Transcriptome analysis unveils the mechanisms of lipid metabolism response to grayanotoxin I stress in *Spodoptera litura*

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Background: *Spodoptera litura* (*S. litura*) is a pest of great economic importance due to being a polyphagous and world-distributed agricultural pest. However, agricultural practices involving chemical pesticides have caused resistance, resurgence and residue problems, highlighting the need for new, environmentally-friendly methods to control the spread of *S. litura*. **Aim:** The aim of this study was to investigate the stomach poisoning of grayanotoxin I, an active compound found in *Pieris japonica*, on *S. litura*, and to explore the underlying mechanisms of these effects. **Methods:** *S. litura* were cultivated in a laboratory setting, and their survival rate, growth and development, pupation time were tested after grayanotoxin I treatment. RNA-Seq was utilized to screen for differentially expressed genes (DEGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted to determine the functions of these DEGs. ELISA was employed to analyze the levels lipase, 3-hydroxyacyl-CoA dehydrogenase (HOAD), and Acetyl-CoA carboxylase (ACC). Hematoxylin and Eosin (H&E) staining was used to detect the development of the fat body. **Results:** Grayanotoxin I treatment resulted in a significant suppression of the survival rate, growth and development, and pupation of *S. litura*. RNA-Seq analysis revealed 285 DEGs following grayanotoxin I exposure, with over 16 genes related to lipid metabolism. These 285 DEGs were enriched in the categories of cuticle development, worm longevity, fat digestion and absorption. Grayanotoxin I treatment also inhibited the levels of FFA, lipase, and HOAD in the hemolymph of *S. litura*. **Conclusion:** The results of this study demonstrated that grayanotoxin I inhibited the growth and development of *S. litura.* The mechanisms might, at least partly, related to the interference of lipid synthesis, lipolysis, and fat body development. These findings provide valuable insights into new, environmentally-friendly

plant-derived insecticide, grayanotoxin I, to control the spread of *S. litura*.

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Abstract

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Methods: *S. litura* were cultivated in a laboratory setting, and their survival rate, growth and development, pupation time were tested after grayanotoxin I treatment. RNA-Seq was utilized to screen for differentially expressed genes (DEGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted to determine the functions of these DEGs. ELISA was employed to analyze the levels lipase, 3-hydroxyacyl-CoA dehydrogenase (HOAD), and Acetyl-CoA carboxylase (ACC). Hematoxylin and Eosin (H&E) staining was used to detect the development of the fat body.

Results: Grayanotoxin I treatment resulted in a significant suppression of the survival rate, growth and development, and pupation of *S. litura*. RNA-Seq analysis revealed 285 DEGs following grayanotoxin I exposure, with over 16 genes related to lipid metabolism. These 285 DEGs were enriched in the categories of cuticle development, worm longevity, fat digestion and absorption. Grayanotoxin I treatment also inhibited the levels of FFA, lipase, and HOAD in the hemolymph of *S. litura*.

Conclusion: The results of this study demonstrated that grayanotoxin I inhibited the growth and development of *S. litura.* The mechanisms might, at least partly, related to the interference of lipid synthesis, lipolysis, and fat body development. These findings provide valuable insights into new, environmentally-friendly plant-derived insecticide, grayanotoxin I, to control the spread of *S. litura*.

1. Introduction

Spodoptera litura (*S. litura*), also named tobacco cutworm pest, is a polyphagous and widely-distributed agricultural pest, that causes damage to over 300 host plants. It is found in Africa, the Middle East, Southern Europe, and Asia [1]. Currently, the control of S. litura relies heavily on the use of chemical pesticides. However, due to the resistance, resurgence, and residue problems caused by unreasonable long-term use of chemical pesticides [2], new environmentally-friendly methods are urgently needed.

One promising approach for developing the environmentally-friendly pesticides is screening bioactive compounds from natural plant products. Compared to synthetic chemical insecticides, botanical insecticides have been considered to be low environmental and mammalian risk, high specificity and safety, low risk of resistance development, and low environmental persistence [3-5]. Several classes of molecules derived from plant products were demonstrated to be with bioactivity, such as terpenes, flavonoids, alkaloids, polyphenols *etc* [6, 7]. These plant-derived

insecticides achieved their effects through mechanisms of affecting nervous system, respiratory and endocrine systems, as well as water balance in insects [7]. For example, Azadirachtin is a series of tetracyclic triterpenoid compounds extracted from plant *Azadirachta indica A. Juss*. It achieved insecticidal effects through deterring feeding, interfering with egg laying, disrupting insect metamorphosis, repelling larvae, and inhibiting their growth [8, 9]. Rotenone induced insect cells necrosis via the cytoplasmic membrane damage and mitochondrial dysfunction [10]. Pyrethrins killed mosquitos through modulating voltage-gated sodium channels [11]. Triterpenoids extracted from plants are important class of compounds extensively studied in the research of plant-based pesticides [12]. Grayanotoxin I is a diterpenoids belongs to grayanotoxin family. Grayanotoxins are commonly found in plants of the *Ericaceae* family, including *Rhododendron* and *Pieris japonic*a [13]. *Pieris japonica*, has been reported to have anti-insect effects[14]. As one of the most abundant and potent toxin in *Pieris japonica*, grayanotoxin was shown to interact with voltage-gated sodium channels, lead to the disruption of neuronal signaling, and cause symptoms such as dizziness, analgesic, weakness, and cardiac effects when ingested [15]. However, the precise effects and mechanisms of grayanotoxin I on agricultural pests are still largely unknown. Our preliminary studies showed that grayanotoxin I significantly inhibited the growth and development of *S. litura*. In order to further explore the mechanisms of this effect, the present study screened the transcriptome of *S. litura*, analyzed the functions of differentially expressed genes (DEGs), detected changes in the development of the fat body, and measured the levels of free fatty acids (FFA), 3-hydroxyacyl-CoA dehydrogenase (HOAD), Acetyl-CoA carboxylase (ACC), and lipase after grayanotoxin I treatment. The present study aims to shed light on the effects and mechanisms of grayanotoxin I on *S. litura*, and contribute to the development of new environmentally-friendly pesticides.

2. Materials & Methods

2.1 Materials and Reagent

Grayanotoxin I was procured from Sichuan Biocrick Biotech Co. Ltd (4720-09-6, Chengdu, China). The free fatty acid assay kit was obtained from Jiancheng Co. Ltd. (Nanjing, China), while the hematoxylin-eosin (H&E) staining solution was obtained from Beyotime Biotechnology (Shanghai, China). The Vazyme® HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) and Vazyme® ChamQ Universal SYBR qPCR Master Mix were purchased from Vazyme Corporation (Nanjing, China). The primers were synthesized by Takara (Dalian, China). Further, Lipase (JM-00078O1), 3-hydroxyacyl-CoA dehydrogenase (JM-00048O1), and Acetyl-CoA carboxylase (JM-00064O1) ELISA kits were procured from Jingmei Biotechnology (Jiangsu, China).

2.2 *Spodoptera litura* culture, treatment, and sample collection

The larvae of *S. litura* were obtained from Keyun Biopesticide Co. Ltd in Henan, China. These larvae were sourced from fields free from heavy metal pollution with no prior application of chemical insecticides. Optimal laboratory culture conditions of a temperature of 25 ± 2 ℃, humidity of 75%-85%, and a light cycle of Light/Dark: 14h/10h were employed for the rearing of the larvae. Only the second instar larvae with uniform size, and normal development were selected for further testing.

