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Identification of heat shock protein genes *hsp70*s and *hsc70* and their associated mRNA expression under heat stress in insecticide-resistant and susceptible diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae)

Lin Jie ZHANG[1,2], Kuan Fu WANG[1,2], Yu Pu JING[1,2], Hua Mei ZHUANG[1,2] and Gang WU[1,*]

[1]Key Laboratory of Biopesticide and Chemical Biology (Ministry of Education), Fujian Agriculture and Forestry University, 350002 Fuzhou, Fujian, China; e-mails: newugan@163.com; zljmeimei6@163.com; wkf456@yahoo.com; zhuanghuamei@yeah.net; yupujing@163.com

[2]Key Laboratory of Integrated Pest Management for Fujian-Taiwan Crops, Ministry of Agriculture, 350002 Fuzhou, Fujian, China

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Abstract. To gain further insight into the molecular features of the ubiquitous Hsp70 family of conserved heat shock proteins, total nine full-length cDNA sequences of inducible *hsp70*s (*Px-hsp69-1*, *-2a*, *-2b*, *-3*, *-4*, *Px-hsp72-1a*, *-1b*, *-2* and *-3*) and one constitutive *hsc70* (*Px-hsc70(C)*) were isolated and characterized in the diamondback moth (DBM), *Plutella xylostella*, collected from Fuzhou, China. The nine *Px-hsp70*s cDNAs encoded the protein of between 629–669 amino acids with molecular weight ranging from 69.00–72.58 kDa and were derived from four *hsp70* genes in the genome of DBM. The *Px-hsc70(C)* cDNA contained 1,953 bp of open reading frame (ORF), which produced a putative protein comprising 650 amino acids with a calculated molecular weight of 71.18 kDa. Whether in adults or larvae of chlorpyrifos-resistant (R_R) and chlorpyrifos-susceptible (S_S) strains of DBM, the basal level (at 25°C) of *Px-hsc70(C)* mRNA expression was high, but no significant up-regulation expression was found under heat stress. However, heat stress facilitated up-regulation expressions of *Px-hsp70*s, and S_S DBM displayed higher up-regulation expression of *Px-hsp70*s than R_R DBM. We suggest that higher up-regulation expression of *Px-hsp70*s in S_S DBM is probably involved in their higher thermal tolerance.

INTRODUCTION

The heat shock proteins or HSPs, a phylogenetically conserved superfamily of proteins, are present in almost all organisms from prokaryotes to eukaryotes (Xu et al., 2010). HSPs play an important ecological and evolutionary role in environmental adaptation and a number of biological processes, including embryogenesis (Cobreros et al., 2008), morphogenesis (Gunter & Degnan, 2007), and diapause (Rinehart et al., 2007). As molecular chaperones, HSPs not only prevent the improper folding or aggregation of proteins, but facilitate the assembly of newly translated proteins and the repair of destroyed proteins (Augustyniak et al., 2009; Xu et al., 2011). Based on molecular mass (MM) and homology of proteins, HSPs are generally divided into several families, including Hsp110, Hsp100, Hsp90, Hsp70, Hsp60 and small Hsps (sHsps, the molecular mass of which ranges from 12 to 43 kDa) (Feder & Hofmann, 1999; Xu et al., 2010). Proteins from different families participate in diverse physiological processes but, in general, they co-operate and complement one another (Augustyniak et al., 2009).

The Hsp70 family, one of the most abundant HSP families, is highly conserved, is characterized by the highest transcript levels, and is the most sensitive to various harmful stimuli (Shu et al., 2011). All proteins of Hsp70 family comprise three distinct domains: an N-terminal adenosine triphosphatase (ATPase) domain (approximately 400 amino acids), a substrate binding domain (approximately 200 amino acids), and a highly variable C-terminal domain (Renner & Waters, 2007). According to subcellular location, eukaryotes possess four types of Hsp70s, each localized to a number of cellular compartments in the cell: cytoplasm, endoplasmic reticulum, mitochondrion, and chloroplast (Renner & Waters, 2007). Moreover, the cytoplasmic Hsp70s are classified into two kinds: one, heat shock inducible protein 70 (Hsp70), is expressed at low basal levels under non-stress conditions but can be quickly induced by heat shock and other environmental stresses; the other, heat shock cognate or constitutive protein 70 (Hsc70), is constitutively expressed under normal conditions and remains unchanged or slightly up-regulated upon exposure to stresses of one form or another (Mahroof et al., 2005; Daugaard et al., 2007). Although their expression patterns are different, both Hsp70 and Hsc70 participate in removing abnormal cellular proteins and help stabilize proteins during folding (Qin et al., 2003).

Since HSPs were first discovered in the fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) by Ritossa in 1962 (Ritossa, 1962), more and more HSPs, especially Hsp70 family genes, have been identified and subsequently well studied in insects, including in Lepidoptera (Sonoda & Tsumuki, 2008; Gkouvitsas et al., 2009; Zhang & Denlinger, 2010; Xu et al., 2011), Diptera (Frydenberg

* Corresponding author.

et al., 2003; Goto & Kimura, 2004), Hymenoptera (Elekonich, 2009), Orthoptera (Qin et al., 2003), and Coleoptera (Mahroof et al., 2005; Dahlhoff & Rank, 2007). Interestingly, almost all eukaryotes have more than one gene that encodes Hsp70 proteins (Daugaard et al., 2007). For example, the fungus *Blastocladiella emersonii* comprises ten putative Hsp70 family members with high homology to their counterparts in yeast (Georg Rde & Gomes, 2007). Yeast has eight Hsp70 homologues, of which six reside in the cytoplasm, whilst two are localized in mitochondria and the endoplasmatic reticulum, respectively (Werner-Washburne & Craig, 1989). In *D. melanogaster*, eight *hsp70* genes are identified through the bioinformatic analysis of the whole genome, and each gene could generate the varying amounts of mRNAs upon alternative splicing (Mou et al., 2011). In the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae), two clusters of six *hsp70* genes are found through the basic local alignment searches of the genome (Gross et al., 2009). The human Hsp70 family contains at least eight proteins with distinct amino acid sequences, expression levels and subcellular localization (Tavaria et al., 1996). However, knowledge concerning the number of Hsp70 family members in lepidopteran insects including the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae) is still far from clear.

Hsp70 is responsive to various environmental stresses, including heat shock (Xu et al., 2011), heavy metals (Shu et al., 2011), radiation (Schmid & Multhoff, 2012), chemical compounds (Rhee et al., 2009), hypoxia (Cheng et al., 2003) and osmotic stress (Spees et al., 2002). For example, up-regulated expression of *hsp70* under heat stress was found in many insects, such as noctuid pest moth species *Helicoverpa zea* (Boddie) (Zhang & Denlinger, 2010), *Spodoptera exigua* (Hübner) (Xu et al., 2011) and *S. litura* (Fabricius) (Shen et al., 2014), hymenopterous wasp parasitoids, *Macrocentrus cingulum* (Xu et al., 2010) and *Cotesia vestalis* Haliday (Braconidae) (Shi et al., 2013) and the fruit fly, *D. melanogaster* (Udaka et al., 2010). Increased expression of *hsp70* was observed when the non-biting midge, *Chironomus yoshimatsui* Martin & Sublette (Diptera: Chironomidae), or *D. melanogaster* was treated with pyrethroid insecticide (Mukhopadhyay et al., 2002; Yoshimi et al., 2002). The insect *hsp70* expression was strongly up-regulated in response to heavy metals, including Zn, Cu, Cd, Ag, Pb and Ni (Sonoda et al., 2007; Augustyniak et al., 2009; Karouna-Renier & Rao, 2009; Ahamed et al., 2010). In addition, cold stress also affected *hsp70* expression (Colinet et al., 2010; Xu et al., 2011). However, knowledge about expression levels of Hsp70 family protein genes (there is more than one *hsp70* gene) under heat stress has been limited in some insect species, more especially in relation to insecticide-resistant and -susceptible strains.

