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

Wen-han Yan¹ · Meng-Ya Wu¹ · Sakhawat Shah¹ · Yu-Chen Yao¹ · Karam Khamis Elgizawy² · Ning Tang¹ · Gang Wu¹ · Feng-Lian Yang¹

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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.

Keywords *Sitotroga cerealella* · Triacylglycerol lipase (TGL) · Apyrene sperm · Spermatophore · Egg laying amount

Introduction

*Sitotroga cerealella* is a global pest of stored grains that has a high reproductive ability and is considered harmful before and after grain harvest [1]. Adults can fly up to 600 m away to infest distantly stored grain reserves [2]. And a female moth can destroy 500 g of hulled cereal in the absence of control measures [3], which seriously threatens the production and quality of stored products [4].

We previously found that when *S. cerealella* males were fumigated with diallyl trisulfide (DAT), the content of energy substances, such as triacylglycerol, glycogen, and trehalose, decreased significantly [5]. Lipid droplets staining showed that the small fat body particles were smaller and

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more empty than those in the control after DAT fumigation; meanwhile, the sperm survival rate, number of sperm in the spermatophore and oviposition decreased significantly; moreover, the transcriptome data results showed that the expression of multiple genes in the lipid metabolism pathway changed significantly, such as the overexpression of the TGL (triglyceride lipase) gene [6, 7]. 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.

Insects store excess nutrients in their fat bodies, such as fat and glycogen [8]. The stored fats are hydrolysed into free fatty acids for the body to absorb and use throughout growth and development to fulfil the energy requirements of insects. Triacylglycerol is the main storage form of fatty acids. In 2004, the lipase ATGL (adipose triglyceride lipase), which hydrolyses triacylglycerol, was discovered in mammals and found to have high substrate specificity for triacylglycerol, to be able to hydrolyse triacylglycerol [9]. Among insects, TGL of Manduca sexta is the only one that has been purified and identified, and research has shown that TGL is the main enzyme that catalyses the first step in triacylglycerol hydrolysis [10, 11]. When TGL gene was knocked down in the yeast, the triacylglycerol content significantly increased compared with the wild type [12]. In addition to triacylglycerol hydrolase activity, TGL is also a phospholipase (type A1) that may hydrolyse phospholipids in the outer layer of lipid droplets, stimulate lipid droplets hydrolysis [10, 13].

In insects and mammals, TGL plays an important role in lipid metabolism and energy balance. However, TGL and other lipases have recently been discovered to be essential in the development and reproduction of insects, as well as pheromone signal transmission, and other processes. The reproduction of Nilaparvata lugens was stimulated 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 [14]. In addition, fatty acids play an important role in the occurrence of Drosophila sperm during spermatogenesis; the accumulation of very-long-chain fatty acids may cause cytokinesis defects, which may also participate in the elongation process of spermatogenesis; and β-oxidation of short-chain fatty acids is essential for sperm development and other processes [15]. TGL, as a main enzyme in the release of fatty acids from triacylglycerol, 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 [16], 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 content of triacylglycerol 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 [17]. 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. In this study, 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 apyrene sperm produced was lower, eventually leading to a significant ovipositional reduction in females mated with dsTGL-injected males. In conclusion, in addition to the known functions involved in the process of energy metabolism [10], our findings indicate that TGL is also linked to the reproductive process of S. cerealella.

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 ± 1 °C, a relative humidity of 75 ± 5% and a photoperiod of L:D = 14:10. The pupae were selected and raised in glass tubes as described by Chang et al. [18]. Newly emerged virgin moths were used to distinguish between males and females for subsequent treatment.
Cloning of the ScerTGL opening reading frame and its analysis of conserved functional domains

Total RNA of the male moth was extracted by TRIzol reagent (TransGen, Beijing, China), and cDNA was synthesized using HiFastr 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 (Biotechnology Co., Ltd, China) for sequencing. The two amplified sequences were then assembled using DNAMAN software (LynnBioSoft, USA) to obtain the complete open reading frame of ScerTGL, which was then translated into an amino acid sequence. SMART (http://smart.embl.de/) and NCBI Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were used to predict conserved functional domains of ScerTGL protein.

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 replicates for each sample.

QTLG gene primers were designed by Primer 5.0 software (Table S2), and 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 pre-denaturation for 5 min; 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 replicates.

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-19 T vector (TAKARA, California, USA) in vitro and transformed into DH5α 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 µL 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 inter-node membrane of the abdomen. The males injected with dsRNA were placed in an artificial climate incubator at a temperature of 28 ± 1 °C, a relative humidity of 75 ± 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 or dsEGFP (EGFP, enhanced green fluorescent protein) 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, females were 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 replicates. Photos were taken by LightTools software.
Statistics of sperm counts before and after mating with ScerTGL-silenced males

The male moths injected with dsTGL or 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 h. 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 µL, 20 µL of 4 ºC precooled DAPI was added, and the mixtures were stored at room temperature under dark conditions for 10–15 min to allow dyeing. Then, 2 µL 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 replicates. Three replicates were set in each group. Male moths injected with dsEGFP were used as the control.