To investigate the effects of grayanotoxin I on *S. litura*, the plant derived insecticide, matrine was used as positive control. The second instar larvae were randomly divided into normal diet, different concentration of grayanotoxin I-containing diet, or matrine-containing diet group. The diets were prepared by adding 7 mL of ddH_2O, 1.25- 6.25 mg/L grayanotoxin I, or 0.4% matrine solution to 5 g diet. The survival rate were calculated at 24-hour, 48-hour, and 72-hour treatment. The midgut of *S. litura* larvae fed on 1.25% grayanotoxin I-contained diet or normal diet (ddH_2O) for 72 hours was collected for RNA-Seq.

For analysis of body weight and developmental time, sublethal concentrations (0.62-1.25 mg/L) of grayanotoxin I were used to treat *S. litura* larvae. The diets were prepared by adding 7 mL of grayanotoxin I solution to 5 g of normal diet. The wet body weight of each larvae was collected at each instar stage until pupation, and the data was recorded.

2.3 Hematoxylin and Eosin (H&E) staining of fat body

The growth rate of insects is largely regulated by the fat body [16]. In order to assess the development of this crucial tissue, we utilized the H & E staining method, as previously described [17]. The specimens were subjected to a 5-hour incubation at 5 °C in 10 % sucrose in 0.01 M phosphate-buffered saline (PBS, pH 7.4), with sucrose concentration gradually increased to 20%. The samples were then embedded in optimal cutting temperature (OCT) compound and instantaneously frozen with dry ice. Further, frozen samples were sectioned at 10 μm and stained by H & E method to obtain images. The images were examined under a microscope to evaluate the development of the fat body.

2.4 RNA extraction and RNA-sequencing

To further explore the impact of grayanotoxin I on the expression of lipid metabolism related genes, RNA-Sequencing using Illumina NovaSeq 6000 plateform (Illumina, San Diego, CA, USA) was carried out at Shanghai Personal Biotechnology Cp., Ltd (Shanghai, China). The methodology was consistent with previously published studies [18, 19]. Briefly, total RNA was extracted using the Trizol reagent. The quality and quantity of total RNA were assessed by measuring the absorbance on wavelength of 260nm and 280nm by NanoDrop spectrophotometer (Thermo Scientific). After removal of rRNA by using poly-T oligo-attached magnetic beads, the total RNA was fragmented by using divalent cations under elevated temperature in an Illumina proprietary fragmentation buffer. The first strand cDNA was synthesized using random oligonucleotides and Super Script II. Subsequently, the second strand cDNA synthesis was performed by using DNA Polymerase I and RNase H. For hybridization preparation, the DNA fragments' 3' ends were adenylated, followed by ligation of Illumina PE adapter oligonucleotides. To obtain cDNA fragments of the desired length (400-500 bp), the library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA). DNA fragments possessing adapter molecules on both ends were selectively enriched through a 15 cycle PCR reaction with the Illumina PCR Primer Cocktail. The resulting products were purified using the AMPure XP system and the quantity was measured using the Agilent high sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent). Finally, the sequencing library was sequenced on the NovaSeq 6000 platform (Illumina) by Shanghai Personal Biotechnology Cp. Ltd.

2.5 Differentially expressed genes (DEGs) identification

The reference genome used in the present transcriptome was "https://www.ncbi.nlm.nih.gov/assembly/GCF_002706865.2". The sequencing data was filtered

to get high quality sequence by using Cutadapt (v1.15) software. The filtered data were mapping to the reference genome using HISAT2 (v2.0.5). The analysis of *S. litura* mRNA expression was performed using HTSeq (0.9.1) statistics. Original expressed readcount value per gene was normalized via the FPKM method. DESeq (1.30.0) was employed to analyze differences in mRNA expression levels. RNAs with |log2FoldChange| > 1.0 and *P*-value < 0.05 were identified as differentially expressed. To perform heatmap clustering, MeV 4.9.0 software was used. Using this method, differentially expressed lipid metabolism related genes were selected and heatmap clustering was conducted.

2.6 RT-qPCR verification of lipid metabolism-related DEGs

In order to verify the expression of four differentially expressed lipid metabolism-related genes, we utilized RT-qPCR as described previously [20]. Total RNAs were extracted using Trizol reagent, followed by reverse transcription to cDNA utilizing the Vazyme®HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper). PCR reactions were carried out using the Vazyme® ChamQ Universal SYBR qPCR Master Mix kit on the Applied Biosystems Quantstudio 5 system. The qPCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60°C for 30 s. The GAPDH and β-actin were used as reference genes. The primers was presented in the Table 1. The non-transcribed RNA was used as negative control. The melting curve analysis was performed to verify the specificity of PCR products. All samples were run in triplicate, and the analyzed using the $2^{(-\Delta\Delta Ct)}$ method.

2.7 Detection of FFA, lipase, ACC, and HOAD

Lipase, HOAD, and ACC are enzymes that play key roles in the metabolism of fatty acids. To investigate the impact of grayanotoxin I on the lipid metabolism of *S. litura*, we employed an ELISA-based approach to measure the levels of lipase, HOAD, and ACC in the hemolymph of 5th instar larvae. Briefly, hemolymph samples were collected in a 1.5 ml tube with 0.1% dithiothreitol (DTT), centrifuged for 5 min (10000 rpm) at 4°C. The supernatant was stored at -80 ℃ for further use [21]. The ELISA analysis was conducted according to the manufacturer's instructions. Specifically, 50 μL of serum samples were added to enzyme-linked immunosorbent plates, mixed with enzyme labeling reagents, and incubated at 37°C for 60 minutes. The liquid was then removed, and each well was washed 5 times with washing solution before adding chromogenic reagent and mixing. The mixture was incubated for 15 minutes at 37°C in the dark, after which the reaction was halted using a stop solution. The absorbance value was then measured to determine the levels of lipase, HOAD, and ACC.

FFA was measured by using the fatty acid assay kit purchased from Jiancheng Co. Ltd. (Nanjing, China) according to the manufacturer's instruction. The assay kit is based on the principal that FFA reacts with copper ions to form fatty acid copper salts, which are soluble in chloroform. By using the copper reagent method to determine the copper ion content, the content of FFA can be estimated by colorimetric assay.

2.7 Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) and protein-protein interaction (PPI) analysis of lipid metabolism-related differentially expressed genes

In order to investigate the functions of differentially expressed genes related to lipid metabolism, we conducted GO enrichment analysis and KEGG pathway analysis. This analysis was carried out using the online tool DAVID (https://david.ncifcrf.gov/) [22, 23]. The top 10 terms from biological process (BP), cell components (CC), molecular function (MF) and KEGG

pathway were visualized, and a P-value < 0.05 was considered significant for both GO terms and KEGG pathways.

To further examine the interactions between the lipid metabolism-related DEGs, we utilized the online tool STRING (website: https://string-db.org/). As *S. litura* data was not available in STRING, we used *Bombyx mori* data as an alternative. We also performed a further analysis of the signal pathways of the lipid metabolism-related DEGs on KEGG pathway (https://www.genome.jp/kegg/).

