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BgFas1: A fatty acid synthase gene required for both hydrocarbon and cuticular fatty acid biosynthesis in the German cockroach, *Blattella germanica* (L.)

Xiao-Jin Pei[a], Nan Chen[a,b], Yu Bai[a,b], Jian-Wen Qiao[a], Sheng Li[b], Yong-Liang Fan[a,c,**], Tong-Xian Liu[a,*]

[a] State Key Laboratory of Crop Stress Biology for Arid Areas and Key Laboratory of Integrated Pest Management on Crops in Northwestern Loess Plateau, Ministry of Agriculture, Northwest A&F University, Yangling, Shaanxi, 712100, PR China
[b] Guangdong Provincial Key Laboratory of Insect Developmental Biology and Applied Technology and Institute of Insect Science and Technology, School of Life Sciences, South China Normal University, Guangzhou, 510631, China
[c] State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China

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ABSTRACT

Insect cuticular hydrocarbons (CHCs), the evolutionary products of aquatic hexapod ancestors expanding to terrestrial environment, are deposited on the surface of insect integument and originally functioned primarily as waterproofing agents. CHCs are derived from the conserved fatty acid synthesis pathway in insects. However, the pivotal fatty acid synthase (FAS) involved in hydrocarbon (HC) biosynthesis remains unknown in many insect orders including the primitive Blattodea. Here, we investigated functional *FAS* genes that modulate cuticular lipid biogenesis in the German cockroach, *Blattella germanica* (L.). Based on our full-length transcriptomic data and the available genomic data, seven *FAS* genes (*BgFas1–7*) were identified from *B. germanica*. Tissue-specific expression analysis revealed that *BgFas1*, *BgFas3*, *BgFas4* and *BgFas7* were highly expressed in the integument, whereas *BgFas2* was dominantly expressed in the fat body. *BgFas5/6* mRNA was almost negligible in the tested tissues. Systemic RNAi screen was performed against *BgFas1–7*, we found that only RNAi knockdown of *BgFas1* caused a dramatic reduction of methyl-branched HCs (mbHCs) and a slight decrease of straight-chain HCs (scHCs) for both internal and external HCs. Significant reduction of cuticular free fatty acids (cFFAs) was also detected within *BgFas1*-repressed cockroaches, while repression of *CYP4G19* resulted in dramatic increase of cFFAs. Moreover, we found that *BgFas1* mRNA levels were correlated with insect molting cycles, and could be induced by long-term mild dryness treatment. Furthermore, desiccation assay revealed that *BgFas1* suppression accelerated water loss and led to early death of cockroaches under desiccation. Our results indicate that *BgFas1* is necessary for both HC and cFFA biosynthesis in *B. germanica*. In addition, our study also confirms that cuticular lipids, particularly mbCHCs, are critical for desiccation resistance in *B. germanica*.

1. Introduction

Excessive water loss is fatal to all organisms, including insects. One major cause of water loss in insects is via evaporation from the cuticle (Hadley, 1994). Because of their small body size and therefore large surface area to body volume ratio, regulation of water loss is critical for insect survival. In order to overcome this defect, insect ancestors have evolved a layer of cuticular lipids during the process of expansion to terrestrial environment (Gibbs, 1998). Insect cuticular lipids comprise a series of aliphatic compounds, such as long-chain hydrocarbons (HCs), free fatty acids (FFAs), wax esters, sterol esters, alcohols, aldehydes and ketones (Blomquist et al., 1987). Insect cuticular lipids especially HCs primarily function as waterproofing agents, and are critical for terrestrial insect survival. However, they also play important roles in resisting chemical xenobiotics, protecting insects from entomopathogen invasion or ultraviolet radiation, and are also informative messengers because

* Corresponding author.
** Corresponding author. State Key Laboratory of Crop Stress Biology for Arid Areas and Key Laboratory of Integrated Pest Management on Crops in Northwestern Loess Plateau, Ministry of Agriculture, Northwest A&F University, Yangling, Shaanxi, 712100, PR China.
E-mail addresses: xiaojinpei@nwafu.edu.cn (X.-J. Pei), nanchen@m.scnu.edu.cn (N. Chen), yubai@nwafu.edu.cn (Y. Bai), jianwen.qiao@nwafu.edu.cn (J.-W. Qiao), lisheng@scnu.edu.cn (S. Li), yfan@nwafu.edu.cn (Y.-L. Fan), txliu@nwafu.edu.cn (T.-X. Liu).

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many of them play complex roles in insect intraspecific or interspecific chemical communications (Howard and Blomquist, 2005; Golebiowski et al., 2011).

Cuticular hydrocarbons (CHCs), as essential waterproofing components, are commonly presented and species-specific in insects. Insect HCs are a blend of straight or methyl-branched long-chain alkanes and alkenes. They are produced in specialized secretory oenocytes (Makki et al., 2014; Fan et al., 2003), deposited into internal organs including the fat body, ovary, and hemolymph (Sevala et al., 1999; Gu et al., 1995), or transported to the epicuticle and oocytes by high-density lipophorin (Schal et al., 1998; Fan et al., 2002). Previous studies on HC biosynthesis were mainly conducted with labeling–tracing technology. Although part of the metabolic pathway for HC biosynthesis within insects has been identified, many genes involved in HC biosynthesis remain unknown (Ginzel and Blomquist, 2016; Blomquist et al., 1987). Studies showed that insect HCs originate from the conservative fatty acid synthesis pathway and involve two important enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). ACC catalyzes the biosynthesis of malonyl-CoA, whereas FAS catalyzes the production of C14, C16, and C18 fatty acyl-CoAs (Dembeck et al., 2015; Wicker-Thomas et al., 2015; Chung et al., 2014). The long-chain fatty acyl-CoA can be desaturated by specific desaturases for further generating of alkenes (Chertemps et al., 2006; Ueyama et al., 2005; Dallerac et al., 2000), or be elongated by several cycles of incorporating malonyl-CoA units which catalyzed by a rate-limiting elongase and cooperated with three other enzymes (Urbanski et al., 2010; Chertemps et al., 2007; Juárez, 2004). This cyclic four-step action generated very long-chain fatty acyl-CoAs can be further reduced to long-chain alcohols by fatty acyl reductases (FARs) and converted to HCs by the P450 oxidative decarbonylase 4G subfamily CYP4Gs (MacLean et al., 2018; Qiu et al., 2012). Study of HC biogenesis pathways in the fruit fly *Drosophila melanogaster* model has been intensive, however, much less is known for other insects.

Cuticular free fatty acids (cFFAs) are not as widely studied as CHCs within insects, but it is undeniable that cFFAs play effective roles in insect stress tolerance (Cerkowniak et al., 2013). Golebiowski et al. (2013, 2008) analyzed the cFFA profiles of several lepidopteran and dipteran insects and revealed both antifungal and antibacterial activities for different cFFAs. Because of their excellent antimicrobial properties, some fatty acids have even been considered for using as antimalarial, antibacterial, or antifungal agents (Golebiowski et al., 2011; Carballeira, 2008). In addition to their antimicrobial ability, cFFAs may be also involved in the prevention of insecticide penetration, due to their rapid increase following induction of chlorpyrifos in *B. germanica* (Paszkiewicz et al., 2016). It is definitive that cFFAs are generated from internal organs, and then transported and deposited on the insect epicuticle; however, the expression site of *FAS* is tissue dependent (Chung et al., 2014). The question of whether cFFAs originate from oenocytes or fat bodies remains unknown in insects.

