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RESEARCH ARTICLE

Using double-stranded RNA to explore the role of heat shock protein genes in heat tolerance in *Bemisia tabaci* (Gennadius)

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SUMMARY

The whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) biotype B, is one of the most destructive invasive pests of field and glasshouse crops, and has a high tolerance to heat. Our previous work found that whitefly females are more heat tolerant than males. In the present study, real-time PCR and double-stranded RNA (dsRNA) methods were used to explore the role of heat shock protein (Hsp) genes in whitefly of both sexes; this provided further evidence of the mechanism underlying the differential heat tolerance abilities of females and males. The results showed that both *hsp23* and *hsp70* mRNA expression levels were higher in females than in males from 37.5 to 42°C, while at the extreme temperature of 44°C the *hsp70* mRNA level was higher in males than in females. There was no significant difference in *hsp90* mRNA expression between females and males under heat shock conditions. Furthermore, the survival rate of females fed *hsp23* or *hsp70* dsRNA significantly decreased following heat shock at 44°C for 1 h, but male survival rate was not significantly affected. Additionally, the survival rate of both females and males showed no significant change after they were fed with *hsp90* dsRNA. Collectively, the present study shows that the optimum mRNA expression of Hsp genes in females promotes a higher survival rate under heat shock conditions; *hsp23* and *hsp70* play a key role for heat tolerance in females but not in males, and *hsp90* shows no significant role in heat tolerance in either females or males. Further, our study indicates that feeding with dsRNA is an effective method by which to study gene function, and the simplicity of this approach opens the way for further research on gene function in different sexes and diverse groups of species.

Key words: *Bemisia tabaci* biotype B, double-stranded RNA (dsRNA), heat shock, heat shock protein gene, heat tolerance, RNA interference (RNAi).

INTRODUCTION

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism through which targeted genes are silenced by the introduction of double-stranded RNA (dsRNA) into living cells or organisms (Fire et al., 1998). Direct injection and oral delivery/feeding of dsRNA are the main methods used to initiate RNAi in insects, as demonstrated in Lepidoptera (Turner et al., 2006), Coleoptera (Araujo et al., 2006), Diptera (Misquitta and Paterson, 1999), Homoptera (Ghanim et al., 2007) and Hymenoptera (Zhou et al., 2008). Most of these studies have focused on the identification of gene function with regard to insect development (reviewed in Lü, 2008). However, one study (Rinehart et al., 2007) used RNAi to explore the relationship between heat and cold tolerance and heat shock protein (Hsp) genes. Rinehart and colleagues found that suppressing expression of either *hsp23* or *hsp70* resulted in the loss of heat tolerance in *Sarcophaga crassipalpis* non-diapausing pupae, and had a significant effect on the pupa's ability to survive exposure to low temperature (Rinehart et al., 2007). Similarly, thermotolerance in *Drosophila* has been shown to be primarily due to Hsp70 (reviewed in Rutherford, 2003).

The heat shock response is one of the most evolutionarily conserved defensive mechanisms against acute exposure to extreme environmental conditions. Experimental manipulation of temperature to create heat shock conditions (reviewed in Lindquist, 1986) has shown that all species respond to heat shock by synthesizing Hsps. Generally, the induction of Hsps coincides with increased heat tolerance and the level of response is assumed to relate to the subsequent level of Hsps expressed. The expression of Hsp genes plays an important role in protecting organisms under stress conditions (reviewed in Hoffmann et al., 2003). Furthermore, heat tolerance involving Hsps can vary according to the sex of the individual (Dahlgaard et al., 1998; Lansing et al., 2000) and patterns of Hsp expression can vary in relation to the kind of high temperature stress exposure (Krebs, 1999).

