MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE

SUMY NATIONAL AGRARIAN UNIVERSITY

Quilified scientific work (Manuscript)

CAO ZHISHAN

UDC: 631.95:632.7:632.937

THESIS

BIOLOGICAL CONTROL OF DISTRBUTION GRAPHOLITA MOLESTA THTROUGH IMMUNOLOGICAL FEATURES

Specialty 202 – Plant Protection and Quarantine

Field of Knowledge: 20 – Agricultiral Sciences and Food Production

for a Doctor Philosophy Degree (PhD)

The dissertation contains the results of own research. The use of ideas, results and texts of other authors are linked to the corresponding source

Supervisor: **Vlasenko Volodymyr**, Doctor of Agriculture Sciences, Professor

Olha Bakumenko, Candidate of Agricultural Sciences, Associate Professor

Sumy 2023

ANNOTATION

Cao Zhishan. Biological Control of Distribution *Grapholitha Molesta* Through Immune Features - Manuscript Thesis for a Doctor Philosophy Degree (PhD): Specialty 202 "Plant Protection and Quarantine. – Sumy National Agrarian University, Sumy, 2023

The oriental fruit moth, *Grapholita molesta* (Busck) is a key pest of tree fruit of Europe, Asia, America, Africa, Australia, and New Zealand, which makes a big damage to apple trees, pear tree and the stone fruit of the peach, plum, apricot, nectarine, cherry and so on. The hidden damage of larvae makes their prevention and control difficult and traditional chemical method is rarely effective. Meanwhile with the increasing demand for food safety and awareness of environmental protection, biological control method to this pest has attracted more and more people's attentions. *Beauveria bassiana* is one of the most widely studied and used entomopathogenic fungi, which can also infect and kill the oriental fruit moth as a biological control agent. The entomopathogenic fungi with a wide range host and they are harmless to the environment, human and animal. Using entomopathogenic fungi to control pests has many advantages and it has been an important part in biological control of pests. However, entomopathogenic fungi as a better alternate control method still with some disadvantages, such as easy to be affected by environmental conditions. In order to make good use of entomopathogenic fungi in the future, it is

necessary to deeply understand their living conditions and infection mechanism to insects. Entomopathogenic fungi can invades the insects from the body wall through contact directly, but also can through the digestive tract, stomata and wounds and other ways into the insect body. But insects have evolved a strong innate immune system to protect themselves from infection by the pathogens and adverse conditions. When insects are infected by entomopathogenic fungi, their innate immune system will firstly be activated. And the insects will resist the infection by their immune response, which will lead to the reduction of infection efficiency and the control effect. So, it is necessary to study the immune response of insects introduced by entomopathogenic fungi, and it has been a study hotspot in pest control. The immune ability of insects to entomopathogenic fungi directly affects the infection efficiency, which is an area of active research in the field of entomopathogenic fungi currently. In this paper, the optimal concentration and infection mode of *B. bassiana* on *G. molesta* larvae were selected under laboratory conditions. Secondly, the transcriptome of *G. molesta* after infected by *B. bassiana* were analyzed，and the immune-related genes of *G. molesta* larva against *B. bassiana* were screened out. Thirdly, the peptidoglycan recognition proteins (GmPGRP-SC), β-1,3-glucan recognition proteins (GmBGRP) and serine protease inhibitor genes (*GmSerpin-2* and *GmSerpin-3*) involved *G. molesta* larva in immune response were identified by molecular methods. We carried out phylogenetic analysis based on amino acid sequences to identify homologous protein in *G.*

molesta belonging to the same clade as that encoding the target proteins. The spatio-temporal expression patterns of the selected target genes in the larva of *G. molesta* after infection with *B. bassiana* were assessed by qPCR. Moreover, we used nanoparticles mediation the *GmSerpin-2* and *GmSerpin-3* genes, which effectively increasing the interference efficiency of RNAi. Finally, we have a good grasp of the occurrence rule of oriental fruit moth in Xinxiang area by investigation of the damage of adult and larva in peach orchard. Meanwhile, the field efficacy test of five different biological pesticides showed that Bt, Spinetoram, Methoxyfenozide, Celastrus angulatus MaXim and *B. bassiana* all have better control effect on oriental fruit moth. But the control effect of Bt and *B. bassiana* are relatively lower than other three agents. We need to do more future research to improve their effectiveness. This study laid a foundation for the exploration of new biological control targets of G. *molesta* IPM system and the enhancement of *B. bassiana* for effectively biological control. The main results and conclusions are as follows:

Laboratory Evaluation of the effect of *B. bassiana* on the vital activity of *G. molesta*: *G. molesta* infected by *B. bassiana* through cuticular infection had higher corrected mortality and better weight inhibition than that of digestive tract infection. Meanwhile, *B. bassiana* at concentration of 1×10^7 conidia/mL may have potential to be used as control measure against *G. molesta* in fruit orchards.

Transcriptome analysis of *G. molesta* larvae 24 h after infected by *B.*

bassiana: The second-generation high-throughput transcriptome sequencing technology were used, the transcriptomic profile of G. molesta larva infected by *B. bassiana* after 24 h were analyzed, the differentially expressed genes (DEGs) were screened and annotated. Totally, 1,755 DEGs were obtained, with 965 up-regulated and 790 down-regulated genes. The fold change (log2 ratio) of the gene expression ranged from -14.224 to 10.718. We focused on the up-regulated genes in *G. molesta* infected by *B. bassiana*, and 14 genes related to immune response of *G. molesta* induced by *B. bassiana* were selected and quantified by qRT-PCR method, and 10 genes were significantly up-regulated. Finally, 1,755 DEGs were enriched in 1,965 GO terms, among which 107 GO terms were significantly enriched, including 62 Biological processes, 7 Cellular components and 38 Molecular functions. These results indicated the *G. molesta* has changed its physiological and biochemical state as defense responses against *B. bassiana*.

Characterization and functional analysis of Immune recognition gene *GmPGRP-SC*, *GmBGRP*, *GmSerpin-2* and *GmSerpin-3*: The complete sequence of *GmPGRP-SC* gene, *GmBGRP*, *GmSerpin-2* and *GmSerpin-3* are obtained and characterized. They have been submitted to NBCI GenBank respectively, and the GenBank accession number is MW773839, ON055286, OQ359960 and OQ35996. The gene sequences, structures, physical and chemical properties were analyzed. To explore the spatio-temporal expression patterns of each gene, the qPCR data form different tissues and developmental

stages were analyzed, besides we analyzed the specific functions of each gene. In order to better understand the molecular interaction mechanism between *B. bassiana* and *G. molesta*, the effect of *B. bassiana* on the regulation of immune-related gene expression were analyzed. Finally, we successfully silenced the target gene using RNAi technology, and the effects of target gene silencing on larvae ability to resist fungal infection were analyzed, which laid a good foundation for further improving the control effect of entomopathogenic fungi. In addition, we analyzed the effects of *GmBGRP* gene silencing on Toll immune signaling pathway related gene expression and PPO enzyme activity. And effectively improve the interference efficiency of *GmSerpin-2* and *GmSerpin-3* genes mediated by nanoparticles M2L and NP5.

Investigation on the occurrence and dynamics of *G. molesta* in peach orchard and evaluation on the control of five different biological pesticides: According to the survey results in the Xinxiang area, overwintered larva began to pupate in late March, the pupal stage was 10-20 days, and first-generation adult appeared in early April, which reaching a high level around 4, May. Then, the number of *G. molesta* began to increase continuously with the rise of temperature. The occurrence of male adult *G. molesta* in Xinxiang has no obvious rule, and with serious overlap generations. There is no obvious boundary between each generation, especially between the 2~3 generation and 3~4 generation. Larvae of oriental fruit moth had boring into the peach shoots from April 18. Meanwhile the larvae have transferring harm habit to peach

shoot. The middle May is the key time for controlling of *G. molesta* in Xinxiang area. The damaged peach shoots can be cut off manually and taken out of the orchard for centralized destruction in order to reduce the occurrence of overwintering generation. The damaged shoots rate by *G. molesta* in peach orchard was higher than that in nectarine orchard. And in the same peach orchard, the damaged shoots are also different due to different planting densities. The shoots damage is higher with planting density of 4×4 meters than that with planting density of 3×3 meters. The five biological agents all had adverse effects on the growth of peach trees. The results of this study indicating that all the five biological agents were safe for the growth of peach trees. And the 5 biological control agents all have certain control effects on *G. molesta*. On day 7 in treatment groups, the control effec of the five agents was 56.3% ~ 86.4%. Its control effect was 63.1% ~ 80.1% on day 14 of treatment. Among them, 60 g/L pinetoram suspension 2000 times liquid and 240 g/L Methoxyfenozide suspension 5 000 times liquid have better control effectiveness with 86.4% and 74.6% control effect on day 7 in the treatment groups. Moreover, the control effect of these two agents was still more than 78% on day 14. The control effects of 0.2% Celastrus angulatus MaXim and 10 billion spores *B. bassiana* were 68.25% and 66.8% respectively on day 7 in treatment groups, and the control effects of these two agents were 61.27% and 67.1% on day 14 in treatment groups which indicating that the two agents had relatively good quick and lasting effect on the control of *G. molesta*.

In summary, all these results lay a foundation for better understanding of the immune mechanism between entomopathogenic fungi and insects, and effectively improve the control effect of entomopathogenic fungi, which lay a solid foundation for the biological control of *G. molesta* pests.

Moreover, the study of immune-related genes, in order to find more suitable target genes, which can also be applied to the development of other biological control agents and improve their control effect. The results providing strong support for better biological control through immune characters of oriental fruit moth.

Key words: Grapholitha molesta Beauveria bassiana , pests, harmfulness, protection measures (plant protection), number of pests, resistance (resilience), biopreparations - elements of biologization, microcoenosis, tillage, yield crop, economic threshold of harmfulness, drug concentration, actinomycetales, genotype.

АНОТАЦІЯ

Цао Чжишань. Біологічний контроль розповсюдження *Grapholitha Molesta* за допомогою імунологічних особливостей – Кваліфікаційна наукова праця на правах рукопису.

Дисертаційна робота на здобуття наукового ступеня доктора філософії за спеціальністю 202 – Захист і карантин рослин. – Сумський національний аграрний університет Міністерства освіти і науки України, м. Суми, 2023 р.

Східна плодова плодожерка, *Grapholita molesta* (Busck) є основним шкідником плодових дерев у Європі, Азії, Америці, Африці, Австралії та Новій Зеландії, який завдає великої шкоди яблуні, груші та кісточковим плодам персика, сливи, абрикоса, нектарина, вишні тощо. Прихована шкода личинок ускладнює профілактику та боротьбу з ними, а традиційні хімічні методи рідко бувають ефективними. Тим часом, зі зростанням попиту на безпеку харчових продуктів та усвідомленням важливості захисту навколишнього середовища, біологічний метод боротьби з цим шкідником привертає все більше уваги. *Beauveria bassiana* є одним з найбільш широко вивчених і використовуваних ентомопатогенних грибів, який також може заражати і знищувати *Grapholita molesta* в якості агента біологічного контролю. Ентомопатогенні гриби мають широкий спектр господарів і є нешкідливими для навколишнього середовища, людей і

тварин. Використання ентомопатогенних грибів для боротьби зі шкідниками має багато переваг і є важливою частиною біологічного контролю шкідників. Однак ентомопатогенні гриби як кращий альтернативний метод боротьби все ще мають деякі недоліки, наприклад, вони легко піддаються впливу умов навколишнього середовища. Для того, щоб ефективно використовувати ентомопатогенні гриби в майбутньому, необхідно детально вивчити умови їхнього існування та механізм зараження комах-шкідників. Ентомопатогенні гриби можуть проникати в організм комах зі стінок тіла безпосередньо через контакт, а також через травний тракт, продихи, рани та інші шляхи. Але комахи мають розвинену вроджену імунну систему, яка їм допомагає захистити себе від зараження патогенами та підвищує стійкість до несприятливих умов існування. Коли комахи заражаються ентомопатогенними грибами, їхня вроджена імунна система активується і комахи протистоять інфекції, що призводить до зниження ефективності інфекції та ефекту контролю над чисельністю шкідників та пошкодженістю рослин. Таким чином, необхідно вивчати імунну реакцію шкідника зараженого ентомопатогенними грибами, що є *актуальним напрямком досліджень* у боротьбі зі шкідниками. Імунна здатність *Grapholita Molesta* до ентомопатогенних грибів безпосередньо впливає на ефективність зараження, що є сферою активних досліджень, на сьогодні, особливо в захисті і карантині рослин. У цій роботі в лабораторних умовах підібрано оптимальну концентрацію та режим

зараження личинок *G. Molesta* біологічно-активними речовинами ентомопатогенних грибів (*B. bassiana*). По-друге, проаналізовано транскриптом *G. molesta* після зараження *B. bassiana* та проведено скринінг генів, пов'язаних з імунітетом личинок *G. molesta* проти *B. bassiana*. По-третє, молекулярними методами ідентифіковано гени пептидогліканових білків (GmPGRP-SC), β-1,3-глюканових білків (GmBGRP) та інгібіторів серинових протеаз (GmSerpin-2 та GmSerpin-3), які залучені до імунітету личинки *G. molesta*. Проведено філогенетичний аналіз на основі амінокислотних послідовностей для виявлення гомологічних білків у *G. molesta*, що належать до тієїж категорії, що й білки-мішені. Просторово-часові параметри експресії обраних генів-мішеней у личинках *G. molesta* після зараження *B. bassiana* оцінювали за допомогою qPCR. Крім того, ми використовували наночастинки, що опосередковують гени GmSerpin-2 і GmSerpin-3, які ефективно підвищують інтерференційну ефективність РНКі. Було визначено стресогенні фактори, які впливають на закономірність появи та розвитку *Grapholita molesta* в районі Сіньсян, досліджуючи морфологію пошкодження імаго та личинок у персиковому саду. Тим часом, польові випробування ефективності різних біологічних пестицидів показали, що *Bt, Spinetoram, Methoxyfenozide, Celastrus Angulatus MaXim і B. Bassiana* мають кращий контроль над *Grapholita molesta*. Але контрольний ефект *Bt та B. Bassiana* є відносно нижчим, ніж у деяких препаратів. Це

дослідження заклало основу для вивчення нових біологічно активних речовин, актиноміцетів, які зменшать чисельність/контролюватимуть поширення *G. molesta* в системі захисту рослин. Окрім того нами розроблена система вдосконалення застосування *B. Bassiana* для ефективного біологічного контролю.

У результаті лабораторної оцінки впливу *B. bassiana* на життєдіяльність *G. molesta* досліджень було виявлено: *G. molesta*, які заражені *B. bassiana* через кутикулярну інфекцію, мали вищу смертність та зниження маси, ніж при інфікуванні травного тракту. *B. bassiana* в концентрації 1×107 конідій/мл може потенційно використовуватися як засіб боротьби з *G. molesta* у плодових та ягідних садах.

Транскриптомний аналіз личинок *G. molesta* через 24 год після зараження *B. bassiana:* за допомогою високопродуктивної технології секвенування транскриптома другого покоління проаналізовано транскриптомний профіль личинок *G. molesta*, інфікованих *B. bassiana* через 24 год, проведено скринінг та анотацію диференційовано експресованих генів (DEGs). Загалом було отримано 1,755 DEGs, з яких 965 – висхідні та 790 – низхідні гени. Коефіцієнт зміни експресії генів (log2) варіював від -14,224 до 10,718. Ми зосередилися на підвищеній регуляції генів у *G. molesta*, інфікованих *B. bassiana*, і відібрали 14 генів, пов'язаних з імунною реакцією *G. molesta,* індукованою *B. bassiana*, та кількісно оцінили їх за допомогою методу qRT-PCR, причому 10 генів

виявилися значно підвищеною регуляцією. Нарешті, 1 755 DEGs були збагачені в 1 965 термінах GO, серед яких 107 термінів GO були значно збагачені, включаючи 62 біологічні процеси, 7 клітинних компонентів і 38 молекулярних функцій. Що свідчить, що *G. molesta* змінила свій фізіологічний та біохімічний стан, морфологію як захисну реакцію проти *B. bassiana*.

Отримано та охарактеризовано повну послідовність генів GmPGRP-SC, GmBGRP, GmSerpin-2 та GmSerpin-3. Вони були передані до NBCI GenBank відповідно з номерами доступу MW773839, ON055286, OQ359960 та OQ35996. Під час вивчення просторово-часових пар експресії кожного гена були проаналізовані дані qPCR з різних тканин і стадій розвитку, а також проаналізовані специфічні функції кожного гена. Для того, щоб краще зрозуміти механізм молекулярної взаємодії між *B. bassiana* та *G. molesta*, було проаналізовано вплив *B. bassiana* на регуляцію експресії генів, пов'язаних з імунітетом. Було успішно знижено імунітет шкідника за допомогою технології РНКі та проаналізовано вплив знижеження імунності личинок проти грибкової інфекції, що заклало хороший фундамент для подальшого покращення ефекту контролю ентомопатогенних грибів. Крім того, було проаналізовано вплив пригнічення гена GmBGRP на експресію генів, пов'язаних з імунною реакцією шляхом Toll, та активність ферменту PPO. А також підвищено

ефективність інтерференції генів GmSerpin-2 та GmSerpin-3 за допомогою наночастинок M2L та NP5.

Досліджено поширення та динаміки *G. Molesta* в персиковому саду в районі Сіньсян та визначено ефективність різних біологічних пестицидів. Личинка, що перезимувала, почала заляльковуватися в кінці березня, стадія лялечки тривала 10-20 днів, а імаго першого покоління з'явилися на початку квітня, досягнувши максимумуи близько 4 травня. Після цього чисельність *G. molesta* почала безперервно зростати з підвищенням температури. Поява імаго *G. molesta* в Сіньсяні не має чіткої закономірності, що зумовлено серйозним перекриттям поколінь. Не виявлено чіткої межі між кожним поколінням, особливо між 2~3 поколінням і 3~4 поколінням. Личинки *G. molesta* вгризалися в пагони персика з 18 квітня. Середина травня виявилася основним періодом для боротьби з *G. molesta* в регіоні Сіньсян. Пошкоджені пагони персика потрібно зрізати і вивозити з саду для централізованого знищення зимуючого покоління. Відсоток пошкоджених пагонів *G. molesta* у персиковому саду був вищим, ніж у нектариновому. Разом з тим, в одному і тому ж персиковому саду, пошкодженість пагонів відрізнялися в залежності від густоти насаджень. Пошкодження пагонів спостерігалася вище при щільності насаджень 4×4 метри, ніж при щільності насаджень 3×3 метри. Всі досліджувані біологічні агенти не мали негативного впливу на ріст персикових дерев, що свідчить про те, що всі п'ять

біологічних агентів були безпечними для росту персикових дерев та мали позитивний ефект над контролем заселення і поширення *G. Molesta*. На 7-й день у досліджуваних групах контрольний ефект препаратів становив 56,3% ~ 86,4%. На 14-й день захисту контрольний ефект становив 63,1% ~ 80,1%. Серед них суспензія пінетораму 60 г/л, розведена у 2000 л. розчину, та суспензія метоксифенозиду 240 г/л, розведена у 5000 л. розчину, мали кращу контрольну ефективність - 86,4% та 74,6% на 7-й день після застосування. Більше того, контрольний ефект цих двох препаратів перевищував 78% на 14-й день. Контрольний ефект 0,2% *Celastrus angulatus MaXim* та 10 мільярдів спор *B. bassiana* становив 68,25% та 66,8% відповідно на 7-й день у групах захисту, а контрольний ефект цих двох препаратів становив 61,27% та 67,1% на 14-й день у групах захисту, що вказує на те, що ці два препарати мали відносно хороший швидкий та тривалий ефект для контролю шкідника – *G. molesta*.

Таким чином, результати досліджень розкривають основні моменти імунного механізму між ентомопатогенними грибами та шкідниками, а також вказують на ефективність контролю ентомопатогенних грибів проти *G. molesta*, що сприятиме розвитку біологічного захисту плодових насаджень.

Крім того, нами досліджено гени, які знижують резистентність (стійкість) шкідників, з метою пошуку більш активних генів-мішеней, які також будуть застосовані для розробки інших агентів біологічного

контролю та покращення їхнього контрольного ефекту. Отримані результати є вагомим внеском в розвиток біологічного контролю шкідників саду.

Key words: Grapholitha molesta, Beauveria bassiana, шкідники, шкідливість, заходи захисту (захист рослин), чисельність шкідників, резистентність (стійкість), біопрепарати-елементи біологізації, мікроценоз, обробіток, врожайність культури, економічний поріг шкодочинності, концентрація препарату, актиноміцети, генотип.

LIST OF THE APPLICANT'S PUBLICATIONS ON THE TOPIC OF THE DISSERTATION

Articles in scientific professional journals citing Scopus

(1). **Cao Zhishan, Cao Jinjun, Vlasenko Volodymyr, Wang Xinfa, Li Weihai.** Transcriptome analysis of *Grapholitha molesta* (Busk) (Lepidoptera: Tortricidae) larvae in response to entomopathogenic fungi *Beauveria bassiana*. Journal of Asia-Pacific Entomology, 25 (2022) 101926. https://doi.org/10.1016/j.aspen.2022.101926

Articles in scientific professional publications of Ukraine

(2). **Cao Zhishan, Cao Jinjun, Zhu Hongxia, Vlasenko Volodymyr.** Molecular identification of a short-type peptidoglycan recognition protein, *GmPGRP-SC* from *Grapholitha molesta*. Bulletin of the Sumy National Agricultural University of Agricultural Cultures, 3 (45), 2021. https://doi.org/10.32845/agrobio.2021.3.7

(3). **Cao Zhishan, Vlasenko Volodymyr, Li Weihai.** Development of biological control of oriental fruit moth and insect immune response induced by entomopathogenic fungi. Bulletin of the Sumy National Agricultural University of Agricultural Cultures, 2 (40), 2020. https://doi.org/10.32782/agrobio.2020.2.10

(4). **Cao Zhishan, Vlasenko Volodymyr.** Application and development prospect of RNA interference technology in pest control. Bulletin of the

Sumy National Agricultural University of Agricultural Cultures. 2 (48), 2022. https://doi.org/10.32845/agrobio.2022.2.1

(5). **Cao Zhishan, Vlasenko Volodymyr.** Laboratory Evaluation of the effect of *Beauveria bassiana* on the vital activity of *Grapholitha molesta* (Lepidoptera: Tortricidae). Bulletin of the Sumy National Agricultural University of Agricultural Cultures. 4 (50), 2022. https://doi.org/10.32845/agrobio.2022.4.1

Articles in scientific journals of other countries

Abstracts

(6). **Cao Zhishan.** Main factors of outbreak damage of oriental fruit moth. International scientific and practical conference dedicated to the 100th anniversary of the birth of Doctor of Biological Sciences, Professor B. M. Lytvynov. October 21-22, 2021.

(7). **Cao Zhishan, Vlasenko Volodymyr.** The Integrated Pest Management Measures for Fruit Borer in Organic Orchard. The 85th anniversary of the birth of Viacheslav Grhyrovych Mikhailov, a prominent scientist in the field of breeding and seeds for agricultural culture. 5 жовтня 2021 року.

(8). **Cao Zhishan, Vlasenko Volodymyr**. Research progress on biological control of oriental fruit moth *Grapholitha Molesta*. «Honcharivski Chytannya» dedicated to the 91th anniversary of Doctor of Agricultural

Sciences Professor Mykolay Dem'yanovych Goncharovian, 5, 25-26, 2020.

(9). **Cao Zhishan, Vlasenko Volodymyr.** Application Research Progress of Entomopathogenic Fungus: *Beauveria Bassiana*. International scientific and practical conference "Potter Readings" dedicated to the 92nd birthday of Doctor of Agricultural Sciences, Professor Mykola Dem'yanovych Goncharovian, 5, 25, 2021.

(10). **Cao Zhishan, Vlasenko Volodymyr.** Research and Application of Nanoparticle-Mediated RNAi Technology in Pest Control. International scientific and practical conference "Potter's Readings" dedicated to the 93rd birthday of Doctor of Agricultural Sciences, Professor Mykola Dem'yanovych Goncharovian, 5, 25, 2022.

ABBREVIATIONS

bp	base pair
ddH_2O	double distilled water
Bb	Beauveria bassiana
BGRP	beta-1,3-glucan recognition protein
cDNA	Complementary deoxyribonucleic acid
d	Day
DGE	digital gene expression
DNA	deoxyribonucleic acid
DOPA	L-3,4-dihydroxyphenylalanine
GST	Glutathione S-Transferase
h	hour
IMD	immunodeficiency
Jak/STAT	Janus kinase/signal transducer and activator of transcriptions
kDa	Kilo-Dalton
LB	Luria-Bertani culture medium
LPS	lipopolysaccharide
mg	milligram
min	minute
mL	milliliter
mol/L	molarity/liter
mRNA	message ribonucleic acid
OD	optical density
ORF	opening reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGN	peptidoglycan
PGRP	Peptidoglycan recognition protein
pI	isoelectric point
PRR	pattern recognition receptor
PAMP	pathogen associated molecular pattern
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
TLR	Toll-like receptor

CONTENTS

INTRODUCTION

Actuality of theme. The oriental fruit moth, *Grapholita molesta* (Busck) is a key pest of tree fruit of worldwide. It is hard to control with traditional chemical method, due to the larvae bored the inside fruit or stem. *Beauveria bassiana* is one of the most studied and applied entomopathogenic fungi, can infected and kill the oriental fruit moth as a biological control agent. In this study, through the study of the molecular mechanism of *B. bassiana* and the molecular study of immune-related genes, all the results laid a foundation for the exploration of new biological control targets of *G. molesta* IPM system and the enhancement of *B. bassiana* for effectively biological control. This research is practical.

Connection of work with scientific programs, plans, themes. Theresearchwas carried out in accordance with the thematic plans of research worksof Sumy National Agrarian University in the framework of the topic «Phytosanitary monitoring and regulation of harmful organisms in agriculture» (state registration number 0123U104019); supported by the Key Scientific and Technological Research Project of Henan Province (№ 202102110220), and the Graduate Education Innovation Training Base Project of Henan Province in 2021 (№ 107020221005).

The purpose and objectives of the study. The aim of the research was to find more efficiency method for biological of *G. molesta* and the enhancement of *B. bassiana* for effectively biological control. The goal was to solve the following tasks:

1. To study the optimal concentration and infection mode of *B. bassiana* on *G. molesta* larvae were selected under laboratory conditions;

2. Analyzed the transcriptome of *G. molesta* after infected by *B. bassiana* were, and screened out the immune-related genes of *G. molesta* larva against *B. bassiana*;

3. Identified the peptidoglycan recognition proteins (GmPGRP-SC), β-1,3-glucan recognition proteins (GmBGRP) and serine protease inhibitor genes (GmSerpin-2 and GmSerpin-3) involved *G. molesta* larva in immune response;

4. To get a good grasp of the occurrence rule of *G. molesta* in Xinxiang area by investigation of the damage of adult and larva in peach orchard. Meanwhile test five different biological pesticides in field.

Object of study. Better understand the harm regularity of *G. molesta*. Explore the molecular mechanism of fungal control. Analysis the effect of biological pesticides on field control. Providing more basis for biological control of *G. molesta*.

Subject of study. Based on the analysis of immune molecular mechanism and immune gene of entomogenous fungi, the foundation of improving the bioloigal control effect for *G. molesta*.

Research methods. General scientific methods: analysis, induction, deduction, synthesis; The field method andthe best concentration and the best way for *B. bassiana* to *G. molesta* were determined by laboratory virulence assay. Biostatistical analysis methods, corrective mortality analysis methods, transcriptome analysis methods, fluorescence quantitative molecular analysis methods - to summarize and determine certain reliability of experimental results

The scientific novelty of the achievements is to solve the important scientific problems of the development of immune genes and improve the effect of biological control for *G. molesta*.

For the first time screened the optimal infection concentration under laboratory conditions. And first time performed the transcriptomics analysis of *G. molesta* larvae after infected by *B. bassiana*. The complete sequence of immune-related genes: *GmPGRP-SC* gene, *GmBGR*P, *GmSerpin-2* and *GmSerpin-3* are firstly identified and characterized. Field investigation and the determination of different biological pesticides provide reliable data and application value for field control of *G. molesta*.

It was improved the control scheme of *G. molesta* using *B. bassiana*. The excavation of the immune genes of *G. molesta* provided support for better development and utilization of biological control work.

The practical significance of the results. According to the findings, the biological control method of *G. molesta* has been improved. And the exploring of immune genes plays an important role in the subsequent development of other more effective biopesticides and implementation in the educational process of Sumy National Agrarian University (Appendix A).

To determine the special role of different immune genes in biological control of *G. molesta*. Meanwhile, the gene silencing effect was effectively improved by nanoparticle mediated method, and the mortality rate of larvae was significantly increased after infection with B. bassiana. These studies have potential application

value. The application of this gene has obtained the certificate of our university.

The personal contribution of the applicant is to plan and carryout research, summarize scientific data of references (literature) on the topic of the dissertation, to do analysis of experimental data, to form conclusions and proposals for production, to prepare and write of scientific papers.

Approbation of dissertation results. The results of the researchwerepublished and discussed at «International scientific and practical conference dedicated to the 100th anniversary of the birth of Doctor of Biological Sciences, Professor B. M. Lytvynov» (2021), at Proceedings of the International Scientific and Practical Conference «Honcharivski Chytannya»" international ones (2020, 2021, 2022). The mainitems,research results and conclusions of the work during 2020-2023 were presentedanddiscussed at the meetings of the Department of Plant Protection Sumy National Agrarian University.

Publications. Based on the results of the research, 4 articles were publishedinprofessional journals, one - in journals indexed in the Scopus database and in the proceedings of five conferences.

The structure and scope of the dissertation. The dissertationstructurecontains an annotation, a list of symbols, introduction, five chapters, conclusions, proposals for production, a list of references, appendexes.

CHAPTER 1

CHAPTER 1 BIOLOGICAL CONTROL OF DISTRBUTION *GRAPHOLITA MOLESTA* (LITERATURE REVIEW)

1.1. Origin and geographical distribution of the oriental fruit moth

Grapholita molesta known as oriental fruit moth, belonged to Tortricidae, Lepidoptera is one of the most severe pests of tree fruits, widely distributed throughout the fruit-growing areas of the world including Asia, Europe, America, Australia, and Africa (Hill, 1987; Rothschild and Vickers, 1991, Natale et al., 2003; Myers et al., 2006; Timm et al., 2008; Bisognin et al., 2012, Ricietto et al., 2016; Kong et al., 2019; Zunic, et al., 2020). The *G. molesta* was thought to a native insect of northwestern China (Rothschild & Vickers, 1991). And it was mentioned in Japan, in 1901 and 1902, as a special pest of sand pear (Chaudry, 1956). In 1906 it has been found in South Australia, as natural enemies of peach, under the popular name Peach Tip Moth. This pest also known by a variety of common names in the past, such as Smaller Pear Borer, Oriental Peach Moth, Oriental Peach Worm and Oriental Fruit Moth, and the last one has been adopted as an officially name. It was reported that *G. molesta* introduced to the United States around 1913, by the cherries imported from Japan (Rings, 1970). Then the pest had spread to many parts of United States, like New York, New Jersey, California and so on. In Europe, *G. molesta* was first recorded in Slovenia in 1920 and it was found in southeastern of France and north-central of Italy in the early 1920s. Then

it has dispersed across to eastern, southern and western of Europe (Kirk et al., 2013). Despite the strict quarantine measures, but now it is widely distributed in temperate and subtropical regions of the world, including Europe (Italy, France, Greece), Asia and other regions (Japan and South Korea), America (USA), South America (Argentina, Uruguay, Chile), north of Africa, Australia, and New Zealand, becoming the Global fruit-boring insect pest (Rothschild and Vickers, 1991, Natale et al., 2003; Myers et al., 2006; Timm et al., 2008; Bisognin et al., 2012; Kirk et al., 2013). It is also an important pest and widely distributed in China, occurred in almost all fruit regions of China except Tibet, especially in the northern and eastern regions (Chen et al., 2009).

1.2. Host and harm of the *G. molesta*

The *G. molesta* is an invasive oligophagous insect (Kong et al., 2020), most of its host plants belong to the family Rosaceae, including many economic fruit trees, such as peach, pear, apple, plum, apricot, cherry, hawthorn, jujube and so on. In addition, the host plants also including many wild and ornamental plants, for example begonia and loquat (Myers, 2006; Varela et al., 2011; Piskorski et al., 2011; Du et al., 2015), and *Myrtaceae*, a special plant found in South America (Rothschild and Vickers 1991). Among all of the host plants, the stone fruits peaches and nectarines sever as primary hosts, while the pome fruits apples and pears are considered as secondary hosts (Rothschild & Vickers, 1991; Myers, et al., 2007).

The *G. molesta* is a host-switching pest species, it always switching from

stone-fruit orchards to pome-fruit orchard during the growing season (Yang et al., 2016; Graillot et al., 2017; Li et al., 2019). The larvae of the first generations can damage the tender shoots of peaches, plums, quinces, and apples in the spring. Each larva damage three or more shoots for its development period, that often leads the tree with little nutrition. More seriously, the attacked tree would wither and die, so the *G. molesta* is also called "folded worms". Sometimes the first-generation of larvae also do harm to small fruit (Rings, 1970). The second and third generations of larvae transferred to fruits and continue to damage the plants, it bore into the fruits and remain there throughout their feeding stages, that causes miscellaneous bacteria, makes fruit rotted and lost its edible value, even drop down. In fruit producing areas, especially peach and pear or apple mixed planting areas, this pest mainly switching from stone fruit trees to kernel fruit trees, for example, in the Emilia-Romagna region of northern Italy, southern Switzerland and other regions, after the peach maturity harvest around July, *G. molesta* switching from the peach orchard to the nearby pear and apple orchards, causing serious damage to the pear and apple; In California, pear orchards near stone fruit orchards are particularly hard hit during the ripening period (Rice et al.,1972; Pollini & Bariselli 1993; Dorn et al., 2001). This pest always causes considerable economic lose (CABI 2016). In some locations even over 45 % of fruits has been infected by the larvae (Kanga et al., 2003), when the *G. molesta* population are high, the incidence of the fruits or shoots damage even reach to 80 % (Zhao, 2004). Due to the characteristics of hidden damage and the transfer of host hazards, it is difficult to

get good results with traditional chemical control that is the main reason for its generally occurrence, spread and seriously harm (Borchert et al., 2004).

The annual generation algebra of *G. molesta* are mainly determined by climate and the length of the growing season, and it was also affected by the nutritional conditions of orchards (Zhang XZ, 1980; Kanga et al., 1999; Ahn et al., 2012). It is reported that *G. molesta* generally has 4-5 seams of fruit trees (Peterson & Haeussler, 1928; Yokoyama et al., 1987). However, in some areas, these pests also have different generations per year, such as in the southern United States and parts of Europe, it has 7~8 generations annually (Reichard & Bodor, 1972). Even in China, *G. molesta* with different annual generation in different parts, it has 3 ~ 4 generations in most areas of northeast China, 4 ~ 5 generations in northwestern China, 5 generations in Xinjiang Province, and 6 ~ 7 generations in south China (Wang CY, 2006). The *G. molesta* also has overlapping generations and wintering phenomenon (Borchert et al., 2004, Magalhaes & Walgenbach, 2011). In general, pupation begins in mid to late March in spring, and the pupal period of this generation is about 15-20d. The pupa begins to emergence when the average temperature reaches 5 ^{o}C for seven consecutive days. When the average temperature reached 11 ~ 12 ^{o}C for five consecutive days, the emergence of adult worms reaches to a peak (Wang YH, 2012). With the occurrence of multi-generations, serious intergenerational overlap, and complex life cycle, it is difficult to predict and control it, which is also the reason for increasing spread and harmfulness year by year.

1.3. Control Technologies of the *G. molesta*

There are many studies and reports on the prevention and control of *G. molesta* around the world, and they are mainly focusing on the following aspects: phytosanitary control, agricultural control, physical and mechanical control, biological control, chemical control and so on.

1.3.1 Phytosanitary control

G. molesta is a quarantine insect in Russia, Mexico and other countries. And it is a potential quarantine method effectively remove the pest by refrigerating the imported fruit at a low temperature. It was reported that 1 ~ 3 instar larvae could not develop to adult after treatment at 0 ºC for 21 days (Yokoyama & Miller 1989). When the temperature is near 0 ºC, eggs and young larvae usually die before the fourth week.