Fatty acid synthase (FAS) is a central enzyme in *de novo* lipogenesis, which produces multiple fatty acids, and are used as the precursors for other functional lipids (Stanley-Samuelson et al., 1998; Semenkovich, 1997). In insects, *FAS* is primarily expressed in the fat body and oenocytes which parallel with adipose tissues and the liver in mammals (Parvy et al., 2012; Gutierrez et al., 2007). Differentially expressed *FAS* may be functionally differentiated, as shown by Chung et al. (2014). There are three distinct *FASs* in *D. melanogaster*, FAS^{CG3524}, FAS^{CG3523}, and $FAS^{CG17374}$. FAS^{CG3524} is expressed in oenocytes and specialized for 2-methyl-branched CHC (mbCHC) synthesis. $FAS^{CG17374}$ is also expressed in oenocytes, but is usually irrelevant with HC biosynthesis, even though RNAi-mediated knockdown of $FAS^{CG17374}$ caused a defect for water tightness within the tracheal system (Garrido et al., 2015). In contrast, FAS^{CG3523} is specifically expressed in fat bodies and modulates the accumulation of triacylglycerols (TAGs); however, this could be sacrificed and meet some HC provisions under less than optimal conditions (Wicker-Thomas et al., 2015). Previous work has demonstrated that the methyl group in mbHCs is formed during the process of long chain fatty acid biosynthesis, which uses methyl-branched primers to start the biosynthesis of 2-methyl branched fatty acid precursors, or incorporates methylmalonyl-CoA during the chain elongation to synthesize 3-methyl or internal branched fatty acid precursors (Ginzel and Blomquist, 2016; Chase et al., 1990; Dillwith et al., 1982; Blailock et al., 1976). Studies on *B. germanica*, *Musca domestica,* and *Triatoma infestans* all confirm that there is a microsomal FAS protein capable of adding methylmalonyl-CoA onto methyl-branched fatty acids more effectively than a cytosolic FAS (Juárez et al., 1996, 1992; Blomquist et al., 1995). However, the specific *FAS* gene involved in mbCHC or scCHC biosynthesis has not been thoroughly studied in insects.

Blattella germanica (L.) is an important model for studying insect ecology, physiology and biology. In this study, we took advantage of its high sensitivity to RNAi (Lin et al., 2017) and performed a systemic RNAi screen for functional *BgFas* genes. We found RNAi knockdown of *BgFas1* caused a dramatic decrease of mbHCs as well as a significant reduction of scHCs and cFFAs, which led to accelerated water loss and early death for *B. germanica* under desiccation. Because of its excellent capacity for insect water balance, *BgFas1* could be regarded as a novel molecular target for cockroach management.

2. Materials and methods

2.1. Insects

A well-established strain of *B. germanica* originally collected in Shanghai and maintained in the laboratory since the 1970s was used in this study. *B. germanica* were reared with rat chow and water in plastic jars at 30 °C with ~70% relative humidity (RH) under a 12: 12 h light–dark photoperiod. Newly hatched nymphs were collected and transferred to new rearing-containers within 12 h to synchronize development. Freshly emerged fifth-instar nymphs or adults were collected within 12 h, and reared for several additional days if needed. Two-day-old fifth-instar nymphs (N5D2) were sexed by observing the female genital plate before administration of dsRNA injection.

2.2. Identification and analysis of BgFas genes

Searching for *FAS* candidate sequences was performed using tBLASTX (Altschul et al., 1990) against our full-length transcriptome data (NCBI accessions: SRR9143014 and SRR9143013) and the recently published *B. germanica* genomic data (Harrison et al., 2018). *FAS* homolog sequences from *D. melanogaster* (Chung et al., 2014) were submitted as query sequences. The *FAS* candidate sequences with *E*-values lower than 1.00E-20 were accepted and further verified by RT-PCR and re-sequencing. Briefly, the first strand cDNA was synthesized using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) with total RNA as templates and Oligo dT as the primers. Several pairs of target specific primers (Table. S1) were synthesized to amplify the complete coding sequences (CDS) using the first-strand cDNA as templates, and the high-fidelity PrimeSTAR GXL DNA Polymerase reagent (Takara, Dalian, China) was used in our amplification experiments according to the supplier's protocol. PCR-amplified fragments were directly purified and supplied for sequencing. Based on our re-sequencing results, the open reading frame (ORF) of each *BgFas* gene was predicted by ORF Finder (http://www.bioinformatics.org/sms2/orffind.html) and translated into protein sequences by ExPASy online translation tools (https://web.expasy.org/translate/). SMART tools (http://smart.embl-heidelberg.de) were used to analyze the deduced protein sequences to identify conserved functional domains. In addition, to identify the gene structure of *BgFas* genes, complete coding sequences (CDS) were mapped to the genomic data (GeneBank: PYGN00000000.1) using BLASTN with *E*-values < 1.00E-30 (Altschul et al., 1990), and the boundaries between exons and introns were determined by the GT-AG rule.

2.3. Phylogenetic analysis of FAS genes

To calculate the genetic relationship of *FAS* genes between *B. germanica* and other representative insects (from different orders), a phylogenic tree was constructed with the FAS protein sequences using MEGA 7.0 software (Kumar et al., 2016). FAS protein sequences for *B. germanica* were obtained as described above and other FAS sequences were downloaded from the NCBI database (accession numbers of these proteins were listed in Table. S2). Sequence alignments were generated with ClustalW, and poorly aligned terminal amino acid sequences were eliminated by the online Gblocks Server (http://www.phylogeny.fr/one_task.cgi?task_type=gblocks) with the most liberal running parameters. Phylogeny was analyzed through the statistical method of neighbor joining and tested by a bootstrap method with 1000 replications.

2.4. Tissue dissection and quantitative PCR analysis

Two-day-old female adults were anesthetized with carbon dioxide, and tissues or body parts including head, thorax, abdominal integument, fat body, gut and ovary were dissected in phosphate buffered solution (PBS) at 4 °C. The intact head and thorax were excised directly, the entire gut and pair of ovaries were isolated followed by two rinses of PBS to remove hemolymph and fat bodies. Sampling of the fat body was carefully performed in order to collect only free fat bodies. The abdominal integument was dissected with the basal membrane attached, but fat bodies tightly associated with the basal membrane were removed as cleanly as possible. Newly collected samples were immediately stored at −80 °C until being used for RNA extraction.

Total RNA was isolated using RNAiso Plus Reagents (Takara, Dalian, China) according to the supplier's protocol. Digestion of genomic DNA and reverse transcription of 800 ng of total RNA were performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Tissue distribution for *BgFas* transcripts were investigated by quantitative PCR (qPCR). Gene-specific qPCR primers with appropriate amplification efficiency (0.95–1.05) were screened by cDNA gradient-based standard curves (Table. S1). All qPCR analyses were performed with three to five biological replicates and technical triplicates. The PCR reaction of 20 μL contained 10 μL of TB Green™ Premix Ex Taq™ (Tli RNase H Plus) (Takara, Dalian, China), 8 μL of cDNA diluted 1:50, and 0.5 μM each of the forward and reverse primers. Thermal cycling and fluorescence detection were performed on a LightCycler 480 system (Roche, Basle, Switzerland) under the control of a 2-step program: 94 °C for 3 min, followed by 45 cycles of 94 °C for 10 s and 61 °C for 30 s. Relative transcription levels of target genes were normalized by the commonly used reference gene *actin5c* (GeneBank: AJ862721.1) (Kamsoi and Belles, 2019; Süren-Castillo et al., 2012) and calculated using the $2^{-\Delta\Delta Ct}$ method when only use one reference gene (Livak and Schmittgen, 2001). The validity of the reference gene *actin5c* was confirmed by using two reference genes, *actin5c* and another reference gene *EF1A* (GeneBank accession: KX228232) as reported by Lin et al. (2017), the results were calculated from the means of both ΔCt determinations. These results were shown in Fig. S1.

2.5. RNA interference

For RNAi knockdown of *BgFas* genes, different RNAi targets were designed based on our re-sequenced results. Gene-specific fragments with the length of 300–500 bp as well as a heterologous fragment from the *Mus musculus* (*Muslta*) as a control were cloned into the pMD™ 19-T Vector (Takara, Dalian, China), recombinant plasmids were transformed into DH5α competent cells (Takara, Dalian, China) and proliferated for sequencing. In order to confirm the function of *BgFas1* in HC biosynthesis, another none overlapping RNAi target (ds*BgFas1B*) specific to *BgFas1* was also cloned. The plasmids containing consistent fragments with RNAi targets were extracted from the monoclonal culture and stored at −20 °C until usage in dsRNA synthesis. Primers with the T7 promoter sequence used to amplify the templates for single-stranded RNA (ssRNA) synthesis are listed in Table. S1. According to the manufacturer's protocol, reverse and forward ssRNAs were transcriptionally synthesized with T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA), and dsRNA were generated by annealing of complementary ssRNA. The newly synthesized dsRNAs were purified and loaded onto a 1% agarose gel and electrophoresis was performed to confirm their identity and integrity. All dsRNA was sub-packaged and stored at −80 °C after being diluted to a working concentration of 2000 ng/μL.

