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The small heat shock protein Hsp20.8 imparts tolerance to high temperatures in the leafminer fly, *Liriomyza trifolii* (Diptera: Agtomyzidae)

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Research Paper

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Abstract

As an environmental factor, temperature impacts the distribution of species and influences interspecific competition. The molecular chaperones encoded by small heat shock proteins (sHsps) are essential for rapid, appropriate responses to environmental stress. This study focuses on *Hsp20.8*, which encodes a temperature-responsive *sHsp* in *Liriomyza trifolii*, an insect pest that infests both agricultural and ornamental crops. *Hsp20.8* expression was highest at 39°C in *L. trifolii* pupae and adults, and expression levels were greater in pupae than in adults. Recombinant *Hsp20.8* was expressed in *Escherichia coli* and conferred a higher survival rate than the empty vector to bacterial cells exposed to heat stress. RNA interference experiments were conducted using *L. trifolii* adults and prepupae and the knockdown of *Hsp20.8* expression increased mortality in *L. trifolii* during heat stress. The results expand our understanding of *sHsp* function in *Liriomyza* spp. and the ongoing adaptation of this pest to climate change. In addition, this study is also important for predicting the distribution of invasive species and proposing new prevention and control strategies based on temperature adaptation.

Introduction

In insects, heat shock proteins (HSPs) are important contributors to temperature stress tolerance and also operate as molecular chaperones (Gehring and Wehner, 1995; Johnston *et al.*, 1998; Feder and Hofmann, 1999; Hu *et al.*, 2014). Small heat shock proteins (sHSPs) provide a level of thermoprotection and have diverse functions and structures (Gehring and Wehner, 1995; Franck *et al.*, 2004). Specifically, sHSPs are known for their chaperone activity and conserved α-crystallin domains (Basha *et al.*, 2012; Haslbeck and Vierling, 2015). They function to promote the correct folding of proteins that accumulate due to different stressors and also inhibit aggregation of proteins (Basha *et al.*, 2012; King and MacRae, 2015). sHsps have been associated with multiple physiological responses, including tolerance to thermal stress (Tsvetkova *et al.*, 2002; Sun and MacRae, 2005; Zhao and Jones, 2012).

sHSPs in insects have gained significant attention over the past few decades because of their involvement in stress tolerance, which fosters adaptation to difficult environmental conditions. The function of sHSPs differs both between and within insect during different developmental stages, physiological states and environmental conditions (Haslbeck and Vierling, 2015; King and MacRae, 2015; Jagla *et al.*, 2018). For example, *sHsp* expression profiles in *Frankliniella occidentalis* varied among different developmental stages and environmental conditions (Yuan *et al.*, 2022). *sHsps* have been studied in fruit flies (Morrow *et al.*, 2006), rice stem borers (Lu *et al.*, 2014; Pan *et al.*, 2018; Dong *et al.*, 2021) and honeybees (Liu *et al.*, 2012; Zhang *et al.*, 2014); unfortunately, their mode of action and function remain unclear. It is essential to study individual sHSPs in insects to better understand their functional diversity and their role in helping pests adapt to climate change.

The leafminer *Liriomyza trifolii* (Diptera: Agtomyzidae) is an insidious pest that causes damage to various crops on a global scale (Spencer, 1973). The larvae of *L. trifolii* produce tunnels in plant foliage, and the adult stage punctures leaves for both feeding and oviposition (Johnson *et al.*, 1983; Parrella *et al.*, 1985; Reitz *et al.*, 1999). The initial report of *L. trifolii* in mainland China occurred after an earlier invasion by other *Liriomyza* spp. (Wen *et al.*, 1996, 1998; Wang *et al.*, 2007). Temperature has a critical role in the *Liriomyza* development and distribution, and minor variations in thermotolerance can disrupt the competitive balance between related species (Kang *et al.*, 2009; Wang *et al.*, 2014a, 2014b). Numerous studies have been conducted to investigate the effects of temperature and thermally regulated interspecific competition on *Liriomyza* spp. (Reitz and Trumble, 2002; Abe and Tokumaru, 2008;

Wang *et al.*, 2014a, 2014b). Additionally, high- and low-temperature stress were shown to induce *Hsp* and *sHsp* expression in three closely related *Liriomyza* species, suggesting their role in heat stress (Huang and Kang, 2007; Chang *et al.*, 2019, 2021b).

