Identification of reference genes and expression analysis of heat shock protein genes in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae), after exposure to heat stress

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Abstract: 【Aim】The brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is a serious rice pest in China and Southeast Asia. The occurrence and migration of *N. lugens* is thought to be related to temperature. This study was conducted to understand the expression patterns of heat shock protein genes (*hsps*) in the adaptation to temperature stress in *N. lugens*. 【Methods】Female and male *N. lugens* adults were exposed to high temperature (30℃ –40℃) for 1 h and 2 h, respectively. Real-time PCR was used to detect the expression of *β-actin*1, *β-actin*2, *β-actin*3, 28S rRNA, 18S rRNA and *α-2-tubluin* in their bodies. The most stable candidate reference gene was identified using geNorm and BestKeeper software. The expression levels of *hsp*70 and *hsp*90 genes in the treated *N. lugens* adults were measured using RT-qPCR. 【Results】The most stable reference gene in both female and male adults of *N. lugens* after exposure to heat stress was *β-actin*1. The expression levels of *hsp*70 after heat stress ranging from 30℃ to 40℃ in both female and male adults were not significantly different compared with those in the control group. The expression level of *hsp*90 displayed significant up-regulation and reached the highest levels in female adults and male adults exposed to 40℃ and 38℃ for 2 h, respectively. 【Conclusions】*β-actin*1 can be used as the reference gene for normalization of gene expression under high temperature stress in *N. lugens* adults. The expression of *hsp*90 is induced by heat shock and the over-expression of *hsp*90 might be involved in the enhancement of thermal tolerance in *N. lugens* adults.

Key words: *Nilaparvata lugens*; heat shock protein; expression pattern; reference gene; qRT-PCR

1 INTRODUCTION

Insects have a weak capability of maintaining and regulating body temperature. Temperature is one of the most important factors for the survival, development, distribution and migration in many insect species. When the environmental temperature is too high or too cold, the life of insects may be affected, and their development was possibly suppressed or even death occurred (Hoffmann, 1985; Asin and Pons, 2001). During the process of evolution, insects have developed abilities to endure various stresses from artificial and natural habitat change including pesticides, extreme temperatures and the invasion of pathogenic bacteria.

Heat shock proteins (Hsps) exist in prokaryotic and eukaryotic organisms and belong to a supergene family. Hsps are highly conserved and act mainly as molecular chaperones, promoting the correct folding of proteins and preventing the aggregation of other proteins (Feder and Hofmann, 1999; Sørensen *et al.*, 2003; Zhao *et al.*, 2012). Previous researches showed that the expression of heat shock protein genes (*hsps*) is important in helping organisms to endure various stresses, especially in extreme changes in temperature (Howrelia *et al.*, 2011; Sakatani *et al.*, 2013). *In vivo* experiments showed that elevated expressions of *hsps* are important in the correct refolding of stress proteins, which can improve the heat tolerance of organism (Heads *et al.*, 1995; Sørensen and Loeschcke, 2007; Colinet *et al.*, 2010). *In vitro* experiments revealed that the expressions of *hsps* could affect the thermotolerance of cells (Riabowol *et al.*, 1988).

The brown planthopper, *Nilaparvata lugens*

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(Stål) (Hemiptera: Delphacidae), is a serious rice pest that frequently causes significant financial loss in China. It is a migratory insect that is mainly distributed in the tropics and subtropics of Asia. Temperature is one of the most primary factors affecting the reproduction, migratory flight and growth and development of *N. lugens*. Previous studies demonstrated that the development, spawning and longevity of *N. lugens* adults increased with the increase of temperature (Ma *et al*., 1998; Dai, 2002; Long, 2010; Piyaphongkul *et al*., 2012). In addition, previous studies also showed that heat stress affected the population dynamics (Zhu *et al*., 1994; Sujithra and Chander, 2013), vitellogenesis (Yi, 2003) and activities of various protective enzymes in *N. lugens* (Feng *et al*., 2001). Few researches at the molecular level have been done for understanding the heat stress mechanisms in *N. lugens*.

In order to understand the function of *hsp* in the heat tolerance process of *N. lugens*, in this study, female and male adults were exposed to different high temperatures (30℃, 32℃, 34℃, 36℃, 38℃ and 40℃) for 1 h and 2 h, respectively. The most stable reference gene was identified, and the relative expression levels of *hsp*70 and *hsp*90 were measured. Our main aim was to contribute a preliminary understanding on the thermostability of *N. lugens*, which may be applicable for integrated pest management (IPM).

2 MATERIALS AND METHODS

2.1 Insects

N. lugens was collected from Nanning, Guangxi Province, China, and was continuously reared for several generations on TN1 rice plants in the laboratory at 25 ± 1℃ and 70% ± 5% relative humidity (RH) with a 14L: 10D light cycle. Only healthy individuals were kept in the laboratory population.