The whitefly, *Bemisia tabaci* (Gennadius) biotype B (*B. argentifolii* Bellows and Perring) (Homoptera: Aleyrodidae), is one of the most destructive invasive pests of field and glasshouse crops throughout the world (Oliveira et al., 2001). It is thought to have come from the Middle East–Asia Minor region (De Barro et al., 2011) where its ability to tolerate exposure to extreme temperatures is central to its survival. Although both sexes possess heat tolerance, adult females have been found to have a higher survival rate than adult males (Cui et al., 2008). Furthermore, through the use of suppressive subtractive hybridization (SSH), variation in heat tolerance with respect to sex has been associated with the differential

expression of heat shock genes (Lü and Wan, 2008). We hypothesized that Hsp genes play a key role in the differential ability of females and males to tolerate heat stress. To further explore the role played by Hsp genes in the heat tolerance of male and female *B. tabaci*, we used both real-time PCR and dsRNA methods to analyse the expression of *hsp23*, *hsp70* and *hsp90* genes and their role in heat tolerance.

MATERIALS AND METHODS

Insects and host plants

Bemisia tabaci biotype B was reared on cotton plants, *Gossypium hirsutum* (L.) (var. Simian No. 3) in the glasshouse at 20–34°C, 50–60% relative humidity (RH) and natural photoperiod (39°55′N, 116°20′E). The plants were individually grown in 9 cm diameter pots under the same conditions as the whitefly.

Heat tolerance test

To determine the role of Hsp genes in the ability to survive heat shock exposure, we analysed the relationship between heat tolerance and Hsp mRNA expression. The heat tolerance test was conducted using the method described previously (Lü and Wan, 2008). As Bowler and Terblanche had observed that adult age resulted in different responses to high temperature stress (Bowler and Terblanche, 2008), we standardized adult age by using only newly emerged whitefly adults that were less than 3 h old. Adult sex was determined visually with the aid of a stereomicroscope. Following sexing, 100 females or males were placed together in a 1.5 ml centrifuge tube. The number of adults chosen was based on preliminary experiments which showed that 100 adults enabled sufficient total RNA to be extracted for reverse transcription. The whiteflies inside the tubes were exposed to heat shock at 35, 37.5, 40, 42 or 44°C (±0.2°C) for 1 h in a constant environment chamber (MHT350, Sanyo Electric Co. Ltd, Osaka, Japan). The temperatures selected were based on the range of summer temperatures experienced in various parts of China. The length of exposure was selected based on preliminary experiments which indicated that a 1 h exposure was sufficient to induce a measurable heat shock response in both males and females. Adults maintained at 25°C were used as untreated controls. Following exposure, the samples were immediately frozen with liquid nitrogen and then stored at –80°C until RNA extraction. Each treatment had three replicates.

Extraction of total RNA and reverse transcription

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA); the RNase-Free DNase Set (Qiagen) was used to remove genomic DNA. The quantity and quality of RNA were assessed by spectrophotometry (Beckman Du 650 spectrophotometer, Fullerton City, CA, USA). RNA quality was also evaluated using 1.0% agarose gel electrophoresis. cDNA was synthesized from 1 μg total RNA using SuperScript™ III Reverse Transcriptase Kit (Invitrogen Life Technologies, Burlington, ON, Canada), following the manufacturer's instructions. The cDNA was stored at –80°C prior to further analysis.

Real-time PCR

hsp23, *hsp70* and *hsp90* were identified from ESTs of whitefly heat shock SSH libraries as described previously (Lü and Wan, 2008). *hsp23*, *hsp70* and *hsp90* mRNA expression under heat shock conditions was examined by absolute quantification real-time PCR. Reactions were performed in a PTC-200 Thermocycler (MJ Research, St Bruno, QC, Canada). The sequences of all primers and probes are listed in Table 1. Primer express software (Applied Biosystems, Foster City, CA, USA) was used to design the primers and probes. Plasmids (pMD18-T vector, TaKaRa, Shiga, Japan) containing an insert of each selected gene were used to make 10-fold serial dilutions from 2.00×10^7 copies of plasmid DNA down to 2.00×10^3 copies. Amplification experiments for each of the three gene dilution sets were replicated three times using 2 μl of the appropriate primers (Table 1) in each experiment for each dilution. The amplification volume was 25 μl, including 1.125 μl forward primer (20 μmol l^{-1}), 1.125 μl reverse primer (20 μmol l^{-1}), 0.625 μl probe (20 μmol l^{-1}), 12.5 μl Applied Biosystems 2×PCR Master Mix, 2.0 μl cDNA sample and 7.625 μl ultra-pure water. Three wells, each containing 2.0 μl ddH_2O, were used as a non-template control. The PCR cycling conditions were: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification; each cycle consisted of 95°C for 30 s and 60°C for 30 s. Plasmid DNA standard curve equations were used to calculate the absolute copy number of the appropriate gene mRNA in each treatment for each sex. The standard curve for *hsp23*, *hsp70* and *hsp90* had a slope of –2.81, –4.12 and –3.67, a correlation coefficient (R^2) of 1.000, 0.990 and 0.999, and a *Y*-intercept of 43.37, 45.58 and 45.34, respectively. Each cDNA sample was assayed in triplicate.

