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# Identification of a heat shock protein 90 gene involved in resistance to temperature stress in two wing-morphs of *Nilaparvata lugens* (Stål)

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ABSTRACT

The brown planthopper, *Nilaparvata lugens*, is one of the most destructive pests damaging rice in Asia and exhibits wing dimorphism, with brachypters possessing severely reduced wings and macropters bearing fully developed wings. Previous studies have shown that macropters are more heat resistant than brachypters. To understand the molecular mechanism underlying the differential thermotolerance abilities of these two morphs, a full-length Hsp gene, *NlHsp90* was cloned from *N. lugen*. Our results showed that the relative expression levels of *NlHsp90* in *N. lugens* females increased with the rise of temperature. Interestingly, *NlHsp90* in macropters females could be induced at lower temperature (32 °C) than that in brachypters (34 °C), and the *NlHsp90* mRNA levels in macropters were significantly higher than those in brachypters from 34 to 40 °C. In addition, the maximum expression levels of *NlHsp90* were achieved much earlier in macropters, and *NlHsp90* mRNA levels in macropters were significantly higher than those in brachypters from 1 to 6 h of recovery after temperature stress. Furthermore, knockdown of *NlHsp90* by dsRNA injection reduced survival in both morphs with a greater reduction in the macropters relative to that of the brachyters. These results indicated that *NlHsp90* plays an important role for thermotolerance in *N. lugens*, and there is difference on induction between two morphs.

## 1. Introduction

Temperature is one of the most important environmental factors that affects many biological processes of species (Bale et al., 2002). Due to the ongoing global warming which is caused by the atmospheric accumulation of carbon dioxide, the influences of climate change on organisms and ecological communities are becoming the focus of research (Ge et al., 2013; Stoeckli et al., 2012). Temperature can significantly affect not only the growth and development of insects, but also their population abundance and geographic distribution (Chen et al., 2014; Zhang and Denlinger, 2010). To deal with climate change-caused impairments, insects have evolved various strategies to obtain thermotolerance, and heat shock proteins (Hsps) are best known for their cell-protecting functions under thermal stress conditions (Huang et al., 2009; Rinehart et al., 2007; Xu et al., 2011).

As their name implies, Hsps are well known for their response to high temperature stress (Feder and Hofmann, 1999; Huang et al., 2007). Inducible Hsps are not expressed in the normal conditions, but are quickly synthesized responding to various environmental stresses, and one of the best known functions is to increase heat tolerance (Lakhotia et al., 2002; Sorensen and Loeschcke, 2001; Zhang and Denlinger, 2010). In addition, Hsps may be the most abundant and the most studied thermal stress induced proteins, and most Hsps function as molecular chaperones to promote correct refolding and prevent aggregation of denatured proteins in response to temperature stress (Huang and Kang, 2007). The expression and synthesis of Hsps increase heat tolerance and protect organisms from thermal damage under stress conditions (Elekonich, 2009). In general, Hsps form a superfamily and can be divided into at least five groups based on the similarity of amino acid sequences and molecular weight: Hsp90, 70, 60, 40 and small Hsps with molecular masses ranging 12–43 kDa (Sun et al., 2014). Of all Hsps, Hsp90 is a highly conserved and abundant chaperone protein in the cell, which consists of three highly conserved domains: an N-terminal ATP-binding domain (25 kDa), a charged linker domain (35 kDa) and a C-terminal dimerization domain (12 kDa) (Taipale et al., 2010). Hsp90 mediates essential activities such as protein folding, localization and degradation (King and MacRae, 2015). Recent studies have confirmed that the expression levels of Hsp90 are up-regulated in response to different kinds of stress conditions, such as heat shock (Jiang et al., 2012; Wang et al., 2012), pesticide treatments (Feng et al., 2010), heavy metal pollution (Shu et al., 2011), diapause (Tachibana et al., 2005) and starvation (Wang et al., 2012). Although Hsp90 is suggested to be involved in thermotolerance, the functional significance of the thermal stress remains obscure. Furthermore, expression profiles of Hsps can vary according to the degree of temperature stress, and thermal

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tolerance involving Hsps can also vary depending on the sex and morph of the individuals (Huang and Kang, 2007; Lu and Wan, 2011).

The brown planthopper, *Nilaparvata lugens*, is one of the destructive rice pests in Asia, causing serious financial losses in many countries (Hu et al., 2014). *N. lugens* has two wing morphs, macropters (long-winged) with a high migratory ability and brachypters (short-winged) with a high reproductive capacity (Tufail et al., 2010; Xu et al., 2015). Temperature is one of the most important abiotic factors determining the growth, development, distribution and population abundance of *N. lugens*, which is often used to forecast population outbreaks (Ge et al., 2013; Piyaphongkul et al., 2012b). Previous studies found that nymphs are less heat tolerant than adults and macropters are more heat resistance than brachypters (Piyaphongkul et al., 2012a; Zhu et al., 1994). However, little is known about the molecular mechanism underlying the differential thermotolerance abilities of these two morphs. We hypothesized that Hsps play significant roles in the differential capacity of these two morphs to temperature stress. In the present study, we first identified a full-length *Hsp90* gene from *N. lugens* and compared the differences in *Hsp90* mRNA expression levels between macropters and brachypters after heat and cold shock. Then, RNAi was applied to examine the role of Hsp90 in relation to temperature tolerance.