2.2 Heat shock

Forty 3 – 5-day-old female and male adults on rice plants were captured in a glass tube (47 mm in diameter, and 220 mm in height), which was subsequently sealed with gauze. Then, the glass tube was exposed to a series of treatment temperatures (30℃, 32℃, 34℃, 36℃, 38℃ and 40℃) for 1 h and 2 h in a light incubator, allowing to recover at 25℃ for 1 h. After treatment, the total RNA from 20 survivors was extracted and stored in liquid nitrogen. Untreated adults were used as a negative control. Each treatment was repeated three times.

2.3 RNA extraction and synthesis of cDNA

Total RNA was extracted using a Trizol Kit (Invitrogen, USA). The amount and quality of the extracted RNA was estimated using a nanoDrop-1000 (Thermo, USA) UV-Vis spectrophotometer. One microgram of total RNA was used as the template for the first strand cDNA synthesis using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Dalin). All operations were performed according to the manufacturer's directions.

2.4 Real-time quantitative PCR

Six candidate reference gene sequences and two *hsps* gene sequences from *N. lugens* were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/), Primer 5.0 was used to design primers (Table 1). Primer specificity was determined by single peak melting curve using Real-time quantitative PCR (RT-qPCR). The amplification length was detected by running 1.5% agarose/EtBr gel. A 25 μL reaction mixture, including 12.5 μL 2 × SYBR® Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, Dalian), 2.5 μL first-strand cDNA and 0.4 μmol/L each of the primers, was used for RT-qPCR. The reactions were performed under the following conditions: pre-heat at 95℃ for 30 s, followed by 40 cycles of 95℃ for 10 s, and 60℃ for 30 s. After the reaction, the melting curves were analyzed from 55℃ to 95℃. In addition, the standard curves were constructed to determinate the PCR efficiency that would be a parameter in quantification data analysis. All reactions were carried out using the Chromo 4 Real-Time PCR Detection System (Bio-Rad, USA).

Table 1 Primer sequences used for real time quantitative PCR

Gene	GenBank accession number	Primer sequence (F/R)	Fragment length (bp)	PCR efficiency (%)	Correlation coefficient (R^2)
*β-actin*1	EU179846	TGTCTCTCACACAGTCCCCATCT/GTCAAGTCACGACCAGCCAAG	80	98.11	0.998
*β-actin*2	EU179849	AGTCGCACCCGAAGAG/AGCCTGGATAGCAACATA	130	93.20	0.998
*β-actin*3	EU179850	TGTGATGGTGGGTATGGG/ATGGCAGGTGAAGCGAAG	270	108.20	0.994
18S rRNA	JF773148	ACCAGGTCCAGACACAATG/CACTCCACCAACTAAGAACG	92	103.80	0.992
28S rRNA	JX556804	ATCAGCGGGGAAAGAAGA/ATCCGAGTAAGTAAGGAAACGA	154	95.10	0.999
α-2-tubulin	FJ810204	GGGCTTCCTCATCTTCC/AACGGCTGTTGATACCTG	145	94.90	0.994
*hsp*70	JQ782193	AAGTCAGGTGGCTATG/CTTTGTGCCGAGGTA	247	108.50	0.991
*hsp*90	GU723300	TGTGAACAACCTGGGAAC/GGACCGTAAACGAACCTC	209	103.70	0.997

2.5 Data analysis

The geNorm (Vandesompele *et al*., 2002) [http://medgen.ugent.be/~jvdesomp/genorm/] applet for Microsoft Excel, which determines the most stable reference gene from a set of candidate genes in a given cDNA sample panel and the BestKeeper excel-based tool, was used to determine the stability of reference genes (Pfaffl *et al*., 2004) [http: //www.wzw.tum.de/gene-quantification/bestkeeper.html]. In addition, the geNorm software also gives an option that can determine the optimal number of reference genes according to the pairwise variation $V_n/V_{n+1} = 0.15$ as a cut-off value, below which inclusion of an additional reference gene is not required. The two softwares were employed to determine the most stable reference gene in different treatments of *N. lugens*.

PCR efficiency (E) was calculated for each pair of primers based on the slope of the standard curve from a 10-fold dilution serial of the first strand cDNA using the Opticon Monitor 3 software for Chromo 4 (Bio-Rad, http://www.bio-rad.com/). PCR efficiencies (E) were calculated according to the formula $E = 10^{-\text{slope}} - 1$.

The relative quantities of *hsp*70 and *hsp*90 were calculated using the $2^{-\Delta\Delta C_T}$ method described by Livak and Schmittgen (2001). The statistical analysis was performed using SPSS 19.0 software. To correct for plate variation, the expression levels of *hsp*70 and *hsp*90 in the control (25℃) were quantified in each plate.

3 RESULTS

3.1 RT-qPCR of candidate reference genes in *N. lugens* adults

The expression levels of six candidate reference genes including *β-actin*1, *β-actin*2, *β-actin*3, 28S rRNA, 18S rRNA and *α-2-tubulin* were detected in male and female adults of *N. lugens* subjected to heat stress at different temperature. The cycle threshold (*Ct*) ranged from 10.13 (28S rRNA) to 32.55 (*β-actin*3). The *Ct* value between replicates was less than 0.5. Standard curves of each pair of primers exhibited correlation coefficients (R^2) higher than 0.99 and PCR efficiencies were very good, ranging from the lowest 93.2% (*β-actin*2) to the highest 108.2% (*β-actin*3) (Table 1). The melting curve of all genes with a single peak and agarose/EtBr gel analyses showed a single band for all PCR products (data not shown), indicating that the primers are target-specific.

