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ORIGINAL ARTICLE

Two putative fatty acid synthetic genes of *BgFas3* and *BgElo1* are responsible for respiratory waterproofing in *Blattella germanica*

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Abstract Water retention is critical for physiological homeostasis and survival in terrestrial insects. While deposition of hydrocarbons on insect cuticles as a key measure for water conservation has been extensively investigated, we know little about other mechanisms for preventing water loss in insects. Here, we report two fatty acid synthetic genes that are independent of hydrocarbon production but crucial for water retention in the German cockroach *Blattella germanica* (L.). First, an integument enriched fatty acid elongase gene (*BgElo1*) was identified as a critical gene for desiccation resistance in *B. germanica*; however, knockdown of *BgElo1* surprisingly failed to cause a decline in cuticular lipids. In addition, RNA interference (RNAi)-knockdown of an upstream fatty acid synthase gene (*BgFas3*) showed a similar phenotype, and transmission electron microscopy analysis revealed that *BgFas3*- or *BgElo1*-RNAi did not affect cuticle architecture. Bodyweight loss test showed that repression of *BgFas3* and *BgElo1* significantly increased the weight loss rate, but the difference disappeared when the respiration was closed by freeze killing the cockroaches. A water immersion test was performed, and we found that *BgFas3*- and *BgElo1*-RNAi made it difficult for cockroaches to recover from drowning, which was supported by the upregulation of hypoxia-related genes after a 10-h recovery from drowning. Moreover, a dyeing assay with water-soluble Eosin Y showed that this was caused by the entry of water into the respiratory system. Our research suggests that *BgFas3* and *BgElo1* are required for both inward and outward waterproofing of the respiratory system. This study benefits the understanding of water retention mechanisms in insects.

Key words elongase; fatty acid synthase; German cockroach; respiratory water loss; very long chain fatty acid; water retention

Introduction

Water balance is essential for the survival and homeostasis of organisms. Terrestrial insects with a high surface-to-volume ratio are in high risk of dehydration (Hadley, 1994; Kühsel *et al*., 2017). Nevertheless, insects are widely distributed in various geographic environments, including deserts, plateau mountains, saline lakes, and

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polar regions (Edney, 1967; Sinclair, 2000; Benoit *et al*., 2007; Teets *et al*., 2012; Botella-Cruz *et al*., 2017). The successful expansion of terrestrial insects in areas of extremely low humidity or high temperature must be partly due to their elaborated water balance strategies, including restricted water loss, enhanced initial water content, extraordinary tolerance of extreme water loss, and behaviors that avoid desiccating conditions (Gibbs *et al*., 2003; Chown *et al*., 2011; Fanning *et al*., 2019). However, the former is the most compelling strategy, as it is universal and is effective for water economy; for most insects, the relentless struggle against desiccation is achieved by reducing water loss through various physiological mechanisms (Gibbs & Matzkin, 2001). Water loss in insects occurs *via* three main routes: evaporation from the cuticle, respiratory water loss, and excretory processes (Hadley, 1994).

A large number of studies have been conducted on the mechanisms underlying water loss reduction. However, cuticular evaporation is mostly studied, as cuticular water loss accounting for the majority of waster budgets; cuticular transpiration generally accounts for > 80% of overall water loss (Gibbs & Rajpurohit, 2010). Insects have developed several characteristics to reduce cuticular permeability. First, a cocktail of aliphatic substances composed of hydrocarbons, fatty acids, wax esters, sterol esters, alcohols, aldehydes, and ketones is deposited on the surface of the epicuticle. The cuticular lipid layer is generally called "envelope" which shows excellent performance in preventing water from escaping through the cuticle (Blomquist *et al*., 1987; Moussian, 2010). The cuticular compositions differ among insects; however, the primary components are generally hydrocarbons. For example, in the German cockroach (*Blattella germanica* L.), cuticular lipids are predominantly composed of hydrocarbons and fatty acids (Paszkiewicz *et al*., 2016). Second, the delicate structures of insect cuticle also play crucial roles in water retention. Insect epicuticle contains a large amount of structural lipids, and the lipids associated with proteins are involved in sclerotization, which increases the hardness and impermeability of the epicuticle (Wigglesworth, 1970, 1975, 1985; Appel & Tanley, 1999). The procuticle (including the exocuticle and endocuticle) is a stratified chitin-protein crosslinking; the loss of function of both cuticular proteins and chitins destroys the choreographed structure of insect cuticle (Zhu *et al*., 2016; Pan *et al*., 2018), and may lead to severe loss of control of cuticular evaporation. Third, insects also reduce the permeability of the cuticle through post-molting processes, and several studies have reported the participation of melanization or pigmentation in cuticular waterproofing (King & Sinclair, 2015; Noh *et al*., 2015; Chen *et al*., 2018, 2020).

Respiratory transpiration is another important route of water loss. Its contribution to overall water loss in insects is controversial and intricate. The arguments especially for the discontinuous gas exchange cycles (DGC) in water conservation have been reviewed, and it is believed that respiratory water loss typically accounts for less than 15% of total water loss (Chown, 2002; Gibbs & Johnson, 2004). However, respiratory water loss is tightly associated with environmental or physiological factors, and may be elevated when insects are in vigorous activities (e.g., flying or running) or exposed to high temperatures (Gefen *et al*., 2009; Terblanche *et al*., 2010; Mullins, 2015). Many studies have been conducted on respiratory water loss based on testing the DGC in water conservation, but studies concerning the role of physiological structure in reducing respiratory water loss are infrequent. Nevertheless, Parvy *et al*. (2012) elucidated the importance of lipids in the fruit fly (*Drosophila melanogaster*) respiratory system: acetyl-CoA carboxylase (ACC), cooperating with its downstream lipid biogenesis enzymes, was shown to generate a putative very long chain fatty acid (VLCFA) and maintained the watertightness of the spiracles and tracheas. RNA interference (RNAi) knockdown of these genes specifically in oenocytes resulted in lethal anoxic issue which is caused by the penetration of food liquids into the larval spiracles and tracheas; later, their function in desiccation resistance was observed, and knockdown of these genes (like $ACC^{CG11198}$ and $FASN^{CG17374}$) was shown to cause even more serious problems than the removal of cuticular hydrocarbons (Wicker-Thomas *et al*., 2015). Jaspers *et al*. (2014) also reported a fatty acyl-CoA reductase gene (*Waterproof*) that produced very long chain fatty alcohols for wax esters or other hydrophobic substance production, and therefore waterproofed the trachea in *D. melanogaster*. However, related knowledge is sorely lacking in other insects.

Excretory water loss accounts for only a small fraction (<6%) of water loss in *Drosophila*. Restriction of excretory water loss is known to be achieved mainly by decreasing the Malpighian tubule activity or increasing the resorption in the hindgut (Park, 2012), and is associated with membrane permeability and functional aquaporins (Spring *et al*., 2009). However, excretory organs may be more important for osmoregulation (Beyenbach *et al*., 2010), and little emphasis has been placed on their role in insect water conservation.

The fatty acid synthesis pathway is an essential route for lipid production, which includes a wide range of

aliphatic substances. Several central upstream enzymes are involved in this pathway. ACC catalyzes the biosynthesis of malonyl-CoA, and fatty acid synthase (FAS) incorporates several malonyl-CoA units into the chain initiator acetyl-CoA, which generate long chain fatty acids (LCFAs, no longer than C18). LCFAs can be further elongated to VLCFAs (longer than C20) by a fatty acid elongation system including a rate-limiting fatty acid elongase and three other different enzymes (Jakobsson *et al*., 2006; Dembeck *et al*., 2015). LCFAs and VLCFAs can be used as substrates for a variety of lipids and related derivatives, including hydrocarbons, wax esters, fatty alcohols, membrane phospholipids, triacylglycerol, structural lipids and others (Spector & Yorek, 1985; Samuels *et al*., 2008; Ginzel & Blomquist, 2016). The fatty acid synthesis pathway is dominantly activated in the fat body and integument in insects. The former is mainly functional in lipid storage, whereas the latter primarily participates in the generation of cuticular lipids and the lipids in respiratory organs (Parvy *et al*., 2012; Chung *et al*., 2014; Wicker-Thomas *et al*., 2015). Several studies have explored the function of the fatty acid synthesis pathway in the biogenesis of cuticular lipids, and therefore, in reducing cuticular permeability (Wicker-Thomas *et al*., 2015; Pei *et al*., 2019; Li *et al*., 2019; Moriconi *et al*., 2019; Zhao *et al*., 2020); however, little is known about its role in respiratory water loss. In this study, we described a putative VLCFA biosynthesis route that is important for desiccation resistance in *B. germanica*; lipid analysis and transmission electron microscope (TEM) test showed that both *BgFas3* and *BgElo1* were irrelevant to cuticular lipid production or maintenance of cuticle structure. However, weight loss rate analysis alongside water immersion and dyeing tests demonstrated the involvement of *BgFas3* and *BgElo1* in the waterproofing of the respiratory system in *B. germanica*. This study highlights the important roles of lipids in keeping water from entering the spiracles, and sheds light on our understanding of waterproofing mechanisms in insects.