We previously reported the occurrence of five *sHsps* in *L. trifolii* and showed that *Hsp20.8* is expressed at significantly higher levels than other *sHsp*s in adults and pupae (Chang *et al.*, 2019, 2021b). Furthermore, RNA interference (RNAi) experiments with heat shock transcription factor 1 (*Hsf1*) resulted in decreased expression of *Hsp20.8* and increased mortality under high temperatures, which suggests a role in adaptation to adverse temperatures (Chang *et al.*, 2021b). However, our understanding of sHsps in *L. trifolii* remains incomplete, and there is a lack of research on their function. This study focuses on the function of Hsp20.8 in *L. trifolii* during high-temperature stress to better understand how *Liriomyza* spp. adapt to thermal stress during climate change.

Materials and methods

Insects

In the laboratory, *L. trifolii* populations were maintained at 25 ± 1°C with a photoperiod of 16:8 h (L:D) as described previously (Chen and Kang, 2002). Bean (*Phaseolus vulgaris*) was used to rear *L. trifolii*, and leaves exhibiting tunnels were gathered for pupation. Pupae were transferred to test tubes until emergence. Both larvae and adults were reared on beans for mating and oviposition.

Heat stress and expression of Hsp20.8

Two-day-old pupae and newly emerged adults were treated with temperatures ranging from 35 to 43°C for 2 h, frozen in liquid nitrogen and stored at −80°C. Controls were incubated at 25°C. The experiment was repeated three times.

The RNA-easy Isolation Reagent (Vazyme, China, #R701) was utilised to isolate RNA from *L. trifolii*, and RNA quality was measured as described (Chang *et al.*, 2019). Total RNA (0.5 µg) was used for reverse transcription in 20 µl volumes as described (Chang *et al.*, 2019). The experiment was performed with triplicate samples, and primers are listed in table 1.

Heterologous expression and validation of recombinant Hsp20.8

The *Hsp20.8* ORF in *L. trifolii* was amplified using primers that incorporated *Eco*RI and *Xho*I sites (table 1). After PCR and *Eco*RI/*Xho*I digestion, the amplification product was ligated into pET-28a (Novagen, Beijing, China) and transformed into *Escherichia coli* BL21(DE3) competent cells. Transformants were cultured in Luria–Bertani (LB) broth with 50 mg l^{-1} kanamycin and incubated at 37°C with agitation at 200 rpm. When the cultures reached an absorbance of 0.5–0.8 at 600 nm, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM. Cultures were then incubated an additional 24 h at 16°C with agitation at 200 rpm. Bacteria cells were pelleted by centrifugation at 6000 × ***g*** for 10 min at 4°C, suspended in lysis buffer (10 mM imidazole, NaCl, $NaH_2PO_4.2H_2O$, pH 7.9–8.1), and sonicated (Sonics, CT, USA). Lysates were centrifuged at 18,000 × ***g*** for 15 min at 4°C, and the collected supernatants were then applied to Ni-NTA Sefinose^{TM} resin as recommended by the manufacturer (Sangon Biotech, Shanghai, China). Proteins were separated by electrophoresis in 12% (v/v) SDS-polyacrylamide gels.

For western blots, proteins were transferred to polyvinylidene difluoride membranes and incubated with anti-His·tag rabbit antiserum horseradish peroxidase-conjugated goat anti-rabbit IgG (1:4000; Sangon Biotech) as described (Dong *et al.*, 2022). Chemiluminescence signals were detected with the ECL western blot kit (Bio-Rad, CA, USA).

