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Research paper

Function of heat shock protein 70 in the thermal stress response of *Dermatophagoides farinae* and establishment of an RNA interference method

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ABSTRACT

Dermatophagoides farinae are an important mite species that cause stored product deterioration and allergic diseases. They widely breed in human habitats because of their strong tolerance to extreme external temperatures. However, mechanisms underlying the stress response and tolerance of *D. farinae* are unclear. We hypothesized that heat shock protein 70 plays an important role in the heat stress response of *D. farinae*. In this study, we determined the survival rates of *D. farinae* at high temperatures (37 °C–45 °C) by performing temperature-gradient experiments in vitro and assessed the expression level of HSP70 by performing RT-qPCR. First, we confirmed that HSP70 regulated the heat stress response of *D. farinae*, with maximum heat stress regulation observed at 41 °C. Next, we confirmed the presence of a Dicer enzyme-mediated RNA interference (RNAi) pathway in *D. farinae* by searching the NCBI database and a Dicer site prediction website. Finally, we performed RNAi in *D. farinae* by using an immersion method with screened dsHSP70 fragments. Moreover, we performed concentration-gradient experiments to determine that 600 ng/μl was the minimal effective concentration of dsHSP70 for silencing *HSP70*. These results confirm that HSP70 regulates the heat stress response of *D. farinae*. The present study is the first to report the use of the non-invasive and highly sensitive immersion method for performing RNAi in *D. farinae*. The results of the present study provide a technical foundation for performing functional gene research and for developing molecular prevention and control strategies against medically important mites.

1. Introduction

Dermatophagoides farinae, a mite species, are small free-living arthropods found worldwide and belong to class Arachnida, subclass Acari, superorder Acariformes, family Pyroglyhidae, and genus *Dermatophagoides*. *D. farinae* can highly adapt to temperature changes in their external environment and therefore extensively breed in stored products and human dwellings. These mites can pollute and destroy food grains, Chinese herbal medicines, feedstuff, etc., resulting in considerable economic losses. Moreover, the excrements, metabolites, and carcasses of these mites have strong allergenicity and cause allergic diseases such as allergic asthma, allergic dermatitis, allergic rhinitis, and allergic cardiac urticaria. Therefore, it is very important to determine mechanisms underlying the thermal stress response of *D. farinae* to effectively control these pests.

Adult *D. farinae* show strong resistance to high and low temperatures. *D. farinae* can tolerate a maximum temperature of 50 °C in water and air but are killed at temperatures above 50 °C (Arlian et al., 2002). Approximately 5% *D. farinae* can survive for 1 day at −18 °C and 15% *D. farinae* can survive for 7 days after acclimation at 2 °C (Sinha and Harasyme, 1974). Mechanisms underlying the stress response and tolerance of *D. farinae* to extreme temperatures are unclear. Previous studies have suggested that HSPs are the most important proteins that regulate biological stress response and that HSP70 is involved in inducing tolerance to various stresses (Li et al., 2009; Roy et al., 2016; Stephanou and Alahiotis, 1983; Yang et al., 2012). Therefore, we hypothesized that HSPs, especially HSP70, participated in the thermal stress response of *D. farinae*.

Since its discovery in *Caenorhabditis elegans* in 1998 (Fire et al., 1998), considerable advances have been made in RNA interference (RNAi) technology in recent years. In the present study, we used the RNAi technology to verify the important role of HSP70 in the thermal stress response of *D. farinae*. However, the application of RNAi technology in mites is still in a nascent stage. A study published in 2007 was the first to use RNAi technology to silence the Distal-less gene in *Tetranychus urticae* (Khila and Grbić, 2007). Since then, only 21 studies

Abbreviations: A, adenosine; C, cytidine; G, guanosine; T, thymidine

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have applied this technology in mites to date. RNAi methods applied in mites are still in the exploration stage. RNAi in mites can be performed using three main methods, namely, injection (Khila and Grbić, 2007; Campbell et al., 2016), immersion (Campbell et al., 2010; Marr et al., 2015; Fernando et al., 2017), and feeding methods (Lee et al., 2013; Wu and Hoy, 2014a; Wu and Hoy, 2014b; Ozawa et al., 2015; Pomerantz and Hoy, 2015; Shi et al., 2015; Tian et al., 2015; Wu and Hoy, 2015; Singh et al., 2016; Kwon et al., 2016; Xia et al., 2016; Xu et al., 2017). Moreover, target genes for RNAi are limited in mites and are mainly of two types, i.e., developmental genes such as Distal-less, HOX, and chitinase (Geng et al., 2016; Hoy et al., 2016) and detoxification enzyme genes such as P450 and glutathione transferase (Campbell et al., 2010; Marr et al., 2015; Xu et al., 2017; Shi et al., 2016). Most studies examining the use of RNAi technology in mites have silenced genes that are necessary for the normal function of mites to prevent their normal growth and development, disrupt their life cycle, and thus observe an obvious RNAi effect. A few studies have examined genes involved in drug resistance and genes expressing drug targets (Shi et al., 2015; Shi et al., 2016). However, no study has examined mechanisms regulating the thermal stress response of and RNAi in *D. farinae*.