Materials and methods

Insect rearing

A laboratory strain of *B. germanica* that was stably kept in the laboratory for more than 40 years was used in this research. The cockroaches were raised in 12-L glass jars supplied with rat chow and tap water. Rearing conditions were maintained at 30 ± 1 °C with a photoperiod of 12 : 12 h and ∼50% relative humidity (RH). Freshly emerged 5th instar nymphs or adult females were collected and used in the experiments.

Bioinformatics and sequence cloning

Informatics analysis and sequence cloning of *BgFas3* were performed in our previous work (Pei *et al*., 2019). The 630 bp complete coding sequence (CDS) of *BgElo1* was first released by Harrison *et al*. (2018), originally tagged as "C0J52_13101" in the scaffold PYGN01001275.1 (National Center for Biotechnology Information [NCBI] accession number). In the present study, we extended its 3′ untranslated region to satisfy the requirements of designing two non-overlapping RNAi targets. We searched this sequence in our third-generation transcriptome data (NCBI accessions: SRR9143014 and SRR9143013) using a local BLASTN tool (Altschul *et al*., 1990) with *E*-values <1.00E-30. Based on the BLASTN results, a pair of specific primers (Table S1) was designed to amplify the *BgElo1* sequence. The polymerase chain reaction (PCR) products were purified, inserted into the pMD^{TM} 19-T vector (Takara, Dalian, Liaoning, China), cloned using *Escherichia coli* DH5α competent cells (Takara, Dalian, Liaoning, China), and subjected to Sanger-based sequencing. The obtained nucleotide sequence was translated into a protein sequence, and sequence alignment against functional elongases in *Mus musculus* and *D. melanogaster* was performed using the DNAMAN program (version 9). The putative domains of elongase were analyzed using SMART tools (http://smart.embl-heidelberg.de), and the transmembrane helices were predicted with the online TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM-2.0). Alternating exons and introns were identified by mapping the messenger RNA (mRNA) sequence to genomic sequence using the BLASTN tool with E-values <1.00E-30 (Altschul *et al*., 1990), and boundaries were determined by the GT-AG rule.

Quantitative reverse-transcription PCR (RT-qPCR)

Total RNA was extracted from various tissues or intact cockroaches using the RNAiso Plus Reagents (Takara, Dalian, Liaoning, China) following the manufacturer's instructions. Eight hundred nanograms of newly isolated total RNA from each sample was used for genomic DNA digestion and reverse transcription with the PrimeScript^{TM} RT reagent Kit (Takara, Dalian, Liaoning, China). Quantitative PCR analysis was conducted using a Roche LightCycler 480 system (Roche, Basel,

Switzerland). The total reaction volume was 20 μL, containing 10 μL of TB Green^{TM} Premix Ex Taq^{TM} (Takara, Dalian, Liaoning, China), 8 μL of 50-fold diluted complementary DNA (cDNA), and 1 μL each of the forward and reverse primers (10 μmol/L). The amplification efficiency of all the primers used in the qPCR analysis was 95%–105%; primer sequences are shown in Table S1. Thermal cycling was set as follows: 94 °C for 3 min, followed by 40 cycles at 94 °C for 10 s each and at 60 °C for 30 s each. The commonly used *B. germanica* housekeeping gene *actin5c* (GenBank: AJ862721.1; Kamsoi and Belles, 2019) was used to calculate the relative gene expression level based on the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Each treatment contained three to five biological replicates and technical triplicates.

Tissue-specific expression analysis

For the *BgElo1* expression profile study, various tissues of *B. germanica* adults were dissected under a stereomicroscope. Two-d-old females were anesthetized and placed on a glass slide that was fixed on ice. An appropriate amount of 4 °C-precooled phosphate-buffered solution was added to submerge the cockroaches. The head, thorax, tracheal trunks, abdominal integument, fat bodies, gut, ovaries, Malpighian tubule, ejaculatory duct (from two-d-old males), and colleterial glands were carefully dissected out. In order to preserve the oenocytes under the epidermis, the abdominal integument was dissected with the basal membrane attached; as for dissecting the tracheas, we considered that the abdominal tracheal trunks may be connected with oenocytes (according to Parvy *et al*., 2012), so only the thoracic tracheal trunks were collected. The various tissues or organs collected were stored at −80 °C and subsequently homogenized in liquid nitrogen for total RNA extraction. The expression profile of *BgElo1* was analyzed with RT-qPCR. In addition, since the Malpighian tubule is an important water retention organ in insects, the expression of *BgFas3* in Malpighian tubules and tracheal trunks was also detected, even though we have found in our previous work that *BgFas3* is dominantly expressed in the integument (Pei *et al*., 2019).

RNAi

Two unique RNAi targets were designed for each target gene. The fragments were amplified by PCR and cloned into the pMD^{TM} 19-T vector (Takara, Dalian, Liaoning, China). The plasmid was stored at −20 °C for further double-stranded RNA (dsRNA) synthesis. Primers embedded with T7 promoter at the 5′ end (listed in Table S1) were used for dsRNA synthesis. All synthetic procedures were conducted using the T7 RiboMAX^{TM} Express RNAi System (Promega, Madison, WI, USA). A heterologous dsRNA fragment from *Mus musculus* (*Muslta*) was also synthesized for use as a control. The quality of the newly generated dsRNA was confirmed with agarose gel electrophoresis. For dsRNA injection, early 5th-instar nymphs (N5D1–N5D3) were anesthetized with carbon dioxide and immobilized on ice. Two microliters of dsRNA (2000 ng/μL) were delivered into the abdomen through a microliter syringe equipped with pulled glass capillary tubes, and a second injection was performed 1 week later to stabilize the gene silencing effects. Newly emerged cockroaches were collected; 2-d-old adults (AD2) were used for determining the RNAi efficiency, and for all subsequent experiments.

Cuticular lipid preparation

The cuticular lipids of *B. germanica* were extracted following the method of Gu *et al*. (1995) with slight modifications. Individual female adult cockroaches, which were sacrificed by freezing at −20 °C overnight, were thawed at room temperature and washed with 1 mL of hexane twice (each wash lasted for 5 min), and *n*-Hexacosane (15 μg) was added as an internal standard during the first extraction. The cockroach and vial were finally rinsed with 1 mL of hexane, and all the cuticular lipid extracts were combined. The extracts were reduced to 300–500 μL with a gentle stream of N_2 and loaded onto a mini Pasteur pipette column packed with ∼500 mg of fresh 70–230 mesh silica gel (Sigma-Aldrich, St Louis, MO, USA). Cuticular hydrocarbons were then eluted with 8 mL of hexane.

Fatty acid methyl ester (FAME) samples were prepared according to the methodology described by Pei *et al*. (2019). The cuticular fatty acids were eluted with 1 mL of petroleum ether (containing 5 μg of tridecylic acid) for 1 min and, subsequently, with 1 mL of dichloromethane for 5 min. The extracts were evaporated with a N_2 flow and then methylated using the sulfate-methanol method. Initially, 2 mL of 1% (v/v) H_2SO_4 in methanol was added to the samples and incubated at 80 °C for 2 h under a N_2 atmosphere. After incubation, 1 mL of saturated NaCl solution was added to the mixture, and FAME fractions were extracted twice by adding 1 mL of *n*-hexane containing 0.01% butylated hydroxytoluene as an antioxidant. The mixture was vigorously vortexed and centrifuged at

1000 × *g* for 5 min, and then the supernatant was collected as FAME samples.

Gas chromatography mass spectrometry (GC/MS) analysis

All the qualitative and quantitative analyses of lipids were performed with GC/MS (Thermo Scientific). HCs were determined as previously described (Pei *et al*., 2019). In this study, FAMEs were separated using a DB-5MS non-polar capillary column (30 m length, 0.25 mm inner diameter; 0.25 μm film thickness; Agilent Technologies). The temperature programing was as follows: 60 °C for 2 min, then heated to 160 °C at a rate of 30 °C/min, then further increased by 3 °C/min up to 250 °C, followed by 10 °C/min up to 320 °C and held at that temperature for 5 min. The ionization was achieved by an electron ionization mode with 70 eV ionization potential. Ion source, inlet, and MS transfer line were set at 280 °C, and the MS scan range was 35–500 m/z at a rate of 5 scan/s. The compound and peak areas were determined using a Xcalibur 2.2 workstation.