For dsRNA delivery, both N5D2 male and female cockroaches were anesthetized with carbon dioxide, and 2 μL of dsRNA (4 μg) specific to either *BgFas* or *Muslta* was directly injected into the abdomen using a manual syringe equipped with pulled glass capillary tubes as micro-injectors. These cockroaches were sampled 2, 4, 6, and 8 days following the first injection to monitor RNAi efficiency. Based on the persistence of RNAi, the treated cockroaches were boosted with a second injection containing the same dose of dsRNA 6 days later. RNAi efficiency was tested again at 14 days post the first injection. For specific analysis of the effect of *BgFas2* on TAG contents, N1D4 cockroaches were used for micro-injection, and 414 ng of dsRNA was injected using a Nanoject II micro-injector (Drummond Scientific, Broomall, PA, USA). After 6 days, TAG contents were determined with a Triglycerides Assay Kit (Nanjing Jiancheng Institute, Nanjing, China) based on the GPO-PAP method (Annoni et al., 1982).

2.6. Extraction and quantification of HCs

B. germanica CHCs were extracted from two-day-old adults following the procedure described by Young et al. (2000), with slight modifications. Briefly, cockroaches were sacrificed by freezing at −20 °C overnight. During the CHC extraction, frozen cockroaches were thawed and immersed in 1 mL of hexane containing 15 μg of *n*-hexacosane as an internal standard followed by gentle shaking for 5 min. The hexane extract was transferred to a new chromatographic vial, and this extraction was repeated again (without *n*-hexacosane). Finally 1 mL of hexane was used for cleaning both the cockroach and vial. Then all solvent extracts were pooled together as cuticular lipid extract. Internal HCs were further extracted from those obtained cockroach remains according to Fan et al. (2002), with slight modifications. The remains were homogenized with a solution of hexane–methanol–ddH_2O (2:1:1 mL) in a glass homogenizer containing 30 μg of *n*-hexacosane for quantification of internal HCs. The homogenate was vigorously vortexed for 5 min and then centrifuged at 2000 *g* for 10 min. One milliliter of the hexane phase supernatant was transferred to a new vial as an internal lipid extract. Both internal and external lipid samples were reduced to ~300 μL with a gentle stream of N_2 and loaded onto mini Pasteur pipette columns packed with ~500 mg of 70–230 mesh silica gel (Sigma-Aldrich, Louis, USA), which had been activated at 120 °C. Finally, the HC fractions were eluted with 7 mL of hexane, and reduced to ~2 mL for chromatography analysis.

HCs were analyzed by a TRACE 1310 gas chromatograph (GC) coupled to an ISQ single quadrupole mass spectrometer (MS) (GC–MS, Thermo Scientific, Waltham, USA). Splitless injection of 1 μL of either the HC samples or C_7–C_{40} normal alkanes (Sigma-Aldrich, Louis, USA) were separated by a 30 m × 0.32 mm × 0.25 μm HP-5MS UI capillary column (Agilent Technologies, Santa Clara, CA, USA) using helium as the carrier gas with a flow of 1 mL/min. Injector and detector temperatures were set at 280 °C and 300 °C, respectively. GC oven temperature progress was set as follows: 60 °C for 2 min, heated to 200 °C at a rate of 30 °C/min, increased 5 °C/min to 320 °C, followed by 10 min hold. The mass detector was set to an EI mode with an electron impact ionization potential energy of 70 eV, and the scan range was 45–650 m/z at a rate of 5 scan/s. Data collection and quantitative analysis of CHCs were performed with the Xcalibur 2.2 software.

2.7. Extraction, derivatization and analysis of FFAs

Both cuticular and internal FFAs were extracted from two-day-old dsRNA-treated male cockroaches, and cFFA samples were prepared according to Paszkiewicz et al. (2016), with slight modifications. Each sample containing two freezing killed males was transferred to a vial. The first extraction was performed with 1 mL of petroleum ether in which 5 μg of tridecylic acid (Sigma-Aldrich, Louis, USA) was added as an internal standard. Vials containing cockroaches were gently stirred with a solvent for 1 min and sucked out with glass pipettes as extracts I. The same cockroach samples were subjected to a second extraction with 1 mL of dichloromethane (Aladdin, Shanghai, China) for 5 min, and these dichloromethane extracts were combined with extracts I and designated as cuticular lipid samples. All extracts were evaporated and used for subsequent methyl esterification. In short, 1 mL of 1% (v/v) H_2SO_4 in methanol was added to the extracts and incubated in an 80 °C water bath with a N_2 atmosphere for 2 h. The sulfuric acid catalysis derived fatty acid methyl esters (FAMEs) were isolated by adding 1 mL of saturated sodium chloride solution and then extracted twice with 1 mL of *n*-hexane. Then the organic phase was transferred into a new vial, dried and resuspended in 500 μL of dichloromethane for subsequent GC–MS analysis. The cuticular lipids-depleted cockroaches were subjected to internal FFAs extraction and derivatization followed by a one-step method as described by Lepage and Roy (1984). A single cockroach was homogenized and directly transmethylated in 2 mL of 1% H_2SO_4 in methanol containing 100 μg of tridecylic acid as an internal standard. The reaction conditions and methods for FAMEs extraction were the same as described above.

A FAME-capable polar chromatographic column, 30 m × 0.32 mm × 0.25 μm TR-WAXms (Thermo Scientific, Waltham, USA), was installed in GC–MS to analyze different FAMEs. The column temperature programming was set as follows: 60 °C for 3 min, heated to 160 °C at a rate of 10 °C/min, increased by 3 °C/min up to 240 °C, followed by a 5 min hold. The inlet and MS transfer line were set at 250 °C, ion source was set at 280 °C, and mass detector scan range was set at 35–500 m/z. Other parameter settings were the same as those for HC determination. Qualitative analysis for FAME peaks was performed by comparing the retention times with those of the Supelco 37 Component FAME Mix additional standard (Sigma-Aldrich, Louis, USA).

2.8. Effect of desiccation stress and molting on BgFas1 expression

We studied whether *BgFas1* mRNA could be induced by some conditions that likely to involve cuticular lipid biosynthesis. Firstly, we reared cockroaches in either ~30% RH or ~70% RH for > 1 year, and then *BgFas1* mRNA levels of N6D2 (2-day-old sixth-instar) female cockroaches were compared by qPCR. In addition, we investigated whether *BgFas1* could be induced by short-term but extreme dehydration stress. Accordingly, we held N6D2 female cockroaches in conditions of either ~5% RH or ~70% RH, supplied with ~1 g of rat chow but without water. Expression levels of *BgFas1* between the two groups were compared 24 or 48 h later.

To study the *BgFas1* expression profiles during two complete molting cycles, the newly emerged fifth- or sixth-instar nymphs reared at 70% RH were collected within 4 h and caged in new plastic jars. Cockroaches were sampled every 48 h and newly emerged or two-day-old female adults were also collected. To analyze the *BgFas1* expression levels, the whole abdomens of harvested cockroaches were immediately removed and stored at −80 °C. Total RNA was extracted from two intact abdomens for each sample. The relative expression level of *BgFas1* was monitored by qPCR.

2.9. Desiccation assay

For the desiccation assay, we employed a method of silica gel dehumidification (Marron et al., 2003). Briefly, an aliquot of 150 g silica gel desiccant activated at 120 °C was parceled with double gauzes and placed in ~900 mL plastic jars which were then sealed by fitted covers and maintained at 30 °C. The RH inside the jars was monitored by HOBO Pro v2 (Onset, Bourne, USA) and dropped to 5% within 2 h. To judge the desiccation tolerance for different dsRNA treated cockroaches, each 10–20 of two-day-old females or males were separately added into the jars. Approximately 1 g of rat chow was supplied but without water, and each treatment group contained at least 90 cockroaches. The survival rate was recorded every 4 h until all the cockroaches died. Death was confirmed by tapping wall of the jar. If the cockroaches did not respond, then they were considered dead. To calculate the weight loss rate of differentially treated cockroaches under desiccation, the experiment was repeated. Cockroaches were weighted at the beginning of the experiment as well as 72 h after being subjected to the extreme dry environment. Dead cockroaches were not taken into consideration during weight loss calculations.

