Expression profile of two HSP70 chaperone proteins in response to extreme thermal acclimation in *Xestia c-nigrum* (Lepidoptera: Noctuidae)

*Ling Wang*, *Shuai Yang*, *Lanlan Han*, *Kuijun Zhao*, and *Lefu Ye*

**Abstract**

Heat shock proteins 70 (HSP70s) are highly evolutionarily conserved and play important roles in helping insects survive under extreme temperatures. In this study, *Xestia c-nigrum* (L.) (Lepidoptera: Noctuidae) was stressed at various temperatures, and the impacts on thermotolerance and cold endurance were examined. Two complementary deoxyribonucleic acid (cDNA) clones encoding heat shock cognate 70 (HSC70) and inducible heat shock protein 70 (HSP70), named *Xc-HSC70* and *Xc-HSP70*, respectively, were isolated from *X. c-nigrum* using reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). Amino acid sequence analysis indicated that *Xc-HSC70* and *Xc-HSP70* contained the signature sequences of the HSP70 family, and phylogenetic analysis showed that *Xc-HSP70s* were consistent with the known insect classification. Genomic DNA structure analysis revealed that the *Xc-HSC70* gene contained 8 introns that all conformed to the GT/AG rule, whereas the *Xc-HSP70* gene did not contain any introns in the coding region. Fluorescent real-time quantitative RT-PCR revealed that both genes were significantly up-regulated under thermal stress and cold stress. The expression patterns indicated that *Xc-HSP70* was heat inducible and *Xc-HSC70* was constitutively expressed. The temperature response was more intense for *Xc-HSP70* than for *Xc-HSC70*, regardless of the temperature or developmental stage; however, the expression of *Xc-HSC70* and *Xc-HSP70* was not altered in the pupa. Taken together, the expression profiles of both genes (*Xc-HSP70s*) provide good support to the insect for coping with adverse conditions.

**Key Words:** heat shock proteins 70 (HSP70s); expression profile; thermal stress; *Xestia c-nigrum*

**Resumen**

Proteínas de choque térmico 70 (Hsp70s) están evolutivamente muy conservadas y juegan un papel importante en ayudar a los insectos a sobrevivir las temperaturas extremas. En este estudio, se puso *Xestia c-nigrum* (L.) (Lepidoptera: Noctuidae) bajo el estrés de diferentes temperaturas, y se examinó el impacto sobre la termotolerancia y resistencia al frío. Se aislaron dos clones de ácido desoxirribonucleico (ADNC) complementarios que codifican el cognado choque térmico 70 (HSC70) y la proteína de choque térmico inducible 70 (HSP70), llamado *Xc-HSC70* y *Xc-HSP70*, respectivamente, a partir de *X. c-nigrum* mediante la cadena de polimerasa con transcriptasa inversa reacción (RT-PCR) y la amplificación rápida de extremos de ADNC (RACE). El análisis de la secuencia de aminoácidos indicó que *Xc-HSC70* y *Xc-HSP70* contenían las secuencias de la firma de la familia HSP70, y el análisis filogenético demostró que *Xc-Hsp70* es consistente con la clasificación conocida del insecto. El análisis genómico de la estructura del ADN reveló que el gen *Xc-HSC70* contenía 8 intrones que todos se ajustaban a la regla GT/AG, mientras que el gen *Xc-HSP70* no contenía intrones en la región codificante. El análisis de RT-PCR fluorescente cuantitativa en tiempo real reveló que ambos genes fueron significativamente regulados hacia arriba bajo estrés térmico y estrés por frío. Los patrones de expresión indicaron que *Xc-HSP70* fue inducible por el calor y *Xc-HSC70* se expresó constitutivamente. La respuesta de la temperatura fue más intensa para *Xc-HSP70* que para *Xc-HSC70*, independientemente de la temperatura o el estadio de desarrollo; sin embargo, la expresión de *Xc-HSC70* y *Xc-HSP70* no se alteró en el pupa. Tomados en conjunto, los perfiles de expresión de ambos genes (*Xc-Hsp70s*) proveen un buen apoyo para el insecto para hacer frente a las condiciones adversas.