The diamondback moth (DBM), *Plutella xylostella* is one of the most destructive lepidopteran pests of cruciferous crops worldwide. It is well known that DBM have developed resistance against many insecticides, including organophosphates, pyrethroids, carbamates, avermectin, and fipronil (Wu & Jiang, 2002). In our previous work, a seasonal change of resistance level to insecticides, high in spring and autumn, but low in summer, was found in field DBM populations (Wu & Jiang, 2002). Compared to insecticide-susceptible DBM, insecticide-resistant insects displayed significantly lower ecological and physiological fitness (significantly lower population growth tendency index values and fecundity) under heat stress (Liu et al., 2008). In that study it was suggested that low fitness in insecticide-resistant DBM caused by high temperature might well be involved in the sharp decline in the frequency of the insecticide resistance forms in the field during the summer months, and the evolution of insecticide resistance of DBM could be affected by heat stress (Liu et al., 2008; Zhuang et al., 2011). Although it is well established that Hsp70 family increase heat tolerance and protect organisms from thermal injury and killing (Gehring & Wehner, 1995), study on the effects of heat stress on mRNA expression of *hsp70*s in insecticide-resistant and -susceptible insect strains was generally sparse. In the present study, we cloned ten members of the Hsp70 gene family from *P. xylostella* (nine *Px-hsp70*s and *Px-hsc70(C)*) and investigated their expression profiles under heat stress in order to evaluate the fitness cost in chlorpyrifos-resistant and -susceptible moths affected by such stress.

MATERIAL AND METHODS

Source of insect

A field population of DBM (starting population) was obtained from the commercial crucifer fields located at Shangjie, Fujian, China in November 2005 and subsequently reared on *Brassica oleracea* in an insecticide-free field insectary at FAFU, Fujian, China for one year (about 18 generations). 800 pupae were randomly chosen from the reared population in November 2006 and then reared in two field insectaries, A and B, respectively. These insectaries (4 m × 2 m × 4 m) were constructed with stainless-steel net and a glass roof to prevent contamination of the captive DBM from external DBM populations. Insects in insectary A, the control, were never exposed to insecticides and were highly susceptible to chlorpyrifos after November 2008, and were defined as chlorpyrifos-susceptible (S_i). In contrast, in insectary B, DBM were selected using chlorpyrifos during November 2006–2008 (about 36 generations), the surviving insects being highly resistant to chlorpyrifos, and thereafter designed as chlorpyrifos-resistant (R_c). S_S was created by crossing a male and female insect randomly chosen from the S_i population. R_R was generated by treating R_c population for several generations with a dose of chlorpyrifos that resulted in ~97% DBM mortality at 25°C. In our previous work, three nucleotide substitutions of the *Acetylcholinesterase 1* (*Ace-1*) gene, which resulted in three amino acids mutations, i.e., A201S, G227A and A441G, were found in resistant DBM population from Fuzhou (GenBank acc. no. JQ085429 and JQ085428). Of the three mutations, the G227A mutation was thought to be the most important in terms of conferring resistance of DBM to organophosphate insecticides (Baek et al., 2005). In this earlier paper, and according to our analysis using a large number of individual DBM, the S_S population comprised a susceptible homozygote with 100% *SS* genotypes at the G227A locus *Ace-1* whilst the R_R population comprised a resistant homozygote with 100% *RR* at the same locus. The difference in chlorpyrifos-resistance was >100-fold between R_R and S_S.

Table 1. Sequences of primers used for cloning *hsp70* and *hsc70* cDNAs of DBM.

Names of primers	Sequences of Primers (5'–3')	Tm (°C)	Isolated gene (Samples for extracting total RNAs)	Positions of forward and reverse primers
For initial fragment(s)				
Hsp70-F*	5'-GACATGAAGCACTGGCCKTTCAA-3'	57.9	*Px-hsp69-1* (sample 1)	357–379, 1,629–1,651
Hsp70-R*	5'-TCAATYTCRGCCTGCGAGAGGCG-3'	58.5	*Px-hsp72-1* (sample 1)	355–377, 1,627–1,649
			Px-hsp69-2a (sample 2)	344–366, 1,616–1,638
			Px-hsp69-3 (sample 3)	431–453, 1,703–1,725
Hsp70-F2	5'-GCGGCGAGGACTTTGACA-3'	54.4	*Px-hsp69-4* (sample 2)	778–795, 1,420–1,446
Hsp70-R2	5'-CTTBGTCATSGCKCKCTCKCCCTCGT-3'	59.1		
Hsp70-F3	5'-TBAACGTGCTBCGSATCATCAACGAGC-3'	61.4	*Px-hsp69-4* (sample 2)	592–618, 1,171–1,189
Hsp70-R3	5'-TCTGGGTTGATGGATAGGT-3'	56.2		
For RACE				
Hsp70-3'-a*	5'-GCAAGCAGTCGCAGACGTTCACC-3'	63.1	*Px-hsp69-1* (sample 1)	1,369–1,391, 1,597–1,619
Hsp70-3'-b*	5'-GCAAGAACATCGTSATCAAGAAC-3'	54.2	*Px-hsp72-1* (sample 1)	1,367–1,389, 1,595–1,617
			Px-hsp69-2a (sample 2)	1,356–1,378, 1,584–1,606
			Px-hsp69-3 (sample 3)	1,443–1,465, 1,671–1,693
Hsp70-5'-a*	5'-GAGTCGTTGAAGTAKGCCGGCAC-3'	59.5	*Px-hsp69-1* (sample 1)	537–559, 450–472
Hsp70-5'-b*	5'-AGCACCATGCTGCTGATCTCCTC-3'	59.4	*Px-hsp72-1* (sample 1)	535–557, 448–470
			Px-hsp69-2a (sample 2)	524–546, 437–459
			Px-hsp69-3 (sample 3)	611–633, 524–546
Hsp-3P1	5'-GCTCAACCTATCCATCAACC-3'	50.1	*Px-hsp69-4* (sample 2)	1,166–1,185, 1,391–1,411
Hsp-3P2	5'-CAACCAGCCCGCCGTCACCAT-3'	65.5		
Hsp-5P1	5'-CAGTGCGTCGATCTCGATGGT-3'	57.3	*Px-hsp69-4* (sample 2)	933–953, 787–806
Hsp-5P2	5'-CACGAGGCGGTTGTCGAAGT-3'	57.4		
UPM**	5'-CTAATACGACTCACTATAGGGCAAG CAGTGGTAACAACGCAGAGT-3'	67.9		
NUP**	5'-AAGCAGTGGTAACAACGCAGAGT-3'	57.8		
For ORF				
Hsp72-1-F	5'-AATCAAAGCGAAAATAGGA-3'	46.5	*Px-hsp72-1a* (sample 1)	
Hsp72-1-R	5'-CAAACATTGGCAAAACAA-3'	45.8	*Px-hsp72-1b* (sample 2 and 3)	
Hsp69-2a-F	5'-TACGAAGCGAAGTAAAACCAA-3'	51.7	*Px-hsp69-2b* (sample 1 and 2)	
Hsp69-2a-R	5'-CCAGACGATCAAATTAAGGA-3'	53.4		
Hsp70-21	5'-TGAGAAATCAAAGCGAAAATAGGAG-3'	55.2	*Px-hsp72-2* (sample 1)	1–25 (Hsp70-61)
Hsp70-61	5'-GAAACGCTACGAGTTATTTACGAAG-3'	53.1	*Px-hsp72-3* (sample 3)	1–25 (Hsp70-61)
Hsc70-F	5'-AGTGAAAAGAAGCCGTCA-3'	50.3	*Px-hsc70(C)* (samples 1–3)	1–18, 2,027–2,047
Hsc70-R	5'-CTTTGGAATGTAGTTTAGTCG-3'	51.7		

*The primer sequences were as described by Sonoda & Tsumuki (2008). **The primer sequences were as described in the SMART™ RACE cDNA Amplification Kit. Note: When cloning *Px-hsp72-1a*, *-1b* and *Px-hsp69-2b*, the obtained clones were sequenced using Hsp72-1-F and Hsp72-1-R, Hsp69-2a-F and Hsp69-2a-R, respectively. Therefore, Hsp72-1-F and Hsp72-1-R, Hsp69-2a-F and Hsp69-2a-R could not be found in the nucleotide sequences of *Px-hsp72-1a*, *-1b* and *Px-hsp69-2b*, respectively.

