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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

Dong Hun Kim[1], Sang-Chul Lee[1], Do-Yeon Kwak[2] and Kyeong-Yeoll Lee[1]

[1]School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, Daegu and [2]Yeongnam Agricultural Research Institute, Milyang, Republic of Korea

Abstract Three heat shock protein (HSP) genes (*hsp70*, *hsc70*, *hsp90*) were partially cloned from the brown planthopper *Nilaparvata lugens* and the small brown planthopper *Laodelphax striatellus* (Homoptera: Delphacidae), which are serious pests of the rice plant. Sequence comparisons at the deduced amino acid level showed that the three HSPs of planthoppers were most homologous to corresponding HSPs of dipteran and lepidopteran species. Identities of both heat shock cognate 70 and HSP90 were higher than HSP70 in both species. Identity of the HSP70 between the two planthopper species was only 81%, a value much lower than seen among fly and moth groups. Effects of heat and cold shocks were demonstrated on expression of the three *hsp* genes in the two planthopper species. Heat shock (40 °C) upregulated the *hsp90* level but did not change the *hsc70* level in either the nymph and adult stages of either species. On the other hand, the *hsp70* level was only upregulated in *L. striatellus*. This heat shock response was prompt and lasted only for 1 h after treatment. In contrast, cold shock at 4 °C did not change the expression levels of any *hsp* in either species.

Key words cold shock, gene expression, heat shock, heat shock protein genes, planthoppers, thermal stress

Introduction

Organisms develop abilities to endure various stresses from unfavorable environmental conditions such as temperature extremes, desiccation, toxic substances and pathogens. At the molecular level, heat shock proteins (HSPs) play a significant role in generating tolerance to such stresses (Krebs, 1999; Lansing *et al*., 2000; Denlinger *et al*., 2001; Sørensen *et al*., 2003). HSPs act as molecular chaperones to protect proteins from damage during protein synthesis, folding, assembly and localization within the cell (Beckman *et al*., 1990; Parsell & Lindquist, 1993; Feder & Hofmann, 1999; Hartl & Hayar-Hartl, 2002). During evolution the molecular structures at the gene and protein levels and cellular functions of HSPs are highly conserved in almost all kinds of life from bacteria to humans (Lindquist & Craig, 1988; Boorstein *et al*., 1994).

The HSP family is multigenic and divided into at least three groups based on their protein size and sequence similarity: small heat shock protein (sHSP) with molecular masses ranging 12–43 kDa, heat shock protein 70 (HSP70), approximately 70 kDa, and heat shock protein 90 (HSP90), a higher molecular mass (Lindquist, 1986). The HSP70 family contains two groups based on their expression patterns although they share many common primary structures (Boorstein *et al*., 1994; Karlin & Brocchieri, 1998; Kiang & Tsokos, 1998). The first group, HSP70, is induced rapidly under various stresses, but is expressed at a very low level under normal condition. The second group, heat shock cognate 70 (HSC70), is constitutively expressed in

Correspondence: Kyeong-Yeoll Lee, School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea. Tel: +82 53 950 5759; fax: +82 53 950 6758; email: leeky@knu.ac.kr

normal cells and changes relatively little in stress conditions such as heat or cold shocks. However, some genes in the HSP70 and HSC70 groups change their expression patterns in response to different conditions of stress (Feder & Hofmann, 1999; Denlinger *et al*., 2001).

Molecular analysis of thermal stress has been extensively studied in a model insect, *Drosophila melanogaster*, and shows various responses of each *hsp* at the transcription level (reviewed by Hoffmann *et al*., 2003). Recently, research has been extended to several other insect species which are important in agricultural, medical and industrial fields (Yocum, 2001; Chen *et al*., 2005, 2006; Sonoda *et al*., 2006; Huang & Kang, 2007; Wang *et al*., 2007; Kim *et al*., 2008). Both the brown planthopper *Nilaparvata lugens* and the small brown planthopper *Laodelphax striatellus* are major pest insects that cause damage either directly or indirectly as vectors of plant viral diseases in rice fields of many Asian countries (Dale, 1994; Nault, 1994). In Korea the impact of *N. lugens* depends on populations that migrated mainly from southern China and southeast Asian countries, such as Vietnam and the Philippines (Uhm *et al*., 1988; Kisimoto & Rosenberg, 1994). It is generally known that *N. lugens* cannot overwinter in the Korean peninsula, while *L. striatellus* can survive during winter as nymphs in diapause. Recent global warming raises concerns about the acquisition of thermal tolerance of planthoppers and predicts potential growth of the population at higher latitudes, such as northern China, Japan and Korea. In this study, to evaluate the thermal responses of planthoppers at the molecular level, three hsp genes were cloned from *N. lugens* and *L. striatellus* and examined for their transcriptional responses to thermal stresses.