## 2. Materials and methods

### 2.1. Insects and temperature stress treatments

*N. lugens* was originally collected from Guangzhou, Guangdong Province, China in September 2008. All insects were reared on rice seedlings (*Oryza sativa* L. cv. TN1) in our laboratory as described previously (Lu et al., 2015). Prior to this study, two generations of *N. lugens* were reproduced on TN1 under constant conditions of 26 ± 1 °C, 65% relative humidity and a photoperiod of 16:8 h (light:dark).

For temperature stress treatments, newly emerged brachypterous and macropterous female adults (within 24 h) were collected separately. Thirty adults were put into a 15 ml plastic centrifuge tube and treated for 1 h at different temperatures from 0 to 40 °C using a bath incubator (Polyscience, Niles, IL, USA). Insect maintained at 26 °C was set as untreated control. After the heat shock (40 °C) or cold shock (0 °C) for 1 h, the insects were allowed to recover at 26 °C for 0, 1, 2, 6, 12 and 24 h respectively, and then frozen quickly in liquid nitrogen until use.

### 2.2. RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from fifteen whole bodies or different tissues from thirty three-day-old insects using an Eastep™ Universal RNA Extraction Kit (Promega Corporation, Madison, WI, USA) according to the instructions provided by the manufacturer. The purity and concentration of the RNAs were determined by spectrophotometer Nanodrop2000C (Thermo Fisher Scientific, West Palm Beach, FL, USA). DNase I was used to eliminate the contaminated DNA and then first-strand complementary DNA (cDNA) was synthesized using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan).

### 2.3. Reverse transcriptase PCR (RT-PCR) and rapid amplification of cDNA ends (RACE)

In order to amplify the partial cDNA fragments of *Hsp90*, two pairs of degenerate primers were designed based on multiple alignments of published *Hsp90* sequences from other insect species. PCR reactions were performed in a 25 μl total reaction volume using Taq™ HS DNA Polymerase (TaKaRa, Tokyo, Japan). PCR conditions were set as follows: 1 cycle of 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min; 1 cycle of 72 °C for 10 min. The PCR products obtained were purified using a gel extraction kit (Tiangen, Beijing, China) and subcloned into pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and transformed into *E. coli* DH5α-competent cells. Positive clones were selected using a blue–white screen and sequenced (Invitrogen, Guangzhou, China).

The full-length cDNA was obtained using the SMART™ RACE (rapid amplification of cDNA ends) cDNA amplification kit (Clontech, Mountain View, CA, USA) following the manufacturer's instructions. Gene-specific primers used for 5′ and 3′ RACE were designed based on the sequence of partial fragment. PCR reactions were performed in a 25 μl total reaction volume using LA Taq™ DNA Polymerase (TaKaRa, Tokyo, Japan). The nest PCR conditions for RACE were: 1 cycle of 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1.5 min; 1 cycle of 72 °C for 10 min. DNA bands with expected sizes were excised from the agarose gel and purified, then cloned as described above. To verify that the RACE fragments were from the same gene, the overlapping sequences were assembled and specific primers flanking ORF (open reading frame) were designed, and then used to amplify the full-length cDNA.

### 2.4. Sequence and phylogenetic analysis

The cDNA sequences of *NlHsp90* were used as queries to search against GenBank database by BLAST software available at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ORF was identified by using the ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf/) and the molecular weight of predicated protein was calculated by the ExPASy-COMPUTE pI/Mw tool (http://web.expasy.org/compute_pi/). The sequences of known sixty different insect species in Fig. 1 were obtained from GenBank database. Multiple alignments of Hsp90 amino acid sequences were performed with ClustalW software and the phylogenetic tree was constructed by MEGA 6.0 using the neighbor-joining (NJ) method with 1000 bootstrap replicates (Tamura et al., 2013).

### 2.5. Quantitative real-time reverse transcriptase PCR (qRT-PCR)

To quantify the expression levels of *NlHsp90*, qRT-PCR was performed in a 10 μl total reaction volume including 5 μl 2× SYBR Premix$^{EX}$ Taq II master mix (TaKaRa, Tokyo, Japan), 4 μM of each gene-specific primer (Table 1) and 1 μl cDNA template (equivalent to 50 ng RNA). All qPCR reactions were performed with three biological replicates in a Light Cycler 480 detection system (Roche Diagnostics, Switzerland), and the cycling conditions were as follows: 1 cycle of 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 20 s. A calibrator sample was prepared, and samples without cDNA template were set as negative controls in the same run. For each cDNA sample, three technical replicates were run. The melting curve was conducted to judge the specificity of the PCR product at each end of the qPCR reaction. The standard curves of *Hsp90* and *β-actin* (**EU179850**, internal control) were constructed from a 10-fold dilution series of cDNA samples to calculate amplification efficiencies ($E$) and correlation coefficient ($R^2$). The relative expression levels of *Hsp90* (fold change) was normalized to the abundance of *β-actin* using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

**Fig. 1.** Phylogenetic tree based on the amino acid sequence alignment of heat shock protein 90 (Hsp90) in insects. Sequences were obtained from GenBank protein database and the accession numbers are shown in parentheses. The phylogenetic tree was constructed with MEGA6.0 software using the neighbor-joining (NJ) method. Bootstrap values in percent from 1000 replicates are shown in the cladogram. The scale bar indicates distance (the number of amino acid substitutions per site). The ratio (%) on the right side indicates the overall identity compared with the *Nl*Hsp90.