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Wen-han Yan

Huazhong Agricultural University: Huazhong Agriculture University

Meng-Ya Wu

Huazhong Agricultural University: Huazhong Agriculture University

Sakhawat Shah

Huazhong Agricultural University: Huazhong Agriculture University

Yu-Chen Yao

Huazhong Agricultural University: Huazhong Agriculture University

Karam Khamis Elgizawy

Benha University

Ning Tang

Huazhong Agricultural University: Huazhong Agriculture University

Gang Wu

Huazhong Agricultural University: Huazhong Agriculture University

Feng-Lian Yang (✉ yangfenglilian@mail.hzau.edu.cn)

Huazhong Agricultural University: Huazhong Agriculture University <https://orcid.org/0000-0001-8980-9495>

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Silencing the *triacylglycerol lipase (TGL)* gene decreases the number of apyrene sperm and inhibits oviposition in *Sitotroga cerealella*

Wen-han Yan^{1,†}, Meng-Ya Wu^{1,†}, Sakhawat Shah¹, Yu-Chen Yao¹, Karam Khamis Elgizawy², Ning Tang¹, Gang Wu¹, Feng-Lian Yang^{1,*}

† These authors contributed equally to this work;

¹Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, P.R.China;

²Plant Protection Department, Faculty of Agriculture, Benha University, Moshtohor, Toukh 13736, Egypt. E-mail: karam.elgizawy@fagr.bu.edu.eg

Wen-Han Yan, wenhanyan97@163.com

Meng-Ya Wu, wumengya2021@163.com

Sakhawat Shah, Shahentomology@webmail.hzau.edu.cn

Yu-Chen Yao, ycyao1997@163.com

Karam Khamis Elgizawy, karam.elgizawy@fagr.bu.edu.eg

Ning Tang, tangning98@126.com

Gang Wu, wugang@mail.hzau.edu.cn

*To whom correspondence should be addressed: Tel/fax +86-27-87287207;

E-mail: yangfenglian@mail.hzau.edu.cn

Abstract

Triacylglycerol lipase (TGL) is an essential lipid metabolism enzyme that also plays a critical role in energy metabolism; however, how it regulates other life processes is unknown. To investigate the functional role of TGL in moth reproduction, males *Sitotroga cerealella* were used as a model. The *TGL* gene was cloned and analysed. The results showed that the open reading frame of *TGL* was 1968 bp long and contained three conserved regions. *TGL* gene expression was higher in the larval and early adult stages than in the pupal stage, with the highest levels observed in the fat body, testis and accessory glands during the early adult stage. Moreover, after *TGL* in male adults was silenced through RNAi, the protein content in male accessory glands remained unchanged, and the spermatophore transferred into females mated with *TGL*-silenced males became small and empty; meanwhile, the number of apyrene sperm in the spermatophore was significantly reduced due to the reduction of apyrene sperm in males, which eventually led to the significant reduction of egg laying amount. All of the findings suggest that TGL regulates the amount of sperm in male moths as well as the morphology and quality of spermatophores transferred to females after mating with treated males, implying that TGL is critical for *Sitotroga cerealella*'s reproductive process.

Key words: *Sitotroga cerealella*; triacylglycerol lipase (*TGL*); apyrene sperm; spermatophore egg; laying amount

Introduction

Insects store sufficient nutrients in their fat bodies, such as fat and glycogen [1]. The stored fats are finally hydrolysed into free fatty acids (FAs) for the body to absorb and use throughout growth and development to fulfil the energy requirements of insects. Triacylglycerol (TG) is the main storage form of FAs. When food is sufficient, TGs are stored in fat body cells in the form of cytoplasmic lipid droplets (LDs). When insects need energy, the stored FAs are released from TG through lipase hydrolysis [1]. Triacylglycerol lipase (TGL) is the enzyme that catalyses the first step in TG hydrolysis. In 2004, the lipase ATGL, which hydrolyses TGs, was discovered and found to have high substrate specificity for TG, to be able to hydrolyse TG and to be related to LDs [2]. Subsequent studies have shown that the TG content in the yeast mutant *Atg13* is substantially higher than that in the wild type, suggesting that TGL has esterase activity and hydrolyses TG into diacylglycerol (DG) and free FAs [3]. In addition to TG hydrolase activity, TGL is also a phospholipase (type A1) that may hydrolyse phospholipids in the outer layer of LDs, which is supposed to allow the entry of TGL into the LD core of TG molecular localization, stimulate LDs hydrolysis, and provide energy for various physiological activities [4, 5]. The interaction between TGL and HDLp (high-density lipophorin), the apolipoprotein component of lipid transporters, can drive the intracellular transport of DG, indicating that TGL may be directly involved in lipoprotein assembly and DG loading and is not limited to TG hydrolase activity [6].