Production of dsRNA transcription templates and synthesis of dsRNA

hsp23, *hsp70* and *hsp90* transcription templates were produced from total whitefly cDNA using gene-specific primers containing a T7 promoter sequence; the T7 primer was as described previously (Ghanim et al., 2007). The primer sequences are listed in Table 2. Amplification reactions were conducted in 13.4 μl ddH_2O, 2 μl 10×buffer, 0.4 μl dNTPs (10 mmol l^{-1} for each nucleotide), 1 μl forward primer (10 μmol l^{-1}), 1 μl reverse primer (10 μmol l^{-1}), 2 μl cDNA template and 0.2 μl Taq DNA polymerase (5 U μl^{-1}, NEB, Ipswich, MA, USA) in a final volume of 20 μl. The PCR cycling conditions were 94°C for 3 min, 35 cycles of 94°C for 45 s, 57°C for 30 s and 72°C for 45 s, and a final extension step of 72°C for 10 min. Amplification of PCR products was confirmed by separation on 1.5% agarose gels and visualized by staining with ethidium bromide under UV light. The PCR products were purified using a Qiaquick PCR purification kit (Qiagen) according to the

Table 1. Primer and probe sequences for absolute quantification real-time PCR

Target gene	Outer primer sequence (5′–3′)	PCR primer sequence (5′–3′)	Probe sequence (5′–3′)	Product (bp)
hsp23	TCCGCACTAACTGACCAGAACT AGGCTGGAACTGCTGAACATC	GTTGAACTCACGTCCCCGTTA CCTGCAGCCAAGGGAAGA	CAACACACAGCTCTC	213, 57
hsp70	TCGACTACTACACCAAGGTCTCAAGA GGGTGCTACGTCGACAAGGA	CGATTCTCAGCGGTGATACCA GGGTGCTACGTCGACAAGGA	CTCAGCTATCCAAGATG	323, 62
hsp90	GGTATGAAAGAGAGCCAGAAGCA TTCGTCCTCAGGCAGTTCTAATC	CACTGGTGAAAGCAAAGATCAAGTA CGATGGGTTCGGTCATGTAGA	CTCCTCATTTGTCGAGCG	219, 95

Product sizes for the outer primer (larger value) and for the real time PCR primer are shown.

Table 2. Primer sequences

Target gene	Primer sequence (5′–3′)	Product (bp)
hsp23	TAATACGACTCACTATAGGGAGACCACTCCGCACTAACTGACCAGAACT	213, 267
	TAATACGACTCACTATAGGGAGACCACAGGCTGGAACTGCTGAACATC	
hsp70	TAATACGACTCACTATAGGGAGACCACGCGCCAAGATAGCTGCCA	225, 279
	TAATACGACTCACTATAGGGAGACCACTCTTGCTCATGATGGGCGA	
hsp90	TAATACGACTCACTATAGGGAGACCACGGTATGAAAGAGAGCCAGAAGCA	219, 273
	TAATACGACTCACTATAGGGAGACCACTTCGTCCTCAGGCAGTTCTAATC	
β-*actin*	CGCTGCCTCCACCTCATT	129
	ACCGCAAGATTCCATACCC	

Primer sequences (no underline) are shown for comparative quantification PCR; primer sequences plus T7 promoter sequences (underlined) are shown for production of dsRNA transcription templates.
Product sizes for the primers with T7 promoter sequences (larger value) and for the primers without T7 promoter sequences are shown.

manufacturer's instructions. The PCR products were stored at –80°C prior to synthesis of dsRNA.

dsRNA was synthesized using the MEGAscript® RNAi Kit (Ambion, Austin, TX, USA) and 1 μg PCR product was used as the transcription template. dsRNA was resuspended in RNase-free water; it was analysed by agarose gel electrophoresis and quantified spectrophotometrically. The dsRNA was stored at –80°C prior to further use.