TEM analysis

To analyze the cuticle structure and thickness, AD2 (2-d-old adults) cockroaches were treated with different dsRNAs and dissected. The integument was collected and fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer for 48 h at 4 °C, thoroughly rinsed thrice with phosphate buffer, and post-fixed in 1% osmium tetroxide in 0.1 mol/L phosphate for 2 h at room temperature. The samples were then dehydrated in an ethanol gradient (50%, 70%, 80%, 90%, 95%, 100%) and 100% acetone each for 15 min, and embedded in Epon resin at room temperature for 2 h. Ultrathin sections (70 nm) were prepared using a UC7 ultramicrotome (Leica, Solms, Germany). Sections were collected on copper grids and counterstained with 2% uranium acetate in saturated alcohol solution and lead citrate, each for 15 min. The images were captured with an HT7700 TEM (Hitachi, Tokyo, Japan).

Desiccation resistance and weight loss assay

In order to set up an arid microenvironment, approximately 120 g of fresh silica gel was wrapped by gauze and placed in a ∼900 mL sealed plastic bottle. The RH in the bottle dropped to approximately 5% after 2 h at 30 °C. For the desiccation assay, AD2 female cockroaches were treated with ds*BgFas3*, ds*BgElo1*, or ds*Muslta* (control), the cockroaches were placed in an arid microenvironment and supplied with ∼1 g of dried rat chow, but no water. The survival rate was calculated every 24 h. Each treatment contained ∼20 cockroaches and was repeated three times. Additionally, to confirm that the death of cockroaches was caused by dehydration, different dsRNA-treated cockroaches were reared at 70% RH without changing any other rearing conditions, and the survival rate was calculated 5 d later.

Because direct analysis of respiratory water loss was difficult to achieve, we applied an alternative method by comparing the difference in water loss rate between the living cockroach (with breathing) and dead cockroach (without breathing) groups. For the living cockroach group, weight loss assay experiments were performed similarly to the desiccation tolerance assay. However, these cockroaches were first weighed using a Sartorius MSA3.6P0TRDM microgram balance (Sartorius, Goettingen, Germany) before being placed in the above-mentioned "arid microenvironment" bottles, and the weight loss rate was calculated 48 h later. In order to analyze the initial water content and critical water content, this experiment was repeated, after these cockroaches died in the drying bottles, their body weight was recorded, the dead cockroaches were further dried thoroughly in a 56 °C oven for 48 h and weighted again. The initial water content was represented by the ratio of total weight loss to initial body weight, and the critical water content at the time of insect death was calculated by the ratio of weight loss after death to initial body weight. To analyze the water loss rate in the dead cockroach groups, the cockroaches injected with different dsRNA were first killed by freezing at −20 °C for 30 min to stop the breathing. The dead cockroaches were then weighed and transferred to the drying bottle. In order to avoid unexpected water loss caused by decomposition, the water loss rate was analyzed 6 h before the dead cockroaches turned black.

Water immersion and Eosin Y staining tests

To test the possibility of *BgFas3* and *BgElo1* being involved in the waterproofing of the respiratory system, cockroaches were injected with ds*BgFas3*, ds*BgElo1*, or ds*Muslta* separately, and then separately caged in 50-mL centrifuge tubes. The cockroaches were submerged in water by filling the tubes with tap water through the holes on the lid. After immersion in water for 10 min at 30 °C, the cockroaches were scooped out and placed on filter paper. The wet cockroaches were dried with blotting paper. Comatose cockroaches were woken up by blowing

air twice per minute, and the waking time was recorded every minute. The cockroaches that had awakened or were comatose for more than 20 min were moved to the rearing containers. In addition, to explore whether the decreased recovery ability was caused by a blockage of the respiratory system, we analyzed the expression level of hypoxia-associated genes (Shoshani *et al*., 2002; Reiling & Hafen, 2004; Centanin *et al*., 2008). The homologous genes of *D. melanogaster* Hypoxia-inducible factor 1α (NP_524584.2) and its target Scylla (NP_648456.2) were analyzed in the German cockroaches. A BLASTP search in the NCBI database revealed a Hypoxia-inducible factor (PSN42112.1) and a Scylla-like protein (PSN58152.1) in the German cockroach. Their expression levels in the drowned cockroaches were analyzed after a 10-h recovery period, and dead cockroaches were excluded during analysis.

In order to observe the intrusion of water into the respiratory system, a water-soluble dye solution of 1% Eosin Y (W/V) containing 0.1% Triton X-100 was modified and applied in this experiment according to Chen *et al*. (2020). Briefly, cockroaches injected with different dsRNA were immersed in the dye solution for 10 min at 30 °C, after which the cockroaches were rinsed thrice in tap water and dissected under a stereoscopic microscope for detailed observation of the tracheal trunks that connected directly to the spiracle. The number of defective spiracles was recorded, as determined by the dye filling conditions of its internal tracheas. If any of the tracheas were stained by the dye solution, the corresponding spiracle was considered defective.

Results

Sequence analysis

The *BgElo1* nucleic acid sequence was cloned and re-sequenced; the verified sequence is provided in Table S2. Since we had found some differences between our result and the old version (PSN36108.1) in the GenBank database, the newly obtained sequence in our study was re-submitted to the GenBank database under accession number MT925720. The mRNA sequence mapped well with the genomic data; the exon-intron structure is shown in Figure 1A. The complete CDS of *BgElo1* encoded a putative protein consisting of 264 amino acid residues. The protein contained a single Elo domain (pfam: PF01151) and included the conserved HXXHH and YXYY motifs, as confirmed by sequence alignment (Fig. 1B and Fig. 1D). BgElo1 was determined as being a typical transmembrane protein, as seven transmembrane helices were predicted in the Elo domain (Fig. 1C). These results indicate that the *BgElo1* gene indeed encodes an elongase protein.

Tissue expression analysis

The expression profiles of *BgElo1* across different tissues or organs were analyzed with RT-qPCR. Our results showed that *BgElo1* was dominantly expressed in the integument and was almost undetectable in other tissues or organs. The expression level of *BgElo1* in the integument was approximately 150 times higher than in other tissues and organs (Fig. 2B). This result suggests that *BgElo1* is a cuticle gene and may play important roles in the integument and its peripheral tissues. In order to analyze whether *BgFas3* might express in the Malpighian tubules and tracheal trunks in *B. germanica*, we compared the expression level of *BgFas3* in these three different kind of tissues. We found that the expression of *BgFas3* in the integument was approximately 70 times higher than in the Malpighian tubule and nearly undetectable in the tracheas (Fig. 2A). Combined with our previous research result that *BgFas3* is mainly expressed in the integument and is almost undetectable in other tissues (Pei *et al*., 2019), we re-confirmed that *BgFas3* is a cuticle gene.

Involvement of BgFas3 and BgElo1 in desiccation tolerance

Injection of *dsBgFas3* or *dsBgElo1* significantly downregulated the mRNA levels of target genes (Fig. 3A, Fig. 3B, and Fig. S1), and subsequent desiccation assays showed that knockdown of either *BgFas3* or *BgElo1* significantly decreased the survival rate of *B. germanica* under desiccation conditions (Fig. 3D). Before 48 h, no significant difference was observed; however, the survival rates of *BgFas3*- as well as *BgElo1*-RNAi groups declined rapidly at 72 h, and were significantly different from those of the control group ($P = 0.028$ for *BgFas3*-RNAi, 0.006 for *BgElo1*-RNAi). This difference was widened at 96 h, and all the cockroaches injected with either *dsBgFas3* or *dsBgElo1* died at 120 h, whereas ~75% of the cockroaches in the control group were still alive. To confirm that the death of cockroaches was due to desiccation, this experiment was repeated at an RH of 70%. Our results showed that the survival rate of cockroaches was similar after being treated with different dsRNAs, and the cockroaches rarely died at 70% RH after 5 d (Fig. 3C). These results indicate that both the cuticle genes *BgFas3* and *BgElo1* are necessary for desiccation tolerance in *B. germanica*.