3. Results

3.1. Sequence analysis of BgFas genes

In our study, we obtained a total of seven *BgFas* genes in *B. germanica*. The CDS were submitted to the Genebank database (GeneBank accession numbers: MK605588–MK605594), and the gene locations, exon-intron structure, nucleic acid sequences and putative amino acid sequences were listed in supplementary materials 2. The CDS of different *BgFas* genes were translated into amino acid sequences, and their functional domains speculated by SMART tools are shown in Fig. 1A. *BgFas1*, *BgFas2* and *BgFas7* contain all seven of the functional domains required for FA biosynthesis, including β-ketoacyl synthase (KS), acetyl transferase (AT), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), phosphopantetheine-binding domain (PB), and thioesterase (TE). However, *BgFas3*, *BgFas4*, and *BgFas5* lacked PB domain, and *BgFas6* was missing the TE domain. The phylogenetic analysis results revealed that different FAS protein sequences were grouped into five clusters (Fig. 1B). Combined with our protein analysis results we found that the FAS proteins were grouped according to their domain structures. Most FAS proteins within clusters I and II have complete domains, but FAS within clusters III-V all had certain missing domains. The FAS in cluster III was lacking an AT domain, with a missing TE domain or irregular domains in cluster IV and a missing PB domain in cluster V. Moreover, we found that clusters I and II were branched into two groups even though they possessed similar domain structures, and Cluster I contained only one FAS from each of the representative insects except for *D. melanogaster*, but cluster II did not. Similarly, *BgFas1*, *BgFas2*, and *BgFas7* all had complete domains, but *BgFas1* and *BgFas7* were grouped into cluster II and distinguished from *BgFas2,* which was designated to cluster I. This difference indicates that the biological functions of these *BgFas* genes may be specialized.

3.2. Tissue expression profiles of BgFas genes

In order to provide some reference for the functional study of *BgFas* genes, we analyzed the gene expression patterns in different tissues and body segments. The expression levels of different genes among different body sites varied significantly (Fig. 2; $F_{41,\ 126} = 25.34$, $P < 0.001$). *BgFas1*, *BgFas3*, *BgFas4* and *BgFas7* were noted to have high expression in the integument and with extreme low expression in other tested tissues. The most highly expressed *BgFas* genes in the integument and fat body were *BgFas3* and *BgFas2*, respectively, although *BgFas2* was also highly expressed in other tissues especially in the integument and thorax. The *BgFas5* and *BgFas6* transcripts were very weak but still detectable in the fat body or integument. The tissue-dependent expression of different *BgFas* indicates their function may be specialized in the integument or fat bodies.

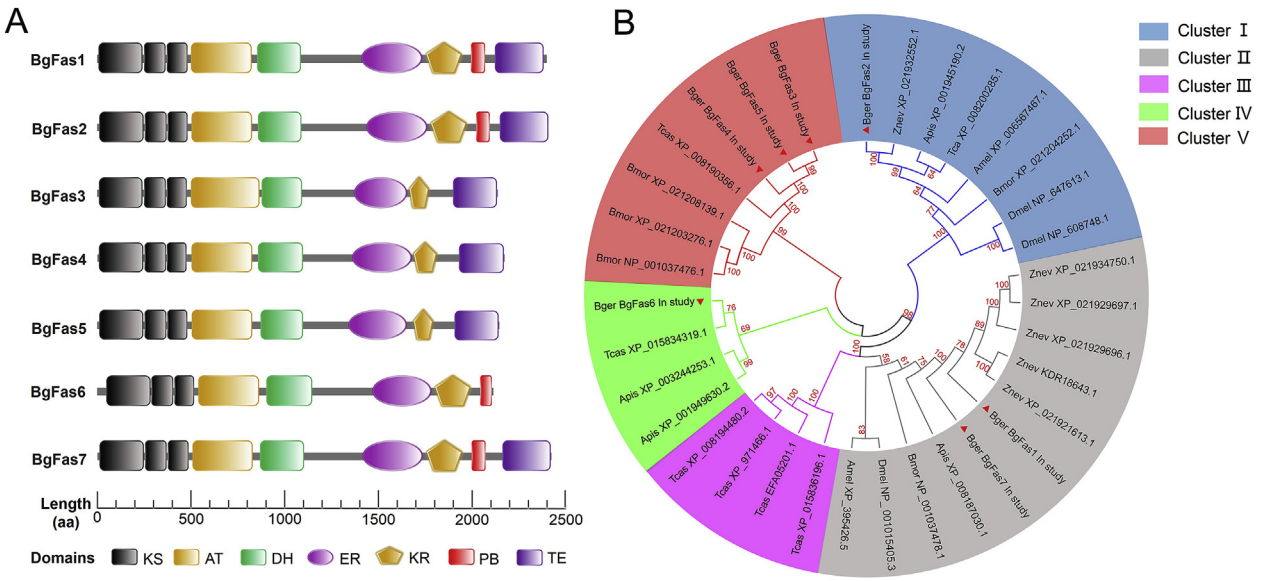

Fig. 1. Sequence analysis. (A) Domain structure analysis of BgFas protein. BgFas protein sequences are deduced by the ExPASy translation tool, and domains are predicted by the SMART tool. The different domain names are abbreviated as follows: β-ketoacyl synthase (KS), acetyl transferase (AT), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), phosphopantetheine-binding domain (PB) and thioesterase (TE). The lengths of different amino acid sequences are measured according to the scale. (B) Phylogenetic classification of FAS proteins in some representative insects. Different FAS protein sequences are grouped into five clusters, which were assigned with different colors. Red numbers at the nodes are bootstrap values. Different sequences are indicated by the abbreviations of the corresponding scientific names followed by their gene names or accession numbers. The *BgFas* sequences studied in our study are indicated by solid red triangles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. *BgFas1* modulates CHC biosynthesis

To identify which *BgFas* gene is functional during cuticular lipid biogenesis, we knocked down the transcripts of different *BgFas* genes using an RNAi approach. Considering *FAS* as an upstream distant gene in HC synthesis, we used a double-injection RNAi strategy to realize long-term repression of *BgFas* function. Firstly, we monitored inhibition of *BgFas1* expression every two days after the first dsRNA injection (Fig. S2A). The highest RNAi efficiency was 80% which appeared 4 days after dsRNA injection; however, the repressed mRNA level started to rebound on day 6 after the first injection, and a faster recovery of target mRNA levels were detected on day 8, so a second injection was performed on day 6 post the initial dsRNA delivery. Based on a double-injection RNAi strategy, the significant down-regulation of *BgFas* genes (all with *P*-values < 0.01) could persist for at least 14 days (Fig. 3A).

The CHCs of female cockroaches with different *BgFas* genes knocked-down were analyzed by GC–MS. We found that only knockdown of *BgFas1* by ds*BgFas1* or ds*BgFas1B* resulted in a ~60% reduction of CHCs (Fig. 3B and Fig. S3). RNAi of *BgFas2*, *BgFas4*, *BgFas5* and *BgFas6* all led to a slight but statistically significant increase in CHC levels ($P < 0.01$ or $P < 0.05$), and RNAi knockdown of *BgFas3* or *BgFas7* also presented an increasing trend. The detailed content of