Feeding with dsRNA and detection

Newly emerged whitefly females and males were fed a diet containing dsRNA diluted to 0.3–0.5 μg μl^{-1} in a 10% w/v RNase-free sucrose solution. Feeding was done using the Parafilm clip nutrient solution method (Miles, 1965). Parafilm was pre-treated with 0.1% diethyl pyrocarbonate (DEPC) solution to remove any RNases, then RNase-free water was used to clean the DEPC from the Parafilm. Two-hundred newly emerged whitefly females were collected and put into a glass tube (3 cm diameter, 8 cm high); this was repeated with 200 males. The tube opening was covered with two layers of Parafilm and 200–250 μl dsRNA solution was injected into the gap between the two layers. The other end of the tube was covered with gauze to enable ventilation. Brown paper was wrapped around the tube, leaving the Parafilm-enclosed end exposed to light. This encouraged the adults to move towards the diet and feed. Each treated tube was placed in a constant environment chamber for 3 h at 25±0.2°C. Afterwards, some of the samples were frozen immediately with liquid nitrogen and then stored at –80°C until RNA extraction. The others were exposed to 44±0.2°C in a chamber for 1 h. After the heat shock, the whiteflies were placed into another chamber at 25±0.2°C for 1 h to allow them to recover; the number of whiteflies recovered was simultaneously counted. The heat shock temperature of 44°C was selected based on preliminary experiments which showed that this was the discrimination point for male and female heat tolerance. The control was whiteflies fed 10% w/v RNase-free sucrose solution only. Each treatment had six replicates.

hsp23, *hsp70* and *hsp90* mRNA expression after feeding with dsRNA was analysed by comparative quantification real-time PCR. The primer sequences are listed in Table 2. Reactions were performed using a PTC-200 Thermocycler. The amplification volume was 12.5 μl, including 0.25 μl forward primer (10 μmol l^{-1}), 0.25 μl reverse primer (10 μmol l^{-1}), 6.25 μl SYBR mix (SYBR Green, dNTPs, Mg^{2+} and TaqDNA polymerase), 0.25 μl Rox (passive reference dye), 1.0 μl cDNA sample and 4.5 μl ultra-pure water. The PCR cycling conditions were 95°C for 10 s followed by 40 cycles of amplification; each cycle consisted of 95°C for 5 s, 63°C for 20 s and 72°C for 20 s. The standard curve for *hsp23*, *hsp70*, *hsp90* and β-*actin* had a slope of –3.13, –3.20, –2.99 and –3.04, an amplification efficiency of 108.9, 105.6, 115.9 and 113.5%, a correlation coefficient (R^2) of 1.000, 1.000, 0.998 and 0.996, and a *Y*-intercept of 18.05, 15.91, 17.37 and 16.47, respectively. A standard curve was derived from a serial dilution to quantify the copy numbers of target mRNA, and β-*actin* was used as the housekeeping gene. The relative level of each Hsp mRNA was defined by comparison with the amount of β-*actin*. Each sample was assayed in triplicate.

RESULTS

Hsp mRNA expression pattern in female and male whiteflies under heat shock conditions

The analysis of *hsp23* expression showed a significant interaction between the different exposure temperatures and whitefly sex