3.2 Expression stability of reference genes in *N. lugens* adults

geNorm was used to calculate the gene expression stability by measuring the M value in all candidate reference genes. The lowest M value indicates the highest stability in expression of *hsps* in all test samples. In this study, the rank of the candidate reference genes for females based on their average M values was as follows, *β-actin*2 ($M = 0.933$) > 18S rRNA ($M = 0.800$) > *α-2-tubulin* > ($M = 0.731$) > 28S rRNA ($M = 0.655$) > *β-actin*1 = *β-actin*3 ($M = 0.571$), and the pairing of *β-actin*1 + *β-actin*3 with the lowest M value were supposed to be the most stable reference genes in females (Fig. 1: A). In males the rank of the average M values are *β-actin*2 ($M = 0.542$) > 18S rRNA ($M = 0.460$) > 28S rRNA ($M = 0.413$) > *α-2-tubulin* ($M = 0.371$) > *β-actin*1 = *β-actin*3 ($M = 0.276$) (Fig. 1: B), and the pairing of *β-actin*1 + *β-actin*3 with the lowest M value displayed the highest stability (Fig. 1: B). In this experiment, the V_n/V_{n+1} values were calculated (Fig. 2). The V_2/V_3 value was 0.132 in females and the minimum value was 0.160 in males. This indicated that the normalization factor should contain two reference genes in females, but in males, increasing the number of reference genes could not reduce the pairwise variation value below 0.15.

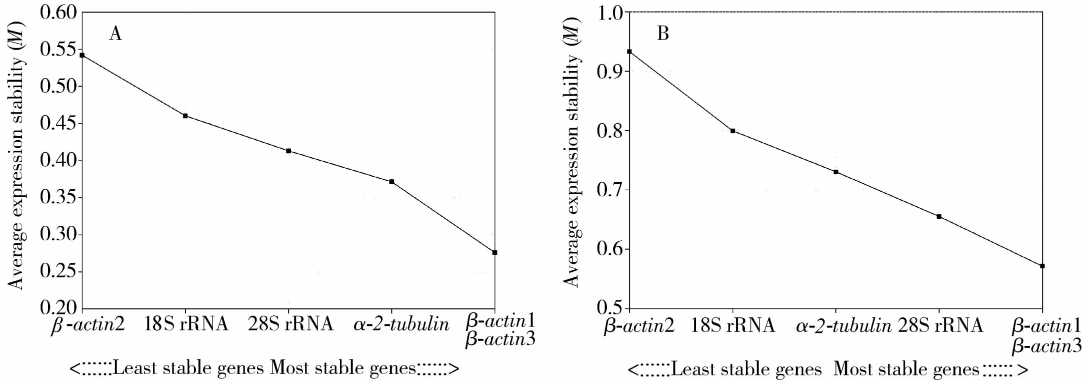

Fig. 1 Determination of the most stable reference gene in female (A) and male (B) adults of *Nilaparvata lugens* under heat stress using the geNorm software

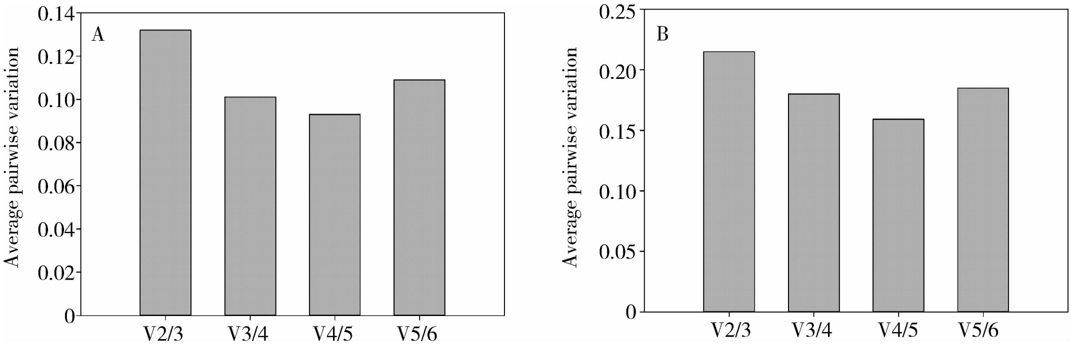

Fig. 2 Determination of the optimal number of reference genes in female (A) and male (B) adults of *Nilaparvata lugens* under heat stress using the geNorm software

BesterKeeper software was also used to determine the most stable reference genes in different treatment samples. According to the standard deviation of *CP* [*SD* ± *CP*] and the standard deviation of the absolute regulation coefficient *SD* [± *x*-fold] values, the most stable reference gene was determined, which has the lowest values suggesting that the gene expression level is the most stable. Data from the BestKeeper analysis for candidate reference genes are shown in Table 2. The results suggested that *β-actin*1 had the greatest stability both in female and male adults of *N. lugens*.