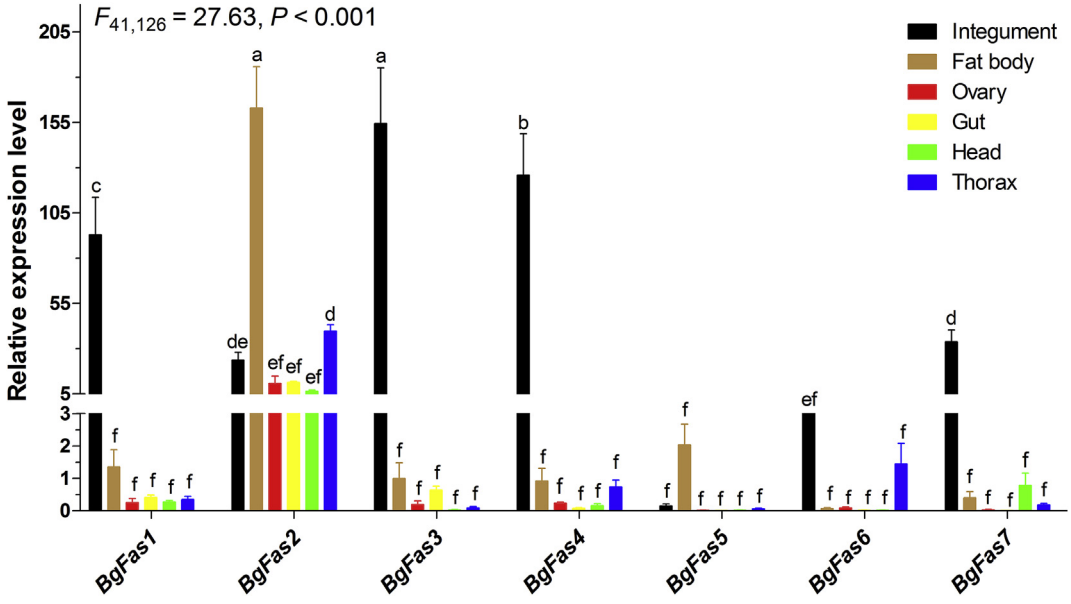

Fig. 2. Tissue expression analysis of different *BgFas* genes. Relative expression levels of seven *BgFas* genes across the integument, fat body, ovary, whole gut, head, and thorax from two-day-old females were detected by qPCR. All data are shown as means ± SE and calculated from four biological replicates. Lowercase letters above the error bars indicate significant differences in corresponding expression levels (ANOVA, LSD, $P < 0.05$).

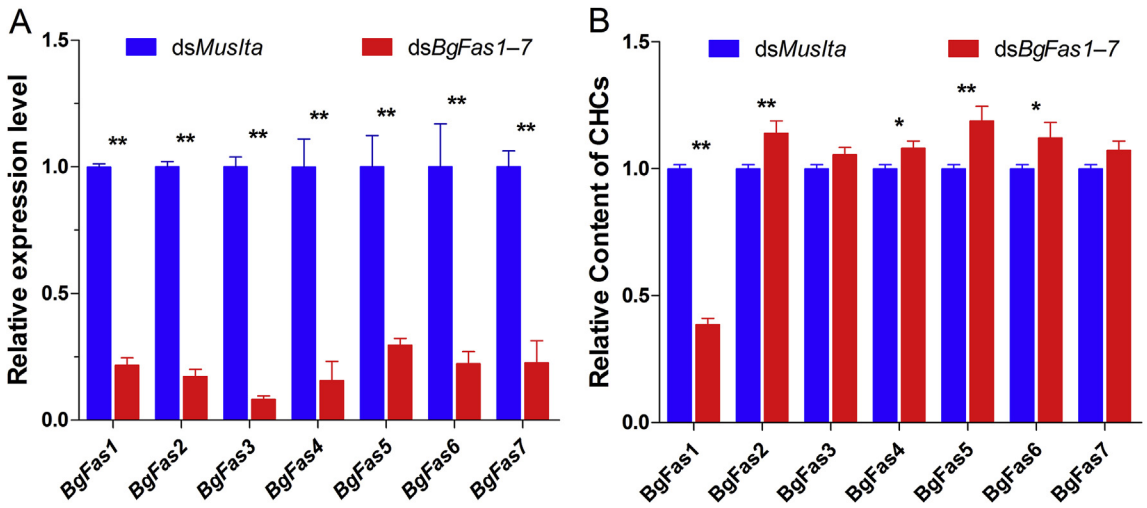

Fig. 3. RNAi screen of different *BgFas* genes involved in CHC synthesis. (A) RNAi efficiency of different *BgFas* genes. Based on a double-injection strategy of RNAi, response of different *BgFas* genes to their corresponding dsRNA was detected on day 14 following the first injection. Relative expression levels for treatments (ds*BgFas1–7*) or for controls (ds*Muslta*) were detected by qPCR using *actin5c* as a reference gene. All data were calculated from three to five biological replicates and shown as means ± SE. ** represents a significant difference at $P < 0.01$ (Student's t-test). (B) Effect of *BgFas1–7* RNAi on the CHCs of female German cockroaches. Relative CHC contents from two-day-old female cockroaches are shown as means ± SE. *$P < 0.05$ and **$P < 0.01$ with Student's *t*-test; n = 10 or 14.

individual CHCs from different dsRNA-injected female cockroaches could be found in Table. S3.

3.4. BgFas1 primarily modulates mbHC biosynthesis

CHCs of both female and male cockroaches after ds*BgFas1* injection were analyzed by GC–MS. Representative chromatographic results from female cockroaches are shown in Fig. S4. GC peaks were annotated in Table. S3, and a consistent quantitative result was presented in Fig. 4D. In total, thirty different CHCs with chain lengths of C27–C32 were detected. HCs with a chain length of 27 or 29 made up a bulk of the composition. All CHCs were saturated, and primarily methyl-branched with only three different types of chain length *n*-alkanes (*n*-C_{27}, peak 1; *n*-C_{28}, peak 7; and *n*-C_{29}, peak 16). For simplification, total amount of CHCs were divided into mbCHC and scCHC fractions (Fig. 4A). We found that the amount of mbCHCs in *BgFas1*-RNAi-treated males or females were dramatically decreased (*P*-values < 0.01), albeit only a slight reduction in scCHCs was detected (*P*-values < 0.01). This difference was also reflected in the percentage fold change as shown in Fig. 4B. The percentages of mbCHCs in *BgFas1*-RNAi-treated males or females were significantly decreased (*P*-values < 0.01) and were increased by two-fold for scCHCs (*P*-values < 0.01). Internal HCs from *BgFas1*-RNAi-treated females showed a similar result (Fig. 4C and Fig. S5). These data strongly suggest that *BgFas1* modulates the HC biosynthesis and shows a preference for regulating mbHC biosynthesis.

3.5. Suppression of BgFas1 reduces cFFAs

As FFAs are the second most abundant cuticular lipids in *B. germanica* (Paszkiewicz et al., 2016) and also the pivotal precursors in HC biosynthesis, we verified the effect of *BgFas1*-RNAi on FFAs. RNAi knockdown of *BgFas1* caused a significant reduction of cFFAs but did not change the amount of internal FFAs (Fig. 5A and Fig. 5B). To further clarify the influence of *BgFas1*-RNAi on cFFAs, we studied the relationship between CHC and cFFA biosynthesis. As CYP4Gs are usually the terminal genes in the HC biosynthesis pathway, they may be more specific to HC biosynthesis. We performed systemic RNAi knockdown against the only CYP4G gene (*CYP4G19*) within *B. germanica* (Fig. S2B). Our previous research has identified that *CYP4G19* is involved in HC biosynthesis (Chen et al., 2019), and in this study we found that blocking of CHCs by *CYP4G19*-RNAi resulted in a two-fold up-regulation of cFFAs (Fig. 5A). This indicates that CHC and cFFA formations compete for resources during their genesis in *B. germanica*.

BgFas2 was the only *FAS* gene that was dominantly expressed in fat bodies, we therefore studied the function of *BgFas2* in FFAs or TAGs biosynthesis. Indeed, knockdown of *BgFas2* led to a 32% reduction of internal FFAs as well as a 57% reduction of TAGs (Fig.5B and Fig. S6). But at the same time, we found that *BgFas2* suppression had no significant influence on cFFAs (Fig. 5A). This indicates that *BgFas2* mainly mediates internal lipid biosynthesis in *B. germanica*. The detailed fatty acid components from both internal tissues and the cuticular surface after ds*BgFas1*, *BgFas2*, or ds*CYP4G19* injections are shown in Table. S4.

3.6. Effect of desiccation or molting on BgFas1 expression

To further study the function of *BgFas1* in HC biosynthesis, we analyzed the expression levels of *BgFas1* under different patterns of desiccation treatments. A long-term adaption of slight dehydration (30% RH) resulted in 40% higher expression of *BgFas1* as compared to that of cockroaches reared under conditions of 70% RH (Fig. 6B), but *BgFas1* could not be induced by 24 or 48 h of precipitous dryness (5% RH) treatment (Fig. 6A). As cuticular lipids are largely translocated during molting, we investigated the expression patterns of *BgFas1* within the last two molting cycles (Fig. 6C). We found that the *BgFas1* gene was slightly expressed in newly molted cockroaches, but expression exploded two days later and then dropped consistently till the next molting cycle.

