Expression Profile of Two HSP70 Chaperone Proteins in Response to Extreme Thermal Acclimation in Xestia c-nigrum (Lepidoptera: Noctuidae)

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

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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-Hsp70s 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 la 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 primarily act 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. The HSP70 family is

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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 developmental processes 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 (RXZ-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

Table 1. Primers for the experiment in this study.

Primer type	Sequence (5′ to 3′)	Use of primers
P1	GAGGGMRTCGACTTCTAYACBTCCATCAC	PCR for *Xc-HSC70*
P2	CTTGTACTTCTCTGCNTCYTGBACCAT	
P3	AACGARCCBACYGCYGCYGC	PCR for *Xc-HSP*70
P4	GCGTCSATSTCRAADGTGAC	
P5	CGTGAAAAGAAGCCGTCAATCG	For full-length and gDNA sequence of *Xc-HSC*70
P6	GAGTTTCAATCATTTATTGGAGAGACTGTAC	
P7	CACCTTTGCTGAGTTACTCTACGAGTTAAG CTGAAG	For full-length and gDNA sequence of *Xc-HSP70*
P8	CAATATCAGTAAAAGCTTGCTGTAATGTTG	
5′*HSC70*GSP1	CGTTACGTTCTCCACTCCCCTTCTTGTCAAGGCCG	5′ RACE for *Xc-HSC*70
5′*HSC70*GSP2	GTTGGAAGCGAGGTCCTTCTTG	
5′*HSP70*GSP1	ATCAACCCCGACGAGGCCGTAGCTTA	5′ RACE for *Xc-HSP70*
5′*HSP70*GSP2	AGGTACAGGATCTGCTGCTGCTCGA	
3′*HSC70*GSP1	GAACGCTCTGGAATCTTACTGCTTCAACATG	3′ RACE for *Xc-HSC70*
3′*HSC70*GSP2	GAAGTCTACCAACAAGGAGAACAAG	
3′*HSP70*GSP1	GTCGGTGGTTCTACCCGTATTCC	3′ RACE for *Xc-HSP70*
3′*HSP70*GSP 2	TACCGTCGTTCCACTAGTGATTT	
HSC70 F	CTTGCACGGTGACAAGTCTGAG	RT-qPCR for *Xc-HSC*70
HSC70 R	GAATGTCTGGGTTTGCTTGGTG	
HSP70 F	GTGACGCGAAGATGGACAAGTC	RT-qPCR for *Xc-HSP70*
HSP70 R	CACCGTAAGCTACAGCCTCGTC	
β-actin F	CGCGACCTCACAGACTACCTG	RT-qPCR for β-actin
β-actin R	CGTAGGACTTCTCCAGGGAGC	

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

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 5′*HSC70*GSP1, 5′*HSC70*GSP2, 5′*HSP70*GSP1, 5′*HSP70*GSP2, 3′*HSC70*GSP1, 3′*HSC70*GSP2, 3′*HSP70*GSP1, and 3′*HSP70*GSP2 (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 5th 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 $2^{-\Delta\Delta Ct}$ comparative threshold cycle (CT) method (Livak & Schmittgen 2001). The mean and standard deviation were calculated from experi-

ments performed in triplicate and presented as *n*-fold differences in expression. Differences in the transcriptional features of *Xc-HSC70* and *Xc-HSP70* in different developmental stages were analyzed using SPSS 16.0. Statistical significance was determined using one-way analysis of variance (ANOVA) and post-hoc Duncan multiple range tests. Significance was defined as $P < 0.05$.