2.10 Statistical analysis

All the statistics of were presented in the form of mean ± S.D. The significance of the differences was analyzed by ANOVA followed by the Newman-Student-Keuls test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Influence of grayanotoxin I on *S. litura* growth and development

To investigate the impact of grayanotoxin I on *S. litura*, we monitored the survival rate, growth and development of the insects after being subjected to grayanotoxin I-contained, metrine-contained, or normal diet. As depicted in Figure 1A, the application of positive control drug, 0.4% matrine, reduced the survival rate to 18.8% after a 72-hour treatment. While 72-hour treatment with 6.25 mg/L grayanotoxin I reduced the survival rate to 40.0 %, as compared to the normal diet (ddH_2O, survival rate of 96.7%). Additionally, lower concentrations of grayanotoxin I (0.62-1.25 mg/L) significantly hindered the growth of *S. litura* (Figure 1 B-C). Compared to ddH_2O group, the 0.2% matrine hindered 95.3% body weight of *S. litura* on day 14. The suppression rate was 90.65% for 1.25 mg/L grayanotoxin I, and 56.29% for 0.65 mg/L grayanotoxin I after 14-day treatment (Fig 1 B-C). Furthermore, we observed a significant delay in pupation time of *S. litura* because of grayanotoxin I (Figure 1 D). The average pupation time for ddH_2O group was 14.72 days. While it was 20.23 days for 1.25 mg/L grayanotoxin I treatment, and was 18.25 days for 0.62 mg/L grayanotoxin I treatment (Figure 1 D).

3.2 Inhibition effect of grayanotoxin I on *S. litura* fat body development

In the present study, H & E staining was conducted to investigate the relationship between fat body development and the growth of *S. litura*. As illustrated in Figure 2, a noticeable accumulation of fat in the fat body was observed in the ddH_2O control group (Fig 2 A). However, treatment with grayanotoxin I resulted in a significant inhibition of fat body development (Fig 2 B).

3.3 Gene expression profiles of *S. litura* under grayanotoxin I treatment

To investigate the mechanisms of grayanotoxin I, we analyzed the transcriptome alteration after 72-hour 1.25% grayanotoxin I treatment by using the RNA-Seq method. The statistical power of this RNA-Seq data calculated in "RNASeqPower" was 0.855 (sequencing depth: 60, sample size: 3). As a result, 285 DEGs were identified. Among them, 151 were upregulated and 134 were downregulated (Figure 3 A-B).

3.4 GO and KEGG enrichment of differentially expressed lipid metabolism related genes

In order to get further insight into the functions of the 285 DEGs, we carried out KEGG pathway enrichment and GO enrichment analysis. In the GO enrichment analysis, these DEGs were mostly enriched in the MF terms related to the structural constituents of chitin-based cuticle; BP terms associated with cuticle development; and CC terms related to the extracellular matrix (as depicted in Figure 3C). The KEGG analysis (Figure 3D) revealed that these DEGs were enriched in several pathways including the Organismal System terms of longevity regulating pathway, cytosolic DNA-sensing pathway, and fat digestion and absorption pathway; the Metabolism terms of cutin, suberin, wax biosynthesis, linoleic acid metabolism, insect hormone biosynthesis, and unsaturated fatty acid synthesis; the Cellular Process terms of peroxisome.

3.5 The effects of grayanotoxin I on lipid metabolism-related genes profile expression, lipid metabolism related enzymes activities in the hemolymph, and FFA level in *S. litura*

In our RNA-Seq analysis, we discovered many DEGs related to lipid metabolism. Specifically, we observed an upregulation of genes such as acyl-CoA desaturase, esterase E4, and phospholipase, and downregulated genes such as fatty acid elongase, fatty acid-binding protein, and pancreatic-like lipase following treatment with grayanotoxin I (Figure 4A). The results of RNA-Seq were verified by qPCR analysis, which were shown in Figure 4B.

Besides, grayanotoxin I (1.25mg/L) treatment dramatically decreased the level of FFA in the hemolymph of *S. litura* (Figure 4 C). Further ELISA analysis revealed a significant decrease in lipase and HOAD mRNA levels after treatment with grayanotoxin I, compared to the normal group ($P<0.05$). A slight decrease of ACC mRNA was also found after grayanotoxin I treatment (Figure 4D-F).

3.6 PPI analysis of lipid metabolism-related DEGs analysis

The PPI of the lipid metabolism-related genes was shown in Figure 5 A. Red circles (CG12268 and Desat1) were upregulated genes in *S. litura* after grayanotoxin I treatment, while green circles (CG5278, CG6271, fabp, and wat) were downregulated genes.

The LOC111354773 (putative fatty acyl-CoA reductase), LOC111355891 (acyl-CoA desaturase 1 like), LOC111350394 (ELOVL fatty acid elongase), LOC111349277 (elongation of very long chain fatty acids protein 7 like), and LOC111360381 (fatty acid-binding protein 2 like) were connected clearly in the network.

Further analysis revealed that LOC111354773 (putative fatty acyl-CoA reductase), LOC111355891 (acyl-CoA desaturase 1 like), LOC111355893 (acyl-CoA desaturase 1 like), LOC111352061 (putative fatty acyl-CoA reductase), and LOC111356581 (fatty acyl-CoA reductase wat like) were enriched in the longevity regulating pathway and were relevant to the aging of the larvae. The aforementioned genes along with LOC111350394 (ELOVL fatty acid elongase), LOC111348151 (phospholipase A1 like), and LOC111356581 (fatty acyl-CoA reductase wat like) were found to be associated with lipid metabolism. Additionally, LOC111355891 (acyl-CoA desaturase 1 like), LOC111360381 (fatty acid-binding protein 2

like), and LOC111355893 (acyl-CoA desaturase 1 like) were found to be relevant to the PPAR signaling pathway, as documented in Table 2 and Figure 5B.

4. Discussion

The impact of grayanotoxin I on *S. litura* was evaluated in the present study, revealing a significant reduction in the survival rate, larvae growth, and delayed pupation. Transcriptome analysis identified 285 DEGs responding to grayanotoxin I treatment. GO enrichment and KEGG pathway enrichment indicated grayanotoxin I affected the expression of genes related to cuticle development, extracellular matrix, wax biosynthesis, insect hormone biosynthesis, fat digestion and absorption *etc*. Notably, over sixteen of these DEGs were linked to lipid metabolism, with a significant decrease in FFA, lipase, and HOAD levels. These findings implicated grayanotoxin I probably interfered in lipid synthesis, lipolysis, lipid trafficking, and fat body development, ultimately restraining the growth of *S. litura*.

Traditional Chinese Medicine (TCM) has long been recognized for its low resistance and high efficiency, making it a popular remedy for a wide range of human ailments as well as agricultural insect infestations [6, 24-26]. Grayanotoxin I is a diterpenoids belongs to grayanotoxin family. Grayanotoxins are commonly found in plants of the *Ericaceae* family, including *Rhododendron* and *Pieris japonica* [13]. Previously, grayanane diterpenoid glucosides were recognized as potent analgesics [15]. Our study found under grayanotoxin I stress, the growth and development of *S. litura* were significantly inhibited. Employing RNA-Seq, we have analyzed the transcriptome of *S. litura* to explore the molecular mechanisms responsible for the actions of grayanotoxin I. Many lipid metablolism-related genes were responded to the treatment of grayanotoxin I, such as elevated expression of acyl-CoA desaturase, esterase E4, lipase H, and phospholipase A, and decreased expression of elongation of very long chain fatty acids protein, fatty acid-binding protein, acyl-CoA reductase wat, and pancreatic-like lipase. We also observed a significant reduction in the FFA level, activities of lipase, and HOAD after grayanotoxin I treatment. Based on these observations, we conclude that grayanotoxin I exerts its effects through, at least partly, modulating of lipid metabolism related genes expression in *S. litura*.