Materials and methods

Insects

Strains of *N. lugens* and *L. striatellus* are supplied from Yeongnam Agricultural Research Institute, Milyang, Korea. Both species were reared separately on rice seedlings (4–5 cm tall) in a plate (13 cm × 19 cm × 2.5 cm) in a rearing room at 28 °C, 70% relative humidity (RH) and a 16 h light/8 h dark (16L/8D) day-long photoperiod. Density of planthoppers was maintained at approximately 200 individuals per plate.

Heat and cold shock

For heat and cold shock experiments, fifth instar nymphs and adults were collected separately. Planthoppers ($n = 30$) attached to rice plants in a Petri dish (4 cm diameter) within a meshed cage (30 cm × 30 cm × 45 cm) were exposed to either 40 °C or 4 °C for 1 h in an incubator (Sanyo, Tokyo, Japan) and then transferred to 25 °C for recovery. At various times after these treatments planthoppers were collected into 1.5 mL microcentrifuge tubes. Total RNA was extracted immediately and stored at −70 °C until used.

Partial hsp70, hsc70, *and* hsp90 *cDNA cloning and sequencing*

Total RNAs were extracted from whole bodies using the total RNA isolation system (Promega, Madison, WI, USA). Reverse transcriptase (RT) reactions were performed with 2 μg total RNA as a template for cDNA synthesis using RETROscript (Ambion, Austin, TX, USA). Pairs of primers were designed using consensus mRNA sequences of insect *hsp*. Nucleotide sequences of the primers for *hsp70* and *hsc70* were 5' -GAT CAT CGC CAA CGA CCA-3' for forward; 5' -TTC ATC TTG GTA AGC ACC AT-3' for reverse. Those for *hsp90* were 5' -CTS ATC ATC AAC ACV TTY TAC TC-3' for forward; 5' -ACR ATC TCY TTG ATY TTG TG-3' for reverse. Polymerase chain reaction (PCR) was performed with 0.1 μg of cDNA as a template in PCR buffer containing 3.5 mmol/L $MgCl_2$, 0.2 μmol/L dNTPs, 0.4 μmol/L of each primer set and 2 units of Taq2000 (Stratagene, La Jolla, CA, USA) in 50 μL solution. The mixtures were amplified in a thermal cycler for 35 cycles (*hsp70* and *hsc70* – 94 °C for 30 sec, 52 °C for 30 sec, 72 °C for 40 sec; and *hsp90* – 94 °C for 30 sec, 48 °C for 30 sec, 72 °C for 40 sec), followed by a 5-min final extension at 72 °C. The PCR products were purified using a PCR purification kit (Nucleogen, Siheung, Korea) and subcloned into pGEM® T Easy Vector (Promega, Madison, WI, USA). Plasmid sequences were determined using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Forster City, CA, USA) and analyzed at the Solgent sequencing facility (Solgent, Daejeon, Korea). Computer analysis of DNA sequences was done using DNASIS version 2.1 (Hitachi Software Engineering America, San Francisco, CA, USA). Databases were searched using the BLAST P algorithm (Altschul *et al*., 1997; Schäffer *et al*., 2001) and amino acid sequences were aligned using CLUSTAL W available by internet access (Thompson *et al*., 1994).