1.3.2. Agricultural control

Avoid mixed planting of peach and pear trees in orchard. When establishing orchards, avoid the mixed planting of peaches, apricots and plums trees with cherries trees, apple trees with pear trees. This will effectively reduce the source of this pest transferred by host. Meanwhile tied the grass to orchard trunks in order to attract mature larvae and reduce the cardinal number of overwintering insects. Removal of dropped fruits from the orchard floor resulted in a significantly lower disease incidence on fruit in the tree; thus, drop-removal may be useful as a *G. molesta* management practice. Clean up the storage place after harvest the fruit (Ma et al., 2016).

1.3.3. Physical and Mechanical control

G. molesta has tendency to sweet-sour liquid. We can hang the bowls with sweet and sour liquid on the fruit trees in order to trap and kill the adult of this pest in the growing season. It was demonstrated that the liquid decoy works best when the ratio of sugar, acetic acid, alcohol, water was 3:1:3:80 (Li et al., 2006; Zhai et al., 2019).

The fruit bagging also has a certain effect on the control of *G. molesta*. It is reported that the damage rate of fruits bagged are below 1 %, while the average insect fruit rate of unbagged fruits reaches 8 %, when they are with the same pesticide treatment (Zhi, 2008).

1.3.4. Chemical control

The pesticide should be applied during the peak period of overwintering adults and the first-generation adults. According to the literatures, the mixture of 48 % Lorsban missible oil, beta-cypermethrin, 2.5 % cyhalothrin, and 5 % jipronil SC shows a better effect to control the *G. molesta* (Chen, 2007).

1.3.5 Biological control

The biological control of *G. molesta* has been widely studied, but mainly focus on natural enemies, sex pheromone and Plant source volatiles (Stelinski et al., 2007; Rodrigues et al., 2011; Barros-Parada et al., 2018; Robledo et al., 2018; Li et al., 2019; Guo et al., 2019; Chen et al., 2020; Liu et al., 2020). Additionally, it was reported that *B. bassiana* can kill this pest and it can parasitic in wintering larvae of *G. molesta*, parasitic rate up to 20–40 %, even as high as 80 % when the

conditions are suitable (Feng et al., 1988; Sarker et al., 2020). And the mortality rate of *G. molesta* caused by *B. bassiana* was 47.2 % (Song et al., 1993). Mix *B. bassiana* with sulfur as a gelatinizing agent is more effective than using this fungus alone on controlling of *G. molesta* (Zhao et al., 2010). However, there were a few studies on the biological control with *B. bassiana* and the studies were not in-depth at present to our knowledge.

1.4. Entomopathogenic fungus: *Beauveria bassiana*

1.4.1. Entomopathogenic fungus

Entomopathogenic fungi are a kind of fungi that can directly invade the host body under normal physiological conditions to reproduce and cause disease or death, mainly including the Ascomycetes and Fungi imperfecti (Xiao et al., 2001). According to reports, there are more than 100 genera and 800 species of entomopathogenic fungi worldwide. And there are 405 species, among them, 239 species are directly parasitic insects (Wang & Ma, 2009). To date, more than 20 species of entomopathogenic fungi have been developed and utilized to control pest insects in agriculture, such as *Beauveria bassiana*, *Metarhizium anisopliae*, *Trichophyla*, *Acanthopylospora*, *Penicillium rosiformis*, *Fusarium*, *Trichopylospora Tompelospora* and so on, and some of which have been used in commercial production (Shah & Pell, 2003). Due to the variety of entomopathogenic fungi and activity of the strains, the host is not easy to develop resistance, and harmless to the environment and human and animal characteristics and gradually received people's attention. Therefore, "control insects with fungi"

will be the focus for biological control work in the future. Although entomopathogenic fungi have many advantages in pest control, it still has some natural defects such as long effective time and easy to be affected by environmental conditions, which lead to many limitations in its practical application. Therefore, in order to better utilize entomopathogenic fungi for biological control, it is necessary to further study the infection process and invasion mechanism of entomopathogenic fungi in order to improve their virulence.

1.4.2 Overview of *Beauveria bassiana*

Entomopathogenic fungi can infect their host insects with directly contact and do not need to be consumed by their host, this always cause infection under normal physiological conditions. It can cause disease or death to the insects by proliferation in insects' body, and play an important role in biological control throughout the world (Ferron, 1978; Mora et al., 2017). *B. bassiana* is one of the most studied and applied entomopathogenic fungi (Clark, 1982). According to the field investigation of overwintering insects, among all fungal diseases, 21 % of deaths are caused by *B. bassiana* (Li et al., 1983). It was reported that *B. bassiana* has been used as control agent against as many as 149 families and more than 700 species of pest insects in agriculture, forestry and veterinary (Zimmermann, 2007). As a broad host rang insect pathogen, *B. bassiana* has been a widely used biological insecticide at present. *B. bassiana* has a significant broad prospect of development in the future with the biotechnological innovations, due to its safety and less dosage. Meanwhile *B. bassiana* not only as a pest biological control agent,

but also as a model organism that be used to examine unique aspects of entomopathogenic fungal growth and development such as the host pathogen interactions (Lewis et al., 2001; Wanchoo et al., 2009).

1.4.3 Biological characteristics of *Beauveria bassiana*

B. bassiana is a globally distributed Hyphomycete, and its strains can infect a range of insects. Colonies of *B. bassiana* grow relatively slowly, and with colors ranging from white to yellow. The hyphae are hyaline, smooth and septate. The aerial conidia are spherical or nearly spherical single spore, smooth and hyaline (De H, 1972, Huang et al., 2002). Germination of the conidia with an optimum temperature of 22 ~ 26 ^{o}C, and when the temperature reaches to 32 ^{o}C, it cannot germinate any more (Kuang et al., 2005). The most suitable pH for its own sporulation is 5.5 ~ 6.5. Conidia germinate in an environment with high relative humidity. The germination percentage reaches to the top when the relative humidity is above 95 %. With the humidity decreases, the rate significantly slow down, and it nearly not grow any more when the humidity decreases to 53 % (Guo et al., 2010). Humidity plays a vital role in activation of the conidia independent of a host (Boucias et al., 1988). Ferron found that insects are more easily be infected by *B. bassiana* at high ambient relative humidity. *B. bassiana* conidia are also easily influenced by light and other factors (Ferron, 1977). Meanwhile the destruction effect of ultraviolet light on the spores will cause unstable characters of strains, which will cause the field control effect become worse.

1.5. Insect defense responses to entomopathogenic fungi

Insects are the largest groups and most diverse animals on the earth (Daly et al.,1978). There are more than one million species of insects, which play an important role in the ecological systems. In the process of evolution, insects have formed a highly effective defense system to resist the invasion and parasitization of microbes and fugus. But insects have no T and B lymphocytes, and lacking adaptive immunity system which is found in mammals. Insects mainly rely on a strong innate immune system to protect the host, which serves as a first-line defense (Kimbrell & Beutler, 2001). The defense system of insects mainly consists of humoral immunity and cellular immunity (Ning et al., 2009). Humoral immunity mainly relies on a variety of active factors in hemolymph, such as antimicrobial peptides, insect defenders, lysozyme, protease inhibitors and other factors to fight against foreign pathogens. Cellular immunity mainly relies on blood cells to resist the invasion of foreign microorganisms through phagocytosis, nodules, cysts and coagulation (Hou et al., 2012). Currently, there are a lot of reach on innate immune response of the insects, but mainly focus on the model insects such as *Drosophila melanogaster*, *Anopheles* gambia*e*, *Bombyx mori* and so on (Hoffmann et al., 2002; Christophides et al., 2002; Hiromitsu et al., 2008). However, there is relatively little research on agricultural and forestry pests.

1.5.1 The body wall defense

The body wall of insects is their first barrier against infection of the entomological fungi. There are three main ways that insect body wall prevents pathogens: firstly, the body wall has some components that inhibit fungal

germination, such as the chitin in the body wall which is not suitable for the germination of some fungi, or even disordered growth; secondly, the body wall has tight structures that prevent fungal secretions from penetrating the walls and entering the insect; thirdly, the molting of body wall could also prevent the invasion of fungi, that is, when the fungus has just invaded the body wall, it could prevent the invasion of foreign toxins and pathogens to organisms (Roberts & Humber, 1981; Hou et al., 2012). These modes may be spontaneous or induced, and they are closely related to the structure and composition of insect body wall and the characteristics of fungi themselves (Wang & You, 1999).

1.5.2 Cellular defense

Cellular defense in insects refers to the defense response such as phagocytosis and encapsulation that are mediated by hemocytes (Strand & Clark, 1999; Irving et al., 2005). There are plasma blood cells, granular blood cells, bead blood cells and granulosa cells in insect hemolymph. These cells can recognize and resist entomopathogenic fungi invasion. Lin (1998) found that most spores could not germinate after cytophagy of blood cells in the not yet dead larva of *Dendrolimus spectabilis* infected with *B. bassiana* (Lin et al., 1998). The number of blood cells in the insects increased after infected with *B. bassiana*, which was mainly because of the increasing of plasma and granular blood cells that participated in cellular immunity (Ren et al., 2013).

1.5.3 Humoral immune defense

Humoral defense refers to the humoral immune factors that exists in insects

normally or induced by fat bodies and blood cells, including the antimicrobial peptides, phenol oxidase, and lysozyme, then it will activate Toll, immune deficiency (IMD) pathways protecting hosts from fungal infection (Gillespie et al., 2000). Humoral immunity is mainly divided into four parts: (1) pattern recognition molecules; (2) signaling modulation-related molecules; (3) immune signaling pathway-related molecules; (4) immune responsive effector genes.

1.5.4. Signal identification

Insects always rely on humoral immunity to defense against or even eliminate the invasion of fungi, bacteria and other pathogens. The immune response is induced by the specific recognition of common components bearing on the microbial surfaces, known as pathogen associated molecular patterns (PAMPs), by pattern recognition receptors (PRRs) (Kang et al. 1998; Ochiai & Ashida 1999; Werner et al., 2000). Peptidoglycan recognition proteins (PGRPs) are the most important PRRs in insects, which can recognize the peptidoglycan (PGN) on the surface of pathogenic microorganisms and activated the Toll and IMD pathways. Then this can trigger the production of antimicrobial peptides and play a crucial role in the innate immunity of insects against microorganisms (Hultmark, 2003; Beutler, 2004; Lu et al., 2020).

PGRPs as an important part of recognition receptor in insects, play an important role in the immune defense signaling pathway (Toll and IMD), which are natural immunity molecules found in insects, mollusks, echinoderms, and vertebrates, but not present in nematodes or plants (Kang et al., 1998; Dziarski &

Gupta, 2006; Gerardo et al*.,* 2010;). Nowadays more than 100 kinds of PGRPs in insects and mammals have been identified, PGRPs can be categorized into two types: long (L) types and short (S) types based on their length. At present, there are nearly 100 members of PGRPs family have been identified, 6 long and 6 short forms have been found in *Bombyx mori*, 6 long and 7 short forms in *D. melanogaster*, and 4 long and 4 short types in *Anopheles gambiae* 4 PGRP genes in Homo-sapiens and Musmusculus (Kang et al., 1998; Tanaka et al., 2008). At present, studies on PGRPs mainly focus on *D. melanogaster* and *B. mor*i, and fewer studies have been performed in other Lepidoptera insects.

We have also identified several PRRs in insects including peptidoglycan recognition proteins (PGRPs), LPS and β-1,3-glucan binding protein (LGBP), C-type lectins, beat-1,3-glucan recognition proteins (BGRPs), Gram-negative bacteria binding proteins (GNBPs), lipopolysaccharide and beta-1,3-glucan binding protein (LGBP), scavenger receptors (SCRs), thioester-containing proteins (TEP), Toll like receptor (TLRs), Hemolin (Yu et al., 2002; Rao et al., 2014).

The BGRPs are one of the major PRRs which have strong specific affinity for β-1,3-glucan, and serve as a biosensor against fungi (Ochia et al., 2000). Its interaction with β-1,3-glucan stimulate the prophenoloxidase (PPO) system, which is an important defense response in insects (Ma et al., 2012). And the PPO activating system (PPO-AS) involves the activation of serine proteinases, which is triggered by PAMPs to enhances the PPO cascade (Sivakamavalli & Vaseeharan, 2014). Till date, BGRPs have been described in many insects, such as the *Blaberus*

craniifer (Soderh et al. 1988), *B. mori* (Ochiai et al., 2000), *Manduca sexta* (Ma et al., 2000), *Tenebrio molitor* (Zhang et al., 2003), *Spodoptera exigua* (Bang et al., 2012), *Locusta migratoria manilensis* (Zheng & Xia, 2012), *Plutella hypostyle* (Huang et al., 2015),

1.5.5. Signal conditioning

When the insect is infected by pathogen, the extracellular immune cascade is activated, and always starting with the extracellular processing signals of serine protease and serpins (Söderhäll et al., 2009). The intracellular immune signaling pathways have been extensively studied in *Drosophila*, with most information having been obtained by injection of bacteria or fungus directly into the fly hemolymph. Toll pathway activation occurs through pathogen detection by soluble peptidoglycan recognition proteins (PGRPs) that stimulate a serine protease cascade, culminating in the proteolytic activation of the extracellular ligand, Spätzle. Activation of a second pathway, the immune deficiency (IMD) pathway, occurs when a pathogen is detected by a membrane-bound class of PGRPs (show as in Fig. 1).

Serpins are a large family of insect proteases, mainly involved in signal transduction, a cascade of amplification, ultimately leads to the activation of specific defense mechanisms, such as melanism, agglutination, and the induction of antibacterial skin. At present, it is known to be involved in the regulation of hemolymph coagulation, antibacterial peptide synthesis and melanism encapsulation and other innate immune responses of insects and it may be the key

link to regulate PPO reactive activity (Liu et al., 1997; Yang et al., 2005). When insect is infected by pathogen, a series of hemolymph serine proteases are rapidly activated, converting PPO into active phenol PO. PO catalyzes the production of melanin at the injured site or around the invading pathogen, causing melanization of the invading pathogen and producing toxic by-products such as o-quinone (Zheng et al., 2009). In this process, serpins are regulated negatively by inhibiting the activity of serine protease in order to maintain the dynamic balance of immune pathways in insects (An & Kanost, 2010). They have been studied in many insects, including *Hyphantria cunea*, *D. melanogaster*, *Mamestra configurata*, *B. mori*, *M. sexta*, *Mythimna unipuncta*, *A. pernyi* etc. (Jiang et al., 1994; Jiang et al., 1996; Zou et al., 2009; Zheng et al.,2009).

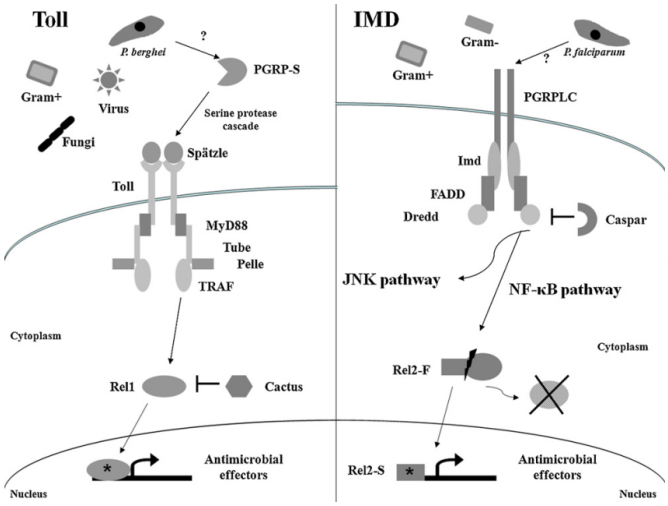


Fig.1. Toll and IMD immune signaling pathways involved in anti-Plasmodium defense. Following recognition of a microbe, or unknown Plasmodium ligand, by soluble PGRP molecules, the Toll pathway is stimulated by binding of the ligand Spätzle with the Toll transmembrane receptor. This triggers a series of molecular events that culminate in the activation (*) and translocation of Rel1 into the nucleus, up-regulating transcription of immune genes that are responsible for microbial killing. The IMD pathway is stimulated when the transmembrane PGRP-LC receptor binds peptidoglycan or an unknown Plasmodium ligand that leads to the cleavage of Rel2-F

and translocation of active Rel2-S (*) into the nucleus. A different set of anti-Plasmodium genes are up-regulated when the IMD pathway is stimulated. Branching of the IMD pathway is indicated, but the JNK pathway has not been extensively characterized in Anopheles mosquitoes.

1.6. Research Ideas of the study

The oriental fruit moth, *G. molesta* (Busck) (Lepidoptera: Tortricidae) is a key pest of tree fruits worldwide (Kong et al., 2019; Kong et al., 2020). The larvae feed on the shoots and fruits of the stone and pome plants mainly belonging to the Rosaceae family, including many economic fruit trees, such as peach, pear, apple etc. (Rothschild & Vickers, 1991; Myers et al., 2006). It has been the most headachy insect pest that threatens fruits quality worldwide, causing great economic losses in many fruit-growing regions of China (Lu et al., 2012; Zhao et al., 2013; Kirk et al., 2013). Thus, the larvae of this insect must be reduced to a low population or virtually eliminated from orchards to avoid major economic losses (Chant, 1964). However, it is difficult to be eradicated with traditional chemical control methods due to the larvae's habits of drilling into fruits or twigs (Borchert et al., 2004). Furthermore, *G. molesta* has developed resistance to some of broad-spectrum insecticides such as carbamates and pyrethroids, and the overuse of insecticides have caused severe harms to the environment (Kanga et al., 2003). Therefore, more environmental-friendly and non-toxic biological control methods are urgently needed to control *G. molesta*.

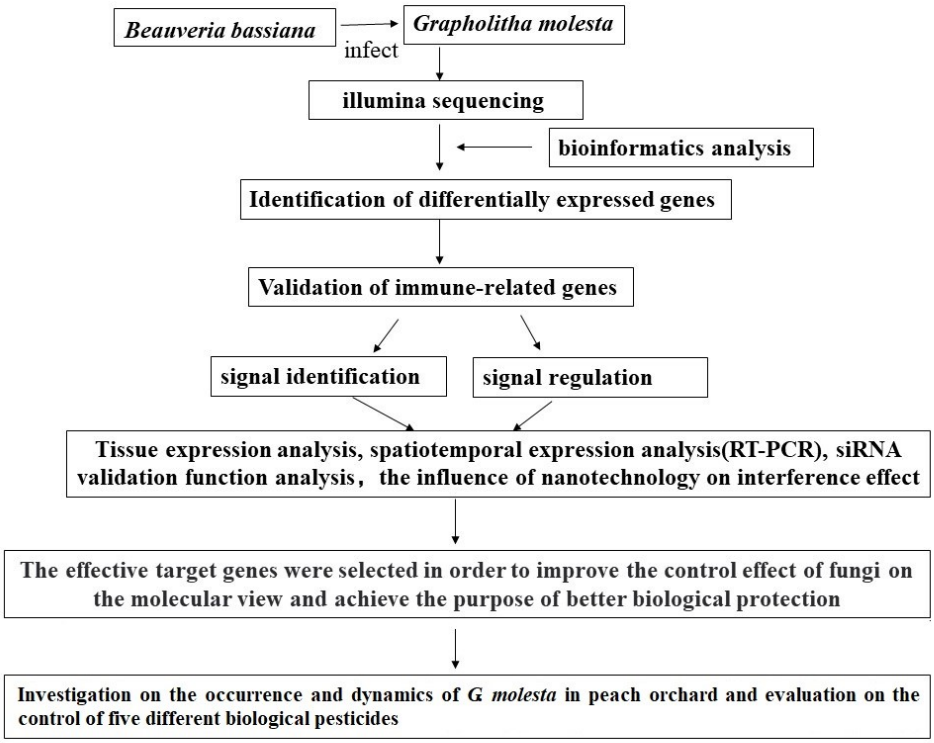
The entomopathogenic fungus, *Beauveria bassiana*, is an efficient bioinsecticide against a variety of pests and an alternative to chemical pesticides for pest control (Ferron, 1978; Feng et al., 2008; Saranraj & Jayaprakash, 2017). *B. bassiana* was effective against larvae of *G. molesta*, and its parasitic rate can reach

to 20–40%, or even to 80% under suitable conditions (Ran et al., 2016; Sarker et al., 2020). *B. bassiana* infect their hosts by penetration of the cuticle directly, but insects rely on an efficient and potent innate immune system to prevent the invasion of pathogens (Lemaitre & Hoffmann, 2007). Therefore, the entomopathogenic fungi always play a role as elicitors of insect immune responses (Lu & St. Leger, 2016). An understanding of fungal induced immune responses could provide strategies for the development of more efficient mycoinsecticide for controlling destructive pest (St. Leger & Bidochka, 1996; Zibaee et al., 2011). Several reports have been published about the immune response to *B. bassiana* infection in insects, such as *Eurygaster integriceps* (Zibaee et al., 2011), *Bemisia tabaci* (Xia et al., 2013; Wang et al., 2015), *Ostrinia furnacalis* (Liu et al., 2014), *D. melanogaster* (Francesco et al., 2015), *Plutella xylostella* (Zhang et al., 2018), *Bombyx mori* (Hou et al., 2011; Geng, 2016), *Riptortus pedestris* (Jin et al., 2019) et al. However, there are few research on *G. molesta* have been reported, and the molecular interactive and responsive mechanisms about *G. molesta* against *B. bassiana* remains poorly understood.

In order to get a better understanding on the interaction mechanism between *B. bassiana* and *G. molesta*, improving the control effectiveness of *G. molesta* against *B. bassiana*, necessary to analysis the transcriptome data of *G. molesta* larvae the immune-related genes involved in this process were identified. Then we analysis the role of pattern recognition receptors *GmPGRP-SC*, *GmBGRP*, and *GmSerpins* in the innate immune response signaling pathway of *G. molesta* from the

perspective of the immune defense response of *B. bassiana*. These results will provide a prospect to help us better understand host-pathogen interactions and provides a foundation for the engineering of entomopathogenic fungi and the discovery of insecticidal targets to control insect pests. And it prospected the improvement of effective on biological control of *G. molesta* by *B. bassiana*, which provide a theoretical basis for supply better services to plant protection in the future.

Research technical courses:



Conclusions to Chapter 1

Due to the characteristics of hidden damage and the transfer of host hazards, it is difficult to get good results with traditional chemical control for *G. molesta*

that is the main reason for its generally occurrence, spread and seriously harm. The entomopathogenic fungus, *Beauveria bassiana*, is an efficient bioinsecticide against a variety of pests and an alternative to chemical pesticides for pest control. *B. bassiana* was effective against larvae of *G. molesta*, however the control effect of *B. bassiana* is always affected by many factors, and results in relatively low control efficiency. Therefore, it is necessary to strengthen the research on the molecular mechanism of immune response of entomogenic fungi and *G. molesta*.

The study of immune genes plays an important role in the signaling pathway of insect resistance to fungal infection. A deeper understanding of the function of these genes can enhance understanding and exploitation.

The biotechnological approach is relevant and promising. These methods greatly speed up the selection process. The use of molecular biological methods to develop more effective target genes provides a promising path for the development of biopesticides.

CHAPTER 2

CHAPTER TWO MATERIALS AND METHODS

2.1. The selected material

2.1.1. Insect rearing

In the laboratory, the insects were reared on artificial diet for more than 30 generations before conducting the experiment. The adults were reared in beaker (2 L in volume) with one fresh fuji apple and a spawning paper inside for egg laying and fed with 10% honey solution. Neonate larvae were reared in the apples, and late instar larvae (third to fifth instar) were picked out from the rotten apples (Wang et al*.,* 2017; Zhang et al., 2021). The late instar larvae were reared on artificial diet until pupation following the methods of Du et al (2009). All of them were reared under a photoperiod of 15: 9 L: D at 85% relative humidity at 26.5 ºC to the appropriate assay instar.

2.1.2. Instrument and equipment

The main software and instrument used in this study are DNAMAN, MEGA 7.0, Graph Pad Prism. 7.00, SPSS 18.0, Bio-Rad PCR instrument (American), Eppendorf 5425 R small high speed refrigerated centrifuge, ABI QuantStudio5 Q5 (American), Nexcelom Cellometer-MIni automatic cell counter (American), THERMO Varioskan Flash all-wavelength multifunctional enzyme label instrument (England), Drummond Nanoject III Drummond Fully Automatic Microinjector (American), HPD-250A Artificial climate Chamber (China),

autoclave (China), NanoDrop one ultrafine spectrophotometer (American) 96-well PCR tubes (TempPlate PCR plate, 0.2 mL thin-wall standard wells, USA Scientific), and etc.

2.1.3. The test reagent

ChamQ Universal SYBR real-time PCR Master Mix (Vazyme, Nanjing, China); HiScript® II Reverse Transcriptase (Vazyme, Nanjing, China); RNAprep Pure Tissue Kit (TIANGEN, Beijing, China); Taq DNA Polymerase (Solarbio, Beijing, China), In vitro Transcription T7 Kit (Takara, Japan), Bradford Protein concentration assay kit (Solarbio, Beijing, China), L-DOPA, Phosphate Buffered Saline, Other reagents are domestic or imported analytical pure reagents.

2.2. Experimental method

2.2.1. Laboratory evaluation of the effect of *Beauveria bassiana* on the vital activity of *Grapholita molesta*

2.2.1.1. Fungal pathogen and preparation of conidial suspension

B. bassiana BNCC 111705 was from BeNa Culture Collection, and cultured on potato dextrose (PDA) plates at 28 °C, 95% humidity under complete darkness. Conidia (spores) used for the infection were harvest form 5–7 days old cultures by scraping the surface of the mycelia with 40 mL ddH_2O, filtered with sterile gauze, and then washed with ddH_2O for third times. The spore concentrations were adjusted to 1×10^5 conidia/mL, 1×10^6 conidia/mL, 1×10^7 conidia/mL. The viability of conidia was determined before the bioassay, and greater than 95% conidia germination was observed in all tests.

2.2.1.2. Larva bioassays

Fourth instar larvae with consistent growth were selected and used for two different modes of infection. Immersion method: To be infected insects were soaked in the spore suspension of 1×10^5 conidia/mL, 1×10^6 conidia/mL, 1×10^7 conidia/mL, for 10 s, while the insects of control group were treated with ddH_2O for the same duration. Feeding method: The same size of artificial diet was smeared with the suspensions of conidia with different concentrations of *B. bassiana*, and place on absorbent paper for a few times to dry excess water surface. The treated group of insects were feed with the artificial diet coated with *B. bassiana*. The control groups were feeding with asepsis artificial diet.

A total of 120 insects for each treatment with three biological replicates, and each biological replicate with 30 larvae respectively. Insects were further dried with sterile filter paper, and reared separately in a dactylethrae with artificial diet and kept in Artificial Climate Chamber, at 26.5 ºC, 95 % RH, under a photoperiod of 15 L: 9 D.

Mortality was observed every 24 h for larvae and, until all insects in the control had died. Every larva in control group and treatment group was numbered, and the body weight of it was recorded on the first day of the fifth instar and pupal stages in each group.

2.2.1.3. Statistical analysis

Statistical analyses were all performed using SPSS 18.0 Statistics software and graphs were constructed using Graph Pad Prism. 7.00. software. One-way

analysis of variance (ANOVA) and Tukey's test were used, $P < 0.05$ was considered significant (Zar, 2010).

Corrected mortality (%) = (treatment mortality - control mortality) /(1 - control mortality) ×100%.

2.2.2 Transcriptome analysis of *G. molesta* larvae in response to entomopathogenic fungi *Beauveria bassiana*

2.2.2.1 Sample preparation for transcriptome sequencing

A total of 60 fifth-instar newly emerged larvae were collected for two different treatment groups. To infect insects were exposed to the spore suspension of 1×10^7 conidia/mL for 10 s, while the insects of control group were treated with ddH_2O. Insects were further dried with sterile filter paper, and reared separately in a dactylethra with artificial diet. All the treatments were performed with three biological replicates. After 24 h, these samples were frozen immediately, grounded in liquid nitrogen and stored at −80 ◦C until use.

2.2.2.2. Process of total RNA extraction from *G. molesta*

Follow the instructions provided by TIANGEN company for RNAprep Pure Tissue Kit:

(1) Homogenate treatment: Add 300 μL lysate RL for every 10-20 mg tissue (please check whether β-mercaptoethanol has been added before use), and grind the tissue thoroughly with a grinding pestle (if the tissue is difficult to be thoroughly ground, electric or glass homogenizer can be used); Then, 590 μL RNase-Free ddH_2O and 10 μL Proteinase K were added into the homogenate,

which was mixed and treated at 56 ºC for 10-20 min;

(2) Centrifuge at 12,000 rpm (~13,400×g) for 2-5 min and take the supernatant for the following operations;

(3) Slowly add 0.5 times the supernatant volume of anhydrous ethanol, mix (precipitation may occur at this time), the resulting solution and precipitation, the adsorption column was transferred to CR3 (the adsorption column was placed in the collection tube) and centrifuged at 12,000 rpm (~13,400×g) for 30-60 s, discard the waste liquid in the collection tube and put the adsorption column back into the collection tube;

(4) Adsorption column CR3 was added with 350 μL deproteinizing solution RW1, centrifuged at 12,000 rpm (~13,400×g) for 30-60 s, and abandoned waste liquid, put the adsorption column back into the collection tube;

(5) Preparation of DNase I working solution: 10 μL DNase I storage solution was put into a new RNase-Free centrifuge tube and added

70 μL RDD buffer, gently mixed.

(6) DNase I working solution (80 μL) was added into the center of adsorption column CR3 and placed at room temperature for 15 min;

(7) Adsorption column CR3 was added with 350 μL deproteinizing solution RW1, centrifuged at 12,000 rpm(~13,400×g) for 30-60 sec, and abandoned waste liquid, put the adsorption column back into the collection tube;

(8) Add 500 μL bleach solution RW to adsorption column CR3 (please check whether ethanol has been added before use) and let stand at room

temperature for 2 min, centrifuge at 12,000 rpm(~13,400×g) for 30-60 s, discard the waste liquid, and put the adsorption column CR3 back into the collection tube;

(9) Repeat Step (8).

(10) Centrifuge at 12,000 rpm (~13,400×g) for 2 min and drain the waste solution. Adsorption column CR3 was placed at room temperature for several minutes to thoroughly dry the residual bleach solution in the adsorbed material;

(11) Adsorption column CR3 was transferred into a new RNase-Free centrifuge tube, and 50 μL RNase-Free ddH2O was added to the middle part of the adsorption membrane. The adsorption column was placed at room temperature for 2 min, and centrifuged at 12,000 rpm (~13,400×g) for 2 min to obtain the results RNA solution.

RNA degradation and contamination was monitored on 1% agarose gels. The concentration and purity of extracted RNA was determined using ultrafine spectrophotometer (NanoDrop One, Thermo Fisher). All samples were kept in -80 ^{o}C for any further study to prevent hydrolysis of RNA.

2.2.2.3 Digital gene expression (DGE) library construction and sequencing

For each sample, 800 ng of total RNA was used to isolate mRNA using oligo(dT) magnetic beads. Sequencing libraries were generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, Ispawich, USA) according to the manufacturer’s recommendations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads (Life technologies,

CA, USA). Fragmentation was carried out using divalent cations under elevated temperature in NEB proprietary fragmentation buffer. DNA fragments with ligated adaptor molecules on both ends were selectively enriched using NEB Universal PCR Primer and Index primer in a 10 cycles PCR reaction. The library was paired-end sequenced using PE150 strategy on Illumina HiSeqTM 2000 (Illumina, San Diego, CA, USA) in the Novogene company (Tianjin, China). The challenged and control libraries were sequenced in one lane then raw reads were sorted out by barcodes.

2.2.2.4 Assemble and annotation of transcriptomes

Raw data (raw reads) in fastq format were firstly processed through a self-written perl scripts (Novogene company, Tianjin, China). In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data. At the same time, Q20, GC- content and sequence duplication level of the clean data was calculated. All the downstream analyses were done on the clean data with high quality. De novo transcriptome assembly was carried out with a short-read assembling program-Trinity (Grabherr et al., 2011). The generated unigenes were analyzed by searching the GenBank database with the BLASTX algorithm (https://www.ncbi.nlm.nih.gov/). Gene Orthology (GO) and KEGG Orthology (KO) annotations of the unigenes were determined using Blast2go (https://www.blast2go.org/) and InterProScan software.

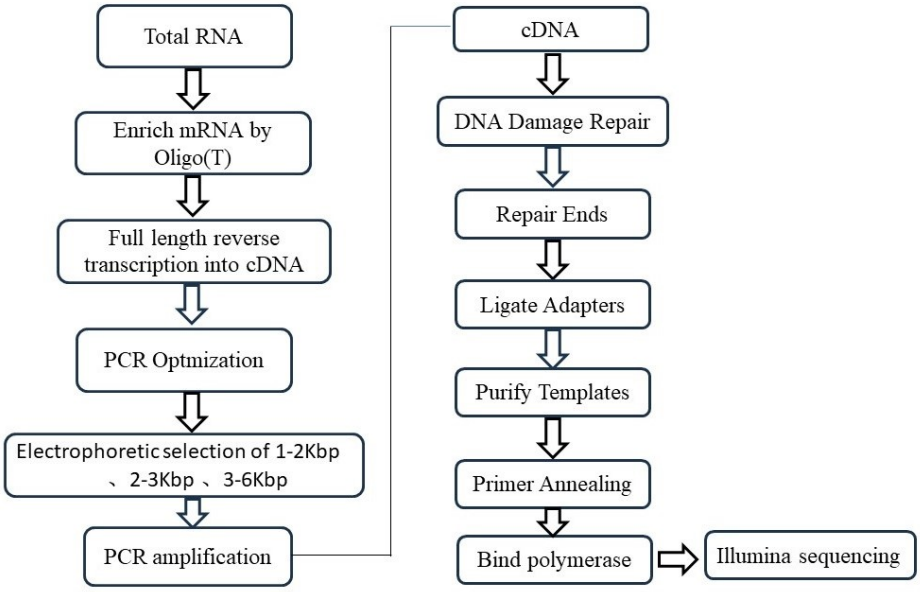


Fig.2. Transcriptome resequencing experimental process

2.2.2.5. Identification of differentially expressed genes

The FPKM (Fragments per kb per million fragments) method was used to calculate the expression of unigene. It is generally considered that when FPKM > 0.3 the unigene are expressed (Mortazavi et al., 2008). The differential gene expression analysis was performed using *P* value and the DESeq2 package (Love et al., 2014). The *P*-value of hypothesis testing was calculated by statistical model and analyzed by the Benjamini and Hochberg's approach (Benjamini & Hochberg, 1995). We screened genes with a *P*-value < 0.05, considering them as significant differentially expressed genes.

2.2.2.6. Reverse transcription

Follow the instructions provided by Vazyme company for HiScript® II Q RT SuperMix for qPCR (+gDNA wiper):

(1) Removal of genomic DNA:

Name of the reagent	usage amount

RNase-free ddH_2O	12μL
4 × gDNA wiper Mix	4 μL
Templet RNA	1μL

Gently use the pipette repeatedly blowing mixed, then the mix was incubated at 42 ºC for 2 min in Bio-Rad PCR instrument.

(2) Preparation of retro-transcriptional response system:

Add 5 x HiScript II qRT SuperMix II directly to the reaction tube in Step 1

Name of the reagent	usage amount
5 × HiScript II qRT SuperMix II	4 μL
The reaction liquid from step 1	12 μL

Gently use the pipette repeatedly blowing mixed, then the mix was incubated at 50 ºC for 15min, 85 ºC for 15 s in Bio-Rad PCR instrument. The product can be immediately used for qPCR reaction, or stored at -20 ºC and used within half a year; Long-term storage: it is recommended to store at -80 ºC after subpackaging. Repeated freeze-thaw should be avoided for cDNA.