Table 2 Stability analysis of the candidate reference genes in *Nilaparvata lugens* adults after exposure to high temperatures using BestKeeper software

Factor	Female						Male					
	*β-actin*1	*β-actin*2	*β-actin*3	18S rRNA	28S rRNA	*α-2-tubultin*	*β-actin*1	*β-actin*2	*β-actin*3	18S rRNA	28S rRNA	*α-2-tubulin*
N	13	13	13	13	13	13	13	13	13	13	13	13
geo Mean [CP]	15.67	19.85	31.31	16.28	11.15	18.26	15.28	20.76	30.66	17.08	11.34	19.32
ar Mean [CP]	15.67	19.88	31.31	16.29	11.17	18.27	15.29	20.80	30.67	17.09	11.36	19.34
min [CP]	15.15	18.72	30.49	15.59	10.13	17.72	14.77	18.87	29.63	16.31	10.52	18.40
max [CP]	16.74	21.90	32.39	17.45	12.46	19.02	16.22	22.77	32.55	18.29	12.53	20.35
Std dev [± CP]	±0.39	±0.88	±0.55	±0.45	±0.59	±0.44	±0.29	±1.24	±0.62	±0.37	±0.63	±0.61
CV [% CP]	2.46	4.45	1.77	2.75	5.29	2.43	1.91	5.94	2.02	2.15	5.59	3.16
min [*x*-fold]	−1.43	−2.19	−1.76	−1.62	−2.04	−1.45	−1.42	−3.70	−2.04	−1.70	−1.77	−1.90
max [*x*-fold]	2.11	4.13	2.12	2.25	2.47	1.69	1.91	4.04	3.71	2.30	2.28	2.04
Std dev [± *x*-fold]	±1.31	±1.85	±1.47	±1.36	±1.51	±1.36	±1.22	±2.35	±1.54	±1.29	±1.55	±1.53
Stability ranking	1	5	3	2	4	2	1	6	5	2	4	3

3.3 Expression of *hsp*70 and *hsp*90 in *N. lugens* adults after heat shock

The expression level of *β-actin*1 was stable under heat stress at different temperatures in *N. lugens* adults, indicating that *β-actin*1 can be used as a suitable reference gene in RT-qPCR. The relative gene expression levels of *hsp*70 and *hsp*90 in *N. lugens* adults after exposure to heat shock were analyzed with RT-qPCR (Fig. 3). In females, the relative expression level of *hsp*70 (Fig. 3: A) was the highest at 32℃ for 2 h (1.47-fold as high as that in the control) and the lowest at 36℃ for 1 h (0.95-fold as high as that in the control). In males, the highest relative expression level of *hsp*70 (Fig. 3: B) was the highest at 34℃ for 2 h (1.75-fold as high as that in the control) and the lowest was at 32℃ for 1 h (1.02-fold as high as that in the control). Under all heat stress condition, the relative expression levels of *hsp*70 increased significantly both in females and males.

The relative expression level of *hsp*90 showed different patterns in response to heat shock. In females the relative expression levels (Fig. 4: A) increased following temperature and time increase. The relative expression level of *hsp*90 in females at 40℃ (3.13-fold for 1 h and 5.42-fold for 2 h), 38℃ (3.89-fold for 1 h and 4.01-fold for 2 h) and 36℃ (3.74-fold for 2 h) were significantly different, compared with the control. Males and females had similar expression pattern, and the expression levels of *hsp*90 at 40℃ (6.84-fold for 1 h and 7.59-fold for 2 h), 38℃ (6.59-fold for 1 h and 7.79-fold for 2 h), 36℃ (2.68-fold for 2 h and 4.20-fold for 2 h) and 34℃ (2.78-fold for 1 h and 4.47-fold for 2 h) were also significantly different from the control (Fig. 4: B).

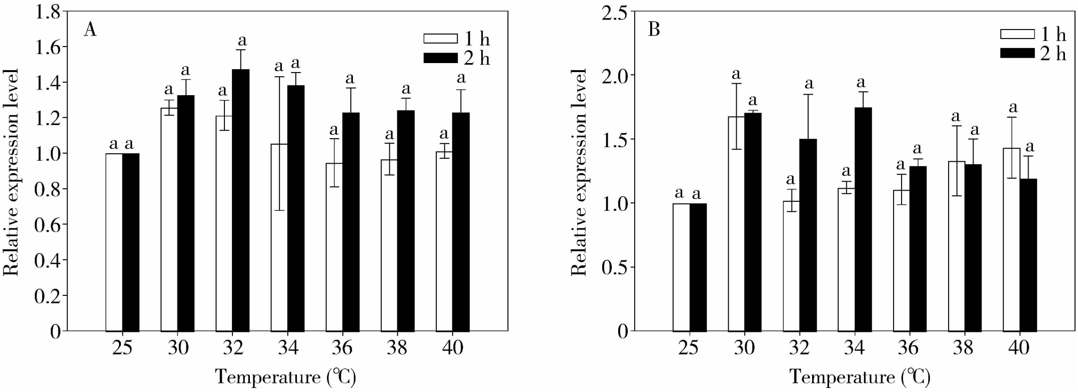

Fig. 3　Relative expression levels of *hsp*70 in female (A) and male (B) adults of *Nilaparvata lugens* after exposure to heat stress

Adults were exposed to high temperatures at 30℃, 32℃, 34℃, 36℃, 38℃ and 40℃, for 1 h or 2 h, respectively, and then transferred to 25℃ for recovery 1 h. The control group remained at 25℃. Data in the figure represent mean ± *SE* of three replicates, and different letters above bars indicate significant difference at the 0.05 level (Tukey's test). Relative expression quantity of genes was calculated using the $2^{-\Delta\Delta C_T}$ method. The same for Fig. 4.