Cloning and nucleotide sequencing of *hsp70*s and *hsc70*

Temperature shock of samples

For cloning *hsp70*s and *hsc70*, second instar larvae from S_i and R_c populations were collected and reared at 25°C. F_1 progenies of new 4th instar larvae and unsexed newly emerged adults were used for the experiments. The DBM were pretreated at different temperature by rearing larvae at 42°C (sample 1), and adults at 25°C (sample 2) and 42°C (sample 3) for 3 h prior to insect total RNA extraction.

Amplification of the initial fragments of *hsp70*

Total RNAs were extracted according to the manufacturer's instructions for the RNA Simple Total RNA Extraction Kit (Tiangen Biotech Co., Ltd., Beijing, China). First-strand cDNAs were synthesized from 1 μg of total RNAs using SuperScript™III First-Strand System (Life Technologies, Carlsbad, California, USA). RT-PCR was conducted according to the method of Sonoda & Tsumuki (2008). Three initial *hsp70* fragments were amplified from the samples 1–3, respectively, by PCR using degenerate primers as detailed in Sonoda & Tsumuki (2008) (Table 1). PCR conditions were as follows: 94°C denaturation for 3 min, followed by 40 cycles of 94°C for 30 s, an annealing step at 55°C for 1 min, an extension step at 72°C for 2 min, and a final extension step at 72°C for 7 min. The initial fragments of *Px-hsp69-1* (from sample 1), *Px-hsp69-2a* (from sample 2) and *Px-hsp69-3* (from sample 3) were obtained, although the same primers and PCR reaction conditions were used. In addition, two internal fragments with lengths 601 and 668 bp of *Px-hsp69-4* were obtained from sample 2 using the degenerate primers (Table 1). PCR conditions here were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 90 s, finally 72°C for 5 min. Based on the two fragments, an internal cDNA fragment (857 bp) of *Px-hsp69-4* was edited and assembled.

Table 2. Sequences of primers used for qPCR of *hsp70*s and *hsc70* of DBM.

Primers	Sequences of primers (5'–3')	Tm (°C)	Gene names	Positions of forward and reverse primers
β-actin-F	5'-ACCGGTATCGTGCTGGACTC-3'	53.8	*β-actin*	448–467, 667–686
β-actin-R	5'-GCCATCTCCTGCTCGAAGTC-3'	53.7		
Pxh1-F	5'-CTGCTGGTGGATGTGGCT-3'	51.3	*Px-hsp69-1*	1,278–1,295, 1,391–1,408
Pxh1-R	5'-TGGTTGTCCGCGTAGGTC-3'	51.4		
Pxh2-F	5'-CGGCATCGACTACTACACCA-3'	51.0	*Px-hsp69-2a*	958–977, 1,089–1,108
Pxh2-R	5'-GCCTCCGACTAAGACCACAT-3'	50.6		
Pxh3-F	5'-GCGTACCTCGGGACTACTG-3'	52.6	*Px-hsp69-3*	572–590, 687–704
Pxh3-R	5'-TGGGCTCGTTGATGATGC-3'	52.3		
Pxh4-F	5'-TCGCCTTCACCGACACC-3'	52.1	*Px-hsp69-4*	217–233, 372–391
Pxh4-R	5'-TTGCCTCCATCACTGACCAC-3'	53.0		
Pxh5-F	5'-CGACGGCATCGACTACTACA-3'	51.2	*Px-hsp72-2*	907–926, 987–1,006
Pxh5-R	5'-GAGAGCCTTTTCAACGGGTT-3'	52.5		
Pxh6-F	5'-CGAAGCGAATTAAAACCA-3'	53.4	*Px-hsp72-3*	21–38, 161–178
Pxh6-R	5'-TCCGTGAAAGCCACATAT-3'	52.6		
Pxhsc70-F	5'-CTCCGTATTATCAACGAACC-3'	50.5	*Px-hsc70(C)*	591–610, 755–774
Pxhsc70-R	5'-CACCTCCCAAGTGAGTGTCA-3'	51.3		

Rapid Amplification of cDNA Ends (RACE) of *hsp70*

For 3'- and 5'-RACE, the first-strand cDNAs were separately constructed from 1 μg of total RNA according to the SMART™ RACE cDNA Amplification Kit (TaKaRa Bio Inc., Otsu, Japan). In RACE of *Px-hsp69-1*, *-2a* and *-3*, the RACE was conducted according to the method of Sonoda & Tsumuki (2008). cDNAs were amplified using specific primers (Hsp70-3'-a or Hsp70-5'-a) and Universal Primer A Mix (UPM) (Table 1). The PCR products were used for re-amplification using Hsp70-3'-b or Hsp70-5'-b and the nested universal primer (NUP) (Table 1). PCR conditions for 3'- and 5'-RACE were 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, finally 72°C for 7 min. The full-length cDNAs of *Px-hsp69-1*, *Px-hsp72-1*, *Px-hsp69-2a*, and *Px-hsp69-3* were edited and assembled from the initial fragments and the fragments obtained from 3'- and 5'-RACE. Because there were two fragments (573 and 690 bp) obtained from sample 1 in 3'-RACE, two sequences, i.e., *Px-hsp69-1* and *Px-hsp72-1*, were edited.

In RACE of *Px-hsp69-4*, Hsp-3P1 and Hsp-3P2 were used for 3'-RACE, and Hsp-5P1 and Hsp-5P2 for 5'-RACE accompanied by UPM and NUP, respectively. PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min, finally 72°C for 7 min. Two fragments with 736 bp (3'-RACE) and 806 bp (5'-RACE) were obtained. The full-length cDNA of *Px-hsp69-4* was edited and assembled by the internal fragment (857 bp), 3'- (736 bp) and 5'-RACE fragment (806 bp).

Amplification of ORFs

To identify the edited full-length sequences, we amplified the ORFs by using forward and reverse primers corresponding to the 5' and 3'-ends of the full-length sequences, respectively. To amplify the ORF of *Px-hsp72-1*, by using the primers (Table 1) designed based on the full-length sequence of *Px-hsp72-1*, three full-length ORF fragments were amplified from the samples 1–3, respectively. PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 43°C for 30 s, and 72°C for 2 min, finally 72°C for 10 min. To amplify the ORF of *Px-hsp69-2a*, using the primers (Table 1) designed based on the full-length sequence of *Px-hsp69-2a*, two full-length ORF fragments were amplified from the sample 1 and 2 among the samples 1–3, respectively. PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 47°C for 30 s, and 72°C for 2 min, finally 72°C for 10 min. To amplify the ORF of *Px-hsp69-3*, two forward primers (Hsp70-21 and Hsp70-61) (Table 1) were designed according to the sequence of *Px-hsp69-3*. 3'-RACE was conducted using 3'RACE-Ready cDNAs from the samples 1–3 using the primer Hsp70-21 and UPM. The PCR products were used for re-amplification using the primer Hsp70-61 and NUP. Two full-length ORF fragments were obtained from sample 1 and 3 among the samples 1–3, respectively. PCR conditions were 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, finally 72°C for 7 min.

For cloning the ORF of *Px-hsc70*, three full-length ORF fragments were amplified from the samples 1–3 using the primers, Hsc70-F and Hsc70-R (Table 1), which were designed based on the nucleotide sequence of *hsc70* of DBM reported by Sonoda et al. (2006). PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 46°C for 45 s, and 72°C for 2 min, finally 72°C for 10 min.

The initial fragments, 3'- and 5'-RACE fragments and the ORF fragments were cloned and sequenced by Shanghai Biosune Biotechnology Co., Ltd., Shanghai, China.

Real-time quantitative PCR (qPCR) for expression of *hsp70*s and *hsc70* after heat stress

Temperature shock

The adults or larvae of R_R or S_S DBM were pretreated at 25, 37 and 42°C for 3 h. After heat-stress treatment, the survival rates of adults and larvae were 100%. The adults and larvae after heat stress were allowed to recovery for 1 h at 25°C before being used for detecting mRNA expression.