Bacterial survival assays

Ten millilitres of *E. coli* BL21 cells harbouring pET28a-LtHsp20.8 or the empty vector pET28a were cultured at 200 rpm in LB medium at 37°C. When the cells reached OD_{600} = 0.3, 0.5 mM IPTG was added and bacteria cultivated at 45°C for 6 h; the OD_{600} was measured hourly. Cells were then diluted 2000-fold

Table 1. Primers used in recombinant protein amplification, dsRNA synthesis, and real-time quantitative PCR

Primer name		Primer sequences (5′→3′)	Length (bp)	Tm (°C)
Primer for recombinant protein amplification				
Hsp20.8-EcoRI	F	CCGGAATTCATGGCAGTAATTCCATTATT	547	58.5
Hsp20.8-Xhol	R	CCGCTCGAGTTAACTTAGGCTTTCTTCTT		
Primers for dsRNA synthesis				
dsHsp20.8	F	**TAATACGACTCACTATAGGG**AGCAAAGTTTTTACAAGTGT	421	60.5
	R	**TAATACGACTCACTATAGGG**TCCACATCTTTAGGAATCTT		
dsGFP	F	**TAATACGACTCACTATAGGG**CCTCGTGACCACCCTGACCTAC	314	68.2
	R	**TAATACGACTCACTATAGGG**CACCTTGATGCCGTTCTTCTGC		
Primers for RT-qPCR				
qPCR-Hsp20.8	F	ATGGTATGTTGACCTTACGGGC	83	57.6
	R	GTCTGCTCAATGGGGACTACAC		
qPCR-Actin	F	TTGTATTGGACTCTGGTGACGG	73	59.2
	R	GATAGCGTGAGGCAAAGCATAA		

EcoRI and Xhol restriction enzyme sites are underlined; the T7 promoter sequences are in bold.

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in fresh LB, and 200 µl aliquots were distributed to LB agar containing 50 mg l^{-1} kanamycin. Bacteria were incubated at 37°C overnight and colony-forming units (CFUs) were recorded. The experiments were repeated three times.

dsRNA synthesis and RNAi

Small interfering RNA sequences were identified in the *LtHsp20.8* sequence with siDirect v. 2.0 (http://sidirect2.rnai.jp/) and used for designing dsRNA primers. A T7 promoter sequence (TAATACGACTCACTATAGGG) was integrated into to the 5′ ends of primers to facilitate transcription from sense and antisense cDNA strands. A dsRNA specific for green fluorescence protein (GFP) was included as a control (table 1). Synthesis of dsRNA was accomplished using purified DNA template (1.5 µg), and the products were purified using the Transcript Aid T7 High Yield Transcription Kit (Thermo, USA, #K0441). Gel electrophoresis and spectrophotometry were used to evaluate the quality and quantity of dsRNA.

To prepare insects for microinjection, adults were anaesthetised with CO_2 and a Nanoliter Injector (WPI, FL, USA) was used to deliver a 5 nl aliquot (50 ng) of dsHsp20.8 into *L. trifolii*. The insects were supplied with a honey/water solution and dead insects were removed as needed (Chang *et al.*, 2021a).

Newly emerged prepupae collected from leaf tissue were also used in RNAi experiments. Prepupae were immersed for 30 s in a solution containing 1% RNATransMate (Sangon Biotech) and 500 ng μl^{-1} dsHsp20.8 or dsGFP (control). Excess solution containing dsRNA was removed using a soft brush to prevent blockage of stomata and potential interference with pupation (Chang *et al.*, 2022). Leaf samples were collected, RNA was extracted and the efficiency of silencing was analysed by qPCR.

To measure silencing efficiency, adults and pupae were exposed to 39°C for 2 h. The efficiency of dsRNA silencing was then measured at 24 h post-injection (adults) and 48 h post-immersion (pupae) by qRT-PCR; primers are shown in table 1. Additionally, survival rates were determined for treatments containing ten injected adults and ten immersed pupae. The numbers of viable adults and eclosed pupae were tallied after exposure to 39°C for 2 h. Treatments were repeated four times.

Statistical analyses

Hsp20.8 expression was determined at different temperatures using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and *Actin* was used as a reference gene (Chang *et al.*, 2017b). One-way ANOVA (Tukey's multiple comparison) was used to identify significant differences among temperature treatments. The Student's *t* test was utilised to identify differences in OD_{600} values and CFUs in Hsp20.8 and the control, and the relative abundance of survival rates and target genes and were compared to the dsGFP control. SPSS v. 16.0 (SPSS, Chicago, IL, USA) was used to transform data for homogeneity of variances. Differences were considered statistically significant at $P < 0.05$.