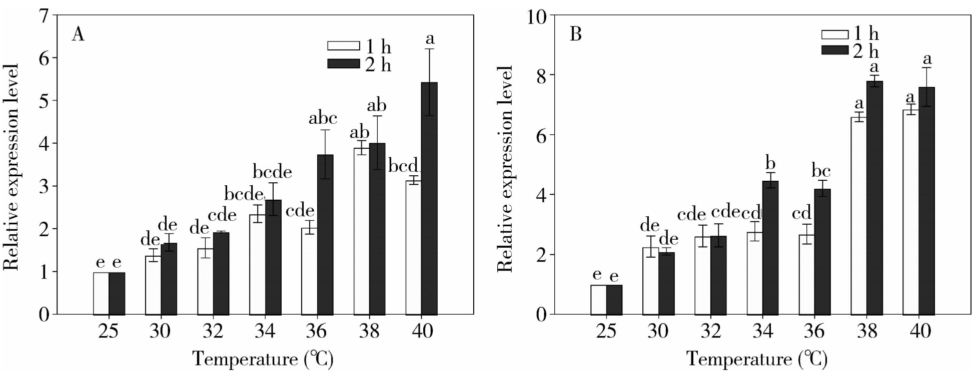

Fig. 4　Relative expression levels of *hsp*90 in female (A) and male (B) adults of *Nilaparvata lugens* after exposure to heat stress

4　DISCUSSION

Studies on gene expression pattern in different samples and tissues contribute to the understanding of the function of genes that are relevant to complex biological processes, such as the processes of immune, disease, development and heat adaptation (Vandesompele *et al*., 2002; Lü and Wan, 2008; Tao *et al*., 2013). RT-qPCR is an important method to investigate gene expression (Pfaffl, 2001) and has been widely used in functional gene studies. Internal control genes are required to normalize the gene expression in different experimental conditions and tissues. Internal control refers to a reference or housekeeping gene, which should have stable expression level in tissues and during the experimental treatment. Therefore, it is important to detect the relative expression level of potential reference genes with RT-qPCR and evaluate the stability of reference genes.

In this study, we evaluated six candidate reference genes from *N. lugens* adults subjected to heat shock using geNorm and Bestkeeper softwares. Data from both softwares suggested that *β-actin*1 was the most stable reference gene in females and males. This conclusion is similar to an earlier study, which indicated that *β-actin*1 was a stable reference gene under different treatment conditions (Jiang *et al*., 2010). The results analyzed by the two softwares also showed that *α-2-tubulin* was more stable genes with a ranked two in females and three in males. Although 18S rRNA and 28S rRNA were highly expressed, the *M* value of 18S rRNA is one of the largest in females and males and the *SD*[±*CP*] and *SD*[±*x*-fold] values of 28S rRNA also were one of the largest in all samples, suggesting that 18S rRNA and 28S rRNA are not suitable as a reference gene under our experimental condition. This is consistent with earlier studies that suggested that rRNA was not appropriate to be used as reference genes (Jiang *et al.*, 2010; Shen *et al.*, 2010). In addition, data from the geNorm software suggested that the optimal combination of reference genes was *β-actin* 1 + *β-actin*3 in females and males. *β-actin*3 had the *Ct* values from the lowest 29.36 to the highest 32.55 in all samples, with low expression abundance, and the degree of variation of the *Ct* values was high. For this reason, we do not consider *β-actin*3 as a suitable reference gene. *β-actin*2 has the highest *M* value, *SD* [± *CP*] and *SD* [± *x*-fold] values, indicating that *β-actin*2 was a less stable gene. In conclusion, the present study suggested that *β-actin*1 was the most suitable reference gene in all test samples. If two genes are required as the internal control, we propose the combination of *β-actin*1 + *α-2-tubulin*.

