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Oral ingestion of heat shock protein 70 dsRNA is lethal under normal and thermal stress conditions in the sweetpotato whitefly, *Bemisia tabaci*

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ABSTRACT

The sweetpotato whitefly, *Bemisia tabaci*, is a serious pest of various horticultural crops worldwide. Since 1990s, *B. tabaci* has been invaded into many countries due to climate change and global trades of agricultural products. Due to the rapid development of pesticide resistance, alternative techniques have been required for the control of *B. tabaci*. Here, we investigated whether oral ingestion of dsRNA induced knockdown in whiteflies. Double-stranded RNA (dsRNA) targeting heat shock protein 70 (*hsp70*) mRNA was produced from cDNA using specific primers. Adult whiteflies (less than 12 h post-eclosion) were allowed to ingest 20% (*w*/*v*) sucrose solution containing *hsp70* dsRNA in a 2-layered membrane feeding tube. Individual whiteflies ingested 16.9 ng dsRNA on an average in 24 h. Quantitative real-time PCR analysis showed that dsRNA ingestion decreased the mRNA level of *hsp70* in a dose-dependent manner, and the *hsp70* knockdown was sustained for at least 3 days. Furthermore, dsRNA-treated whiteflies showed increased mortality after 3 days of incubation at 25 °C. Mortality was prompted by heat shock but not by cold shock treatments. Our data suggest that the oral delivery of dsRNA had excellent efficacy for the RNAi treatment of whiteflies and that the expression of *hsp70* was critical for the survival of *B. tabaci*, regardless of the temperature conditions.

Introduction

The sweetpotato whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) is a species complex including at least 24 biotypes that express different ecological and physiological characteristics (De Barro et al., 2011). *B. tabaci* is one of the most serious pests of horticultural crops worldwide. *B. tabaci* infests over 600 plant species and damages plants directly by ingestion and indirectly by honeydew excretion, which promotes fungal decomposition of plants (Henneberry et al., 2002; Navas-Castillo et al., 2011). Moreover, whiteflies transmit more than 100 plant viruses, especially begomoviruses. Most notably, *B. tabaci* is the only known vector of *Tomato yellow leaf curl virus*, which extensively damages tomato cultivars worldwide (Czosnek et al., 2002).

The control of *B. tabaci* via chemical pesticides has proven to be troublesome. Recently, both the B- and Q-biotypes of *B. tabaci*, which have spread worldwide, have developed a remarkable level of resistance to neonicotinoid pesticides, such as imidacloprid, due to their overuse for the last decade in many countries (Basit et al., 2013; Gnankiné et al., 2013; Sun et al., 2013). Therefore, it is necessary to develop alternative control techniques for whitefly management, such as the use of biopesticides that target natural enemies, microbial pathogens, and insecticidal plant molecules (Stansly and Natwick, 2010). Furthermore, the recent development of molecular techniques provides great potential for developing new methods of pest management (Scott et al., 2013).

RNA interference (RNAi) naturally regulates cellular and physiological processes in insects via modulating the expression of specific genes, and artificial use of RNAi has potential applications in pest control, as well as the protection of beneficial insects such as the honey bee (Price and Gatehouse, 2008; Xue et al., 2012). Recently, RNAi-based techniques have been used in various groups of insect species. However, Scott et al. (2013) suggest that the efficiency of RNAi varies among different species, life stages, target genes, and experimental parameters such as the RNAi molecule, delivery method, and dosage chosen.

RNAi molecules such as dsRNA and siRNA can be delivered through injection, which typically shows the best efficiency, and also through

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oral ingestion and transgenesis. Particularly, the oral route is a practical delivery system for sapping pest species such as whiteflies, aphids, thrips, psyllids, and mites, most of which have a small body size (Upadhyay et al., 2011; Wuriyanghan et al., 2011). RNAi molecules can be incorporated into the diet or into vectors such as host plants and viruses engineered to express dsRNA (Scott et al., 2013).

In *B. tabaci*, the use of RNAi had firstly been evaluated using injection of dsRNA that silenced genes in a tissue-specific manner (Ghanim et al., 2007). Recently, oral ingestion of both dsRNA and siRNA has been found to be more efficient than injection in this species (Lu and Wan, 2011; Upadhyay et al., 2011). Particularly, Upadhyay et al. (2011) demonstrated the effectiveness of orally delivered RNAi against 5 different genes of whiteflies. The effect of those RNAi treatments on adult mortality ranged from 29 to 97%, and RNAi treatments against the *V-ATPase A* and *RPL9* genes produced more effective gene knockdown than did those against the *actin*, *ADP/ATP translocase*, and *tubulin* genes. *B. tabaci* is particularly amenable to RNAi treatment because the mechanisms of its siRNA machinery are relatively well understood (Upadhyay et al., 2013). Furthermore, transgenic tobacco plants expressing dsRNA of *V-ATPase* showed highly increased resistance against whiteflies (Thakur et al., 2014).

Here we determined the effect of knocking down *hsp70* by oral ingestion of specific dsRNA on the vitality of adult *B. tabaci* using a simple 2-layered membrane feeding chamber.