Results

Hsp20.8 expression during heat stress

Expression of *Hsp20.8* was evaluated in *L. trifolii* pupae and adults during heat stress. Results showed that *Hsp20.8* expression was significantly higher in temperatures ranging from 35 to 43°C in both development stages as compared to the control at 25°C (pupae: $F_{5,12} = 163.706$, $P < 0.05$; adults: $F_{5,12} = 64.948$, $P < 0.05$). Expression of *Hsp20.8* was highest at 39°C; at this time point, transcript levels were 38.72- and 14.35-fold greater than the control in pupae and adults, respectively (fig. 1). In general, *Hsp20.8* expression was significantly higher in pupae as compared to adults.

Protective effects of LtHSP20.8 against heat stress

The function of LtHSP20.8 was investigated in *E. coli* by generating a recombinant protein fused with a 6 × His·tag (fig. 2a). Western blot analysis revealed the presence of LtHSP20.8 at approximately 20–25 kDa (fig. 2b). Survival assays after exposure to 45°C were conducted with *E. coli* cells containing LtHsp20.8 and the empty vector. Cells that were overproducing LtHspP20.8 exhibited significantly higher tolerance to elevated temperatures as compared to the control cells. The OD_{600} values of *E. coli* containing *LtHsp20.8* began to increase significantly after 4 h of heat shock as compared to control cells ($t_{4h} = -3.283$, $P < 0.05$; $t_{5h} = -3.916$, $P < 0.05$; $t_{6h} = -4.984$, $P < 0.05$), whereas the OD_{600} values of the control group remained unchanged (fig. 3a). The viability of *E. coli* cells subjected to high-temperature stress was determined by counting CFUs after exposure to thermal stress. Survival rates of the *E. coli* control decreased significantly after heat stress, whereas cells that overexpressed *LtHsp20.8* exhibited higher survival rates ($t = -6.625$, $P < 0.05$) (fig. 3c).

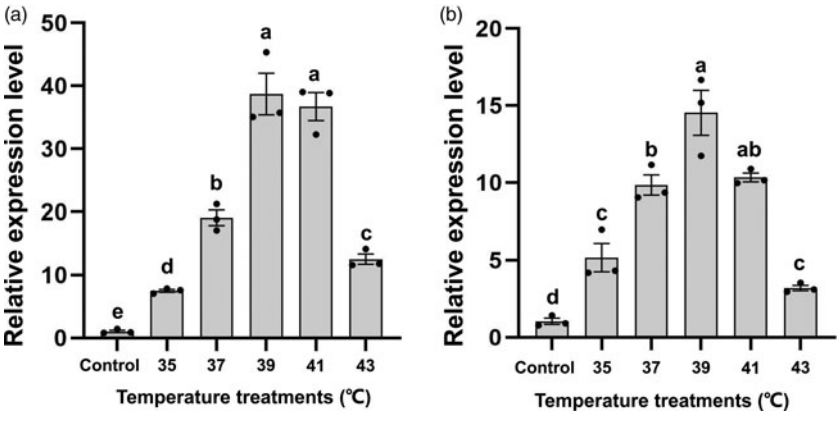

Figure 1. Relative expression levels of *LtHsp20.8* under high-temperature treatments. The relative level of *Hsp* expression represented the fold increase as compared with the expression in controls. (a) Relative expression levels for pupae; (b) relative expression levels for adults. The data were denoted as mean ± SE. One-way analysis of variance (ANOVA) was used to analyse the relative expression levels of *Hsp20.8* under high-temperature treatments. For the ANOVA, data were tested for homogeneity of variances and normality. Different lowercase letters indicate significant differences among different temperature treatments. Tukey's multiple range test was used for pairwise comparison for mean separation ($P < 0.05$).