Lipids play crucial roles in the growth, development, and reproduction of insects. Fatty acid-derived wax esters, fatty alcohols, and hydrocarbons are essential components of the insect epidermis [27]. Very long chain fatty acids serve as the precursors of sphingolipids and glycerolipids, two fundamental components of cell membranes. Unsaturated fatty acids and fatty acid content are also crucial for the cold tolerance of insects [28]. Furthermore, lipids serve as an essential energy source for insect activities [29, 30]. Due to the vital role lipids play in insects, lipid synthesis and lipolysis have become attractive targets for agriculture pest control. For instance, an in vitro enzyme kinetic experiment showed the pesticide spirotetramat binded to the carboxyltransferase (CT) domain of ACC and inhibited the fatty acid biosynthesis in *Myzus persicae*, *Spodoptera frugiperda*, and *Tetranychus urticae* [31]. ACC is the rate-limiting enzyme in the initial step of fatty acid synthesis, responsible for insect lipid accumulation and epidermal function [32]. *Piper aduncum* (*Piperaceae*) essential oil, when delivered to insect thorax by micropipette, effectively depleted lipid content in fat body cells of brown stink bug *Euschistus*

heros (*Heteroptera: Pentatomidae*), leading to the inhibition of bug development and reproduction [33]. Similarly, *S. frugiperda* larvae, fed with corn leaf pieces immersed with citronella oil from *Cymbopogon winterianus*, increased glycogen, but decreased protein, lipid, and total sugar content in leading to diminished reproduction [34]. Our study observed a significant decrease in insect survival rate, suppression of larvae growth, and delay in pupation following grayanotoxin I treatment. Additionally, hemolymph FFA content and fat body lipids were notably decreased. These phenotypes strongly suggested the involvement of lipid metabolism in the effects of grayanotoxin I on *S. litura.*

Lipase is an enzyme that catalyzes the hydrolysis of triglycerides into fatty acids and glycerol, playing a crucial role in the digestion and transportation of lipids. Insects possess several types of lipase, including pancreatic-like lipase, which hydrolyzes most dietary fats. Fatty acid-binding proteins (FABPs) are a group of small, soluble intracellular proteins responsible for efficient lipid trafficking and signaling within cells [35]. In our current study, we observed a significant decrease in FABP mRNA following grayanotoxin I treatment. FABPs are involved in regulating long-term memory, sleep, and lipid accumulation in insects [36]. Two FABP subtypes, slFABP1 (MFB2) and slFABP2 (MFB1), were found in the midgut of *S. litura*, and they are known to participate in starving stress and body development [37]. HOAD is a crucial enzyme involved in the beta-oxidation of lipids, which is responsible for the energy supply in insects. Grayanotoxin I treatment was found to suppress lipase and FABP activity, potentially disrupting the formation and trafficking of FFA in *S. litura.* Additionally, decreased HOAD activity may hinder fatty acid utilization and subsequent energy supply for the pest.

Our study uncovered a decrease in elongation of very long chain (ELOVL) fatty acids elongase after grayanotoxin I treatment. ELOVL fatty acid elongase is primarily located on the endoplasmic reticulum (ER) and promotes the synthesis of C18-26 fatty acids from the C16 chain. ELOVL fatty acid elongases are widely existed in different insects, such as *Bombyx mori*, *Locusta migratoria,* and *Ericerus pela Chavannes* [38-40]. The Very long chain fatty acids, including saturated and unsaturated fatty acids, are crucial source of accumulated fat in the fat body of insects. Our present study found significant decrease of ELOVL fatty acid elongase mRNA expression after grayanotoxin I treatment. Considering the important roles of ELOVL elongase in the fat body development, we presumed that the effects of grayanotoxin I on *S. litura* growth and development might, at least partly, related to the inhibition of ELOVL fatty acid elongase. Furthermore, our research revealed an increase in phospholipase A expression. Phospholipases hydrolyze phospholipids and participate in cell signaling pathways. The elevation of phospholipase A levels suggested the involvement of inflammation under grayanotoxin I stress.

In our studies, the stomach poisoning of grayanotoxin I on *S. litura* was tested by diet mixed method according to the book “Standard Operation Practice for Pesticide Biological Activity Testing” written by Baogen Gu and Xue Liu [41]. For the pesticide bioassay testing on *S. litura*, “diet mixed with insecticide” and “leaf dip feeding” methods were two commonly used methods for testing stomach poisoning, while spray application was used for contact toxicity studies [41,

42]. In the lab bioassay of insecticide, “diet mixed withi insecticide” method was widely used because this method is simple, cost-effective, time-saving, and reliable. It is suitable for long-term medication and particularly appropriate for insecticides that are insoluble in water or have poor palatability [43-45].

Besides *S. litura*, we have screened the insecticidal effects of grayanotoxin I on *Diamondback moth, Beet armyworm*, and *Budworm*. *S. litura* was the most sensitive insect to grayanotoxin I, followed by *Diamondback moth. Beet armyworm*, and *Budworm* were not sensitive to grayanotoxin I stress. Therefore, we selected *S.litura* as target insect.

5. Conclusions

The results of this study demonstrated that grayanotoxin I inhibited the growth and development of *S. litura.* The mechanisms might, at least partly, related to the interference of lipid synthesis, lipolysis, and fat body development. These findings provide valuable insights into new, environmentally-friendly plant-derived insecticide, grayanotoxin I, to control the spread of *S. litura*.

6. Data Availability

The RNA-Seq raw data are available at the SRA: PRJNA957576.

8. References

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Figure legends:

Figure 1. Effects of grayanotoxin I on survival rate, the growth & development of *S. litura*. The 2nd instar larvae of *S. litura* were fed with normal diet, grayanotoxin I containing diet, or matrine containing diet. The survival rate, body length, body weight, and pupation time were measured. (A) The survival rate of *S. litura* after ddH_2O, 1.25-6.25 ml/L grayanotoxin I, or 0.4% matrine treatment in 24, 48, and 72 hours; (B) the body length of *S. litura* between ddH_2O or 0.62 mg/L grayanotoxin I treatment on day 14; (C) the bodyweight-time curve after 0.62-1.25 ml/L

grayanotoxin I, ddH_2O, or sublethal matrine (0.2%) treatment; the body weight of each worm was measured every 2 days. (D) the pupation time after grayanotoxin I, ddH_2O, or sublethal matrine (0.2%) treatment. All data were presented in mean ± SD, $^{**}P<0.01$; $^{*}P<0.05$ vs ddH_2O group.

Figure 2. The development of fatty body after treatment of grayanotoxin I. After treatment of grayanotoxin I for 14 days, the larvae of *S. litura* specimens were sectioned and stained by Hematoxylin and Eosin. The images were examined under a microscope to evaluate the development of the fat body. A, *S. litura* treated by ddH_2O; B, *S. litura* treated by grayanotoxin I.

Figure 3. The transcriptomic analysis, GO enrichment, and KEGG enrichment of differentially expressed genesafter grayanotoxin I treatment in *S. litura*. (A) The number of upregulated and downregulated genes after grayanotoxin I treatment; (B) The heatmap of all differentially expressed genes after grayanotoxin I treatment; (C) GO enrichment of differentially expressed genes; (D) KEGG enrichment of differentially expressed genes

Figure 4. Effects of grayanotoxin I on lipid metabolism-related genes, lipid metabolism related enzymes activities, and FFA levels in in *S. litura*. The 2nd instar larvae of *S. litura* were treated with ddH_2O (control group) or 1.25 mg/L grayanotoxin I containing diet for 72 hours following which the midgut of *S. litura* was collected for RNA-Seq. A, the heatmap of differentially expressed lipid metabolism related genes; B, qPCR verification of 4 randomly chosen lipid metabolism related genes; C-F, the level of free fatty acid, lipase , acetyl-CoA carboxylase, and HOAD in hemolymph of *S. litura*. All data were presented in mean ± SD, $^{**}P<0.01$ vs control group.