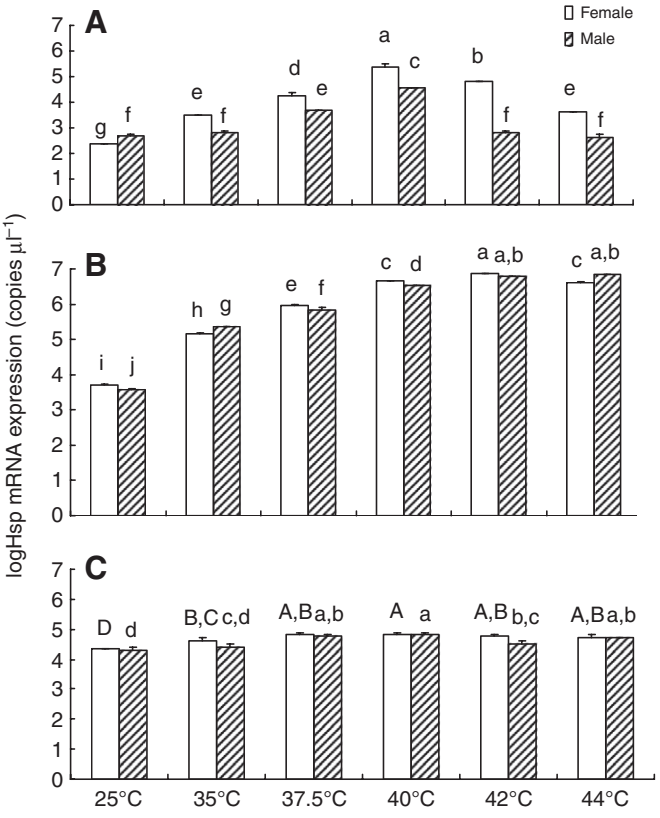

Fig. 1. Changes in expression of Hsp mRNA (A, *hsp23*; B, *hsp70*; C, *hsp90*) in *Bemisia tabaci* females and males following a 1 h treatment at the temperature indicated. Values are means + s.e.m. Different lowercase and uppercase letters indicate significant difference at $P<0.05$.

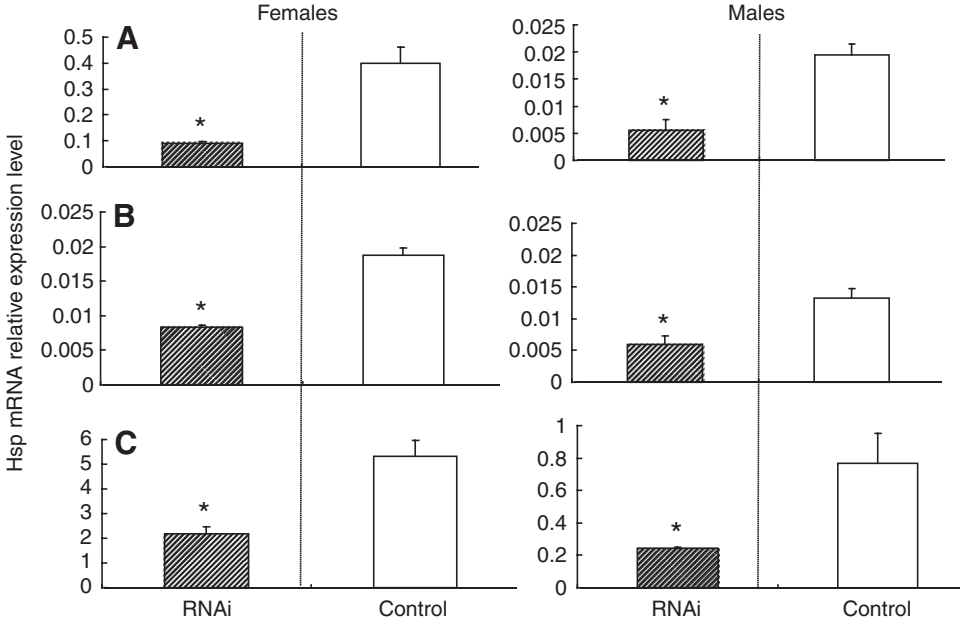

Fig. 2. The effect of dsRNA treatment (RNA interference, RNAi) on Hsp mRNA expression in *B. tabaci* females and males exposed to 44°C for 1 h (A, *hsp23*; B, *hsp70*; C, *hsp90*). Values are means + s.e.m. *Significantly different at $P<0.05$.

($F_{5,35}$=60.7, P<0.001) (Fig. 1A). At 25°C, males had a higher mRNA expression level of *hsp23* than females (P<0.001). However, the *hsp23* mRNA level in females was significantly higher than that in males from 35 to 44°C (P<0.05). In addition, *hsp23* mRNA expression level was significantly increased at 35–44°C when compared with the 25°C control temperature in females (P<0.05), and reached an expression peak at 40°C. For males, *hsp23* mRNA expression was significantly increased at 37.5–40°C when compared with that at 25°C (P<0.05) (Fig. 1A).