Materials and methods

Insects

The colony of Q-biotype *B. tabaci* was maintained on tomato plants (*Lycopersicon esculentum* Mill.) within insect-proof net cages ($45 \times 60 \times 90$ cm^3) in an insect-rearing room under conditions of 25 ± 1 °C, 60 ± 5% relative humidity, and a 16 h light/8 h dark (16L:8D) photoperiodic cycle. For the experiment, to collect newly eclosed 0-day-old adult whiteflies (less than 12 h post-eclosion), tomato leaves containing many pupae were detached from the plant in the evening and kept in a Petri dish until the following morning, when newly emerged adults were collected using a fine glass sucking tube and released into a Parafilm feeding chamber for experiments.

Synthesis of dsRNA of hsp70 of B. tabaci

Total RNA was isolated from adult whiteflies aged less than 12 h using Trizol® reagent (Ambion, Austin, TX, USA). The cDNA synthesis reaction for each total RNA (2 μg) sample was completed using a Reverse Transcriptase System Kit (Applied Biosystems, Foster City, CA, USA) in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA). The PCR reaction was performed with primers that contained a T7 RNA polymerase promoter sequence for dsRNA specific to *hsp70* of *B. tabaci* (Table 2). The PCR product was run on a 1.5% low melting point agarose gel and purified using the Wizard® PCR Preps DNA Purification System (Promega, Madison, WI, USA). The PCR product was used for preparation of dsRNA using the MEGAscript® RNAi kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). The quantity of dsRNA was determined using a NanoPhotometer® (Implen, Munich, Germany). The dsRNA was diluted into 20% (*w*/*v*) sucrose solution at desired concentrations for experiments.

Oral ingestion of dsRNA using the Parafilm feeding chamber

An artificial feeding chamber was made using 2 pieces of Parafilm membrane and a glass tube (length 12 cm length, 3 cm diameter) as described by Jahan et al. (2014) with some modification. Two pieces of Parafilm membrane (2×2 cm^2) were stretched out by hand until they were each 2-fold their original length. A tube was sealed with 2 layers of membrane containing 20% (*w*/*v*) sucrose solution (200 μL) between them. One side of the tube was covered with a piece of meshed net to allow aeration. Adult whiteflies (aged less than 12 h) were released into the Parafilm chamber before covering it with a meshed net. A Parafilm sandwich was positioned into the top of the tube and the tube was incubated at 25 °C. Mortality was determined by counting the number of dead whiteflies within the tube. For RNAi experiments, various amounts (0, 2, 20, and 200 ng/μL) of dsRNA were diluted into 20% (*w*/*v*) sucrose solution. Each set of experiments was conducted 3 times under the same conditions.

The amount of sucrose solution ingested by each individual whitefly was determined. Parafilm feeding tubes were prepared with 10 μL of 20% (*w*/*v*) sucrose solution containing 0–250 ng/μL *hsp70* dsRNA, and the whiteflies ($n = 20$) were allowed to feed for 24 h at 25 °C. The amount of ingested sucrose solution was determined by drawing the remainder of the solution into 1–10 μL microcapillary calibrated pipets (Sigma-Aldrich, St. Louis, MO, USA) via capillary action.

Quantitative real-time reverse transcription (RT)-PCR

Real-time PCR primers were designed based on the sequences of each gene fragment (Table 1). PCR amplifications were performed in a 25 μL reaction volume containing 0.2 μL cDNAs as a template, 0.2 μL of gene-specific primers at a concentration of 80 nM, 11.9 μL of distilled water, and 12.5 μL of Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The amplified signals were monitored continuously with the 7300 Sequence Detection System (Applied Biosystems). The amplification protocol was as follows: 1 cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 35 s, and then 1 final cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s to dissociate DNA duplexes into single strands. Threshold cycle (Ct) values were used to calculate the relative quantities of *hsp70* and *actin*, and *hsp70* mRNA levels were normalized to those of *actin* in the same samples.

Statistical analysis

Analysis of variance and multiple mean comparisons were performed using the general linear model function of Statistical Analysis System software version 9.1 (SAS Institute, Cary, NC, USA). Differences among mean values were determined using Duncan's new multiple range test at $P \leq 0.01$.

Results and discussion

Here, the RNAi efficacy of *hsp70*-dsRNA delivered through oral ingestion was determined by measuring the expression of the target gene *hsp70* by real-time RT-PCR and mortality under normal and thermal stress conditions. Our study indicated that oral ingestion of dsRNA

Table 1
Primer information of *hsc70* and *actin* genes of *Bemisia tabaci*.

	Target gene	Primer	Sequence (5′ → 3′)	Length (bases)	Product (bp)
RT-PCR	*hsp70*	Forward	GATATGAAGCACTGGCCCTTC	21	1112
		Reverse	AGATTGTTATCCTTCGTCATTGC	23	
RNAi	*hsp70*	Forward	TAATACGACTCACTATAGGGAGAC CACGCGCCAAGATAGCTGCCA[a]		279
		Reverse	TAATACGACTCACTATAGGGAGAC CACTCTTGCTCATGATGGGCGA[a]		
Realtime	*hsp70*	Forward	CAATTCTTAGCGGCGACACCA	21	62
		Reverse	AGGCGCCACGTCCAGGA	17	
Realtime	*actin*	Forward	GACGGACAGGTCATCATAATCG	22	78
		Reverse	CATACCCAAGAAGGATGGCTG	21	

[a] T7 promoter seq underlined.