In insects and mammals, TGL plays an important role in lipid metabolism and energy balance. TGL and other lipases have recently been discovered to be essential in the development and reproduction of insects and mammals, as well as their resistance to pathogens, pheromone signal

transmission, and other processes. In mammals, ATGL is the main lipase in adipose tissue and plays an indispensable role in the distribution of FAs in the liver and signal transduction in the regulation of energy metabolism. The heart function of mice with *ATGL* gene deletion degenerates prematurely as a large amount of lipids accumulate in the heart, ultimately leading to premature death. Among the TGLs of insects, that of *Manduca sexta* is the only one that has been purified and identified, and research has shown that it is the main lipase involved in the hydrolysis of TG [5, 7]. The reproduction of *Nilaparvata lugens* was stimulated by using the fungicide jinggangmycin (JGM), the contents of protein and glycerin in the ovaries and fats required for reproduction were significantly reduced in females treated with JGM due to ATGL knockout, and the content of soluble sugar was increased at the same time, resulting in a decrease in egg laying amount. This confirms that ATGL is one of the key enzymes involved in the reproductive process of *Nilaparvata lugens* stimulated by JGM [8]. TGL regulates the sex difference in TG homeostasis in *Drosophila*, and the loss of the *TGL* gene nearly removes the sex difference in TG storage [9]. In addition, FAs play an important role in the occurrence of *Drosophila* sperm during sperm development; the accumulation of very-long-chain FAs may cause cytokinesis defects, which may also participate in the elongation process of spermatogenesis; and β -oxidization of short-chain FAs is essential for sperm development and other processes [10]. TGL, as a main enzyme in the release of FAs from TG, may also play an important role in normal sperm production. Recent research has uncovered that the endocrine system can control the lipid metabolism pathway where TGL is found, influencing insect reproduction and the diapause process of insects. In *Aedes aegypti*, juvenile hormone (JH) promoted the accumulation of lipids in the ovary to increase the reproductive ability of females [11], and TGL, which is the key lipid metabolism pathway enzyme, was

involved in this process. In summary, studying the regulation of TGL expression will help us better understand the regulation of lipid metabolism in insects and mammals, as well as the energy regulation mechanism during insect reproduction. The functional role of TGL in the process of lipid accumulation has been confirmed in many insects, but the regulatory mechanism during insect reproduction is still unclear.

The content of TG that accumulates and is reserved in the body of the larva of *M. sexta* during the feeding period reaches a maximum at the end of the pupal stage [12]. Similar to *M. sexta*, *S. cerealella* does not feed during its adult stage. The energy required for reproductive activities depends on accumulation in the early stage. Therefore, lipid metabolism-related pathways are critical to *S. cerealella*'s reproductive behaviour. We previously found that when *S. cerealella* males were fumigated with diallyl trisulfide (DAT), the content of energy substances such as TG, glycogen and trehalose decreased significantly. LDs staining showed that the small fat body particles were smaller and more empty than those in the control after DAT fumigation. At the same time, the sperm survival rate, number of sperm and oviposition decreased significantly[13]. To better understand the molecular mechanism of DAT underlying ovipositional inhibition, we determined the transcriptomes of males after DAT treatment. The results showed that the expression of multiple genes in the lipid metabolism pathway changed significantly, such as the overexpression of the *TGL* gene. In view of our previous results mentioned above, we speculate that TGL could be involved in the reproductive process of male moths, but the specific role of TGL is still unknown.

Lipid metabolism is required for insect sperm production. Exploring the lipid metabolism mechanism will help us better understand moths' reproductive activities. As a key enzyme in lipid

metabolism, TGL must play an important role in the process of sperm development. To explore the specific function of TGL in the reproductive activities of *S. cerealella*, we conducted a series of studies. First, we conducted a preliminary study on the temporal and spatial expression patterns of *ScerTGL* and found that in addition to that in fat body, *ScerTGL* expression in testis tissues was also very high, indicating that TGL may participate in the reproductive process of the moth apart from the process of lipid metabolism. Afterwards, we used RNAi technology to silence the expression of *ScerTGL* to initially explore the specific functions of TGL in the reproductive process of the moth. The results showed that compared with that in the control group, the probability of forming normal spermatophores in females mated with dsTGL-injected males was significantly reduced, and the spermatophore was smaller and emptier. The total amount of sperm was lower, which eventually led to a significant ovipositional reduction in females mated with dsTGL-injected males. In this process, silencing *ScerTGL* mainly affected the production and transfer of apyrene sperm in males. In conclusion, our findings indicate that TGL is not only involved in natural energy metabolism but is also linked to the development of sperm and the contents of spermatophores transported to females during *S. cerealella* mating and has an effect on final egg production.

Materials and Methods

Test insects

The moth used in the experiment was a long-term laboratory breeding strain, which was reared in a round-bottom transparent glass bottle (10 cm height, 4 cm radius). The bottle was filled with clean wheat kernels to a level of approximately 2-3 cm and placed in an artificial climate incubator with a temperature of $28\pm1^{\circ}\text{C}$, a relative humidity of $75\pm5\%$ and a photoperiod of L:D=14:10. The pupae

were selected and raised in glass tubes as described by Chang et al [14]. Newly emerged virgin moths were used to distinguish between males and females for subsequent treatment.

Cloning of the *ScerTGL* opening reading frame and its bioinformatic analysis

Total RNA of the male moth was extracted by TRIzol reagent (TransGen, Beijing, China), and cDNA was synthesized using Hifair II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (YEASEN, Shanghai, China). Then, the *ScerTGL* 1 and *ScerTGL* 2 primers designed by Primer 5.0 software were used to amplify the *ScerTGL* fragment of the moth (Table S1).