Determination of mRNA expression

Extraction of total RNAs and the synthesis of the cDNAs were as described above. The primers used for qPCR of *Px-hsp70*s and *Px-hsc70(C)* are listed in Table 2, whilst the primers for *β*-actin (housekeeping gene) were used as the endogenous control. qPCR was performed using a Bio-Rad Miniopticon Real-Time PCR System (Bio-Rad Laboratories, Hercules, California, USA) with SYBR Premix Ex Taq™ kit (TaKaRa Bio Inc.) as follows: 95°C for 10 s; 40 cycles of 95°C for 6 s, 60°C for 25 s, read plate 10 s. Subsequently, the homogeneity of the PCR products was confirmed by melting curve analysis. The expression level of each gene was calculated according to the threshold cycle (Ct) and equation of the standard curve. Therefore, the normalized expression value of the target gene was calculated by comparing the

Table 3. Analysis of nucleotide and inferred amino acid sequences of *hsp70*s cDNA of DBM.

	Px-hsp69-1	*Px-hsp69-2a*	*Px-hsp69-2b*	*Px-hsp69-3*	*Px-hsp69-4*	*Px-hsp72-1a*	*Px-hsp72-1b*	*Px-hsp72-2*	*Px-hsp72-3*	*Px-hsp72-J*
GenBank acc. no.	HM370509.1	HM370510.1	JQ693014.1	HM370511.1	HM212645.1	JQ693015.1	JQ711194.1	HQ107971.1	HQ107972.1	AB325801.1
Full-length (bp)	2,171	2,146	2,019	2,248	2,172	2,092	2,070	2,245	2,222	2,258
ORF	111–2,000	98–1,987	85–1,974	185–2,074	90–1,988	62–2,053	39–2,030	50–2,059	51–2,054	107–2,101
Putative protein (aa)	629	629	629	629	632	663	663	669	667	664
Molecular weight (kDa)	69.02	69.07	69.00	69.05	69.27	72.16	72.13	72.58	72.39	72.26
Polyadenylation signal. The 3'untranslated regions of these *hsp70*s genes contained several typical motifs, such as the polyadenylation signal.										
ATTAAA	1,996–2,001	1,983–1,988	1,970–1,975	2,070–2,075	1,984–1,989	2,049–2,054	2,026–2,031	–	2,050–2,055	–
AATAAA	2,120–2,125	2,104–2,109	–	2,192–2,197	2,099–2,104	–	–	2,193–2,198	2,181–2,186	2,239–2,244
AT-rich element/RNA instability motif. The AT-rich element (ATTTA) has been shown to afford greater mRNA stability at normal temperatures and to contribute to the maintenance and re-establishment of basal levels of gene expression.										
ATTTA	2,001–2,005 2,132–2,136	2,041–2,045 2,116–2,120	–	2,204–2,208	2,111–2,115 2,121–2,125	–	–	2,120–2,124 2,209–2,213	2,113–2,117 2,197–2,201	2,162–2,166

Note: *Px-hsp72-J* was identified in Japanese DBM (Sonoda & Tsumuki, 2008).

expression value of the target gene with that of *β*-actin (Larionov et al., 2005; Zhuang et al., 2011). All data obtained from qPCR were analyzed using the Statistical Product and Service Solutions (SPSS). mRNA expression was replicated three times with 12 insect individuals per replication.

RESULTS

Cloning and sequencing analysis of *Px-hsp70*s

For 3'-RACE, two fragments of 573 and 690 bp were obtained from sample 1. Based on these initial fragments and 3'- and 5'-RACE amplification fragments, four full-length cDNA sequences of *hsp70*, named *Px-hsp69-1*, *Px-hsp72-1* (sample 1), *Px-hsp69-2a* (sample 2), and *Px-hsp69-3* (sample 3), were edited and assembled. The four full-length cDNA sequences were 2,171 (with 629 amino acids, aa), 2,284 (with 667 aa), 2,146 (with 629 aa), and 2,248 bp (with 629 aa), respectively, with calculated molecular weight 69.02, 72.39, 69.07 and 69.05 kDa, respectively (Table 3). The full-length cDNA sequence of *Px-hsp69-4* was 2,172 bp (with 632 aa), the calculated molecular weight 69.27 kDa (Table 3).

For cloning the ORF of *Px-hsp72-1*, three full-length ORF fragments from samples 1–3 were estimated at 2,092, 2,070, and 2,070 bp, respectively (663 aa), with calculated molecular weights of 72.16, 72.13 and 72.13 kDa, respectively. The amino acid sequences from sample 2 and 3 were the same, but showed 98.5% identity to the sequence from sample 1. Therefore, the two sequences from sample 1 and 2 were named as *Px-hsp72-1a* and *Px-hsp72-1b*, respectively. For cloning the ORF of *Px-hsp69-2a*, two full-length ORF fragments from sample 1 and 2 were estimated at 2,031 and 2,019 bp, respectively (629 aa) and with the same calculated molecular weight, 69.00 kDa. The two amino acid sequences shared 100% similarity, and named as *Px-hsp69-2b* because of 93.5% similarity with *Px-hsp69-2a*. No ORF fragment was amplified from sample 3. For cloning the ORF of *Px-hsp69-3*, two full-length ORF fragments from sample 1 and 3 were estimated at 2,245 (with 669 aa, 72.58 kDa) and 2,222 bp (with 667 aa, 72.39 kDa), respectively and were named as *Px-hsp72-2* and *Px-hsp72-3*, respectively. The two sequences shared 98.5% identity with *Px-hsp72-1*, respectively. No sequence was amplified from sample 2.

Total nine full-length cDNA sequences of *Px-hsp70*s (*Px-hsp69-1*, *-2a*, *-2b*, *-3*, *-4*, *Px-hsp72-1a*, *-1b*, *-2* and *-3*), encoding the protein of 629–669 amino acids with molecular weight of 69.00–72.58 kDa, were identified in this study and named according to their molecular weight (Table 3). Nucleobase positions of polyadenylation signals (ATTAAA or AATAAA) and the AT-rich element (ATTTA), which has been shown to afford greater mRNA stability at normal temperatures and to contribute to the maintenance and re-establishment of basal levels of gene expression (Lindquist & Petersen, 1990) at nucleotide sequences of *Px-hsp70*s, are shown in Table 3. The nine amino acid sequences of *Px-hsp70*s contained three highly conserved Hsp70 family signatures (IDLGTTYS, IFDLGGGTFDVSIL, and VVLVGGSTRIPKIQT) (Gupta & Singh, 1994), a putative ATP/GTP binding site (AEAYLGTS) (Saraste et al., 1990), and two putative nuclear localization signal motifs (KRKYHKDLTGNARALRR and ARALRRLRTAAERAKRT) (Knowlton & Salfity, 1996) (Fig. 1).

The nine *Px-hsp70*s showed a high degree of homology with *Px-hsp72-J* identified from Japanese DBM (Sonoda & Tsumuki, 2008) (Fig. 1). The amino acid sequence similarity of *Px-hsp72-J* was 92.9% with *Px-hsp69-1*, 92.2% with *Px-hsp69-2a*, 87.3% with *Px-hsp69-2b*, 90.6% with *Px-hsp69-3*, 82.5% with *Px-hsp69-4*, 97.3% with *Px-hsp72-1a*, 98.2% with *Px-hsp72-1b*, 97.7% with *Px-hsp72-2*, and 98.0% with *Px-hsp72-3*, respectively. *Px-hsp69-1*, *-2a*, *-2b*, *-3* and *-4* shared about 88.4–98.1% similarity with each other. *Px-hsp72-1a*, *-1b*, *-2* and *-3* shared about 96.8–98.2% similarity. Phylogenetic analysis revealed *Px-hsp70*s from *P. xylostella* (nine *Px-hsp70*s identified in this study and *Px-hsp72-J* from Japanese DBM) formed a sister subgroup with other lepidopteran insects (Fig. 2), and they shared an identity of 83–95%.