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

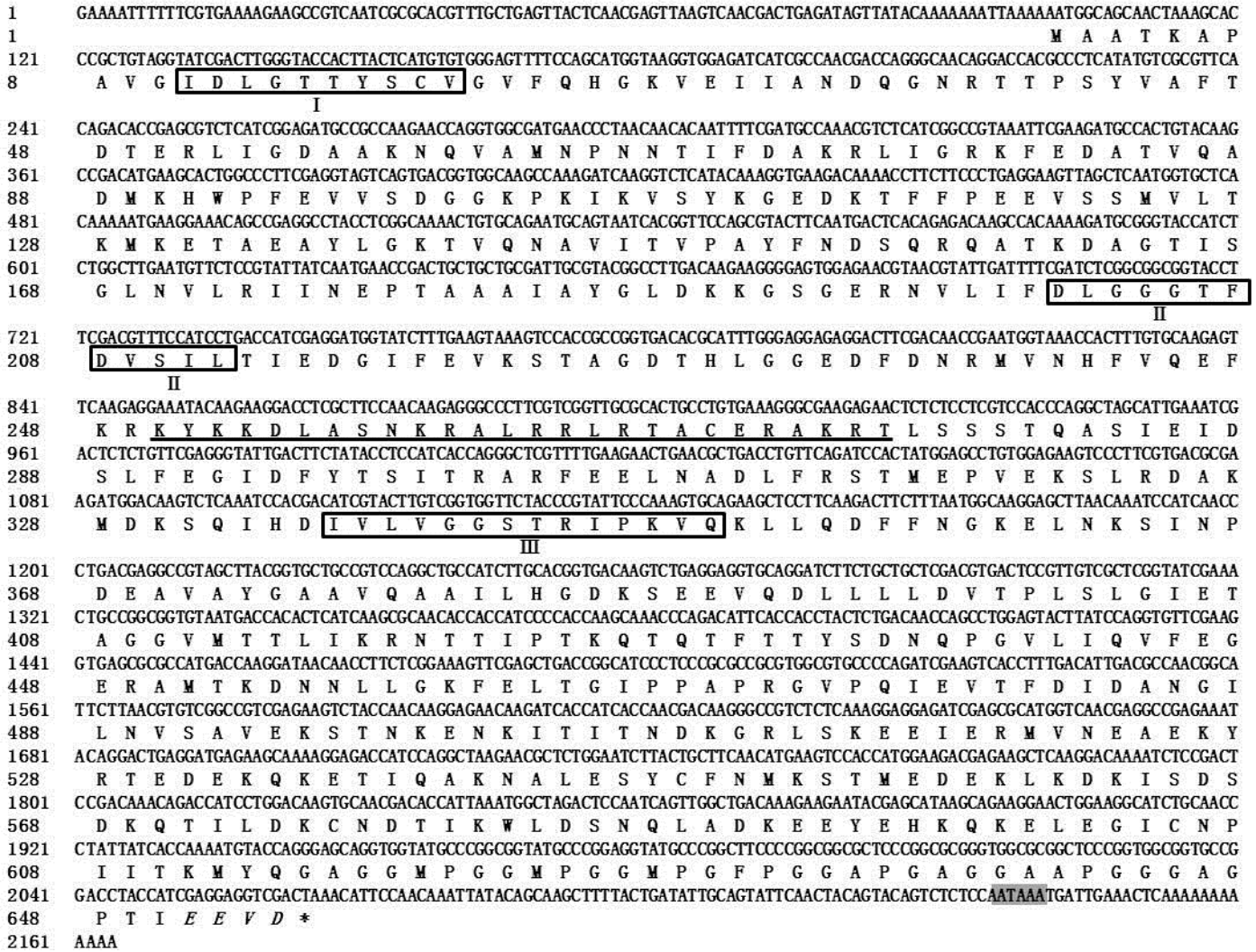

Fig. 1. Nucleotide and deduced amino acid sequences of *Xc-HSC70.* The signature sequences of the *HSP70* family are shown in boxes, the nuclear localization signal sequence is underlined, the consensus sequence EEVD at the C-terminus is indicated in italics, and the start and stop codons are in bold. The nucleotides and amino acids are numbered along the left and right margins. The sequence encoding Xc-HSC70 has been deposited in GenBank under accession no. KC844151.