**Palabras Clave:** proteínas de choque térmico 70 (Hsp70s); perfil de expresión; estrés térmico; *Xestia c-nigrum*

**Cellular stress response is involved in protecting organisms from damage caused by exposure to a variety of stressors, including temperature, heavy metals, and other xenobiotics. The stress response entails the rapid synthesis of heat shock proteins (Hsps) to protect cellular proteins against denaturation (Feder & Hofmann 1999; Boutet et al. 2003; Rinehart et al. 2007). In normal conditions, heat shock proteins (Hsps) are termed as molecular chaperones involved in protein metabolism, cell cycle regulation, and apoptosis (Welch 1993; Ming et al. 2010). On the basis of molecular weight and sequence similarity, Hsps can be divided into several families, including HSP90, HSP70, HSP60, HSP40, and small heat shock proteins (molecular weights ranging from 12 to 43 kDa) (Sørensen et al. 2003; Huang et al. 2008; Shen et al. 2014). Among the Hsps, HSP70s are the most conserved and important protein families and have been studied extensively.**

*Northeast Agricultural University, College of Agriculture, Heilongjiang Province, Harbin 150030, China*

*Virus-Free Seedling Research Institute, Heilongjiang Academy of Agricultural Sciences, Heilongjiang Province, Harbin, 150086, China*

*Corresponding author; E-mail: kjzhao@163.com*
encoded by 2 different genes, constitutive type HSC70 (heat shock cognate 70) and inducible type HSP70 (Boutet et al. 2003; Deane & Woo 2005; Franzellitti & Fabbri 2005). In normal conditions, HSC70 is constitutively expressed in all cells, whereas HSP70 is not expressed or is expressed only at a low level. Under stress conditions, HSC70 expression remains unchanged or is slightly up-regulated, whereas HSP70 expression is highly induced (Lindquist & Craig 1988; Franzellitti & Fabbri 2005). These 2 heat shock proteins play key roles in the cell as molecular chaperones. However, HSC70 is mainly involved in physiological processes, such as cell division, multiplication, and development (Park et al. 2001; Kregel 2002).

In insects, it is well known that HSP70s increase heat tolerance and provide protection against thermal injury and death. Many types of HSP70 genes are up-regulated in response to heat or cold stress in various insects, such as Diptera (Huang & Kang 2007; Huang et al. 2009; Gray 2013), Lepidoptera (Jiang et al. 2012; Choi et al. 2014; Shen et al. 2014), and Coleoptera (Mahroof et al. 2005). Because of the important roles of HSP70s in thermal stress, it is important to investigate the function and expression characteristics of HSP70 genes under temperature stress. In Drosophila melanogaster Meigen (Diptera: Drosophilidae), thermotolerance was found to be significantly improved in a strain with extra copies of HSP70s (Bettencourt et al. 2008; Jensen et al. 2014). The suppression of HSP70 mRNA levels by RNAi decreased the heat and cold tolerance in Pyrrhocoris apterus (L.) (Heteroptera: Pyrrhocoridae) and Spodoptera exigua Hübner (Lepidoptera: Noctuidae) (Kostal & Tollarova-Borovanska 2009; Choi et al. 2014). In addition, HSP70s may be involved in the development of some insects and in fecundity (Jensen et al. 2014), longevity (Zhang et al. 2010; Choi et al. 2014), diapause (Rinehart et al. 2007), development (Huang et al. 2009), and metamorphosis (Zheng et al. 2010). These studies have provided direct evidence of the roles of HSP70s in cellular activity and development and have elucidated important biological functions.

Xestia c-nigrum L. (Lepidoptera: Noctuidae) is an important polyphagous pest of vegetables, cotton, wheat, maize, soybean, and ornamental plants. It is a worldwide pest that occurs in tropical, subtropical, and temperate regions (Mukawa & Goto 2011). In South China, including Guangdong, and Taiwan, X. c-nigrum is adapted to high temperatures that occur throughout the year. Severe crop damage occurs when the temperature is high in the summer and autumn, indicating that X. c-nigrum has a significantly positive response to high temperature (Zheng & Wang 2010; Zhang et al. 2013). In addition to heat tolerance, X. c-nigrum exhibits cold resistance, overwintering as larvae and pupae in temperate zones without entering diapause. The pupae have an average supercooling point of approximately −17 °C with a low of −21 °C in some individuals (Mukawa & Goto 2010; Landolt et al. 2011). In the southern region of North America and in Central and East Asia, overwintering pupae have been observed in fields (Xi et al. 2002). In China, the infesting population reaches northward to Heilongjiang Province in Northeast China. Notably, this species undertakes a long-distance migration between or within tropical, subtropical, and temperate regions, which significantly increases the geographic range over which infestations occur (Jiang et al. 2012). Furthermore, X. c-nigrum is known for its rapid development of resistance to many chemical pesticides and its lack of susceptibility to transgenic Bt crops (Landolt et al. 2011; Liu et al. 2011). Therefore, it can be presumed that X. c-nigrum has a high potential to tolerate or acquire tolerance to various environmental stresses. However, the biochemical and molecular mechanisms of such tolerance are unknown, and no information is available regarding stress-induced HSP70 expression in X. c-nigrum; therefore, the mechanisms underlying this species’ ecological adaptability and stress tolerance remain unclear.