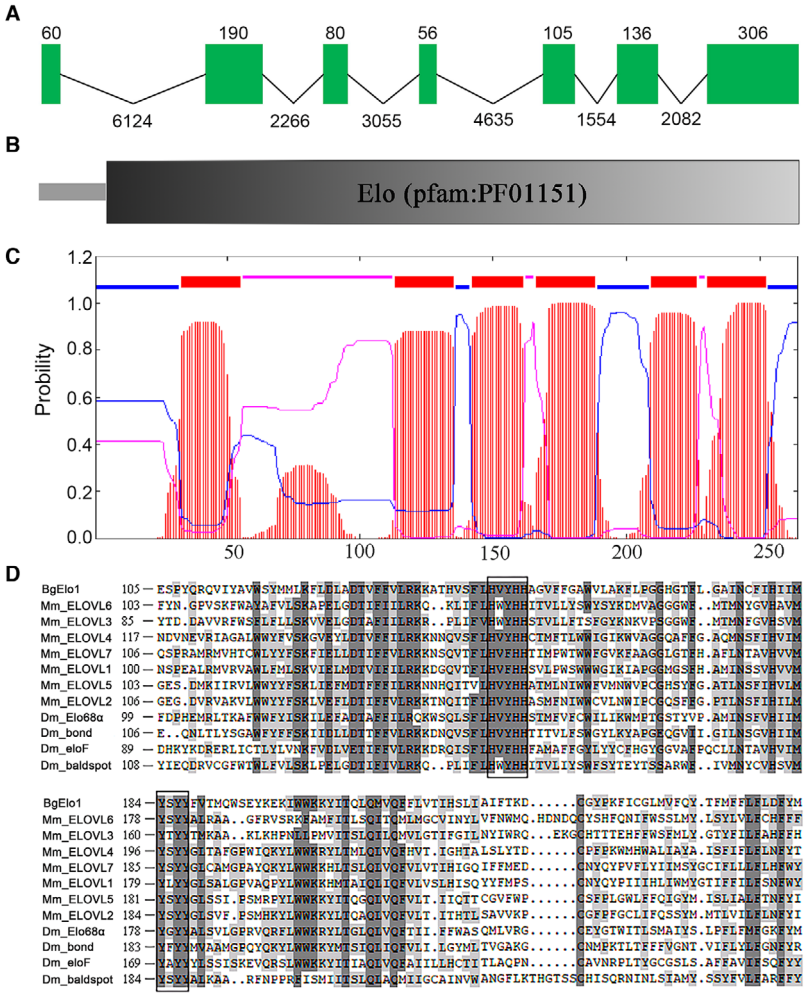

Fig. 1 Bioinformatics analysis of *BgElo1*. (A) Exons (green rectangles) and introns (black lines) of *BgElo1* were analyzed by comparing the re-sequenced transcript with genomic data. Numbers indicate the length (bp) of each exon or intron. (B) The domain structure of BgElo, which contains a single Elo domain. The accession number in the Pfam database is given in parentheses. (C) Prediction of transmembrane helices in the BgElo1 protein. The numbers at the bottom of the picture represent the locations of amino acid residues. The blue line represents the possibility of being inside the membrane, and the red line represents the possibility of being outside the membrane. Possible transmembrane regions are represented by red thick lines or red vertical stripes. (D) Multiple sequence alignment of BgElo1 with functional BgElos in Mm (*Mus musculus*) and Dm (*Drosophila melanogaster*). The C-terminal and N-terminal sequences are omitted. The numbers in front of the sequence represent the location of the first amino acid residue in the diagram. Homologous fragments are highlighted in light or dark gray, and conserved motifs are labeled with rectangles, including a HXXHH motif and a YXYY motif.

Effects of BgFas3- and BgElo1-RNAi on cuticular lipids and cuticle structure

Considering that cuticular lipids play an important role in water conservation, the cuticular hydrocarbons (CHCs) and cuticular free fatty acids (cFFAs) were analyzed with GC/MS. The CHC profile was similar to that previously identified (Jurenka *et al*., 1989). In this study, RNAi of *BgFas3* using a new target showed similar results to those reported in our previous study (Pei *et al*., 2019). We confirmed that *BgFas3* did not participate in CHC biosynthesis (Fig. 4A and Fig. S2). As for cFFAs, we found that the cFFAs of *B. germanica* were mainly composed of C16 and C18 FAs, and repression of *BgFas3* significantly increased the amount of cFFAs, especially for palmitic acid, oleic acid, and linoleic acid (Fig. 4B). RNAi of *BgElo1* slightly increased the total amount of CHCs and caused significant increases in short chain (C27 and C28) CHCs

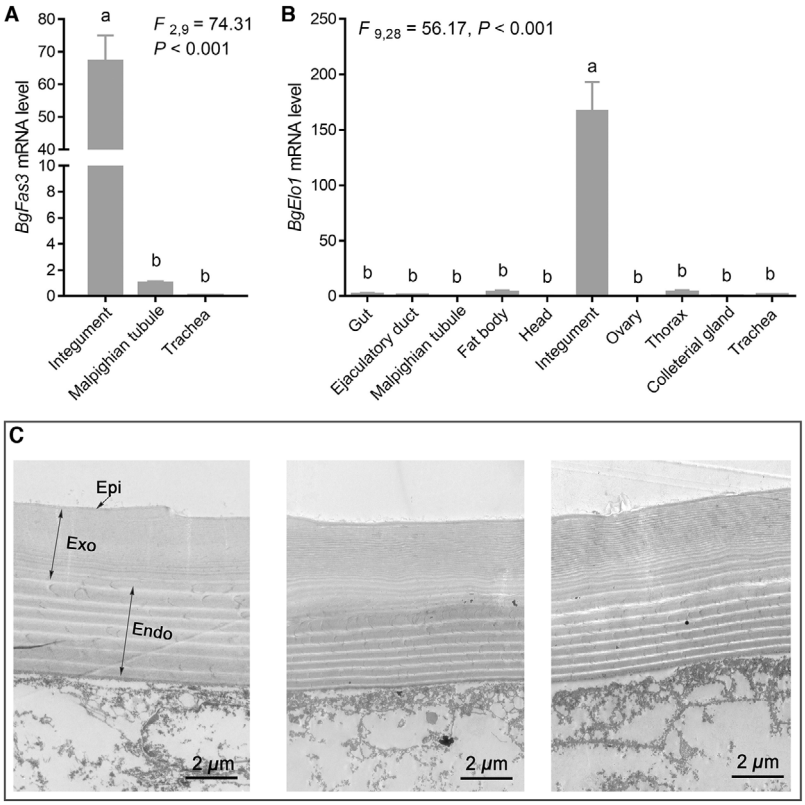

Fig. 2 Tissue expression and transmission electron microscope (TEM) analysis. (A) Relative expression level of *BgFas3* in the integument and Malpighian tubule. Data are shown as mean ± SE. ***$P < 0.001$ (Student's *t*-test, $n = 4$). (B) Tissue expression profile of *BgElo1*. Relative expression levels of *BgElo1* were compared among the gut, ejaculatory duct, Malpighian tubule, fat body, head, integument, ovary, thorax, colleterial gland. Data are shown as mean ± SE and calculated from four independent samples. Letters above the error bars indicate significant differences in corresponding expression levels (analysis of variance, Least Significant Difference, $P < 0.05$). (C) TEM analysis of abdominal cuticle structure from ds*Muslta*-, ds*BgFas*-, and ds*BgElo1*-treated 2-d-old female cockroaches. Different cuticle layers were distinguishable: Epi, epicuticle; Exo, exocuticle; Endo, endocuticle. Scale bars are equal to 2 μm.

(Fig. 4A and Fig. S2). However, repression of *BgElo1* had no effect on the total cFFAs ($P = 0.69$), although *BgElo1*-RNAi downregulated the amount of minor FA components with chain lengths longer than C20 (Fig. 4B). Overall, inhibition of either *BgFas3* or *BgElo1* resulted in a slight upregulation of cuticular lipids.

In addition, we analyzed the influence of *BgFas3*- or *BgElo1*-RNAi on cuticle structure via TEM. *B. germanica* cuticle typically contains three layers: the epicuticle, exocuticle, and endocuticle (Fig. 2C). The different layers could be distinguished easily, and the thickness of the exocuticle, endocuticle, and epicuticle among differently treated cockroaches showed no obvious difference. These results suggest that *BgFas3* and *BgElo1* may not be involved in epicuticle and procuticle structure maintaining, and combined with lipid analysis and TEM results, we speculate that *BgFas3* and *BgElo1* may not be involved in preventing cuticular water loss.