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 µl of the standard solution was mixed with 200 µl of BCA working solution, and the mixture was incubated at 37 ºC 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 ºC 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

Using SPSS19.0 (SPSS Inc., Chicago, IL) software for One-Way ANOVA analysis, 2-sample t test and 2 × 2 Contingency tables (Pearson χ² test), the qRT-PCR expression data were calculated using the 2−∆∆CT method, and GraphPad Prism 5 software was used to create figures.

Results

Cloning and bioinformatic analysis of ScerTGL

Based on the 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 78.0%, and the overall average coefficient of hydrophobicity was -0.419, indicating that the protein was hydrophilic (Fig. S1B).
After using SMART and NCBI-CDD Search to predict and analyze the conserved domains of ScerTGL protein, we found two conserved regions of lipids in the ScerTGL amino acid sequence, namely, N-terminal 60–127 WWE conserved domain (Pfam: PF002825), and the C-terminal 416–611 DDHD conserved domain (Pfam: PF02862) (Fig. 1). In addition, the 106–381 amino acid residues, the central region containing the lipase consensus sequence GHSGLG, Ser367, Asp457 and His590 were the active sites of the protein.

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 testes 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 may be mainly used to decompose triacylglycerol; however, the expression in the testes 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 h of the adult stage were not very different, and that at 48 h 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 may be mainly involved in the energy required for the growth and development of larvae at this stage and energy storage, like M. sexta. 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 or dsEGFP revealed that different morphologies of the spermatophores (Fig. 3B). The morphology of most spermatophores was obviously abnormal in the dsTGL-injected groups compared with that in control groups under the microscope. A total of 60 females after mating were dissected in each group, after statistics, the absence of a...
spermatophore in females after mating occurred at the same frequency for both groups of males, no significant difference was found between the two groups (the number of spermatophores: dsEGFP = 46, dsTGL = 44; \( \chi^2 = 0.18, \ df = 1, \ P = 0.673 \)), which showed that dsTGL did not affect the mating of S. cerealella. However, the frequency of abnormal spermatophores in the dsTGL-injected group was significantly higher than that in the dsEGFP group (the number of abnormal spermatophores: dsEGFP = 6, dsTGL = 31; \( \chi^2 = 30.62, \ df = 1, \ P < 0.0001 \)) (Fig. 3C).

**Reduction of apyrene sperm number in RNAi-treated males**

After observing the morphological change of the spermatophores, considering the importance of sperm to moth reproduction, we counted the sperm numbers in the spermatophores in mated females. 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 = 97,437 ± 9046; dsTGL = 39,218 ± 3139; \( P = 0.004 \)). A significant decrease was observed in the number of apyrene sperm in spermatophores of females mated with dsTGL-injected males compared with dsEGFP (dsEGFP = 96,098 ± 8702; dsTGL = 37,929 ± 3201; \( P = 0.003 \)). In contrast, no significant difference was found in eupyrene sperm transferred to the spermatophores between the control and dsTGL-injected groups (dsEGFP = 1310 ± 399; dsTGL = 1062 ± 107; \( P = 0.579 \)) (Fig. 4A). To explore the main reason for sperm reduction in spermatophores, we counted the sperm numbers from both the entire male reproductive system before mating and male reproductive organs after mating subsequently. 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 = 117,538 ± 3046; dsTGL = 71,418 ± 1821; \( P < 0.0001 \)), and the number of apyrene sperm was significantly decreased (dsEGFP = 114,351 ± 7543; dsTGL = 68,586 ± 2272; \( P = 0.004 \)), while the number of eupyrene sperm exhibited no difference from that in the control groups (dsEGFP = 2182 ± 166; \( P = 0.384 \)), which was the same as the number of sperm transferred to the spermatophores (Fig. 4B). Further statistical analysis of the number of sperm remaining in the reproductive
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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 groups.
and the number of sperm in the dsTGL-injected groups was extremely significantly greater than that in the dsEGFP groups (dsEGFP = 88 ± 4; dsTGL = 203 ± 9; P < 0.0001) (Fig. 4E). Combining the analyses of sperm number in all reproductive tissues, including spermatophores, the decrease in the total number of sperm in the 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 spermatophores.