In the present study, we determined the survival rates of *D. farinae* at high temperatures (37 °C–45 °C) by performing temperature-gradient experiments in vitro and assessed the expression level of HSP70 by performing RT-qPCR. Moreover, we determined the temperature at which HSP70 showed the maximum ability to regulate the heat stress response of *D. farinae*. Moreover, we confirmed the presence of a Dicer-mediated RNAi pathway in *D. farinae* by searching the NCBI database and predicted Dicer enzyme cleavage sites in dsHSP70 by using a Dicer enzyme prediction website. Finally, we established an immersion method for performing RNAi in *D. farinae* and determined the function of HSP70 in regulating the thermal stress response of *D. farinae* under high temperatures. The RNAi method established in the present study will provide technical support for performing additional studies on the functional genes of *D. farinae* and will lay a foundation for assessing stress response mechanisms and gene functions in medically important mites that have small bodies and are difficult to be isolated.

2. Materials and methods

2.1. RNA extraction and cDNA synthesis

2.1.1. Mite culture and collection

D. farinae were acquired from a culture maintained in the dark at a temperature of 25 °C ± 1 °C and relative humidity of 70%~75% in an artificial climate incubator at our laboratory in Xi'an, China. Next, the acquired *D. farinae* were separated and collected using a sieve vibration method.

2.1.2. RNA extraction

Approximately 500 active adult female *D. farinae* were placed in a sterile, RNase-free 15 ml centrifuge tube and were thoroughly ground in liquid nitrogen. RNA was extracted using RNeasy Micro Kit (Qiagen, Germany), according to the manufacturer's instructions. RNA concentration and purity were measured using an ultraviolet spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.1.3. cDNA synthesis

First-strand cDNA was synthesized using reverse transcriptase M-MLV (TaKaRa, Shiga, Japan), according to the manufacturer's instructions, and was stored at −20 °C.

2.2. HSP70 cloning and sequence analysis

2.2.1. Full-length HSP70 cloning

Full-length *HSP70* CDS of *D. farinae* (KM009992) was retrieved from GenBank and was used as a template. The following primers were designed and synthesized by GENEWIZ Co., Ltd. (Suzhou, China): ATGCCGTCGAAAACATTGA (forward primer) and TTAATTCACTTCTT CGACTACTGG (reverse primer). PCR was performed as follows: initial denaturation at 94 °C for 1 min; 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 15 s, and extension at 68 °C for 90 s; and final extension at 68 °C for 7 min. PCR products were separated by performing agarose gel electrophoresis on a 1.0% gel and were purified using Gel Extraction Kit (OMEGA Bio-Tek, Norcross, GA, USA). The purified *HSP70* DNA fragment was cloned into pMD19-T vector (Takara, Dalian, China) and was transformed into DH5a competent cells (Tiangen, Beijing, China). Positive clones were screened and sent to GENEWIZ Co., Ltd. for sequencing.

2.2.2. Bioinformatics analysis

The nucleotide and amino acid of HSP70 sequences of *D. farinae* were edited using DNAMAN (Lynnon, Pointe-Claire, Quebec, Canada). *HSP70* ORF was identified using ORF Finder (http://www.bioinformatics.org/sms2/orf_find. html). The molecular weight and theoretical isoelectric point (PI) of HSP70 were predicted using ExPASy (https://web.expasy.org/compute_pi/). HSP70 signal peptides were predicted using SignalP-2.0 (http://genome.cbs.dtu.dk/sevices/signalP-2.0), and HSP70 motifs, binding sites, and structural and functional domains were analyzed using PFSCAN (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html), PROSITE (http://www.expasy.org/prosite), and SMART (http://smart.embl-heidelberg.De/), respectively.

2.3. Heat stress experiments

2.3.1. Temperature-gradient experiments and survival rate calculation

Some active adult female *D. farinae* were placed in a sterile, RNase-free 1.5 ml microcentrifuge tube containing 20 µl DEPC-treated water. The tubes were placed in water baths maintained at 37 °C, 39 °C, 41 °C, 43 °C, or 45 °C (high temperature) or at 25 °C (control) for 1 h. Next, the mites were recovered by cooling the tubes to room temperature (25 °C ± 1 °C) for 1 h. Each experiment was performed in triplicate. Next, the *D. farinae* were placed on a clean glass slide and were observed under a light microscope (Motic, Xiamen, China). The number of living mites was recorded, and survival rates were calculated. The survival rates were compared using chi-square test with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA), and a P value of < 0.05 was considered to be statistically significant.

2.3.2. HSP70 expression detection

Primers against *HSP70* (forward primer: GCCAAGAATCAAGTTTC CATG; reverse primer: CTTTCCAGATTCGCTTACAAC) and reference gene α-tubulin (forward primer: CGTCTATCCAGCACCACAAG; reverse primer: CAGATGTCGTAGATGGCTTCA) were designed using Primer Premier 5.0 (Premier Biosoft Interpairs, Palo Alto, CA). RT-qPCR was performed in a 20 µl reaction mixture containing 1 µl first-strand cDNA, 10 µl SYBR Premix Ex Taq (Takara), 8 µl ddH_2O, and 0.5 µl of each primer (10 µM). Each experiment was performed in three biological replicates. PCR was performed using a real-time thermal cycler (Agilent Technologies, Santa Clara, California, USA) with the following procedure: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. A melting curve was recorded at 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. Relative expression levels were calculated using $2^{-\triangle\triangle Ct}$ method. Differences between two groups were analyzed using Student's *t*-test, with a P value of < 0.05 being considered statistically significant.