Northern blot hybridization

RNAs from each sample (10 μg) were separated by 1% agarose formaldehyde gel electrophoresis, stained with ethidium bromide and transferred onto a Hybond-N nylon membrane (Amersham, Piscataway, NJ, USA). To make

probes of each gene the *hsp70, hsc70* and *hsp90* cDNA inserts were isolated from the cDNA clones by restriction endonuclease digestion, purified by electrophoresis on an Low Melting Point (LMP) agarose gel, and labeled with ^{32}P-dCTP (3000 Ci/mmol, Perkin Elmer Life Science, Waltham, MA, USA) using random hexamer priming (Feinberg & Vogelstein, 1983). The blots were hybridized to each probe at 42 °C overnight at 10^6 cpm/mL in a solution containing 40% (vol/vol) formamide, 5 × Saline-Sodium Citrate (SSC), 10 × Denhardt's solution, 100 μg/mL sheared and denatured herring sperm DNA, 50 mmol/L sodium phosphate (pH 7.0), and 10% (w/v) dextran sulfate. The filters were washed at 65 °C successively for 30 min each in 4 × SSC, 0.1% Sodium Dodecyl Sulfate (SDS); 2 × SSC, 0.1% SDS; 0.5 × SSC, 0.1% SDS; and 0.2 × SSC, 0.1% SDS. The filters were exposed to X-ray film (Kodak X-Omat) at −70 °C. As a control for RNA loading, the ethidium bromide-strained gel was photographed before blotting to visualize the rRNAs. Northern blots were triplicated in each experiment.

Results

Homology comparisons of hsp70, hsc70 *and* hsp90

Amplification of cDNAs from *N. lugens* and *L. striatellus* using each primer set resulted in a single band corresponding to a size of 304 bp for *hsp70* and *hsc70*, and 527 bp for *hsp90* in both species. Sequence comparisons, at deduced amino acid level, using BLAST X in the NCBI database showed that HSP70, HSC70 and HSP90 of the two planthopper species were highly similar to corresponding genes from dipteran and lepidopteran species (Table 1). Among them, HSC70 was the most highly conserved, 94%, then HSP90 at 89%−90% and HSP70 at 85%−89%. Partial sequences of the HSC70 clones of the two planthopper species were completely identical and were most similar to the lepidopteran insects. HSP90 was also highly similar to sequences of moth and locust species. HSP70 of *N. lugens* has high identities with the mosquito *Anopheles albimanus*, the vegetable leafminer *Liriomyza sativae*, and the silkworm *Bombyx mori*, at 89%, 86% and 85%, respectively. HSP70 sequence of *L. striatellus* was most similar to *L. sativae*, 88% identity, but has only 81% identical to HSP70 in *L. striatellus*. Sequences for *hsp70*, *hsc70* and *hsp90* of *N. lugens* were deposited at GenBank as accession numbers ABV44275, ABV44277 and ABV44280, respectively. Those of *L. striatellus* are deposited as ABV44276, ABV44278 and ABV44279, respectively.

Effects of cold and heat shock on the expression of hsp70, hsc70 *and* hsp90

To determine the effects of thermal stress on *hsp* gene expression, nymphs and adults of the two planthoppers

Table 1 Comparisons of the deduced amino acid sequences of *hsp70*, *hsc70* and *hsp90* of *N. lugens* and *L. striatellus* with those of other insects.

HSPs	Species	Accession numbers	Identities (%)	References
HSP70	*Nilaparvata lugens*	ABV44275	–	Present study
	Anopheles albimanus	AAC41542	89	Benedict *et al*., 1993
	Liriomyza sativae	AAW32099	86	Huang & Kang, 2007
	Bombyx mori	NP_001037396	85	NCBI database
	Laodelphax striatellus	ABV44276	81	Present study
HSC70	*Nilaparvata lugens*	ABV44277	–	Present study
	Laodelphax striatellus	ABV44278	99	Present study
	Plutella xylostella	BAE48743.1	94	Sonoda *et al*., 2006
	Trichoplusia ni	AAB06239.1	94	NCBI database
	Lonomia obliqua	AAV91465.1	94	Veiga *et al*., 2005
HSP90	*Nilaparvata lugens*	ABV44280	–	Present study
	Laodelphax striatellus	ABV44279	98	Present study
	Bombyx mori	NP_001036876.1	90	NCBI database
	Locusta migratoria	AAS45246.2	90	NCBI database
	Mamestra brassicae	BAF03554.1	89	Sonoda *et al*., 2007

The homology was searched using the BLAST X algorithms in NCBI database, and amino acid sequences were aligned using CLUSTAL W available by internet access (Thompson *et al*., 1994). HSP, heat shock protein; HSC, heat shock cognate.