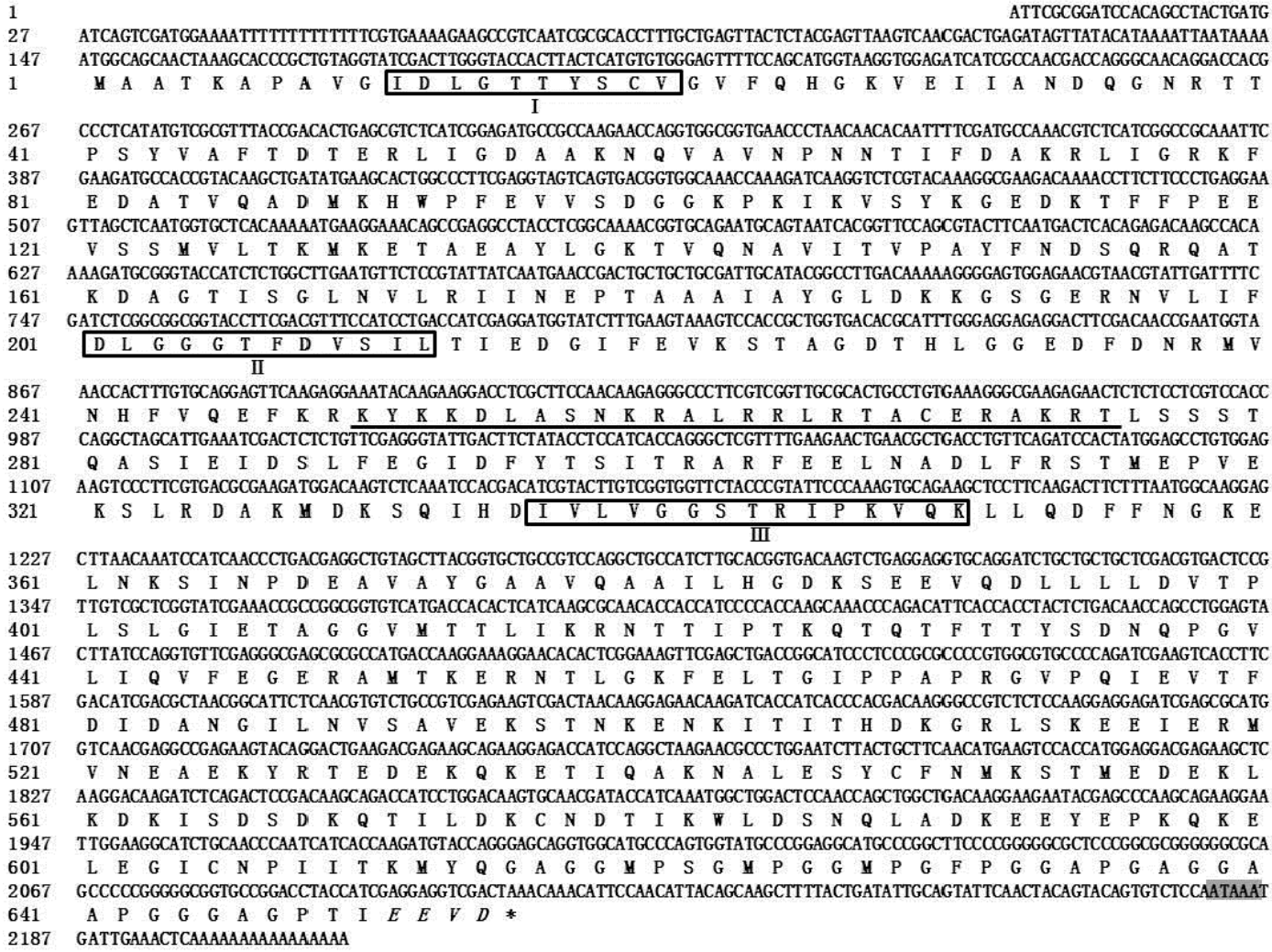

Fig. 2. Nucleotide and deduced amino acid sequences of the *Xc-HSP70* gene. The signature sequences of the *HSP70* family are shown in boxes, the nuclear localization signal sequence is underlined, the consensus sequence EEVD at the C-terminus is indicated in italics, and the start and stop codons are 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.

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**tg**TTC**ca**G**aa) 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 phylogenic tree were in general agreement with traditional taxonomy.

Fig. 3. Schematic structure of the *Xc-HSC70* gene. Exons are shown as boxes in which white boxes represent untranslated regions, whereas the black boxes are the protein-coding exons; introns are indicated as lines between the boxes. The numbers above and below the drawing represent the sizes (base pairs) of each exon and intron, respectively. The start codon (ATG) and stop codon (TAA) are also indicated. The genomic DNA sequence of *Xc-HSC70* has been deposited in GenBank under accession no. KF731994.