Figure 5. The protein-protein interactions and signal pathways of lipid related DEGs. A, the protein-protein interaction of lipid-related DEGs analyzed by STRING online software; B, the visualization of PPAR signaling pathway obtained from KEGG pathway online software.

Table 1 The primers used in the present analysis

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Figure 1

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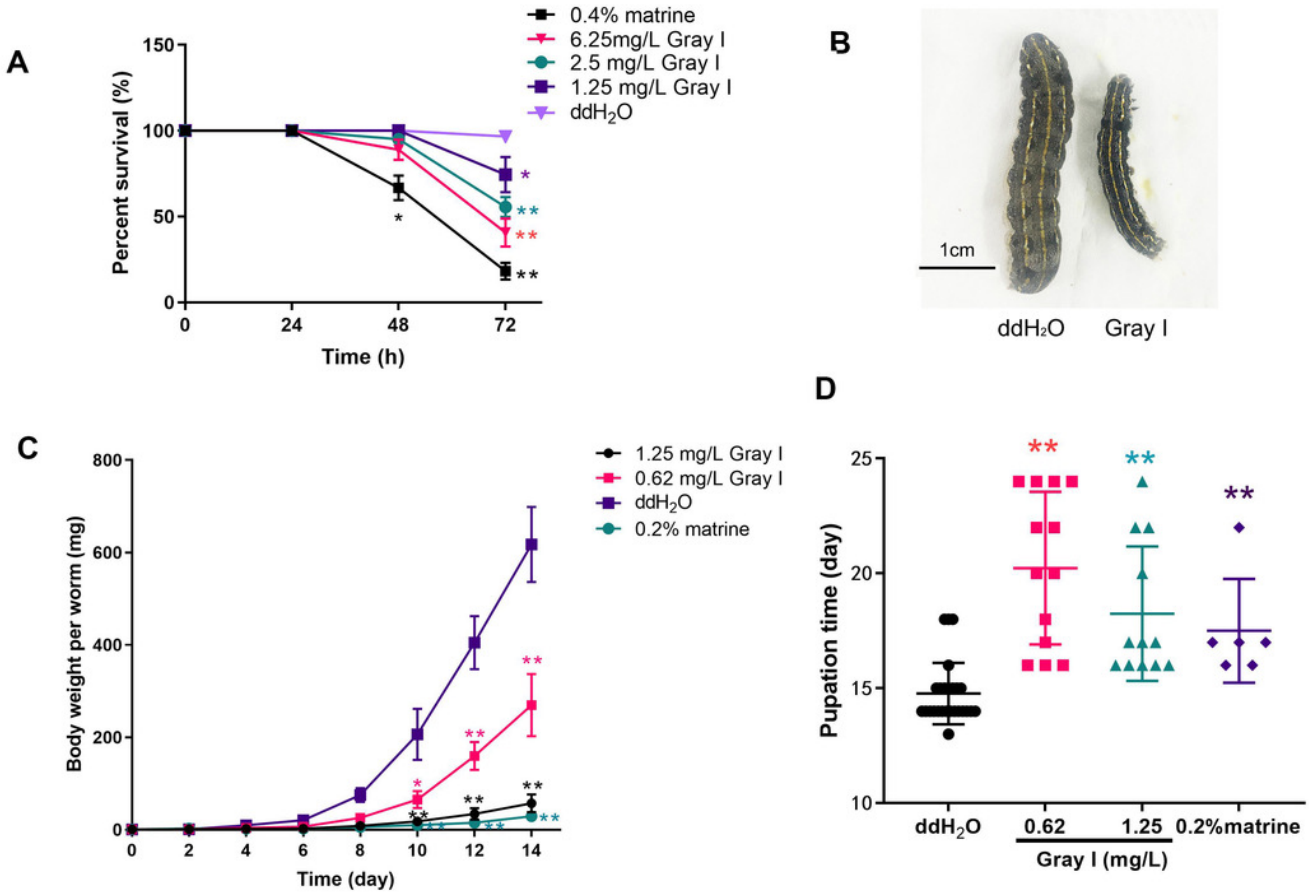
A
0.4% matrine
6.25mg/L Gray I
2.5 mg/L Gray I
1.25 mg/L Gray I
ddH2O
Percent survival (%)
Time (h)
B
1cm
ddH2O
Gray I
C
1.25 mg/L Gray I
0.62 mg/L Gray I
ddH2O
0.2% matrine
Body weight per worm (mg)
Time (day)
D
Pupation time (day)
ddH2O
0.62
1.25
0.2%matrine
Gray I (mg/L)

Figure 2

The development of fatty body after treatment of grayanotoxin I.

Figure 2. The development of fatty body after treatment of grayanotoxin I. After treatment of grayanotoxin I for 14 days, the larvae of *S. litura* specimens were sectioned and stained by Hematoxylin and Eosin. The images were examined under a microscope to evaluate the development of the fat body. A, *S. litura* treated by ddH_2O; B, *S. litura* treated by grayanotoxin I.