2.2.2.7. Design and completion of primers

Table 1

Lists of the primer sequences

Primer name	Primer Sequence (5' to 3')
SRCBF1	AGACGACCTTCTCTTTCTGC
SRCBR1	GCTGAATGTTGCCGATAGA
PLAF1	TGAAACCCGTCAAGTCCT
PLAR1	GCACTCATTTGTCTGGCA
SERF1	TGTGATTGTTCTTCTGTGCG
SERR1	GGCTTGTCTTATTCTCTCTGCT
CarEF1	CCCTGGGCGATACTAAATC
CarER1	CTGCTATTACAAGACGGGTGT
SERF2	TTTAGCCCTACCAACCCA
SERR2	GCACCATCAGTCCGTTAGA
P450F2	TGGCTTGTATGGAGGAGAC
P450R2	CAGGATTACGGTTCAAATGG

P450-3F1	TAGTGTTCTTTGCTGCGG
P450-3R1	CATCTCCTTTACGGCATTG
P450F1	ATGAAGGCTGAGGTTGGAC
P450R1	TGTGTGCCCAGGTCTATTG
CPBF1	CAGAACAGGAAGAATAGGATGG
CPBR1	TTGGAGGTGTCGGTAAAGTT
SERF3	ATCTCTGCGGATGGTGAA
SERR3	GCGGGTGGTTGAAATACTC
DPP-4F1	CCCATTGAAAGAAGGAGACG
DPP-4R1	TACTGAAGGTCCGAGCACTG
EF-1a F	CATCACAGTAAAGGACGGTAAG
EF-1a R	AGAACAAGACCAGAGCATCC
PGRPF1	TCAAGTGCGGAGTGACCAA
PGRPR1	ATGCCATCAAGATTGTCGG
BGRPF1	CAACCAACCGCTACCATAG
BGRPR1	TTCTCAGCATCCCAAAGAC
TollF1	CCAACATTTCACTGGCATAC
TollR1	GAATAGTTTCTGCTCCAAGTCC

According to the gene sequence retrieved by *de novo* transcriptome data, the primers were designed and synthesize by Shenggong Bioengineering (Shanghai) Co.

2.2.2.8. Real-time PCR

The RNA samples used for the qRT-PCR analysis were the same as for the DGE experiments and independent RNA extractions were used for biological replicates. The cDNAs were synthesized following the method of HiScript® II Reverse Transcriptase (Vazyme, Nanjing, China). The qRT-PCR was performed on QuantStudio5 Real-Time PCR System (ABI, USA) using the ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), according to the manufacturer's instructions. *Ef-1α* were used as a housekeeping gene from the housekeeping gene selection report (Cao, 2015). Quantitative analysis followed a comparative Ct (ΔΔCT) method $-\triangle\triangle Ct = -(\triangle Ct.q - \triangle Ct.ab)$ (Livak & Schmittgen, 2001). Ct represents

the number of cycles the target amplification product underwent to reach the set threshold, △ Ct means difference of Ct value between target gene and housekeeping gene (q means target gene in this experiment is the target gene and ab means housekeeping gene in this experiment is *Ef-1α* gene, Primers sequences are detailed in Table 1. Follow the instructions provided by Vazyme company for ChamQ Universal SYBR qPCR Master Mix:

(1) The following mixture was prepared in the 96-well PCR tubes.

Name of the reagent	usage amount
2 × ChamQ Universal SYBR qPCR Master Mix	12 μL
Primer F (10 μM)	0.4 μL
Primer R (10 μM)	0.4 μL
Template DNA/cDNA	1 μL
ddH_2O	8.2 μL
Total	20 μL

The real-time PCR was calculated by relative quantitative method follow the instructions of ChamQ Universal SYBR real-time PCR kit. The instruments used are ABI QuantStudio5 Q5 (American) real-time PCR, the reaction conditions are as follows: pre-denaturation at 95 ºC for 30 s, 95 ºC for 10 s, 60 ºC for 30 s, a total of 40 cycles, 95 ºC 15s, 60 ºC 60s, 95 ºC 15 s for recording the dissolution curve.

2.2.3. Characterization and functional analysis of target genes from *G. molesta*

2.2.3.1. Identification of target genes and full-length and validating obtaining the opening reading frame (ORF) of target genes

The full-length of the target cDNA was obtained from *de novo* transcriptome

data constructed previously by our library (Cao et al., 2022). To verify the full-length cDNA acquired from the transcriptome sequences, two gene-specific primers were used to amplify the ORF of the target gene.

Polymerase chain reaction (PCR) was conducted using a 50 μl reaction mixture consisting of 25 μl of 2× Es Taq PCR Master Mix (CWBIO, Jiangsu, China), 2 pmol of each forward and reverse primer and 2 μl of cDNA template (40 ng/μl). The PCR conditions were as follows: an initial incubation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30s, 55 °C for 30s, and 72 °C for 30s, with a final extension at 72 °C for 2 min. The PCR-amplified fragments were gel-purified by electrophoresis using 1% agarose gel. The purified PCR products were sequenced using an ABI Model 3100 automated sequencer (Sangon Biotechnology Co. Ltd., Shanghai, China).

2.2.3.2. The amino acid sequence of target genes of *G. molesta* was compared and the evolutionary tree was constructed

The homology search for protein sequence cDNA sequence of was performed using BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/). The ORF prediction and protein translation were performed by DNAMAN. Protein physical, chemical properties and structural domain prediction using the Expert Protein Analysis System (http://web.expasy.org/protparam/), SignalP-5.0 Server (http://www.cbs.dtu.dk/servic-es/SignalP) and SMART (http://smart.emblheidelberg.-de/) analysis. Amino acid sequence from other insects were retrieved from the NCBI GenBank. A neighbor-joining (NJ)

phylogenetic tree was constructed using Mega7.0 software. The reliability of the branching was tested using the bootstrap resampling (with 1000 pseudo replicates) technique (Appendix B).

2.2.3.3. Temporal and spatial expression analysis of target genes

The samples of RNA were extracted at different development stages (1st, 2nd, 3rd, 4th, 5th instar, pupa, and adult) and sample of different tissue were extracted from the larvae that second days after fifth-instar larva, including hemocyte (70 to 80 samples were taken from each sample, and the hemolymph was collected at 4000 g/min), fat bodies, epidermis, malpighian tubules and midgut, according to the method of RNAprep Pure Tissue Kit, RNA purity and integrity were checked with ultrafine spectrophotometer (NanoDrop One, Thermo Fisher).

2.2.3.4. siRNA synthesis

The 21-nucletide sequence of the siRNA sequences were selected using DSIR (http://biodev.extra.cea.fr/DSIR/DSIR.htmL) which automatically provides a list of potential candidates. Negative CK was a kind of commercial siRNA that showed no RNAi effects in any treatments purchased from Genepharma Company (China, shanghai). The siRNAs were synthesized using an In vitro Transcription T7 Kit (Takara, Japan) according to the to the manufacturer's instructions as follows:

Table 2

Lists of the primer sequences for siRNA

Primer name	Primer Sequence (5' to 3')
PGRP Oligo-1	GATCACTAATACGACTCACTATAGGGCGTGCTTGTGGTATTTCAAGGTT
PGRP Oligo-2	AACCTTGAAATACCACAAGCACGCCCTATAGTGAGTCGTATTAGTGATC
PGRP Oligo-3	AACGTGCTTGTGGTATTTCAAGGCCCTATAGTGAGTCGTATTAGTGATC
PGRP Oligo-4	GATCACTAATACGACTCACTATAGGGCCTTGAAATACCACAAGCACGTT
BGRP Oligo-1	GATCACTAATACGACTCACTATAGGGGGAAGCGATTTATCCTAGAGGTT
BGRP Oligo-2	AACCTCTAGGATAAATCGCTTCCCCCTATAGTGAGTCGTATTAGTGATC

BGRP Oligo-3	AAGGAAGCGATTTATCCTAGAGGCCCTATAGTGAGTCGTATTAGTGATC
BGRP Oligo-4	GATCACTAATACGACTCACTATAGGGCCTCTAGGATAAATCGCTTCCTT
Serpin2 oligo 1	GATCACTAATACGACTCACTATAGGGGGTTGGGTGTCTAATCTTATT
Serpin2 oligo 2	AATAAGATTAGACACCCAACCCCCTATAGTGAGTCGTATTAGTGATC
Serpin2 oligo 3	AAGGTTGGGTGTCTAATCTTACCCTATAGTGAGTCGTATTAGTGATC
Serpin2 oligo 4	GATCACTAATACGACTCACTATAGGGTAAGATTAGACACCCAACCTT
Serpin3 oligo 1	GATCACTAATACGACTCACTATAGGGGGACGAATCTGGAGAAGAAGATT
Serpin3 oligo 2	AATCTTCTTCTCCAGATTCGTCCCCCTATAGTGAGTCGTATTAGTGATC
Serpin3 oligo 3	AAGGACGAATCTGGAGAAGAAGACCCTATAGTGAGTCGTATTAGTGATC
Serpin3 oligo 4	GATCACTAATACGACTCACTATAGGGTCTTCTTCTCCAGATTCGTCCTT

(1) Preparation of double-stranded Oligo DNA

Name of the reagent	usage amount
10× Annealing Buffer	2 μL
100 pmol/μL Oligo A*	2 μL
100 pmol/μL Oligo B*	2 μL
RNase free dH2O	14 μL

a. A and B must be paired. Specific pairing conditions are as follows: Oligo-1 and Oligo-2; Oligo-3 and Oligo-4; n-Oligo-1 and n-Oligo-2; n-Oligo-3 and n-Oligo-4.

b. The Oligo DNA annealing solution was placed on the PCR amplification instrument, treated at 95 ºC for 2 min, cooled to 25 ºC for 45 min, and then kept at 25 ºC for 10 min. At this time, a pair of single-stranded Oligo DNA was annealed to form double-stranded Oligo DNA, and the concentration of double-stranded Oligo DNA was 10 pmol/ μL. The solution can be used as template DNA. Store at -20 ºC when not in use.

(2) In vitro transcriptional reaction

Name of the reagent	usage amount
10× Transcription Buffer	2 μL
ATP Solution	2 μL
GTP Solution	2 μL
CTP Solution	2 μL
UTP Solution	2 μL
RNase Inhibitor	2 μL

T7 RNA Polymerase	0.5 μL
RNase free dH2O	5.5 μL
10 pmol/μL double strands Oligo DNA solution（Oligo-1/-2）*1	1 μL
10 pmol/μ l double strands Oligo DNA solution（Oligo-3/-4）*1	1 μL
Total	20 μL

The above solution was mixed evenly and centrifuged slightly. The transcription reaction solution was collected at the bottom of the reaction tube and reacted for 2 hours at 42 ºC. A portion of the reaction solution was taken for gel electrophoresis to confirm the RNA products after in vitro transcription.

(3) Nuclease treatment:

a. The following solution was added to the reaction tube after the in vitro transcription reaction.

Name of the reagent	usage amount
RNase free DNase I (5 U/μ l)	2 μL
RNase T1 (4 U/μ l)*	1 μL
Total	3 μL

*: 4 U/ μL RNase T1: Dilute the RNase T1 to 4 U/ μL in the RNase T1 Dilution Buffer. (Dilute RNase T1 should be used as soon as possible and should not be stored.)

b. Reaction at 37 ºC for 2 hours.

The injection solution was prepared by dissolving siRNA in RNase free dH_2O for 2000ng/μL and both the treatment group and control group were injected with 1μL. The siRNA was injected into the abdominal intersegment behind the second abdominal segment of the selected fifth-instar larvae using a microinjector with a glass capillary needle. For phenotype observation, 30 larvae were injected for each

treatment, and three replications were made. The injected larvae were reared routinely on artificial diet. After 24 h, 48 h and 72 h, the surviving larvae were selected to measure the silencing effect of siRNA microinjection on target gene transcript levels by qPCR.

2.2.3.5. M2L-siRNA and NP5- siRNA preparation of complex

The Nanocarrier material M2L and NP5 were kindly gifted from Dong jiangtao. The material is described in detail in the article Dong et al (2021), which can effectively bind dinucleotides and prevent their degradation. In order to obtain the nanoparticle complex, dsRNA solution containing 30 ug was combined with the nanomaterial M2L and NP5 (the nanomaterial was completely dispersed by ultrasonic treatment before use), and the complex was mixed with equal volume of DEPC water, treated with 4 ^{o}C and incubated for 2 h, so that the nanomaterial was fully combined with double-stranded RNA. In order to determine the encapsulation efficiency of M2L and NP5, the M2L-SiRNA and NP5-siRNA complex were centrifuged at 21000 g for 20 min, and the content of double-stranded RNA in the supernatant was measured with the spectrometer. The percentage of double-stranded RNA in the complex formed by M2L and NP5 was calculated (that is, the loading rate of double-stranded RNA).

2.2.3.6. siRNA injection

The injection solution was prepared by dissolving siRNA in RNase free ddH_2O for 2000 ng/μL and both the treatment group and control group were injected with 1μL. The siRNA, M2L- siRNA and NP5-siRNA were injected into

the abdominal intersegment behind the second abdominal segment of the selected fifth-instar larvae using a microinjector with a glass capillary needle respectively. For phenotype observation, 30 larvae were injected for each treatment, and three replications were made. The injected larvae were reared routinely on artificial diet. After 24 h, 48 h and 72 h, the surviving larvae were selected to measure the silencing effect of siRNA microinjection on the target gene transcript levels by qPCR.

2.2.3.7. Analysis of PPO enzyme activity

Collection of hemolymphs: after interference treatment, dissected the *G. molesta* in PBS and collected the hemolymphs on ice (30 samples of each treatment and control group, and the hemolymph was collected at 4000 g/min), then stored at -80 ºC for Phenoxylase activity determination.

Phenoxylase activity determination: add 1.4 mL 0.02 mol/L phosphoric acid buffer to 1.5 mL L-DOPA (0.05mol/L), water bath at 30 ºC for 30 min, add 0.1 mL of enzyme solution and the OD values at 475 nm were determined in all-wavelength multifunctional enzyme label instrument. The change of light absorption value at 475 nm was measured with multifunctional enzyme label instrument, and the reading was performed once every minute for 30 min. One unit of enzyme activity is defined as A475- light absorption value change of 0.001 per minute. At the same time will dilute after 10 μL haemolymph supernatant liquid to join Bradford, 10 min, gently shake test 595 nm absorbance reading, according to the determination of protein concentration in the standard curve lie, determine the

protein concentration of the sample, and calculate the specific activity of PO. Protein content was determined by G-250 Coomassie bright blue method.

A PPO enzyme activity unit is defined as ΔA475/min = （ΔA treatment group – ΔA control group） / 10.

SPSS statistical software was used for one-way analysis of variance (ANOVA) and Tukey's test method for multiple comparison between the mean values. $P < 0.05$ indicated significant difference, and $P < 0.01$ indicated extremely significant difference. The data used are mean ± standard deviation (M±SD).

2.2.4. Investigation on the occurrence and dynamics of *G. molesta* in peach orchard and evaluation on the control of five different biological pesticides

2.2.4.1. Dynamic investigation

(1) Description of experimental locations

The location of the survey was the fruit production demonstration park of Henan Academy of Agricultural Sciences modern agricultural science and technology experimental demonstration base. The climate in study area is warm temperate continental monsoon climate with moderate precipitation and mild climate. The average annual temperature is 11.8 °C, with an average of 19°C in January. The average temperature in July is 27.8 °C, the average annual precipitation is 161 mm, and the annual sunshine duration is 2698.4 hours. The surrounding environment of the test site: peach trees planted in the east, covering an area of 2 mu, with a tree age of 10 years, mainly planting new peach varieties

such as Zhongnong Jinhui, Jinming and Jinshuai; Pear trees planted in the west, an area of 5 mu, as a resource conservation nursery, tree age of 10 years, mainly to preserve western pear variety resources. The row spacing of the trees is 2 mx4 m, and the tree shape is evacuation stratification.

(2) Investigation on dynamics of *G. molesta*

Pheromones were used to trap *G. molesta* in a water basin. The birdbath is a plastic basin with a diameter of about 20 cm. In the distance from the top edge of the basin 3 cm evenly play three holes, and then through the hole into the fine wire. The lure was fixed with thin iron wire over the center of the basin and 3 cm above the water. Put a little washing powder into the water. During the investigation and test, add water in time when the water is dry, in order to kept the central lure 1-1.5 cm away from the water surface in the basin. The hand-made water basin bait traps were hung on the outer canopy of peach and pear trees with fine wire, with a height of 1.5 m, 4 in each orchard, and the interval of each trap was more than 20 m。Checking and recording the number of *G. molesta* in the bowl every two days from 6 pm. to 7 pm. The core is replaced once a month. It begins in mid-to-late March 2021 and ends in late October 2021, and it does not end until the *G. mol*esta is not observed twice in a row. Recording the number, morphology and age of all *G. molesta* in feromoncsapda, meanwhile the number and characteristics of affected fruit trees were observed and recorded.

(3) Investigation of damage of *G. molesta* to peach tree shoots

The investigation was in Xinxiang Base of Henan Academy of Agricultural

Sciences peach orchard from mid-April to the end of June 2021. Selecting the representative peach orchard and recording the harm of peach tree shoots regularly. The number of shoots surveyed in each peach orchard is generally not less than 50 trees, then calculated the percentage of damage. After investigation, cutting the damage peach shoots and droughting back to the laboratory. The number and development of the *G. molesta* larva were examined by peeling off the shoots. Finally, compared the harm of the larva in different peach orchards.

2.2.4.2. Study on screening of five kinds of biopesticides for control of *G. molesta* in peach orchard

(1) Experiment design

A total of 6 treatments groups were set up in the experiment, each treatment was repeated 4 times. On each plot, except the control plots, 4 randomly arranged peach tree saplings (10 years old) were planted in one row with four saplings per row. Specific experimental design and pharmaceutical information were shown in Table 3. Expect the different application measures, other management measures of each group are consistent.

Table 3

Test drug information and treatment design

Treatment group	Name of the drug	Dilution ratio	Manufacturer	Active ingredient	Treatment period
1	16 000 IU/mg Bt	200	Jiangsu Dongbao Biochemical Co., LTD	1×10^{10}cfu/g (effective viable bacteria count)	3-7 days
2	6 0 g / L Spinetoram	2000	Dow AgroSciences	Ethyl multicidin -J and ethyl multicidin -L (3:1)	4 hours

3	2 4 0 g / L Methoxyfenozide	5 000	Dow AgroSciences	Methoxyfenozide	3-7 days
4	0.2% Celastrus angulatus MaXim	1000	Xinxiang Dongfeng Chemical Co., LTD	Celastrus angulatus	5-7 days
5	10 billion spores *Beauveria bassiana*	10000	Shanxi Green Sea pesticide technology Co., LTD	*B . bassiana* (*The final concentration is 1x10^7 conidia/mL spore)*	3-7 days
6	CK	0	0	0	

Table 4

Test plot arrangement

Repetition	plot arrangement
Repetition 1	6 5 2 4 3 1
Repetition 2	3 4 6 1 5 2
Repetition 3	5 2 4 3 1 6
Repetition 4	2 3 1 5 6 4

The orientation of the test plot was upper west and lower east, and each plot had a single row of 4 peach trees with an area of 80 m. The middle two strains were selected for investigation. Repeat four times for each agent tested.

(2) Experimental method

We used the sex attractant of *G. molesta* as an indicator of control. When the predicted results reach the control index, control treatment will be carried out. In the treatment area of the test, the amount of drug used in each area was calculated according to the plot area and the dosage of the drug set in the test. Application water of 3kg per plant, 1500kg/hm^2. Mixed spray from low concentration to high concentration in turn. The control area of blank test was sprayed with an equal amount of water.

Description of the duration of efficacy of each agent:

1. 16 000 IU/mg Bt: Bacillus subtilis is a new type of biological pesticide derived from microorganisms. It can not only control a variety of plant diseases,

but also has a better insecticidal effect. Generally, the use of spraying 3-7 days can get a preventive effect, and the efficacy period can last for a month.

2. 6 0 g / L Spinetoram: Ethyl polycidin is a macrolide insecticide developed by Dow Company, which has the properties of contact and stomach toxicity, quick action and long duration. The insecticidal speed of ethyl polycidin is fast, generally a few minutes to a few hours or so can get control effect. The duration of ethyl polycidin is longer, and it still has a good insecticidal effect after 10-14 days of spraying.

3. 2 4 0 g / L Methoxyfenozide: Methoxyfenazide is the second generation dihydrazide insect growth regulator, which has highly selective insecticidal activity against Lepidoptera pests. The insecticidal action is slow but the efficacy period is long, up to 15-20 days.

4. 0.2% Celastrus angulatus MaXim: Picrographe, is a plant source pesticide, mainly with stomach toxicity to control pests, insecticidal effect is strong, the duration of the drug is generally about 20 days, usually after 24 h of application, insects will not harm crops, 5-7 days of significant effect, low toxicity to humans and livestock, birds, aquatic organisms and beneficial biosafety, no harm to crops; stable to light and resistant to pests.

5. 10 billion spores B. bassiana: B. bassiana is a broad-spectrum biological insecticide that can control a variety of pests. Generally, after 3-7 days, it can get control effect, and the efficacy of a long duration, up to 80 days of effect.

(3) Investigation method

The direct investigation method was used as follows: Ten peach trees of each pesticide treatment plot were selected to investigate. Each plant was selected from five directions: east, west, south, north and middle five leaves on the upper branches were investigated from each direction and the number of live insects in leaves were recorded. Five control peach trees that had not been treated with water were investigated at the corresponding time and in the community. Each plant was selected from five east, west, south, north and middle locations. And the same investigation method as treatment groups. The natural insect population reduction rate was calculated to correct the corresponding insect population reduction rate. The live insect count before application was investigated 1 day before application on 15, July, 2021. The live insect numbers after treatment were investigated 1, 3, 7, 10 and 15 days, totally 6 times investigated. The difference of wormhole fruits rate between each treatment and the control group was compared, and the control effect of each biological pesticide on *G. molesta* was summarized.

Calculation method: Relative control effect (%) = (wormhole fruits rate of control group- wormhole fruits rate of treatment group)/ wormhole fruits rate of control group ×100

(4) Calculation method of control effect

Relative control effect (%) = (wormhole fruits rate in control area- wormhole fruits rate in treatment area)/ wormhole fruits rate in control area ×100.

Conclusions to Chapter 2

Experiment 1 was conducted under laboratory conditions. Different

concentrations of B. bassiana were used to infect *G. molesta* through different infection modes. The corrected mortality was calculated by biostatistical analysis. The experiment adopted the common method of calculating corrective mortality in pharmacodynamic analysis.

Experiment 2 was molecular mechanism of the infection of B. bassiana, analyzed comprehensively and comprehensively using transcriptome analysis methods such as fluorescence quantification, gene analysis, gene silencing, evolutionary tree analysis and nanoparticle mediation. The analysis method has comprehensiveness, diversity and sufficiency.

Experiment 3 A five-point sampling method was used to investigate peach orchards in different environments. The analysis method is reliable. At the same time, the biodetermination methods of different biological pesticides are scientific and diverse. Regression model, cluster analysis, factor analysis was used.

CHAPTER 3

LABORATORY EVALUATION OF THE EFFECT OF *BEAUVERIA BASSIANA* ON THE VITAL ACTIVITY OF *G. MOLESTA*

3.1. Morphology of larvae infected with *Beauveria bassiana*

Studies have shown that the insects of treatment groups infected by different concentration of *B. bassiana* suspension, their body size decreased with the increase of the concentration of *B. bassiana* solution after infected 4th days. On the 8th day of treatment with the conidial suspension of *B. bassiana*, the larvae of the control group had pupated, and the treated groups with white mycelia covering the body and continued to moisturize and culture, the insect body was covered with white conidia.

3.2. Larval mortality infected by *B. bassiana*

(1) Infected through body wall

Larvae of *G. molesta* died fast when the spore concentration is 1×10^7 conidia/mL, and the corrected mortality for this was 65.7%. The corrected mortality infected with 1×10^5 conidia/mL suspension *B. bassiana* was 44.9%, which reached the maximum death rate on 7 days. And the lethal effect of these two groups (1×10^6 conidia/mL and 1×10^7 conidia/mL) were significantly higher than that of 1×10^5 conidia/mL *B. bassiana* concentration 11.7%.

Compared with the control group, the body weight of larvae treated with 1×10^5 conidia/mL decreased 24% and 21% at the fifth and pupa ages, the group treated with 1×10^6 conidia/mL, body weight decreased 36% and 31% at the fifth

and pupa ages, and the group treated with 1×10^7 conidia/mL body weight decreased 75% and 74% at the fifth and pupa ages which was significantly lower than that of the control group and the lower concentrations (Fig.3,4).

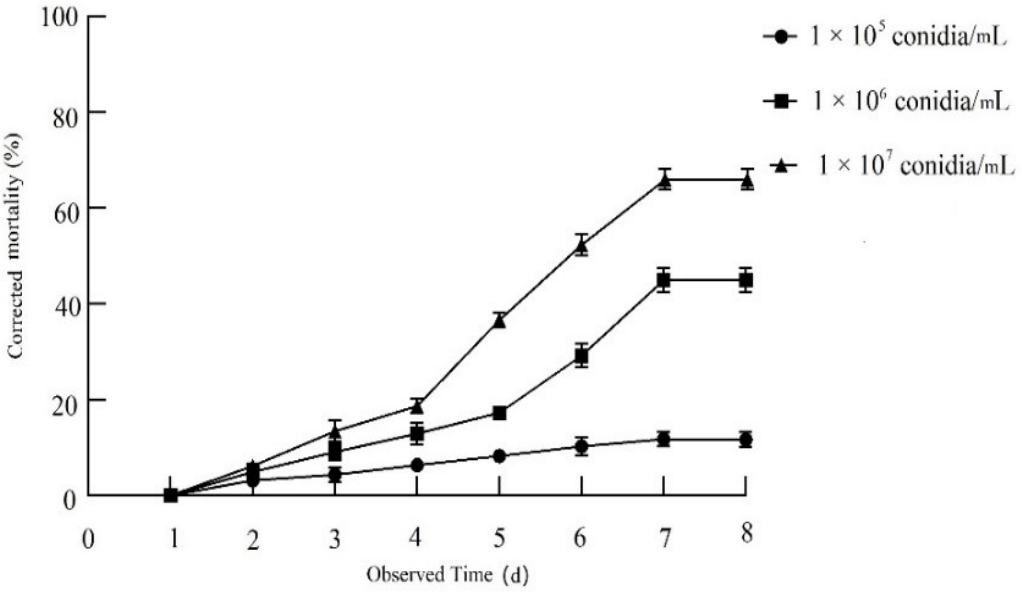


Fig. 3. The Corrected mortality of cuticular infection on *G. molesta* infected by different concentration of *B. bassiana.* The bars represent the mean ± SD (n = 3).

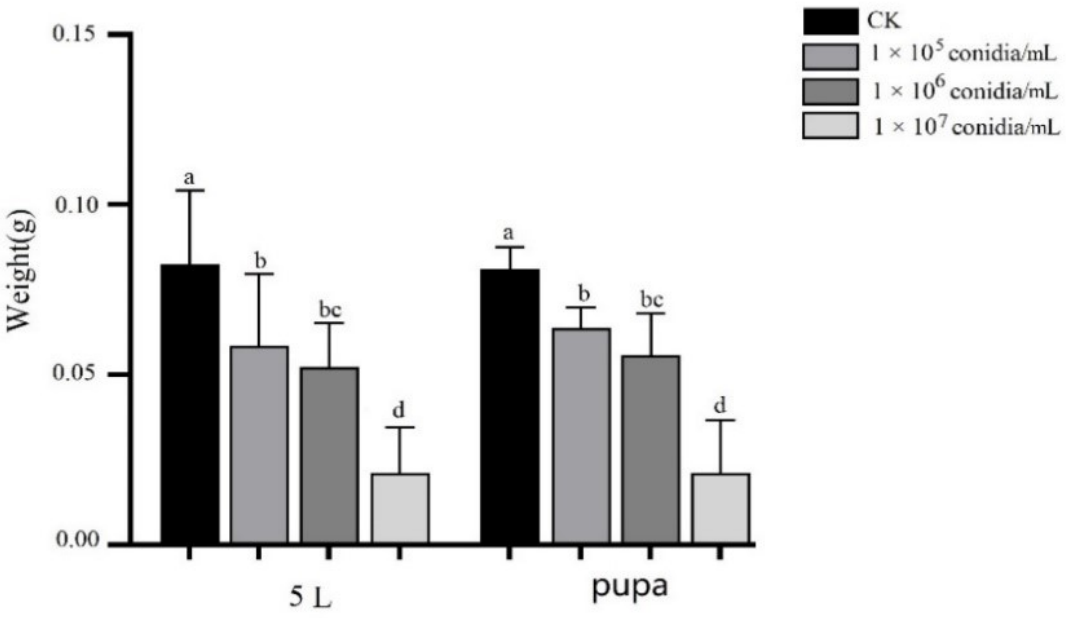


Fig. 4. Weight of cuticular infection on *G. molesta* infected by *B. bassiana*.

Data represent mean ± SD, the same age, different concentrations according to ANOVA and Turkey test method significance difference analysis, means followed by the same letter are not significantly different (α=0.05).

(2). Infected by feeding

The results showed that the corrected mortality of *B. bassiana* was up to 22.7% when the concentration of conidia suspension was 1×10^7 conidia/mL, and the corrected mortality of larvae was less than 20% when the concentration was reduced (7.6% and 16.7%). When the concentration of conidia suspension was 1×10^7 conidia/mL, the body weight is significant with others. And there was no significant difference in body weight between different instars and control group, when the concentration of conidia suspension was 1×10^5 conidia/mL and 1×10^6 conidia/mL (Fig. 5-6).

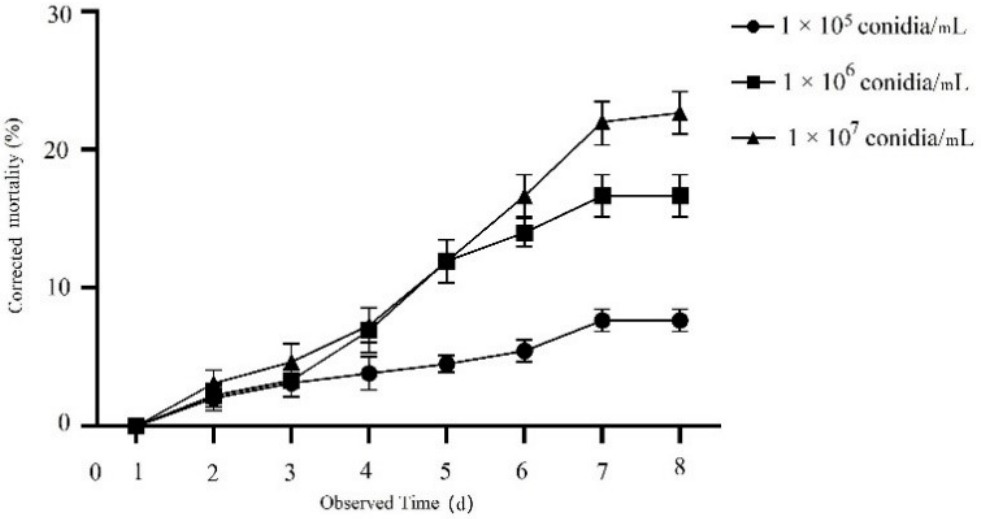


Fig. 5. The Corrected mortality of digestive tract infection on *G. molesta* infected by different concentration of *B. bassiana*. The bars represent the mean ± SD (n = 3).

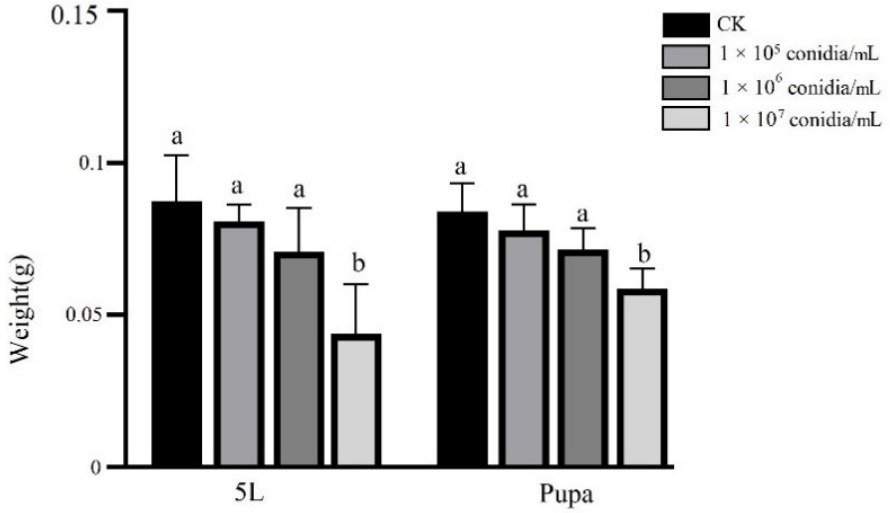


Fig. 6. Weight of digestive tract infection on *G. molesta* infected by *B. bassiana*.

Data represent mean ± SD, the same age, different concentrations according to ANOVA and Turkey test method significance difference analysis, means followed by the same letter are not significantly different (α=0.05).

Conclusion and Discussion. The entomopathogenic fungus *B. bassiana* is well known as a potential alternative to chemical pesticides for control of insect pests and is commercially available for such purpose in numerous countries worldwide (Glare et al., 2008; Sevim et al., 2010; Glare & Inwood, 2014; Saranac & Jayaprakash, 2017). Compared with bacterial and viral insecticides, fungal insecticides have stronger spreading ability and wider control spectrum due to its unique way of infecting insect body walls. In this study, when the concentration is 1×10^7 conidia/mL, the mortality of cuticular infection on *G. molesta* infected by *B. bassiana* is significantly higher than that of digestive tract infection. Previous research reports that other larvae of Lepidopteran also have this similar phenomenon. In *Plutella xylostella*, the correct mortality of cuticular infection by *B. bassiana* Bb02 (1×10^7 conidia/mL) is 73.79%, while that of digestive tract infection is 33.79% (Lei et al., 2010). However, sometimes, for the same insect, due to the different concentration and host strain type, the results will be significantly different. In *Plutella xylostella*, the corrected mortality of digestive tract infection was 80.5%, infected by *B. bassiana* GDS at concentration of 1×10^8 conidia/mL (Yan et al., 2013), while the corrected mortality is 92.4% when infected by cuticular through *B. bassiana* MZ041016 at concentration of 2.3×10^8 conidia/mL (Yuan et al., 2007). In *Helicoverpa armigera*, *B. bassiana* HFW-05 was successfully infected by the digestive tract (feeding method), and the corrected mortality rate was 75.8% after infected 6 days. The body weight and food intake of the insects infected by *B. bassiana* HFW-05 through the cuticle surface were

similar to those of the control group (the corrected mortality rate of 6 days was only 17.3%, and the pathogenic effect could not be achieved through the cuticle surface (Cao et al., 2011). However, Yu et al (2020) reports that in *Helicoverpa armigera*, after tenth day infected by *B. bassiana* at concentration of 1.5×10^8 conidia/mL, the corrected mortality of cuticular infection was 63%, and that of digestive tract infection was only 38% (Yu et al., 2020). It is generally believed that the host relationship between *B. bassiana* and its hosts is established mainly through the germination of conidia on the insect body surface, producing bud tubes and forming appressorium to penetrate the host body wall, and then invading the host body for reproduction (Cao et al., 2013; Holder & Keyhani, 2015). However, the hyphomycetes of *B. bassiana* can also entry through the digestive tract, conidia enter the digestive tract with food, germinate in the digestive tract, grow into mycelia, and then invade and expand into the body cavity through the intestinal wall cells (Ferron, 1978; Huang et al., 2002; Lei et al., 2010). Thus, it is of great significance to better understand its infection mode and to achieve better control effect. Until now, only a few entomopathogenic fungus have been studied to determine its effects on *G. molesta*. And, there was no reports on the infection mode of *G. molesta* and *B. bassiana*.

In this study, *G. molesta* infected by *B. bassiana* through cuticular infection had higher corrected mortality and better weight inhibition than that of digestive tract infection. These results provide reliable theoretical support for the development and production of fungal preparations for the control of *G. molesta*.

Meanwhile, *B. bassiana* at concentration of 1×10^7 conidia/mL may have potential to be used as control measure against *G. molesta* in fruit orchards, but the field experiment needs further verification. Moreover, *B. bassiana* is also affected by many other environmental factors, and it need to be further researched on how to improve its control effect on *G. molesta* in the future.

3.3. Transcriptome analysis of *G. molesta* larvae 24h after infected by *Beauveria bassiana*

3.3.1. Construction of DEG library and unigenes assembly

A total number of 21,330,962 and 22,293,716 raw reads were generated from the samples of control and treated groups respectively. Raw sequence reads have been submitted to NCBI Sequence Read Archive (Bioproject PRJNA759425). A total number of 20,806,484 and 21,633,702 clean reads were obtained respectively for the control and treatment groups. The control and treated groups contained 6,240,000,000 and 6,490,000,000 nucleotides, respectively. Based on the high-quality reads, we finally obtained 115,416 transcripts with an average length of 11,484 bp and 48,980 unigenes with an average length of 1,226 bp. The size distribution indicated that the lengths of most unigenes concentrated in 300-500 bp followed by those 501-1000 bp and the longer genes.