2.4. RNA interference

2.4.1. DsRNA fragments for target gene screening

Three dsHSP70 fragments of different lengths in different positions,

namely, dsHSP70-a, dsHSP70-b, and dsHSP70-c, were designed, and RNAi efficiency of these fragments was evaluated based on *D. farinae* survival rate and HSP70 expression level. The dsHSP70 fragment with the highest RNAi efficiency was selected for performing subsequent experiments.

2.4.2. RNAi pathway confirmation

Eight Ago and three Dicer genes of *D. farinae* were found in the NCBI database, thus confirming the presence of an RNAi pathway in this mite species. Next, Dicer sites in the three dsHSP70 fragments were predicted using a Dicer enzyme prediction website (http://www.gensil.com/business/products/Order2.htm).

2.4.3. Control screening

Two controls were set up simultaneously in this experiment. DEPC-treated water was used as a blank control. *EGFP* and *lacZ* were previouly reported in the RNAi of other mites. These together with the control supplied in TranscriptAid T7 High-Yield Transcription Kit (Thermo Scientific, Wilmington, DE, USA) were used as non-target controls. A suitable non-target control that exerted a minimal effect on the target gene was screened based on *D. farinae* survival rate and HSP70 expression level.

2.4.4. dsRNA preparation

Primers containing a T7 promoter region (TAATACGACTCACTAT AGGG) in both sense and antisense strands were designed for developing dsEGFP, dslacZ, and dsHSP70 fragments by using pEGFP-C3, pMD-19 T, and HSP70 as templates, respectively (Table 1). DNA fragments were recovered after PCR amplification, and dsRNAs were synthesized using the TranscriptAid T7 High Yield Transcription Kit. Transcription reactions were performed by incubating 1 μg DNA, 4 μl 5× TranscriptAid Reaction Buffer, 8 μl NTP mix (four isometrically mixed NTPs), and 2 μl TranscriptAid Enzyme Mix at 37 °C for 2 h. Next, the dsRNAs obtained were purified using hydroxybenzene–chloroform, precipitated using ethanol, and resuspended in 20 μl DEPC-treated water. Concentrations of the obtained dsRNAs were measured using an ultraviolet spectrophotometer after dilution at a ratio of 1:40. Finally, the dsRNAs were mixed with an isopycnic buffer, incubated at 70 °C for 10 min, cooled immediately on ice for 3 min, and detected by performing agarose gel electrophoresis on a 1% gel.

2.4.5. dsRNA concentration-gradient experiments

The dsRNAs were diluted with DEPC-treated water to obtain concentrations of 1200, 600, and 300 ng/μl by using a multi-proportion dilution method. Next, 30 active adult female *D. farinae* were placed in a sterile 1.5 ml microcentrifuge tube, and 20 μl dsHSP70 fragment with the highest RNAi efficiency, screened non-target control, and DEPC-treated water were added to the tube. Next, the microcentrifuge tubes were incubated at 4 °C for 24 h to induce RNAi, followed by washing three times with pre-cooled DEPC-treated water. The mites were given a heat shock treatment at an appropriate temperature, and their survival rate was calculated. HSP70 expression level after the RNAi was determined to verify gene silencing efficiency. Differences among three concentration groups of 1200, 600, and 300 ng/μl were examined with multiple comparisons by using one-way ANOVA (LSD method), and a P value of < 0.05 was considered to be statistically significant. Statistical analysis data was shown in Supplementary material.

Table 1
The dsRNA primers of *D. farinae*.

Primers	Sequences (5′-3′)	Length (position)	GC%
dsHSP70-a	F: GTTGTAAGCGAATCTGGAAAG R: GAGAACATTTTTTTCACCACC	309 (192–439)	44
dsHSP70-b	F: TGGCGGTGAAGACTTTGATA R: GGCAGCACCATAAGCGAC	439 (596–1034)	39
dsHSP70-c	F: AGATAACAACCGCCTTGGTAC R: GTCGACAGGCATTTTCTATTTC	461 (1265–1725)	40
dsEGFP	F: ATGGTGAGCAAGGGC R: TCAGTTATCTAGATCCGGT	798 (613–1410)	61
dslacZ	F: CGGTCGCCGCATACAC R: AGTTCGCCAGTTAATAGTTTGC	321 (2504–1635)	49

F: forward; R: reverse.

3. Results

3.1. HSP70 cloning, sequencing, and analysis

The full-length *HSP70* CDS was successfully amplified and sequenced and was found to contain 1965 bp (Fig. 1a). Similarity and coverage rate of this full-length *HSP70* CDS with KM009992 were 100% each. Bioinformatics analysis showed that *HSP70* encoded a polypeptide containing 654 amino acids, with a predicted molecular weight of 72.6 kDa and a theoretical PI of 5.81. The polypeptide encoded by *HSP70* contained characteristic motifs IDLGTTY, DLGGGTFD, and IVLVGG; a predicted ATP–GTP-binding site AEAYLGQK; a bipartite nuclear localization signal comprising KK and RRLRT; a potential non-organellar eukaryotic consensus motif RARFEEL; and a C-terminal region EEVN (Fig. 1b).