This study examined basal gene expression and thermal responses among different developmental stages, such as the larvae and pupae of X. c-nigrum, using expression profiling of Xc-HSC70 and Xc-HSP70. Furthermore, we evaluated Xc-HSP70 expression at the mRNA level under heat or cold stress in larvae and pupae. We explored the functions of these genes in the context of thermotolerance and development and provided information needed to explore the mechanism of environmental tolerance and ecological adaptation in X. c-nigrum.

Materials and Methods

INSECTS

Adult X. c-nigrum specimens were collected from Xiangfang Farm of Harbin, Heilongjiang Province, China. After oviposition, the eggs were incubated at 25 ± 1 °C with a 12:12 h L:D photoperiod for hatching. Newly hatched larvae were reared at 25 ± 1 °C and 70% relative humidity (RH) under a photoperiod of 14:10 h L:D in a climatic cabinet (RKX-308B, Jiangsu, China), and cabbage leaf was used to feed the different instars. For diapause induction, 6th instars were placed in an 18 ± 1 °C controlled-temperature room with an 8:16 h L:D photoperiod, and the diapause status of the pupae was ascertained as previously described (Liu et al. 2011).

TEMPERATURE EXPOSURE

Based on previous studies of thermotolerance in insects (Jiang et al. 2012; Lyytinen et al. 2012; Franke et al. 2014), developing larvae from the 2nd to 6th instar were chosen for heat and cold shock treatments. For each treatment, 5 individuals were placed into a cryogenic tube and shocked at high temperatures of 28, 31, 34, 37, 40, 43, 45, and 47 °C for 2 h, then allowed to recover at 25 °C for 1 h. Similarly, larvae from the 2nd to 6th instar and 4-d-old pupae were shocked at low temperatures of 5, 0, −4, and −7 °C for 2 h, then transferred to 25 °C for 1 h of recovery. Corresponding untreated larvae and pupae were used as controls. The larvae did not survive at above 47 °C and below −7 °C, and therefore the mRNA was not analyzed. Each treatment was replicated 3 times. To detect the developmental regulation of the HSC70 and HSP70 genes, 2-, 4-, and 6-d-old pupae were reared at 25 °C, with 3 replications. All of the samples were frozen quickly in liquid nitrogen and stored at −80 °C. Temperature control was achieved using environmental chambers (Sanyo, Tokyo, Japan).

CLONING OF THE FULL-LENGTH CDNAs OF XC-HSC70 AND XC-HSP70

Total RNA isolation and first-strand cDNA synthesis: RNA was isolated using an RNA isolation kit (Omega Bio-Tek, Norcross, Georgia, USA) according to the manufacturer’s instructions. The concentration and quality of the RNA were verified by spectrophotometry and electrophoresis on a 1.0% agarose gel. The cDNA was synthesized using a cDNA kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions.

Primer design: The HSC70 and HSP70 genes of other insects were aligned in GenBank to identify the conserved region of the gene family. Primer Premier 5.0 (Premier, Canada) was used to design the primers, and the sequences of the primers are listed in Table 1. The PCR protocol was as follows: 3 min at 94 °C and 35 cycles of 30 s at 94 °C, 30 s at 57.8 °C for Xc-HSC70 or 30 s at 58.0 °C for Xc-HSP70, and 1 min at 72 °C, followed by a 10 min extension at 72 °C. Then, the amplified fragment was isolated using a 1.0% agarose gel and ligated into the pMD18-T vector (TaKaRa, Dalian, China) for sequencing. RACE amplification was
performed based on the sequenced fragment of Xc-HSC70 and Xc-HSP70 using the 3' Full and 5' Full RACE kit (TaKaRa, Dalian, China). We designed the 5' RACE and 3' RACE primers S'HSC70GSP1, S'HSC70GSP2, 5'HSP70GSP1, S'HSP70GSP2, 3'HSC70GSP1, 3'HSC70GSP2, 3'HSP70GSP1, and 3'HSP70GSP2 (Table 1) and combined the 3 single fragments of Xc-HSC70 and Xc-HSP70 to design the full-length primer pairs P5, P6 and P7, P8, respectively.