were exposed to either heat (40 °C) or cold (4 °C) shock for 1 h. In *N. lugens*, heat shock increased *hsp90* transcript level at 1 h after treatment but then decreased again to the basal level (Fig. 1). Levels of *hsp70* and *hsc70* were not changed by heat shock in either nymphs or adults. In *L. striatellus*, heat shock increased levels of both *hsp70* and *hsp90* transcripts at 1 h after treatments (Fig. 2). The *hsp70* level was especially high in the adult stage while it increased slightly in the nymphal stage in response to heat shock. However, the level of *hsc70* was not changed by heat shock in either stages. In contrast, cold shock at 4 °C for 1 h did not change any of the *hsp* levels in either the nymphal or adult stages in the two species (Figs. 1, 2). To determine how quickly the *hsp* genes respond to heat shock, the *hsp* transcript levels were determined at different hours after heat shock. The levels of both *hsp70* and *hsp90* of *L. striatellus* were promptly increased by heat shock but the elevation persisted only for 1 h after the shock (Fig. 3).

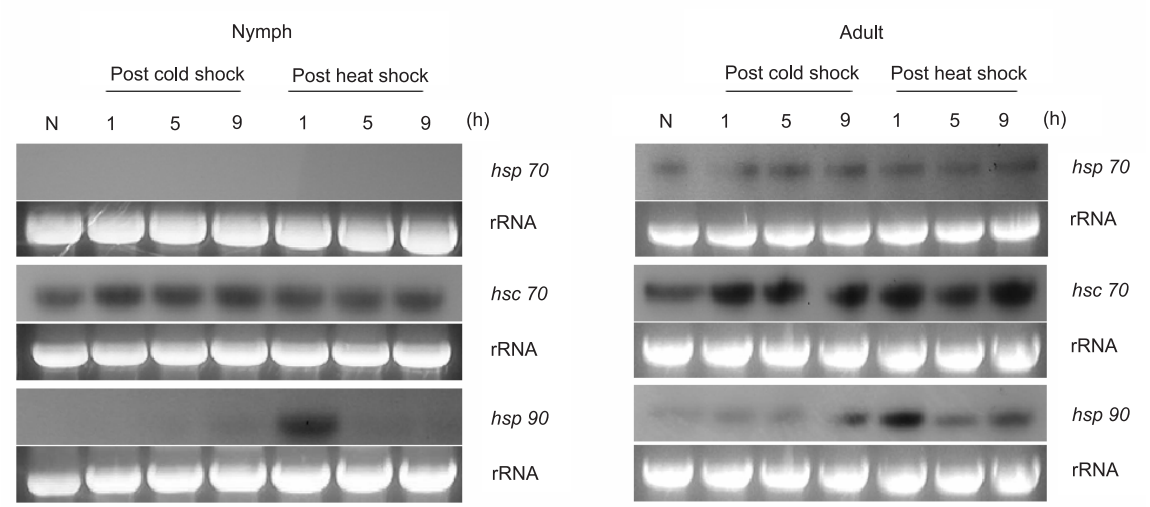

Fig. 1 Effects of cold and heat shock on the expression of *hsp70*, *hsc70* and *hsp90* in *N. lugens*. Both nymphs and adults were exposed at either 4 °C or 40 °C for 1 h and then allowed to recover at 25 °C for 1, 5 and 9 h. Total RNA samples were extracted from whole bodies and 10 μg was loaded in each lane. Ribosomal RNAs stained with ethidium bromide are shown as loading controls.