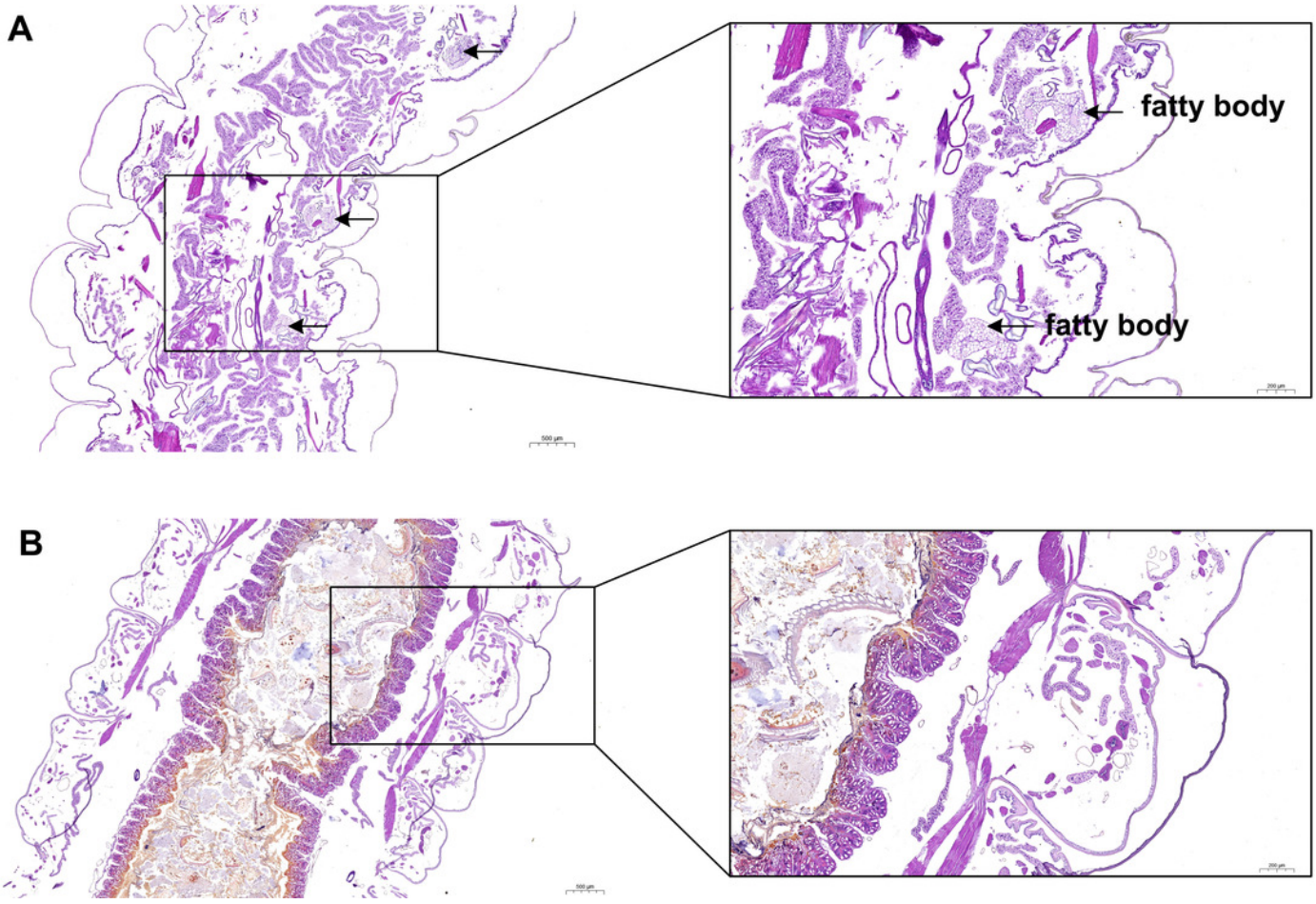

Figure 3

The transcriptomic analysis, GO enrichment, and KEGG enrichment of differentially expressed genesafter grayanotoxin I treatment in *S. litura*.

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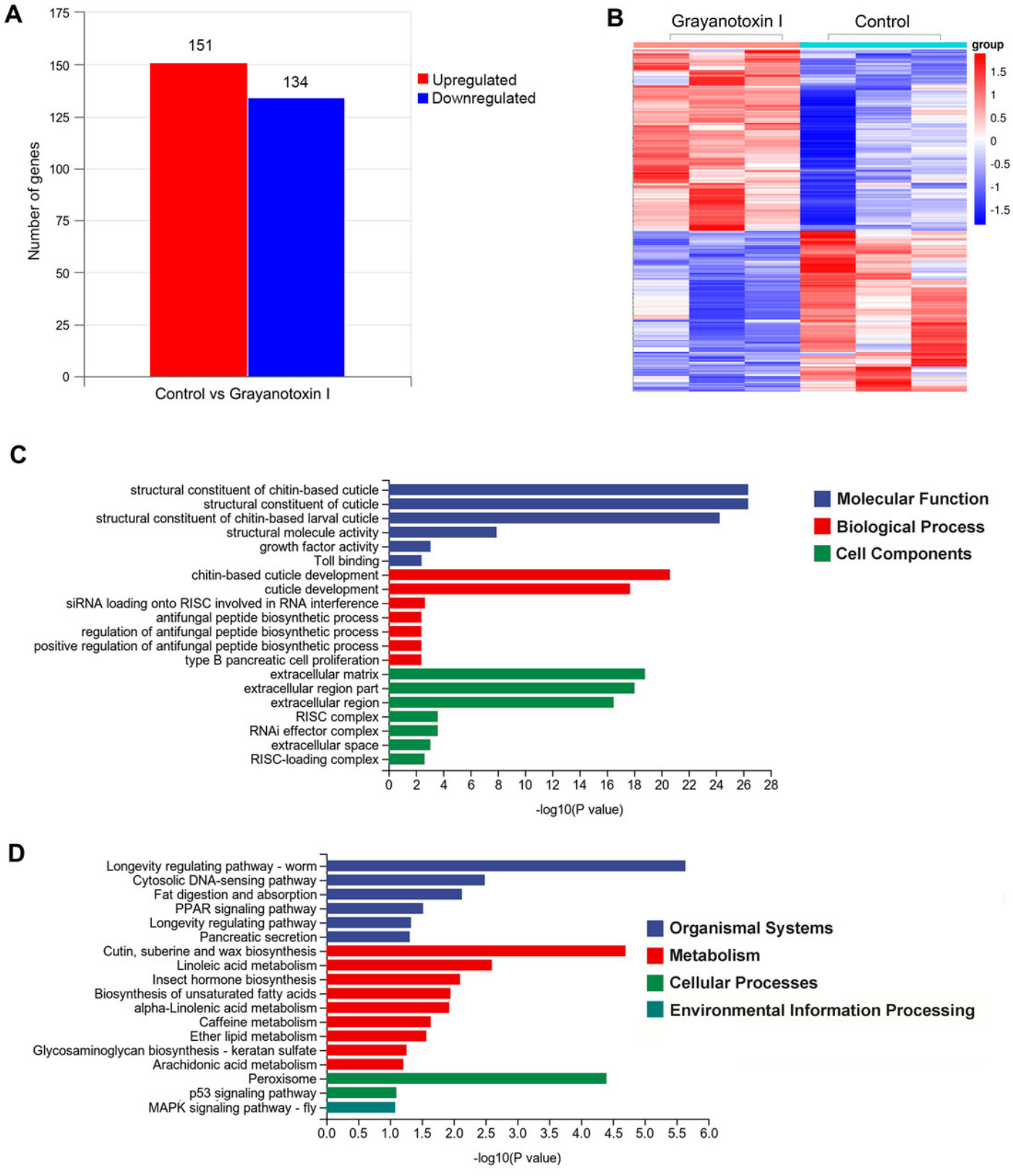
A
175
150
125
100
75
50
25
0
Number of genes
151
134
Upregulated
Downregulated
Control vs Grayanotoxin I
B
Grayanotoxin I
Control
group
1.5
1
0.5
0
-0.5
-1
-1.5
C
structural constituent of chitin-based cuticle
structural constituent of cuticle
structural constituent of chitin-based larval cuticle
structural molecule activity
growth factor activity
Toll binding
chitin-based cuticle development
cuticle development
siRNA loading onto RISC involved in RNA interference
antifungal peptide biosynthetic process
regulation of antifungal peptide biosynthetic process
positive regulation of antifungal peptide biosynthetic process
type B pancreatic cell proliferation
extracellular matrix
extracellular region part
extracellular region
RISC complex
RNAi effector complex
extracellular space
RISC-loading complex
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
-log10(P value)
Molecular Function
Biological Process
Cell Components
D
Longevity regulating pathway - worm
Cytosolic DNA-sensing pathway
Fat digestion and absorption
PPAR signaling pathway
Longevity regulating pathway
Pancreatic secretion
Cutin, suberine and wax biosynthesis
Linoleic acid metabolism
Insect hormone biosynthesis
Biosynthesis of unsaturated fatty acids
alpha-Linolenic acid metabolism
Caffeine metabolism
Ether lipid metabolism
Glycosaminoglycan biosynthesis - keratan sulfate
Arachidonic acid metabolism
Peroxisome
p53 signaling pathway
MAPK signaling pathway - fly
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0
-log10(P value)
Organismal Systems
Metabolism
Cellular Processes
Environmental Information Processing

Figure 4

Effects of grayanotoxin I on lipid metabolism-related genes, lipid metabolism related enzymes activities, and FFA levels in in *S. litura*