3.2. Heat stress experiments

The survival rates of *D. farinae* were inversely correlated with temperature (Fig. 2a). The survival rates of *D. farinae* at temperatures 37 °C, 39 °C, 41 °C, 43 °C, and 45 °C were 92.54%, 86.67%, 80.77%, 72.73%, and 51.36%, respectively. HSP70 showed the highest expression at 41 °C (Fig. 2b), which was statistically different from that in the other five temperature groups ($P < 0.05$). The relatively high survival rate of *D. farinae* at 41 °C, which provided sufficient number of living mites for RNA extraction, indicated that the stress response function of HSP70 was the most obvious at 41 °C. Therefore, 41 °C was chosen as the optimal temperature for detecting gene silencing efficiency after RNAi.

3.3. RNA interference

3.3.1. dsRNA preparation

After confirming that *D. farinae* HSP70 had abundant Dicer sites, the three dsHSP70 fragments were successfully amplified and electrophoresed (Fig. 3a). The dsRNA bands of three non-target controls EGFP, LacZ and control supplied in kit (Fig. 3b) and the three dsHSP70 fragments (Fig. 3c) were single and clear, indicating their successful transcription and purification and no degradation.

3.3.2. dsHSP70 fragment screening

Compared with those of *D. farinae* treated with DEPC-treated water, the survival rates and HSP70 expression levels of *D. farinae* under heat stress at 41 °C for 1 h both decreased significantly after being treated with three fragments of dsHSP70 ($P < 0.05$; $P < 0.05$) (Table 2 and Fig. 4). However, no difference was observed in the survival rates of *D. farinae* treated with the three dsHSP70 fragments ($P > 0.05$). In view of dsHSP70-a was associated with a relatively lower survival rate and lower HSP70 expression level than dsHSP70-b and dsHSP70-c, it was considered to exert a better RNAi effect and was selected for performing subsequent RNAi experiments.

3.3.3. Non-target control screening

No difference was observed in the survival rate of *D. farinae* treated with the three non-target controls and those treated with DEPC-treated water ($X^2 = 1.157$, $P = 0.763$, $P > 0.05$; Table 3). However, HSP70 expression levels were different between *D. farinae* treated with each of the three the non-target controls and DEPC-treated water (Student's *t*-test, $P < 0.05$; Fig. 5a–b). Because the difference in HSP70 expression

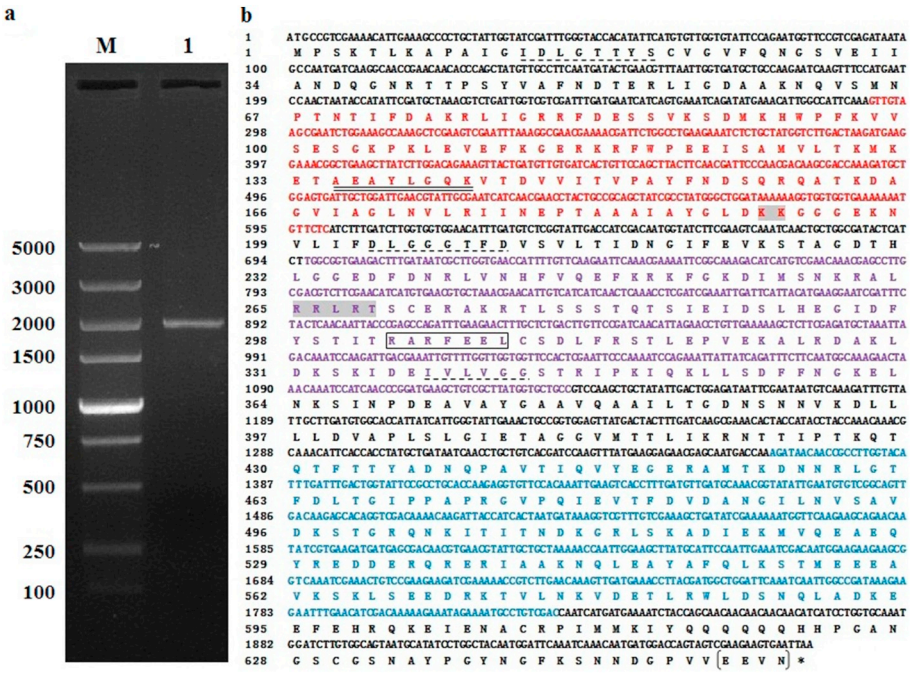

Fig. 1. (a) Electrophoretogram of PCR amplification of *D. farinae* HSP70. Lane M, DL5000 DNA ladder marker; Lane 1, PCR products of HSP70 CDS. (b) The cDNA and deduced amino acid sequence of HSP70 of *D. farinae*. The numbers on the lef are for the positons of nucleotdes and amino acids in the sequences. The characteristic motifs are underlined with dots; the putative ATP-GTP binding sites are double underlined; a putative bipartite nuclear localization signal is shaded in grey; the potential non-organellar eukaryotic consensus motif is boxed; the cytoplasmic HSP70 carboxyl terminal region is shown in a parenthesis; and the stop codon is indicated with an asterisk.

levels between *D. farinae* treated with dslacZ and those treated with DEPC-treated water was minimal and because dslacZ exerted a relatively small non-target effect, dslacZ was used as the non-target control in subsequent experiments.