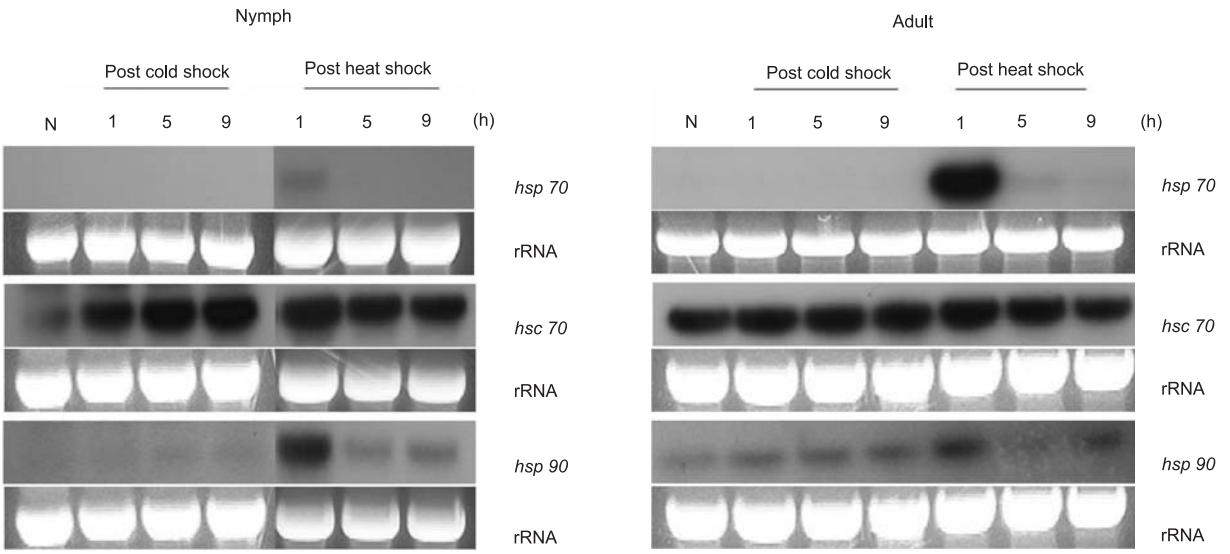

Fig. 2 Effects of cold and heat shock on the expression of *hsp70*, *hsc70* and *hsp90* in *L. striatellus*. Both nymphs and adults were exposed at either 4 °C or 40 °C for 1 h and then allowed to recover at 25 °C for 1, 5 and 9 h. Total RNA samples were extracted from whole bodies and 10 μg was loaded in each lane. Ribosomal RNAs stained with ethidium bromide are shown as loading controls.

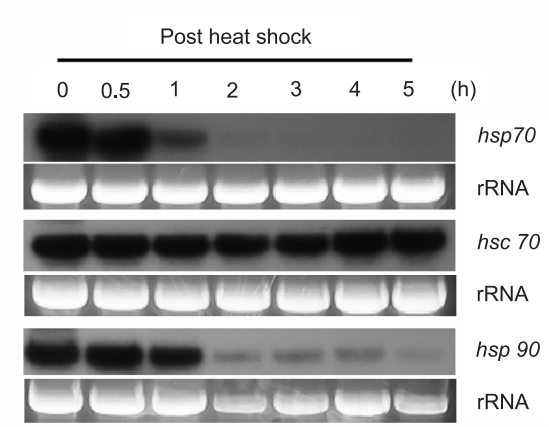

Fig. 3 Expression of *hsp70*, *hsc70* and *hsp90* of *L. striatellus* adults during recovery following heat shock. Planthoppers were exposed to 40 °C for 1 h and then allowed to recover at 25 °C for 0, 0.5, 1, 2, 3, 4 and 5 h. Total RNA samples were extracted from whole bodies and 10 μg was loaded in each lane. Ribosomal RNAs stained with ethidium bromide are shown as loading controls.

Discussion

Heat shock proteins are highly conserved molecules in aspect of genomic and proteomic organizations throughout evolution. Although no similar HSP sequences of the related hemipteran species are yet listed in the current NCBI database, our comparison analysis at the deduced amino acid level showed that partial sequences of HSP70, HSC70 and HSP90 of two planthopper species were highly similar with those of dipteran and lepidopteran species. Our results showed that sequences of both HSC70s and HSP90s of *N. lugens* and *L. striatellus* were most identical between the two planthoppers. However, the HSP70 identity between the two planthopper species was lower than with those of lepidopteran or dipteran species, despite their belonging to the same taxa. Although our partial clones of *hsp70* are too short to compare overall similarity between the species, the low identity between the two planthopper *hsp* genes suggests that they may be generated from different members within the HSP70 family. Generally, the HSP70 family is multigenic, consisting of several isoforms derived from different genes within a species. For example, *D. melanogaster* has at least 13 genes encoding five different HSP70s, one HSP68 and seven different HSC70s (Ingolia *et al*., 1982; Craig *et al*., 1983; Palter *et al*., 1986). Further studies are required to determine whether planthoppers also have several isoforms of HSP70.