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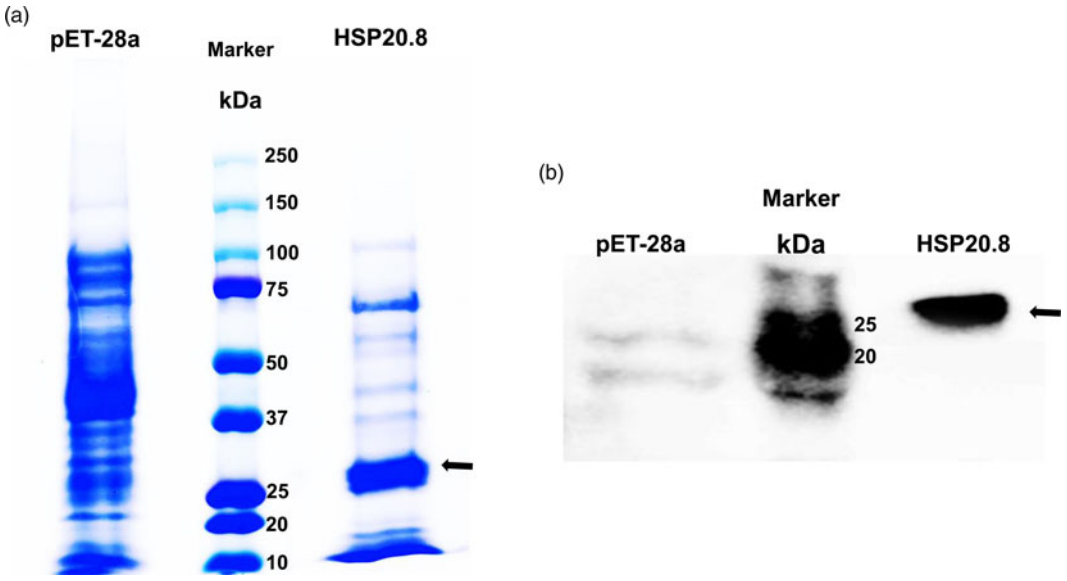

Figure 2. Recombinant *Lt*HSP20.8 protein was (a) heterologously expressed in *Escherichia coli* and (b) verified by Western blotting. 'pET-28a' represents the empty vector. The arrowheads indicate the position of *Lt*HSP20.8.