The cDNA sequences of *Px-hsp70*s were used as queries to perform BLAST searches against the whole genome of DBM in the National Centre for Biotechnology Informa-

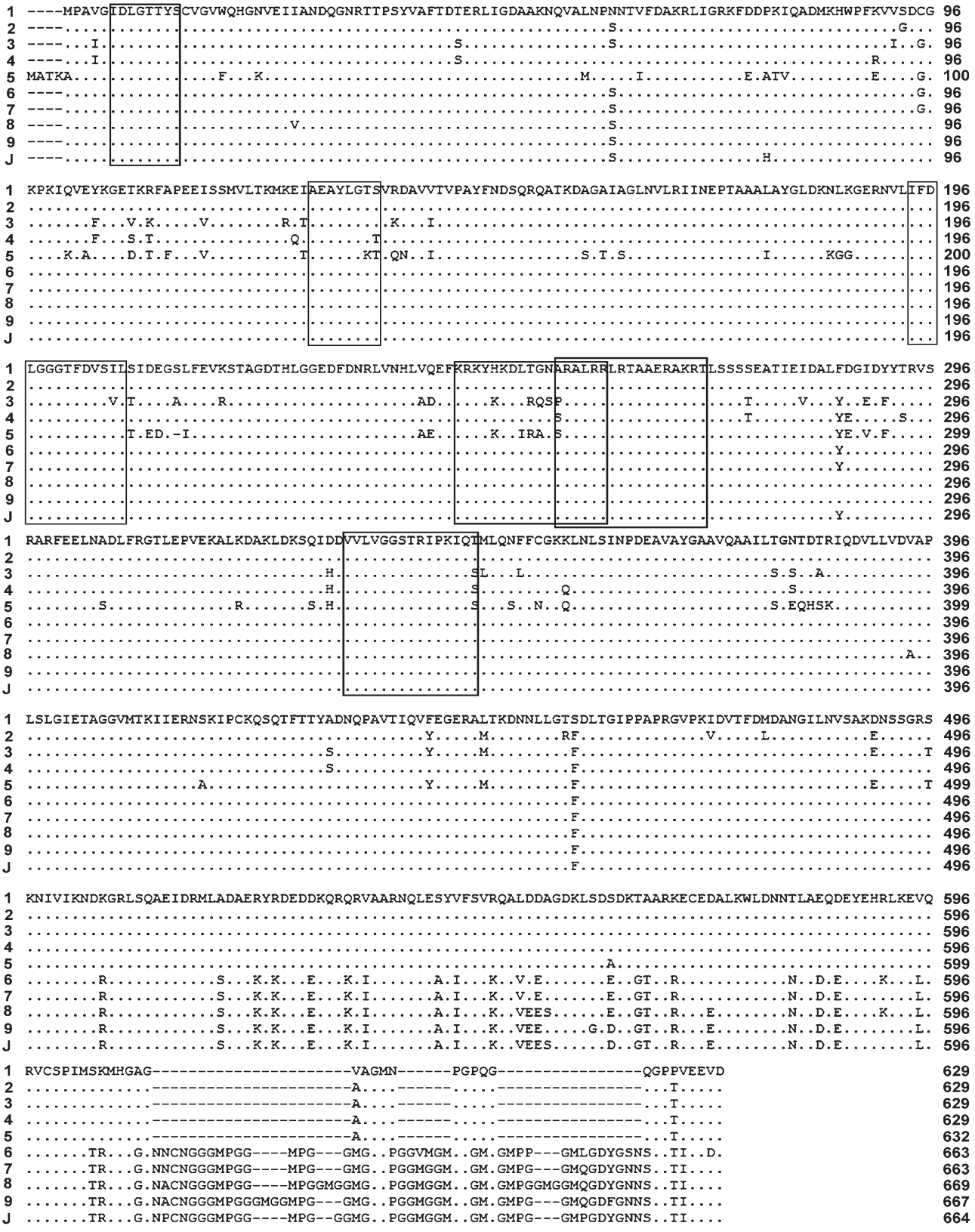

Fig. 1. The amino acid sequences alignment of *Px-hsp70*s in DBM. The protein alignment was created using Lasergene v7.1 (DNASTAR, Inc., Madison, Wisconsin, USA). 1–9: *Px-hsp69-1* (ADK94697.1), *Px-hsp69-2a* (ADK94698.1), *Px-hsp69-2b* (AFQ37587.1), *Px-hsp69-3* (ADK94699.1), *Px-hsp69-4* (ADK39311.1), *Px-hsp72-1a* (AFQ37588.1), *Px-hsp72-1b* (AFQ33498.1), *Px-hsp72-2* (ADV58254.1), *Px-hsp72-3* (ADV58255.1) were in this study identified in Fujian DBM. J: *Px-hsp72-J* (BAF95560.1) identified in Japanese DBM (Sonoda & Tsumuki, 2008). Three highly conserved Hsp70 family signatures (IDLGTTYS, IFDLGGGT-FDVSIL, and VVLVGGSTRIPKIQT), a putative ATP/GTP binding site (AEAYLGTS) and putative nuclear localization signal motifs (KRKYHKDLTGNARALRR and ARALRRLRTAAERAKRT) are shown boxed. Identical amino acid residues are indicated by dots. Gaps (indicated by dashes) were added to improve the alignment.

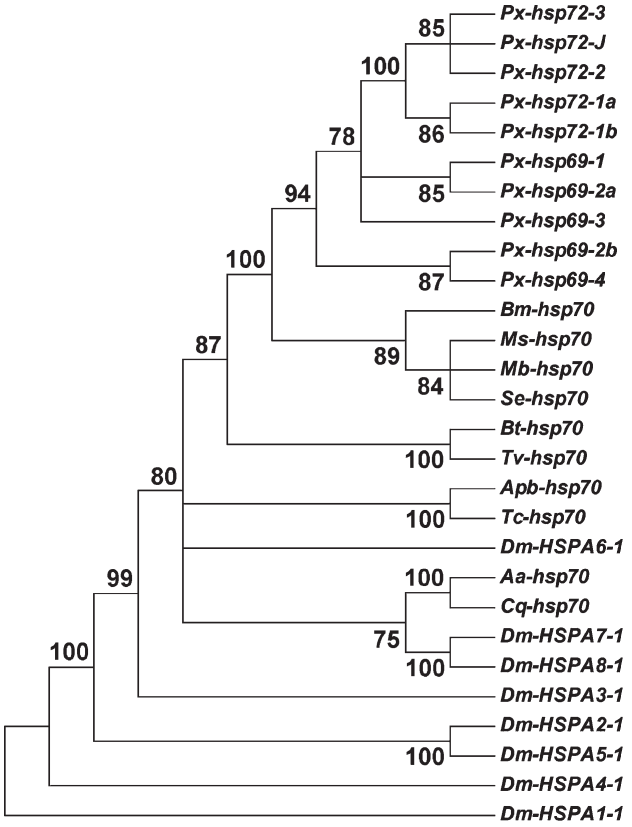

Fig. 2. The phylogenetic analysis of *Px-hsp70s* in DBM and other insect species using the neighbor-joining method (MEGA 4.0). The bootstrap cut-off value was 70%. *Bm-hsp70*: *Bombyx mori* (NP-001037396.1); *Mb-hsp70*: *Mamestra brassicae* (BAF03555.1); *Se-hsp70*: *Spodoptera exigua* (ACN78407.1); *Ms-hsp70*: *Manduca sexta* (AAO65964.1); *Aa-hsp70*: *Aedes aegypti* (ACJ64194.1); *Cq-hsp70*: *Culex quinquefasciatus* (XP_001861436.1); *Apb-hsp70*: *Anatolica polita borealis* (ABQ39970.1); *Tc-hsp70*: *Tribolium castaneum* (NP_001164199.1); *Bt-hsp70*: *Bemisia tabaci* (ACZ52196.1); *Tv-hsp70*: *Trialeurodes vaporariorum* (ACH85201.1); *hsp70* family of *P. xylostella* included *Px-hsp69-1*, *-2a*, *-2b*, *-3*, *-4*, *Px-hsp72-1a*, *-1b*, *-2*, *-3* and *-J*; *hsp70* family of *Drosophila melanogaster* (Dm) included *Dm-HSPA1-1* (NP-523741.2), *Dm-HSPA2-1* (NP-524063.1), *Dm-HSPA3-1* (NP-524339.1), *Dm-HSPA4-1* (NP-727563.1), *Dm-HSPA5-1* (NP-524356.1), *Dm-HSPA6-1* (NP-524474.1), *Dm-HSPA7-1* (NP-731651.1) and *Dm-HSPA8-1* (NP-731716.1).

tion (NCBI) database. It was found that the nine cDNA sequences of *Px-hsp70*s were derived from four *hsp70* genes in the genome of DBM.