3.7. Effect of BgFas1-RNAi on desiccation resistance

To investigate the role of *BgFas1* in water protection we subjected the ds*BgFas1*-injected cockroaches under desiccation conditions. We found that RNAi knockdown of *BgFas1* led to an early death for both males and females. As shown in Fig. 7A and Fig. 7B, there was a significant difference in the survival rates between *BgFas1*-suppressed cockroaches and controls under desiccation (*P*-values < 0.0001, Mantel–Cox test). The ds*BgFas1*-injected cockroaches were died earlier than controls, with the median survival time significantly reduced from 108 h to 76 h in females and from 76 h to 48 h in males. Furthermore, to understand whether the early death of cockroaches induced by *BgFas1*-RNAi was correlated with loss control of water evaporation, we

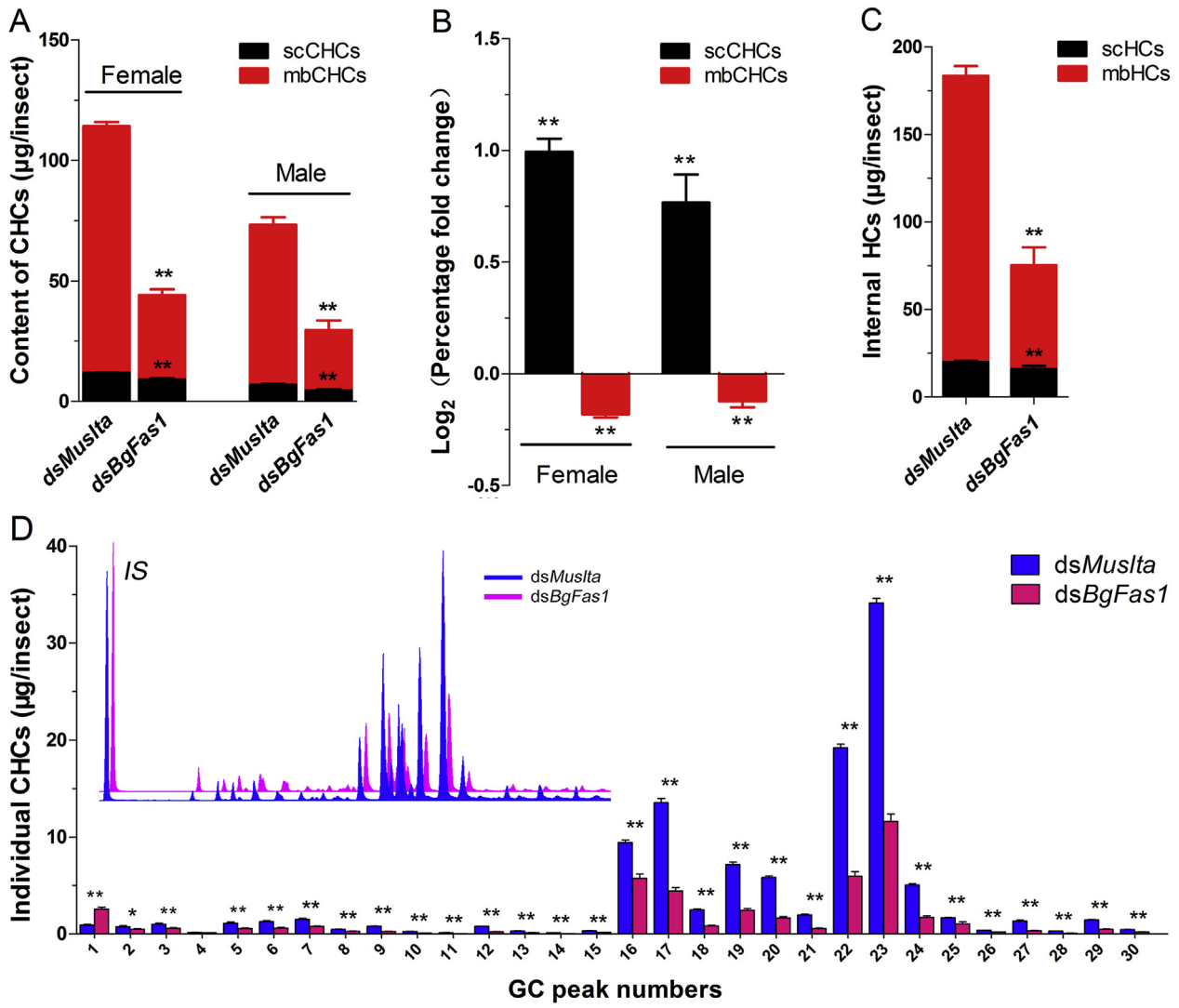

Fig. 4. Changes of HCs from *BgFas1*-suppressed *B. germanica*. Effect of *BgFas1* suppression on cuticular (A) or internal (C) HCs (μg/cockroach). Total CHCs are divided into mbCHCs (red box) and scCHCs (black box). Data are shown as means ± SE. ** represents a significant difference in mbCHC or scCHC levels between treatments and controls ($P < 0.01$ with Student's *t*-test; n = 10 or 14). (B) Effect of *BgFas1* suppression on the percentage of CHC composition. Percentage fold change of mbCHCs or scCHCs after *BgFas1*-RNAi treatment is presented on the log2 scale; error bars illustrate variability within 10 biological replicates. *P*-values were calculated by Student's *t*-test based on the null hypothesis; $^{**}P < 0.01$. (D) Effect of *BgFas1* suppression on CHCs of individual female. An overlapped chromatogram of CHCs from controls (blue) or treatments (pink) is shown. *IS* represents the peak of internal standard (equal values). Different GC peak numbers are consistent with those in Fig. S4 and annotated in Table. S3. Data are shown as means ± SE. $^{**}P < 0.01$ with Student's *t*-test; n = 10. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

calculated the weight loss rate of female cockroaches under extremely dry conditions (Fig. 7C). We found that the weight of ds*BgFas1*-treated females was reduced by 45.5% in 72 h while control lost a significantly lower percentage of 35.2% ($P < 0.01$). The increased mortality and accelerated weight loss rate of *BgFas1*-RNAi-treated cockroaches demonstrated that the insects defective in cuticular lipids were more susceptible to desiccation which might be caused by an excessive water evaporation.

4. Discussion

In this study, we cloned and characterized seven potential fatty acid synthesis genes in *B. germanica* and performed a systemic RNAi screen of their functions. Functional study revealed that *BgFas1* is required for biosynthesis of both HCs and cFFAs, and our desiccation assay further confirmed that *BgFas1* is involved in the desiccation tolerance of *B. germanica*.

In our study, total of 30 different HC components from *B. germanica* were detected. The major HC components are the same as previously reported (Jurenka et al., 1989). RNAi knockdown of *BgFas1* dramatically reduced both the internal and cuticular HCs of *B. germanica*. This result strongly suggests that *BgFas1* is involved in HC biosynthesis. Furthermore, analysis of individual HC components found that knockdown of *BgFas1* dramatically decreased the amount of mbHCs and surprisingly caused a significant increase of C27 *n*-alkane. Combined with previous studies that integument-rich microsomal FAS protein participated in methyl-branched HC synthesis (Juárez et al., 1996, 1992; Blomquist et al., 1995), we speculate that *BgFas1* encodes a microsomal FAS enzyme. Working on the fruit fly, Chung et al. (2014) found another microsomal FAS (FAS^{CG3524}) that participates in 2-MeCHC biosynthesis, but our results indicate that *BgFas1* has no preference for the methyl branch position. An interesting result showed that one of the scCHC component C27 *n*-alkane increased by two-fold after suppression of *BgFas1*. This indicates that *BgFas1* is not associated with C27 *n*-alkane biosynthesis. A possible explanation for this increase in C27 *n*-alkane is feedback regulation, or, in other words, there may be