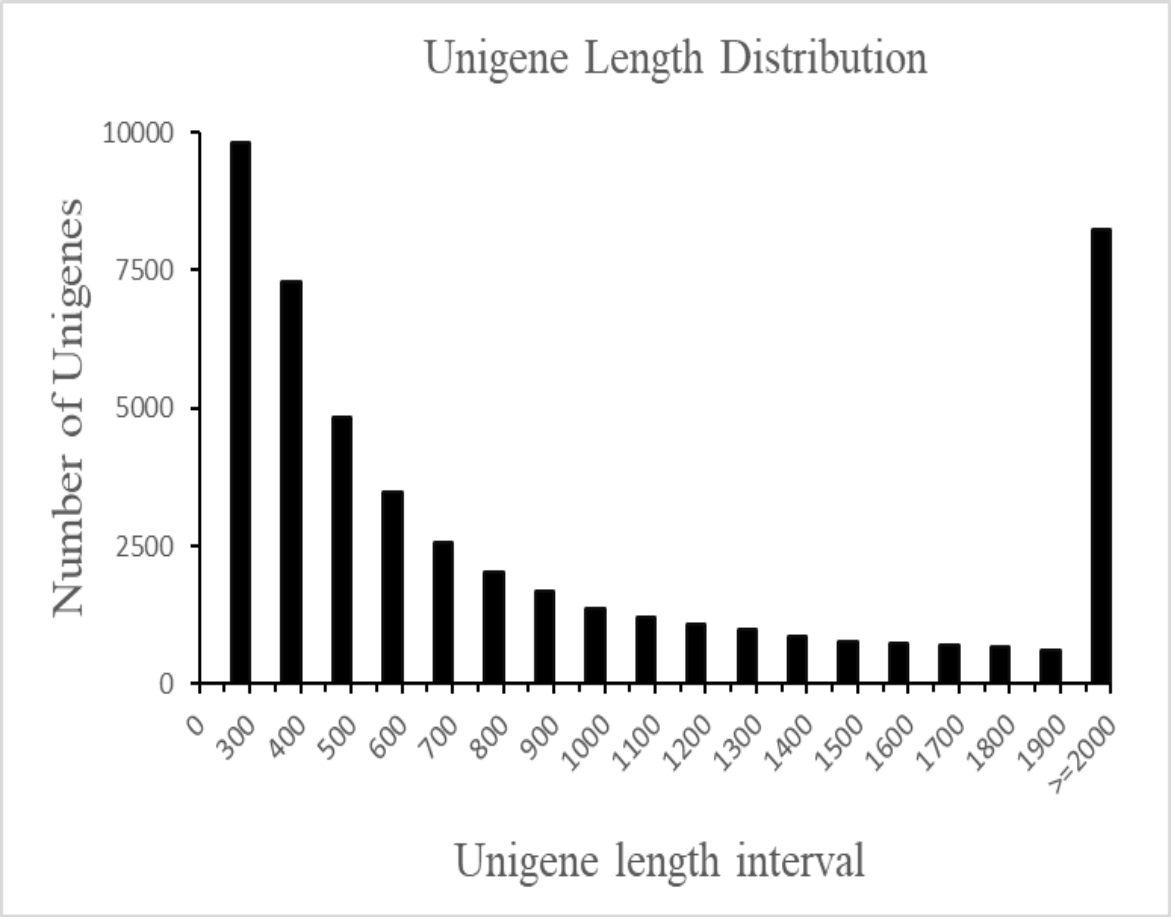


Fig. 6. Length distribution of *G. molesta* unigenes. Histogram presentation of sequence-length distribution for significant matches that was found. The x-axis indicates sequence sizes from 300 nt to >2000 nt. The y-axis indicates the number of uingenes for every given size. The proportion of sequences with matches (with a cut-off E-value of 1.0E-5) in NCBI Nr databases is greater among the longer assembled sequences.

Table 5

Summary of control and treatment transcriptomes

Summary	Control[a]	Parasitized[b]	All[c]
Total number of raw reads	21330962	22293716	-
Total number of clean reads	20806484	21633702	-
Total number of clean	6240000000	6490000000	-
nucleotides	299.9	300	-
Average length of clean reads	-	-	115416
(bp)	-	-	1484bp

Summary	Control[a]	Parasitized[b]	All[c]
Total number of transcripts	-	-	2380bp
Mean length of transcripts	-	-	607bp
N50 of transcripts	-	-	48980
N90 of transcripts	-	-	1226bp
Total number of unigenes	-	-	2031bp
Mean length of unigenes	-	-	478bp
N50 of unigenes	98.27%	98.43%	-
N90 of unigenes	0.02%	0.02%	-
Q20 percentage			
Error rate			

[a] The results were average of samples Control groups (C-1, C-2, C-3).

[b] The results were average of samples Treatment groups (T-1, T-2, T-3)

[c] The results of mixed assembled clean reads from Control groups and Treatment groups (C-1, C-2, C-3 and T-1, T-2, T-3)

3.3.2. Annotation and functional classification

All unigenes were annotated by using BLASTX against the six main databases with a cut-off E-value of 10-5, including NCBI Nr, NT, KO, Swiss-Prot, GO and KOG. Using this approach, 48,980 unigenes returned an above cut-off BLAST result (Table 6). The E-value distribution of the top hits in the Nr database ranged from 0 to 1.0E-100, approximately 35.3% of the mapped sequences ranged smaller than 1.0E-60, showing a strong homolog (Fig. 7A). The similarity distribution of the top BLAST hits for each sequence ranged from 18% to 100%, and most of the mapped sequences having a similarity higher than 45% (Fig. 7B). For species distribution, it has top matches (first hit) trained with sequences of *Ostrinia furnacalis* (11.1 %), *Papilio Xuthus* (8.6 %) and *Helicoverpa armigera* (7.0 %), *Chilo suppressalis* (6.5 %), and *Hyposmocoma Kahamano* (6.4 %) (Fig. 7)

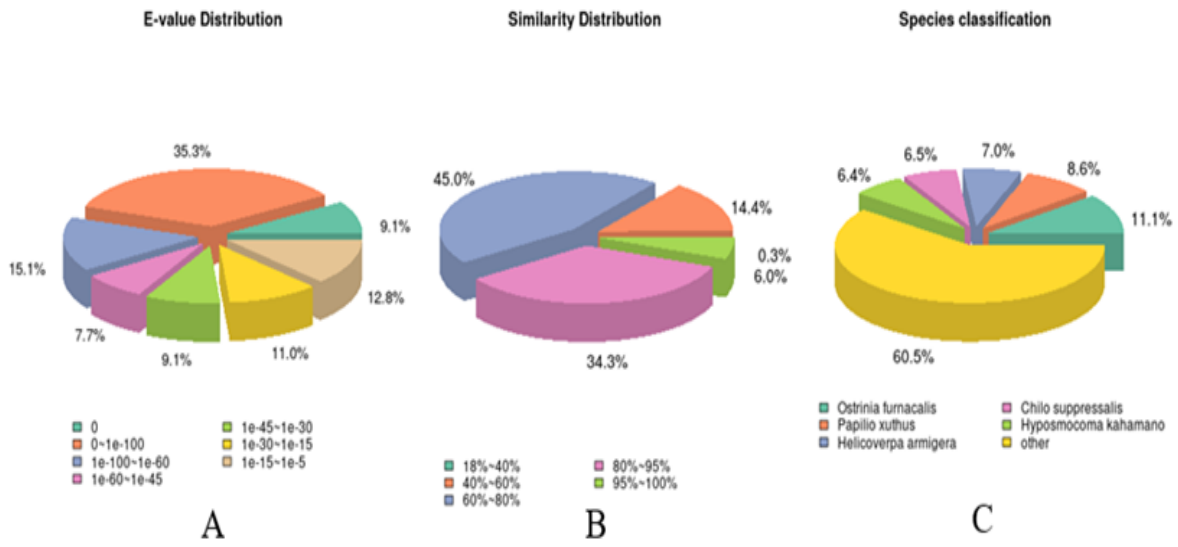


Fig. 7. Characteristics of homology search of Illumina sequences against the Nr database. (A) E-value distribution of BLAST hits for each unique sequence with a cut-off E-value of 1.0E-5. (B) Similarity distribution of the top BLAST hits for each sequence. (C) Species distribution is shown as a percentage of the top 10 homologous sequences with an E-value of at least 1.0E-5. We used the first hit of each sequence for analysis.

Table 6

Summary for the G. molesta transcriptome date. (Annotation of Unigene in Nr, NT, Swiss-Prot, PFAM, KOG, and KO databases).

Database	Number of Unigenes	Percentage (%)
Annotated in Nr	17,719	36.17
Annotated in NT	12,083	24.66
Annotated in KO	7725	15.77
Annotated in Swiss-Prot	11,739	23.96
Annotated in PFAM	14,038	28.66
Annotated in GO	14,038	28.66
Annotated in KOG	6,789	13.86
Annotated in all Databases	3,093	6.31
Annotated in at least one Database	23,825	48.64
Total Unigenes	48,980	100

3.3.3. Functional classification by GO, KOG and KEGG

GO assignments were used to classify the functions of the predicted *G. molesta* genes. Based on sequence homology, 48,980 sequences can be categorized

into 40 GO terms, including 23 biological processes, 5 cellular components and 12 molecular functions. In each of the three main categories (biological process, cellular component and molecular function) of the GO classification, the terms ‘cell process’, ‘cellular anatomical entity’ and ‘binding’ were prominently represented (Fig. 8).

The euKaryotic Orthologous Groups (KOG) is a database where the orthologous gene products were classified. To further evaluate the effectiveness of our annotation process, we searched the annotated sequences for the genes involved in KOG classifications. In 17,719 Nr hits, 6,789 sequences have a KOG classification (Figure 8).

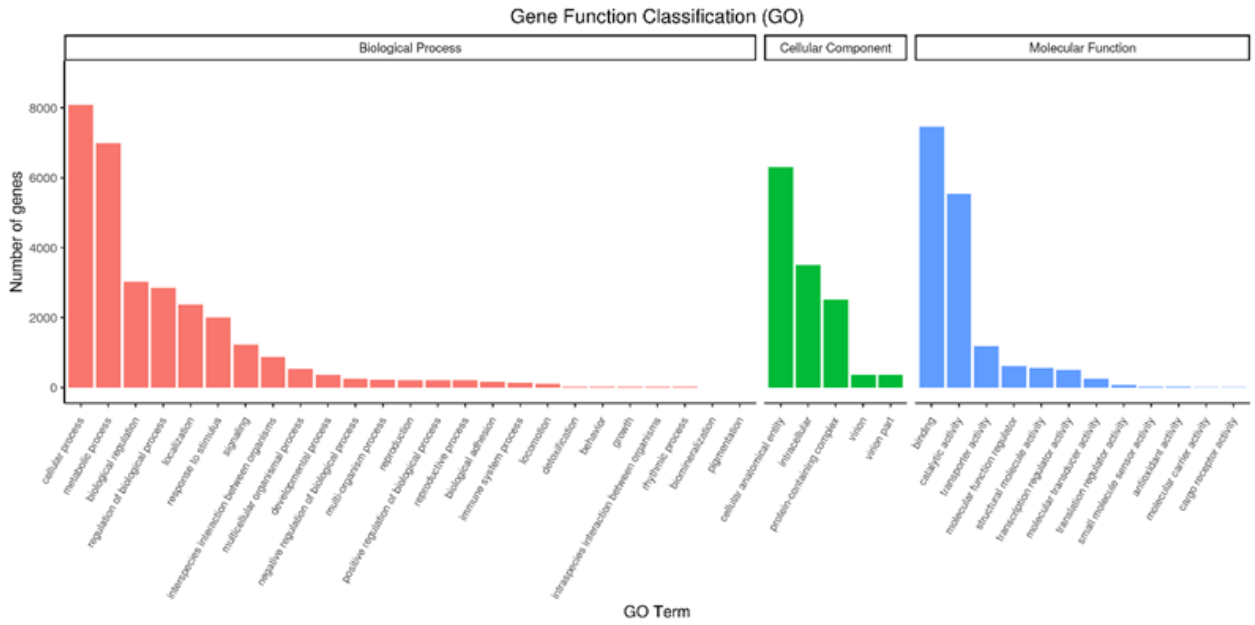


Fig. 8. Histogram presentation of Gene Ontology classification based on level two. The results are summarized in three main categories: biological process, cellular component and molecular function. The y-axis indicates the number of genes in a category.

Among the 25 KOG function categories, the cluster for General Functional Prediction only (1,108, 16.3%) represented the largest group, followed by Signal Transduction (827, 12.2%), defense mechanisms (45, 6.62%), nuclear structure (28, 4.12%), and with cell motility (13, 0.19%) as the smallest groups (Fig. 9).

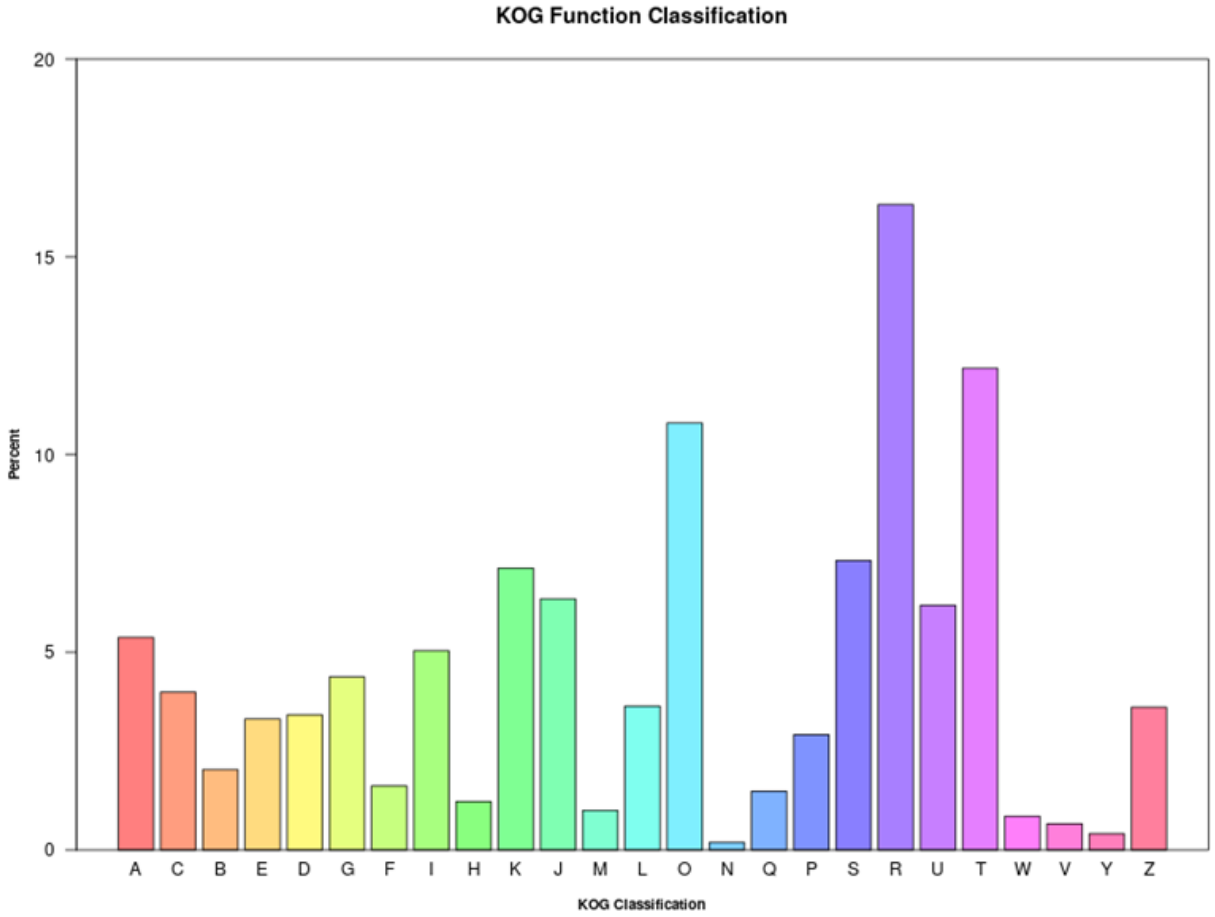


Fig. 9. Histogram presentation of euKaryotic Orthologous Groups (KOG) classification. There are totally 6,789 sequences have a KOG classification among the 25 categories.

Note:[A] RNA processing and modification; [B] Chromatin structure and dynamics; [C] Energy production and conversion; [D] Cell cycle control, cell division, chromosome partitioning; [E] Amino acid transport and metabolism; [F] Nucleotide transport and metabolism; [G] Carbohydrate transport and metabolism; [H] Coenzyme transport and metabolism; [I] Lipid transport and metabolism;[J] Translation, ribosomal structure and biogenesis; [K] Transcription; [L] Replication, recombination and repair; [M] Cell wall/membrane/envelope biogenesis; [N] Cell motility; [O] Posttranslational modification, protein turnover, chaperones; [P] Inorganic ion transport and metabolism; [Q] Secondary metabolites biosynthesis, transport and catabolism; [R] General function prediction only; [S] Function unknown; [T] Signal transduction mechanisms; [U] Intracellular trafficking, secretion, and vesicular transport; [V] Defense mechanisms; [W] Extracellular structures; [Y] Nuclear structure; [Z]

Cytoskeleton

The Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database records the networks of molecular interactions in the cells, and their variants specific to particular organisms (Kanehisa et al., 2004). To identify the biological pathways that are active in the *G. molesta*, we mapped the 5,037 annotated unigenes to the reference canonical pathways in the KEGG database. The pathways most represented by unique sequences included signal transduction (967 genes), transport and catabolism (605 genes), endocrine system (493 genes), folding, sorting and degradation (477 genes), immune system (348 genes), lipid metabolism (361 genes) and cell growth and death (313 genes) (Fig. 10).

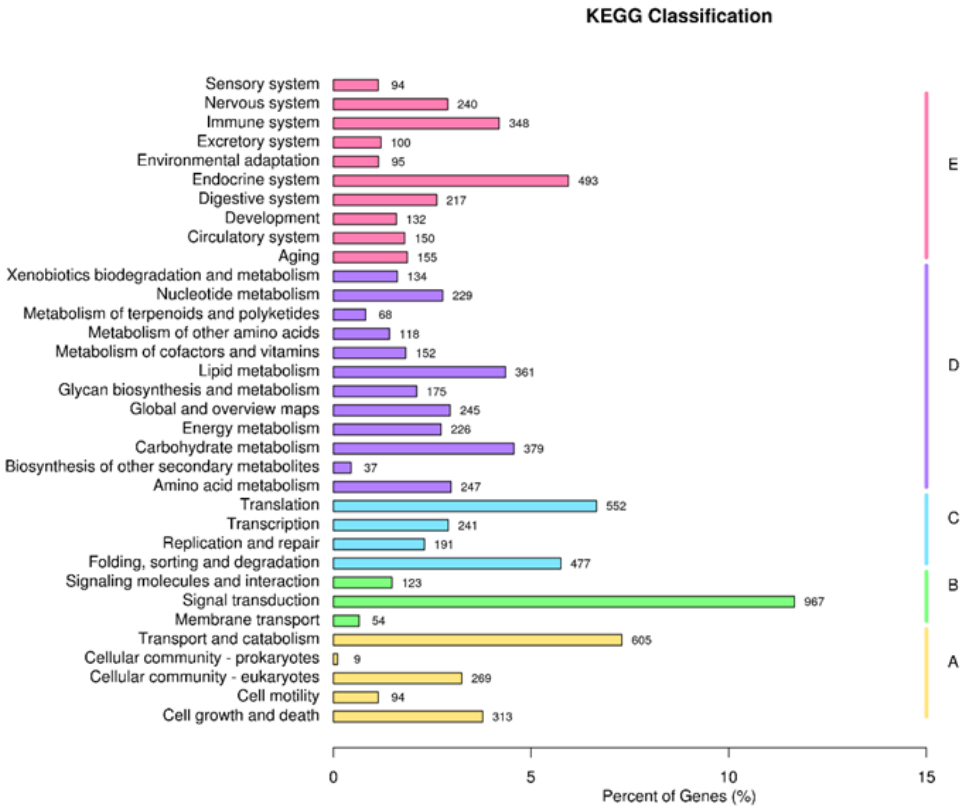


Fig. 10. Histogram presentation of the kyoto encyclopedia of genes and genomes (KEGG) classification. There are totally 5,037 sequences have a KEGG

classification. The y-axis indicates the name of KEGG pathway. The x-axis indicates the percent of uingenes for every given size.

Note: [A] Cellular Processes; [B] Environmental Information Processing; [C] Genetic Information Processing; [D] Metabolism; [E] Organismal Systems.

3.3.4. Identified of DEGs related to immune response and qRT-PCR Validation

To gain detailed information about the transcriptional changes in *G. molesta* larvae infected with *B. bassiana*, the non-infected and *B. bassiana*-infected libraires were compared and differentially expressed genes (DEGs) were identified. Totally, 1,755 DEGs were obtained, with 965 up-regulated and 790 down-regulated genes. The fold change (log2 ratio) of the gene expression ranged from -14.224 to 10.718. We focused on the up-regulated genes in *G. molesta* infected by *B. bassiana*, and with a special emphasis on the immune response related genes, which were up-regulated within seven folds (log2 ratio) (Table 5). Totally, 14 gene related to immune response of *G. molesta* induced by *B. bassiana* were selected and quantified by qRT-PCR method, and 10 genes were significantly up-regulated ($P<0.05$) (Fig. 11). These results showed the concordant changes with the transcriptome data. The immune-related gene that selected were PGRP-SC1 (peptidoglycan recognition protein), β-GRP-3 (β-1,3-glucan recognition gene), SRCB (scavenger receptor gene), Serine, Serpin, Toll, P450, PLA2 (phospholipase A), CarE (Carboxylesterase), CPB (carboxypeptidase B), and DPP4 (dipeptidyl peptidase 4) (Table 9).

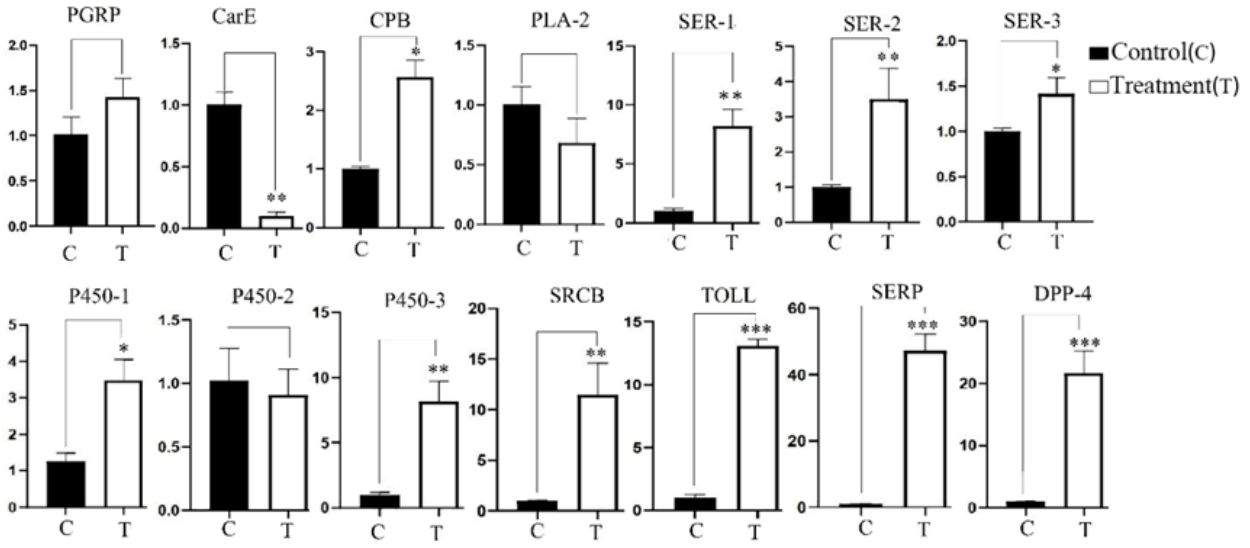


Fig. 11. Verification of differentially expressed genes by qRT-RC. *G. molesta*. EF-1a was used as an internal standard to normalize the templates. The bars represent the mean 6 S.D. (n = 3). Asterisks indicate means that are significantly different from the control (unpaired t test, * means $P<0.05$, ** means $P<0.01$, *** means $P<0.001$). Lack of asterisk indicates the difference is not significant (unpaired t test, $P>0.05$).

Table 9

Parts of the putative immune-related unigenes identified in *G. molesta*

Designated name	Unigene ID	Nucleotide length(nt)	Fold change	P value	Identity to best hit (%)
PGRP-SC1	CL7985.11379	707[b]	0.15908	0.018041	78.76
β-GRP-3	CL7985.16243	1691[b]	0.26806	0.026752	82.10
SRCB	CL7985.21037	4275[b]	1.1614	0.040743	69.17
PLA2	CL7985.1282	1914[b]	1.224	0.00094056	73.28
Serine 1	CL10639.0	1075[b]	6.6868	0.0001522	61.34
Serine 2	CL7985.295	876[b]	2.9299	0.040407	62.5
Serine 3	CL7985.22014	1466[b]	3.1805	0.024143	64.36
P450-1	CL9817.0	1772[b]	3.3163	0.029803	63.08

Continued from Table 9

P450-2	CL7985.1289	1629[b]	3.8859	0.0026496	61.17
CPB	CL7985.10992	1426[b]	1.8735	0.045841	62.62
DPP	CL7985.25031	3152[b]	1.8683	0.038989	66.7
TOLL	CL8824.0	3905[b]	0.43007	0.031052	57.45
Serpin	CL7985.13346	3202[b]	0.18546	0.044137	80.09
P450-4	CL7985.8575	512[a]	3.841	0.049999	77.87
Toll receptor-6	CL7985.8742	413[a]	2.5241	0.047125	37.04
P450-5	CL7985.4244	1681[b]	2.0653	0.046794	68.30
Serine easter-	CL7985.15956	1748[b]	1.9373	0.07063	50.28
like	CL7985.5291	1052[a]	3.0457	0.0009221	48.35
CarE					
P450-3	CL7985.25130	2298[b]	1.9026	0.033423	73.57

[a] The sequence was not completed.

[b] The sequence was completed, the result is the length of the coding area.

3.3.5. Enrichment analysis of GO function and KEGG pathway of DEGs

In order to further reveal the functions of DEGs, GO annotation was conducted. Totally, 1,755 DEGs were enriched in 1,965 GO terms, among which 107 GO terms were significantly enriched ($P<0.05$), including 62 Biological processes, 7 Cellular components and 38 Molecular functions. In biological processes, most DEGs were enriched in Oxidation-reduction process (GO:0055114), carbohydrate metabolic process (GO:0005975), DNA integration (GO:0015074), response to external biotic stimulus (GO:0043207), and defense response (GO:0006952) (Table 10). These results indicated the *G. molesta* has changed its physiological and biochemical state as defense responses against *B. bassiana*.

Table 10

Parts of GO terms which enriched in biological

GO term	GO ID	Total genes	GEGs	P-value
sensory organ development	GO:0007423	15	6	0.0000149
Oxidation-reduction process	GO:0055114	1002	61	0.000000236
regulation of multi-organism process	GO:0043900	25	6	0.00040666
regulation of conjugation	GO:0046999	25	6	0.00040666
carbohydrate metabolic process	GO:0005975	453	33	0.00049172
G-protein coupled receptor signaling	GO:0007186	323	21	0.0010606
pathway	GO:0015074	263	20	0.0024607
DNA integration				
Response to external biotic stimulus	GO:0043207	193	14	0.01916
Negative regulation of immune system	GO:0002683	3	1	0.011779
process				
Immune effector process	GO:0002376	132	7	0.026031
Immune response	GO:0006955	91	4	0.048517
Negative regulation of T cell activation	GO:0050868	3	1	0.011779
T cell activation	GO:0042110	4	1	0.02668
Defense response	GO:0006952	210	14	0.0349
Toll signaling pathway	GO:0008063	3	1	0.01838

Lastly, we also conducted KEGG pathway analysis for the DEGs. The result showed that 1,755 DEGs were enriched in 15 KEGG pathway ($P< 0.05$), and most of the DEGs were enriched in protein processing in endoplasmic reticulum (ko04141), insect hormone biosynthesis (ko00981), antigen processing and presentation (ko04612), vitamin digestion and absorption (ko04977), ascorbate and aldarate metabolism (ko00053) (Table 11), these pathways are always related in the

energy metabolism and immunity.

Table 11

Parts of enriched pathways in KEGG

Pathway ID	Description	DEGs	Total genes	P-value
ko04141	Protein processing in endoplasmic reticulum	16	165	2.11E-05
ko00981	Insect hormone biosynthesis	5	22	0.00058866
ko04612	Antigen processing and presentation	5	31	0.002268852
ko04977	Vitamin digestion and absorption	3	12	0.006326743
ko00053	Ascorbate and aldarate metabolism	5	41	0.006674414
ko00040	Pentose and glucuronate interconversions	6	66	0.011088359
ko04146	Peroxisome	8	118	0.017320985
ko00260	Glycine, serine and threonine metabolism	4	36	0.019817494
ko00500	Starch and sucrose metabolism	5	61	0.028616039
ko05134	Legionellosis	4	41	0.029215551
ko00511	Other glycan degradation	3	25	0.035529692
ko00983	Drug metabolism - other enzymes	5	66	0.037580557

Lastly, we also conducted KEGG pathway analysis or the DEGs. The result showed that 1,755 DEGs were enriched in 15 KEGG pathway ($P< 0.05$), and most of the DEGs were enriched in protein processing in endoplasmic reticulum (ko04141), insect hormone biosynthesis (ko00981), antigen processing and presentation (ko04612), vitamin digestion and absorption (ko04977), ascorbate and

aldarate metabolism (ko00053) (Table 12), these pathways are always related in the energy metabolism and immunity.

Table 12

Parts of enriched pathways in KEGG

Pathway ID	Description	**DEGs**	Total genes	P-value
ko04141	Protein processing in endoplasmic reticulum	16	165	2.11E-05
ko00981	Insect hormone biosynthesis	5	22	0.00058866
ko04612	Antigen processing and presentation	5	31	0.002268852
ko04977	Vitamin digestion and absorption	3	12	0.006326743
ko00053	Ascorbate and aldarate metabolism	5	41	0.006674414
ko00040	Pentose and glucuronate interconversions	6	66	0.011088359
ko04146	Peroxisome	8	118	0.017320985
ko00260	Glycine, serine and threonine metabolism	4	36	0.019817494
ko00500	Starch and sucrose metabolism	5	61	0.028616039
ko05134	Legionellosis	4	41	0.029215551
ko00511	Other glycan degradation	3	25	0.035529692
ko00983	Drug metabolism - other enzymes	5	66	0.037580557

B. bassiana is a promising entomopathogenic fungus for *G. molesta* control, and also as a model organism can be used to study the mechanism of interaction with insects and entomopathogenic fungal (Lewis et al., 2001; Wanchoo et al., 2009). Fungal infection is a complex process that involved several factors as well as the activation of immune responses of insects to combat infection (Gillespie et

al., 2000). The transcriptional analysis of *B. bassiana*-infected *G. molesta* larvae helps better understand the *G. molesta* immune responsive mechanism to fungal infection and has also revealed and identified several candidate immune-related genes in *G. molesta.*

Insects depend on an efficient innate immune system to fight against fungal infection (Gillespie et al.,1997; Gillespie et al., 2000). PGRP, β-GRPs and SRCB belong to the PRPs (pattern recognition receptor) which are the innate immune sensors in insects and serve as the first line of defense against the infection of entomopathogenic fungi (Wang et al., 2011; Keehnen et al., 2017). Insect immune responsive is activated depended on a various of PRRs that trigger the Toll, IMD and JAK/STAT signal pathways (Kim et al., 2020). It is reported that in *Aedes* and *Drosophila*, the Toll pathways are activated when infected by entomopathogenic fungi, and also lead to the expression of various of anti-microbial peptide (Lemaitre et al., 1996; Xi et al., 2008). However, none of the anti-microbial peptide genes family was significantly up-regulated in this study. It is not clear whether this was due to the immune specificity of *G. molesta* to *B. bassiana*, or the failure to induce the expression of these genes at the appropriate time, this needs to be further studied. The Toll pathways in insects play important roles in immunity response against fungal infection and involved complex proteins interactions. The serine protease and serpin are considered as the modulator of the Toll pathway and have been reported in *Aedes aegypti, Tenebrio molitor,* and *Manduca sexta* (Lemaitre et al., 2007; Barillasmury et al., 2007; An et al., 2009; Zou et al., 2010).

We observe three serine proteases were up-regulated as shown in the DEGs result, however, one of them was not significantly different in qRT-PCR results. This may due to the specificity of different serine genes. The PLA2 was also involved in the activation of the Toll pathway (Liu & Yuan, 2018), however, there was no report about it in the interaction between insect and entomopathogenic fungi. Therefore, more work is need to clarify the role of PLA2 in insects against fungal infection.

P450 family genes are the important detoxification enzymes involved in insect resistance that have been reported to be up-regulated in response to infection by *B. bassiana* in *Bombyx mori* (Hou et al., 2004; Zhang et al., 2018). these reports are consistent with our findings that three P450 genes are up-regulated in *G. molesta* upon infection with *B. bassiana*. Besides P450s, CarE is also the detoxification enzymes in insects, however, it was associated with resistance for carbamate and pyrethroid in several insects (Wheelock et al., 2005). There is no report that CarE is related to the immunity of fungal infection. In this study, CarE is up-regulated in *G. molesta* infected with *B. bassiana* that was not consistent with the qRT-PCR results. Generally, the qRT-PCR results are considered more accurate, so we suppose that this may be caused by insect specificity or differences in enzyme activity.

In addition, CPB and DPP4 were also up-regulated in *G. molesta* infected by *B. bassiana*. The CPB gene of *Scylla paramamosain* can participate in the immune response of *Vibrio alginolyticus* by affecting cell phagocytosis, cell apoptosis and total number of blood cells (Qian & Zhu, 2019). The study of DPP4 gene always

focuses on application of disease diagnose in mammals and humans (Piao, et al., 2021). However, these two genes were rarely reported in insects. We supposed that the results may cause by the wrong annotation under the condition of lacking reference genome analysis.

In summary, we have firstly sequences and characterized the transcriptome from non-injected and *B. bassiana*-infected *G. molesta* larvae, which made a significant contribution for better understanding the interactions between *G. molesta* and *B. bassiana*. In addition, it provides the comprehensive sequence resources of the immune-related genes of *G. molesta* larvae, presenting valuable information for deeply understanding the macular mechanism of innate immunity process in *G. molesta* against *B bassiana.* Such knowledge can be very helpful for exploring target genes involved in the response to *B. bassiana*, and improving efficiency of *B. bassiana* in the management of *G. molesta*.

Conclusions to Chapter 3

1. *G. molesta* infected by *B. bassiana* through cuticular infection had higher corrected mortality and better weight inhibition than that of digestive tract infection. Meanwhile, *B. bassiana* at concentration of 1×10^7 conidia/mL may have potential to be used as control measure against *G. molesta* in fruit orchards.

2. The second-generation high-throughput transcriptome sequencing technology were used, the transcriptomic profile of *G. molesta* larva infected by *B. bassiana* after 24 h were analyzed, the differentially expressed genes (DEGs) were

screened and annotated. Totally, 1,755 DEGs were obtained, with 965 up-regulated and 790 down-regulated genes. We focused on the up-regulated genes in *G. molesta* infected by *B. bassiana*, and 14 genes related to immune response of *G. molesta* induced by *B. bassiana* were selected and quantified by qRT-PCR method, and 10 genes were significantly up-regulated. These results indicated the *G. molesta* has changed its physiological and biochemical state as defense responses against *B. bassiana*.

CHAPTER 4

CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF GENE: *GMPGRP; GMBGRP; GMSERPIN-2 AND GMSERPIN-3*

4.1. Structure analysis and phylogenetic analysis of *GmPGRP*

The complete sequence of *GmPGRP-SC* gene was obtained by analyzing the data of the transcriptome data in our laboratory (Cao et al.,2021). And it has been submitted to NBCI GenBank, the GenBank accession number is MW773839 (Appendix B). The results showed that the full length of the *GmPGRP-SC* cDNA Sequence is 707 bp, the length of the open reading frame (ORF) is 621bp, and it encoded 206 amino acid residues. The predicted signal peptide is located between 1 ~ 33 amino acids, the transmembrane region is between 7~26 amino acids. The molecular weight is predicted to 22.75 KD. Total number of negatively charged residues (Asp + Glu) is 15 and total number of positively charged residues (Arg + Lys) is 21. The instability index (II) is computed to be 26.60, and this classifies the protein as stable. The theoretical pI (isoelectric point) is 9.19. The Grand average of hydropathicity (GRAVY) is -0.1. The domain structure was analyzed by SMART software online, the result showed that the PGRP domain structure is located between 35 ~ 177 amino acids (Fig. 12). Blast search results showed that the amino acid sequence of *GmPGRP-SC* was highly consistent with that of other insects. These features indicated *GmPGRP-SC* belong to the PGRP-S family.