3.3.4. Concentration-gradient experiments

For this, dslacZ was double diluted to obtain 1200, 600, and 300 ng/μl dslacZ. The survival rates and HSP70 expression levels were not statistically different among *D. farinae* treated with the three concentrations of dslacZ ($X^2 = 1.466$, $P = 0.690$; ANOVA, $P = 0.9777$; Table 4 and Fig. 5c–d). Therefore, 1200 ng/μl was used as the concentration of the non-target control dslacZ.

The survival rates of *D. farinae* treated with 1200, 600, and 300 ng/μl dsHSP70-a were significantly different from those of *D. farinae* treated with 1200 ng/μl dslacZ ($P < 0.05$; Table 4), indicating that the three dsHSP70-a concentrations effectively silenced *HSP70*. The survival rates of *D. farinae* treated with 1200 ng/μl dsHSP70-a was significantly different from those of *D. farinae* treated with 600 ng/μl and 300 ng/μl dsHSP70-a ($P < 0.05$), while no difference was observed in the survival rate of *D. farinae* treated with 600 ng/μl and 300 ng/μl dsHSP70-a ($P > 0.05$).

Results of RT-qPCR showed that HSP70 expression levels in *D. farinae* treated with 1200 and 600 ng/μl dsHSP70-a were statistically different from those in *D. farinae* treated with the non-target control dslacZ (Student's *t*-test, $P < 0.05$). However, HSP70 expression level in *D. farinae* treated with 300 ng/μl dsHSP70-a was not statistically different from that in *D. farinae* treated with dslacZ (Student's *t*-test, $P > 0.05$; Fig. 6), indicating that both 1200 and 600 ng/μl dsHSP70-a effectively induced RNAi in *D. farinae*. Because no statistical difference was observed in HSP70 expression levels in *D. farinae* treated with 1200 and 600 ng/μl dsHSP70-a, 600 ng/μl was suggested as the lowest effective concentration of dsHSP70 for inducing RNAi.

4. Discussion

The present study is the first to confirm that HSP70 plays an important role in the heat stress response of *D. farinae* and to successfully establish a non-invasive RNAi method (immersion method) for use in *D. farinae*. The experimental design used in the present study has six highlights. First, the Dicer sites in *D. farinae* HSP70 were predicted to ensure the feasibility of the experiment. Second, *D. farinae* survival rate and HSP70 expression level were simultaneously determined to assess the effectiveness of RNAi based on phenotypic change and gene expression level. Third, the three dsHSP70 fragments were generated to ensure RNAi efficiency. Fourth, the three non-target controls and blank control (DEPC-treated water) were established to objectively determine the RNAi efficiency. Fifth, the concentration-gradient experiments were performed to select the lowest effective dsHSP70 concentration and to improve RNAi efficiency. Sixth, three technical and five biological replicates were established for each experimental group to ensure the reliability of the experiments. Thus, the RNAi method established in the present study for use in *D. farinae* is scientifically credible and can be used as a foundation for future research on the functional genes of mites.

The prerequisite for establishing an RNAi method is to ensure the presence of an RNAi pathway in *D. farinae*. At the beginning of this

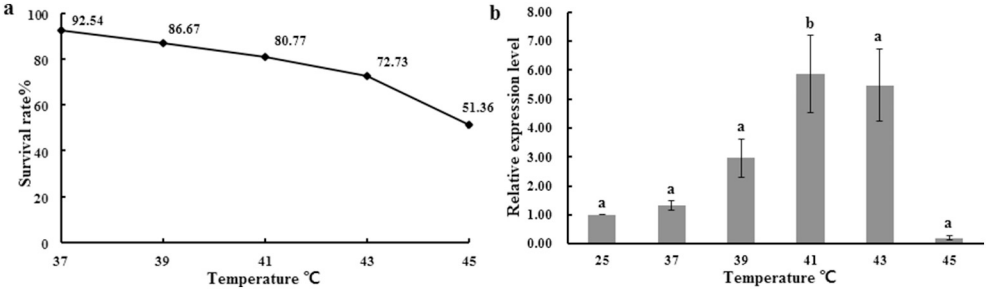

Fig. 2. (a) Survival rates of *D. farinae* after heat shock for 1 h and recover at 25 ± 1 ℃. (b) Relative expression level of HSP70 at high temperatures. Error bars represent the standard error of the calculated mean based on three biological replicates. Different letters on the error bars show significant difference according to *t*-test ($P < 0.05$). No statistical difference between "a" and "a"; significant difference between "a" and "b".

