *E. onukii* adults under different temperature treatments. Males and females of *E. onukii* were exposed to each temperature ranging from −5°C to 44°C for 1 h. Different letters on the error bars indicate significant differences among the different temperature treatments, and the lower case letters are used for males and the capital letters for females (*P* < 0.05).

Fig. 6. Relative expression of *Eohsp90* (A) and *Eohsp70* (B) in males and females of *E. onukii* adults under different temperature treatments. The fold changes of each temperature treatment were relative to the calibrator (20°C treatment). Different letters on the error bars indicate significant differences among the different temperature treatments, and the lower case letters are used for males and the capital letters for females (*P* < 0.05).
there was a leveling off of expression for both sexes (Fig. 8A). The expression of Eohsp70 showed a similar expression pattern as that of Eohsp90 with a significant increase for both females and males in 20 min, and a continue increase to 80 min for males and 100 min for females, after that there were no further statistically significant increases in expression in 120 min (Fig. 8B).

**Discussion**

Understanding how E. onukii responds to heat and cold stress is crucially important to understand how they withstand extreme environmental temperatures. Hsps represent a class of molecular chaperones that are well known for their quick responses to environmental stress (Sørensen et al. 2003). This study focused on the full length of an ORF and the encoding sequences for members of two major Hsp families, genes Hsp90 and Hsp70, and the gene expression level in E. onukii Hsps in response to both heat and cold stress conditions.

Hsps are highly conserved in their coding regions (Feder and Hofmann 1999, Sørensen et al. 2003) and our results confirmed this high homology. The deduced amino acid sequences of two full-length transcripts of Hsps in E. onukii showed high similarity compared with their counterparts in other insect species. The identity range was 86–91% for Eohsp90 and 74–81% for Eohsp70. The typical signature structures of the Hsp family and a conserved MEEVD or V/IEEVD motif at the C-terminals was also found in the deduced amino acid sequences. The conserved C-terminal motif MEEVD is suggested to bind to many cochaperones with small helical TRP domains (Pearl and

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**Fig. 7.** Relative expression of Eohsp90 (A) and Eohsp70 (B) transcripts subjected at 41°C heat treatment for different time durations in E. onukii adults. The fold changes of each time treatment were relative to the calibrator (0 min). Different letters on the error bars indicate significant differences between the sexes, and the lower case letters are used for males and the capital letters for females ($P < 0.05$).
Prodromou 2006, Ramya et al. 2006), and this indicates that Eohsp90 is a cytosolic Hsp (Gupta 1995). The conserved V/IEEVD motif enabled Eohsp70 to bind with other cochaperones (Daugarrd et al. 2007). Synthesis of Hsps in response to cold and heat stress is well documented in many organisms (Parsell and Lindquist 1994, Dahlgaard et al. 1998, Sørensen et al. 1999, Kim et al. 2008, Chen et al. 2013, Zhu et al. 2013). However, in eukaryotes, Hsp families comprise multiple members that differ in inducibility and function (Feder and Hofmann 1999, Huang et al. 2009, Garczynski et al. 2011).

In this study, the relative expressions of Eohsp90 and Eohsp70 were upregulated following an increase or decrease of temperature except at the extreme -5°C and 44°C temperature points, and a general increase in expression was observed following prolonged exposure to high or low temperatures. The results suggested that Eohsp90 and Eohsp70 had a relationship with heat and cold temperature changes and perhaps play important roles in surviving temperature stress for E. onukii adults. The relative expression of Eohsp70 was about one order of magnitude higher than that of Eohsp90 at high and low temperatures and even after exposure to extreme temperatures. For example, the maximum expression of Eohsp70 could reach 8.72-fold increase, but it had only 4.87-fold increase for Eohsp90. This finding is consistent with the earlier study by Zhang and Denlinger (2010) in Helicoverpa zea. The Hsp70

Fig. 8. Relative expression of Eohsp90 (A) and Eohsp70 (B) transcripts subjected at 0°C low temperature treatment for different time durations in E. onukii adults. The fold changes of each time treatment were relative to the calibrator (0 min). Different letters above the error bars indicate significant differences between the sexes, and the lower case letters are used for males and the capital letters are used for females (P < 0.05).
family appears to be the most prominent contributor to temperature tolerance in insects, and other members of family, while not as highly expressed, are still a conspicuous element in temperature response (Denlinger and Yocum 1998).

Experiments proved that cold stress induces some Hsp genes in insects. For example, in the flesh fly, Sarcophaga crassipalpis, cold shock at either −10°C or 0°C stimulated the synthesis of several Hsps (Joplin et al. 1990). Cold stress also upregulated expression levels of Hsp90 and Hsp70 (Rinehart and Denlinger 2000). In our study, the expression of Hsps increased as the cold temperature except decreased at the −5°C temperature point, and the expression level when exposed to the high temperature was higher than the expression at the low temperature points. More interestingly, we found that expression levels of both Eohsp90 and Eohsp70 also varied in different sexes of E. onukii. The level of expression in females was much higher than that in males at moderately heat and cold temperatures and at unfavorable extreme temperatures after prolonged (at least 80 min) exposure. These results indicated that induced expression of Hsps in females of E. onukii is perhaps more sensitive than in males. However, the expression of Hsps in E. onukii under different environmental temperatures at the molecular level has not been reported.

Regarding to the survival rates of E. onukii, as the temperature rose (>38°C) or fell (<5°C), the survival rates of females were higher than males, which indicated that female adult is more thermal and cold tolerance than male. The result is consistent with the females adult number are higher than males, whether in hot summer or cold winter in tea plantation (L.Q., unpublished data).

Our results suggested that there was a maximal threshold level for the expression of Hsps. For example, the expression levels of Eohsp90 and Eohsp70 reached a peak at 0°C and 41°C and declined beyond those temperatures (e.g., −5°C and 44°C). The expression reached the highest level at 41°C after 100 min exposure for Eohsp90 and 80 min for Eohsp70 of in both females and males. The Eohsp90 expression reached the highest level at 0°C after 40 min of exposure in males and 60 min of exposure in females. The Eohsp70 expression reached the highest level at 0°C after 80 min of exposure in males and 100 min of exposure in females. It is impossible for the genes encoding Hsps to undergo unlimited amplification in an organism, and Hsps are therefore subjected to strict regulation by multiple molecular mechanisms (Feder and Hofmann 1999). Therefore, the ability of Hsps as molecular chaperones to completely mitigate the damage resulting from extreme thermal or cold stress and increase temperature tolerance and survival rates of insects is limited. To some extent, the death of adults at −5°C and 44°C within 1 h indicated that Hsps expression cannot prevent death in severe temperature stresses, even for a short period of time in either male or female E. onukii adults.

In conclusion, Hsps of E. onukii could be induced by temperature stress, especially heat stress as supported by the dramatic increase in expression for Eohsp90 and Eohsp70, at 35, 38, and 41°C temperatures. Both Hsps in this study were more sensitive to heat than cold based on differential gene expression between heat and cold temperatures. Eohsp90 and Eohsp70 showed different transcriptional expression profiles in response to temperatures stress in E. onukii adults and varied in induction between sexes. The induced expressions of Eohsp90 and Eohsp70 in female were at earlier time than male, which showed that female was more sensitive to high or low temperature than male. Our comprehension of Hsps in response to temperatures stress is crucial for understanding the capability of E. onukii to tolerate future changes to the environmental temperature. Ultimately, these novel molecular findings may aid in the development of protocols to control the E. onukii and other related pests thereby increasing tea yield.

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