The amino sequences of *GmPGRP-SC* protein and other 16 species PGRPs of

insects, including *Bombyx mori* PGRP-S and *Plutella xylostella* PGRP-SC2 multilinked by BLASTX search. The results showed that the amino acid sequence of *GmPGRP-SC* and that of *Leguminivora glycinivorella* PGRP-SC had the highest consistency more than 93 %, and with that of *Papilio machaon* PGRP, *Papilio xuthus* PGRP, *Papilio xuthus*

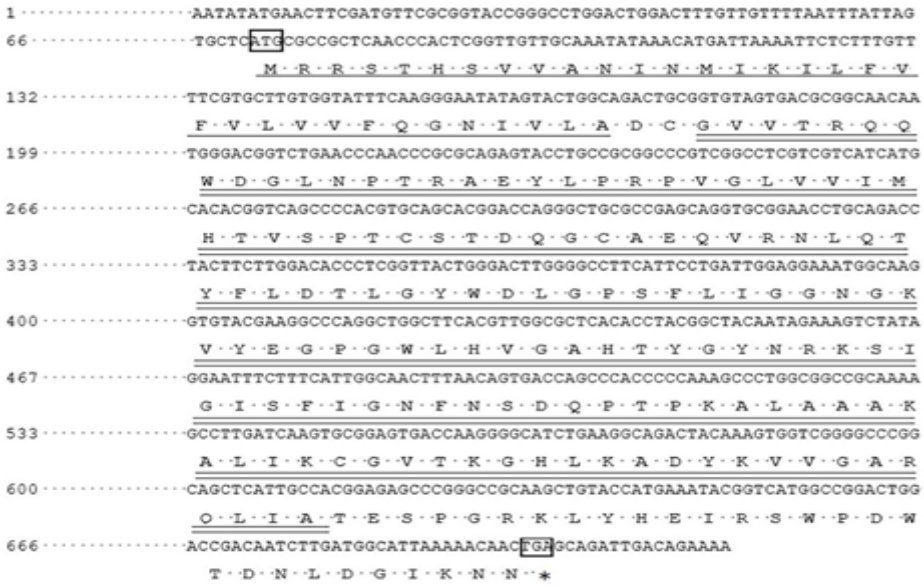


Fig. 12 The cDNA and encoding amino acid sequence of the GmPGRP-SC.

The underlined signal indicates the signal peptide sequence; the double underline indicates the PGRP domain, and the start and stop codon are indicated in the boxes. PGRP-SA are more than 79 %, furthermore the consistency with other 12 insects are more than 50 % (Fig. 13).

An evolutionary phylogenetic tree was constructed with the amino acid sequences of the remaining 16 insect species. The results showed that when it has closer the genetic relationship, the homology with the *GmPGRP-SC* amino acid sequence is higher. For example, the *L. glycinivorella* PGRP-SC form a branch with highest homology, and the confidence coefficient is 100. Meanwhile the amino acid homology of *GmPGRP-SC* with insects such as *Glleria mellonella* PGRP-SC2 and *D. melanogaster* PGRP-SC1A are very low, and the genetic

relationship is relatively distant, which form to different branches (Fig. 14).

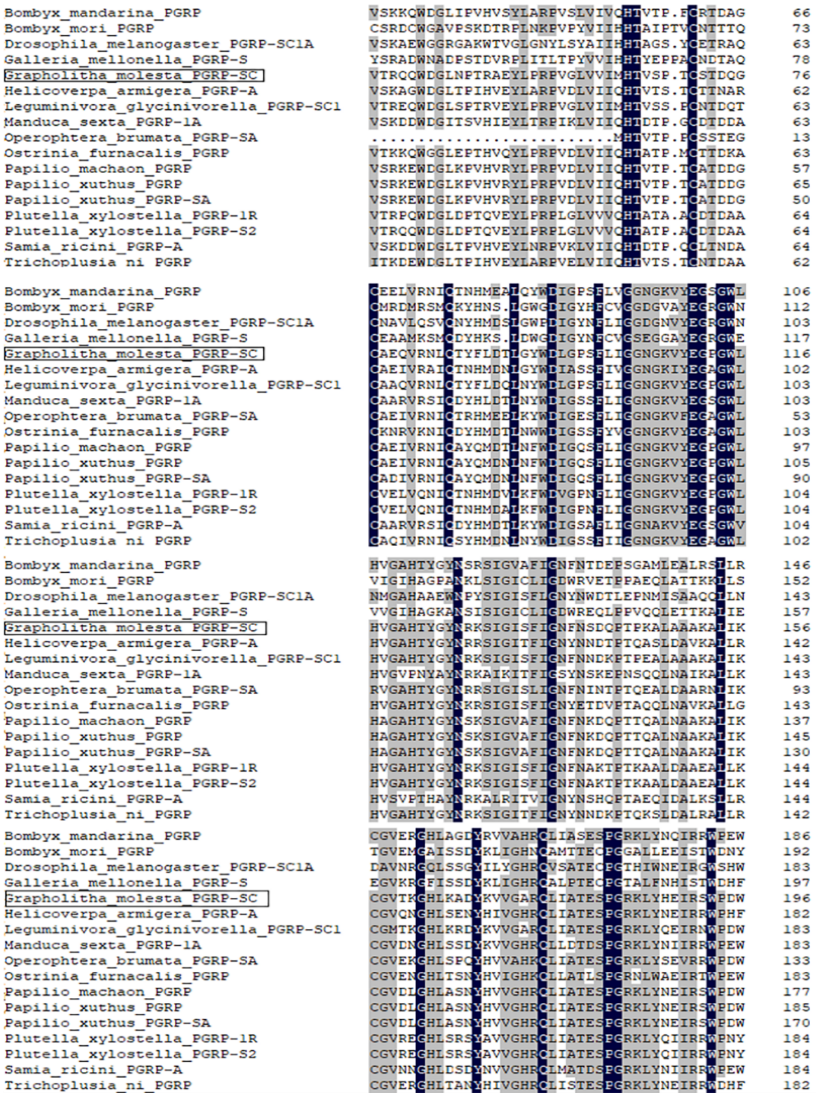


Fig. 13 Multiple sequence alignment of *GmPGRP-SC* with the homologs of other insects based on amino acid sequence. The PGRP and correspond GenBank accession numbers are as follows. *Bombyx mandarina* PGRP: XP_028043866.1; *Bombyx mori* PGRP: NP_001036836.1; *Drosophila melanogaster* PGRP-SC1A: CAD89163.1; *Glleria mellonella* PGRP-SC2: XP_026759339.1; *Helicoverpa armigera* PGRP-A: AHK59818.1; *Leguminivora glycinivorella* PGRP-SC1 AXS59124.1; *Manduca sexta* PGRP-1A: AAO21509.1; *Operophtera brumata* PGRP-SA: KOB63145.1; *Ostrinia furnacalis* PGRP: ABZ81267.1; *Papilio machaon* PGRP: KPJ06010.1; *Papilio xuthus* PGRP: XP_013170473.1; *Papilio xuthus* PGRP-SA: BAM19609.1; *Plutella xylostella* PGRP-1R: QCS60952.1; *Plutella xylostella* PGRP-S2: AUI41055.1; *Samia ricini* PGRP-A: BAF03522.1; *Trichoplusia ni* PGRP: XP_026737257.1. Amino acids with 100% identity are in black box, those with 75% in gray box and with 50% in white box.

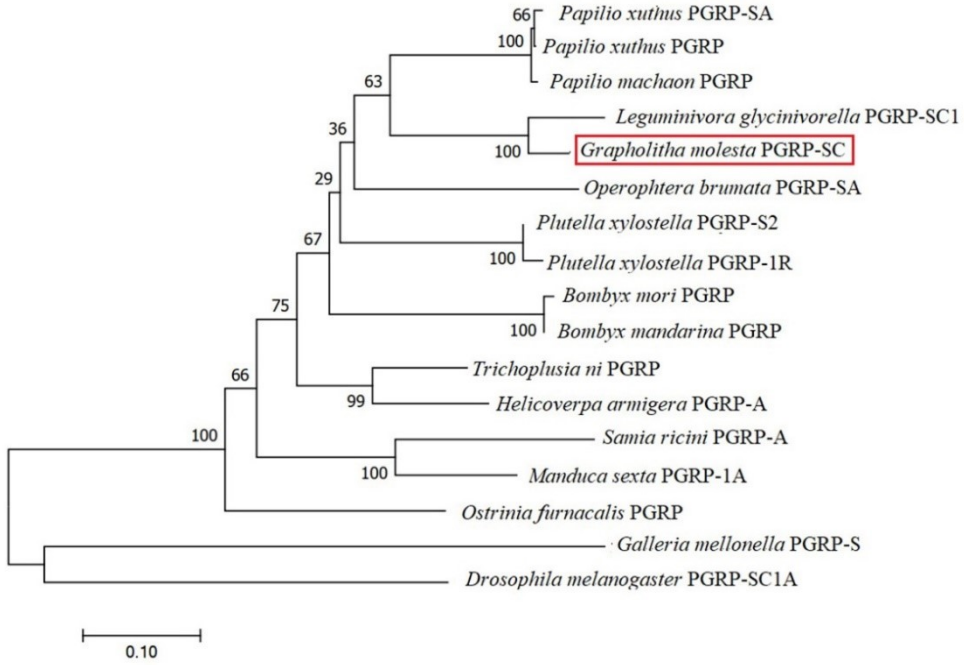


Fig. 14. Phylogenic tree based on amino acid sequence of the PGRP from *G. molesta* and other insects. Scale bar indicates the genetic distance, the numbers on the branches means confidence coefficient.

4.1.2. Developmental stage and tissue expression patterns of *GmPGRP*

To characterize the function of *GmPGRP-SC*, we first analyzed its expression pattern. Samples of *G. molesta* at different developmental stages were taken, and *Ef-1α* was used as the housekeeping gene. The relative expression of *GmPGRP-SC* gene in different developmental stages of *G. molesta* was compared. The real-time PCR result showed that, the *GmPGRP-SC* gene in different developmental stages of *G. molesta* was all expressed, but with different levels of expression. Its expression level was higher at pupa stage of *G. molesta* about 8

times than fifth instars larvae, then in the adult, the relative expression was low, nearly the same as the fifth instars larvae (Fig.15).

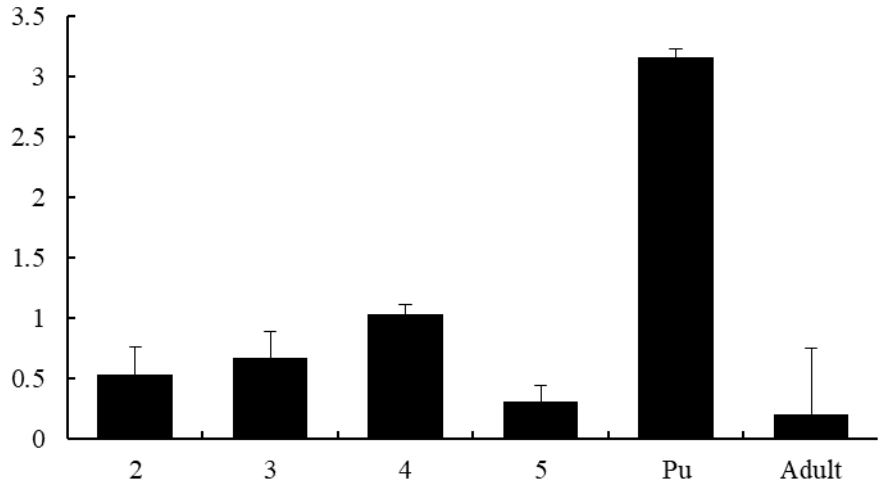


Fig. 15. The relative expression level of *GmPGRP-SC* gene. Data in the figure are mean ± SD.

Different tissues from the larvae of the fifth instar at the second day were taken, and *Ef-1α* was used as the reference gene, the relative expression of *GmPGRP-SC* gene in different tissues of *G. molesta* was compared. The result showed that the relative expression of *GmPGRP-SC* gene in different tissues of larvae was significantly different, with the highest expression in epithelium and hemocyte, that were about 10 times of that in other tissues, and in fat body it is barely expressed, however, the expression levels of *GmPGRP-SC* gene in other tissues with little difference. (Fig.16).

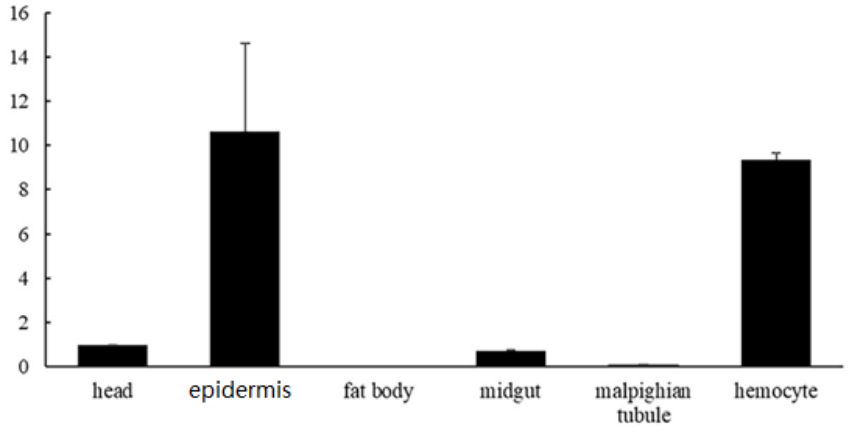


Fig. 16. The relative expression level of *GmPGRP-SC* gene in different tissues of the larva of *G. molesta*. Data in the figure are mean ± SD .

4.1.3. The effect of the expression level of *GmPGRP-SC* in *G. molesta* after infected by *B. bassiana*

In order to analyses expression changes after immune stimulations, we performed real-time PCR to analyses the transcript level reduced by different treatment of *B. bassiana.* The result showed that the infection of *B. bassiana* can induced expression of the *GmPGRP-SC* gene, this is consistent with the results that PGRPs involved in the immune function. The effect on the expression of *GmPGRP-SC* gene is different when the spore concentration and infected times of *B. bassiana* are different. When *B. bassiana* is 10^5 conidia/mL, after 48h of infection, the expression of *GmPGRP-SC* gene was significance between the CK group and Treatment group. But when 24h and 96h after infected without significant difference (Fig. 17). The *B. bassiana* is 10^7 conidia/mL, after 24 h and 48 h, the expression of *GmPGRP-SC* gene was significance between the CK group and Treatment group. While when the *B. bassiana* is 10^9 conidia/mL, there was no

significant difference between the treatment group and the control group, and the expression of *GmPGRP-SC* gene was slightly down-regulated (Fig. 18−19).

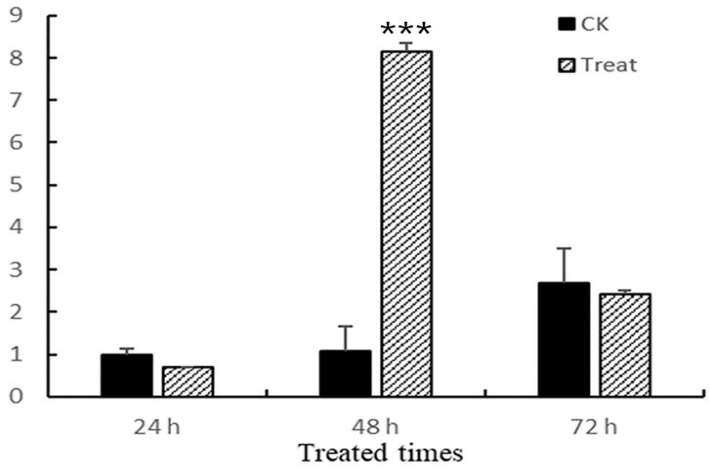


Fig. 17. The relative expression level of GmPGRP-SC gene of different time by in treated with 105 conidia/mL spore suspension of B. bassiana. Data in the figure are mean ± SD. The asterisks above bars indicate significance between the treatment and the CK determined by the student 's t-test, respectively. The same for the following figures.

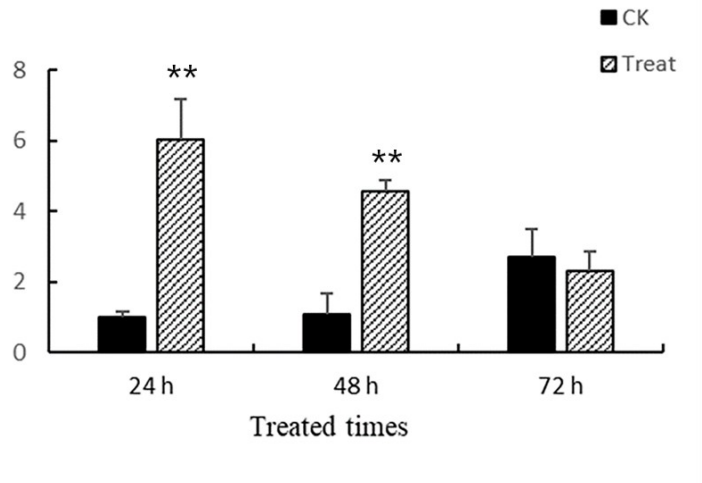


Fig. 18. The relative expression level of *GmPGRP-SC* gene of different time by in treated with 10^7 conidia/mL spore suspension of *B. bassiana*. Data in the figure are mean ± SD.

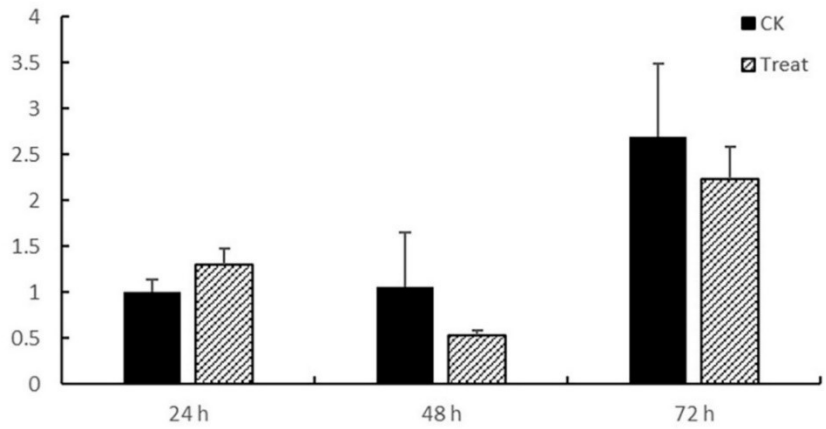


Fig. 19. The relative expression level of *GmPGRP-SC* gene of different time by in treated with 10^9 conidia/mL spore suspension of *B. bassiana*. Data in the figure are mean ± SD.

4.1.4. Effect of siRNA interference of *GmPGRP* gene

The result shows that when siRNA of *GmPGRP* gene was injected into the fifth instar larva, *GmPGRP* gene could be effectively silenced at 24h, 48h and 72h, and the silencing efficiency was 87%, 69% and 22%. Meanwhile the results shows that silencing efficiency is the highest after treated 24 hours (Fig. 20). At the same time, we conducted another group of experiments. After 24h of interference of *GmPGRP* gene with siRNAi, both the treatment group and the control group were infected with *B. bassiana* (1×10^7 conidia/mL). The mortality of the treatment group and the control group was analyzed. There was no significant difference in the corrected morality of siRNAi treatment group (73.23%) and control group (65.90%) (Fig. 21).

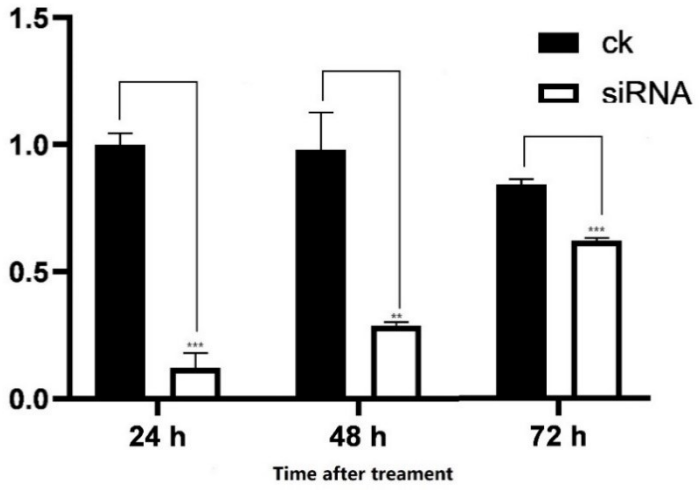


Fig. 20. Effect of RNA interference on relative expression level of *GmPGRP-SC* gene (The tested insects were collected for RNA isolation at 24h, 48h and 72h after siRNA injection，and then the gene silencing efficiency was detected by RT-qPCR.)

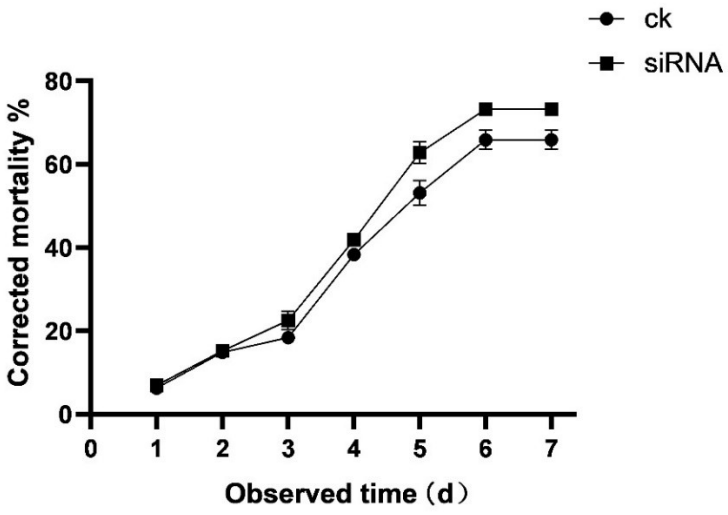


Fig. 21. The corrected mortality of *G. molesta* infected by 1×10^7 conidia/mL of *B. bassiana* (The insects were injected at 24h after injection of gene-specific siRNA，and the corrected mortality were calculated within 7d. The group injected with the same amount of Negative CK was used as the negative control. The significance of difference between the treatment group and the control group was determined via the student ’s t-test (*P < 0． 05;**P<0.01; *** P<0.001) .

In addition, we observed that the pupation time of *G. molesta* was prolonged

after interference treatment, which might be due to the need for more time to accumulation of the gene component after knocked down of *GmPGRP-SC* gene. The mortality rate of larvae treated with *B. bassiana* was higher than that of the group without interference of *GmPGRP-SC* gene, but without was significant difference.

Members of the *PGRPs* family, including insects and mammals, are highly conserved. There is a PGRP domain composed of about 165 amino acids at the C-terminal, which plays a crucial role in the recognition of exogenous substances. Different PGRP domains may be the mechanism for distinguishing and identifying different kinds of microorganisms (Blanco et al., 2008). The results of *GmPGRP-SC* gene in our study are consist with this. The amino acid sequence prediction results showed that *GmPGRP-SC* has a conserved domain structure, this structure also funded in *Helicoverpa armigera*, *Manduca sexta* and *Tribolium castaneum* etc. (Gottar et al*.,* 2002). We explored the evolution and conservation of *GmPGRP-SC* with other insects. The phylogenetic tree shows that *GmPGRP-SC* has high homology and close genetic relationship with *L. glycinivorella.* Except when the data is missing, the PGRP sequences reasonably generalize the tree of hypothetical species in each gene family (Wiegmann et al., 2011).

The report about the *Musca domestica* and *Drosophila melanogaster* has the similar result with the gene *MdPGRP-SC,* it was speculated that this gene may play an important role in the pupal stage (Werner, 2000; Gao, 2013). But *Bd PGRP-SB1* was highly expressed in 3rd larvae and adults of *Bactrocera dorsalis* (Zhang et al.,

2020). All these three are belong to *Diptera*, and the developmental expression pattern of *PGRP* gene in *Lepidoptera* has been reported in *Antherea pernyi,* but without significant change (Liu W, 2019). And in other insects of Lepidoptera, there is no more report, whether it is related to the developmental regulation and immunity, it needs to be further studied. The expression level of *GmPGRP-SC* was greatest in the pupal stage and in epidermis and hemocyte. But in *Antherea pernyi* the *ApPGRP-A*, *ApPGRP-B*, *ApPGRP-C* have no significant change. Epidermis and hemocyte were related to its immune response, so in these tissues were highly expressed. The result is consisting with that of *Antheraea pernyi*, and the *ApPGRP-C* gene was expressed in immune-related groups, such as hemolymph and epidermal (Liu W, 2019). In *Bombyx mori, BmPGRP-S4* is highly expressed mainly in hemolymph, which may be involved in the systemic immune response of *Bombyx mori*, depending on hemolymph circulation (Yang et al., 2017).

And further on determining whether exogenous pathogens can affect the expression of *GmPGRP-SC* gene, the fifth instar larvae of *G. molesta* were injected with *B. bassiana*, and the expression of *GmPGRP-SC* gene at different times was analyzed after the infected with different concentrations of *B. bassiana*. The real time-PCR results showed that the expression of *GmPGRP-SC* gene was up-regulated in different times after infected with 1×10^5 conidia/mL and 1×10^7conidia/mL of *B. bassiana.* And 1×10^9 conidia/mL may inhibit the normal development of *G. molesta*. These are consistent with the results of our study, but the function of *GmPGRP-SC* gene in the immune signaling pathway needs to be

further studied, which will do of great significance for the study of the interaction between *B. bassiana* and *G. molesta.* In *Ostrinia furnacalis*, member of *PGRP* genes is up-regulated when infected by *B. bassiana* with 2×10^5 conidia/μL (Liu et al*.,* 2014). The *MxPGRP-1* in *Manduca sexta* was up-regulate after infected by *Escherichia coli* (Sumathipala & Jiang, 2010). In *Drosophila,* the expression of *DmPGRP-LB*, *DmPGRP-SA*, *DmPGRP-SB1*, *DmPGRP-SC2 and DmPGRP-SD* were strongly up-regulated by *Bacillus subtilis* and purified peptidoglycan (Werner et al*.,* 2000). The Mortality became higher as concentration increase when the *G. molesta* was infected with different concentration of *B. bassiana* ARP14 (Sarker et al., 2020). In this study, we also observed that when infected with 1×10^9 conidia/mL, the growth and development of *G. molesta* were slowly. But due to the small number of insects in our experiment, it does not constitute ecological statistics. So, we supposed that maybe the infection of *B. bassiana* with 1×10^9 conidia/mL, it affects the normal growth and development of the *G. molesta*, thus leading to the immune function of *PGRP* be restrained.

As a major pattern recognition receptor, PGRP plays an important role in the innate immune regulation of *G. molesta*. In this study, it is the first time that we obtained and verified the full sequence of the short types of *PGRP*s gene named it *GmPGRP-SC*, its GenBank accession number is MW773839. The transcriptional expression of *GmPGRP-SC* gene was analyzed in different developmental stages and different tissues of *G. molesta*, result showed some difference with other reported PGRPs from other insects, this may relate to its immunity functions either

at specific times and in specific locations. RNA Interference (RNAi) is a post-transcriptional gene silencing mechanism by which the expression of homologous endogenous mRNA will be inhibited by the introduction of exogenous dsRNA or small interfering RNA (siRNA) (Moritz et al., 2010; Liu et al., 2012; Xu et al., 2016; Itsathitphaisarn et al., 2017). As a gene silencing method, RNAi played an important role in advancing insect science and has helped in identifying functions of many genes involved in physiological, developmental, behavioral and reproductive processes of insects (Palli, 2014; Mallikarjuna et al., 2016). In this study, the RNA interference of *GmPGRP* gene was successful in the fifth instar larvae, and the effect was better 24 h after interference. Continued research laid the foundation. However, the mortality rate of *B. bassiana* infected after interference was not significantly different from that of the undisturbed group, which still needs further study. Meanwhile we have identified the immune response reduced by *B. bassiana,* it is the first time to study the interaction between *B. bassiana* and *G. molesta* on molecular aspect. All these results have provided a good support for better understand the function of *GmPGRP-SC* gene in *G. molesta*, and also lays a foundation for finding target genes and further prevention and control by molecular biology method. This can be applied by interfering the expression of *GmPGRP-SC* gene, the immune ability of the body to resist fungus can be reduced, so as to achieve an effective prevention and control role.

4.2. Characterization and functional analysis of *GmBGRP* gene

4.2.1. Structure analysis and phylogenetic analysis of *GmBGRP*

The completed sequence has submitted to NBCI GenBank, the GenBank accession number is ON055286. The open reading frame of *GmBGRP* was 1458 bp long, encoding a polypeptide of 486 amino acid residues, and with a non-coding region containing 20 bp and 208 bp at its 5′end and 3 ′end, respectively. The signal peptide is located 1-18 residues, and without transmembrane region. The predicted molecular mass was 54.8 kDa. Theoretical pI is 6.14. Conserved Domains were used to analyze the domains contained in the protein, and the results showed that the protein contained two domains. Protein sequence 24-137 aa contains the CBM39 domain and this domain can recognize invading microorganisms. A typical glycoside hydrolase family 16 domain (GLU domain) was found from the 249-420 aa, and this domain has the ability to bind to β-1, 3-glucose (Fig. 22-23).

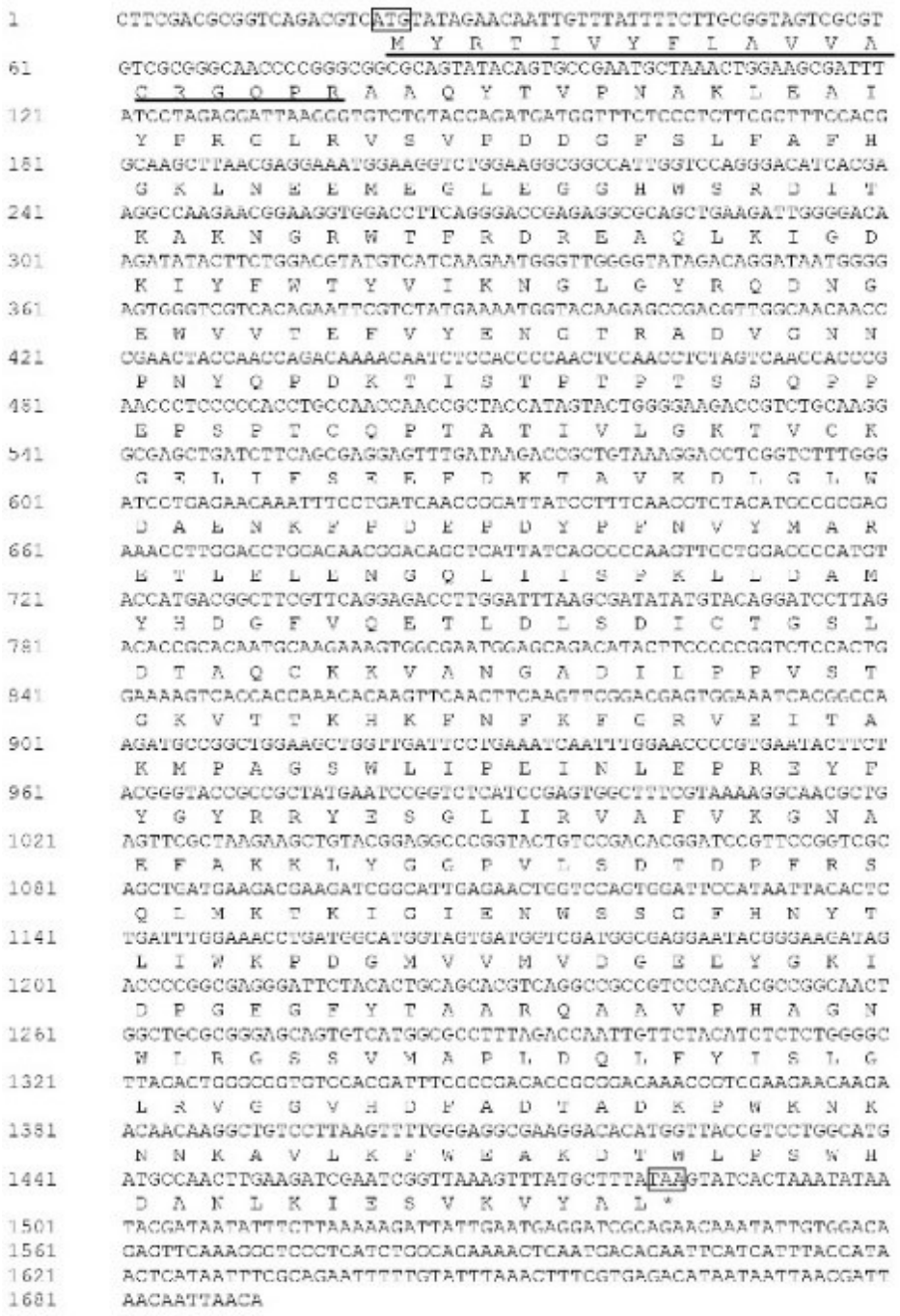


Fig. 22. Nucleotide sequence and the deduced amino acid sequence of *GmBGRP* gene. The nucleotide sequence is numbered from the 5′ end, and the amino acid code is presented below the corresponding codon. The sequence of the signal peptide is underlined. * Termination codon.

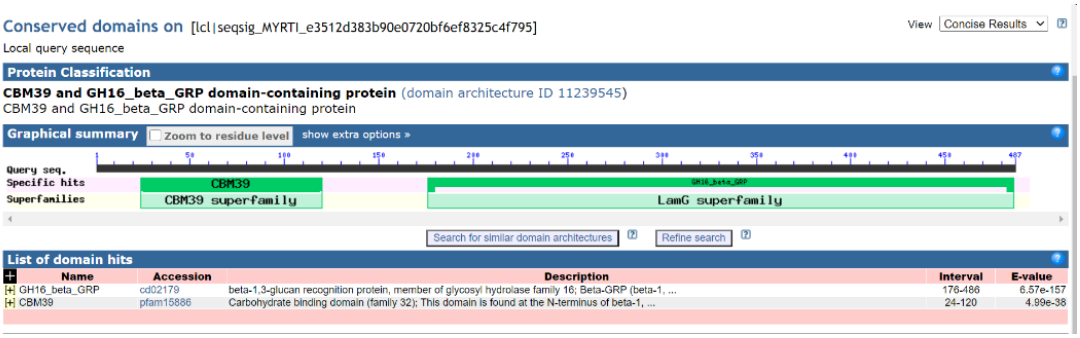


Fig. 23. Analysis of *GmBGRP* protein domain

In order to resist the invading microbes more effectively and better survival of in nature, the immune-related genes have been constantly evolving and changing. In this continuous process of evolution, BGRP families also forming into different family members during evolution. They have different amino acid sequences that perform their respective functions in the insect's immune system, greatly improving the insect's ability to survive. The phylogenetic tree was constructed based on the amino acid sequences of the *GmBGRP* and other 16 species of Lepidoptera insects (Fig. 20) which indicated that the was located in the same phylogenetic branch as that of *L. glycinivorlla*, but was far away from that of *Papilio xuthus*, which belonged to the Papilionidae. However, amino acid sequences are highly similar and may have the same function. The specific functional mechanism still needs further study and discussion.