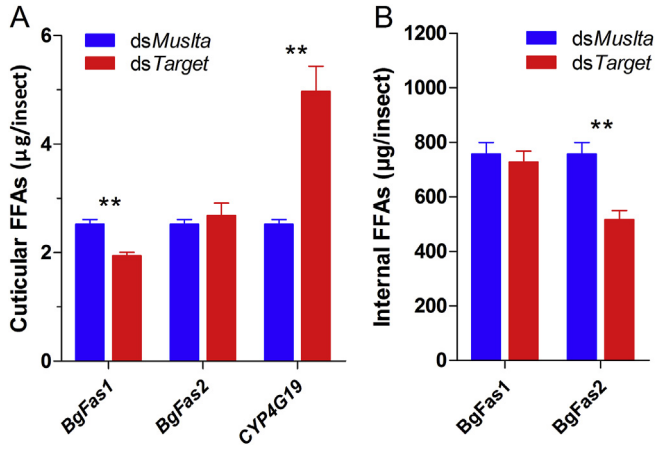

Fig. 5. Effect of *BgFas1–2* and *CYP4G19* knockdown on FFAs. The relative amounts of cuticular FFAs on two-day-old male German cockroaches under ds*BgFas1* or ds*CYP4G19* (A) and ds*BgFas2–7* (B) treatments were analyzed by GC–MS. Averages are presented as means ± SE based on 9 to 13 biological replicates. Significant differences between treatments (ds*Target*) and controls (ds*Control*) are indicated by ** ($P < 0.01$, Student's *t*-test). (C) After ds*BgFas1–7* injection, two-day-old male cockroaches were subjected to internal FFA analysis. Relative FFA amounts are compared between treatments and controls. Error bars represent the mean standard error calculated by 10–14 biological replicates. **$P < 0.01$ with Student's *t*-test.

another *BgFas* gene involved in C27 alkane biosynthesis. Suppression of *BgFas1* may have reserved more lipid substrates, which resulted in an increase of C27 *n*-alkane. In our study, we performed a systematic RNAi screen against *BgFas* genes, but unfortunately the *BgFas* gene involved in scHC biosynthesis escaped our detection. A possible reason is that RNAi could not eradicate the target gene entirely, and using a gene editing technique that does not rely on egg production for gene functional study in *B. germanica* would be required (Chaverrarodriguez et al., 2018). However, another possibility may be that the biosynthesis of scHCs was catalyzed by more than one nonspecific *BgFas* genes. In addition, we found that RNAi knockdown of other *BgFas* genes resulted in a slight increase or increasing trend of CHCs, which might also be caused by feedback regulation of lipid reserves. RNAi of other *BgFas* genes may decrease the consumption of lipid substrates like malonyl-CoA, and therefor shunted more substrates for *BgFas1* to synthesis HCs. And the result that RNAi of *BgFas2* led to an increasing trend of *BgFas1* mRNA level ($P = 0.068$, Fig. S7B) proved this consumption. In addition, RNAi knockdown of *BgFas1* also caused some increasing trends for *BgFas4*, *BgFas5*, and *BgFas7* (Fig. S7A). Even though all of them were not significant, but these results indicate that there may be some compensatory regulations among different *BgFas* genes.

Considering that the direct synthetic products of FAS are FFAs, suppression of *BgFas1* was expected to reduce the amount of FFAs first. We analyzed the effect of *BgFas1*-RNAi on both internal and external FFAs. RNAi of *BgFas1* led to a significant decrease of cFFAs. This result indicates that *BgFas1* is also associated with cFFAs biogenesis. In addition, we found that RNAi knockdown of *CYP4G19* sharply elevated cFFA level. We consider that the increase of cFFAs is caused by providing more precursors necessary for the formation of cFFAs. Qiu et al. (2012) performed a knockdown of *CYP4G1* in *D. melanogaster* and also detected a significant increase of FAs and esters on the cuticle. These results increased the reliability that *BgFas1* was involved in cFFAs biogenesis; otherwise, RNAi of *BgFas1* should have a similar effect to *CYP4G*-RNAi on cFFAs. Our data provided evidence that cFFAs are modulated by *BgFas1* which provided long-chain fatty acyl-CoAs that used for HC biosynthesis, so we speculate that the origins of cFFAs are partly the similar to HCs in *B. germanica*.

Our desiccation assay showed that the defective cuticular lipid significantly reduced the survival time of *B. germanica* under 5% RH conditions for both male and female insects. This is consistent with the previous assumption that cuticular lipids play a critical function in insect desiccation resistance (Howard and Blomquist, 2005; Gibbs, 1998). To judge whether the compromised desiccation resistance was correlated with an accelerated rate of water loss, we continuously recorded the weight loss of *BgFas1*-suppressed *B. germanica* under desiccation stress. Unfortunately, this experiment was difficult to perform as adult *B. germanica* are extremely restless, and repeated anesthetization of insects by CO_2 resulted in physiological abnormalities (Hooper, 1970). However, we successfully completed one time point before the cockroaches died in large numbers. The significantly increased weight loss rate indicated that the waterproofing ability of *BgFas1*-suppressed cockroaches was compromised. Interestingly, the German cockroach seems more drought-tolerant than *D. melanogaster* (Qiu et al., 2012). This difference may be caused by their greatly varied body size; however, other mechanisms, such as limiting excretory or respiratory water losses and increasing water content may also be responsible for the superior drought tolerance of *B. germanica* (Terblanche et al., 2010;

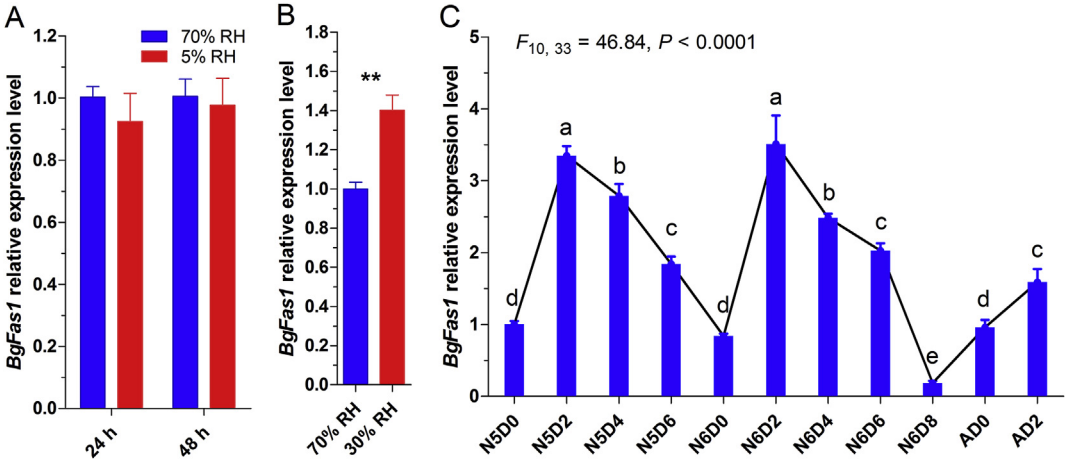

Fig. 6. Induction expression analysis of *BgFas1*. (A) N6D2 namphs were reared at 5% RH for 24 or 48 h and their expression levels of *BgFas1* against controls (70% RH) were compared. Error bars represent the variation of means calculated from 5 to 7 biological replicates. (B) Relative expression levels of N6D2 cockroaches reared at 30% or 70% RH for > 1 year were detected by qPCR. Data are shown as means ± SE. **$P < 0.01$ (Student's *t*-test, n = 4). (C) Expression patterns of *BgFas1* spanning the last two molting cycles were detected. Different instar designations are represented by N5 (fifth-instar), N6 (sixth-instar), or A (adult). Ages are presented as various days of D0–D8. Relative expression levels of *BgFas1* within abdomens are shown as means ± SE based on four independent biological replicates. Different letters above the error bars indicate significant differences between expression levels (ANOVA, LSD, $P < 0.05$).