The amplified PCR product was subjected to low-melting agarose gel electrophoresis and then gel cutting, and the product was purified using the gel recovery kit instructions (Axygen, California, USA). The recovered product was sent to TSKINGKE for sequencing. The two amplified sequences were then spliced using DNAMAN to obtain the complete open reading frame of *ScerTGL*, which was then translated into an amino acid sequence. Homologous amino acid sequences were searched for with the NCBI-BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) comparison function, 33 amino acid sequences of TGL from different sources were selected, and 34 TGL amino acid sequences, including *ScerTGL*, were analysed by MEGA 6.06 software to build a phylogenetic tree. Among them, the TGL amino acid sequences of closely related species were selected for conserved sequence analysis including functional domains using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), SMART (<http://smart.embl.de/>), and Jalview 2.10.5 software with *ScerTGL* amino acid sequences.

Expression patterns of *ScerTGL* in different tissues and developmental stages

The larval, pupal, and adult stages of males at 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h were used for temporal expression analysis, while the accessory glands, testis, head, and fat body of males and

ovaries of females were dissected for tissue expression determination. There were three repetitions for each sample.

According to the QTGL gene primer designed by Primer 5.0 software (Table S2), the cDNA obtained by reverse transcription was diluted 25 times as a template, and the expression of *ScerTGL* in different stages and tissues was analysed using qRT-PCR. The amplification procedure was as follows: Step 1, 95°C predenaturation for 5 minutes; Step 2, Amplification reaction at 95°C for 5 s and 60°C for 30 s for 40 cycles; and Step 3, Melting curve construction at 55°C for 30 s. In each cycle, the temperature was increased by 0.5°C until it reached 95°C. GAPDH was used as an internal reference gene, and each sample had four technical repetitions.

Functional analysis of *ScerTGL*

In vitro synthesis of dsRNA

Using the PCR product of *ScerTGL* as a template, two double-stranded RNAs were amplified with the primers dsTGL1 and dsTGL2 (Table S3), and the amplified dsTGL was recovered according to the gel recovery procedure.

The product recovered from the dsTGL PCR gel was ligated with the PMD-19T vector (TAKARA, California, USA) in vitro and transformed into DH5 α chemically competent cells (YEASEN), and then the recombinants were selected using blue-white spot screening. The colonies selected from the LB medium were inoculated into Amp+LB liquid medium and cultured at 37°C with 200 r/min shaking for 6-8 h until the liquid became turbid. At the same time, using the bacterial solution as the template, PCR amplification was performed again. After the product was identified by gel electrophoresis, the bacterial solution with the predicted size was selected for sequencing. A total of 100 μ L of the correctly

sequenced strain was added to 6 mL of fresh LB medium containing antibiotic Amp⁺, and shaking was performed again to obtain a fresh bacterial solution. At the same time, 1 mL of bacterial solution and 35% glycerol were mixed at a ratio of 1:1. The plasmid that was extracted from the bacterial liquid containing the target fragment was used as a template, and PrimeSTAR (TAKARA) enzyme was used for amplification to check whether the band was correct. Afterwards, DNA in the PCR product was extracted by the phenol-chloroform extraction method, and this extracted product was used as a template to synthesize dsTGL using a T7 dsRNA in vitro synthesis kit (Promega, Wisconsin, USA).

Quantitative detection of the *ScerTGL* silencing effect

dsRNA was injected into newly emerged adult male moths by microinjection. The injection site was the 5th-6th internode membrane of the abdomen. The males injected with dsRNA were placed in an artificial climate incubator at a temperature of $28 \pm 1^{\circ}\text{C}$, a relative humidity of $75 \pm 5\%$, and a photoperiod of L:D=14:10. RNA was extracted from the moths on day 1, day 2, day 3, day 5, and day 7 post-injection. qRT-PCR was used for expression analysis.

Observation and statistics of the morphology of spermatophores transmitted to females mated with *ScerTGL*-silenced males

Males injected with dsTGL and dsEGFP were placed in an artificial climate incubator for two days. On the third day, males were removed to pair with virgin females that had emerged for 12 h, and mating was observed. Then, each spermatophore was dissected immediately after mating completion, and the morphology of the spermatophore was observed and measured under a microscope (Shunyu, Ningbo, China). Twenty spermatophores were observed for each group, with three biological repetitions. Photos were taken by LightTools software.

Statistics of sperm counts before and after mating with *ScerTGL*-silenced males

The male moths injected with dsTGL and dsEGFP were placed in an artificial climate incubator for two days. On the third day, some males were removed, and used to dissect the entire male reproductive system except for the accessory gland and to count the number of sperm in the entire reproductive system before mating. The other males were paired with virgin females that had emerged for 12 hours. After mating, the spermatophores in females and single ejaculatory ducts, double ejaculatory ducts, testis, and seminal vesicles in males were dissected and placed in PBS, and then the fat particles on the surface were removed from organs in the buffer. The organs were poked with a pin, and the contents of the organs were separated with tweezers and distributed evenly into PBS buffer. The PBS buffer containing the contents was pipetted into a 1.5 mL centrifuge tube, and three reproductive organs were placed in each tube. The samples were slowly ground with a grinding pestle, the sperm in different organs was mixed thoroughly, enough PBS buffer was added to reach a volume of 80 μ L, 20 μ L of 4°C pre-cooled DAPI was added, and the mixtures were stored at room temperature under dark conditions for 10-15 minutes to allow dyeing. Then, 2 μ L of the mixed solution was pipetted to make a glass slide, the slide was placed under an inverted fluorescence microscope (OLYMPUS, Tokyo, Japan), the sample was illuminated by UV fluorescence, photographs were taken, and the number of sperm in the square was counted. Three reproductive organs from each tube were used to make two glass slides, which served as two repetitions. Three repetitions were set in each group. Male moths injected with dsEGFP were used as the control.