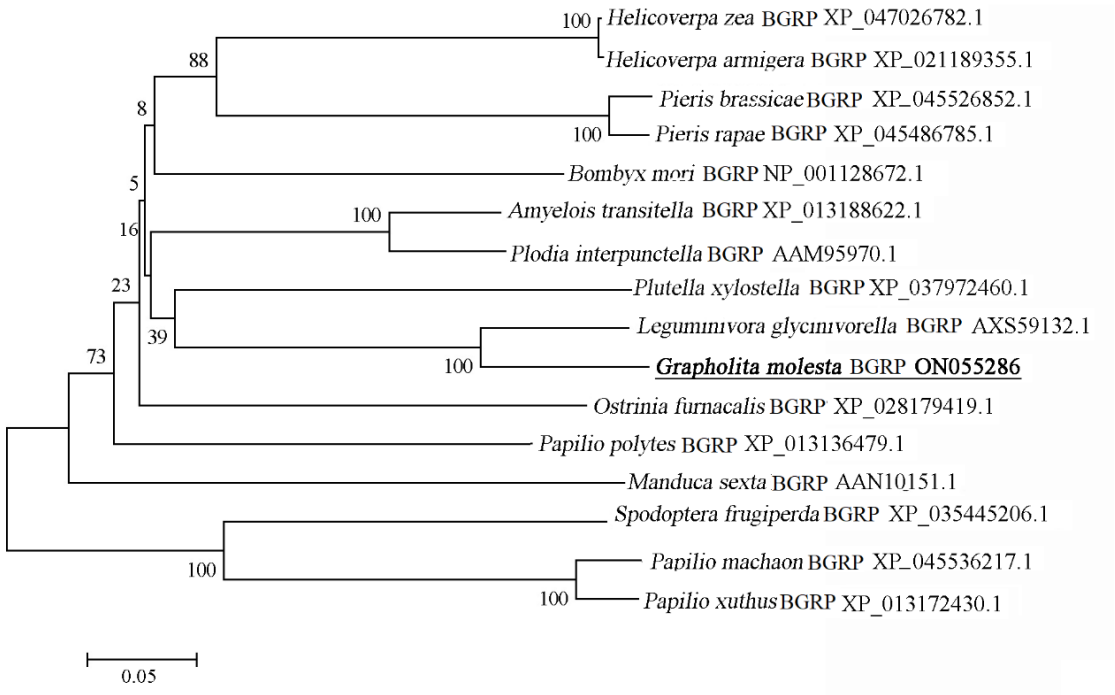


Fig. 24. NJ phylogenetic tree based on amino acid sequence of the *GmBGRP* and other insects. The GenBank accession numbers are behind the Latin names. The numbers above the branch denote bootstrap percentages.

4.2.2. Developmental and tissue expression patterns of *GmBGRP* gene

The qPCR was performed to measure the relative expression of *BGPR* gene transcripts in different developmental stages and tissues of *G. molesta*. The expression levels of *GmBGRP* gene were higher in pupal stage, and subsequently decreased in the adult stage, and it was also expressed in other instars of larva and without significant difference (Fig. 25). The expression levels of *BGPR* gene were very high epidermis, hemolymph and fat body from the second day after 5th instar larvae, but lower in other tissues (Fig. 26).

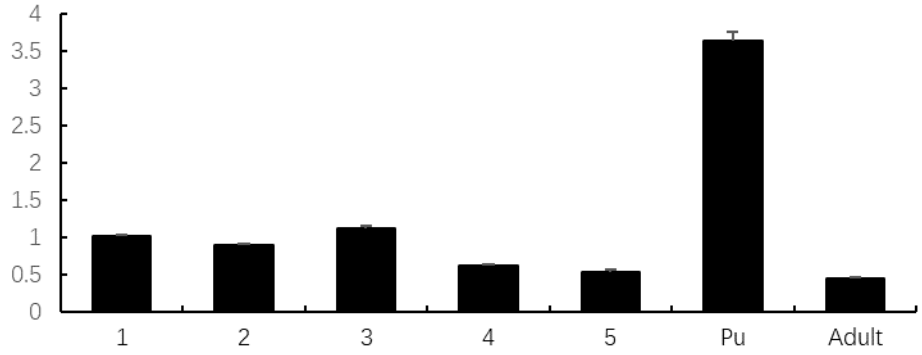


Fig. 25. The temporal expression level of *BGPR* gene Data in the figure are mean ± SD.

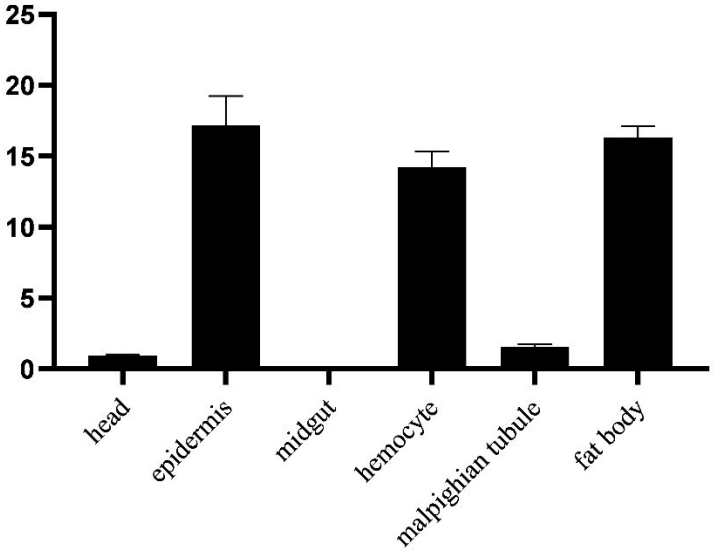


Fig. 26. The relative expression level of *GmBGPR* gene in different tissues of the larva of *G. molesta*. Data in the figure are mean ± SD.

4.2.3. The expression level of G*mBGPR* gene in response to *B. bassiana* infection

Expression levels of *BGPR* gene in day two of the fifth instar larvae at different time points after infected by *B. bassiana* (1 x 10^7 conidia/mL spore suspension) and quantitated by Q-RT PCR. The expression level of *BGPR* gene

were significantly increased at 24h and 48h after infected by *B. bassiana*, and was induced 156-fold and 2.3-fold at 24h and 48h, respectively. But expression level of *BGPR* gene was not significantly difference with that of control group at 72h after infected by *B. bassiana* (Fig. 27).

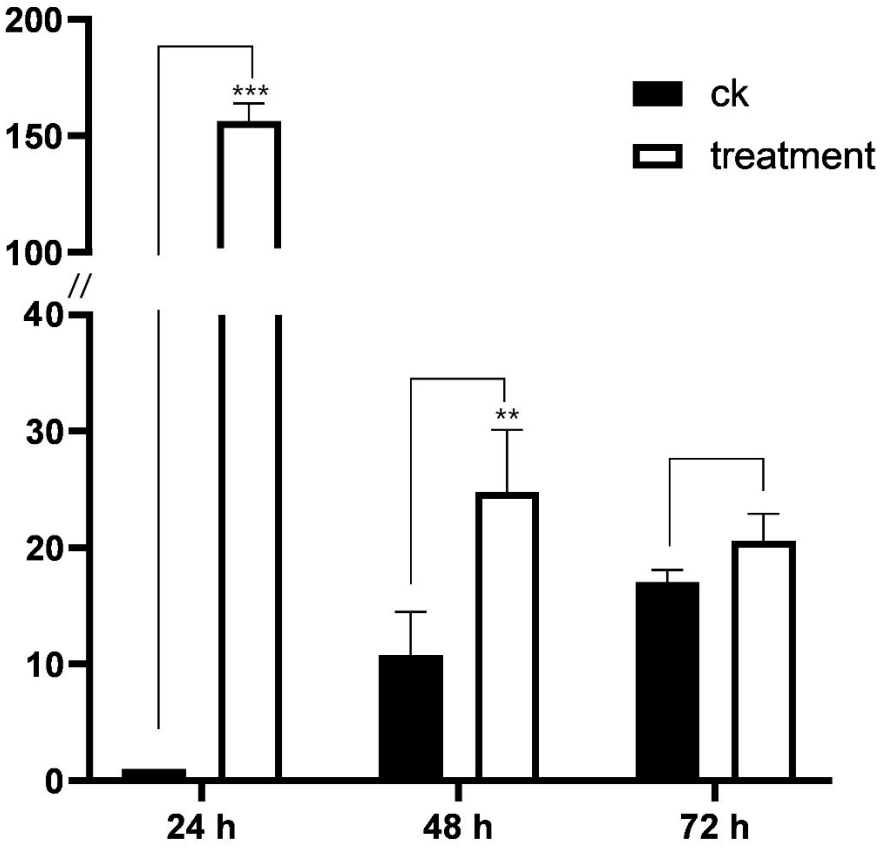


Fig. 27. The temporal expression level of *BGPR* gene of different time by in treated with $1x10^7$ conidia/mL spore suspension of *B. bassiana* (Data in the figure are mean ± SD. The asterisks above bars indicate significance between the treatment and the CK determined by the student 's t-test, respectively)

4.2.4. Effective siRNA-mediated knock-down of *BGPR* gene transcription

The effects of knockdown of *BGPR* gene transcription in *G. molesta* were

investigated by siRNA-mediated. Transcription of *BGPR* gene in fifth instar larvae of *G. molesta* was reduced to approximately 88%, 86% at 24h and 48h post-injection of siRNA, and it is extremely significant different compared with the control group. The expression level *BGPR* gene after 72h of interference treatment was significantly different from that of the control group ($P<0.05$), and decreased by 36% (Fig. 28). Thus, demonstrating effective siRNA-mediated knockdown in *BGPR* gene transcription in *G. molesta* and can be used for further detection of antifungal infection ability.

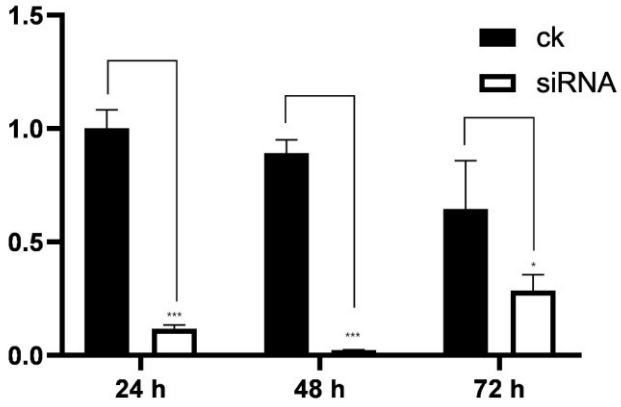


Fig. 28. RNAi efficiency of *GmBGRP* in body. *G. molesta* was injected with BGRP siRNA (20 μg), or equal amounts of universal negative siRNA as control. RNAs were collected to check the silencing efficiency by qPCR at specific time points. Data were representative of two independent repeats.

4.2.5. Analysis of antibacterial activity of larva of *G. molesta* after *GmBGRP* gene silencing

In this study, larvae of *G. molesta* were infected by 1×10^7 conidia/mL of *B. bassiana* after 24h of *GmBGRP* gene silencing by RNAi. The larval mortality rate was calculated, so as to detect the immune defense ability of *GmBGRP* gene against fungal infection in larval body of *G. molesta*. The results shows that the

mortality rate of the control group infected only with *B. bassiana* (66.2%) was significantly lower than that of the group infected with *B. bassiana* after RNAi treatment (75.23%). These results show that the *GmBGRP* gene plays an important role in the immune regulation of the *G. molesta* larva against fungal infection (Fig. 31).

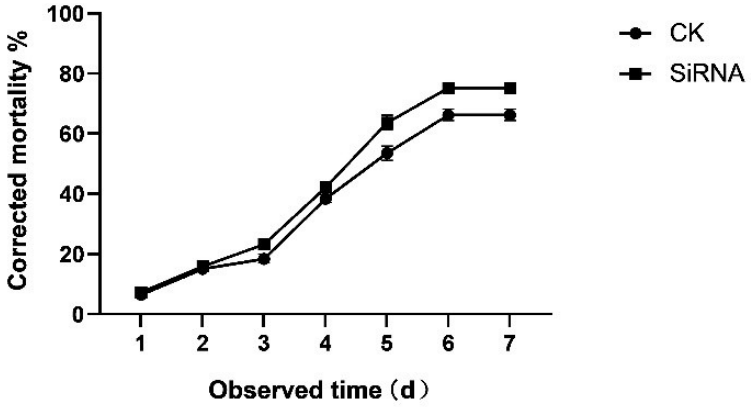


Fig. 31. The corrected mortality of *G. molesta* infected by 1×10^7 conidia/mL of *B. bassiana*

The insects were injected at 24h after injection of gene-specific siRNA，and the corrected mortality were calculated within 7d. The group injected with the same amount of Negative CK was used as the negative control. The significance of difference between the treatment group and the control group was determined via the student 's t-test.

4.2.6. The *GmBGRP* gene mediated Toll pathway and phenol oxidase activation against *B. bassiana* infection

Pattern recognition proteins recognize pathogen-related patterns and activate downstream cellular and humoral immunity to clear pathogens. *GmBGRP* plays an important role in resistance to infestation of *G. molesta*. How does it function? In *Drosophila*, *BGRP3* recognizes beta-1, 3-glucan and activates Toll pathway and PPO pathway then resistance to fungal infection through the serine level

associative activation (Matskevich et al., 2010). In order to further verify the role of *GmBGRP* gene in the immune pathway in the larva of *G. molesta*, we examined its effect on Toll pathway-related genes and PPO activity after 24 h of *GmBGRP* gene RNA silencing. The mRNA expression levels of immune-related genes *SRCB*, *Serine-1*, *Serine-2*, *Serine-3*, *Serpin-2*, *Toll* and *PPO* gene were detected by qPCR after 24h injection of double-stranded RNA. The results showed that the expression of these genes decreased after the interference of *GmBGRP* gene: the difference of *SRCB* and *PPO* genes were extremely significant compared with the control ($p < 0.001$), Serine-1, Serine-2, Serine-3, Serpin-2 and Toll genes were significant compared with the control ($p < 0.05$) (Fig. 29). This may be due to the different roles of different genes in immune signaling pathways.

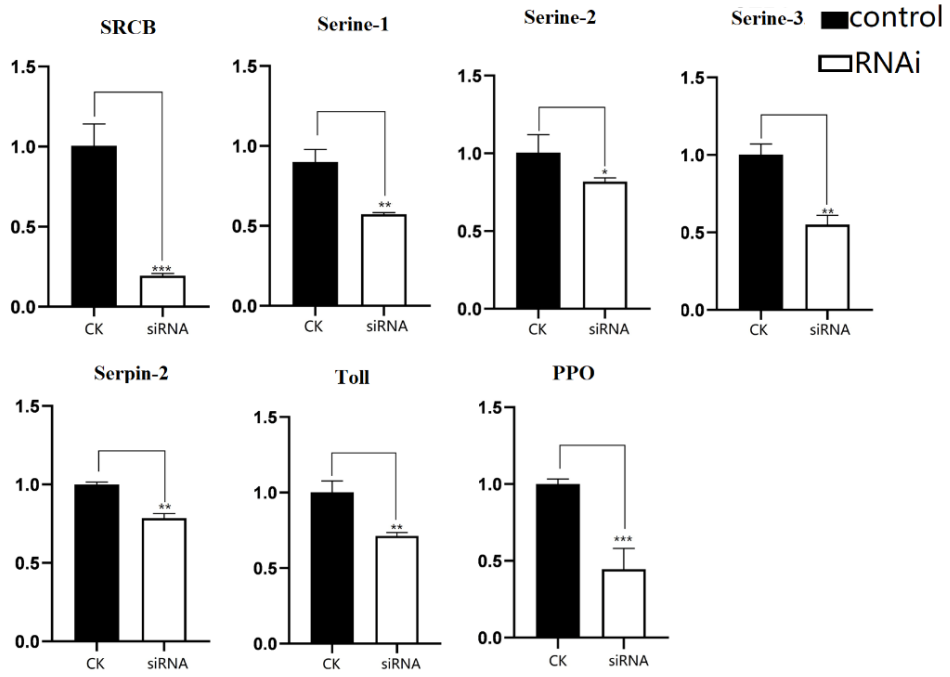


Fig. 32. Relative expression levels of immune-related genes in *G. molesta* larvae after 24h treated by *GmBGRP* gene interference

After the silencing of *GmBGRP* gene, the hemolymph samples of larva were taken periodically from the control and treatment group receptively, then it was used to determine the PPO enzyme activity. The results shows that compared with control group, PPO enzyme activity was significantly down-regulated after *GmBGRP* gene silencing, especially after 24 h and 48 h, the enzyme activity in hemolymph was significantly lower than that in control group ($p < 0.05$) (Fig. 30). Therefore, it is suggested that speculated that *GmBGRP* gene as a pattern recognition protein, plays an important role in the activation system of prophenoloxidase in *G. molesta* larva.

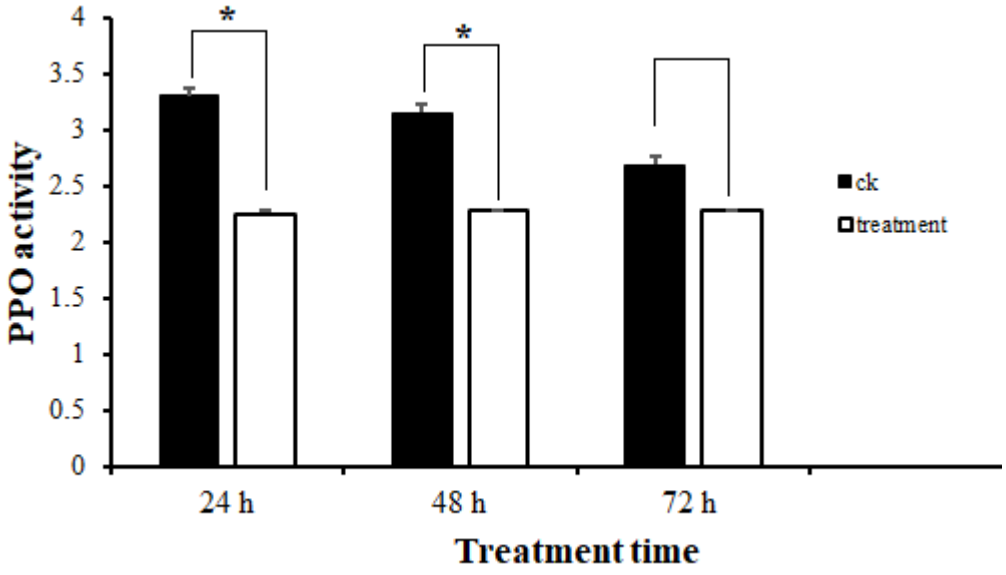


Fig. 33. The PPO enzyme activity in hemolymph of larva of *G. molesta* after 24 h 48 h, 72 h of *GmBGRP* gene silencing

Studies of the insect immune system have shown that pattern recognition receptors can mediate the insect immune response in the insect defense system, and many important advances have been made in the study of their function. On the

one hand, the expression of antimicrobial peptides and other effector factors can be activated by humoral immunity through the Toll pathway or IMD pathway. On the other hand, it can regulate the aggregation and encapsulation reaction of cells and prevent the spread of pathogenic agents in cells. In addition, it can also regulate the melanism cascade reaction in insect body, through the catalytic activation of a series of enzymes, the inactive phenol oxidase progen into active phenol oxidase, causing the melanism of tissues. In this study, we report here the full-length cDNAs encoding *GmBGPR* gene from the *G. molesta* larval. Similar to the *BGRP* genes of other species, such as *Bombyx mori*, *D. melanogaster*, (Ochiai & Ashida, 1988; Kim et al., 2000; Pauchet et al., 2009), the glycosyl hydrolase 16 superfamily domains specific to the BGRP family was also found in the glycosyl hydrolase 16 gene identified in this study. They may belong to the glycoside hydrolase 16 superfamily members. The BGRP family consisting a total of 21 members from 10 species of Lepidoptera had been phylogenetically (Pauchet et al., 2009). In this study, phylogenetic analysis revealed that the *GmBGRP* gene with the closest relationship to *BGRP* gene form *L. glycinivoreua*. The expression levels of *GmBGRP* gene were the highest in pupal stage, and the lowest in adult stage, as well expressed in other instars of larva, but with no significant difference. This result was consisting with *BmPGRP*-L5 in *Bombyx mori*, *BmPGRP*-L5 was highly expresses in transition stage of metamorphosis (Xu et al., 2010). The expression levels of *GmBGRP* gene were highest in epidermis, hemolymph and fat body from the second day after 5th instar larvae, but lower in other tissues. In *Plutella*

xylostella, *BGRP* gene was consistently expressed at all life stages and was upregulated after microbial induction. The expression was mainly distributed in fat bodies, epidermis and hemocyte (Huang et al, 2015). BGRP is used as a pattern recognition receptor in insects to specifically recognize β-1, 3-glucan in fungal cell walls and initiate immune response (Hoffmann, 2003). In this study, infection with *B. bassiana* caused a sharp increase in *GmBGRP* gene expression in *G. molesta*. This result was consisting with reports in other insects of Lepidoptera. In *Bombyx mori*, the third day larvae of the 5th instar larvae were infected by *B. bassiana*, the expressions of *BGPR2* and *BGPR4* were up-regulated and stable, which were about 6 and 10 times that of the control group, respectively (Xu et al., 2010). Other studies have shown that the expression of *BGRP*s gene in other insects at different time after infected by fungi. In *Anopheles Gambiae*, it was up-regulated at 4h, 12h, 24h and 30h after bacterial infection, and the up-regulation was more obvious at 12h and 24h, and reached the peak at 12h, but there was no significant difference between the control group at 30h (Dimopoulos et al., 1997).

Recognition of PAMPs by PRRs is a crucial step in PPO cascade activation. In *Manduca sexta*, when *BGRP* combined with soluble P - 1, 3 glucans, laminarin, then add to dilute the blood, it will induce participation serine protease cascade of PPO activation pathways, and the activity of phenol oxidase can accumulate in the plasma (Ma & Kanost, 2000). The expression of BGRP-3 in the fat body of larvae of *Ostrinia furnacalis* increased significantly after stress from *Escherichia coli* and *Bacillus subtilis*, and then decreased. The activity of PPO in hemolymph was

significantly decreased after silencing of *GmBGRP*, suggesting that BGRP-3 of the *Ostrinia furnacalis* is one of the pattern recognition receptors involved in the activation of PPO (Wu et al, 2018). Based on the role of PPO cascade in melanism reaction, we speculated that *GmBGRP* could promote melanism through PPO cascade activation. In this experiment, PPO activity in hemolymph was reduced after RNA interference treatment, indicating that *GmBGRP* was positively regulating PPO activity. At the same time, re-infection with *B. bassiana* resulted in significantly higher mortality than the control, indicating that *GmBGRP* plays an important role in the immune response against pathogenic fungal. Meanwhile, after *GmBGRP* gene silencing, the expression levels of immune-related genes in the immune signaling pathway decreased, which also proved its recognition role in the signaling pathway and activated the expression of downstream genes. These results will provide a basis for the development and improvement of novel microbial insecticides targeting PRRs against pathogenic fungi.

4.3. Characterization and functional analysis of Serpin genes (*GmSerpin-2* and *GmSerpin-3*)

4.3.1. Structure analysis and phylogenetic analysis of *GmSerpin-2* & *GmSerpin-3*

The completed sequences have submitted to NBCI GenBank, the GenBank accession numbers are OQ359960 and OQ359968 respectively. The complete cDNA fragments of *GmSerpin-2* and *GmSerpin-3* contained 1371 bp and 1554 bp of open reading frame (ORF), potentially encoding a protein of 457 and 518 amino

acids respectively. *ExPASY* analysis revealed that the proteins have a molecular weight of 50.6 kDa and 58.3 kDa, 5.77 and 5.56 isoelectric point respectively. SMART analysis revealed that *GmSerpin*-2 contains a typical SERPIN domain (residues 80-442), and that domain in *GmSerpin*-3 is 41-450 amino acid residues (Fig. 34).

Fig. 34 The cDNA and encoding amino acid sequence of the GmSerpin-2 & GmSerpin-3. (A represents GmSerpin-2 and B represents GmSerpin-3). The underlined indicates the SERPIN domain, and the start and stop codon are indicated in the boxes.

To investigate the evolution of the *GmSerpin-2* and *GmSerpin-3* genes, we analyzed phylogenetic trees based on alignment of their amino acid sequences. The *GmSerpin-2* and *GmSerpin-3* amino acid sequences were searched in NCBI, and the phylogenetic tree was constructed with of serpine genes obtained from other 12 insects. The results show that: the *GmSerpin-2* protein shared greatest similarities with the serpin2 from *Leguminivora glycinivoreua* and *GmSerpin-3* shows most closely related to the serpin3 from *Pectinophora gossypiella* (Fig. 35).

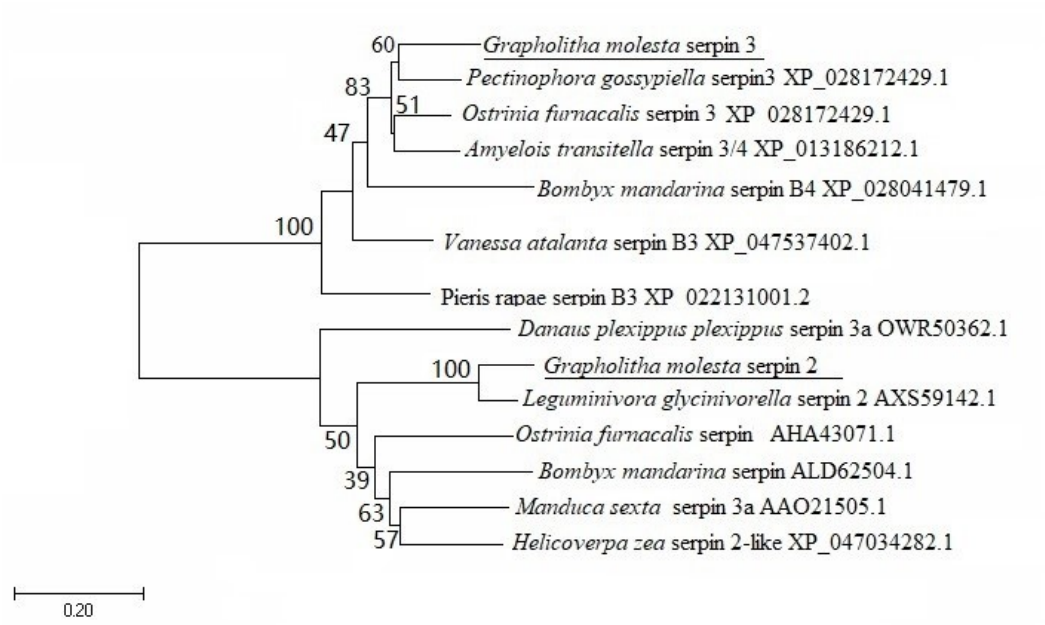


Fig. 36 Phylogenetic analyses of GmSerpin-2 and GmSerpin3 with serpins from other species. The phylogenetic tree was constructed using the neighbor-joining algorithm and bootstrap values (1000 repetitions) of the branches are indicated. The name and GenBank accession numbers of the serpins are indicated.

4.3.2. Developmental expression and tissue distribution of *GmSerpin-2* and *GmSerpin3*

We performed qRT-PCR to determine the expression levels of s *GmSerpin-2* and *GmSerpin3* in different tissues and developmental stages. The qRT-PCR

analysis revealed that *GmSerpin-2* and *GmSerpin-3* were expressed in all developmental stage and examined tissues. Expression pattern of different developmental stages showed that *GmSerpin-2* with greatest expression at the 4th larval stage and lowest expression at the adult; *GmSerpin-3* with greatest expression at the pupa stage and lowest expression at the adult, and there are no insignificant variations at other developmental stages (Fig. 37). The analysis also revealed that *GmSerpin-2* with higher expression levels in fat bodies, hemolymph and epidermis (Fig. 38a), while *GmSerpin-3* with higher expression levels in hemolymph, fat body and malpighian tubule (Fig. 37b). The results indicated that *GmSerpin-2* and *GmSerpin-3* may play an important role in biological processes.

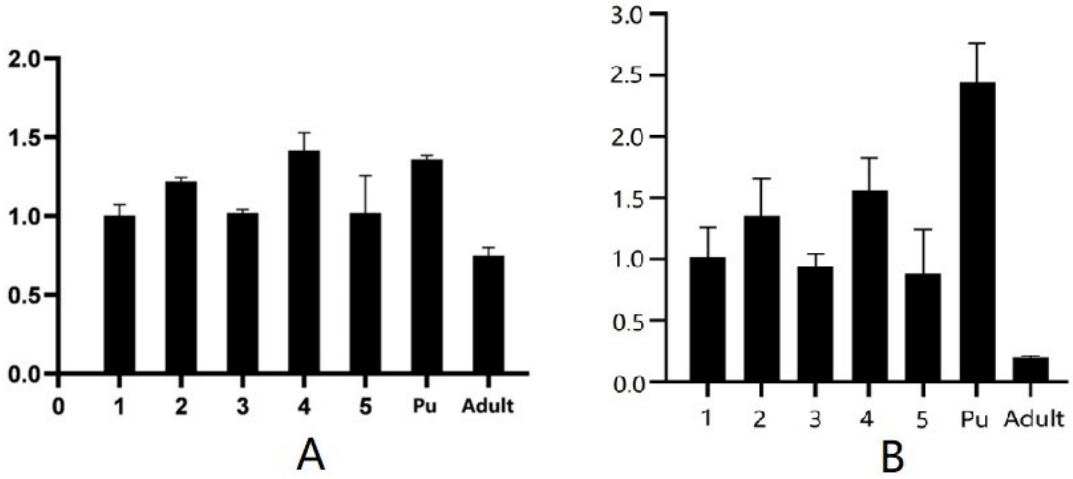


Fig. 37. Expression levels of GmSerpin-2 and GmSerpin-3 during developmental stages. The sequence of period are 1st, 2nd, 3rd, 4th, 5th, pupae and adult. (A represents GmSerpin-2 and B represents GmSerpin-3).

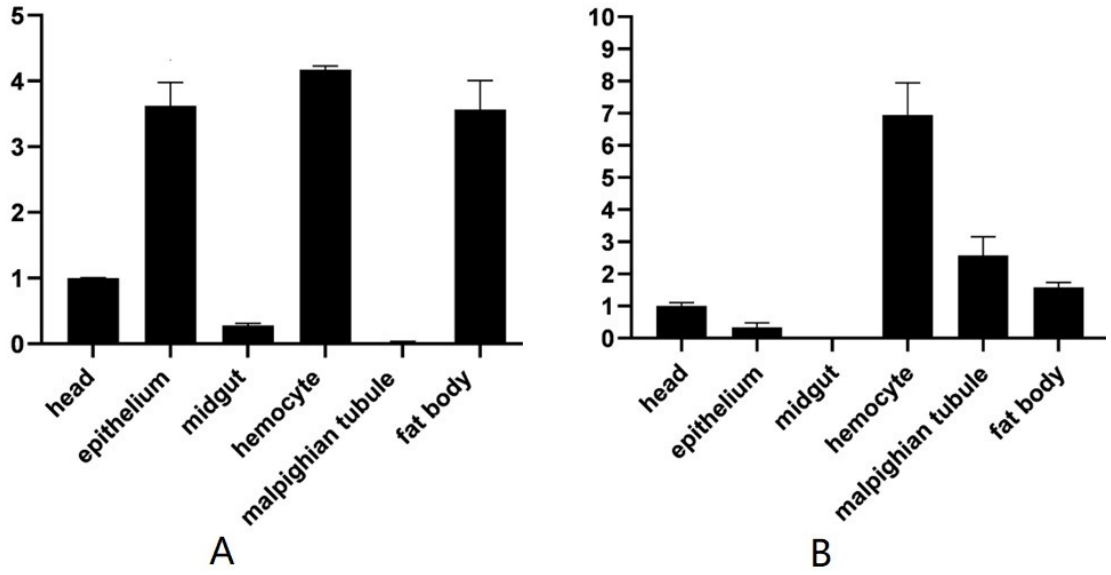


Fig. 38. Tissue distribution of *GmSerpin-2* and *GmSerpin-3* in the second day after 5th instar larvae of *G. molesta*. (A represents *GmSerpin-2* and B represents *GmSerpin-3*)

4.3.3. Effect of nanoparticles on silencing efficiency of siRNA

In order to further optimize the effect of RNAi interference, we adopted nanoparticle mediated siRNA double-stranded to further verify the interference efficiency. After RNAi, the *GmSerpin-2* and *GmSerpin-3* genes were successfully silenced, and the expression level of *GmSerpin-2* and *GmSerpin-3* decreased to different degrees at 6,12 and 48 h of interference, and decreased by 71. 05%, 36. 22%, 21.05%, and 83%, 64%, 27% respectively. There were significant differences compared with the control group at 24h, 48h and 72h ($P<0.05$). In order to improve the interference efficiency and the stability of dsRNA, we use nanoparticles M2L and NP2 mediated. The results show that, for *GmSerpin-2* except 48 h, M2L and NP2 can significantly reduce infection efficiency. For *GmSerpin-3*, NP2 with

significant difference and better effect at 24h and 48 h, but M2L without significantly different compared to unmediated nanoparticles groups (Fig. 39). This indicates that there are some differences in the mediated effects of nanoparticles for different genes.

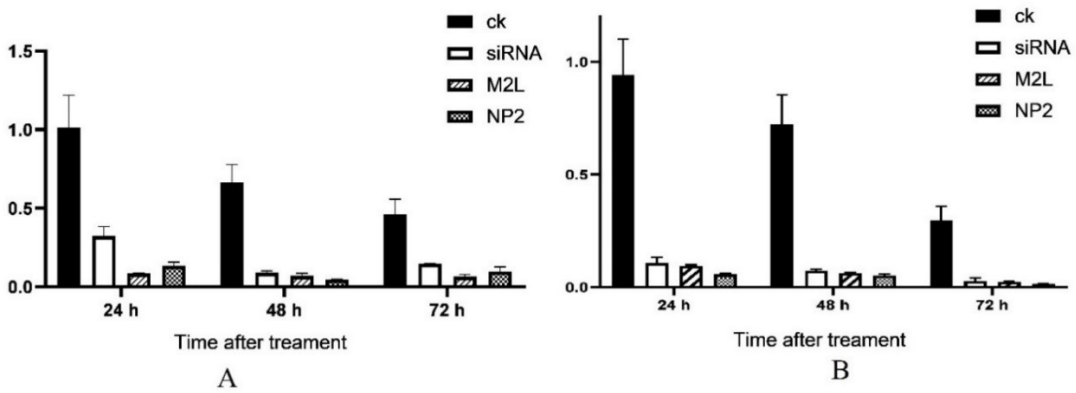


Fig. 39. RNAi efficiency estimation of *GmSerpin-2* and *GmSerpin-3* with nanoparticle M2L and NP2 mediated. (A represents *GmSerpin-2* and B represents and *GmSerpin-3*).

4.3.4. Effect of M2L-mediated *GmSerpin-2* gene RNA silencing on *G. molesta* larval resistance to *B. bassiana* infection

The result shows that *GmSerpin-2* gene interference effect is best at 24h when M2L-mediated RNA of *GmSerpin-2* gene was injected into the fifth instar larva. In order to analysis the *G. molesta* larval resistance to fungi after *GmSerpin-2* gene silencing, both the treatment group and the control group were infected with *B. bassiana* (1×10^7 conidia/mL) after 24h of *GmSerpin-2* gene silencing. The mortality of the treatment group and the control group was analyzed. There was significant difference in the corrected morality of siRNAi-M2L treatment group (84. 3%) and control group (66.2%) (Fig. 40). In conclusion, nanoparticle M2L

mediated *GmSerpin-2* silencing could significantly increase the mortality rate of the 5th instar larvae of *G. molesta* and the reduce the defense ability against *B. bassiana*.

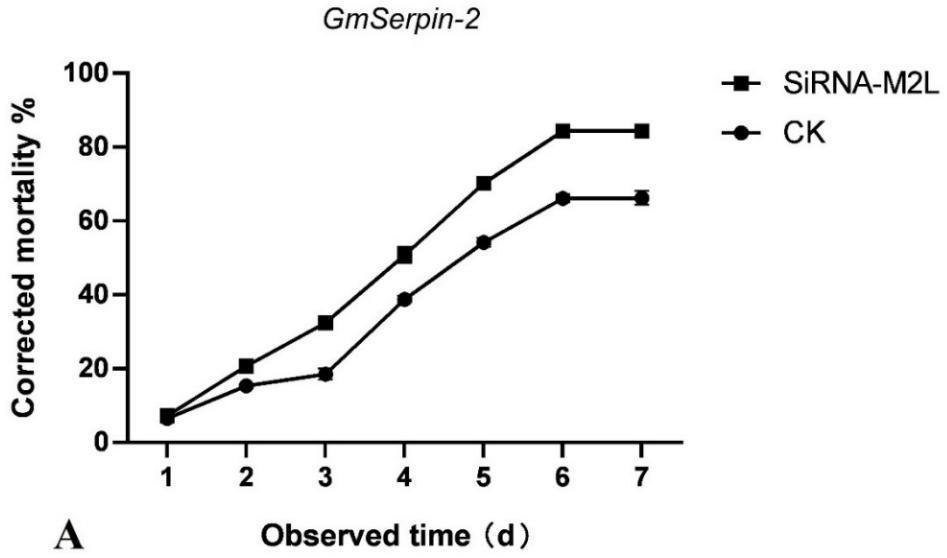


Fig. 40. The corrected mortality of *G. molesta* infected by 1×10^7 conidia/mL of *B. bassiana*

The insects were injected at 24h after injection of siRNA， and the corrected mortality were calculated within 7 d. The group injected with the same amount of Negative CK was used as the negative control. The significance of difference between the treatment group and the control group was determined via the student ’s t-test.