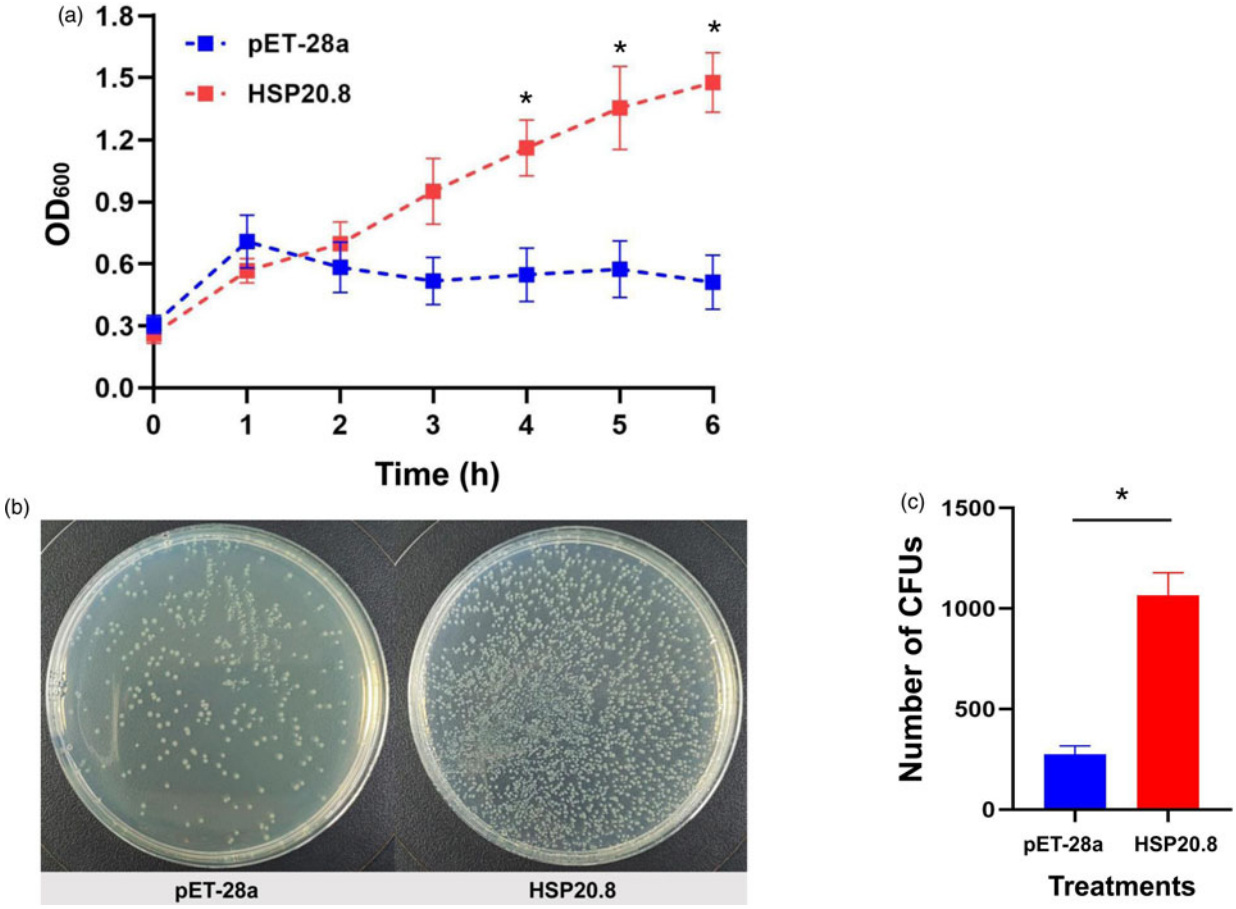

Figure 3. *Lt*HSP20.8 displays protective effect against heat stress. *Escherichia coli* cells containing pET-28a-*Lt*HSP20.8 plasmid and pET-28a empty vector were grown in medium under heat stress (45°C). (a) The growth curve of the bacteria was recorded every hour. (b) After a 6 h culture under heat stress, *E. coli* cells with or without overexpressing *Lt*HSP20.8 were spread on LB agar plates, and grown at 37°C overnight. (c) The number of bacteria colony-forming units (CFUs) on the LB agar plates were counted. Data are means ± SE. '*' denotes a significant difference between two groups (Student's *t* test, $P < 0.05$).

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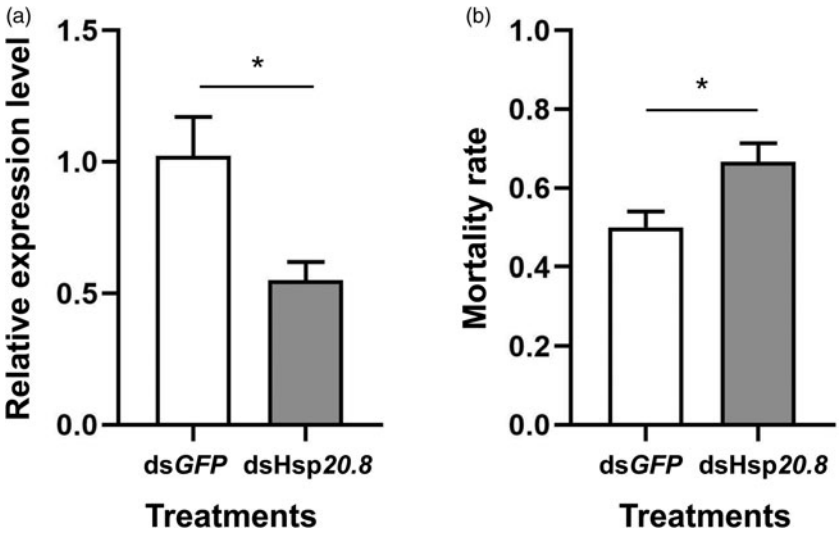

Figure 4. Knockdown of *LtHsp20.8* by RNA interference and its effect on heat tolerance in adult stage. (a) Injection of dsRNA of *LtHsp20.8* significantly reduced the expression level of *LtHsp20.8* compared to the flies injected with dsRNA of green fluorescent protein (*dsGFP*) under heat stress. (b) Mortality of *dsLtHsp20.8*-injected and *dsGFP*-injected flies exposed to heat stress. Data are presented as means ± SE. '*' indicate a significant difference between two groups (Student's *t* test, $P < 0.05$).