Oviposition of female moths after mating with *ScerTGL*-silenced males

Two days after the injection of dsRNA, males were paired with virgin females that had emerged for 12 hours, and mating was observed. After successful mating, egg-laying paper was placed in the tube. After 5 days, the number of eggs was counted. Males injected with dsEGFP were used as the control group, and 20 pairs were counted from each group, with three repetitions.

Determination of male Acp content in *ScerTGL*-silenced male moths

Sample Preparation:

Newly emerged male moths injected with dsTGL and dsEGFP were cultured in an incubator for two days. On the third day, the accessory gland was dissected and collected in a centrifuge tube precooled with liquid nitrogen. The sample was ground in the centrifuge tube, the grinding rod was rinsed with PBS buffer, and the combined washing solution was transferred to a centrifuge tube. The amount of PBS buffer was 20 μ l per insect. Males injected with dsEGFP were used as the control group. Experiments were performed with three repetitions, comprising 6-8 moths.

Production of a standard curve:

First, 0.5 mg/ml BSA protein standard solution was used to prepare standard measurement solutions with final concentrations of 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml, 20 μ l of the standard solution was mixed with 200 μ l of BCA working solution, and the mixture was incubated at 37°C for 30 min. The absorbance of the standard was measured at 562 nm, and a standard curve was drawn.

Sample determination:

The tested samples were centrifuged at 2500 r/min and 4°C for 10 min, and the supernatant was collected. The measurement procedure was the same as that for the standard, and the absorbance of the

sample at 562 nm was recorded. The male Acps content of the sample was calculated according to the standard curve.

Data analysis

The data were analysed with SPSS 19.0 (SPSS Inc., Chicago, IL) statistical software, the significance test used was Tukey's test after one-way ANOVA, the qRT-PCR expression data were calculated using the $2^{-\Delta\Delta CT}$ method, and GraphPad Prism 5 software was used to create figures.

Results

Cloning and bioinformatic analysis of *ScerTGL*

Based on the nonparametric transcriptome data of *S. cerealella* established in our laboratory, ScerTGL was cloned and identified according to BLAST analysis of GenBank. We successfully obtained two gene fragments, *ScerTGL 1* and *ScerTGL 2*, after cloning: *ScerTGL 1* was 1104 bp, and *ScerTGL 2* was 888 bp (Fig. S1A). The two fragments were spliced together, and the open reading frame (ORF) of *ScerTGL* was 1968 bp, encoding 656 amino acids. The predicted molecular weight was 72.96 kDa, the isoelectric point was 5.54, the probability of lipid identification was 77.98%, and the overall average coefficient of hydrophobicity was -0.419, indicating that the protein was hydrophilic (Fig. S1B).

After analysing the phylogenetic tree, it was found that ScerTGL and the TGLs of *Danaus plexippus plexippus* (OWR50376.1), *Manduca sexta* (ACR61720.1), *Aedes aegypti* (EAT38149.1), *Apis mellifera* (XP_003251782.2) and *Tripolium castaneum* (XP_008200243.1) had the highest similarity in amino acid sequence (Fig. 1A), so we selected 4 of these insects for multiple alignment of amino acid sequences including functional domains analysis. We found three conserved regions of

lipids in the *ScerTGL* amino acid sequence, namely, N-terminal 58-128 WWE conserved domain (Pfam: PF02825), the central region containing the lipase consensus sequence GHSLG, and the C-terminal DDHD conserved domain (Pfam: PF02862). Ser367, Asp457 and His590 were the active sites of the protein, which were similar to those in other insects. The amino acid sequences of *ScerTGL* and the TGLs of other insects had high similarity (Fig. 1B).

Expression patterns of *ScerTGL*

qRT-PCR was used to detect the expression level of *ScerTGL* in male moths at different developmental stages and in different tissues. The results showed that *ScerTGL* was expressed at higher levels in the fat body and testis than in the head and male accessory gland (MAG) (Fig. 2A). The higher expression of *ScerTGL*, an important enzyme for lipid catabolism, in the fat body is mainly used to decompose TG; however, the expression in the testis may indicate that TGL plays an important role in the development of testis tissue or sperm development.

A higher expression level of *ScerTGL* was observed in the larval and adult stages than in the pupal stage, while the expression levels in the larval stage and the first 24 hours of the adult stage were not very different, and that at 48 hours in the adult stage was significantly higher than that in the larval stage (Fig. 2B). The higher expression of *ScerTGL* in the larval stage indicates that TGL plays a major role in TG decomposition, participates in the body's energy metabolism, and promotes the growth and development of moths. The higher expression in the adult stage indicates that TGL may have a role in the reproductive activities of adult moths.

Morphological alteration of spermatophores in females mated with RNAi-treated males

To clarify the role of *ScerTGL* in moth reproduction, we synthesized *dsTGL* and injected it into male moths. The expression of *ScerTGL* was reduced significantly within 5 days compared with that in the control group injected with *dsEGFP* (Fig. 3A), indicating successful use of RNAi to silence the expression of *ScerTGL* in male moths.