Repression of either BgFas3 or BgElo1 increased respiratory water loss

To confirm that the observed decrease in desiccation resistance was caused by an excessive water loss rate, we compared the weight loss rate of cockroaches under 5% RH. The rate of water loss in the control cockroaches was 0.48% of body weight per hour; in contrast, the cockroaches with suppressed *BgFas3* or *BgElo1* showed a significantly increased weight loss rate (both $P < 0.01$) of approximately 0.7% of body weight per hour (Fig. 5A). In addition, we examined the initial water content and critical death water content in differently treated cockroaches; the initial water content showed no significant difference (Fig. S3A), which suggested that RNAi of *BgFas3* or *BgElo1* could not change the initial water storage. However, RNAi of either *BgFas3* or *BgElo1* showed a trend of downregulated critical water content (Fig. S3B),

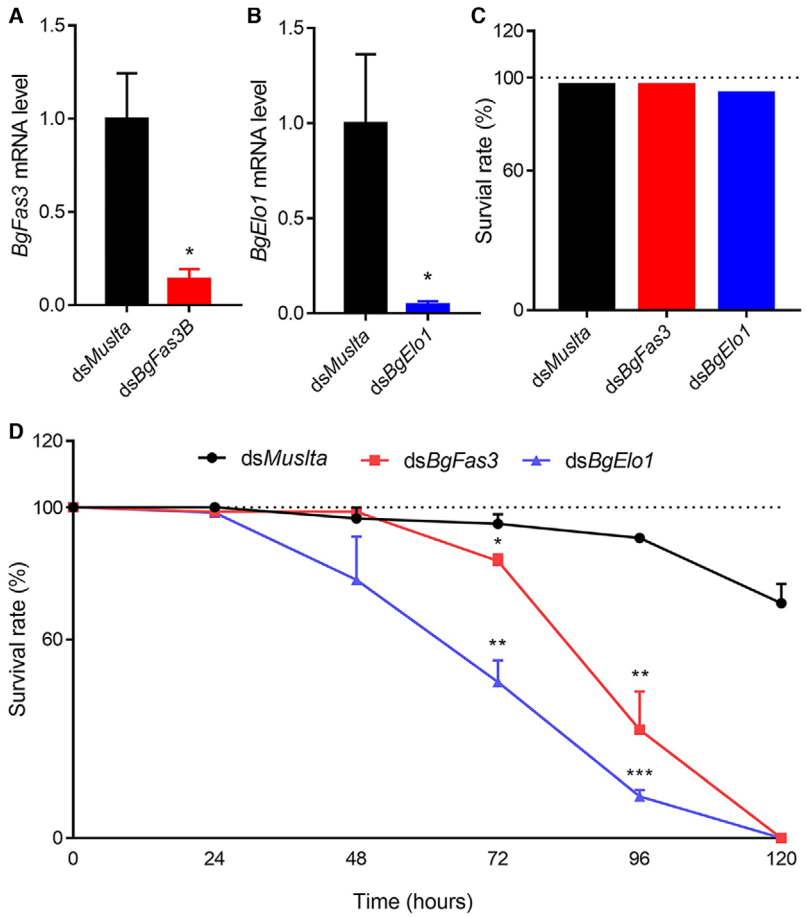

Fig. 3 *BgFas3* and *BgElo1* affect desiccation resistance. Relative expression levels of *BgFas3* (A) and *BgElo1* (B) after injection of target double-stranded RNA (dsRNA) or control. The expression levels were detected with quantitative polymerase chain reaction and normalized using the reference gene *actin5c*. Data are calculated from four biological replicates and shown as means ± SE. *Represents a significant difference at $P < 0.01$ (Student's *t*-test). (C) Survival rate of cockroaches during 5 d after injection with ds*Muslta* (control), ds*BgFas3*, and ds*BgElo*1 under relative humidity (RH) of 70%. The average survival rate is calculated from the survival rates of 30 cockroaches. (D) The effects of repressing *BgFas3* or *BgElo1* on desiccation resistance. The control (black line), ds*BgFas3* (red line), and ds*BgElo1* (blue line) treated cockroaches were kept at 5% RH, and the survival rates were calculated every 24 h for a total of 5 d. Each treatment contains three biological replicates, and each replicate contains 20–25 cockroaches. The survival rate is shown as mean ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t*-test).

which reflected the increased tolerance to water loss. And we suspect that decrease in the ability of controlling water loss will result in an increased physiological tolerance to water loss. Whatever, this result indicates that the early death of *dsBgFas3*- or *dsBgElo1*-treated cockroaches resulted from the increased water loss rate, which severely dehydrated the cockroaches.

As the RNAi of neither *BgFas3* nor *BgElo1* influenced cuticular lipids or disrupted the cuticle structure, we subsequently tested the differences between the treated groups in terms of respiratory water loss. We blocked breathing by killing the cockroaches, and repeated the weight loss experiment upon which we no longer found differences among the various treatment groups ($P = 0.71$ for *BgFas3*-RNAi, 0.48 for *BgElo1*-RNAi); the control group showed an average weight loss rate of 0.77%, while the *BgFas3*- and *BgElo1*-RNAi groups showed weight loss rates of 0.79% and 0.81%, respectively (Fig. 5B). This result suggests that breathing through the respiratory system accounted for the difference in water loss rates. In other words, repression of either *BgFas3* or *BgElo1* increased water loss due to breathing.

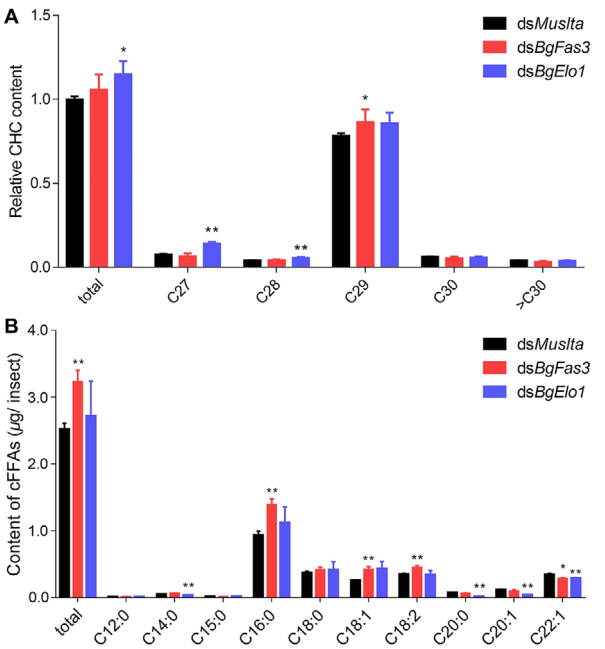

Fig. 4 Effect of *BgFas3*-RNAi and *BgElo1*-RNAi on cuticular lipids. Cuticular hydrocarbons (CHCs) (A) and cuticular free fatty acids (cFFAs) (B) of 2-d-old female cockroaches were analyzed after injecting with ds*Muslta* (black columns), ds*BgFas3* (red columns), and ds*BgElo1* (blue columns), respectively. The total amounts of CHCs or cFFAs were compared, and lipids with different chain lengths or saturation levels were also compared. All data are shown as means ± SE. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test, $n = 10–15$).

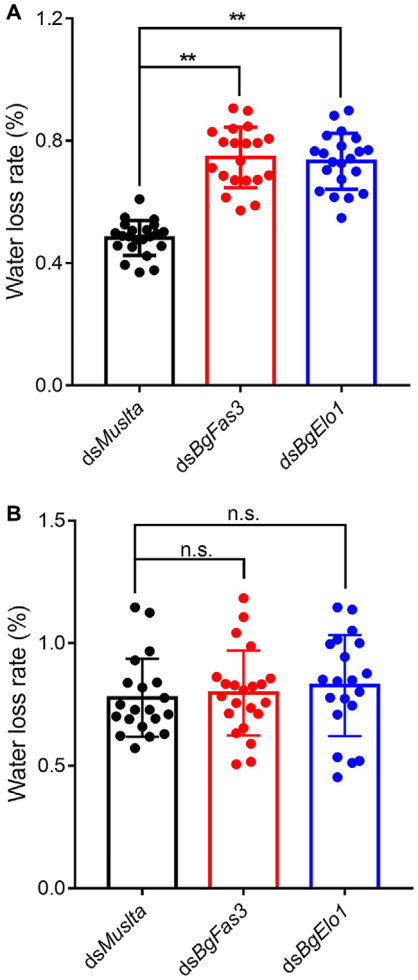

Fig. 5 Water loss rate of cockroaches under desiccation. After injection of different double-stranded RNA (dsRNA), 2-d-old females were collected, placed into the dehumidified bottles directly or after being frozen to death. Water loss rates of the living (A) and dead (B) cockroaches under 5% relative humidity were calculated, and data are presented as percentages of weight lost per hour; each dot represents a data point calculated from a single cockroach. Average water loss rates are shown as mean ± SE. $**P < 0.01$ (Student's *t*-test, $n = 20–22$).