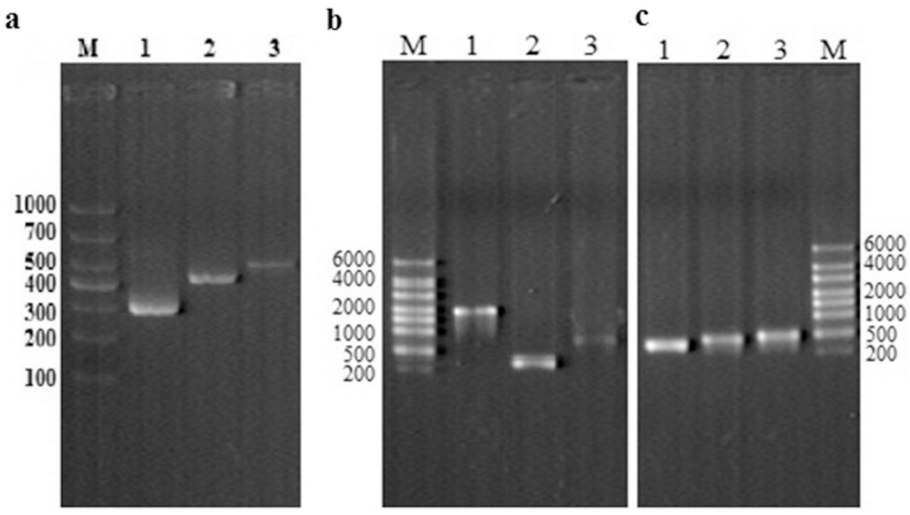

Fig. 3. (a) Electrophoretogram of PCR amplification of three HSP70 DNA fragments. Lane M, DL1000 DNA ladder marker; Lane 1, HSP70-a; Lane 2, HSP70-b; Lane 3, HSP70-c; (b) Electrophoretogram of transcription products of non-target genes. Lane M, RNA ladder; Lane 1, dsControl; Lane 2, dslacZ; Lane 3, dsEGFP; (c) Electrophoretogram of transcription products of HSP70. Lane M, RNA ladder; Lane 1, dsHSP70-a; Lane 2, dsHSP70-b; Lane 3, dsHSP70-c.

study in 2017, the establishment of an RNAi method to verify the role of HSP70 in the heat stress response of *D. farinae* was met with two major challenges. First, it was unclear whether an RNAi pathway existed in *D. farinae*. To overcome this, we searched the NCBI database and found that *D. farinae* had eight Ago (KY794591–KY794598) and three Dicer (KY794588–KY794590) genes, thus confirming the presence of an RNAi pathway in *D. farinae*. Next, we predicted Dicer sites in the three dsHSP70 fragments by using the Dicer site prediction website and found that the three dsHSP70 fragments were rich in Dicer sites. Based on these results, we established an RNAi method to verify the role of HSP70 in regulating the heat stress response of *D. farinae*. A recently published study has also shown that the RNAi pathway of *D. farinae* is mediated by Ago and Dicer genes (Mondal et al., 2018), which is consistent with the search results obtained in the present study. Second, although HSPs involved in the thermal stress response of mites have been reported, most studies have verified HSP expression levels by performing RT-qPCR (Shim et al., 2006; Chen et al., 2015; Xu et al., 2016; Lu et al., 2017). Only one study has used an RNAi method to examine the function of HSP90 under different temperatures and in the presence of different avermectin concentrations in *Panonychus citri*. This was the first study on thermal stress in mites validated by RNAi and filled an important knowledge gap in this field of research; therefore, it is of profound significance. However, the design of this study had two obvious limitations. First, the three selected temperatures of 18 °C, 25 °C, and 32 °C were very moderate to determine the effect of heat stress on HSP90. Second, simultaneous treatment with different temperatures and ivermectin may have exerted overlapping or antagonistic effects, thus impairing the credibility of the conclusion that HSP90 regulated heat stress response of *P. citri*.

Therefore, three improvements were made to the design of the present study. First, HSPs are a large gene family that regulate thermal stress response and include six subfamilies, namely, HSP110, HSP90, HSP70, HSP60, HSP40, and sHSP. Because HSP70 is the most reported gene involved in thermal stress response, it was selected for the first time as the target gene for performing RNAi in the present study. Second, changes in *D. farinae* survival rate and HSP70 expression level were determined in vitro by performing the temperature-gradient experiments. The temperature that exerted the most obvious regulatory effect on the heat stress response of *D. farinae* was determined to ensure the maximum stress response of HSP70 and a more obvious RNAi effect. Third, high temperature was the only factor in the study design to prevent the interaction of multiple factors. Results of the in vitro temperature-gradient experiments performed at 37 °C~45 °C showed that HSP70 expression was upregulated at 37 °C, 39 °C, and 41 °C and that HSP70 expression increased with an increase in temperature. Moreover, we observed that HSP70 expression decreased at 43 °C and was lost at 45 °C. These results validated our hypothesis that HSP70 is involved in the heat stress response of *D. farinae* and confirmed that HSP70 expression is the highest at 41 °C. Moreover, our results suggested that the heat stress mechanism of *D. farinae* was completely activated at 41 °C, which is the optimal temperature for verifying RNAi effect. The survival rate of *D. farinae* was relatively high (80.77%) at 41 °C, which provided the required number of living mites for subsequent RNA extraction.

4.1. Selection of the RNAi method

Feeding, injection, and immersion are the three methods for performing RNAi in mites. The feeding method and the injection method are the most widely used and efficient RNAi method, respectively, both mainly used for inducing RNAi in agricultural mites with a larger body, such as *Tetranychus* and *Varroa* mites. Although the immersion method of RNAi is less frequently used compared with the other two methods, it has been used for inducing RNAi in *Varroa* mites and medical mites such as *D. pteronyssinus* and *Sarcoptes scabiei*. So in this study, the immersion method, in which exogenous dsRNA was mainly uptaked through mouthparts and epidermis (Marr et al., 2015; Fernando et al., 2017; Mondal et al., 2018), was selected for inducing RNAi of HSP70 in *D. farinae*, and achieved satisfactory interference effects, suggesting that the immersion method was feasible for inducing RNAi in *D. farinae*. It needed to be pointed out that compared with the 100% survival rate of *S. scabiei* after immersion in 2500 ng/μl dsGST for 24 h at 4 °C (Fernando et al., 2017), the survival rates of *D. farinae* were lower, with

Table 2
The survival rates % of *D. farinae*.

	con	mites	RNAi	41 °C for 1 h	Total
DEPC	0	240	87.92% (211/240)	74.41% (157/211)	65.42% (157/240)
dsHSP70-a	1200	120	37.50% (45/120)	44.44% (20/45)	16.67% (20/120)
dsHSP70-b	1200	120	40.00% (48/120)	47.92% (23/48)	19.17% (23/120)
dsHSP70-c	1200	120	37.50% (45/120)	48.89% (22/45)	18.33% (22/120)

con: concentration, ng/μl.