Dissection of female moths mated with males injected with *dsTGL* revealed that the spermatophores were significantly smaller in the *dsTGL*-injected groups than in the control groups under the microscope (Fig. 3B). The number of abnormal spermatophores in the *dsTGL* injection group was greater than that in the *dsEGFP* control group. The rate of abnormal spermatophores in the *dsTGL* group was 5-fold higher than that in the *dsEGFP* group ($dsEGFP=10.00 \pm 2.89\%$; $dsTGL=51.67 \pm 4.41\%$; $P<0.01$), and the rate of normal spermatophore formation dropped by approximately 67.50% ($dsEGFP=66.67 \pm 1.67\%$; $dsTGL=21.67 \pm 4.41\%$; $P<0.01$). At the same time, females in both groups did not produce spermatophores after mating, and no significant difference was found between the two groups ($dsEGFP=23.33\% \pm 1.67\%$; $dsTGL=26.67\% \pm 1.67\%$; $P>0.05$), which showed that *dsTGL* did not affect the mating of *S. cerealella* (Fig. 3C).

Reduction of apyrene sperm number in RNAi-treated males

Considering the importance of sperm to moth reproductivity, we counted the sperm numbers from both the entire male reproductive system before mating and the spermatophores in mated females and male reproductive organs after mating. The results showed that the total number of sperm in the male reproductive system of *dsTGL*-injected groups was significantly lower than that in control groups before mating ($dsEGFP=117538.20 \pm 3045.52$; $dsTGL=71417.50 \pm 1821.42$; $P<0.001$), and the number of apyrene sperm was significantly decreased ($dsEGFP=114350.80 \pm 7542.53$; $dsTGL=68586.00 \pm$

2272.26; $P < 0.01$), while the number of eupyrene sperm exhibited no difference from that in the control groups (dsEGFP=2094.17 $\pm$ 172.58; dsTGL=2181.67 $\pm$ 165.65; $P > 0.05$) (Fig. 4A). The total number of sperm transferred to the spermatophores after mating in the dsTGL-injected groups was also significantly lower than that in the control groups without affecting mating (dsEGFP=97436.66 $\pm$ 9046.23; dsTGL=39217.67 $\pm$ 3138.97; $P < 0.01$). A significant decrease was observed in the number of apyrene sperm in spermatophores of females mated with dsTGL-injected males compared with dsEGFP (dsEGFP=96097.90 $\pm$ 8701.53; dsTGL=37929.11 $\pm$ 3200.89; $P < 0.01$). In contrast, no significant difference was found in eupyrene sperm transferred to the spermatophores between the control and dsTGL-injected groups (dsEGFP=1310.30 $\pm$ 398.62; dsTGL=1061.57 $\pm$ 106.87; $P > 0.05$) (Fig. 4B), which was the same as the number of sperm before mating. Further statistical analysis of the number of sperm remaining in the reproductive tissues of males in dsTGL-injected groups after mating showed that the total number of sperm remaining in the testis and seminal vesicles, including the numbers of apyrene sperm and eupyrene sperm, was not significantly different from that in the dsEGFP groups (the total sperm: dsEGFP=26092.50 $\pm$ 4013.14; dsTGL=26199.25 $\pm$ 5118.75; apyrene sperm: dsEGFP=25891.83 $\pm$ 5502.64; dsTGL=26612.25 $\pm$ 4177.25; eupyrene sperm: dsEGFP=200.67 $\pm$ 30.40; dsTGL=336.00 $\pm$ 118.04; $P > 0.05$) (Fig. 4C). Although no significant difference was found in the numbers of the two types of sperm remaining in the double ejaculatory ducts between the two groups (total sperm: dsEGFP=1785.00 $\pm$ 755.27; dsTGL=6364.32 $\pm$ 2896.27; apyrene sperm: dsEGFP=1701.00 $\pm$ 711.61; dsTGL=6359.6560 $\pm$ 2898.38; eupyrene sperm: dsEGFP=84.00 $\pm$ 43.72; dsTGL=4.67 $\pm$ 4.67; $P > 0.05$), the total number of sperm and the number of apyrene sperm in the dsTGL-injected groups showed an increasing trend compared with that in the dsEGFP groups, while

eupyrene sperm showed the opposite result (Fig. 4D). This trend became more significant in the single ejaculatory ducts connected to the external genitalia directly. In the single ejaculatory ducts, the presence of eupyrene sperm was not observed in either group, and the number of sperm in the dsTGL-injected groups was extremely significantly greater than that in the dsEGFP groups (dsEGFP=87.50 ± 3.50; dsTGL=203.00 ± 9.26; P<0.001) (Fig. 4E). Combining the analyses of sperm number in all reproductive tissues, including spermatophores, the decrease in the total number of sperm in spermatophores was mainly due to the number of apyrene sperm, and the reason for the decrease in the number of apyrene sperm was mainly caused by *ScerTGL* silencing, which reduced the production of apyrene sperm in males, although in the process of sperm transfer from males to females, single and double ejaculatory ducts would play a certain obstructive effect, with the effect of a single ejaculatory duct being more significant, but this is not the main reason for the decrease in the number of apyrene sperm in the seminal vesicles.

To clarify whether there are other reproduction-related activities that affect the morphology of spermatophores in addition to the decrease in the number of apyrene sperm after silencing *ScerTGL*, the content of male accessory gland proteins (Acps) transferred to females during mating was determined after dsRNA injection. The results of the measurement showed that there was no significant difference in the content of Acps between the two groups (Fig. S2).

Silencing of the *ScerTGL* gene can reduce the number of eggs laid by females after mating with males

To further clarify whether the decrease in the contents of the spermatophore, including the variation in the number of apyrene sperm, would affect the reproduction of *S. cerealella*, two days post

injection of dsTGL, the number of eggs laid by females after mating with the males injected with dsTGL was counted. Significant decreases were observed in eggs from the experimental group compared with the control group (dsEGFP=52.77 ± 5.38; dsTGL= 28.82 ± 4.56; P<0.01) (Fig. 5).