Silencing Hsp20.8 decreases heat tolerance in L. trifolii

When newly emerged adults were injected with dsHsp20.8, expression of *LtHsp20.8* showed a significant decrease (55.13%) as compared to that with *dsGFP* under 39°C for 2 h treatment ($t = 2.909$, $P < 0.05$). After exposure to 39°C for 2 h, mortality significantly increased in *L. trifolii* injected with dsHsp20.8 (66.67%) as compared to dsGFP (50.00%) ($t = -2.673$, $P < 0.05$) (fig. 4).

RNAi was also performed by immersing *L. trifolii* prepupae in dsHsp20.8 and dsGFP. The expression *Hsp20.8* in *L. trifolii* decreased significantly when exposed to 500 ng μl^{-1} of dsHsp20.8, and expression levels were 66.87% of the dsGFP control ($t = 3.799$, $P < 0.05$). Furthermore, the mortality rate of *L. trifolii* pupae (69.38%) was significantly higher when immersed in dsHsp20.8 and exposed to 39°C for 2 h as compared to the dsGFP control (43.33%) ($t = -3.398$, $P < 0.05$) (fig. 5).

Discussion

This study shows that *LtHsp20.8* was significantly upregulated during high-temperature stress in *L. trifolii* adults and pupae, and the expression patterns were consistent with other *L. trifolii Hsps* (Chang *et al.*, 2017a, 2017b, 2019, 2021b). *Hsp20.8* is more highly expressed than other *sHsps* of *L. trifolii* in both pupal and adult stages (Chang *et al.*, 2019, 2021b). Previous studies have shown that a 2 h exposure results in maximal expression of *Hsps* in *L. trifolii* (Chang *et al.*, 2021b); however, research is lacking on the effects of a 2 h exposure to different temperatures. In this study, we show that *Hsp20.8* expression was highest at 39°C, and this temperature was used for functional verification. Expression of *Hsp20.8* was higher in pupae than adults, which is consistent with our research and that of the highest expression level during the pupal stage under 1 h treatment is about five times that of the adult stage (Chang *et al.*, 2019, 2021b). In nature, *L. trifolii* pupae must resist environmental stress, and it has been reported that *L. trifolii* overwinters in the pupal form (Kang *et al.*, 2009).

The production and assay of recombinant proteins is commonly used to verify protein function *in vitro* (Baneyx, 1999). For example, the role of *Hsps* in peach aphids exposed to reactive oxygen species (ROS) was investigated by overproducing recombinant Hsps in *E. coli* and exposing bacterial cells to ROS