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,

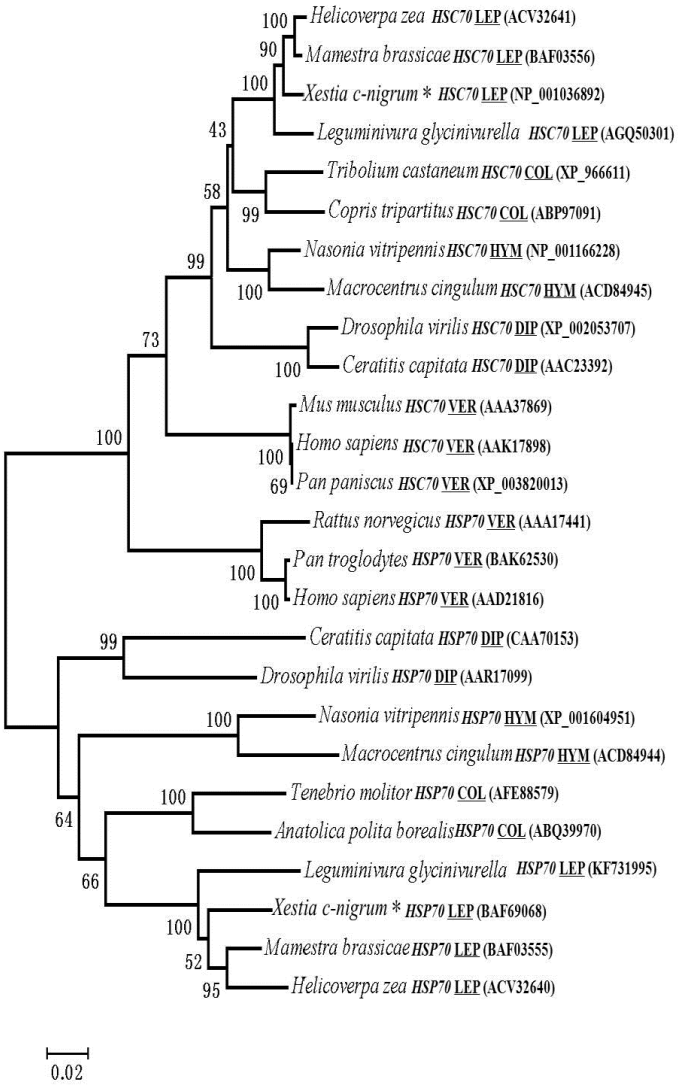

Fig. 4. Phylogenetic tree of Xc-HSC70 and Xc-HSP70 amino acid sequences from different species. A 3-letter code has been included to indicate the order name of the corresponding insect and vertebrate orders (COL = Coleoptera, LEP = Lepidoptera, DIP = Diptera, HYM = Hymenoptera, and VER =Vertebrata). The values indicated on the branches correspond to bootstrap percentages (BP).

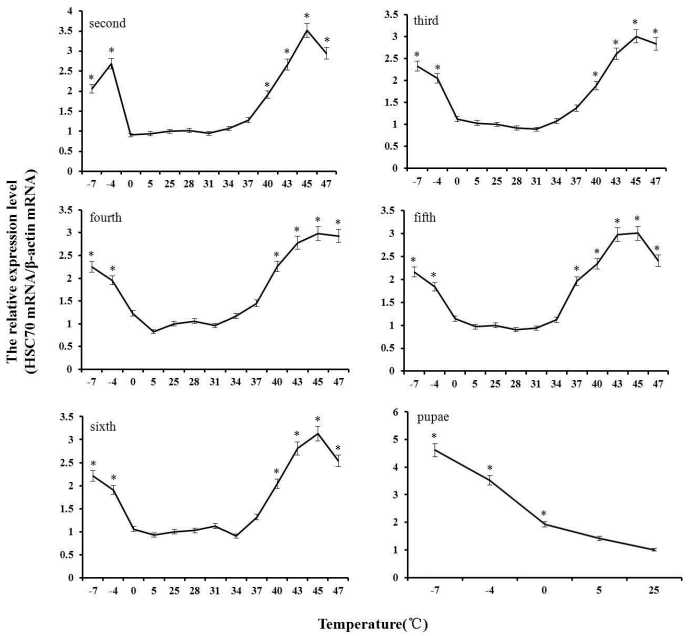

Fig. 5. *Xc-HSC70* mRNA expression profiles induced by cold (−7 to 5 °C) and heat (37 to 47 °C) in 2nd, 3rd, 4th, 5th, and 6th instars and pupae of *Xestia c-nigrum*. The relative quantities indicate the levels of the *HSC70* gene transcript normalized against transcript levels of β-*actin* as an internal standard and compared with the transcript levels of the untreated control at 25 °C. An asterisk indicates a significant difference between the control and heat shock conditions (significant, * $P < 0.05$). The data are denoted as the mean ± SEM (error bar).