Overall, female moths in the dsTGL group produced significantly smaller spermatophores and reduced numbers of eggs within 5 days compared with those in the control. This indicates that TGL affects the formation of the spermatophore during the mating process, which further affects the number of sperm and egg production and affects the reproductive ability of *S. cerealella*.

Discussion

S. cerealella is an important stored-product pest worldwide that has a high reproductive ability and is considered harmful before and after grain harvest, which seriously threatens the production and quality of stored products [15]. *S. cerealella* is a typical bisexual insect whose reproductive behaviour is complex and variable and includes mating and egg laying [16]. After the insect enters the adult stage and reaches sexual maturity, it completes the production of offspring through a series of reproductive processes. However, these reproductive processes are usually regulated by internal physiological factors [17]. TGL is required for the decomposition and utilization of TG in moths and releases free FAs to provide energy. The energy required by the adult moth, which does not feed, is only supplied by the energy stored during the larval stage, so the function of TGL is particularly important in the adult stage of the moth. In this study, we confirmed that TGL not only participates in the lipid metabolism process of *S. cerealella* but also plays an important role in regulating the formation of apyrene sperm in males, the morphology of spermatophores transported to females and the number of eggs produced by females after mating.

During mating, males use spermatophores to transfer mating contents such as seminal fluid, sperm, accessory gland contents and nutrients to the female [18, 19]. The contents of the spermatophore are transferred to the spermatheca of female moths, where they mix with the materials of the spermatheca to promote ovulation of the female and complete the process of fertilization and egg production [20]. In addition, some studies have shown that some of the substances in the spermatophore also play a role in the development of the ovaries, thus enhancing the female's reproductive ability [21]. Therefore, the spermatophore plays an important role in the mating of male and female moths and the process of female egg laying. Our research shows that when *ScerTGL* in males is silenced, the morphology of spermatophores formed after mating with females become significantly small and empty without affecting mating (Fig. 3B). After statistical analysis, it was found that the rate of male moths forming normal spermatophores after silencing *ScerTGL* was only approximately 32.50% of that of the control group (Fig. 3C) ($P < 0.01$). This shows that the deletion of *ScerTGL* caused a decrease in the mating contents transferred from the male to the female moth's bursa copulatrix during the mating process, which has many effects on the subsequent reproductive process.

To clarify the reason for the formation of abnormal spermatophores and the substances that cause the morphology of the spermatophore to become empty, we preliminarily determined the number of sperm in the entire male reproductive system before mating and in the spermatophore of mated females and the number of sperm remaining in male reproductive organizations after mating with dsTGL-injected males. The results showed that the number of sperm in the male reproductive system was much lower than that in the dsEGFP groups, and the number was only 60.76% of that in the control group before mating ($P < 0.001$) (Fig. 4A). The total number of sperm in the spermatophore

formed after mating was significantly lower than that in the control groups ($P < 0.01$), i.e., only approximately 40.00% of that in the control groups (Fig. 4B). Further analysis showed that the decrease in the total number of sperm in the entire reproductive system before mating and in spermatophores after mating was mainly caused by the decrease in apyrene sperm ($P < 0.01$). There was no significant difference in the number of eupyrene sperm between the two groups in the entire male reproductive system before mating or in spermatophores after mating (Fig. 4A&B). Except for a single ejaculatory duct, which had a certain resistance to the transfer of apyrene sperm from males to females, the number of sperm, including apyrene and eupyrene sperm, remaining in males after mating was not different from that in the control groups (Fig. 4C&D&E). Summarizing the number of sperm in reproductive organs before and after mating, the silencing of *TGL* reduced the amount of apyrene sperm produced, and it also played a certain role in hindering the transfer of apyrene sperm from males to females. Sperm dimorphism is widely found in lepidopteran insects. Lepidopteran insects, including *S. cerealella*, produce two coexisting sperm: nucleated sperm (eupyrene sperm) that can be fertilized and nonnucleated sperm (apyrene sperm) that cannot be fertilized [22, 23]. Eupyrene sperm can fertilize an egg [24]. There are many hypotheses about the function of apyrene sperm [25, 26], such as reducing sperm competition, filling the spermatheca, and providing an advantage for the first mating sperm [25, 27]. It promotes the separation of eupyrene sperm bundles and plays an important role in the formation or transport of eupyrene sperm [28, 29]. Studies in silkworms have shown that the movement of apyrene sperm is a necessary condition for the transfer of eupyrene sperm from the bursa copulatrix to the spermatheca [30]. Combining the functional roles of apyrene sperm, we speculated that there is no significant difference in the number of eupyrene sperm between the two groups after mating, and the

decrease in apyrene sperm may affect the transport from the bursa copulatrix to the spermatheca and fertilization process of eupyrene sperm in females and ultimately affect female egg production. Therefore, we continued to determine the number of eggs laid by females mated with male moths with silenced *ScerTGL*. The results showed that compared with that in the control group, the number of eggs laid by females in the dsTGL-injected group was significantly reduced ($P < 0.01$) (Fig. 5). This showed that the decrease in *ScerTGL* expression in males caused a decrease in the number of apyrene sperm transferred to females, which will affect the fertilization process of eupyrene sperm in females and ultimately affect the number of eggs in females.