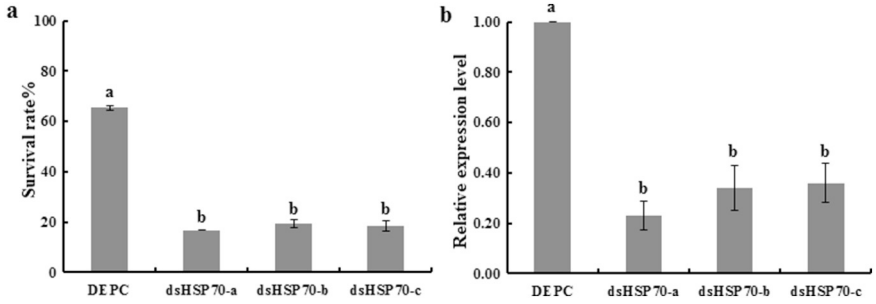

Fig. 4. Survival rates of *D. farinae* (a) and Relative expression level of HSP70 (b) after immersion in three dsHSP70 fragments and heat shock at 41 °C for 1 h. Error bars represent the standard deviation of the calculated mean based on three biological replicates. Different letters on the error bars show significant difference according to statistic analysis ($P < 0.05$). i.e. Significant difference between "a" and "b", between "b" and "c", between "c" and "d", between "a" and "d".

Table 3
The survival rates of *D. farinae*.

	con	mites	RNAi	41 °C for 1 h	Total
DEPC	0	240	87.92% (211/240)	74.41% (157/211)	65.42% (157/240)
dsControl	1200	120	81.67% (98/120)	73.47% (72/98)	60.00% (72/120)
dslacZ	1200	120	85.00% (102/120)	74.50% (76/102)	63.33% (76/120)
dsEGFP	1200	120	83.33% (100/120)	73.00% (73/100)	60.83% (73/120)

con: concentration, ng/μl.

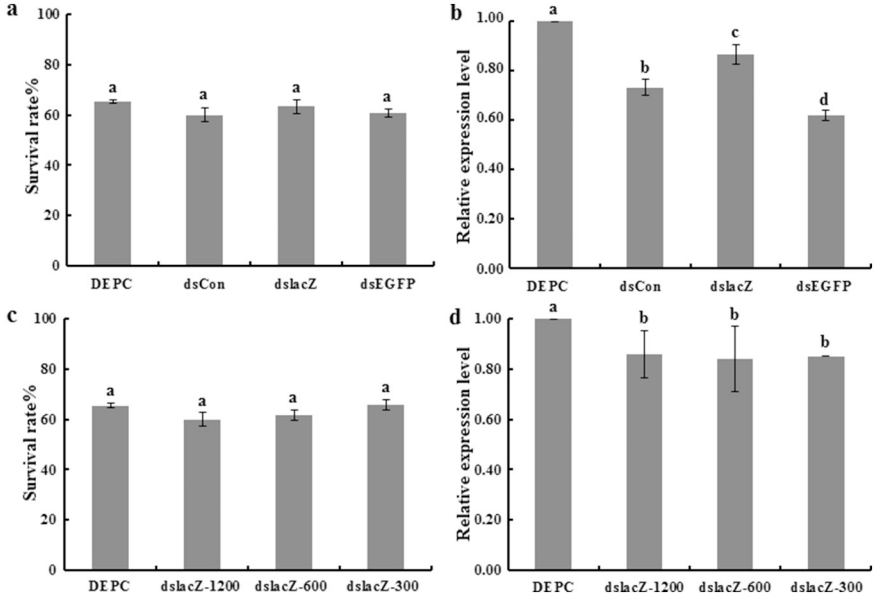

Fig. 5. Survival rates of *D. farinae* (a) and Relative expression level of HSP70 (b) after immersion in three non-target controls and heat shock at 41 °C for 1 h. Survival rates of *D. farinae* (c) and Relative expression level of HSP70 (d) after immersion in dslacZ of different concentrations and heat shock at 41 °C for 1 h.