Cloning and sequencing analysis of *Px-hsc70(C)*

For cloning the ORF of *Px-hsc70*, three full-length ORF fragments from samples 1–3 were the same: 2,047 bp with 650 aa and 71.18 kDa. The three sequences shared 100% identity, and were named *Px-hsc70(C)* (GenBank acc. no. JN676213.1). There were three highly conserved Hsp70 family signatures, IDLGTTYS, IFDLGGGTFDVSIL, and IVLVGGSTRIPKVQK, at amino acid positions 10–17, 198–211, and 335–349, respectively (Fig. 3). A putative ATP/GTP binding site, AEAYLGKT (Saraste et al., 1990), was identified at amino acid positions 132–139 (Fig. 3).

Table 4. Inferred amino acid sequence identities of *Px-hsc70(C)* from DBM with its homologs from other insects.

Species	Amino acid identity (%)	GenBank acc. no.
Lepidoptera		
Bombyx mori	97.5	NP_001036892.1
Mamestra brassicae	96.3	BAF03556.1
Melitaea cinxia	96.6	AGR84220.1
Sesamia nonagrioides	96.2	AAY26452.2
Spodoptera litura	96.3	ADK55518.1
Xestia cnigrum	96.5	AGQ50302.1
Diptera		
Aedes aegypti	90.9	ABF18332.1
Chironomus tentans	90.3	AAN14525.1
Chironomus yoshimatsui	90.2	AAN14526.1
Drosophila melanogaster	89.7	NP_524356.1
Hymenoptera		
Bombus terrestris	92.0	XP_003397462.1
Camponotus floridanus	91.8	EFN65945.1
Nasonia vitripennis	91.2	NP_001166228.1

Two putative nuclear localization signals, KRKYKK-DLTTNKRALRRL and KRALRRLRTACERAKRTL (Knowlton & Salfity, 1996), were located at amino acid positions 247–264 and 258–275 (Fig. 3). The amino acid sequence of *Px-hsc70(C)* was the same as, but 24 amino acid residues longer at the 3'-end, than that of *Px-hsc70(J)* identified from Japanese DBM (Sonoda et al., 2006) (Fig. 3) and also displayed a high degree of homology with those of other insects. The *Px-hsc70(C)* showed 96.2–97.5%, 89.7–90.9%, and 91.2–92.0% amino acid identity with *hsc70* from Lepidoptera, Diptera, and Hymenoptera, respectively (Table 4). Phylogenetic analysis revealed that *Px-hsc70(C)* from *P. xylostella* was clustered together with other Lepidoptera (Fig. 4).

Expression of *Px-hsp70*s and *Px-hsc70(C)* under heat stress

The Ct values of *β*-actin and target genes (*Px-hsp70*s and *Px-hsc70(C)*) in adults and larvae of both R_R and S_S DBM under heat stress are provided in supplementary files (Table S1 and S2). In adults or larvae of both R_R and S_S DBM, the basal levels (at 25°C) of *Px-hsc70(C)* mRNA expression were high, but no significant up-regulation expression was found whatsoever under increasing heat stress (Fig. 5B and D). However, the basal mRNA expression levels of *Px-hsp70*s in adults or larvae of R_R and S_S DBM were lower than those of *Px-hsc70(C)*, but up-regulation expression levels of *Px-hsp70*s were higher under heat stress. The basal levels of mRNA expression of the six *Px-hsp70*s in S_S adults or larvae were lower than those in R_R adults or larvae in most cases. Even so, the induced mRNA expressions (at 37 and 42°C) of the *Px-hsp70*s in S_S were in general, higher than those in R_R, although there were several exceptions, such as in *Px-hsp69-2a* and *Px-hsp72-3* (Fig. 5). Compared to the control (25°C), the mRNA expression levels of *Px-hsp70*s were up-regulated under heat stress (37 and 42°C) in adults or larvae of both R_R and S_S DBM. To compare the extent of up-regulation expression of *Px-hsp70*s between R_R and S_S DBM under heat stress, the ra-

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Px-hsc70 (C) MATKAPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPNNTIFDAKRLIGRKFEDATVQADMKHWPFEVVSDGG 100
Px-hsc70 (J) ..........................................................................................................  100

Px-hsc70 (C) KPKIKVAYKGEDKTFFPEEVSSMVLTKMKETAEAYLGKTVQNAVITVPAYFNDSQRQATKDSGTISGLNVLRIINEPTAAAIAYGLDKKGGGERNVLIFD 200
Px-hsc70 (J) ..........................................................................................................  200

Px-hsc70 (C) LGGGTFDVSIITIEDGIFEVKSTAGDTHLGGEDFDNRMVNHFVQEFKRKYKKDLTTNKRALRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTSITR 300
Px-hsc70 (J) ..........................................................................................................  300

Px-hsc70 (C) ARFEELNADLFRSTMEPVEKSLRDAKMDKAQIHDIVLVGGSTRIPKVQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILHGDKSEEVQDLLLLDVTPL 400
Px-hsc70 (J) ..........................................................................................................  400

Px-hsc70 (C) SLGIETAGGVMTTLIKRNTTIPTKQTQTFTTYSDNQPGVLIQVFEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGILNVSAIEKSTNKEN 500
Px-hsc70 (J) ..........................................................................................................  500

Px-hsc70 (C) KITITNDKGRLSKEDIERMVNEAEKYRNEDEKQKETIGAKNALESYCFNMKSTMEDEKLKDKITDSDKQIILDKCNDTIKWLDSNQLADKEEYEHKQKEL 600
Px-hsc70 (J) ..........................................................................................................  600

Px-hsc70 (C) EGICNPIITKLYQGAGGPPGGMPGFPGGAPGAGGAAPGAGGAGPTIEEVD                                                  650
Px-hsc70 (J) ..........------------------------..............                                                  626
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Fig. 3. The amino acid sequences alignment of *Px-hsc70(C)* and *Px-hsc70(J)*. The protein alignment was created using Lasergene v7.1 (DNASTAR, Inc.). *Px-hsc70(C)* (JN676213.1) was in this study identified in Fujian DBM, while *Px-hsc70(J)* (AB214973.1) was identified in Japanese DBM (Sonoda et al., 2006). Three highly conserved Hsp70 family signatures (IDLGTTYS, IFDLGGGT-FDVSIL, and IVLVGGSTRIPKVQK), a putative ATP/GTP binding site (AEAYLGKT), and putative nuclear localization signals (KRKYKKDLTTNKRALRRL and KRALRRLRTACERAKRTL) are shown boxed. Identical amino acid residues are indicated by dots. Gaps (indicated by dashes) were added to improve the alignment.