To investigate whether silencing *ScerTGL* caused other changes in other reproductive process-related substances in males and whether other reproductive processes changed the number of apyrene sperm transported to females during the process of silencing *ScerTGL*, we measured the amount of male Acps. The MAG is an important part of the male reproductive system. It contains substances that can increase the number of eggs laid by females, reduce the attractiveness of females to males, shorten the lifespan of females, and store sperm that cause physiological changes in insects [18, 31, 32]. For male moths, these accessory gland contents and sperm are mixed to form seminal fluid, which is transferred into the female body with the spermatophore, and it can dilute seminal fluid, prevent the loss of seminal fluid, activate sperm, and promote sperm motility to protect the sperm from being degraded [18, 33]. The MAG contents input into the female during mating can promote ovulation, and there are antibacterial proteins that can protect fertilized eggs, improve female reproductive tract immunity and provide a suitable environment for the oviposition process [34-38]. The contents of the accessory glands are rich in proteins and have many functions. They are generally collectively referred

to as male Acps, but their function is not limited to male reproduction; they play a critical role in the entire process of insect reproduction [39, 40]. Studies have shown that Acps is the main functional factor in the contents of the accessory gland [40]. Our research shows that the content of Acps in a male moth is not affected by the expression of *ScerTGL*. After interfering with the expression of *ScerTGL*, the content of Acps was not significantly different from that of the control group (Fig. S2). Given the function of Acps, we believe that TGL does not affect the secretion of the accessory gland content when regulating the reproductive process of the male moth. From this perspective, the resistance of apyrene sperm during the transfer of single and double ejaculatory ducts of males to females is not caused by changes in the content of Acps. Summarizing the above experimental results, our research showed that in addition to participating in the energy metabolism process of adult male moths, TGL also played a critical role in regulating the reproductive process of males. Changes in *ScerTGL* expression affected the production of apyrene sperm, the ability to transfer single and double ejaculatory ducts, the morphology of spermatophores transported to females and the number of apyrene sperm transferred to spermatophores. There have been some advances in the study of sperm dimorphism in lepidopteran insects. Apyrene and eupyrene sperm of lepidopteran insects have mitochondrial derivatives, but their specific functions are not clear yet [41, 42]. The absence of *sex-lethal* in the silkworm caused defects in the mitochondrial derivatives of apyrene sperm, causing apyrene sperm to lose their motility, and mitochondrial derivatives were considered the key host of the mitochondrial energy metabolism pathway. Simultaneously, the motility of apyrene sperm was a necessary condition for the transfer of eupyrene sperm from the bursa copulatrix to spermatheca [30]. In addition, research showed that the release of apyrene sperm in *Helicoverpa armigera* occurred later

than that of eupyrene sperm. Therefore, given the above research progress, we suspected that in the process of sperm formation, the start time of apyrene sperm may be later than that of eupyrene sperm, which led to a decrease in the production of apyrene sperm due to an insufficient energy supply in males lacking *TGL*. At the same time, the movement of apyrene sperm in the single and double ejaculatory ducts may have been blocked because of interference with *TGL*, making mitochondrial derivatives of apyrene sperm abnormal and reducing their motility. Finally, the number of eggs laid by females was significantly reduced, which seems to verify our hypothesis. The interference of *TGL* significantly reduced the number of apyrene sperm in the bursa copulatrix, and its mitochondrial derivatives may be abnormal, which together caused the abnormal transfer of eupyrene sperm dependent on apyrene sperm from the bursa copulatrix to spermatheca, eventually leading to a decrease in egg production. However, how the loss of function of *TGL* affects the formation of apyrene sperm and whether it affects the normal morphological structure of the mitochondrial derivatives of apyrene sperm, resulting in a partial loss of motility of apyrene sperm and ultimately a decrease in egg production, still need to be studied further.

Declarations

Conflict of interest

The author(s) declare(s) that they have no competing interests.

Ethical approval and consent to participate

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material (data transparency)

The author(s) declare(s) that all data and material are available in the manuscript.

Author Contributions

Conceived of and designed the experiments: Wen-Han Yan, Meng-Ya Wu, and Feng-Lian Yang. Performed the experiments: Wen-Han Yan, Meng-Ya Wu, Ning Tang; Analysis of the data: Wen-Han Yan and Gang Wu; Drafted and revised manuscript: Wen-Han Yan, Sakhawat Shah, Yu-Chen Yao, Karam Khamis Elgizawy, and Feng-Lian Yang. All authors approved the final version of the article, including the authorship list.

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Figure Legends

Fig. 1 Phylogenetic tree analysis of homologous sequences and multiple alignment of the predicted amino acid sequence of *ScerTGL* with the TGLs of other insects. (A) Phylogenetic tree analysis of homologous sequences of *ScerTGL* and sequences from other species. *ScerTGL* is marked with “▲”. (B): Multiple alignment of the predicted amino acid sequence of *ScerTGL* with those of other insects. MS: *Manduca sexta*; SR: *Sitotroga cerealella*; TC: *Tribolium Castaneum*; AA: *Aedes aegypti*; AM: *Apis mellifera*; Red dotted frame: Alignment of the N-terminal WWE domains (Pfam: PF02825); Yellow dotted frame: Alignment of the central regions containing the lipase consensus sequence GHSLG; Orange dotted frame: GHSLG; Light blue dotted frame: Alignment of the C-terminal domains that are homologous to the DDHD domain (Pfam: PF02862). Partial sequences of *Aedes aegypti* and *Apis mellifera* were omitted in the alignment for brevity. The different degrees of blue in each column represent different degrees of conservativeness, the darker the blue, the more conservative, and vice versa