Table 4
The survival rates % of *D. farinae*.

	con	mites	RNAi	41 °C for 1 h	Total
DEPC	0	240	87.92% (211/240)	74.41% (157/211)	65.42% (157/240)
	1200	120	81.67% (98/120)	73.47% (72/98)	60.00% (72/120)
dslacZ	600	120	83.33% (100/120)	74.00% (74/100)	61.67% (74/120)
	300	120	85.83% (103/120)	76.70% (79/103)	65.83% (79/120)
	1200	120	33.33% (40/120)	52.50% (21/40)	17.50% (21/120)
dsHSP70-a	600	120	56.67% (68/120)	69.12% (47/68)	39.17% (47/120)
	300	120	60.83% (73/120)	75.34% (55/73)	45.83% (55/120)

con: concentration, ng/μl.

only 33.33% after immersion in 1200 ng/μl dsHSP70-a and 87.92% after immersion in DEPC-treated water. This result may be influenced by two factors. First, *GST* is involved in the detoxification metabolism of *S. scabiei*; in contrast, *HSP70* is involved in stress response as well as normal growth metabolism of *D. farinae*. Second, the habitats of *S. scabiei* and *D. farinae* are different; therefore, these mites may show

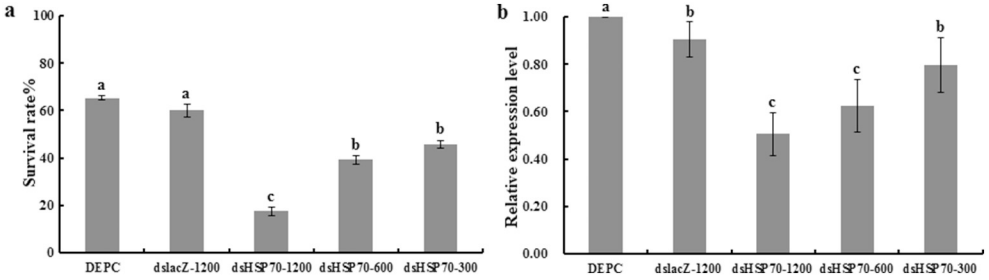

Fig. 6. Survival rates of *D. farinae* (a) and Relative expression level of HSP70 (b) after immersion in dsHSP70-a of different concentrations and heat shock at 41 °C for 1 h.

different tolerance levels in liquid media.

4.2. dsRNA fragment screening

To obtain the dsRNA fragment with best efficiency to silence *HSP70* expression, three dsHSP70 fragments, namely, HSP70-a, HSP70-b, and HSP70-c, with different lengths at different positions were designed. These three dsHSP70 fragments showed good RNAi efficiency, with no statistical difference. This may be because these fragments were strictly in accordance with the study design. First, the initial positions of HSP70-a, HSP70-b, and HSP70-c at 192, 596, and 1265 bp, respectively, downstream of the *HSP70* initiation codon were after the required 75–100 bp sequence. Second, the GC% content of HSP70-a, HSP70-b, and HSP70-c was 40%, 39%, and 40%, respectively, which was within the range of 40%~60%. Third, the lengths of HSP70-a, HSP70-b, and HSP70-c were 309, 439, and 461 bp, respectively, which were within the required length of 200–1000 bp. Finally, HSP70-a was selected as the target fragment for performing subsequent RNAi experiments because its GC% content and length were better than those of HSP70-b and HSP70-c and because its RNAi efficiency (77%) was higher than that of HSP70-b (66%) and HSP70-c (64%).

4.3. Control selection

This study used a blank control and non-target controls. These controls were designed to rule out the effects of water and dsRNA on mites. Selection of an appropriate non-target control in RNAi experiments is important to improve the reliability of gene silencing results. Refer to the literatures of RNAi in mites (Campbell et al., 2010; Campbell et al., 2016; Marr et al., 2015; Fernando et al., 2017; Ozawa et al., 2015; Wu and Hoy, 2014a, 2014b; Pomerantz and Hoy, 2015; Shi et al., 2015; Tian et al., 2015; Wu and Hoy, 2015; Singh et al., 2016; Kwon et al., 2016; Xia et al., 2016; Shi et al., 2016; Xu et al., 2017; Mondal et al., 2018), DEPC-treated water was selected as the blank control, and dslacZ, dsEGFP, and the control provided in the kit were selected as the non-target controls in this study. The three non-target controls exerted a slight effect on HSP70 expression relative to the blank control. However, dslacZ had the least effect on HSP70 expression; therefore, it was used as the non-target control for performing subsequent RNAi experiments in *D. farinae*.

4.4. Confirmation of the minimal effective dsRNA concentration for effective gene silencing

The immersion method used for performing RNAi in mites generally involves the use of 2500 ng/μl dsRNA. Because non-target dsRNAs exert some effect on the survival rate of *D. farinae* and because dsRNA concentration is negatively correlated with the survival rate of *D. farina*, we performed dsRNA concentration-gradient experiments to screen the lowest effective dsRNA concentration for inducing an RNAi effect, to prevent the influence of dsRNA on RNAi effect, and to decrease the cost of synthesizing high dsRNA concentrations. The RNAi efficiency of dsHSP70-a was 36% at 600 ng/μl concentration and 45% at 1200 ng/μl concentration, without any statistical difference. However, the RNAi efficiency of 600 ng/μl dsHSP70-a (36%) was significantly higher than that of 300 ng/μl dsHSP70-a (24%). Therefore, 600 ng/μl was confirmed to be the minimal effective dsRNA concentration for inducing an RNAi effect. The RNAi sensitivity reported in the present study was > 4-fold higher than that reported in previous studies.

5. Conclusion

The present study is the first to establish a simple, efficient, and non-invasive immersion method for performing RNAi in *D. farinae*. The results of the present study showed that HSP70 was involved in regulating the heat stress response and in maintaining the normal activities of *D. farinae*. The present study lays a foundation for the application of RNAi and the determination of gene functions in *D. farinae* in the future and provides technical support for promoting functional research on the molecular control of medically important mites.

Conflicts of financial interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2019.04.032.

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