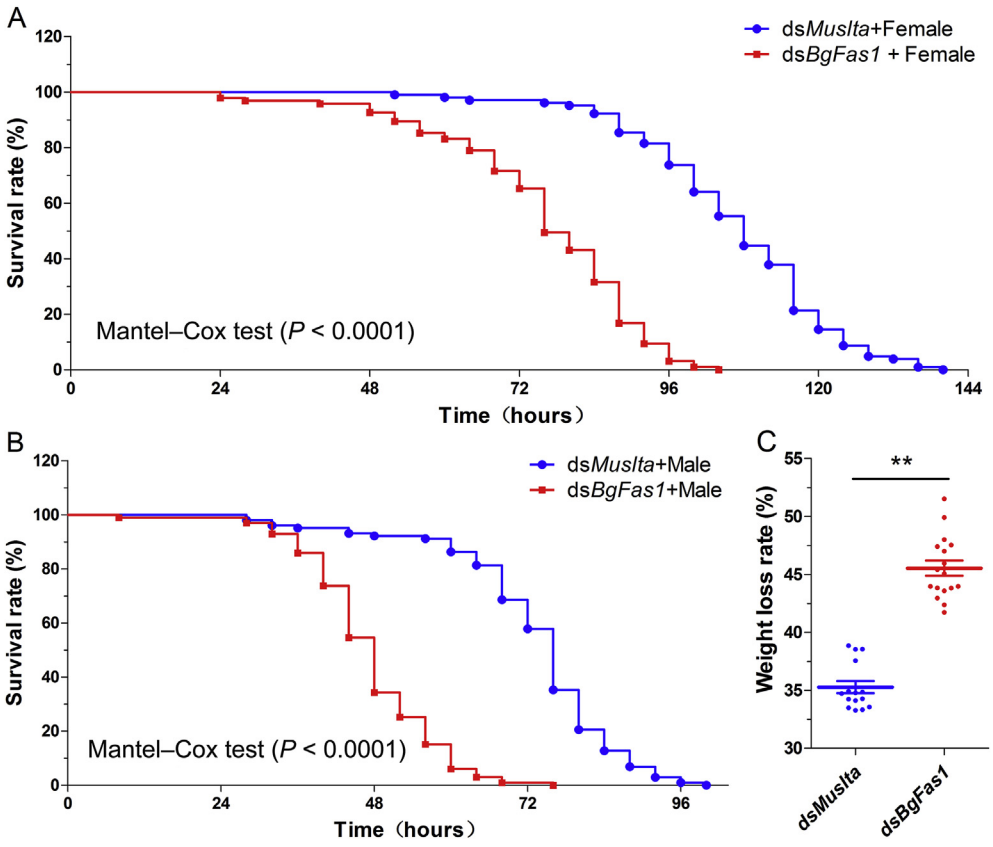

Fig. 7. Effect of *BgFas1* suppression on desiccation tolerance in *B. germanica*. Two-day-old female (A) or male (B) cockroaches were held at 5% RH following injection of ds*Muslta* (blue curves) or ds*BgFas1* (red curves). The time course was recorded every 4 h; survival rates were calculated for at least 90 cockroaches; *P*-values were calculated with the Mantel–Cox test. (C) Weight loss rate was calculated from different female cockroach treatment groups under 5% RH. Each data point represents the weight loss rate of an individual cockroach. Average values are presented as means ± SE and represented as a heavy line coupled with error bars; $^{**}P < 0.01$ with Student's *t*-test; n = 15 or 17. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Gibbs et al., 1997).

We monitored the expression levels of *BgFas1* in the last two molting cycles and detected dramatically increased expression of *BgFas1* mRNA within newly molted insects. Molting consumes quite a bit HCs, as some CHCs were lost on insect exuviae (Chen et al., 2016; Buckner et al., 1999), and the CHCs on the new epicuticle were rebuilt at the expense of internal HC pools (Flaven-Pouchon et al., 2016; Schal et al., 1998, 2001). We suspect that turnover of internal HC pools may provoke a cascade of HC biosynthesis, and this may be the reason why *BgFas1* is induced by molting. *ABCH-9C* functioning as a cuticular lipid transporter is also highly expressed in the newly molted migratory locust *Locusta migratoria* (Yu et al., 2017), and a FAR gene involved in CHC biosynthesis is highly expressed in the newly molted brown planthopper *Nilaparvata lugens*, too (Li et al., 2019). However, whether other genes involved in HC biosynthesis present a similar expression pattern in insect molting needs to be further explored. CHCs could be easily influenced by many environmental factors (Otte et al., 2018). The finding that CHC biosynthetic genes could be induced by a long-term dryness was not surprising, but the result that the expression of *BgFas1* was not influenced by a 24 h or 48 h extreme dryness surprised us. A possible explanation is that induction of *BgFas1* is a long-term adaptive process rather than an immediate stress response.

Our sequence analysis showed that all *BgFas1, BgFas2*, and *BgFas7* encoded very large compound polypeptides containing all seven functional domains required for fatty acid biosynthesis. This is consistent with type I FAS in animals or fungi (Maier et al., 2008). Other *BgFas* genes within *B. germanica* possessed seemingly incomplete functional domains and are not particular to insects as previously reported (Finck et al., 2016). However, whether the absence of functional domains means the missing of biological functions remains to be further explored. Analysis of internal lipids showed that *BgFas2*-RNAi led to a significant reduction of both FFAs and TAGs, suggesting that *BgFas2* is functionally associated with internal lipid production, as reported by many other studies (Tan et al., 2017; Li et al., 2016; Alabaster et al., 2011). In addition, this also confirms that the functions of *BgFas1* and *BgFas2* are specialized, which is consistent with phylogenetic and tissue-specific analysis results.

Phylogenetic analysis showed that *BgFas1* and *BgFas2* were divided into two branches. Further study indicated that *BgFas1* was dominantly expressed in the integument and involved in HC biosynthesis. In contrast, *BgFas2* was mainly expressed in fat bodies and mediated internal FFA and TAG accumulations (Fig. S6). This supports the hypothesis that internal and external lipids are generally synthesized by two distinct FAS enzymes. What's more, cluster I contains only one *FAS* for each representative insect except for *D. melanogaster*, which is consistent with previous research (Finck et al., 2016). So, we believe that the FASs in cluster I are more conservative and may be necessary for insect survival. However, more studies on other insects are needed to identify whether this branch is grouped by their similar function participating in internal lipid biosynthesis.

Tissue expression analysis indicated that *BgFas* genes were dominantly expressed in the integument and fat bodies. Similarly, studies on *D. melanogaster* found that two out of three *FASs* were expressed in oenocytes, with the third *FAS* expressed in fat bodies (Chung et al., 2014). Functional study showed that oenocyte-expressed FAS^{CG3524} were involved in HC biosynthesis, and FAS^{CG3523} specifically expressed in fat bodies is responsible for internal lipid biosynthesis within *D. melanogaster* (Garrido et al., 2015; Wicker-Thomas et al., 2015). In our study, the expression level of *BgFas1* in the integument was almost one hundred times higher than that of other tissues, and *BgFas2* was dominantly expressed in the fat body. At the same time, their functions are paralleled with that of FAS^{CG3524} and FAS^{CG3523}, respectively. So, we speculate that *BgFas1* and *BgFas2* may be also specifically expressed in the integument and fat bodies respectively, in *B. germanica*. As for why *BgFas2* mRNA was also largely detected in the integument and other tested tissues? We think that this may be due to the interference of remaining fat body tissues, as fat bodies are widely distributed among internal organs and some of them are tightly attached to the integument

and just segregated by a basement-membrane film in cockroaches (Makki et al., 2014). Further study using mRNA-specific fluorescence *in situ* hybridization or immunohistochemistry tools is needed to ultimately answer this question. In the recent work, Moriconi et al. (2019) characterized three *FAS* genes from *Rhodnius prolixus*, they found a similar result as it in *D. melanogaster* that two *FAS* genes were mainly expressed in the integument and one of the integument expressed *FAS* gene was involved in integument fatty acid and hydrocarbon synthesis, while the third *FAS* gene was mainly expressed in the fat body. All these results from *D. melanogaster, R. prolixus,* and *B. gerimica* indicated that different *FAS* genes in insects may be tissue specifically expressed and functionally specialized.

In summary, here we investigated the gene family of *FAS* in *B. germanica*, and we found that only *BgFas1* out of the seven *BgFas* genes is simultaneously required for both HC and cFFA biogenesis and presented a preference for mbHC formations. *BgFas1* therefore modulates the waterproofing ability of *B. germanica*. Moreover, our results support the hypothesis that *BgFas1* encodes a microsomal FAS enzyme that presumably modulates mbHC biosynthesis in *B. germanica*. We also provide evidence that the synthesis sites of cFFAs and CHCs are partially the similar and presumably in the oenocytes within *B. germanica*. Study of genes participating in different classes of HC biosynthesis would be helpful for understanding the molecular mechanisms underlining HC diversity.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103203.

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