tios of the mRNA expression between the high temperature treatment and control (25°C) were calculated. Compared to those at 25°C, the mRNA expression of *Px-hsp69-1* increased 14.0-fold at 37°C and 137.0-fold at 42°C in R_R adults, 60.0-fold at 37°C and 1180.0-fold at 42°C in S_S adults, 19.2-fold at 37°C and 6994.6-fold at 42°C in R_R larvae, 23.6-fold at 37°C and 1734.1-fold at 42°C in S_S larvae, respectively. The mRNA expression of *Px-hsp69-2a* increased 74.6-fold at 37°C and 1254.6-fold at 42°C in R_R adults, 5.1-fold at 37°C and 286.4-fold at 42°C in S_S adults, 14.9-fold at 37°C and 1561.7-fold at 42°C in R_R larvae, 5.3-fold at 37°C and 1598.9-fold at 42°C in S_S larvae, respectively. The mRNA expression of *Px-hsp69-3* increased 15.0-fold at 37°C and 78.3-fold at 42°C in R_R adults, 120.7-fold at 37°C and 1844.8-fold at 42°C in S_S adults, 1.5-fold at 37°C and 235.4-fold at 42°C in R_R larvae, 3.1-fold at 37°C and 304.9-fold at 42°C in S_S larvae, respectively. The mRNA expression of *Px-hsp69-4* increased 1.3-fold at 37°C and 1.2-fold at 42°C in R_R adults, 1.6-fold at 37 and 42°C in S_S adults, 1.0-fold at 37 and 42˚C in R_R larvae, 2.2-fold at 37°C and 2.1-fold at 42°C in S_S larvae, respectively. The mRNA expression of *Px-hsp72-2* increased 17.9-fold at 37°C and 85.4-fold at 42°C in R_R adults, 183.0-fold at 37°C and 1319.2-fold at 42°C in S_S adults, 6.5-fold at 37°C and 780.9-fold at 42°C in R_R larvae, 34.4-fold at 37°C and 3258.8-fold at 42°C in S_S larvae, respectively. Lastly, the mRNA expression of *Px-hsp72-3* increased 21.7-fold at 37°C and 46.3-fold at 42°C in R_R adults, 450.0-fold at 37°C and 2933.3-fold at 42°C in S_S adults, 12.1-fold at 37°C and 2097.6-fold at 42°C in R_R larvae, 28.0-fold at 37°C and 5756.1-fold at 42°C in S_S larvae, respectively (Fig. 5). Therefore, whether in adults or larvae DBM, the extent of up-regulation expression of the six *Px-hsp70*s in S_S was higher than that in R_R.

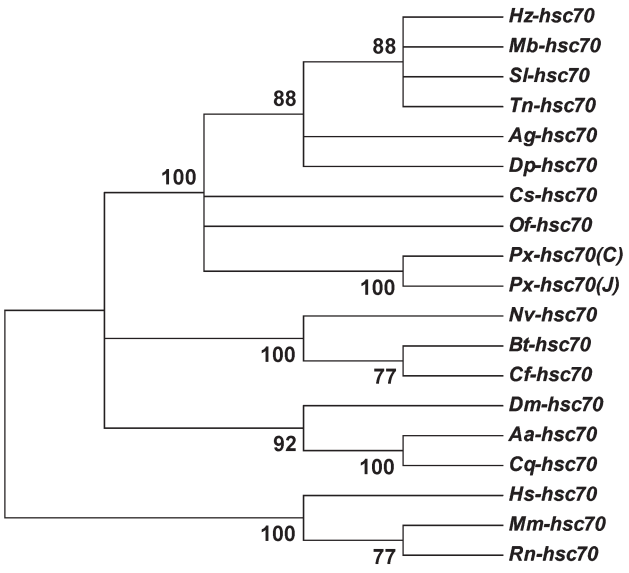

Fig. 4. The phylogenetic analysis of *Px-hsc70(C)* in DBM and other species, mostly insect, plus two mammalian species (house mouse and brown rat) using the neighbour-joining method (MEGA 4.0). The bootstrap cut-off value was 70%. *Aa-hsc70*: *Aedes aegypti* (ABF18332.1); *Ag-hsc70*: *Anticarsia gemmatalis* (ADO32621.1); *Bt-hsc70*: *Bombus terrestris* (XP_003397462.1); *Cf-hsc70*: *Camponotus floridanus* (EFN65945.1); *Cs-hsc70*: *Chilo suppressalis* (BAE44308.1); *Cq-hsc70*: *Culex quinquefasciatus* (XP_001850527.1); *Dp-hsc70*: *Danaus plexippus* (EHJ68380.1); *Dm-hsc70*: *Drosophila melanogaster* (NP_524356.1); *Hz-hsc70*: *Helicoverpa zea* (ACV32641.1); *Hs-hsc70*: *Homo sapiens* (NP_006588.1); *Mb-hsc70*: *Mamestra brassicae* (BAF03556.1); *Mm-hsc70*: *Mus musculus* (NP_112442.2); *Nv-hsc70*: *Nasonia vitripennis* (NP_001166228.1); *Of-hsc70*: *Ostrinia furnacalis* (ADR00357.2); *Px-hsc70(C)* (AFC38439.1) identified in Fujian DBM; *Px-hsc70(J)* (BAE48743.1) identified in Japanese DBM (Sonoda et al., 2006); *Rn-hsc70*: *Rattus norvegicus* (NP_077327.1); *Sl-hsc70*: *Spodoptera litura* (ADK55518.1) and *Tn-hsc70*: *Trichoplusia ni* (AAB06239.1).

DISCUSSION

In this study, nine cDNA sequences of inducible *hsp70*s (*Px-hsp69-1*, *-2a*, *-2b*, *-3*, *-4*, *Px-hsp72-1a*, *-1b*, *-2* and *-3*) and one constitutive *hsc70* (*Px-hsc70(C)*) were identified from the diamondback moth, *P. xylostella*. The nine cDNA sequences of *Px-hsp70*s were derived from four *hsp70* genes in the genome of DBM. For instance, the cDNA