Roles of BgFas3 and BgElo1 in the recovery from drowning

In order to confirm that the repression of *BgFas3* and *BgElo1* indeed affected water loss from the respiratory organs, we next analyzed the inward watertightness of the respiratory system, as the waterproofing abilities of aliphatic substances are reflected in dual (outward and inward) directions. As predicted, repression of either *BgFas3* or *BgElo1* increased the recovery time after drowning, and the recovery rates were discernibly lower than those of the control group within 20 min (Fig. 6A). Furthermore, after a recovery period of 10 h, both the hypoxia response genes *BgHIF-1α* and *BgScylla-like* showed significant upregulation (Fig. 6B and Fig. 6C). RNAi of either *BgFas3* or *BgElo1* caused approximately 40% upregulation of *BgHIF-1α* (both $P < 0.01$). Repression of *BgFas3* and *BgElo1* resulted in 68% ($P < 0.01$) and 37% upregulation ($P < 0.05$) of the *BgScylla-like* gene, respectively. These results suggest that inhibition of *BgFsa3* and *BgElo1* made it more difficult for the cockroaches to recover from drowning, and this was caused by hypoxia which might be due to the entry of water into the spiracles.

Effect of BgFas3 and BgElo1 repression on preventing the entry of Eosin Y

In order to observe the entry of water into the respiratory system, we adopted a red water-soluble Eosin Y dye

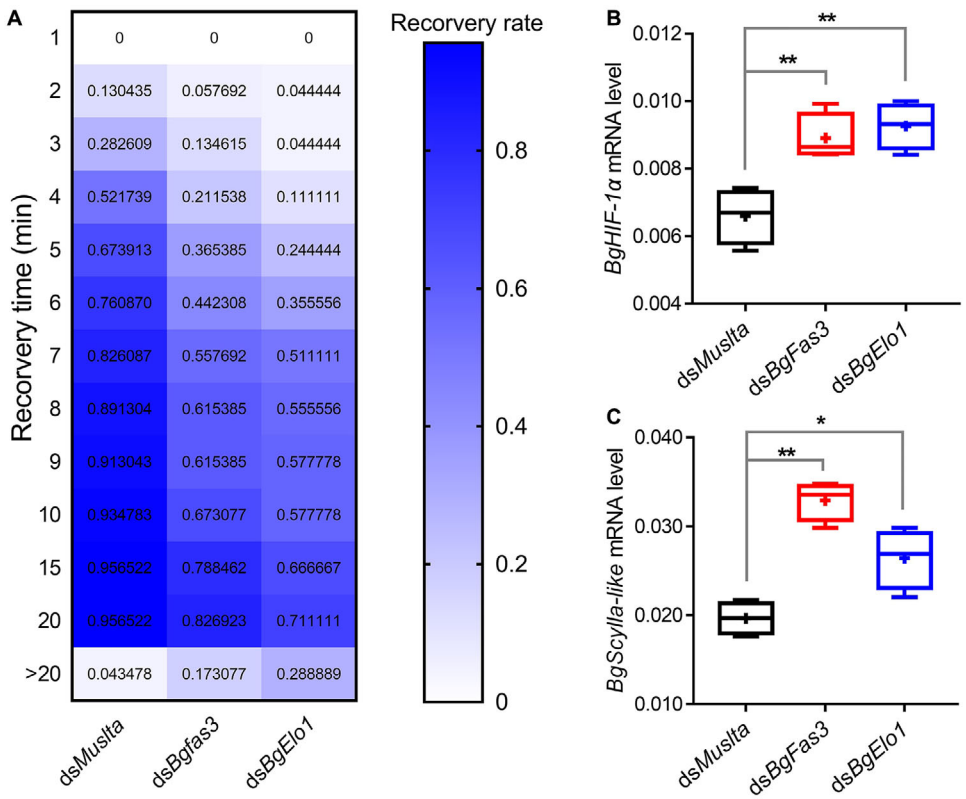

Fig. 6 Effect of *BgFas3* and *BgElo1* repression on recovery from drowning. (A) Differently treated cockroaches were immersed in water for 10 min. The drowning cockroaches were then pulled out, and the recovery time and recovery rate of ds*Muslta*-, ds*BgFas3*-, or ds*BgElo1*-treated cockroaches were recorded every minute. The recovery time is listed on the left *y*-axis; the recovery rates are represented by gradient color changes and numbers in each cell, and the color change rulers are listed on the right. The average recovery rates were calculated from 46 (ds*Muslta*), 52 (ds*BgFas3*), and 45 (ds*BgElo1*) cockroaches. The relative expression level of hypoxia-associated genes *BgHIF-1α* (B) and *BgScylla-like* (C) after a 10-h recovery from drowning. Data are shown as box-plot; the horizontal line in the box represents the median and the averages are represented by plus sign. Significant differences were determined by Student's *t*-test (*$P < 0.05$, **$P < 0.01$, $n = 4$).

staining method. First, we identified the location of the thoracic and abdominal spiracles as well as the connected internal tracheal trunks (Fig. S4). There are two pairs of thoracic spiracles and six pairs of abdominal spiracles in *B. germanica*. Representative photos after staining are shown in Figure 7. The Eosin Y polluted thoracic tracheal trunks could be observed after removing the legs, and RNAi of either *BgFas3* or *BgElo1* resulted in more staining by the Eosin Y solution. The area of the tracheal trunk was markedly redder in the *BgFas3*- or *BgElo1*-RNAi cockroaches when compared to the control cockroaches; further, upon enlarging the pictures, the polluted tracheal trunks could be seen more clearly. Lastly, the infusion of red dye in spiracles could be recognized externally. The polluted and un-polluted spiracles are marked by blue and black arrows, respectively, in the enlarged photos. The rates of affected spiracles in the different treatment groups were calculated (Fig. 8A). In the control cockroaches, about 28% of thoracic spiracles and 18% of abdominal spiracles were polluted; however, for the cockroaches that underwent *BgFas3*- and *BgElo1*-RNAi, about 46% and 48% of the thoracic spiracles were polluted, respectively. Twenty-eight percent and 36% of abdominal spiracles in *BgFas3*-RNAi and *BgElo1*-RNAi cockroaches, respectively, were polluted. Moreover, the influence of *BgFas3*- and *BgElo1*-RNAi on spiracle structural integrity was not only reflected in the numbers of affected cockroaches, but was also indicated by the number of damaged spiracles in each cockroach. When the intensity of spiracles in individual cockroaches was compared, we found that the percentages of affected cockroaches in the *BgFas3*- or *BgElo1*-RNAi groups were markedly higher than those in the control group (Fig. 8B and Fig. 8C). More importantly, the numbers