**CLONING OF THE XC-HSC70 AND XC-HSP70 GENES FROM GENOMIC DNA**

Genomic DNA was isolated from fifth instars using the Universal Genomic DNA Extraction kit (Takara, Dalian, China) according to the manufacturer’s instructions. The concentration and quality of the DNA were verified by spectrophotometry and electrophoresis on a 1.0% agarose gel.

To determine whether the Xc-HSC70 and Xc-HSP70 genes contained introns in their coding regions, the genomic DNA fragments for Xc-HSC70 and Xc-HSP70 were amplified from genomic DNA (100 ng) as a template, and their nucleotide sequences were analyzed. For Xc-HSC70 intron analysis, the amplification was performed using a pair of specific primers (P5, P6; Table 1), and the amplification conditions were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 4 min, with a final elongation step at 72 °C for 10 min. For Xc-HSP70 intron analysis, the genomic DNA fragment was amplified using a pair of specific primers (P7, P8; Table 1), and the amplification conditions were as described above. The DNA fragments were then cloned and sequenced.

**SEQUENCE ANALYSIS**

The bioinformatic software DNASTar (DNASTar, USA) was used to split joint the full-length sequences of Xc-HSC70 and Xc-HSP70. BLAST software was used to analyze the homology of the sequences (http://www.ncbi.nlm.nih.gov/blast/). Biology WorkBench (http://workbench.sdsc.edu/) was used to identify the open reading frames (ORFs), ProtParam (http://http://web.expasy.org/protparam/), ScanProsite (http://prosite.expasy.org/scanprosite/), and SWISS-MODEL (http://www.swissmodel.expasy.org/SWISS-MODEL.html) were used to analyze the protein sequence characteristics. PSORT II (http://psort.hgc.jp/form2.html) was used to calculate the nuclear localization signal fragment. CLUSTALW and MEGA 4.0 were used to draw the cladogram. Spidey (http://www.ncbi.nlm.nih.gov/spidey/) was used to analyze the genomic introns.

**QUANTITATIVE ANALYSIS OF XC-HSC70 AND XC-HSP70 MRNA EXPRESSION**

Total RNA isolation and first-strand cDNA synthesis were isolated as above. Real-time quantitative PCR (qRT-PCR) primers were designed using the Xc-HSC70 (HSC70 F, HSC70 R) and Xc-HSP70 (HSP70 F, HSP70 R) sequences, and the resulting products had lengths of 145 bp and 141 bp, respectively. The reference gene β-actin was used as an endogenous control to quantify the expression of the target genes. This gene is an appropriate control for studies on HSP70s in insects during this wide developmental window as determined in our previous studies (Jiang et al. 2012; Yu et al. 2012), with a resulting product of 156 bp. Xc-HSC70, Xc-HSP70, and β-actin sequences were amplified from each of the instars under each treatment condition using specific primers, Thunderbird® Sybr qPCR Mix (ToYoBo, http://www.toyobo-global.com, Japan), and a Chromo4™ Real-Time PCR instrument (Bio-Rad, Hercules, California, USA). All of the amplifications were performed in triplicate. The final volume of each qRT-PCR reaction was 20 μl, which contained 10 μl of 2 × SYBR Mix (ToYoBo, http://www.toyobo-global.com, Japan), 1 μl of diluted cDNA template, 7.8 μl of PCR-grade water, and 0.6 μl of each primer at 10 μM. PCR conditions were as follows: 95 °C for 30 s and 40 cycles of 95 °C for 10 s and 60 °C for 30 s.

Xc-HSC70 and Xc-HSP70 expression levels were calculated using the 2^-ΔΔCt comparative threshold cycle (CT) method (Livak & Schmittgen 2001). The mean and standard deviation were calculated from experiments.