Heat stress upregulates various *hsp* genes in many insect species. Upregulation of *hsp70* by heat shock has been demonstrated in three *Drosophila* species (Goto & Kimura, 1998), the flesh fly *Sarcophaga crassipalpis* (Rinehart *et al*., 2000), the silverleaf whitefly *Bemisia argentifolii* (Salvucci *et al*., 2000), the red flour beetle *Tribolium castaneum* (Mahroof *et al*., 2005), the blow fly *Lucilia sericata* (Tachibana *et al*., 2005), and the solitary bee *Megachile rotundata* (Yocum *et al*., 2005). This gene responds to heat shocks in the range of 35−40 °C after 1 h or even 30 min. Our present study showed that the *hsp70* level of *L. striatellus* is upregulated by heat shock in both the nymphal and adult stages. Interestingly, the level of expression in adults was much higher than in the nymphs. However, the *hsp70* of *N. lugens* did not respond to the same heat shock. This result suggests that thermal responses of the *hsp70* gene may be different between the two planthopper species. On the other hand, this difference, together with the sequence information, may be due to the responses of different isoforms of *hsp70*. However, it is necessary to investigate heat shock response of the *hsp70* at broader temperature range because the *hsp70* genes of the two species may respond at different temperatures. *Hsc70*, a cognate form of *hsp70*, is generally expressed constitutively in normal cells and does not respond to heat stress (Kiang & Tsokos, 1998; Denlinger *et al*., 2001; Kregel, 2002). Similarly, those levels in the two planthopper species were high but not changed by heat shock.

The *hsp90* gene of both species was rapidly induced by heat shock. Similar upregulation of this gene has been noticed in flies, bees and moths including *D. subobscura* (Arbona *et al*., 1993), *D. auraria* (Yiangou *et al*., 1997), *S. crassipalpis* (Rinehart & Denlinger, 2000), *Delia antique* (Chen *et al*., 2005), *L. sericata* (Tachibana *et al*., 2005), *M. rotundata* (Yocum *et al*., 2005), and *C. suppressalis* (Sonoda *et al*., 2006). In those insects, *hsp90* is usually induced by heat shock in the range 35−40 °C. Our data showed that upregulation of both *hsp70* and *hsp90* is immediate in response to heat treatment and extends to at least 1 h after treatment. The rapidity of *hsp70* induction by heat shock is recorded as 15 min in *S. crassipalpis* (Rinehart *et al*., 2000).

Cold stress induces some *hsp* genes in insects. For example, in the flesh fly, *S. crassipalpis*, cold shocks at either −10 °C or 0 °C stimulate the synthesis of several heat shock proteins (Joplin *et al*., 1990). This cold stress also upregulates transcript levels of *hsp70*, *hsc70* and *hsp90* (Rinehart *et al*., 2000; Rinehart & Denlinger, 2000). However, our results showed that cold shocks at 4 °C for 1 h did not change the levels of any hsp transcripts of the two planthopper species. This suggests that the range of cold shock temperature is important for the induction of *hsp* genes. Goto and Kimura (1998) showed that cold shocks at mild temperature such as 4 °C do not significantly changed *hsp70* level but that shocks at lower temperatures, down to

−8 °C, proportionally upregulated its level in *Drosophila* species. Similarly, Huang and Kang (2007) reported no inductions of five different *hsp* genes when two leafminer species were shocked at temperatures 2.5−27.5 °C, whereas high induction appeared at temperature shocks outside of that range. To detect the effect of cold shock on planthoppers, further study is required at subzero or lower temperature ranges. However, recently Yocum *et al*. (2006) showed in diapausing prepupae of the solitary bee, *M. rotundata*, that long periods of cold storage at 4 °C maintain high *hsp70* levels. Thus, *hsp* genes may respond to mild chilling when insects are exposed for a long period.

The present study determined partial sequences of *hsp70*, *hsc70* and *hsp90* genes and demonstrated the effects of heat and cold shocks in two rice planthoppers, *N. lugens* and *L. striatellus*. Our results indicate that the thermal responses of *hsc70* and *hsp90* are similar, but *hsp70* is different between the two planthoppers. In addition, our probes potentially can be used to monitor seasonal changes of thermal stress levels in planthopper populations. Further study is required to understand the tolerance mechanism to thermal stress of planthoppers.

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