The expression of *hsp70* showed a significant interaction between the different exposure temperatures and whitefly sex ($F_{5,35}$=19.8, P<0.001) (Fig. 1B). At 25, 37.5, 40 and 42°C, females had a higher mRNA expression level of *hsp70* than males (P<0.01). However, females had a lower mRNA expression level of *hsp70* than males at 35 and 44°C (P<0.01). In addition, *hsp70* mRNA expression level was significantly increased at 35–44°C when compared with the 25°C control temperature in both sexes (P<0.05), reaching an expression peak at 42°C in females and 44°C in males (Fig. 1B).

There was no significant interaction between temperature and whitefly sex in the mRNA expression of *hsp90*. However, there was a significant difference in the expression of *hsp90* mRNA for different exposure temperatures in both sexes (female, $F_{5,16}$=11.2, P<0.01; male, $F_{5,16}$=7.66, P<0.01). For females, the expression of *hsp90* mRNA was significantly increased at 35–44°C when compared with the 25°C control temperature. For males, *hsp90* mRNA expression was significantly increased at 37.5–44°C when compared with the control (25°C). In addition, we did not detect a difference between females and males at any temperature (Fig. 1C).

The role of Hsp genes during heat shock treatment

Compared with the controls, *hsp23*, *hsp70* and *hsp90* mRNA expression decreased significantly in both females and males after feeding with dsRNA for 3 h: *hsp23*, female: t_4=–5.20, P=0.007; male: t_4=–4.92, P=0.008 (Fig. 2A); *hsp70*, female: t_4=–9.28, P=0.001; male: t_4=–3.62, P=0.022 (Fig. 2B); *hsp90*, female: t_4=–4.32, P=0.012; male: t_4=–2.87, P=0.045 (Fig. 2C).

Furthermore, for adults fed with either *hsp23* or *hsp70* dsRNA and then exposed to 44°C for 1 h, female survival rate decreased significantly compared with the control whereas males showed no significant change: *hsp23*, female: t_{10}=–5.43, P=0.000 (Fig. 3A); *hsp70*, female: t_{10}=–3.61, P=0.005) (Fig. 3B). These results show that *hsp23* and *hsp70* influenced adult female, but not male, heat tolerance. In contrast, there was no significant change in the survival of either sex as a result of feeding with *hsp90* dsRNA followed by exposure to 44°C for 1 h (Fig. 3C).

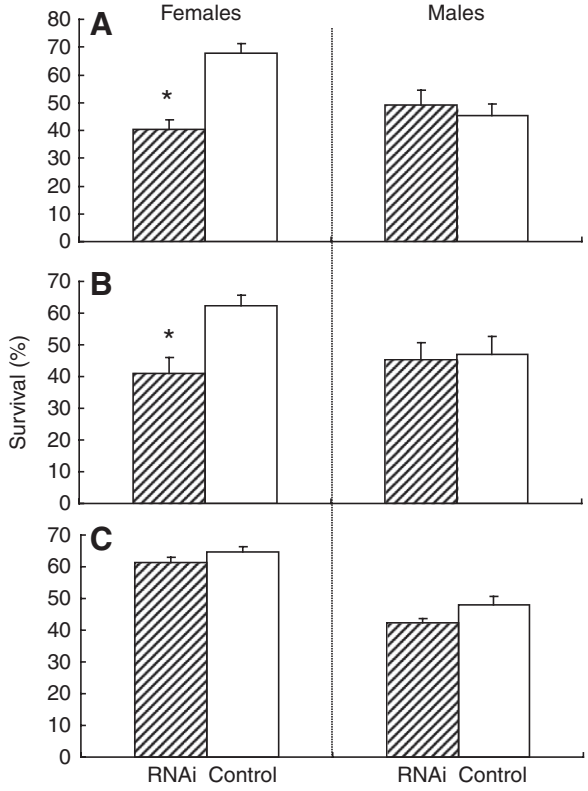

Fig. 3. The effect of dsRNA treatment (A, *hsp23*; B, *hsp70*; C, *hsp90*) on heat tolerance of *B. tabaci* females and males exposed to 44°C for 1 h. Values are means + s.e.m. *Significantly different at P<0.05.