Serpins play important roles in insect immunity (Gan et al., 2001; Irving et al., 2000). Serpins are widely distributed in eukaryotes and considered to be involved in the regulation of several protease mediated biological processes such as the immune response, metamorphosis and embryogenesis (Jiang & Kanost, 1997; De Gregorio et al., 2002; Zhao et al., 2012). To maintain environmental homeostasis in

insects, serpins function by regulating the activity of serine proteases and inactivating proteinases irreversibly by forming covalent complexes (Tripathi & Sowdhamini, 2008; Van et al., 2003). Studies have shown that Serpin coding genes usually exist in the form of multi-gene families, and their number and biological functions vary among different insect groups (Meekins et al., 2017). At present, these gene have been successfully identified from *Bombyx mori*, *Apis cerana*, *Locusta migratoria*, *Anatolica polita boreali*, *Nilaparvata lugen* and other insects, and biological functions of some serpin genes have been studied (Li et al., 2016; Li et al., 2017; Yang et al., 2017; Li et al., 2020; Wu et al., 2022). In this study, two full-length cDNA for *G. molesta*, *GmSerpin-2* and *GmSerpin-3* were analyzed and sequenced, and characterization of their biochemical properties. Sequence characterization showed that *GmSerpin-2* and *GmSerpin-3* both contained conserved serpin domains, indicating that they belonged to a typical member of the serpin protein family. Multiple sequence alignment showed that the *GmSerpin-2* protein shared greatest similarities with the serpin2 from *Leguminivora glycinivoreua* and *GmSerpin-3* shows most closely related to the serpin3 from *Pectinophora gossypiella* indicating that they are closely related to each other and may play similar biological functions in these insects. *GmSerpin-2* with greatest expression at the 4th larval stage and lowest expression at the adult, while the *Musca domestica* serpin encoding gene SP2 was highly expressed in the second and third instars of larva (Wei et al., 2017), these results suggesting that there may be significant differences in their physiological function among different insects.

The expression patterns are different between *GmSerpin-2* and *GmSerpin-2*, that indicates different serpin genes in the same insect may have different biological functions. The expression of *GmSerpin-2* and *GmSerpin-3* in larva of *G. molesta* has spatiotemporal specificity obviously. Our qRT-PCR analysis showed that *GmSerpin-2* was expressed in all examined tissues, with the highest expression levels in the fat body, epidermal and hemocyte. while *GmSerpin-3*3 shows higher expression levels in hemolymph, fat body and malpighian tubule. The relatively high expression of *GmSerpin-2* and *GmSerpin-3* genes in larval hemolymph may be due to that serpin is involved in the regulation of serine protease cascade in hemolymph. While in *Manduca sexta*, the expression levels of serpin-2 and serpin-3 were low in larval hemolymph, but with high expression after being stimulated by pathogenic bacteria (Gan et al., 2001; Zhu et al.2003).

Biological control based on entomopathogenic fungi is one of the optimal strategies for green control of *G. molesta*, among which *B. bassiana* has been developed into commercial preparations for the control of *G. molesta*. Under natural conditions, the host infection process of *B. bassiana* begins with conidia attachment on the host body wall, then germinates and penetrates the host body wall, enters the blood cavity and turns into blastospore, and rapidly proliferates through the way of germination until the blood cavity nutrient exhaustion causes host stiffness (Butti et al., 2016). Obviously, overcoming the host insect immune defense response is a key link and an important basis for *B. bassiana* to exert its pathogenicity. Studies have shown that suppressing insect immune system can

significantly reduce its ability for resisting pathogenic agents' infection. For example, when silencing the serpin gene of *D. melanogaster*, that can significantly decrease its ability against *B. bassiana* infection (Yang et al., 2014; Han et al., 2014).

RNAi as an emerging biological technology for silencing gene expression has become a potentially powerful tool develops rapidly in recent years. However, the biggest challenge for using RNAi for effectively pest control is how to overcome the low efficient of dsRNA delivery. With the rapid development of nanotechnology, nanomaterials have been widely used in the many fields as well as applied in pest control. Conjugation of dsRNA of the target gene with nanoparticles can protect the dsRNA from nucleolytic degradation and keep its stability in the gut long enough to allow enough cellular uptake by the midgut cells. Nanoparticles show great advantages in improving RNAi efficiency, and promoting the development of RNAi-based pest management strategies. Under most conditions, the nanoparticle combines with dsRNA into the nanoparticle/dsRNA complex by the electrostatic interactions between the cationic groups between the nanoparticle and the phosphate groups in the dsRNA. Nowadays, there are many nanoparticles have been used in various insects to enhance the RNAi effect, such as cationic core-shell fluorescent nanoparticles (FNP), quantum dots, branched amphiphilic peptide capsules (BAPCs), chitosan (CS) etc. He et al., (2013) firstly reported when the larvae of *Ostrinia furnacalis* were fed with FNP/CHT10-dsRNA mixture artificial, it will successfully silence

the expression of CHT10 gene then lead to the hindered of growth and development of larvae (Yan et al., 2020). The nanoparticle mediated dsRNA technology has been successfully applied in different pests, such as *Anopheles gambiae*, *Blattella germanica*, *Acyrthosiphon pisum*, *Tribolium castaneum*, *Spodoptera exigua* (He et al., 2013). Zheng et al (2019) used a fluorescent nanoparticle mediated dsRNA to penetrate the *Aphis glycines* cuticle within 1h, the result showed the RNAi efficiency reached to 95.4%, and aphid population control effect reached to 80.5% (Zhang et al., 2019). In this study, we used nanoparticles M2L and NP5 mediate *GmSerpin-2* and *GmSerpin-3* genes respectively, which significantly improved the gene silencing effect and reduced its immune capacity, thus improving the fungal control effect. However, there are many questions remaining to be answered. The mechanisms involved in the interaction between *GmSerpin-2* and *GmSerpin-3* and its target proteinases remain to be discovered. Further research is needed on how to popularize it widely in the field. We need to conduct more studies to evaluate these molecular and signaling mechanisms in order to provide a clearer understanding of its possible functions in *G. molesta*.

Conclusions to Chapter 4

The complete sequence of *GmPGRP-SC* gene, *GmBGRP*, *GmSerpin-2* and *GmSerpin-3* are obtained and characterized. They have been submitted to NBCI GenBank respectively, and the GenBank accession number is MW773839, ON055286, OQ359960 and OQ35996.

The effect of *B. bassiana* on the regulation of immune-related gene

expression were analyzed. Finally, we successfully silenced the target gene using RNAi technology, and the effects of target gene silencing on larvae ability to resist fungal infection were analyzed, which laid a good foundation for further improving the control effect of entomopathogenic fungi.

The effects of GmBGRP gene silencing on Toll immune signaling pathway related gene expression and PPO enzyme activity were analysed. And effectively improve the interference efficiency of GmSerpin-2 and GmSerpin-3 genes mediated by nanoparticles M2L and NP5.

CHAPTER 5

INVESTIGATION ON THE OCCURRENCE AND DYNAMICS OF *G. MOLESTA* IN PEACH ORCHARD AND EVALUATION ON THE CONTROL OF FIVE DIFFERENT BIOLOGICAL PESTICIDES

5.1. Dynamics of adult *G.. molesta*

The results of the survey on the number of attracting moths of *G. molesta* are shown in Fig.41. According to the survey results in the Xinxiang area, we can indicate that the overwintered larva began to pupate in late March, and the pupal stage was 10-20 days. The first-generation adult appeared in early April and reaching a high level around 4, May. Then, the number of *G. molesta* began to increase continuously with the rise of temperature. The 1~ 2 generation larvae generally occurred from late April to late June. In the mixed planting area of peach fruit trees, the larvae mainly damaged the shoot of peach trees, and the 3~ 4 generation larvae mainly harmed fruits of peach and pear trees.

As shown in Fig 41, the occurrence of male adult *G. molesta* in Xinxiang has no obvious rule, and with serious overlap generations. There is no obvious boundary between each generation, especially between the 2~3 generation and 3~4 generation. It has been observed that the first-generation of larva is from end-April to the end-May; the second-generation of larva is from early June to late June; the third-generation of larva is from early July to mid-July; the fourth-generation of larva is from early August to late August; and the fifth-generation of larva is from late August to mid-September. The occurrence period of adult of overwintering

generation was from early April to early May; the first-generation adult occurrence period from mid-May to mid-June; the second-generation adult occurrence from late June to early July; the third-generation adult occurrence from late July to early August; and fourth-generation adult occurrence from late August to mid September. However, the fifth-generation could not complete the complete generation due to fruit picking and other reasons, and some climbed up the trunk after dropping the fruit to overwinter under the old bark or the foundation. Because of the serious overlapping of generations, we should take integrated control method, not only rely on chemical pesticides.

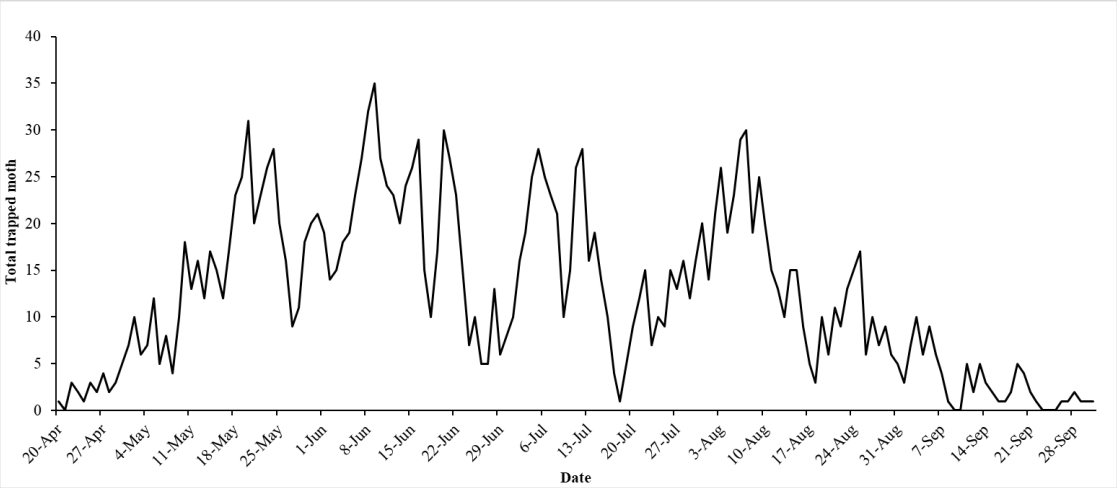


Fig. 41. Amount of day moths attracted by *G. molesta* from March, 2021 to October, 2021

5.2. Investigation result of *G. molesta* on damage of peach shoot

The investigation results were shown in Table 13, it indicating that *G. molesta* larvae had boring into the peach shoots from April 18. Generally, one *G. molesta* larvae can harm 2-3 peach tree shoots, and it causes the tender tip of the damage shoots with gummosis phenomenon, dry slowly. It is necessary to further study

whether the *G. molesta* transfer damage habits are related to the gummosis phenomenon of fruit trees. By comprising the number of damaged shoots and the number of living larvae, we can indicate that larvae continued to damage peach shoots from late April to late June. Meanwhile, the harm by *G. molesta* reached to peak from early May to early June, which was also the time the number of old larvae reach to high, while the young and middle larvae significantly decreased after mid-June. Therefore, the middle May is the key time for controlling of *G. molesta* in Xinxiang area. The damaged peach shoots can be cut off manually and taken out of the orchard for centralized destruction in order to reduce the occurrence of overwintering generation.

Table 13

Investigation of *Grapholitha molesta* larvae damaged shoot

Investigated Data	Damaged shoots	Larvae in damaged shoots	Percentage (%)	Larvae devolvement stage		
				Low	Middle	Mature
4.18	15	1	3.33	1	0	0
4.21	30	1	3.33	1	0	0
4.30	30	3	10.00	2	1	0
5.12	101	25	24.75	18	7	0
5.15	105	44	41.90	21	10	13
5.19	100	39	39.00	19	9	11
5.23	120	45	37.50	9	23	13
5.29	104	24	23.08	6	9	9
6.2	86	23	26.74	2	9	12
6.8	102	29	28.43	2	7	20
6.14	101	31	30.69	4	14	13
6.19	101	27	26.73	4	15	8
6.23	103	17	16.50	3	12	2
6.29	82	8	9.76	0	1	7
7.4	86	7	8.14	0	1	6
7.8	76	5	6.58	0	0	5
7.14	71	5	7.04	0	0	5

Note: Low stage larva is white, head and pronotum is brown, with age increased, body changed slightly pink, mature larva length is 10-13 millimeters, light red.

According to the investigation on the damaged shoots of peach orchard with different peach tree varieties (Table 14), the damaged shoots rate by *G. molesta* in peach orchard was higher than that in nectarine orchard. And in the same peach orchard, the damaged shoots are also different due to different planting densities. The shoots damage is higher with planting density of 4×4 meters than that with planting density of 3×3 meters (Table 15). The results also indicated that the damaged shoots of peach orchard had the following rules: the damaged shoots rate of peach trees on the edge of peach orchard is higher than that in the middle; the damaged shoots rate of peach trees with extensive management is higher than that of peach trees with fine management; and damaged shoots rate of peach fruit trees with young fruit trees is higher than that of old fruit trees. These results indicate that *G. molesta* harm to peach shoots is greatly related to the variety and growth environment of peach trees. We can greatly reduce the occurrence number of overwintering larvae and control it effectively, when we pay more attention to this pest in ordinary agricultural production (Appendix C).

Table 14

Investigation of larvae damaged shoot in different peach orchard

Investigated date	Peach			Nectarine		
	Damaged shoots	Larvae in shoot	Percentage（%）	Damaged shoots	Larvae in shoot	Percentage（%）
5.9	50	8	16.00	50	10	20.00
5.14	50	13	26.00	50	14	28.00
5.16	50	9	18.00	50	16	32.00
5.20	50	11	22.00	50	14	28.00
6.4	50	12	24.00	50	11	22.00
6.10	50	8	16.00	50	10	20.00

Table 15

Investigation of larvae damaged shoot in different planting density orchard

Investigated date	Planting density of 3 ×3 m			Planting density of 4 ×4 m		
	Damaged shoots	Larvae in shoot	Percentage （%）	Damaged shoots	Larvae in shoot	Percentage （%）
5.9	50	7	14.00	50	3	6.00
5.14	50	14	28.00	50	5	10.00
5.16	50	25	50.00	50	10	20.00
5.20	50	18	36.00	50	9	18.00
6.4	50	17	34.00	50	4	8.00
6.10	50	14	28.00	50	7	14.00

Fig. 42. Investigation situation and the damaged shoot of the peach orchard.

17，May 2021

5.3. Safety investigation

According to the investigation, the growth of peach trees in the treatment groups was basically the same as that in the control groups. The five biological agents all had adverse effects on the growth of peach trees. These results indicating that all the five agents were safe for the growth of peach trees.

5.4. Field control efficiency

Table 15

Comparison of the control effect of different biological agents on *G. molesta* in peach orchard

Name of the drug	Dilution ratio	Control effect (%)	
		7 days after treatment	14 days after treatment
16 000 IU/mg Bt	200	56.3a	62.0a
6 0 g / L Spinetoram	2000	86.4b	79.2b
2 4 0 g / L Methoxyfenozide	5000	74.6c	80.1c
0.2% Celastrus angulatus MaXim	1000	68.25d	61.27d
10 billion spores *Beauveria bassiana*	10000	66.8e	67.1e

Note: Different letters after the same column of data indicate the extremely significant and significant difference of the efficiency between treatments respectively.

The results were shown on Table 15, and it indicated that the 5 biological control agents all have certain control effects on *G. molesta*. On day 7 after treatment, the control effectiveness of the five agents was 56.3% ~ 86.4%. Its control effect was 63.1% ~ 80.1% on day14 in treatment groups. There was no significant difference between the five drug treatments. Among them, 60 g/L Spinetoram suspension 2000 times liquid and 240 g/L Methoxyfenozide suspension 5 000 times liquid have better control effective. On day 7 in treatment groups, the control effect was 86.4% and 74.6%, respectively. Moreover, on day 14 in treatment groups, the control effect of these two agents was still more than 78%, indicating that these two agents had good quick and persistent effect on *G. molesta*. The control effects of 0.2% Celastrus angulatus MaXim and 10 billion spores *B. bassiana* were 68.25% and 66.8% respectively on day 7 after treatment, and the control effects of these two agents on day 14 after treatment were 61.27% and 67.1%, and without significantly difference, all the results indicating that the two

agents had relatively good quick and lasting effect on the control of *G. molesta*. The effect of 16 000 IU/mg wettable powder of Bacillus 200 times liquid on the control of *G. molesta* on day 7 and 14 aftertreatment were 56.3% and 62.0%, it indicated that performance of prevention was relatively stable. In summary, the five commonly used biopesticides have their own advantages and disadvantages in the process of fild control for *G. molesta*. 60 g/L Spinetoram suspension 2000 times liquid has higher and faster control effect.

Biological pesticides are far less toxic than traditional chemical pesticides and have no residue in the soil, which is a green technology beneficial to soil and environment (Cycoń et al., 2017). And it mainly inhibits growth and development of the pests, so as to achieve the purpose of effective pest control. However, the activity against pests may be different at different doses and experimental environments. Compared with chemical pesticides, the effect of biopesticides on pests is generally slower, and generally does not directly kill pests, but prevents feeding and laying eggs or inhibit the growth and development of larvae, thereby inhibiting the growth of pest populations. Biopesticides is not the first choice in the production of fruit farmers, and it need to be future promote vigorously. Moreover, studies found that pesticide residues with fruits do harmful to human health. With the improvement of living standard, organic fruit has become the object of people's pursuit and as an increase in human vitamins, amino acids and other need to be eaten often. Therefore, biological pesticides should be use in orchards urgently. The *G. molesta* of is widely distributed, and make serious harm to fruit orchard.

The rate of insect fruit in reproducing orchards exceeded 50%, and resulting in a decline in fruit yield, quality, even a complete loss of edible value, which usually cause serious economic losses. The *G. molesta* has become an important limiting factor for green orchard production, such as peach orchard and pear orchard (Liu et al., 2013).

The use of chemical pesticides can lead to occurrence of resistant strains and can pose environmental hazards, accumulation in the food chain, high and acute toxicity, prolonged degradation, and increased potency to eliminate beneficial and harmful pests (Barnard et al., 1997). At present, the production of pollution-free green fruit, the first concern is whether the selected pesticides are beneficial to the surrounding biosafety, whether there are pesticide residues, according to the results of this study, biopesticides are safe for natural enemies of peach orchard, will not pollute the fruit and the environment, and are suitable for the control of *G. molesta*. In this study, we also predicted the occurrence in Xinxiang area of adult *G. molesta* by using sex attractors. And the investigated the larval occurrence by surveying the peach trees shoots, the results provide scientific basis for the prediction and comprehensive management of this pest. Therefore, the above five kinds of biopesticide can be used as the main drugs to prevent and control *G. molesta* in peach orchard. In order to ensure the control effectiveness, in the prevention and control of this pest in peach orchard, it is recommended that the appropriate period of control should be held 7~10 d after the peak of adult emergence. And we should pay more attention to the rotation of the use of agents to delay the generation of

resistance to this pest. However, the efficacy of biopesticides is easily affected by many factors. We can also do more research on immune-related genes, and explore more suitable target genes, so as to improve the efficacy of biopesticides, and provide more powerful support for the scientific and green control of *G. molesta*.

Conclusions to Chapter 5

1. The survey results in the Xinxiang area shows that overwintered larva began to pupate in late March, the pupal stage was 10-20 days, and first-generation adult appeared in early April, which reaching a high level around 4, May. Larvae of *G. molesta* had boring into the peach shoots from April 18. The middle May is the key time for controlling of *G. molesta* in Xinxiang area.

2. The damaged peach shoots can be cut off manually and taken out of the orchard for centralized destruction in order to reduce the occurrence of overwintering generation. The damaged shoots rate by *G. molesta* in peach orchard was higher than that in nectarine orchard. And in the same peach orchard, the damaged shoots are also different due to different planting densities. The shoots damage is higher with planting density of 4×4 meters than that with planting density of 3×3 meters.

3. The five biological agents all had adverse effects on the growth of peach trees. The results of this study indicating that all the five biological agents were safe for the growth of peach trees. And the 5 biological control agents all have certain control effects on *G. molesta*. On day 7 in treatment groups, the control effec of the five agents was 56.3% ~ 86.4%. Its control effect was 63.1% ~ 80.1%

on day 14 of treatment. Among them, 60 g/L pinetoram suspension 2000 times liquid and 240 g/L Methoxyfenozide suspension 5 000 times liquid have better control effectiveness with 86.4% and 74.6% control effect on day 7 in the treatment groups. Moreover, the control effect of these two agents was still more than 78% on day 14. The control effects of 0.2% Celastrus angulatus MaXim and 10 billion spores *B. bassiana* were 68.25% and 66.8% respectively on day 7 in treatment groups, and the control effects of these two agents were 61.27% and 67.1% on day 14 in treatment groups which indicating that the two agents had relatively good quick and lasting effect on the control of *G. molesta*.

CONCLUSION

The dissertation provides a theoretical generalization and a new solution to the scientific task of improving the biological control of *G. molesta* by using immune genes as target genes, based on the interaction and immune response of *B. bassiana* and *G. molesta*, the effects of *B. bassiana* on the infection concentration and infection mode of *G. molesta*, the immune response of *G. molesta* larvae to *B. bassiana* and the molecular identification and functional analysis of immune-related genes of *G. molesta*, and the investigation on the occurrence and dynamics of *G. molesta* in peach orchard and evaluation on the control of five different biological pesticides.

1. As a result of studying the laboratory evaluation of the effect of *B. bassiana* on the vital activity of *G. molesta*: it was clear that *B. bassiana* mainly infected *G. molesta* through epidermal penetration, meanwhile the optimal infection concentration of *B. bassiana* was determined. When the concentration of *B. bassiana* is 1×10^7 conidia/mL, it caused higher mortality of *G. molesta* larvae. These results provided a theoretical basis and practical guide for better biological control of *G. molesta* by *B. bassiana* in field control.

2. As a result of molecular mechanism of *B. bassiana* and *G. molesta* through transcriptome analysis of *G. molesta* larvae 24 h after infected by *B. bassiana*, the immune-related genes were effectively screened. This is the first time that performed the transcriptomics analysis of *G. molesta* larvae after infected by *B. bassiana*. Research shows that gene of *G. molesta* after infected by *B. bassiana*,

mainly involved in cellular processes, metabolism, biological control, development and biological process. By comparing the treatment group to controls, with 965 up-regulated and 790 down-regulated genes. The up-regulated genes in *G. molesta* infected by *B. bassiana* were analysed, and 14 gene related to immune response of *G. molesta* induced by *B. bassiana* were selected and quantified by qRT-PCR method, the results show consistent with the transcriptome analysis and the reliability of transcriptome sequence was verified. The immune response of *G. molesta* larva infected by *B. bassiana* were firstly studied and analyzed. These results given an advancing knowledge of the mechanisms involved in entomogenous fungal and *G. molesta* and will be helpful in reaching a better understanding of the fungal infections and hosts insects interactions, which lays a good foundation for better use of fungi for biological control of *G. molesta.* These research results laid a good foundation for further improving the control effect of entomogenous fungi through molecular mechanism research, searching for effective target genes, and developing new pesticides, and provided solid and reliable data support for subsequent research.

3. As a result of characterization and functional analysis of Immune recognition gene *GmPGRP-SC*, *GmBGRP*, *GmSerpin-2* and *GmSerpin-3*, the complete sequence of immune-related genes: *GmPGRP-SC* gene, *GmBGRP*, *GmSerpin-2* and *GmSerpin-3* are firstly identified and characterized. They have been submitted to NBCI GenBank respectively, and the GenBank accession number is MW773839, ON055286, OQ359960 and OQ35996 respectively, these

genes studied have potential applications and are in the process of being certified. The physical and chemical properties, gene structure, amino acid sequence, systematic evolution, and expression patterns of the gene family were analysed which laying a better foundation for further investigation of their functions. The target immune-related gene expression changes in *G. molesta* in response to infection with *B. bassiana* were described using qPCR method. Finally, the effective targeted gene silencing strategy to reduces insect immunity against fungal infection using RNAi technology were demonstrated, which laid a good foundation for further improving the control effect of fungi. Immune gene silencing has potential application value, because it can reduce the immune ability of *G. molesta*, so as to better use biological control means to achieve the best control effect.

All results could contribute to abetter understanding of the resistance mechanism of *B. bassiana* on *G. molesta* and provided a foundation for designing the IPM strategy for the effectively controlling *G. molesta* in the field. 4. As a result of investigation on the occurrence and dynamics of *G. molesta* in peach orchard and evaluation on the control of five different biological pesticides: the best control period of *G. molesta* was better understood, and more effective biological control reagents were selected. The result indicated that middle May is the key time for better controlling of *G. molesta* in Xinxiang area. The damaged shoots rate by *G. molesta* in peach orchard was higher than that in nectarine orchard. The shoots damage is higher with planting density of 4×4 meters than that with planting density of 3×3 meters. The five biological agents all had adverse

effects on the growth of peach trees. And the 5 biological control agents all have certain control effects on *G. molesta*, but among them, 60 g/L pinetoram suspension 2000 times liquid has better control effectiveness with 86.4% on day 7 in the treatment groups. And the effects were still over 78 % after 14 days of treatment. These research results provide theoretical and reliable data support for better understanding the occurrence rule of *G. molesta* and effective use of biological control methods in field control. It laid the foundation for the research of biological control of *G. molesta*.

PROPOSALS FOR PRODUCTION

1.The use of *B. bassiana* for field control needs to be taken at the end of the larva outbreak, and the appropriate concentration should be taken for body wall at the concentration of *B. bassiana* is 1×10^7 conidia/mL to achieve better control effect.

2. The exploring of immune genes has potential application value in the development of new pesticides to improve the control of *B. bassiana* and other entomopathogenic fungi for *G. molesta* control. Especially for Serpin genes, the research in this area should be strengthened to provide an effective way to better improve the biological control of *G. molesta*. At the same time, the mediation of nanoparticles should be better used for strengthen the effect of biopesticides.

3. In order to seclcted the right time and take more effective measures for contrl of *G. molesta*, it is necessary to effectively grasp the law of its occurrence harm and accurately grasp the law of occurrence. For the test of five originally used biopesticides, 60 g/L pinetoram suspension 2000 times liquid should be preferred.

REFERENCES

1. Ahn, J.J., Yang, C.Y. & Jung, C. (2012). Model of *Grapholita molesta* spring emergence in pear orchards based on statistical information criteria. *Journal of Asia-Pacific Entomology*,15(4):589–593.

2. Akira, S., Uematsu, S. & Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell*, 124 (4): 783–801. https://doi.org/10.1016/j.cell.2006.02.015

3. An, C. & Kanost, M.R. (2010) *Manduca sexta* serpin-5 regulates prophenoloxidase activation and the Toll signaling pathway by inhibiting hemolymph proteinase HP6. *Insect Biochemistry and Molecular Biology*, 40(9): 683–689.

4. Barnard, C., Padgitt, M. & Uri, N. (1997). Pesticide use and its measurement. *Intonational Pest Control*. 39: 61–164.

5. Barros-Parada, W., Ammagarahalli, B. Basoalto, E. Fuentes Contreras, E., & Gemeno, C. (2018) Captures of oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae), in traps baited with host-plant volatiles in Chile. *Applied entomology and zoology*, 53(2): 193–204. https://doi.org/10.1007/s13355-017-0543-7

6. Bisognin, M., Zanardi, O.Z., Nava, D.E., Arioli, C.J., Botton, M., Garcia, M.S., & Cabezas, M.F. (2012) Burrknots as food source for larval development of *Grapholita molesta* (Lepidoptera: Tortricidae) on apple trees. *Environmental Entomology*, 41: 849–854. https://doi.org/10.1603/EN11119

7. Borchert, D.M., Stinner, R.E., Walgenbach, J.F., & Kennedy, G. G. (2004)

Oriental fruit moth (Lepidoptera: Tortricidae) phenology and management with methoxyfenozide in North Carolina apples. *Journal of Economic Entomology*, 97(4): 1353–1364. http://dx.doi.org/10.1603/0022-0493-97.4.1353

8. Bulet, P., & Stöcklin, R. (2005) Insect antimicrobial peptides: structures, properties and gene regulation. *Protein Protn & Peptide Letters*, 12: 3–11. https://doi.org/10.3724/SP.J.1141.2010.01027

9. Butt, T.M., Coates, C.J., Dubovskiy, I.M. & Ratcliffe, N.A. (2016) Entomopathogenic fungi: new insights into host-pathogen interactions. *Advances in Genetics*, 94: 307–364.

10. Chaudry, G.U. (1956) The development and fecundity of the oriental fruit moth, *Grapholotha molesta* (Busck) under controlled temperatures and humilities. *Bulletin of Entomological Research*, 46(04), 869–898. http://dx.doi.org/10.1017/S0007485300037111

11. Chen M.X., Luo Y.Q. & Zhao, C.J. (2009) Progress in the research on the oriental fruit moth. *Journal of the North Garden*, 8: 144–147 (in Chinese).

12. Chen, J.H. (2007) Effects of 48 % Lorsban missible oil on the prevention and control of oriental fruit moth. *Deciduous Fruit Tree*, (4): 39–40. (In Chinses).

13. Chen, L.H., Tian, K.; Xu, X.L., Fang, A.S., Cheng, W.N., Wang, G.R., Liu, W. & Wu, J.X. (2020) Detecting Host-Plant Volatiles with Odorant Receptors from *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae). *Current Microbiology*, 68(9): 2711–2717. https://doi.org/10.1021/acs.jafc.9b07305

14. Christophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S. & Blass, C. (2002) Immunity-related genes and gene families in *Anopheles gambiae*. *Science*, 298: 159–165. http://dx.doi.org/10.1126/science.1077136

15. Clark, R.A., Casagrande, R.A. & Wallace, D.B. (1982) Influence of pesticides on *Beauveria bassiana,* a pathogen of the Colorado potato beetle. *Environmental Entomology*, 11: 67–70.

16. Colinet, D., Dubuffet, A. & Cazes, D. (2009) A serpin from the parasitoid wasp *Leptopilina boulardi* targets the *Drosophila* phenoloxidase cascade. *Developmental and Comparative Immunology*, 33(5): 681–689.

17. Cudic, M., Condie, B.A., Weiner, D.J., Lysenko, E. S., Xiang, Z., Insug O, Bulet, P. & Otvos, L.J. (2002) Development of novel antibacterial peptides that kill resistant isolates. *Peptides*, 23(12): 2071–2083.

18. Cycoń, M., Mrozik A. & Piotrowska-Seget, Z. (2017). Bioaugmentation as a strategy for the remediation of pesticide-polluted soil: A review. *Chemosphere*. 172: 52–71. doi: 10.1016/j.chemosphere.2016.12.129

19. De Gregorio, E., Han, S.J., Lee, W.J., Baek, M.J., Osaki, T., Kawabata, S., Lee, B.L., Iwanaga, S., Lemaitre, B. & Brey, P. T. (2002) An immune-responsive Serpin regulates the melanization cascade in *Drosophila*. *Developmental Cell*, 3: 581–592. https://doi.org/10.1016/S1534-5807(02)00267-8

20. De Hoog, G. S. (1972) The genera *Beauveria*, *Isaria*, *Tritirachium* and *Acrodontium* gen. nov. *Transactions of the British Mycological Society*, 1:1–41.

21. Dimopoulos, G., Richman, A., Müller, H.M. & Kafatos, F.C. (1997) Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proceedings of the National Academy of Sciences of the United States of America*, 94(21): 11508–11513. doi: http://dx.doi.org/10.1073/pnas.94.21.11508

22. Dimopoulos, G., Richman, A., Muller, H.M., & Kafatos, F. (1997). Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Immunology*, 94: 11508–11513. doi: http://dx.doi.org/10.17221/71/2019-PPS

23. Dong, J.T., Chen, W., Feng, J.G, Liu, X.Q, Xu, Y., Wang, C., Yang, W.C. & Du, X.Z. (2021) Facile, Smart, and Degradable Metal−Organic Framework Nanopesticides Gated with FeIII-Tannic Acid Networks in Response to Seven Biological and Environmental Stimuli. *ACS Applied Materials & Interfaces*, 13: 19507–19520. doi: https://doi.org/10.1021/acsami.1c04118

24. Dorn, S., Hughes, J., Molinari, F. & Cravedi, P. (2001) *Cydia molesta* and *Cydia pomonella*: comparison of adult behavior. *IOBC wprs Bull*, 24:133–137.

25. Du, J., Li, G.W., Xu, X.L., & Wu, J.X. (2015) Development and Fecundity Performance of Oriental Fruit Moth (Lepidoptera: Tortricidae) Reared on Shoots and Fruits of Peach and Pear in Different Seasons. *Environmental Entomology*, 44(6): 1522–1530. doi: http://dx.doi.org/10.1093/ee/nvv124

26. Faria, M., & Wraight, S.P. (2001) Biological control of *Bemisia tabaci* with fungi. *Crop Protection*, 20(9): 767–778.

27. Feng, J.G. & Zhang, Y. (1988) Study on control of fruit tree pests by *Trichogramma dendrolimi*. *Knowledge of insects*, 6: 344–347. (in Chinese)

28. Ferron, P. (1977). Influence of relative humidity on the development of fungal infection caused by *Beauveria bassiana* in imagines of *Acanthoscelides obtectus* (Coleoptera: Bruchidae). *Entomophaga*, 2: 393–396. doi: https://doi.org/10.5772/intechopen.81431

29. Ferron, P. (1978) Biological control of insect pest by entomopathogenic fungi. *Annual Review of Entomology*, 23: 409–420. https://doi.org/10.1146/annurev.en.23.010178.002205

30. Gan, H., Wang, Y., Jiang, H.B., Mita, K. & Kanost, M.R. (2001) A bacteria-induced, intracellular serpin in granular hemocytes of *Manduca sexta*. *Insect Biochemistry Molecular Biology*, 31: 887–898. doi: http://dx.doi.org/10.1016/S0965-1748(01)00034-0

31. Gillespie, J. P., Bailey, A. M., Cobb, B. & Vilcinskas, A. (2000). Fungi as elicitors of insect immune responses. *Archives of Insect Biochemistry and Physiology*, 44: 49–68. https://doi.org/10.1002/1520-6327(200006)44:2<49::AID-ARCH1>3.0.CO;2-F

32. Govind, S. (2008) Innate immunity in Drosophila: Pathogens and pathways. *Insect Science*, 15: 29–43. https://doi.org/10.1111/j.1744-7917.2008.00185.x

33. Graillot, B., Blachere-Lopez, C., Besse, S., Siegwart, M. & Lopez-Ferber, M. (2017) Host range extension of *Cydia pomonella* granulovirus:

adaptation to Oriental Fruit Moth, *Grapholita molesta*. *Biocontrol*, 62, 19–27. http://dx.doi.org/10.1007/s10526-016-9772-x

34. Guo, X.J., Di, N., Chen, X., Zhu, Z.Y., Zhang, F., Tang, B., Dai, H.J., Li, J.T., Guo, R. & Wang, S. (2019) Performance of *Trichogramma pintoi* when parasitizing eggs of the oriental fruit moth *Grapholita molesta. Entomologia Generalis*, 39, 239–249.

35. Guo, Z.F., Shao, H.B. & Liu, H.L. (2010) Study on biological characteristics of *Beauveria bassiana* strain B 11. *Research on Hebei Forest Fruit*, 25 (1): 57–61. (in Chinese)

36. Han, P., Fan, J., Liu, Y., Cuthbertson, A.G., Yan, S., Qiu, B.L., Ren, S. (2014) RNAi-mediated knockdown of serine protease inhibitor genes increases the mortality of *Plutella xylostella* challenged by Destruxin A. *PLoS ONE*, 9(5), e97863. doi: http://dx.doi.org/10.1371/journal.pone.0097863

37. He, B. C., Chu, Y. & Yin, M., Z. (2013) Fluorescent nanoparticle delivered ds RNA toward genetic control of insect pests. *Advanced Material*, 25(33), 4580–4584.