**Table 1. Primers for the experiment in this study.**

<table>
<thead>
<tr>
<th>Primer type</th>
<th>Sequence (5' to 3')</th>
<th>Use of primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>GAGGGMRTGCGACTTCTGCTACTGTCATC</td>
<td>PCR for Xc-HSC70</td>
</tr>
<tr>
<td>P2</td>
<td>CTTGACTTCTCTCAGCTGTGAC</td>
<td>PCR for Xc-HSP70</td>
</tr>
<tr>
<td>P3</td>
<td>AACGARCCBACYYGCYGC</td>
<td>For full-length and gDNA sequence of Xc-HSC70</td>
</tr>
<tr>
<td>P4</td>
<td>GCGTCAATSAACCTTGTTGAT</td>
<td>For full-length and gDNA sequence of Xc-HSP70</td>
</tr>
<tr>
<td>P5</td>
<td>CGTTAAGAAGGCAGGCACTCAG</td>
<td>5' RACE for Xc-HSC70</td>
</tr>
<tr>
<td>P6</td>
<td>AGTCTGAGTCTACCTAGGTTAAC</td>
<td>5' RACE for Xc-HSP70</td>
</tr>
<tr>
<td>P7</td>
<td>CACCCATGCTAGCTGCTGCTGAAG</td>
<td>3' RACE for Xc-HSC70</td>
</tr>
<tr>
<td>P8</td>
<td>CAAATCTGAAAACCTGGCTCTAAGT</td>
<td>3' RACE for Xc-HSP70</td>
</tr>
</tbody>
</table>

Note: All primers were synthesized at Shanghai Generay Biotech Co., Ltd. (Shanghai, China).
Results

NUCLEOTIDE ANALYSIS AND DEDUCED AMINO ACID SEQUENCES OF Xc-HSC70 AND Xc-HSP70

Total RNAs of Xc-HSC70 and Xc-HSP70 were isolated from larvae of X. c-nigrum which reared at room temperature and treated at 37 °C respectively. Full-length cDNAs of Xc-HSC70 and Xc-HSP70 were 2,152 bp (published in Wang et al. 2014) and 2,213 bp (published in Wang et al. 2015), respectively. The sequence of Xc-HSC70 contains a 5′-terminal untranslated region (UTR) of 101 bp, a 3′-terminal UTR of 86 bp, and an ORF of 1,965 bp that encoded a protein of 654 amino acids with a calculated molecular weight of 71.59 kDa (Fig. 1). The sequence of Xc-HSP70 also contains a 5′-terminal UTR of 147 bp, a 3′-terminal UTR of 86 bp, and an ORF of 1,965 bp that encoded a protein of 654 amino acids with a calculated molecular weight of 71.62 kDa (Fig. 2). Amino acid sequence analysis indicated that Xc-HSC70 and Xc-HSP70 contained the cytoplasmic characteristic motif EEVD (Boutet et al. 2003) and 3 signature sequences of the HSP70 family (Figs. 1 and 2). At the carboxyl terminal region, Xc-HSC70 contained 3 consecutive repeats of the tetrapeptide motif GGMP (617 to 628 amino acids), whereas Xc-HSP70 contained 2 tetrapeptides of GGMP (617 to 620 amino acids, 625 to 628 amino acids). Putative bipartite nuclear localization signals (NLS) were also observed in the two HSP70s using the online software PSORT II (Figs. 1 and 2).

ANALYSIS OF THE GENOMIC DNA STRUCTURE OF Xc-HSC70 AND Xc-HSP70

For Xc-HSC70 intron analysis, a genomic DNA fragment of 3,710 bp was isolated by specific primers (P5, P6; Table 1). Comparison of the sequence isolated from genomic DNA with the cDNA sequence of Xc-HSC70 revealed 9 exons and 8 introns in the Xc-HSC70 fragment. The coding region of the Xc-HSC70 gene contained 8 exons.
and 7 introns (Fig. 3). The first intron (561 bp) was located in the 5' non-coding region, whereas the other introns were in the coding region, and their lengths were as follows: 157, 213, 87, 86, 135, 101, and 218 bp. All of the donor and acceptor sites of these introns were GT and AG, respectively, following the GT/AG rule as described by Breathnach & Chambon (1981) and Ming et al. (2010).

For Xc-HSP70 intron analysis, a 2,043 bp genomic DNA fragment was isolated by specific primers (P7, P8; Table 1), with a sequence identical to that of the Xc-HSP70 cDNA, indicating that there was no intron in the coding region of the Xc-HSP70 gene. Otherwise, compared with the cDNA and gDNA sequences of the cloned Xc-HSC70 gene, the Xc-HSC70 gene contained 8 introns (Fig. 3), wherein the longest intron lay in the 5' UTR, which also contained an heat shock element (HSE)–like core sequence (gaatatg C a GAA tgg TTC ca Gaa) and other introns (with different lengths from 86 to 218 bp). This is the first report on the specific amount and sites of introns in HSC70 of X. c-nigrum.