The over-expression of *hsp* genes in enhancing thermotolerance of insects have been detected in early studies (Gehring and Wehner, 1995; Sørensen *et al.*, 2003; Yin *et al.*, 2006; Huang and Kang, 2007; Liu *et al.*, 2013). However, the earlier studies were often limited to model insects, few crop pests were studied. The relationship of the over-expression of *hsps* with temperature in many insects is poorly understood. The over-expression of *hsp*70 and *hsp*90 genes during heat stress was detected in some insects (Sonoda *et al.*, 2006; Bettencourt *et al.*, 2008; Kalosaka *et al.*, 2009; Bernabò *et al.*, 2011). In this paper we have shown the expression patterns of *hsp*70 and *hsp*90 in adults of *N. lugens* after exposure to heat over time. Our results indicated that the expression levels of *hsp*70 ranging from 30℃ to 40℃ in both female and male adults of *N. lugens* were not significantly different compared with the control, but the expression levels of *hsp*90 at higher temperatures were significantly different from the control. The HSP70 family contains two groups, HSP70 and heat shock cognate 70 (HSC70). It is generally recognized that the relative expression level of HSP70 is low under normal conditions but is over-expressed under various stresses and HSC70 has stable expression in normal conditions and is not induced by stress (Kim *et al.*, 2008). In this study, *hsp*70 was not over-expressed under heat stress in *N. lugens*. Ge *et al.* (2013) showed that when exposed to sub-lethal concentrations of the triazophos insecticide, the thermotolerance of *N. lugens* was enhanced, and the *hsp*70 transcripts both in the third-instar nymphs and brachypterous adult females were up-regulated, RNAi silencing also demonstrated that *hsp*70 gene are essential for survival and tzp-increased thermotolerance. These results suggested that the expression pattern of *hsp*70 in *N. lugens* was correlated with an inducing factor. The expression of *hsps* can be induced by bisphenol A, cold hardening, insecticides and ecdysone (Huang *et al.*, 2009; Shashikumar and Rajini, 2010; Wang *et al.*, 2011; Michail *et al.*, 2012), but the functional connection between thermal tolerance and heat shock protein regulation needs further investigation.

Here, the expression of *hsp*90 gene in the treated female and male adults of *N. lugens* was up-regulated during heat shock. In insects, *hsp*90 is usually induced by heat stress in the range of 35℃ to 40℃ (Kim *et al.*, 2008). Our study showed that the expression of *hsp*90 in *N. lugens* adults was induced significantly by high temperature from 36℃ and 34℃ in females and males, respectively. This result was consistent with the earlier studies. It was confirmed that heat shock induced up-regulated expression of *hsp*90 in *N. lugens* adults and as a consequence enhanced their tolerance to heat.

The heat shock protein gene family includes *hsp*100, *hsp*90, *hsp*70, *hsp*60, *hsp*40 and small *hsps*. In this study, *hsp*70 and *hsp*90 expression patterns after heat stress in *N. lugens* adults were shown; however, the expression patterns of *hsp*100, *hsp*60 and small *hsps* were unclear. Cloning and characterization of *hsps* from *N. lugens* are needed to further understand the heat adaptation mechanism.

In summary, our results showed that the best stable reference gene was *β-actin*1 in *N. lugens* adults after exposure to high temperatures. High temperature did not change the expression levels of *hsp*70, but induced the up-regulation of *hsp*90 expression. These results will help us to understand the mechanism of thermal tolerance, and supply basic information for crop pest forecasting.

References

Asin L, Pons X, 2001. Effect of high temperature on the growth and reproduction of corn aphids (Homoptera: Aphididae) and implications for their population dynamics on the northeastern Iberian peninsula. *Environmental Entomology*, 30(6): 1127 – 1134.

Bernabò P, Rebecchi L, Jousson O, Martínez-Guitarte JL, Lencioni V, 2011. Thermotolerance and *hsp*70 heat shock response in the cold-stenothermal chironomid *Pseudodiamesa branickii* (NE Italy). *Cell Stress and Chaperones*, 16(4): 403 – 410.

Bettencourt BR, Hogan CC, Nimali M, Drohan BW, 2008. Inducible and constitutive heat shock gene expression responds to modification of *Hsp*70 copy number in *Drosophila melanogaster* but does not compensate for loss of thermotolerance in *Hsp*70 null flies. *BMC Biology*, 6: 5.

Colinet H, Lee SF, Hoffmann A, 2010. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult

Drosophila melanogaster. *FEBS Journal*, 277(1): 174 – 185.

Dai HG, 2002. Effect mechanism of high temperature on growth, development and reproduction in brown planthopper, *Nilaparvata lugens*. *Science Technology and Engineering*, 2(5): 75 – 76. [戴华国, 2002. 高温对褐飞虱生长发育与繁殖的影响机制. 科学技术与工程, 2(5): 75 – 76]

Feder ME, Hofmann GE, 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*, 61(1): 243 – 282.

Feng CJ, Dai HG, Wu SW, 2001. Stress response of *Nilaparvata lugens* at high temperature and activities of its protective enzyme systems. *Chinese Journal of Applied Ecology*, 12(3): 409 – 413. [冯从经, 戴华国, 武淑文, 2001. 褐飞虱高温条件下应激反应及体内保护酶系活性的研究. 应用生态学报, 12(3): 409 – 413]

Ge LQ, Huang LJ, Yang GQ, Song QS, Stanley D, Gurr GM, Wu JC, 2013. Molecular basis for insecticide-enhanced thermotolerance in the brown planthopper *Nilaparvata lugens* Stål over-circlel (Hemiptera: Delphacidae). *Molecular Ecology*, 22(22): 5624 – 5634.