To investigate whether there were male secretions that were affected and thus affected the morphology of the spermatophores after silencing ScerTGL, the content of male accessory gland proteins (Acps) 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 = 52.8 ± 5.4; dsTGL = 28.8 ± 4.6; P = 0.001) (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**

The reproductive behaviour of a typical bisexual insect, including mating and egg-laying, is complex and variable [19]. *S. cerealella* is also one of the typical bisexual insects. 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 [20]. TGL is required for the decomposition and utilization of triacylglycerol in moths and releases free fatty acids 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, in addition to the known function of TGL to be involved in energy metabolism process [10], we confirmed that TGL 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 [21, 22]. 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 [23]. 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 [24]. 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, spermatophores formed after mating with females become significantly small and empty without affecting mating (Fig. 3B). After statistical analysis, it was found that the frequency of male moths forming normal spermatophores significantly reduced after silencing ScerTGL (Fig. 3C). This showed 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.
The basic function of mating is to transfer sperm to females. The spermaphore is a carrier for transporting sperm to the female [25]. In view of the above experimental results, we would like to know whether the silencing of ScerTGL would affect the number of sperm transferred to spermaphores while affecting the morphology of spermaphores? First, we determined the number of sperm in the spermaphore. The results showed that the number of sperm, which is mainly apyrene sperm, was significantly lower than that in the dsEGFP group in the spermaphore. Further experimental results showed that the reason why the number of apyrene sperm transferred to the spermaphore after mating were much lower was mainly that the amount of apyrene sperm produced in male reproductive organs was significantly reduced after silencing ScerTGL (Fig. 4A&B). In addition, the hindered transfer of apyrene sperm from males to females in the single ejaculatory duct was also a reason for the decrease in the number of sperm in the spermaphore (Fig. 4C&D&E).

Sperm dimorphism is widely found in lepidopteran insects. Lepidopteran insects, including S. cerealella, produce two coexisting sperm: nucleated sperm (eupyrene sperm) that fertilize eggs and nonnucleated sperm (apyrene sperm) that do not [25, 26]. There are many hypotheses about the function of apyrene sperm [27, 28], such as reducing sperm competition, filling the spermatheca, and providing an advantage for the first mating male [27, 29]. It promotes the separation of eupyrene sperm bundles and plays an important role in the formation or transport of eupyrene sperm [30, 31]. 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 [32]. 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 by eupyrene sperm in females and ultimately affect female egg production. Therefore, we determined 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 (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 affects fertilization by eupyrene sperm in females and ultimately affects the number of eggs in females.

To investigate the substance that caused the change in the morphology of the spermaphore, we also measured the amount of male Acps before mating. 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 cause physiological changes in insects [21, 33, 34]. For male moths, these accessory gland contents and sperm are mixed to form seminal fluid, which is transferred into the female body with the spermaphore, 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 [21, 35]. 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 [36–40]. 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 [41, 42]. Studies have shown that Acps is the main functional factor in the contents of the accessory gland [42]. 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). 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 Acps. In addition, this result also shows that there might be other reproductive-related substances, such as other accessory gland secretions, that have caused the change in the morphology of the spermaphores after silencing ScerTGL, which should be studied further in the future.

Summarizing the above experimental results, in addition to participating in the energy metabolism process [10], our research showed that 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 spermaphores transported to females and the number of apyrene sperm transferred to spermaphores. 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 [43, 44]. The absence of sex-let al 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 [32]. In addition, research showed that the start time of apyrene sperm formation was later than that of eupyrene sperm in lepidopteran insects [45], which may be
lead 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. 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. The spermatophores are mainly composed of an outer envelope, a bolus of sperm and an inner matrix [25]. In addition to Acps, the main component of the matrix also contains other accessory gland secretions and other seminal fluid proteins. In our experiment, we only measured the Acps content in the male accessory gland before mating. In the process of mating and transferring to spermatophores, whether there is any hindrance to the transfer of accessory gland proteins and whether there are other male secretions that make the spermatophores empty, we need to further explore. Most importantly, in many insect species, females use nutrients from spermatophores to support egg production and somatic cell maintenance [46, 47]. Therefore, the drop in egg production is not only related to sperm, but may also be related to the inability of the empty spermatophores to provide enough nutrition for the females. In summary, our subsequent work will focus on exploring the main reasons for the changes in the morphology of the spermatophores and the decrease in the production of apyrene sperm after silencing ScerTGL, so as to provide more possibilities for environmentally friendly plant protection methods of S. cerealella.

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**Author Contributions** Conceived of and designed the experiments: W-HY, M-YW, and F-LY. Performed the experiments: W-HY, M-YW, NT. Analysis of the data: W-HY and GW. Drafted and revised manuscript: W-HY, SS, Y-CY, KKE, and F-LY. All authors approved the final version of the article, including the authorship list.

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

**Declarations**

**Conflict of interest** The author(s) declare(s) that they have no competing interests.

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

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