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 2th 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 development 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 *HSP*70 and *HSC*70, respectively. Some

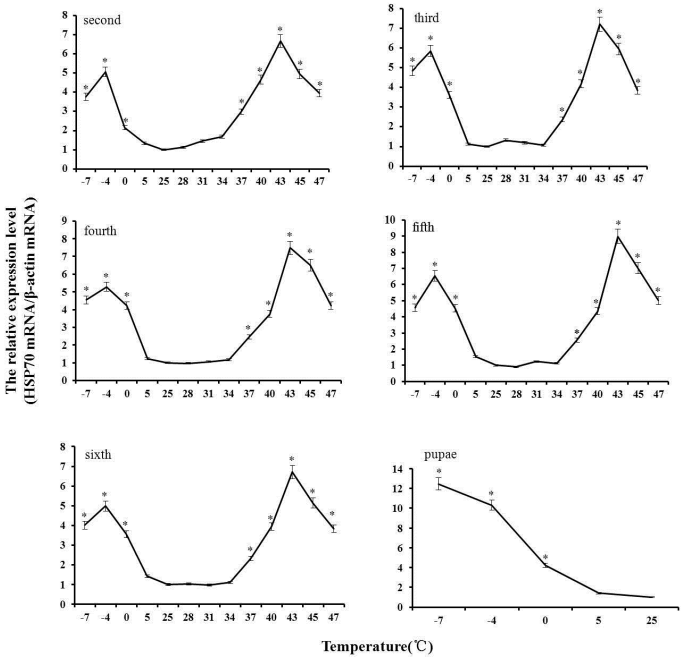

Fig. 6. *Xc-HSP70* mRNA expression profiles induced by cold (−7 to 5 °C) and heat (37 to 47 °C) in 2nd, 3rd, 4th, 5th, and 6th instars and pupae of *Xestia c-nigrum*. The relative quantities indicate the levels of the *HSP70* gene transcript normalized against transcript levels of β-*actin* as an internal standard and compared with the transcript levels of the untreated control at 25 °C. An asterisk indicates a significant difference between the control and heat shock conditions (significant, * $P < 0.05$). The data are denoted as the mean ± SEM (error bar).

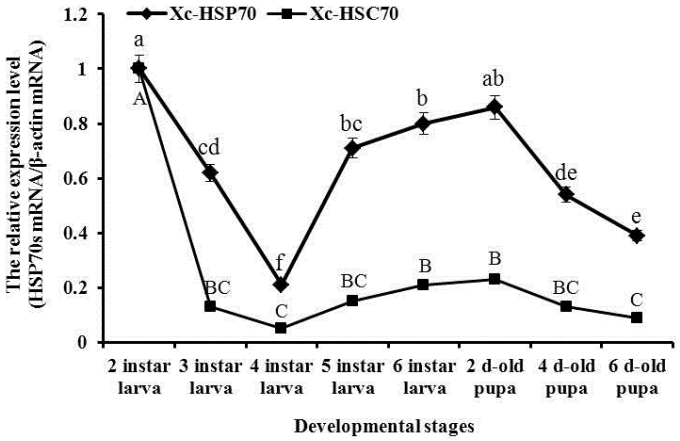

Fig. 7. Expression levels of 2 *HSP70s* at different developmental stages relative to expression levels in 2nd instars at 25 °C. The data are denoted as the mean ± SEM (error bar), and the different lowercase or uppercase letters indicate a significant differenwce in the means as assessed using multi-comparison tests ($P < 0.05$).

studies have found that expression of *HSP*70 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 (a**GAA**tatgca**GAA**tgttcca**GAA**a). 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 (Sonna et al. 2002; Huang & Kang 2007). In *X. c-nigrum*, *Xc-HSC70* and *Xc-HSP70* were positively induced during development when the insect was stressed, regardless of heat or cold, suggesting that up-regulation of these genes is essential for thermotolerance in *X. c-nigrum*.

The level of *Xc-HSP70* expression and transcription was increased after thermal stress treatments in different developmental stages, which suggests that *Xc-HSP70s* are closely associated with insect resistance to thermal stress (Sonoda et al. 2006; Zhang & 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 & Loeschcke1994), 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 *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 and 37 °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 & Loeschcke 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 progress, 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

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