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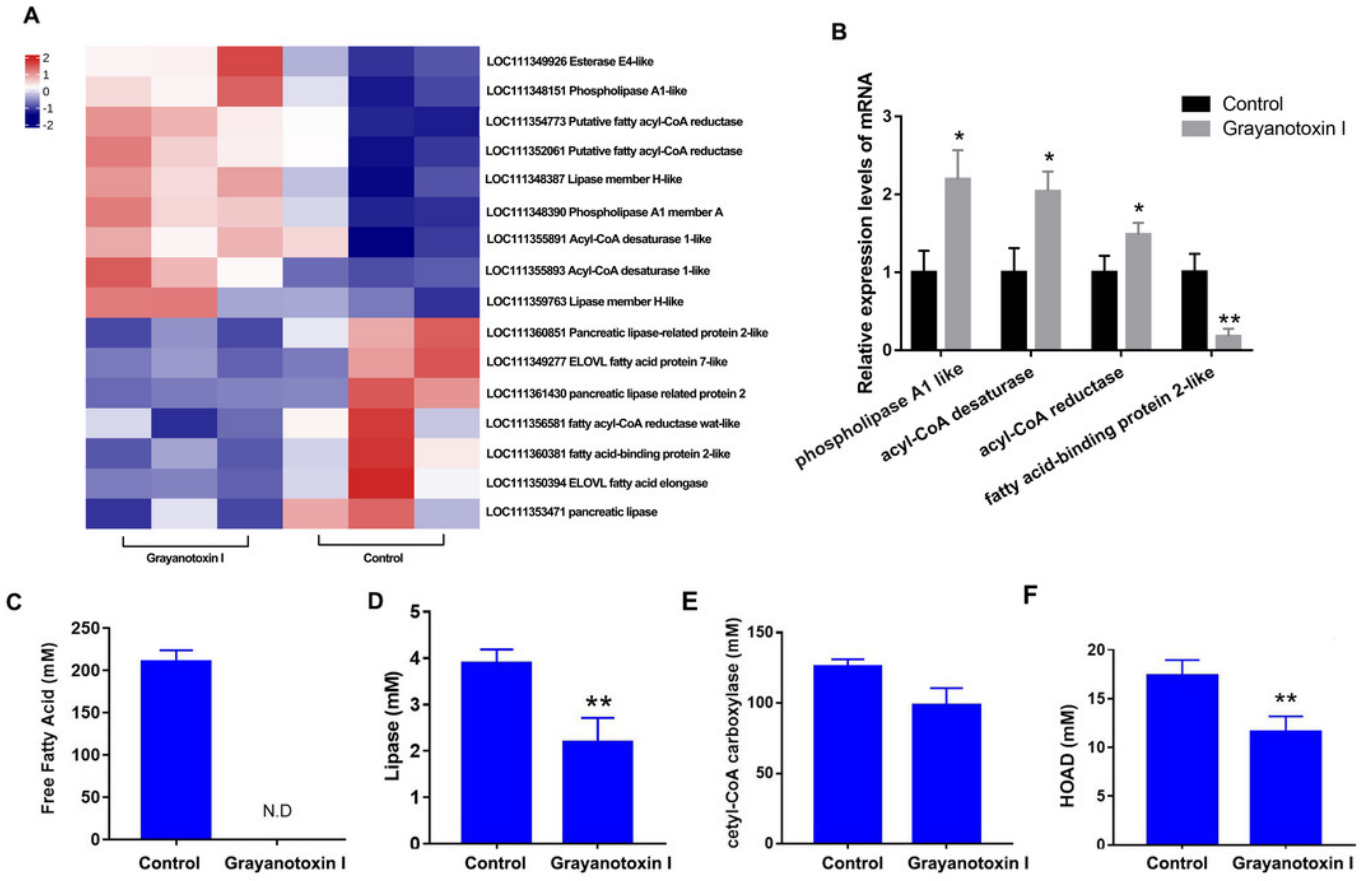
A
2
1
0
-1
-2
LOC111349926 Esterase E4-like
LOC111348151 Phospholipase A1-like
LOC111354773 Putative fatty acyl-CoA reductase
LOC111352061 Putative fatty acyl-CoA reductase
LOC111348387 Lipase member H-like
LOC111348390 Phospholipase A1 member A
LOC111355891 Acyl-CoA desaturase 1-like
LOC111355893 Acyl-CoA desaturase 1-like
LOC111359763 Lipase member H-like
LOC111360851 Pancreatic lipase-related protein 2-like
LOC111349277 ELOVL fatty acid protein 7-like
LOC111361430 pancreatic lipase related protein 2
LOC111356581 fatty acyl-CoA reductase wat-like
LOC111360381 fatty acid-binding protein 2-like
LOC111350394 ELOVL fatty acid elongase
LOC111353471 pancreatic lipase
Grayanotoxin I
Control
B
Relative expression levels of mRNA
Control
Grayanotoxin I
phospholipase A1 like
acyl-CoA desaturase
acyl-CoA reductase
fatty acid-binding protein 2-like
C
Free Fatty Acid (mM)
N.D
Control
Grayanotoxin I
D
Lipase (mM)
Control
Grayanotoxin I
E
cetyl-CoA carboxylase (mM)
Control
Grayanotoxin I
F
HOAD (mM)
Control
Grayanotoxin I