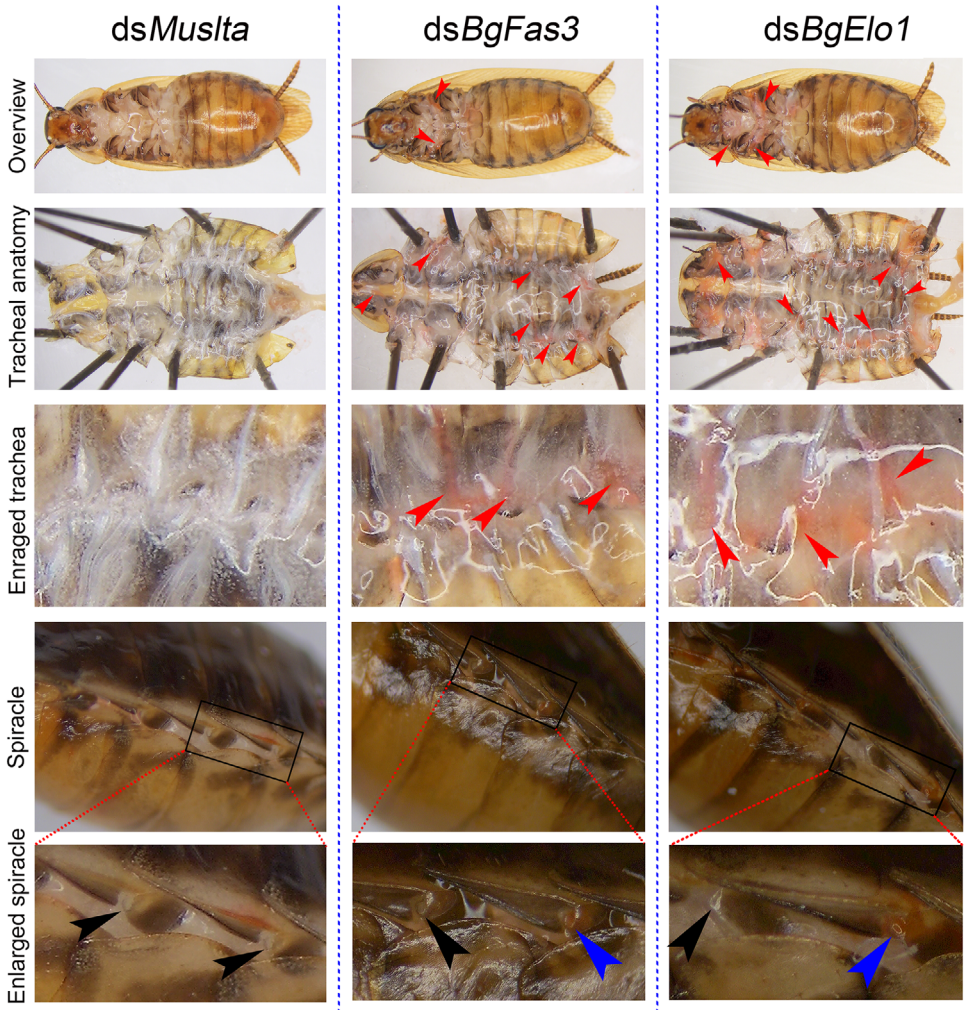

Fig. 7 Phenotypes for the invasion of Eosin Y into the respiratory system. In the overviews, the Eosin Y polluted tracheas in the thorax are marked by red arrows. In the tracheal anatomy pictures, the polluted tracheal area is significantly redder than the control. Enlarged images of the tracheas are provided in order to display the effect of the dye more clearly; the polluted tracheal trunks are marked with red arrows. An overview of the spiracles in the cockroaches that received the various treatments is shown. The area in the black box is enlarged, and the polluted and un-polluted spiracles are marked with blue and black arrows, respectively.

of polluted thoracic or abdominal spiracles in a single *BgFas3*- or *BgElo1*-RNAi cockroach were also greater than those in the control cockroaches. These data indicate that inhibition of either *BgFas3* or *BgElo1* decreased the inward waterproofing ability of the respiratory system of *B. germanica*.

Discussion

In this study, we identified a novel fatty acid elongase gene (*BgElo1*), which plays a crucial role in water conservation in *B. germanica*. A functional study revealed that *BgElo1* did not participate in cuticular lipid biogenesis or cuticle structure maintenance. However, our results strongly suggested the involvement of *BgElo1* in respiratory watertightness. Furthermore, an upstream FAS gene (*BgFas3*), which showed a similar function to that of *BgElo1* in the respiratory system, was also identified in this study. Based on these findings, we speculate that the two genes, *BgFas3* and *BgElo1*, in the VLCFA biosynthetic pathway may be involved in a putative VLCFA biosynthesis, which is functional in respiratory watertightness in *B. germanica*.

Bioinformatic analysis revealed that the putative BgElo1 amino acid sequence showed high homology

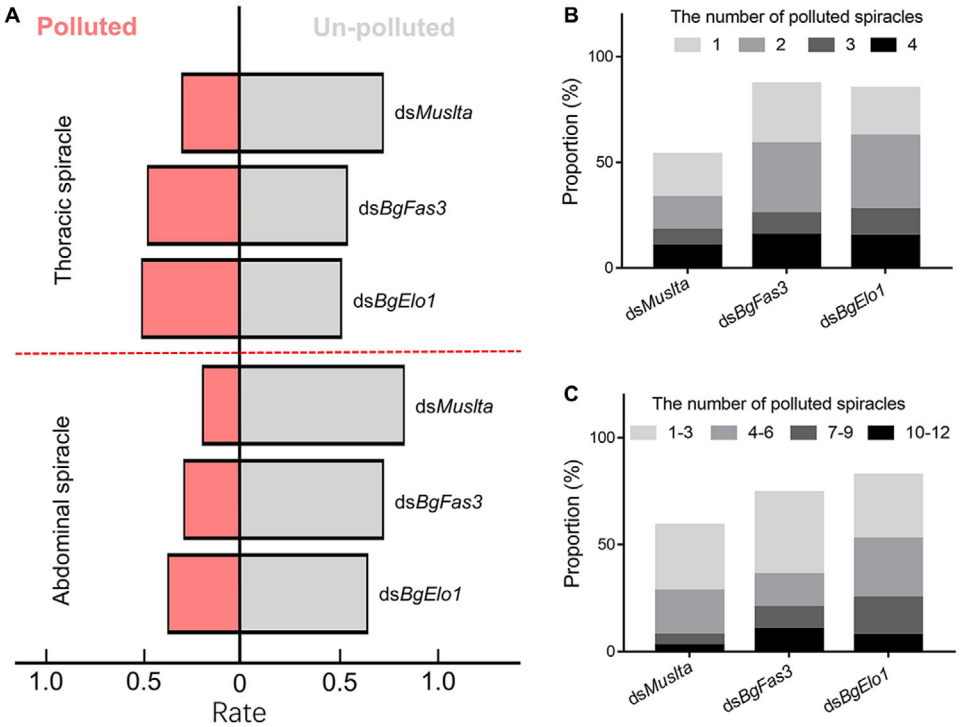

Fig. 8 The effect of *BgFas1* and *BgElo1* repression on the respiratory system with regard to inward waterproofing ability. (A) The proportions of polluted (light red) and un-polluted (light gray) spiracles were analyzed. For thoracic spiracles, data were calculated from 156 (ds*Muslta*), 156 (ds*BgFas3*), and 160 (ds*BgElo1*) spiracles. For abdominal spiracles, data were collected from 468 (ds*Muslta*), 468 (ds*BgFas3*), and 480 (ds*BgElo1*) spiracles. Pollution levels of the thoracic spiracles (B) and abdominal spiracles (C) in single cockroaches were analyzed. The proportions of cockroaches with different numbers of polluted spiracles are shown; the number of polluted spiracles is represented by different colors. Data were calculated from 39 (ds*Muslta*), 39 (ds*BgFas3*), and 40 (ds*BgElo1*) cockroaches.

with functional fatty acid elongases in *D. melanogaster* and *M. musculus*. In addition, the BgElo1 protein showed strong transmembrane properties and contained the conserved Elo domain with HXXHH and YXYY motifs. These data strongly suggest that *BgElo1* is a fatty acid elongase gene, as it fits the typical characteristics of elongase proteins (Jakobsson *et al*., 2006). Tissue expression analysis showed that *BgFas3* and *BgElo1* were both dominantly expressed in the integument, and were almost undetectable in the excretory organs, such as the gut and Malpighian tubules. This expression pattern suggests that *BgFas3* and *BgElo1* are unlikely to be involved in excretory water conservation. Furthermore, these results are consistent with those of previous studies on *D. melanogaster* and some other insects, which suggest that there are some integument-specific *FAS* and *ELO* genes, and some of these genes are involved in cuticular lipid generation (Chung *et al*., 2014; Wicker-Thomas *et al*., 2015; Li *et al*., 2019; Moriconi *et al*., 2019; Pei *et al*., 2019). Based on these findings, we conclude that both *BgFas3* and *BgElo1* are cuticle genes that may have important functions in the cuticle and its peripheral tissues.

RNAi of *BgElo1* significantly reduced the survival time of *B. germanica* in arid environments, but did not influence the survival rate under high RH conditions, indicating that these genes are involved in desiccation resistance. The calculation of weight loss rate in a dry environment revealed that repression of *BgElo1* significantly increased the weight loss rate, which largely reflected the increased water loss rate. These results prompted us to test the possible changes in cuticular lipids, as they play crucial roles in reducing cuticular evaporation of internal water (Blomquist *et al*., 1987). A large number of studies have reported the involvement of fatty acid elongases in cuticular lipid biosynthesis (Juárez, 2004; Chertemps *et al*., 2007; Li *et al*., 2019; Zhao *et al*., 2020). However, GC/MS analysis of the main cuticular lipid components, which function as waterproofing agents, showed that RNAi of *BgElo1* marginally increased the total amount of cuticular lipids. Moreover, the repression

of a previously studied FAS gene (*BgFas3*) showed a similar result that the desiccation resistance was severely affected. In our previous study, we found that *BgFas3* did not directly participate in HC biosynthesis (Pei *et al*., 2019). In this study, we designed another RNAi target and re-confirmed that *BgFas3* was indeed irrelevant to HC biosynthesis. We further found that RNAi of *BgFas3* significantly increased the amount of cFFAs in *B. germanica*. These results not only demonstrated that *BgFas3* and *BgElo1* were not involved in the biogenesis of the main groups of cuticular lipids, but also suggested that *BgFas3* and *BgElo1* may have functions in the generation of other lipids. Repression of *BgFas3* and *BgElo1* may provide more precursor substances used for the formation of cuticular lipids. However, it is worth noting that RNAi knockdown of *BgElo1* significantly decreased the amount of minor cFFA components the chain lengths of which were longer than C20; the downregulation of miner cFFA components cannot reveal the reason why the desiccation resistance is drastically affected, but it may partially imply the possible functions of *BgElo1* in VLCFA biosynthesis. Independent of the cuticular lipid layer, the internal cuticle structure is also important for water loss control. In this study, envelop, the outermost layer of insect cuticle was illegible. So, its thickness is difficult to compare, but its function in water retention is largely reflected by cuticular lipids that have been first analyzed in this study; Moreover, TEM analysis showed that neither *BgFas3*- nor *BgElo1*-RNAi influenced the thickness or structure of the epicuticle and procuticle, which suggests that *BgFas3* and *BgElo1* are largely excluded from maintaining cuticle permeability. Combined with the results of lipid analysis and TEM, we speculate that *BgFas3* and *BgElo1* may not be involved in cuticular water loss but may affect water retention via other routes.