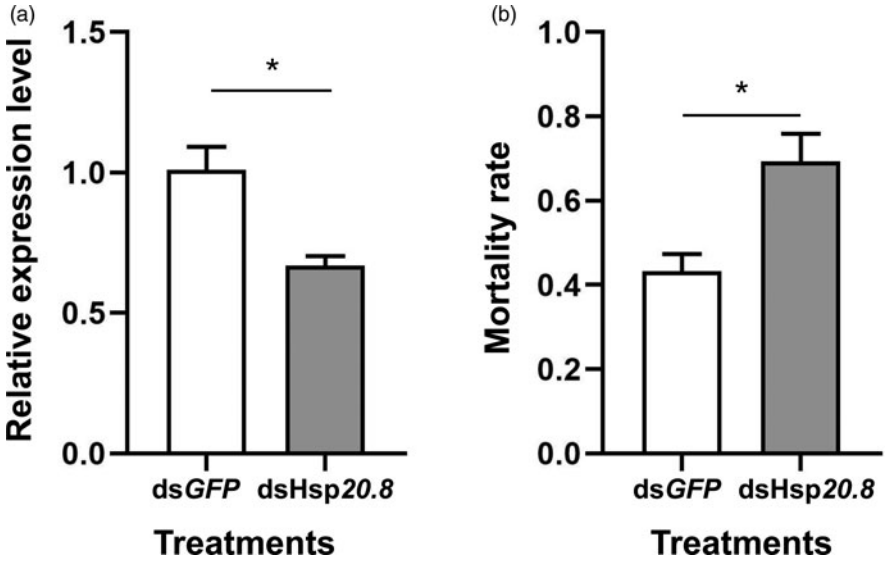

Figure 5. Knockdown of *LtHsp20.8* by RNA interference and its effect on heat tolerance in pupae stage. (a) Immersion of dsRNA of *LtHsp20.8* significantly reduced the expression level of *LtHsp20.8* compared to the flies immersed with dsRNA of green fluorescent protein (*dsGFP*) under heat stress. (b) Mortality of ds *LtHsp20.8*-immersed and *dsGFP*-immersed flies exposed to heat stress. Data are presented as means ± SE. '*' indicate a significant difference between two groups (Student's *t* test, $P < 0.05$).

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generators (Dong *et al.*, 2022). To study the role of *Accs*Hsp22.6 in *Apis cerana cerana*, the protein was overproduced in *E. coli* and disc diffusion assays were used to evaluate the protective activity of the protein during oxidative stress (Zhang *et al.*, 2014).

Several reports have documented the accumulation of sHSPs in cells, and these were shown to protect cytoplasmic proteins from damage due to thermal stress (Derocher *et al.*, 1991; Pacheco *et al.*, 2009). Furthermore, *in vitro* studies have demonstrated that several sHSPs possess the ability to prevent thermal aggregation under various conditions (Pérez-Morales *et al.*, 2009; Li *et al.*, 2012; Liu *et al.*, 2012). In this study, the induction of *LtHsp20.8* led to a higher survival rate in *E. coli* during heat stress, which is similar to results with other sHSPs. We recognise that *in vitro* results need to be combined with *in vivo* functions to verify protein function in the cell.

Using established methods for delivering dsRNA (microinjection and immersion), we obtained similar levels of interference for *Hsp20.8* during high-temperature stress. In the model species, *Caenorhabditis elegans*, soaking in a dsRNA solution was similar to results obtained with amending the diet with dsRNA; however, in both cases RNAi was less potent than delivery by microinjection (Tabara *et al.*, 1998). Although microinjection is technically more difficult and results high in mortality, the adult stage of pests is easy to collect (Chang *et al.*, 2021a). Microinjection has been widely used to evaluate gene functions in the corn planthopper, Western flower thrips and other species (Yao *et al.*, 2013; Badillo-Vargas *et al.*, 2015; Joga *et al.*, 2016). Although the soaking method is simple and has been utilised in some species as an dsRNA delivery method, it has primarily been used with larvae (Tabara *et al.*, 1998; Zhang *et al.*, 2015). The delivery of dsRNA by immersion is more effective for insect cells than intact insect bodies, and this is possibly due to physical barriers in adults such as the cuticle. In this study, we soaked prepupae of *L. trifolii* in dsRNAs. The pre-pupation period for dipteran insects is short, which makes them difficult to collect (Spencer, 1973). Even with the extra barriers present in insect bodies, the uptake of dsRNA is possible (Yu *et al.*, 2013). For example, *Ostrinia furnalalis* larvae were sprayed with a dsRNA solution, which resulted in substantial mortality (Wang *et al.*, 2011). This application method suggests that dsRNAs can penetrate the integument and elicit RNAi, which suggests that RNAi-based pest management is a possible.

In this study, *Hsp20.*8 was induced in both pupae and adults during thermal stress. The role of Hsp20.8 in the heat stress response was verified *in vitro* by expressing recombinant *Hsp20.*8 in *E. coli* and exposing cells to heat shock. Furthermore, the exposure of *L. trifolii* adults and prepupae to dsHsp20.8 increased mortality during heat stress. With respect to climate change, the adaptation of insects to thermal stress presents obstacles to preventing their dissemination. It is critical to study the adaptation pests to environmental changes, and this is particularly important for *L. trifolii*, which is adaptable to various temperatures and is highly invasive. This study expands our knowledge of sHsp function in *Liriomyza* spp. and provides theoretical guidance on the ongoing adaptation of invasive pests to global climate change.

Data availability statement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Competing interests. None.

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