DISCUSSION

In recent years, RNAi technology has been successfully applied to the study of gene silencing in whiteflies (Ghanim et al., 2007). In the study by Ghanim and colleagues (Ghanim et al., 2007), target mRNA expression decreased significantly in whiteflies after the injection of dsRNA; however, the technique is time consuming, difficult to undertake and subject to constraints such as the need for micromanipulation equipment. In contrast, the dsRNA feeding method is simple and easy to do (Baum et al., 2007; Mao et al., 2007). In some insects such as *Drosophila*, feeding with dsRNA is not effective because they lack the transmembrane protein SID-1, which therefore prevents transport across the cell membrane (Feinberg and Hunter, 2003; Roignant et al., 2003). However, in insects that have this protein, e.g. *Schistocerca gregaria*, *Apis mellifera* and *Bombyx mori*, dsRNA feeding is effective (Dong and Friedrich, 2005; Turner et al., 2006). Our work indicates that whiteflies have this cross-membrane transport mechanism and as a result feeding with dsRNA leads to the inhibition of Hsp mRNA expression.

Previous studies have shown that Hsp expression can be induced to varying levels depending on the degree of heat shock exposure (Feder et al., 1996; Krebs and Feder, 1998; Krebs, 1999). Generally, mRNA expression of Hsp genes reaches a peak at a moderate heat shock temperature and then decreases following more extreme temperature exposure. This was the case in our study, e.g. the expression level of *hsp23* in both sexes increased from 35 to 40°C, peaked at 40°C and then decreased once temperatures exceeded 40°C. There are several possible explanations for this. For example, stress intensity may exceed the regulation capacity of the organism (Li et al., 2004). Alternatively, it may be a real-time regulation process for organisms to maintain energy balance (Sørensen et al., 2003).

Our results show that adult female whiteflies have a greater tolerance to 44°C than adult males – this has also been observed for other insect species. Female *D. melanogaster* (Dahlgaard et al., 1998; Lansing et al., 2000), *Drosophila buzzatii* (Sørensen et al., 1999; Sørensen et al., 2005; Sarup et al., 2006) and *A. aegypti* (Andersen et al., 2006) all tolerate high temperature stress better than males. In these species tolerance to heat stress has been shown to be related to the level of dopamine expression (Gruntenko et al., 2004; Andersen et al., 2006). Our results show that this difference is related, at least in part, to the level of expression of *hsp23* and *hsp70*. For adult females, feeding with both *hsp23* and *hsp70* dsRNA decreased survival rates significantly, but had no effect on male survival. This supports the idea that *hsp23* and *hsp70* play a key role in heat tolerance in females, but not in males. In the case of *hsp23*, the expression level in females exceeded that in males and indicated that higher expression was responsible for the increased tolerance. This result is consistent with previous studies where the level of expression was found to be positively related to heat tolerance (Downs et al., 1998; Malik et al., 1999). We therefore conclude that the higher *hsp23* expression plays a key role in female heat tolerance. In contrast, the expression level of *hsp70* was lower in females than in males at 44°C, but despite this female survival was higher than male survival. Dahlgaard and colleagues reported a similar finding for *D. melanogaster* (Dahlgaard et al., 1998). The exact reason for this phenomenon is not clear and further experiments are required to resolve this.

Interestingly, the survival rate of both females and males showed no significant change after feeding with *hsp90* dsRNA. This suggests that *hsp90* plays no significant role in heat tolerance. The *hsp90* gene has various roles in different species (Siriani et al., 2005; Yamada et al., 2007). While it can be associated with heat shock tolerance (Rinehart and Denlinger, 2000; Yamada et al., 2007), it has also been shown to play a role in developmental regulation. For instance, *hsp90* has been identified as a capacitor for morphological evolution in *Drosophila* (Rutherford and Lindquist, 1998) and plays a key role in organism development (Rutherford and Lindquist, 1998; Rutherford et al., 2007). More work is therefore needed to uncover the role of *hsp90* in whiteflies.

In summary, the present study shows that feeding with dsRNA is an effective method by which to study gene function. The simplicity of this approach should open the way for further studies on gene function in diverse groups of species.

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