By comparing the weight loss rates in the live and dead cockroach groups, we found that the difference among the differently treated live groups disappeared in the dead cockroaches. This suggests that the differences between the variously treated live cockroaches may be caused by air breathing through the respiratory system. Although we found that the blockage of breathing in dead cockroaches eliminated the difference in water loss rate, this conclusion was drawn largely based on the assumption that respiratory water loss was entirely blocked in dead cockroaches. We noted that 6 h after the implementation of the desiccation condition, the water loss rate of control cockroaches in the dead group was approximately 60% higher than that of the live group. Gibbs and Rajpurohit (2010) observed that killing of some insects, for example *D. melanogaster*, with organic solvent may cause the water loss rate to double, and they deduced that the significantly increased water loss rate was not only due to the removal of cuticular lipids, but also the opening of spiracles after organic solvent treatment in some species. However, we believe that the increased water loss rate in our study was not caused by the opening of spiracles. First, we killed the cockroaches by freezing, which is more moderate than the use of organic solvents; organic solvents kill the insects immediately but freezing is likely to halt insect respiration gradually. Second, the calculation of water loss rate during the prolonged drying time showed that the water loss rate increased. For example, within 12 h the water loss rate in the dead control cockroaches was approximately 1.5 times higher in the living cockroaches (data not shown), and this result is similar to that of a previous study (Appel & Tanley, 1999) where freshly killed *B. germanica* showed a water loss rate of 1.99%–2.33%/h in 24 h, which is about three times higher than that of living cockroaches in our study. If the increase of water loss rate in dead cockroaches is caused by the opening of spiracles, then the water loss rate will remain stable, and will not increase over time. Therefore, it is reasonable to assume that the spiracles are closed when the cockroaches freeze to death, and the increased water loss rate may be caused by decay.

Although we have found that the difference in the rate of water loss among the variously treated live cockroaches was eliminated by killing the cockroaches, we draw the conclusion cautiously. Future studies using a flow-through respirometry, which is able to measure respiratory water loss directly, should be performed to confirm this result (Gibbs *et al*., 2003); however, it was unavailable to us for use in this study. It has been shown that hydrophobic substances in insects are useful not only for serving as an outward barrier for preventing water loss, but also as an inward barrier to prevent unwanted water penetration (Blomquist & Bagnères, 2010). Therefore, the absence of lipid substances in the respiratory system, which is caused by the repression of *BgFas3* or *BgElo1*, may also affect the inward waterproofing ability. The results of our study verified that knockdown of *BgFas3* or *BgElo1* made cockroaches more susceptible to drowning and increased their recovery time from drowning. It might be caused by the leaking of water into the trachea through the spiracles, which hinder the ability of the cockroaches to breath fresh air; on the contrary, as the control cockroaches were able to keep their respiratory system unobstructed, they were able to receive oxygen shortly after they were pulled out of the water. And this assumption was verified by the leakage of water into the respiratory system which was further visualized with the Eosin Y dyeing experiment. The dye was able to only slightly penetrate the spiracles of the

control cockroaches. This result showed a little difference with *D. melanogaster* and the mealworm (*Tenebrio molitor*), in which Eosin Y could not stain the spiracles under temperature conditions below 50 °C (Wang *et al*., 2016). More importantly, we found that the knockdown of *BgFas3* or *BgElo1* via RNAi increased the intensity of the leakage of water-soluble substances; this was particularly so for the thoracic spiracles, as we found that they were much larger in size than the abdominal spiracles (Fig. S4), which might be the reason why thoracic spiracles were more easily affected. Similarly, after a 10-h recovery, analysis of hypoxia-associated genes showed a significant increase, which indicated that the prolonged recovery time was due to a lack of oxygen. Moreover, the water that leaked into the respiratory system may be difficult to remove immediately, resulting in cockroaches persistently suffering from inadequate oxygen intake. This could be why hypoxia-associated genes were upregulated after a recovery of 10 h. These results suggest that both *BgFas3* and *BgElo1* are involved in inward waterproofing of the respiratory system in the German cockroach.

In the present study, we provide strong evidence that *BgFas3* and *BgElo1* are involved in both inward and outward waterproofing of the respiratory system in *B. germanica*. In *D. melanogaster*, acetyl-CoA-carboxylase shows a similar function of watertightness, and its functions largely rely on the downstream oenocyte-specific FAS and fatty acid elongase genes, which generate putative VLCFAs that play vital roles in waterproofing the respiratory system (Parvy *et al*., 2012; Garrido *et al*., 2015; Wicker-Thomas *et al*., 2015). In our study, we found that *BgFas3* and *BgElo1* are enriched in the integument, but whether they are specifically expressed in spiracle-associated oenocytes needs further exploration. In addition, it is certain that FAS is involved in fatty acid synthesis, and fatty acid elongase further elongates fatty acids to VLCFAs (Semenkovich, 1997; Amanda *et al*., 2004). However, it is difficult to determine whether some specific VLCFAs or other derivatives of VLCFAs play hydrophobic roles in the spiracular respiratory system. Parvy *et al*. (2012) have shown that some VLCFAs are generated in oenocytes; however, the defective spiracles are caused by failed transport of lipids from spiracular glands to the point of air entry, and that unicellular glands are rich in smooth endoplasmic reticula, which is a key organelle in the biosynthesis of lipids (Jarial & Engstrom, 1995). These results suggest that the VLCFAs may be further processed in the spiracular glands before they are secreted into the respiratory system. Moreover, it has been shown by Jaspers *et al*. (2014) that a fatty acid reductase, Waterproofing, is able to reduce C24 and C26 VLCFAs to very long chain alcohols, which mediate hydrophobic coating of the tracheal envelope in *Drosophila*; however, it is unknown whether the alcohols are functional substances. Therefore, we speculate that VLCFAs do not function directly in respiratory watertightness. This biosynthetic pathway is important and may be conserved. Respiratory watertightness is crucial for both aquatic and terrestrial insects, and even in higher mammals. In addition, the insect spiracle is a main penetration route for some insecticides (Sugiura *et al*., 2008); therefore, the lipids in the spiracle may also play a role in insecticide resistance. More studies are required for further identification of potential downstream genes, such as fatty acid reductase and esterase genes, which generate alcohols and wax esters.

In summary, we identified a putative fatty acid-based pathway that plays crucial roles in water conservation in *B. germanica*. We performed a systemic analysis of the potential involvement of both *BgFas3* and *BgElo1* in different water loss routes. We provided evidence that *BgFas3* and *BgElo1* are irrelevant to cuticular or excretory water loss, and we further revealed that *BgFas3* and *BgElo1* are functional in preventing water loss in the respiratory system. Furthermore, we also found that the putative fatty acid-based pathway sustained by *BgFas3* and *BgElo1* is essential for preventing the entry of hydrotropic substances into the respiratory system in *B. germanica*. Our findings improve the understanding of mechanisms that restrict respiratory water loss in insects, and also provide a reference for how insects waterproof their spiracles.

Acknowledgments

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Disclosure

The authors have no conflicts of interest to declare.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 The response of *BgElo1* messenger RNA level to ds*BgElo1B*.

Fig. S2 Detailed changes of cuticular hydrocarbons for *BgFas3*- and *BgElo1*-RNAi.

Fig. S3 Initial and critical water content of differentially treated cockroaches.

Fig. S4 The position of spiracles and their connected tracheas in *Bllattella germanica*.

Table S1 Gene-specific primers used in this research.

Table S2 *BgElo1* messenger RNA sequence obtained in this study.