Figure 5

The protein-protein interactions and signal pathways of lipid related DEGs

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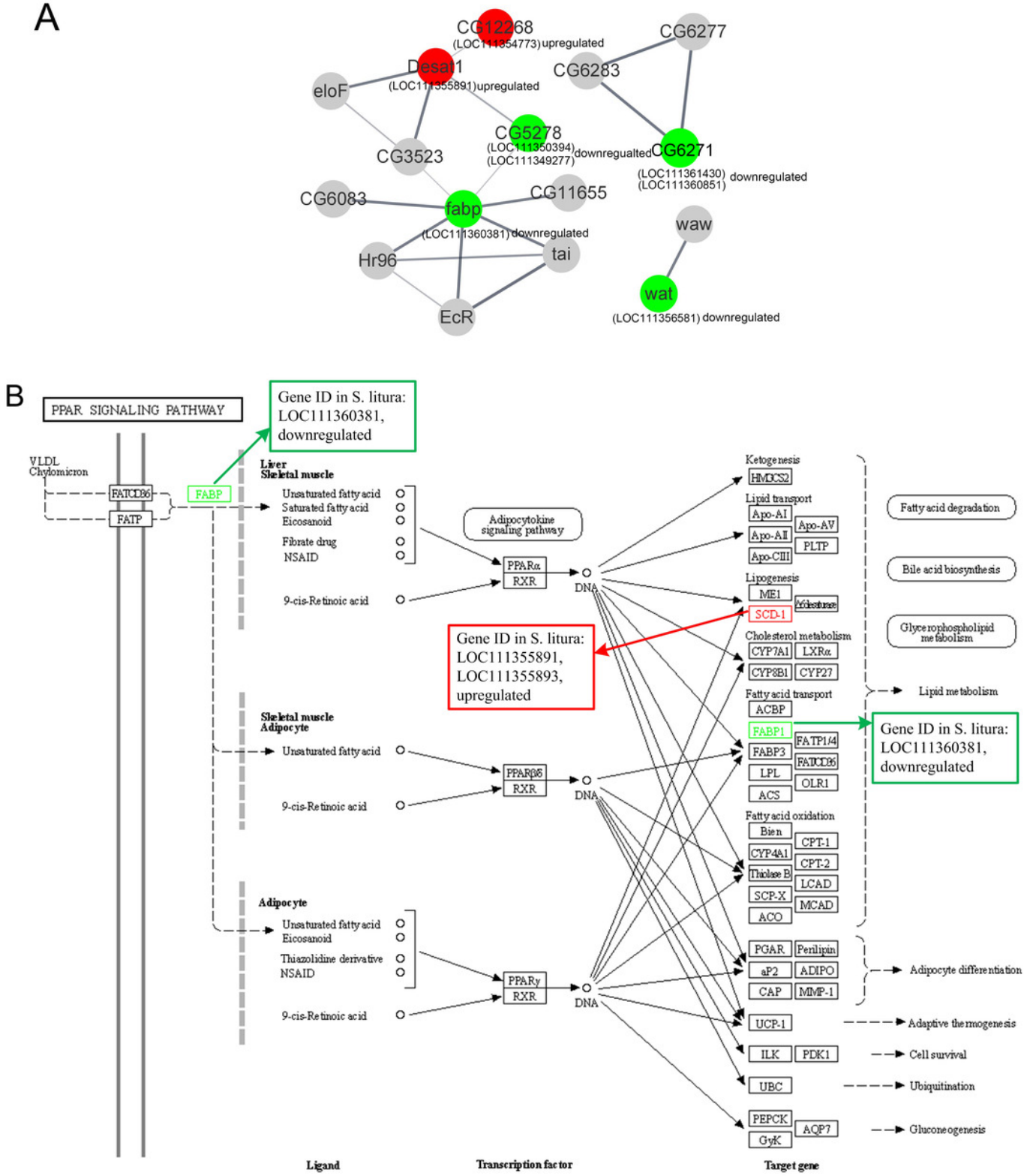
A
CG12268
(LOC111354773) upregulated
Desat1
(LOC111355891) upregulated
eloF
CG6277
CG6283
CG5278
(LOC111350394)
(LOC111349277) downregulated
CG3523
CG6271
(LOC111361430)
(LOC111360851) downregulated
CG6083
CG11655
fabp
(LOC111360381) downregulated
waw
Hr96
tai
wat
(LOC111356581) downregulated
EcR
B
PPAR SIGNALING PATHWAY
Gene ID in S. litura: LOC111360381, downregulated
VLDL
Chylomicron
FATCD86
FATP
FABP
Liver
Skeletal muscle
Unsaturated fatty acid
Saturated fatty acid
Eicosanoid
Fibrate drug
NSAID
9-cis-Retinoic acid
Adipocytokine signaling pathway
PPARα
RXR
DNA
Gene ID in S. litura: LOC111355891, LOC111355893, upregulated
Skeletal muscle
Adipocyte
Unsaturated fatty acid
9-cis-Retinoic acid
PPARβδ
RXR
DNA
Adipocyte
Unsaturated fatty acid
Eicosanoid
Thiazolidine derivative
NSAID
9-cis-Retinoic acid
PPARγ
RXR
DNA
Ligand
Transcription factor
Target gene
Ketogenesis
HMGCS2
Lipid transport
Apo-AI
Apo-AII
Apo-CIII
Apo-AV
PLTP
Lipogenesis
ME1
Acetoacetyl-CoA synthetase
SCD-1
Cholesterol metabolism
CYP7A1
LXRα
CYP8B1
CYP27
Fatty acid transport
ACBP
FABP1
FABP3
LPL
ACS
FATP1/4
FATCD86
OLR1
Fatty acid oxidation
Bien
CYP4A1
Thiolase B
SCP-X
ACO
CPT-1
CPT-2
LCAD
MCAD
PGAR
aP2
CAP
Perilipin
ADIPO
MMP-1
UCP-1
ILK
PDK1
UBC
PEPCK
GyK
AQP7
Fatty acid degradation
Bile acid biosynthesis
Glycerophospholipid metabolism
Lipid metabolism
Gene ID in S. litura: LOC111360381, downregulated
Adipocyte differentiation
Adaptive thermogenesis
Cell survival
Ubiquitination
Gluconeogenesis

Table 1(on next page)

Table 1 The primers used in the present analysis

1 **Table 1 The primers used in the present analysis**

Gene name	Forward primer	Reverse primer	Product lenghth	Amplified gene regions
Phospholipase A1like	TCCTTGTCCACT CAGATATGT	GTTGATAACCG TGCGATGTA	102 bp	Coding region
Acyl-CoA reductase	CTGGTTGATGCT CTGCTGTT	TGCCATTCCTTC GTTGTGTAAT	113 bp	Coding region
Acyl-CoA desaturase	GCTTCTTCTTCT GCCACATC	ACATCACCATC CAATCACCTT	111 bp	Coding region
Fatty acid-binding protein 2 like	TTCCTTAACAAG AACTACAA	AGTATCTCCATC CTTAGTC	138 bp	Coding region
β-actin	GCATCCACGAGA CCACTTACAA	CTGTGTTGGCGT ACAAGTCCTTA	75 bp	Coding region
GAPDH	GGGTATTCTTGA CTACAC	CTGGATGTACTT GATGAG	184bp	Coding region

2
3

Table 2(on next page)

Table 2 The KEGG pathway terms of Lipid metabolism-related DEGs

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Pathway ID	Pathway	Level 1	Level 2	P-value	DGE ID	Up/down regulation
ko04212	Longevity regulating pathway - worm	OS	Aging	2.30E-06	LOC111354773	Up
ko04212	Longevity regulating pathway - worm	OS	Aging	2.30E-06	LOC111355891	Up
ko04212	Longevity regulating pathway - worm	OS	Aging	2.30E-06	LOC111355893	Up
ko04212	Longevity regulating pathway - worm	OS	Aging	2.30E-06	LOC111352061	Up
ko04212	Longevity regulating pathway - worm	OS	Aging	2.30E-06	LOC111356581	Down
ko04975	Fat digestion and absorption	OS	Digestive system	0.007337	LOC111360381	Down
ko03320	PPAR signaling pathway	OS	Endocrine system	0.030415	LOC111355891	Up
ko03320	PPAR signaling pathway	OS	Endocrine system	0.030415	LOC111360381	Down
ko03320	PPAR signaling pathway	OS	Endocrine system	0.030415	LOC111355893	Up
ko00073	Cutin, suberin, and wax biosynthesis	M	Lipid metabolism	1.99E-05	LOC111354773	Up
ko01040	Biosynthesis of unsaturated fatty acids	M	Lipid metabolism	0.011254	LOC111355891	Up
ko01040	Biosynthesis of unsaturated fatty acids	M	Lipid metabolism	0.011254	LOC111350394	Down
ko00062	Fatty acid elongation	M	Lipid metabolism	0.327476	LOC111350394	Down
ko01040	Biosynthesis of unsaturated fatty acids	M	Lipid metabolism	0.011254	LOC111355893	Up
ko00561	Glycerolipid metabolism	M	Lipid metabolism	0.559342	LOC111348151	Up
ko00073	Cutin, suberin, and wax biosynthesis	M	Lipid metabolism	1.99E-05	LOC111352061	Up
ko00073	Cutin, suberin, and wax biosynthesis	M	Lipid metabolism	1.99E-05	LOC111356581	Down
ko04152	AMPK signaling pathway	EIP	Signal transduction	0.268133	LOC111355891	Up
ko04146	Peroxisome	CP	Transport and catabolism	4.00E-05	LOC111354773	Up

ko04146	Peroxisome	CP	Transport and catabolism	4.00E-05	LOC111352061	Up
ko04146	Peroxisome	CP	Transport and catabolism	4.00E-05	LOC111356581	Down

OS: Organismal Systems, CP: Cellular Processes, M: Metabolism, EIP: Environmental Information Processing