SIMILARITY ANALYSIS AND PHYLOGENETIC TREE CONSTRUCTION

Homology analysis revealed that 2 Xc-HSP70s were highly conserved in insects. The deduced amino acid sequences were highly similar to those of other known HSP70s. Xc-HSC70 had higher than 88.1% similarity with other insect HSC70 genes, 97.8% similarity with HSC70 of Mamestra brassicae L. (Lepidoptera: Noctuidae) and 75% similarity with HSC70 of vertebrates. Through the similarity analysis, we found that the Xc-HSP70 genes had higher than 76.1% similarity with other insect HSP70 genes, e.g., 88.8% identity with HSP70 of M. brassicae and 86.3% identity with HSP70 of Helicoverpa zea Boddie (Lepidoptera: Noctuidae), and 71.5% similarity with HSP70 of vertebrates. The similarity between Xc-HSC70 and Xc-HSP70 was 83.5%. We also found that the sequences of Xc-HSC70 and Xc-HSP70 were more closely related to those of other HSC70 and HSP70 genes, respectively, in insects than to each other.

Phylogenetic tree construction showed that the HSP gene family could be divided into 2 main clusters: HSC70 and HSP70. This classification was supported by a high degree of confidence, as shown in the evolutionary tree cluster (Fig. 4). Xc-HSC70 belonged to the HSC70 cluster, which also contained all 11 other Xc-HSC70 genes. Xc-HSP70 belonged to the HSP70 cluster, which only contained all of the other HSP70 genes. Therefore, this finding supports that Xc-HSC70 is a constitutively expressed gene and that Xc-HSP70 is an inducible gene, with a bootstrap value of 100% in 1,000 replicates. The relationships displayed in the phylogenetic tree were in general agreement with traditional taxonomy.

Fig. 2. Nucleotide and deduced amino acid sequences of the Xc-HSP70 gene. The signature sequences of the HSP70 family are shown in boxes, the nuclear localization signal sequence is underlined, the consensus sequence EEVD at the C-terminus is indicated in italics, and the start and stop codons are in bold. The nucleotides and amino acids are numbered along the left and right margins. The sequence encoding Xc-HSP70 has been deposited in GenBank under accession no. HQ698836.
EXPRESSION OF XC-HSC70 AND XC-HSP70 RNA IN RELATION TO THERMAL STRESS DURING DEVELOPMENT

The relative mRNA expression levels of Xc-HSC70 induced by low and high temperatures during development were quantified by qRT-PCR (Fig. 5). Xc-HSC70 expression was significantly up-regulated at temperatures of ≤ −4 or ≥ 40 °C in all developmental stages (P < 0.05), and the Xc-HSC70 expression level induced by a temperature of 37 °C in 5th instars was significantly different from that in the controls (P < 0.05). Therefore, the onset temperatures at which the expression level of Xc-HSC70 was significantly higher than that of the controls during cold and heat shock in most developmental stages were −4 and 40 °C, respectively. The intensity of the temperature response of Xc-HSC70 varied among the developmental stages. Higher increases of 2.69 and 4.61 fold appeared in 2nd instars and pupae at −4 and −7 °C, respectively, under cold shock, whereas the greatest increases were observed in 2nd instars under heat shock, with a maximum increase of 3.52 fold (Fig. 5).

No significant increase in relative mRNA expression levels of Xc-HSP70 were observed when larvae and pupae were stressed by temperatures of ≥ 5 °C or when larvae were stressed by temperatures of ≤ 34 °C (Fig. 6, P > 0.05). However, Xc-HSP70 was significantly up-regulated at temperatures of ≤ 0 °C or ≥ 37 °C (P < 0.05). Therefore, the onset temperatures for Xc-HSP70 up-regulation during cold and heat shock in all of the tested developmental stages were 0 and 37 °C, respectively. Higher increases of 6.54 and 12.47 fold appeared in 5th instars at −4 °C and pupae at −7 °C, respectively, under cold shock, whereas the greatest increases were observed in 5th instars under heat shock, with a maximum increase of 8.98 fold (Fig. 6). The intensity of the temperature response of Xc-HSP70 varied among developmental stages,
showing expression profiles similar to those of Xc-HSC70 during development. Xc-HSC70 was significantly up-regulated at temperatures of ≤−4 °C or ≥ 40 °C (P < 0.05). And the onset temperatures for Xc-HSC70 up-regulation during cold and heat shock in all of the tested developmental stages were −4 and 40 °C, respectively. Higher increases of 2.7 and 4.6 fold appeared in 2nd instars at −4 °C and pupae at −7 °C, respectively, under cold shock, whereas the greatest increases were observed in 5th instars under heat shock, with a maximum increase of 3.5 fold (Fig. 5).