Gehring WJ, Wehner R, 1995. Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. *Proceedings of the National Academy of Sciences of the United States of America*, 92(7): 2994 – 2998.

Heads RJ, Yellon DM, Latchman DS, 1995. Differential cytoprotection against heat stress or hypoxia following expression of specific stress protein genes in myogenic cells. *Journal of Molecular and Cellular Cardiology*, 27(8): 1669 – 1678.

Hoffmann KH, 1985. Metabolic and enzyme adaptation to temperature. In: Hoffmann KH ed. Environmental Physiology and Biochemistry of Insects. Springer-Verlag, Berlin. 1 – 32.

Howrelia JH, Patnaik BB, Selvanayagam M, Rajakumar S, 2011. Impact of temperature on heat shock protein expression of *Bombyx mori* cross-breed and effect on commercial traits. *Journal of Environmental Biology*, 32(1): 99 – 103.

Huang LH, Kang L, 2007. Cloning and interspecific altered expression of heat shock protein genes in two leafminer species in response to thermal stress. *Insect Molecular Biology*, 16(4): 491 – 500.

Huang LH, Wang CZ, Kang L, 2009. Cloning and expression of five heat shock protein genes in relation to cold hardening and development in the leafminer, *Liriomyza sativa*. *Journal of Insect Physiology*, 55(3): 279 – 285.

Jiang HB, Liu YH, Tang PA, Zhou AW, Wang JJ, 2010. Validation of endogenous reference genes for insecticide-induced and developmental expression profiling of *Liposcelis bostsrychophila* (Psocoptera: Liposcelididae). *Molecular Biology Reports*, 37(2): 1019 – 1029.

Kalosaka K, Soumaka E, Politis N, Mintzas AC, 2009. Thermotolerance and HSP70 expression in the Mediterranean fruit fly *Ceratitis capitata*. *Journal of Insect Physiology*, 55(6): 568 – 573.

Kim DH, Lee SC, Kwak DY, Lee KY, 2008. Cloning of heat shock protein genes from the brown planthopper, *Nilaparvata lugens*, and the small brown planthopper, *Laodelphax striatellus*, and their expression in relation to thermal stress. *Insect Science*, 15(5): 415 – 422.

Liu QN, Zhu BJ, Dai LS, Fu WW, Lin KZ, Liu CL, 2013. Overexpression of small heat shock protein 21 protects the Chinese oak silkworm *Antheraea pernyi* against thermal stress. *Journal of Insect Physiology*, 59(8): 848 – 854.

Livak KJ, Schmittgen TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods*, 25(4): 402 – 408.

Long Y, 2010. Effect of Temperature on Reproduction Behavior in Brachypterous Females of the Brown Planthopper, *Nilaparvata lugens*. MSc Thesis, Chinese Academy of Agricultural Sciences, Beijing. [龙颖, 2010. 温度对褐飞虱短翅型雌虫繁殖行为的影响. 北京: 中国农业科学院硕士学位论文]

Lü ZC, Wan FH, 2008. Differential gene expression in whitefly (*Bemisia tabaci*) B-biotype females and males under heat-shock condition. *Comparative Biochemistry and Physiology D – Genomics & Proteomics*, 3(4): 257 – 262.

Ma JF, Hu GW, Cheng JA, 1998. Ecological performances of three planthopper species at simulated fluctuating temperature. *Entomological Journal of East China*, 7(2): 85 – 90. [马巨法, 胡国文, 程家安, 1998. 三种稻飞虱在高温变温下的生态表现. 华东昆虫学报, 7(2): 85 – 90]

Michail X, Kontogiannatos D, Syriou V, Kourti A, 2012. Bisphenol-A affects the developmental progression and expression of heat-shock protein genes in the moth *Sesamia nonagrioides*. *Ecotoxicology*, 21(8): 2244 – 2253.

Pfaffl MW, 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29(9): e45.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP, 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. *Biotechnology Letters*, 26(6): 509 – 515.

Piyaphongkul J, Pritchard J, Bale J, 2012. Can tropical insects stand the heat? A case study with the brown planthopper *Nilaparvata lugens* (Stål). *PLoS ONE*, 7(1): e29409.

Riabowol KT, Mizzen LA, Welch WJ, 1988. Heat shock is lethal to fibroblasts microinjected with antibodies against hsp70. *Science*, 242(4877): 433 – 436.

Sakatani M, Bonilla L, Dobbs KB, Block J, Ozawa M, Shanker S, Yao J, Hansen PJ, 2013. Changes in the transcriptome of morula-stage bovine embryos caused by heat shock: relationship to developmental acquisition of thermotolerance. *Reproductive Biology and Endocrinology*, 11: 3.

Shashikumar S, Rajini PS, 2010. Cypermethrin elicited responses in heat shock protein and feeding in *Caenorhabditis elegans*. *Ecotoxicology and Environmental Safety*, 73(5): 1057 – 1062.

Shen GM, Jiang HB, Wang XN, Wang JJ, 2010. Evaluation of endogenous references for gene expression profiling in different tissues of the oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae). *BMC Molecular Biology*, 11: 76.