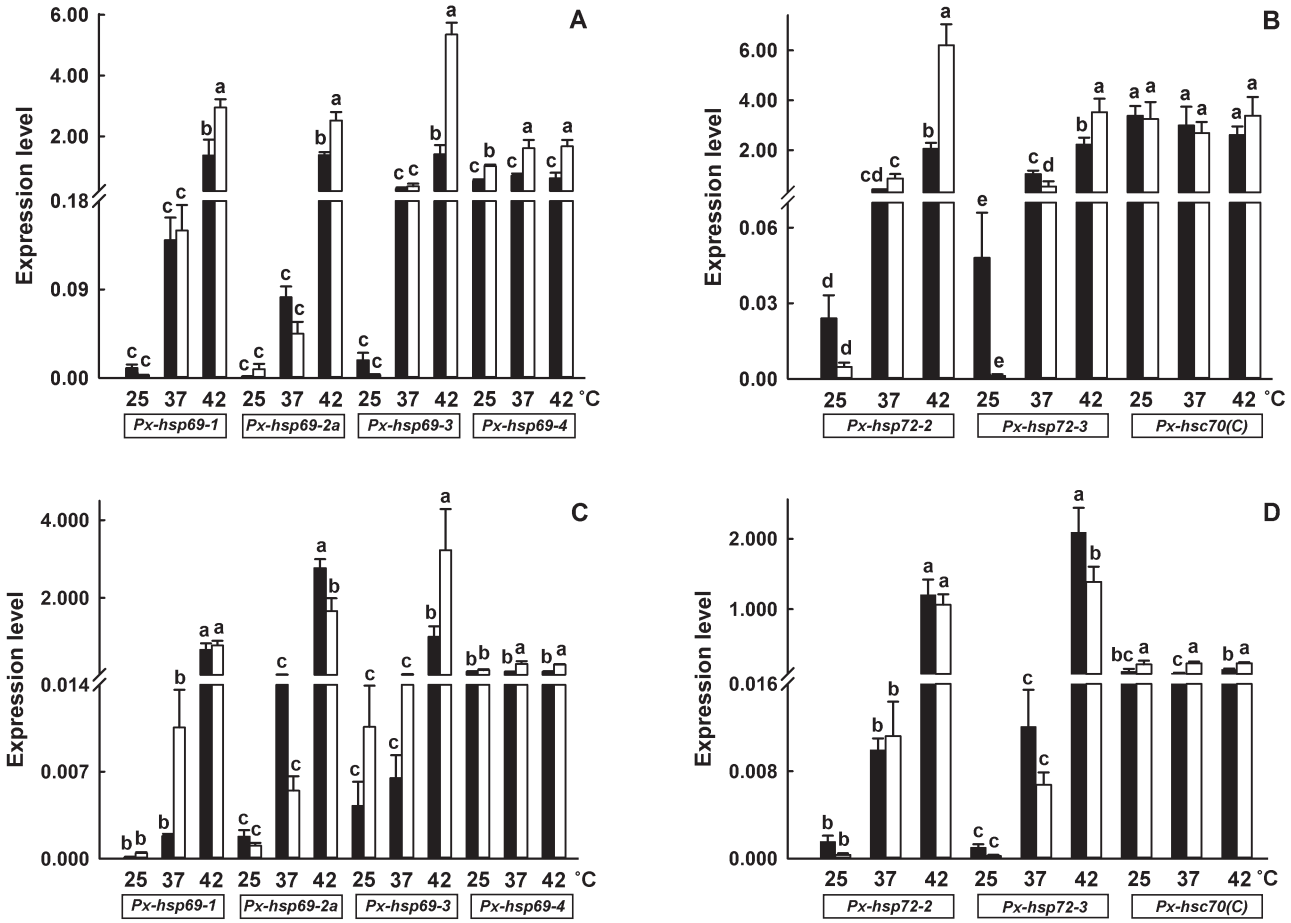

Fig. 5. Effects of heat stress on expression of *Px-hsp70*s and *Px-hsc70(C)* in R_R (black) and S_S (white) adults (A and B) and larval (C and D) DBM. Samples of total RNA were extracted from R_R or S_S adults or larval DBM after the insects were reared at 25, 37, or 42°C for 3 h. Abscissa: temperature (°C) (for 3 h). Each bar represents the mean ± SE of three independent experiments. Different lowercase letters indicate significant differences among the expressions of mRNA (Duncan's-test, $P \leq 0.05$).

sequences of *Px-hsp69-1*, *-2a* and *-2b* were derived from the same *hsp70* gene which was located at scaffold_372. The cDNA sequences of *Px-hsp69-3* and *-4* were derived from two different *hsp70* genes which were located at scaffold_274 and _50, respectively. The cDNA sequences of *Px-hsp72-1a*, *-1b*, *-2* and *-3* were derived from the same *hsp70* gene which was located at scaffold_162. In addition, when conducting the cloning experimentation of *Px-hsp70*s, more than three biological replicates were performed, and the DNA polymerase (Pyrobest DNA polymerase, TaKaRa Bio Inc.) used for PCR amplification was of high fidelity. Thus, copying errors during the amplification process resulting in the sequence differences in the nine *Px-hsp70* cDNAs was largely excluded. In light of this, the cDNAs corresponding to the same *hsp70* gene were thought to be different mRNA products. A similar situation was also observed in the European flat oyster, *Ostrea edulis* (Mollusca: Ostreoida; Ostreidae) and fruit fly, *D. melanogaster*. Thus, in *O. edulis*, four different cDNA sequences of *hsp70*s, *Oedcl5*, *OedclD2*, *OedclF2*, and *OedclL8*, were obtained and were derived from two different *hsp70* genes (Piano et al., 2005). In *D. melanogaster*, eight distinct *hsp70* genes (*HSPA1-8*) were identified and each gene could generate the varying amounts of mRNAs upon alternative splicing (Mou et al., 2011). The requirement for multiple, highly homologous though different Hsp70 proteins is still far from clear, but many studies show that the different Hsp70 proteins have distinct biological tasks under both normal and stressful conditions (Daugaard et al., 2007). The *hsp70* responses (mRNA expression levels) were known to vary considerably according to tissue, developmental stage and stressor (Feder et al., 1996). In the present study, samples 1–3 varied with the pretreated temperature and developmental stages before the insects were used for total RNA extraction. Therefore, the *hsp70* with high expression among the Hsp70 family at a given temperature in a developmental stage would be amplified preferentially if same primers were used.

Both *Px-hsp70*s and *Px-hsc70(C)* as here identified were highly conserved and exhibited the characteristic structural features of the Hsp70 family, i.e., three highly conserved Hsp70 family signatures (IDLGTTYS, IFDLGGGTFDVSIL, and V/IVLVGGSTRIPKI/VQT/K) (Gupta & Singh, 1994), the putative ATP/GTP binding site (AEAYLGT/KS/T) (Saraste et al., 1990), and putative nuclear localization signal motifs (KRKYH/KKDLTG/TNA/KRALRR and A/KRALRRLRTAA/CERAKRT), the last playing an important role in the selective translocation of Hsp70

into the nucleus (Knowlton & Salfity, 1996) (Figs 1 and 3). Furthermore, the motif EEV/DD, which is essential for ATPase and peptide-binding activity (Pockley et al., 2008), resides at the C-terminal tail.

A common physiological response of organisms to environmental stresses is the up-regulation expression of HSP genes, especially Hsp70 family genes, which are thought to help organisms cope with different potentially adverse conditions by refolding of damaged proteins, preventing the aggregation of denatured proteins, and promoting protein transport to intracellular locations for degradation (Leu et al., 2009; Colinet et al., 2010). In insects, this process has been widely studied for heat shock. In our present study, no significant up-regulation expression of *Px-hsc70(C)* was found in adults or larvae of both R_R and S_S DBM under heat stress. Likewise, unaltered expression level of *hsc70* under heat stress was observed in *D. melanogaster*, *H. zea* (maize) and the flesh fly, *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) (Rinehart et al., 2000; Bettencourt et al., 2008; Zhang & Denlinger, 2010). The absence of transcriptional change in expression of *hsc70* suggests that it does not contribute to the heat repair or heat acclimation machinery. In contrast to the above situation, the induction of *hsc70* expression in response to heat stress was found in such wasp parasitoid species as *Pteromalus puparum* (L.) (Hymenoptera: Pteromalidae), *C. vestalis* and *M. cingulum* (Wang et al., 2008; Xu et al., 2010; Shi et al., 2013). Therefore, in insects, *hsc70* displays species-specific transcriptional changes in response to heat stress, being either induced or not induced.

Unlike the expression pattern of *Px-hsc70(C)*, the expression levels of *Px-hsp70*s were up-regulated under heat stress in adults or larvae of both R_R and S_S DBM. This phenomenon was observed in almost all organisms tested so far (Piano et al., 2005; Renner & Waters, 2007; Udaka et al., 2010; Burger et al., 2014). It indicates that the inducible *hsp70* plays a critical role in cell protection and the enhancement of heat tolerance. In our experiments evaluating the extent of up-regulation expression of *Px-hsp70*s between R_R and S_S DBM under heat stress, we found that whether in adults or larvae DBM, the extent of up-regulation expression of *Px-hsp70*s in S_S was higher than that in R_R. In our previous results (Liu et al., 2008), insecticide-resistant DBM displayed considerable disadvantages in terms of life-history parameters (particularly in adult's longevity and fecundity among the developmental stages) under high temperature condition, compared with insecticide-susceptible insects. Based on the present results, higher up-regulation expressions of *Px-hsp70*s in S_S DBM are probably involved in the higher thermal tolerance of these particular strains.

In conclusion, in this study the molecular characterization of nine inducible *Px-hsp70*s and one constitutive *Px-hsc70(C)* in the DBM, *P. xylostella* were revealed, along with investigation of their associated expression patterns under heat stress. In adults or larvae of both R_R and S_S DBM, *Px-hsc70(C)* was expressed at higher level under normal conditions and remained unchanged in response to heat stress. In contrast, *Px-hsp70*s were expressed at lower level under normal conditions but could be induced by heat stress. This latter finding indicates that *Px-hsp70*s are more sensitive to, and supply more important protection under, heat stress. Furthermore, the induced responses of *Px-hsp70*s in S_S DBM were higher than those in R_R DBM, which suggests that S_S DBM is more heat tolerant than R_R DBM.

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S1 (http://www.eje.cz/2015/039/S01.pdf). The Ct values of β-actin and target genes in adults of both R_R and S_S DBM.

S2 (http://www.eje.cz/2015/039/S02.pdf). The Ct values of β-actin and target genes in larvae of both R_R and S_S DBM.