Comparative mRNA expression profiles of Xc-HSC70 and Xc-HSP70 induced by cold and heat during development indicated 3 obvious characteristics (Figs. 5 and 6). First, the onset temperature varied between Xc-HSC70 and Xc-HSP70, depending on developmental stage in the case of heat shock but not cold shock, with a value of 40 °C for Xc-HSC70 in most developmental stages and 37 °C for Xc-HSP70 in all tested stages. Second, the temperature responses of Xc-HSP70 at any given temperature or developmental stage were more intense than those of Xc-HSC70.

The expression levels of the 2 X. c-nigrum heat shock protein genes in different developmental stages at 25 °C were determined relative to the expression levels in 2nd instars. Xc-HSC70 and Xc-HSP70 expression decreased with the development of the larva and pupa without thermal stress (Fig. 7). Expression decreased substantially with larval age, and the lowest expression level was observed in 4th instars and then increased in 5th instars and 6th instars again. Expression of Xc-HSC70 and Xc-HSP70 did not change significantly in 4d and 6d-old pupa.

Discussion

Cluster analysis supported the finding that Xc-HSC70 and Xc-HSP70 belong to 2 different branches of HSP70 and HSC70, respectively. Some studies have found that expression of HSP70 increased significantly after the induction of environmental stress, but HSC70 expression was lower than HSP70 expression (Deane & Woo 2005). It has been suggested that the lack of introns in inducible HSP70 genes could help to circumvent blocks in RNA splicing and enable preferential expression of heat shock proteins during periods of stress (Huang et al. 1999), thus protecting cells against harmful insults. In this study, genomic DNA sequence analysis revealed 8 introns in the coding region of the Xc-HSC70 gene, representative of constitutive features, whereas the Xc-HSP70 gene did not contain any introns, representative of inducible features. All of the intron borders of the Xc-HSC70 gene start and end with the consensus GT and AG splicing signals (Breathnach & Chambon 1981). Xc-HSP70 was significantly different from Xc-HSC70 with regard to expression amount and expression time, and it displayed an increased rate of expression. The qRT-PCR results suggested that Xc-HSC70 was constitutively expressed and that Xc-HSP70 was induced, possibly because the Xc-HSP70 gene had no introns and Xc-HSC70 had introns, which affect the splicing and joining of mRNA (Yost & Lindquist 1991; Chuang et al. 2007).

The heat shock response is characterized by the induction of numerous HSPs (Balch et al. 2008) and is mostly regulated at the transcription level by heat shock transcription factors (HSFs), which can specifically bind to heat shock elements (HSEs) in the promoters of heat shock genes (Morimoto 1998). HSEs are composed of at least 3 inverted repeats of the consensus sequence nGAAn (Fernandes et al. 1994). The longest intron of Xc-HSC70 lies in the 5' UTR, which also contains an HSE-like core sequence (gGAAatagcGAAgtgctcGAAA). The HSFs bind to the HSEs and initiate the transcription of the HSP70s (Morimoto 1993). Although we did not measure the HSC70 or HSP70 protein level in this experiment, the precondition of higher levels of HSC70 or HSP70 is the higher abundance of HSC70 or HSP70 mRNA, respectively, because the synthesis of HSP70s is primarily regulated at the transcriptional level (Molina et al. 2000). To sustain a proper level of HSP mRNA, the transcription of HSP genes can be controlled by negative regulation of HSPs. When HSPs reach high levels, HSFs bind to the HSPs, which blocks DNA binding to HSE genes (Morimoto 1993). The difference in mRNA expression between the Xc-HSC70 and Xc-HSP70 genes may be related to unique structures (Ali et al. 2003), functions, and regulatory characteristics (Park et al. 2001; Kregel 2002).