Fig. 2 Expression pattern of *ScerTGL* in different tissues and developmental stages. (A) *ScerTGL* expression pattern in different tissues of males. MAG: male accessory gland. (B) Expression pattern of *ScerTGL* in different developmental stages. 0, 6, 12, 18, 24, 48, 72 h: 0, 6, 12, 18, 24, 48, and 72 h after the emergence of males. Note: Data in the figure are presented as the mean $\pm$ SE. The different letters indicate significant differences according to one-way ANOVA ($P < 0.05$)

Fig. 3 Observation and statistics of the morphology and quantity of spermatophores after dsTGL injection. (A) Silencing effect within 7 days after injection of dsTGL. (B) The morphology of spermatophores after dsTGL injection; a: dsEGFP; b and c: dsTGL. (C) Morphological statistics of

spermatophores after dsTGL injection. a: Normal spermatophore rate; b: abnormal spermatophore rate; c: no spermatophore rate after mating. Note: Data in the figure are presented as the mean $\pm$ SE. * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$, and NS means no significant difference according to Tukey's test

Fig. 4 Number of sperm in the entire male reproductive system before mating and in spermatophore and other male reproductive tissues after mating with dsRNA injection. (A): The number of sperm in the entire male reproductive system except for the accessory gland before mating. (B): The number of sperm in spermatophores of females mated with RNAi-treated males. (C) The number of sperm in the testis and seminal vesicles after mating. (D) The number of sperm in the double ejaculatory ducts after mating. (E) The number of sperm in the single ejaculatory duct after mating. Apyrene sperm: non-nucleated sperm; eupyrene sperm: nucleated sperm; total sperm: sum of the two kinds of sperm. Note: Data in the figure are presented as the mean $\pm$ SE. *** means $P < 0.001$, ** means $P < 0.01$, and NS means no significant difference according to Tukey's test

Fig. 5 The number of eggs produced by females mated with males with *ScerTGL* interference.

Note: Data in the figure are presented as the mean $\pm$ SE. ** indicates $P < 0.01$ according to Tukey's test

Fig. 1

Figure 1

Phylogenetic tree analysis of homologous sequences and multiple alignment of the predicted amino acid sequence of ScerTGL with the TGLs of other insects. (A) Phylogenetic tree of homologous sequences of ScerTGL and sequences from other species. ScerTGL is marked with “ ”. (B): Multiple

alignment of the predicted amino acid sequence of ScerTGL with those of other insects. MS: *Manduca sexta*; SR: *Sitotroga cerealella*; TC: *Tribolium Castaneum*; AA: *Aedes aegypti*; AM: *Apis mellifera*; Red dotted frame: Alignment of the N-terminal WWE domains (Pfam: PF02825); Yellow dotted frame: Alignment of the central regions containing the lipase consensus sequence GHSLG; Orange dotted frame: GHSLG; Light blue dotted frame: Alignment of the C-terminal domains that are homologous to the DDHD domain (Pfam: PF02862). Partial sequences of *Aedes aegypti* and *Apis mellifera* were omitted in the alignment for brevity. The different degrees of blue in each column represent different degrees of conservativeness, the darker the blue, the more conservative, and vice versa

Fig. 2

Figure 2

Expression pattern of ScerTGL in different tissues and developmental stages. (A) ScerTGL expression pattern in different tissues of males. MAG: male accessory gland. (B) Expression pattern of ScerTGL in different developmental stages. 0, 6, 12, 18, 24, 48, 72 h: 0, 6, 12, 18, 24, 48, and 72 h after the emergence of males. Note: Data in the figure are presented as the mean ± SE. The different letters indicate significant differences according to one-way ANOVA (P<0.05)

Fig. 3

Figure 3

Observation and statistics of the morphology and quantity of spermatophores after dsTGL injection. (A) Silencing effect within 7 days after injection of dsTGL. (B) The morphology of spermatophores after dsTGL injection; a: dsEGFP; b and c: dsTGL. (C) Morphological statistics of 31 spermatophores after dsTGL injection. a: Normal spermatophore rate; b: abnormal spermatophore rate; c: no spermatophore rate after mating. Note: Data in the figure are presented as the mean $\pm$ SE. * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$, and NS means no significant difference according to Tukey's test

Fig. 4

Figure 4

Number of sperm in the entire male reproductive system before mating and in spermatophore and other male reproductive tissues after mating with dsRNA injection. (A): The number of sperm in the entire male reproductive system except for the accessory gland before mating. (B): The number of sperm in spermatophores of females mated with RNAi-treated males. (C) The number of sperm in the testis and seminal vesicles after mating. (D) The number of sperm in the double ejaculatory ducts after mating. (E) The number of sperm in the single ejaculatory duct after mating. Apyrene sperm: non-nucleated sperm; eupyrene sperm: nucleated sperm; total sperm: sum of the two kinds of sperm. Note: Data in the figure are presented as the mean $\pm$ SE. *** means $P<0.001$, ** means $P<0.01$, and NS means no significant difference according to Tukey's test

Fig. 5

Figure 5

The number of eggs produced by females mated with males with ScerTGL interference. Note: Data in the figure are presented as the mean $\pm$ SE. ** indicates $P < 0.01$ according to Tukey's test

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