Sonoda S, Ashfaq M, Tsumuki H, 2006. Cloning and nucleotide sequencing of three heat shock protein genes (*hsp*90, *hsc*70, and *hsp*19.5) from the diamondback moth, *Plutella xylostella* (L.) and their expression in relation to developmental stage and temperature. *Archives of Insect Biochemistry and Physiology*, 62(2): 80-90.

Sørensen JG, Kristensen TN, Loeschcke V, 2003. The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, 6(11): 1025-1037.

Sørensen JG, Loeschcke V, 2007. Studying stress responses in the post-genomic era: its ecological and evolutionary role. *Journal of Biosciences*, 32(3): 447-456.

Sujithra M, Chander S, 2013. Simulation of rice brown planthopper, *Nilaparvata lugens* (Stal.) population and crop-pest interactions to assess climate change impact. *Climatic Change*, 121(2): 331-347.

Tao Y, Pan L, Zhang H, Liu N, 2013. Identification of genes differentially expressed in clams *Ruditapes philippinarum* in response to endosulfan after different exposure time. *Ecotoxicology and Environmental Safety*, 89: 108-116.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F, 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3(7): research0034.

Wang H, Lei Z, Li X, Oetting RD, 2011. Rapid cold hardening and expression of heat shock protein genes in the B-biotype *Bemisia tabaci*. *Environmental Entomology*, 40(1): 132-139.

Yi WX, 2003. Characterization of Vitellin and Effectation of High Temperature on Its Vitellongenesis in Brown Planthopper, *Nilaparvata lugens* (Stål). MSc Thesis, Nanjing Agricultural University, Nanjing. [衣维贤, 2003. 褐飞虱(*Nilaparvata lugens* Stål)卵黄蛋白的性质及高温对卵黄发生的影响. 南京: 南京农业大学硕士学位论文]

Yin X, Wang S, Tang J, Hansen JD, Lurie S, 2006. Thermal conditioning of fifth-instar *Cydia pomonella* (Lepidoptera: Tortricidae) affects HSP70 accumulation and insect mortality. *Physiological Entomology*, 31(3): 241-247.

Zhao LM, Jones WA, 2012. Expression of heat shock protein genes in insect stress responses. *ISJ - Invertebrate Survival Journal*, 3(9): 93-101.

Zhu SD, Lu ZQ, Hang BB, Xu H, 1994. Effect of temperature on the population regulation in brown planthopper, *Nilaparvata lugens*. *Entomological Journal of East China*, 3(1): 53-59. [祝树德, 陆自强, 杭杉保, 徐海, 1994. 温度对褐飞虱种群调控作用研究. 华东昆虫学报, 3(1): 53-59]

褐飞虱热胁迫下内参基因的筛选及热激蛋白基因表达分析

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摘要:【目的】褐飞虱 *Nilaparvata lugens* (Stål)是为害水稻的重要害虫之一,温度是影响其暴发、迁飞的主要环境因子之一。本研究旨在探讨研究褐飞虱对高温胁迫适应性的热激蛋白基因表达调控模式。【方法】分别以不同的高温(30℃~40℃)处理褐飞虱雌、雄虫 1 h 和 2 h,利用荧光定量 PCR 技术检测其体内的 *β-actin* 1, *β-actin*2, *β-actin*3, 28S rRNA, 18S rRNA 和 *α-2-tubulin* 6 个内参基因的表达量,用 geNorm 和 BestKeeper 软件分析确定最稳定表达的内参基因,并检测热胁迫后 *hsp*70 和 *hsp*90 基因在处理褐飞虱成虫体内的表达模式。【结果】geNorm 软件分析结果表明,热胁迫后褐飞虱内参基因稳定性在雌虫体内为: *β-actin*1 = *β-actin*3 > 28S rRNA > *α-2-tubulin* > 18S rRNA > *β-actin*2; 在雄虫体内为: *β-actin*1 = *β-actin*3 > *α-2-tubulin* > 28S rRNA > 18S rRNA > *β-actin*2。BestKeeper 软件分析结果显示,在热胁迫的雌、雄虫体内 *β-actin*1 均最稳定,18S rRNA 次之, *β-actin*2 最不稳定。两种软件分析结果基本一致。以 *β-actin*1 为校正内参基因,荧光定量 PCR 分析 *hsp*70 和 *hsp*90 在不同热胁迫条件下的表达模式,结果表明,各高温处理下 *hsp*70 表达量与对照 26℃ 下的表达量没有显著性差异;而 *hsp*90 基因表达模式表现为被高温诱导上调表达,在雌、雄虫体内表达量达到最高的处理条件分别为 40℃ 和 38℃ 处理 2 h。【结论】*β-actin*1 基因可以作为热胁迫下褐飞虱雌雄虫体内基因表达模式分析的校正内参基因使用。褐飞虱 *hsp*90 基因能被高温诱导表达,该基因可能在褐飞虱适应热胁迫过程中起着重要的作用。

关键词: 褐飞虱; 热激蛋白; 表达模式; 内参基因; 荧光定量 PCR

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