Although heat shock was the first stress shown to elicit synthesis of HSPs, it is now evident that various environmental, physiological, and
genetic factors (Sørensen et al. 2003), even cold shock (Goto & Kimura 1998), can regulate the expression of these same proteins. Therefore, the regulation of inducible HSP70 genes plays an important role in cellular responses. The function of HSP genes in thermotolerance has been confirmed in various organisms (Sonoda et al. 2002; Huang & Denlinger 2010). The intensity of the temperature responses also varies among HSP70 genes in different insects. Heat induction resulted in an expression increase of approximately 6 fold for the Xc-HSP70 gene (Figs. 5 and 6). This finding is peculiar because in many other insects, including some Lepidoptera species, heat up-regulates HSP70 expression by more than 100 fold. Regarding the 6-fold induction, we consider that the experimental materials, which were taken from the northernmost province of China in Heilongjiang Province (Habin, 130°10’N, 46°40’E), may be an important factor. Some studies have shown that production of HSP70 in response to temperature shock is less intense in organisms that are more frequently exposed to unfavorable temperatures in their habitat than in organisms in benign conditions (Sørensen et al. 2001; Lyytinen et al. 2012). As the synthesis of HSP70s requires considerable energy and may thus occur at the cost of the synthesis of other proteins (Krebs & Loeschke1994), northern populations might have evolved a less costly way to resist cold stress (Lansing et al. 2000; Sørensen et al. 2003). HSP70s may not be the only proteins involved in heat and cold resistance; other proteins such as HSP20, HSP60, and HSP90 may also be involved in heat resistance (Zhang & Denlinger 2010; Wang et al. 2012) or in the accumulation of low-molecular-weight cryoprotectants (Crowe et al. 1988), synthesis of antifreeze proteins (Duman 2001), and remodeling of the structure of the cell membrane (Tomcala et al. 2006). Some studies have found Xc-HSP70 to be up-regulated 2–5 fold in insects (Colinet et al. 2010; Morales et al. 2011; Shu et al. 2011; Wang et al. 2012; Yu et al. 2012; Luo et al. 2014).

The onset temperature at which HSP70 gene expression is up-regulated, whether low or high, varies among organisms (Garbuz et al. 2003). Tomanek & Somero (1999) suggested that the threshold temperature may be a useful way to identify temperature tolerance limits and that a higher onset temperature is associated with high heat tolerance, and vice versa. Findings in Drosophila (Garbuz et al. 2003) and leaf miner species (Huang & Kang 2007) support this idea. In X. c-nigrum, the relatively high onset temperature of 40 °C for Xc-HSC70 and Xc-HSP70, respectively, in response to heat shock may represent an indicator of heat tolerance. In contrast, the values of −4 and 0 °C for Xc-HSC70 and Xc-HSP70, respectively, under cold shock may suggest only weak cold tolerance for this species. Furthermore, the difference in onset temperature between the 2 genes under heat shock, i.e., 40 °C in most developmental stages for Xc-HSC70 and 37 °C in all of the tested developmental stages for Xc-HSP70, may reflect different functions. That is, inducible expression of HSP70 but not HSC70 at “normal” temperatures in organisms may play a negative role in cell growth and division. Expression of Xc-HSP70 was not significantly different across a wide temperature range from 26 to 40 °C, which is consistent with the negative effect of HSP70 overexpression on growth, survival, and fecundity observed in other insects (Krebs & Loeschke 1994).

Finally, the basal HSP70 gene expression profiles varied with developmental stage, suggesting that these genes may be involved in development (Huang et al. 2009). Interestingly, the relative accumulated amounts of mRNA from large HSP70s, including HSC70 and HSP70, may increase or decrease as larval and pupal development progresses, depending on the species (Sonoda et al. 2006). In X. c-nigrum, the relative transcript levels of Xc-HSC70 and Xc-HSP70 decreased with the developmental progress of the pupa. These results are consistent with research conducted on Pteromalus puparum (L.) (Hymenoptera: Pteromalidae) and the pyrrhocorid P. apterus (Kostal & Tollarova-Borovanska 2009; Wang et al. 2012). Furthermore, gene expression profiles for induced HSP70s also varied after heat or cold shock during development. Mahroof et al. (2005) suggested that increased mRNA abundance induced under thermal stress during different developmental stages may contribute to the increased thermotolerance in those stages. Increased thermotolerance in young larvae of Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae) was suggested to result from increased expression of HSP70s (Mahroof et al. 2005). In X. c-nigrum, higher Xc-HSC70 and Xc-HSP70 expression were observed in heat and cold shocked young larvae and pupae, consistent with high heat resistance in larvae and stronger cold tolerance in young larvae and pupae. Therefore, our findings that correspond to thermal acclimation for X. c-nigrum during development based on HSP70 gene expression levels are consistent with temperature tolerances observed in field populations.

Acknowledgments

This work was supported by the Special Fund for Modern Agro-Industry Technology Research System: Soybean Technology Research System (CARS-04) and the Fund of Common Wealth Industry (Agriculture) Special Research (201103002).

References Cited