38. Hill, D. S. (1987) Agricultural insect pests of temperate regions and their control. Cambridge University Press, Cambridge, United Kingdom, 459pp.

39. Hiromitsu, T., Ishibashi, J., Kosuke, F., Nakajima, Y., Sagisaka, A., Tomimoto, K., Suzuki, N., Yoshiyama, M., Kaneko, Y, Iwasaki, T., Sunagawa, T., Yamaji, K., Asaoka, A., Mita, K. & Yamakawa, M. (2008) A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori. Insect*

Biochemistry and Molecular Biology, 38(12): 1087–1110. doi: https://doi.org/10.1016/j.ibmb.2008.09.001

40. Hoffmann, J.A. & Reichhart, J.M. (2002) *Drosophila* innate immunity, an evolutionary perspective. *Nature Immunology*, 3: 121–126. doi: https://doi.org/10.1038/ni0202-121

41. Hoffmann, J.A. (2003) The immune response of *Drosophila*. *Nature*, 426(6962), 33–38. doi: http://dx.doi.org/10.1038/nature02021

42. Hou, C.X., Tan, G.X., Liu, T. & Guo, X.J. (2012) Advances in defense mechanisms of insects against pathogenic fungi. *Anhui Agricultural Sciences*, 40(23): 11649–11652. https://doi.org/10.17221/71/2019-PPS

43. Hu, J.J. & Fan, M.Z. (1996) Relation between Extracellular Protease of *Beauveria bassiana* and Its Virulence to *Dendrolinus Punctatus*. *Journal of Anhui Agricultural University*, 23(3): 273–278. (in Chinese)

44. Huang, B., L. Chun, L. Zhen Gang, F. Mei Zhen & Z. Li. (2002). Molecular identification of the teleomorph of *Beauveria bassiana. Mycotaxon*, 81: 229–236. doi: https://doi.org/10.1002/iub.144

45. Huang, W., Xu, X., Freed, S., Zheng, Z., Wang, S., Ren, S. & Jin, F. (2015) Molecular cloning and characterization of a β-1,3-glucan recognition protein from *Plutella xylostella* (L.). *New Biotechnology*, 32(2): 290–299. doi: http://dx.doi.org/10.1016/j.nbt.2015.01.002

46. Irving, J.A., Pike, R.N., Lesk, A.M. & Whisstock, J.C. (2000) Phylogeny of the serpin superfamily: implications of patterns of amino acid

conservation for structure and function. *Genome Research*, 10, 1845–1864. doi: http://dx.doi.org/10.1101/gr.14780

47. Irving, P., Ubeda, J., Doucet, D., Troxler, L., Lagueux, M., Zachary, D., Hoffmann, J., Hetru, C. & Meister, M. (2005) New insights into *Drosophila* larval haemocyte functions through genome-wide analysis. *Cellular Microbiology*, 7(3): 335–350. doi: https://doi.org/10.1111/j.1462-5822.2004.00462.x

48. Itsathitphaisarn, O., Thitamadee, S., Weerachatyanukul, W. & Sritunyalucksana K. (2017) Potential of RNAi applications to control viral diseases of farmed shrimp, *Journal of Invertebrate Pathology,* 147, 76 − 85. doi: http://dx.doi.org/10.1016/j.jip.2016.11.006

49. Jiang, H, Wang, Y. & Kanost, M.R. (1994) Mutually exclusive exon uses and reactive center diversity in insect serpins. *Journal of Biological Chemistry*, 269(1):55–81. doi: http://dx.doi.org/10.1016/S0021-9258(17)42311-8

50. Jiang, H. & Kanost, M.R. (1997) Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. *Journal of Biological Chemistry*, 272: 1082–1087. doi: http://dx.doi.org/10.1074/jbc.272.2.1082

51. Jiang, H., Wang, Y., Huang, Y. et al. (1996) Organization of serpin gene-1 from *Manduca sexta*. Evolution of a family of alternate exons encoding the reactive site loop. *Journal of Biological Chemistry*, 271(45): 28017.

52. Johnson, D.L. & Goettel, M.S. (1993) Reduction of grasshopper populations following field application of the fungus *Beauveria bassiana*.

Biocontrol Science Technology, 3:165–175. doi: https://doi.org/10.1080/09583159309355273

53. Kanga, L.H.B., Pree, D.J., van Lier, J.L., Walker, G.M. (1999). Monitoring for resistance to organophosphorus, carbamate, and pyrethroid insecticides in the oriental fruit moth (Lepidoptera: Tortricidae). *Canadian Entomologist*, 131(4): 441–450.

54. Kanga, L.H.B., Pree, D.J., Van Lier, J.L. & Walker, G.M. (2003) Management of insecticide resistance in Oriental fruit moth (*Grapholita molesta*; Lepidoptera: Tortricidae) populations from Ontario. *Pest Management Science*, 59:921–927. doi: https://doi.org/10.1002/ps.702

55. Khush, R. S. & Lemaitre, B. (2000) Genes that fight infection: what the Drosophila genome says about animal immunity. *Genetics of Drosophila immunity*, 16(10): 442–449. doi: https://doi.org/10.1016/S0168-9525(00)02095-3

56. Kim, Y.S., Ryu, J.H., Han, S.J., Choi, K.H., Nam, K.B. & Jang, I.H. (2000) Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and β-1,3-glucan protein that mediates the signaling for the induction of innate immune gene in *Drosophila melanogaster* cells. *Journal of Biological Chemistry*, 275, 32721–32727.

57. Kirk, H., Dorn, S. & Mazzi, D. (2013) Worldwide population genetic structure of the oriental fruit moth (*Grapholita molesta*), a globally invasive pest. *BMC Ecology*, 13: 12. doi: https://doi.org/10.1186/1472-6785-13-12

58. Kong, W.N., Wang, Y., Guo, Y.F., Chai, X.H., Li, J. & Ma, R.Y. (2020)

Importance of Preovipositional Period of an Oligophagous Moth in Predicting Host Suitability. *Journal of Economic Entomology*, 113(1): 222–229. doi: https://doi.org/10.1093/jee/toz278

59. Kong, W.N., Wang, Y., Jia, X.T., Gao, Y., Fan, R.J., Li, J. & Ma, R.Y. (2019) Emergence and mating behavior of the oriental fruit moth *Cydia molesta* (Lepidoptera: Tortricidae) and its potential for reproduction. *Annales De La Societe entomological De France*, 55(5): 446–453. doi: https://doi.org/10.1080/00379271.2019.1657361

60. Kuang, Z. B., Lv, L. H. & Feng, X. (2005) Effects of temperature and common pesticides on the biological characteristics of *Beauveria bassiana*. *Journal of South China Agricultural University*, 26(3): 26–29. (in Chinese)

61. Lee, S.J., Yang, Y.T., Kim, S., Lee, M.R., Kim, J.C., Park, S.E., Hossain Muktadir, S., Shin, T.Y., Nai, Y.S. & Kim, J.S. (2018) Transcriptional response of *bean bug* (Riptortus pedestris) upon infection with entomopathogenic fungus, *Beauveria bassiana* JEF-007. *Pest management Science*, 75(2): 333–345. doi: https://doi.org/10.1002/ps.5117.

62. Lemaitre, B. & Hoffmann, J. (2007) The Host Defense of Drosophila melanogaster. *Annual review of immunology*, 25: 697–743.doi: https://doi.org/10.1146/annurev.immunol.25.022106.141615

63. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. & Hoffmann, J.A. (1996) The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*, 86,95. doi:

https://doi.org/10.1016/S0092-8674(00)80172-5

64. Levashina, E. A., Ohresser, S., Bulet, P., Rechhart, J. M., Hetru, C. & Hoffmann, J.A. (1995) Metchnikowin, a novel immune inducible praline-rich peptide from *Drosophila* with antibacterial and antifungal properties. *European Journal of Biochemistry*, 233(2): 694–700.

65. Lewis, L.C., Bruch, D.J., Gunarson, R.D. & Bidne, K.G. (2001) Assessment of plant pathogenicity of endophytic *Beauveria bassiana* in *Bt* transgenic and non-transgenic corn. *Crop Science*, 41, 1395–1400. doi: https://doi.org/10.2135/cropsci2001.4151395x

66. Li B., Qin Y.C. & He, L. (2006) Control of pear small heart worms with different attracting core and sweet and sour liquor. *Chinese journal of plant protection*, 35(6): 285–286. (in Chinese).

67. Li, B., Yu, H.Z., Ye, C.J., Ma, Y., Li, X., Fan, T., Chen, F.S. &Xu, J.P. (2017) *Bombyx mori* Serpin-6 regulates prophenoloxidase activity and the expression of antimicrobial proteins. *Gene*, 610: 64–70.

68. Li, B.B., Tian, Y., Du, G.L., Yue, F.Z., Nong, X.Q., Liu, T.H., Zang, Z.H. & Wang, G.J. (2020) Effect of serine protease inhibitor Serpin1 from *Locusta migratoria* on the infection of *Metarhizium anisopliae*. *Chinese Journal of Biological Control*, 36(5): 729–736. (in Chinese).

69. Li, G.W., Chen, X.L., Sun, Y. Chen, Y.X., Xu, S.C. & Wu, J.X. (2019) Expression profiles and ligand-binding properties of two odorant-binding proteins from *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae). *Journal of Asia*

Pacific Entomology, 22(4): 1098–1108. doi: https://doi.org/10.1016/j.aspen.2019.09.003

70. Li, R.S. & Luo S.B. (1983) Microbial Control Pests. Beijing. *Science Press*, 27–45. (in Chinese).

71. Li, Y.S., Liu, H.W., Zhu, R., Xia, Q.Y. & Zhao, P. (2016) Protease inhibitors in *Bombyx mori* silk might participate in protecting the pupating larva from microbial infection. *Insect Science*, 23(6): 835–842.

72. Li, Y.S., Zhao, P., Liu, S.P., Dong, Z.M., Chen, J.P., Xiang, Z.H. & Xia, Q.Y. (2012) A novel protease inhibitor in *Bombyx mori* is involved *Beauveria bassiana. Insect Biochemistry and Molecular Biology*, 42(10): 766–775. doi: https://doi.org/10.1016/j.ibmb.2012.07.004

73. Lin, H. F., Hu, Z. & Li, Z.Z. (1998) Biological characteristics of *Beauveria bassiana* and its application in forest. *Journal of Anhui Agricultural University*, 26 (1): 54–58 (in Chinese).

74. Liu, C.M. & Kainoh, Y. (2020) Laboratory rearing of *Lytopylus rufipes* (Hymenoptera: Braconidae: Agathidinae), a parasitoid wasp of the oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae), using apple and a commercially available diet. *Applied entomology and zoology*, 55(2): 271–276. doi: https://doi.org/10.1007/s13355-020-00671-0

75. Liu, C.T., Hou, R.F. & Ashida, M. (1997) Effects of inhibitors of serine protease phenoloxidase and dopa decarboxylase on the melanization of *Dirofilaria immitis* microfilariae with *Armigeres subalbatus* haemolymph in vitro.

Parasitology, 115(1): 57–68. doi: https://doi.org/10.1017/S0031182097001108

76. Liu, Q., Zhou, H., Zhang, K., Shi, X. X., Fan, W. Zhu, R. X., Yu, P. S. & Cao, Z.W. (2012) *In Silic* Target-Specific siRNA Design Based on Domain Transfer in Heterogeneous Data. *PLoS One*, 7(12): e50697. doi: http://dx.doi.org/10.1371/journal.pone.0050697.

77. Liu, Y.G., Gu, S.D., Li, L.L., Zhang, B., Yu, Y. & Zheng, C.Y. (2013). The frequency of occurrence of *Grapholitha molesta* (Busck) in single pear, peach and mixed pear-peach orchards in Laiyang. *Chinese Journal of Applied Entomology*, 50(6): 1538–1545.

78. Lu, P.F., Huang, L.Q. & Wang, C.Z. (2012) Identification and Field Evaluation of Pear Fruit Volatiles Attractive to the Oriental Fruit Moth, *Cydia molesta*. *Journal of Chemical Ecology*, 38(8): 1003–1016. https://doi.org/10.1007/s10886-012-0152-4

79. Ma, A.H., Li, W. L. & Lu, Z.Y. (2016) The Occurrence Dynamics and Damage Characteristics and Integrated Control of Oriental Fruit Moth in Peach Orchard. *Journal of Hebei Agricultural Sciences*, 20 (4): 27–29 (in Chinese).

80. Ma, C. & Kanost, M.R. (2000) A β-1,3-glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade. *Journal of Biological Chemistry*, 275(11): 7505 –7514.

81. Magalhaes, L.C. & Walgenbach, J.F. (2011) Life Stage Toxicity and Residual Activity of Insecticides to Codling Moth and Oriental Fruit Moth (Lepidoptera: Tortricidae). *Journal of Economic Entomology*, 104(6): 1950–1959.

doi: https://doi.org/10.1603/EC11070

82. Mallikarjuna, R.J., Moises, J.Z., Guy, S. & Olivier, C. (2016) RNAi Efficiency, Systemic Properties, and Novel Delivery Methods for Pest Insect Control: What We Know So Far. *Fronters in Physiology*, 7: 553. doi: http://dx.doi.org/10.3389/fphys.2016.00553

83. Matskevich, A.A., Quintin, J. & Ferrandon, D. (2010) The *Drosophila* PRR GNBP3 assembles effector complexes involved in antifungal defenses independently of its Toll-pathway activation function. *European Journal of Immunology*, 40(5): 1244–1254. doi: https://doi.org/10.1002/eji.200940164

84. Meekins, D.A., Kanost, M.R. & Michel, K. (2017) Serpins in arthropod biology. *Seminars in Cell and Developmental Biology*, 62: 105–119. doi: http://dx.doi.org/10.1016/j.semcdb.2016.09.001

85. Monteiro, L.B., Witt, L.G., Guiloski, I.C., Regis Silvori, D.S. & Helena, S.A. (2020) Evaluation of Resistance Management for the Oriental Fruit Moth (Lepidoptera: Tortricidae) to Insecticides in Brazilian Apple Orchards. *Journal of Economic Entomology*, 113(3): 1411–1418. doi: http://dx.doi.org/10.1093/jee/toaa023

86. Mora, M.A.E., Castilho, A.M.C. & Fraga, M.E. (2017) Classification and infection mechanism of entomopathogenic fungi. *Agricultural Microbiology*, 84: 1–10. doi: https://doi.org/10.1590/1808-1657000552015

87. Morisato, D. & Anderson, K.V. (1994) The spätzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral

pattern of the *Drosophila* embryo. *Cell*, 76(4): 677–688. doi: https://doi.org/10.1016/0092-8674(94)90507-X

88. Moritz, G., Thomas, H., Zeynep, A., Oliver, P., Evgeny, K. & Michael, B. (2010) Genome RNAi: a database for cell-based RNAi phenotypes. 2009 update. *Nucleic Acids Research*, 38: D448–D452. doi: http://dx.doi.org/10.1093/nar/gkp1038

89. Myers, C.T., Hull, L. A. & Krawczyk, G. (2006) Seasonal and cultivar-associated variation in oviposition preference of oriental fruit moth (Lepidoptera: Tortricidae) adults and feeding behavior of neonate larvae in apples. *Journal of Economic Entomology*, 99(2): 349–358. doi: https://doi.org/10.1603/0022-0493-99.2.349

90. Myers, C.T., Hull, L.A. & Krawczyk, G. (2007) Effects of orchard host plants (apple and peach) on development of oriental fruit moth (Lepidoptera: Tortricidae). *Journal of Economic Entomology*, 100(2): 421–430. doi: https://doi.org/10.1603/0022-0493(2007)100 [421: EOOHPA]2.0.CO;2

91. Natale, D., Mattiacci, L., Hern, A., Pasqualini, E. & Dorn, S. (2003) Response of female *Cydia molesta* (Lepidoptera: Tortricidae) to plant derived volatiles. *Bulletin of Entomological Research*, 93: 335–342. doi: http://dx.doi.org/10.1079/BER2003250

92. Navarro-Roldan, M.A., Bosch, D., Gemeno, C. & Siegwart, M. (2020) Enzymatic detoxification strategies for neurotoxic insecticides in adults of three tortricid pests. *Bulletin of Entomological Research*, 110(1): 144–154. doi:

https://doi.org/10.1017/S0007485319000415

93. Ning, Y.Y., You, M.S. & Wang, C.S. (2009) Advances in research on immune recognition and immune escape mechanism of pathogenic agents in insects. *Acta Entomological Sinica*, 52(5): 567–575.

94. Ochiai, M. & Ashida, M. (1988) Purification of a *beta*-1,3-glucan recognition protein in the prophenoloxidase activating system from hemolymph of the silkworm, *Bombyx mori*. J*ournal of Biological Chemistry*, 263(24): 12056–12062. doi: http://dx.doi.org/10.1016/S0021-9258(18)37892-X

95. Palli, S.R. (2014). RNA interference in Colorado potato beetle: steps toward development of dsRNA as a commercial insecticide. *Current Opinion in Insect Scienc*e, 6: 1–8. doi: http://dx.doi.org/10.1016/j.cois.2014.09.011

96. Paparazzo, F., Tellier, A., Stephan, W. & Hutter, S. (2015) Survival Rate and Transcriptional Response upon Infection with the Generalist Parasite *Beauveria bassiana* in a World-Wide Sample of Drosophila melanogaster. *PLOS One*, 10(7): e0132129. doi: https://doi.org/10.1371/journal.pone.0132129 J.

97. Pauchet, Y., Freitak, D., Heidel-Fischer, H.M., Heckel, D.G. & Vogel, H. (2009) Immunity or digestion: glucanase activity in a glucan-binding protein family from Lepidoptera. *The Journal of Biological Chemistry*, 284(4): 2214–2224. doi: http://dx.doi.org/10.1074/jbc.M806204200

98. Peterson, A. & Haeussler, G.J. (1928) Some observations on the number of larval instars of the Oriental peach moth *Laspeyresia molesta* Busck. *Economic Entomology*, 21: 843–852. doi: https://doi.org/10.1093/jee/21.6.843

99. Pinero, J.C. & Dorn, S. (2009) Response of female oriental fruit moth to volatiles from apple and peach trees at three phenological stages. Entomologia Experimentalis Et Applicata, 131(1): 67–74. doi: https://doi.org/10.1111/j.1570-7458.2009.00832.x

100. Piskorski, R., Ineichen, S. & Dorn, S. (2011) Ability of the Oriental Fruit Moth *Grapholita molesta* (Lepidoptera: Tortricidae) to Detoxify Juglone, the Main Secondary Metabolite of the Non-host Plant Walnut. *Journal of Chemical Ecology*, 37(10): 1110–1116. doi: https://doi.org/10.1007/s10886-011-0015-4

101. Piskorski, R., Ineichen, S. & Dorn, S. (2011) Ability of the Oriental Fruit Moth *Grapholita molesta* (Lepidoptera: Tortricidae) to Detoxify Juglone, the Main Secondary Metabolite of the Non-host Plant Walnut. *Journal of Chemical Ecology*, 37(10): 1110–1116. doi: https://doi.org/10.1007/s10886-011-0015-4

102. Portilla, M., Snodgrass, G., Luttrell, R. & Jaronski, S. (2014) A novel bioassay to evaluate the potential of *Beauveria bassiana* strain NI8 and the insect growth regulator novaluron against *Lygus lineolaris* on a non-autoclaved solid artificial diet. *Journal of Insect Science*, 14: 115–117. doi: http://dx.doi.org/10.1093/jis/14.1.115

103. Reichart, G. & Bodor, J. (1972) Biology of the oriental fruit moth (*Grapholita molesta* Busck) in Hungary. *Acta Phytopathologica Academiae Scientiarum Hungaricae*, 7: 279–295.

104. Ren, J., Han, X.M., Liu, Z. & Lei, Z.G. (2013) Defense reaction of blood cells of whitefish against white fungus rigor. *China vegetable*, 12: 61–65. (in

Chinese)

105. Rice, R.E., Doyle. J. & Jones, R.A. (1972) Pear as a host of the oriental fruit moth in California. *Journal of Economic Entomology,* 65:1212–1213.

106. Ricietto, A.P.S., Gomis-Cebolla, J., Vilas-Boas, G.T. & Ferre, J. (2016) Susceptibility of *Grapholita molesta* (Busck, 1916) to formulations of Bacillus thuringiensis, individual toxins and their mixtures. *Journal of Invertebrate Pathology*, 141: 1–5. doi: http://dx.doi.org/10.1016/j.jip.2016.09.006

107. Rings, R.W. (1970) Economic aspects of the biological and control of the oriental fruit moth, *Grapholita molesta* Busk, in the United States. *The Ohio Journal of Science*, 70(1): 58.

108. Roberts, D.W. & Humber, R.A. (1981) Entomogenous fungi. *Biology of Conidial Fungi*, 2: 201–236.

109. Robledo, N., Rzuffi, R., & Reyes-Prado, H. (2018) Modification of behavioral response in *Copitarsia decolora* (Lepidoptera: Noctuidae) due to pre-exposure to sex pheromone and host plant volatiles. *Florida entomologist*, 101(1): 69–73. doi: https://doi.org/10.1653/024.101.0113.

110. Rodrigues, M.L., Garcia, M.S., Nava, D.E., Botton, M., Parra, J.R.P. & Guerrero, M. (2011) Selection of *Trichogramma prestiosun* linages for control of Grapholita molesta in peach. *Florida Entomologist*, 94(3): 398–403. doi: https://doi.org/10.1653/024.094.0303

111. Rothschild, G.H.L. & Vickers, R.A. (1991) Biology, ecology and control of the oriental fruit moth. Tortricid Pests: Their Biology, natural Enemies

and Control. *Elsevier Science publication*, 72: 389–412. doi: https://doi.org/110.1038/ng.2007.60

112. Ryu, J.H., & Ha, E.M. (2006) An essential complementary role of NF-kB pathway to microbicidal oxidants in *Drosophila* gut immunity. *The EMBO Journal*, 25: 3693–3701. doi: https://doi.org/10.1038/sj.emboj.7601233

113. Ryu, J.H., Ha, E.M. & Lee, W.J. (2010) Innate immunity and gut–microbe mutualism in *Drosophila*. *Developmental and Comparative Immunology*, 34: 369–376. doi: https://doi.org/10.1016/j.dci.2009.11.010

114. Sarker, S., Woo, Y.H. & Lim, U.T. (2020) Laboratory Evaluation of *Beauveria bassiana* ARP14 Against *Grapholita molesta* (Lepidoptera: Tortricidae). *Current Microbiology*, 77(9): 2365–2373. doi: https:// doi.org/10.1007/s00284-020-02012-4

115. Shah, P.A. & Pell, J.K. (2003) Entomopathogenic fungi as biological control agents. *Applied microbiology and biotechnology*, 61(5-6): 413–423.

116. Shen, D.X., Liu, Y., Zhou, F., Wang, G.R. & An, C.J. (2014). Identification of Immunity-Related Genes in *Ostrinia furnacalis* against Entomopathogenic Fungi by RNA-Seq Analysis. *PLOS One*, 9(1): e86436. doi: https://doi.org/10.1371/journal.pone.0086436

117. Söderhäll, I., Wu, C.L., Novotny, M., Lee, B. L. & Söderhäll, K. (2009) A Novel Protein Acts as a Negative Regulator of Prophenoloxidase Activation and Melanization in the Freshwater Crayfish *Pacifastacus leniusculus*. *The Journal of biological chemistry*, 284: 6301–6310. doi:

https://doi.org/10.1074/jbc.M806764200

118. Song, X.B., Zheng, W.F. & Ren, S.T. (1993) Investigation and prevention and control of the overwintering rule of oriental fruit moth. Shan xi Forest Science and Technology, 59–61 (in Chinese).

119. Srivastava, N., Maurya, P., Sharma, P. & Mohan, L. (2009) Prospective role of insecticides of fungal origin: *Review. Entomological Research*, 39: 341–355. doi: https://doi.org/10.1111/j.1748-5967.2009.00244.x

120. Strand, M.R. & Clark, K.D. (1999) Plasmatocyte spreading peptide induces spreading of plasmatocytes but represses spreading of granulocytes. *Archives of Insect Biochemistry and Physiology*, 42: 213–223. doi: https://doi.org/10.1002/(SICI)1520 6327(199911) 42:3<213: AID-ARCH5>3.0.CO;2-4.

121. Suwanchaichinda, C., Ochieng, R. & Zhuang, S. (1999) Molecular cloning and biochemical characterization of *Manduca sexta* serpin-7, a regulator of prophenoloxidase activation. *Pathologica*, 37(3): 1530–1560. doi: https://doi.org/10.1007/s00259-013-2371-5

122. Tian, Z., Li, Y., Xing, Y.J., Li, R.C. & Liu, J.Y. (2019) Structural Insights into Two Representative Conformations of the Complex Formed by *Grapholita molesta* (Busck) Pheromone Binding Protein 2 and Z-8-Dodecenyl Acetate. *Journal of Agricultural and Food Chemistry*, 67(16): 4425–4434. doi: https://doi.org/10.1021/acs.jafc.9b00052

123. Timm, A.E., Geertsema, H. & Warnich, L. (2008) Population genetic

structure of *Grapholita molesta* (Lepidoptera: Tortricidae) in South Africa. *Annals of the Entomological Society of America*, 101: 197–203.

124. Torriani, M.V.G., Mazzi, D., Hein, S. & Dorn, S. (2010) Structured populations of the oriental fruit moth in an agricultural ecosystem. *Molecular Ecology*, 19(13): 2651–60. doi: https://doi.org/10.1111/j.1365-294X.2010.04711.x

125. Tripathi, L.P. & Sowdhamini, R. (2008) Genome-wide survey of prokaryotic serine proteases: analysis of distribution and domain architectures of five serine protease families in prokaryotes. *BMC Genomics*, 9, 549. doi: http://dx.doi.org/10.1186/1471-2164/9/594

126. Van, G.D., Sharp, P., Morgan, K. & Kashers, N. (2003) Serpins: structure, function and molecular evolution. *International Journal of Biochemistry & Cell Biology*, 35: 1536–1547. doi: http://dx.doi.org/10.1016/s1357-2725(03)00134-1

127. Varela, N., Avilla, J., Anton, S. & Gemeno, C. (2011) Synergism of pheromone and host-plant volatile blends in the attraction of *Grapholita molesta* males. *Entomological experiment and application*, 141(2): 114–122. doi: https://doi.org/10.1111/j.1570-7458.2011.01171.x

128. Vella, H. & Engstr, M.Y. (2007) A multilayered defense against infection: combinatorial control of insect immune genes. *Trends in Genetics*. 23(7), 342–349. doi: https://doi.org/10.1016/j.tig.2007.05.003

129. Vey, A., Matha, V. & Dumas, C. (2002) Effects of the peptide mycotoxin destruxin E on insect haemocytes and on dynamics and efficiency of

the multicellular immune reaction. *Journal of Invertebrate Pathology*, 80: 177–187. doi: https://doi.org/10.1016/S0022-2011(02)00104-0

130. Wanchoo, A., Lewis, M.W. & Keyhani, N.O. (2009) Lectin mapping reveals stage-specific display of surface carbohydrates of in vitro and haemolymph derived cells of the entomopathogenic fungus *Beauveria bassiana*. *Microbiology*, 155: 3121–3133. doi: https://doi.org/10.1099/mic.0.029157-0

131. Wang C.Y. (2006) Comprehensive prevention and control of pyridae in yellow pear producing areas in Qinghai. *Qinghai Agricultural Technology Extension*, (2): 43–44. (in Chinese)

132. Wang, C.S. & Leger, R.J.S. (2006) A collagenous protective coat enables Metarhizium anisopliae to evade insect immune response. *Proceedings of the National Academy of Sciences of the United States of America*, 103(17): 6647–6652. doi: https://doi.org/10.1073/pnas.0601951103

133. Wang, H.C. & You, M.S. (1999) Mechanism of insect invasion by *Metarhizium anisopliae*. *Bulletin of Microbiology*, 26(1): 71–73.

134. Wang, H.L., He, H.P. & Gong, L.Z. (2012) Observation on the occurrence dynamic of Grapholita molesta Busk in peach orchard. *Hubei Agricultural Science*, 51: 4784–4786.

135. Wang, J.X. & Ma, L.J. (2009) Application of entomogenous fungi in biological control of agroforestry pests. *Journal of Zhejiang A&F University*, 26(2): 286–291.

136. Wei, C.C., Xiu, J.F., Hu, Y., Shang, X.L., Zhang, Y.C., Wu, J.W. (2017)

Cloning, sequence analysis and expression pattern of three serine protease inhibitors (Serpin) in *Musca domestica*. *Journal of Environmental Entomology*, 39(6): 1334–1341. (in Chinese) doi: http://dx.doi.org/10. 3969 /j. issn. 1674 — 0858. 2017. 06. 20

137. Wu, T., Zhao, Y., Wang, Z., Song, Q., Wang, Z., Xu, Q., Wang, Y., Wang L., Zhang, Y. & Feng, C. (2018) β-1,3-Glucan recognition protein 3 activates the prophenoloxidase system in response to bacterial infection in *Ostrinia furnacalis* Guenée. *Developmental and Comparative Immunology*, 79: 31–43. doi: http://dx.doi.org/10.1016/j.dci.2017.10.004

138. Wu, W., Xu, H.L., W, Z.L. & Yu, X.P. (2022) Cloning and Function Analysis of a Serine Protease Inhibitor Gene Nlserpin2 in *Nilaparvata lugens*. *Scientia Agricultura Sinaca*, 55(12): 2338–2346. doi: http://dx.doi.org/10.3864/j.issn.0578-1752.2022.12.006

139. Xia, J., Zhang, C.R., Zhang, S., Li, F.F., Feng, M.G., Wang, X.W. & Liu, S.S. (2012) Analysis of Whitefly Transcriptional Responses to *Beauveria bassiana* Infection Reveals New Insights into Insect-Fungus Interactions. *PLOS One*, 8(7): e68185. doi: https://doi.org/10.1371/journal.pone. 0068185.

140. Xiao, Y., Ren, S.X. & Qiu, B.L. (2001) Application of entomogenic fungi to control *Bemisia tabaci*. *Journal of Environmental Entomology*, 23(1): 30–34.

141. Xu, J., Wang, X. F., Chen, P., Liu, F. T., Zheng, S. C., Ye, H. & Mo M.H. (2016) RNA Interference in Moths: Mechanisms, Applications, and Progress. *Gene*,

7(10): 88. doi: https://doi.org/10.3390/genes7100088

142. Xu, P.Z., Zhang, M.R. & Cheng, Y.C., Huang, L., Tan, X. & Xia, Q.Y. (2010) Molecular Cloning and Expression Profile Analysis of Genes Encoding Pattern Recognition Receptors PGRP and βGRP in the Silkworm, *Bombyx mori*. *Science of Sericulture*, 36(3): 0383 − 0390. doi: http://dx.doi.org/10.13441/j.cnki.cykx.2010.03.024

143. Yan, S., Ren, B. Y. & Shen J. (2021) Nanoparticle-mediated double-stranded RNA delivery system: A promising approach for sustainable pest management. *Insect Science,* (1): 21–34.

144. Yan, S., Ren, B. Y. & Shen, J. (2020) Improving RNAi efficiency for pest control in crop specie. *Biotechniques*, 68(5): 283–290.

145. Yang, J., Lee, K.S., Kim, B.Y., Choi, Y.S., Yoon, H.J., Jia, J. & Jin, B.R. (2017) Anti-fibrinolytic and anti-microbial activities of a serine protease inhibitor from honeybee (*Apis cerana*) venom. Comparative Biochemistry and Physiology. *Toxicology and Pharmacology*, 201: 11–18.

146. Yang, L, Keyhani, N.O., Tang, G., Tian, C., Lu, R., Wang, X., Pei, Y., Fan, Y. (2014) Expression of a toll signaling regulator serpin in a mycoinsecticide for increased virulence. *Applied and Environmental Microbiology*, 80(15): 4531–4539.

147. Yang, S., Huang, F.S. & Duan, J.H. (2005) Cloning of the serine protease gene from *Anopheles stephensi* and its relationship with melanization of *Plasmodium yoelii* oocysts. *Chinese Journal of Zoonoses*, 21(12): 1059–1063.

148. Yang, X.F., Fan, F., Wang, C. & Wei, G.S. (2016) Where does *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) overwinter in adjacent peach, pear and apple orchards? *Bulletin of Entomological Research*, 106(1): 135–140. doi: http://dx.doi.org/10.1017/S0007485315000887

149. Yokoyama, V.Y., Miller, G.T. & Harvey, J.M. (1987) Development of oriental fruit moth (Lepidoptera: Tortficidae) on a laboratory diet. *Journal of Economic Entomology*, 80: 272–276. doi: http://dx.doi.org/10.1093/jee/80.1.272

150. Zhai, H., Yu, X.M., Ma, Y.N., Zhang, Y. & Wang, D. (2019) Sugar-Acetic Acid-Ethanol-Water Mixture as a Potent Attractant for Trapping the Oriental Fruit Moth (Lepidoptera: Tortricidae) in Peach-Apple Mixed-Planting Orchards. *Plants*, 8: 401. doi: https://doi.org/10.3390/plants8100401

151. Zhang, X.Z. (1980) Preliminary report on a study of the oriental fruit moth. Journal of plant protection, 7(4), 254–256 (in Chinese)

152. Zhang, Y., Tang, M.Y., Dong, Z.M., Zhao, D.C., An, L.N., Zhu, H.T., Xia, Q.Y. & Zhao, P. (2020) Synthesis, secretion, and antifungal mechanism of a phosphatidylethanolamine-binding protein from the silk gland of the silkworm *Bombyx mori. International Journal of Biomacromolecules*, 149, 1000–1007. doi: https://doi.org/10.1016/j.ijbiomac.2020.01.310

153. Zhao, J. L., Zhang Y. Z., Li J. L., Guo F. Z. & Chao D. (2010) IPM Measures of Fruit Borer in Organic Orchard. *Journal of Shanxi Agricultural Sciences*, 38(5), 39–43 (in Chinese).

154. Zhao, P., Dong, Z., Duan, J., Wang, G., Wang, L., Li, Y., Xiang, Z. &

Xia, Q. (2012) Genome-wide identification and immune response analysis of serine protease inhibitor genes in the silkworm, *Bombyx mori. PLoS ONE*, 7, e31168. doi: https://doi.org/10.1371/journal.pone.0031168

155. Zheng, Y., Hu, Y., Yan, S. (2019) A polymer/detergent formulation improves dsRNA penetration through the body wall and RNAi-induced mortality in the *soybean aphid Aphis glycines*. *Pest Management Science*, 75(7): 1993–1999.

156. Zheng, Y., Peng, X., Liu, G.M., Pan, H.Y., Dorn, S. & Chen, M.H. (2013) High Genetic Diversity and Structured Populations of the Oriental Fruit Moth in Its Range of Origin. *PLOS ONE*, 8(11): 1–12. doi: https://doi.org/10.1371/journal.pone.0078476

157. Zheng, Y.P., He, W.Y. & Beliveau, C. (2009) Cloning, expression and characterization of four serpin-1 cDNA variants from the spruce budworm, *Choristoneura fumiferana*. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 154(2): 165–173. doi: https://doi.org/10.1016/j.cbpb.2009.05.016

158. Zhi, Y.R., Ye, X.H. & Lan, Y. (2008) Occurrence and prevention measures of the oriental fruit moth in fruit tree mixed planting area. *Journal of Southwest Agriculture*, 21(4): 1006–1009 (in Chinese).

159. Zhu, Y., Wang, Y. & Gorman, M.J. (2003) *Manduca sexta* serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases. *Journal of Biological Chemistry*, 278(47): 46556.

160. Zimmermann, G. (2007) Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii. Biocontrol Science and Technology*, 17(5/6): 553–596. doi: http://dx.doi.org/10.1080/09583150701309006

161. Zou, Z., Zhao, P., Weng, H., Mita, K. & Jiang, H.B. (2009) A comparative analysis of serpin genes in the silkworm genome. *Genomics*, 93(4): 367–75. doi: http://dx.doi.org/10.1016/j.ygeno.2008.12.010.

162. Zunic, A., Vukovic, S., Lazic, S., Sunjka, D. & Baskovic, D. (2020) The efficacy of novel diamide insecticides in *Grapholita molesta* suppression and their residues in peach fruits. *Plant protection Science*, 56(1): 46–51.

APPENDICES

Appendix A

ЗАТВЕРДЖУЮ
Проректор з науково-педагогічної роботи
д.біол.н., професор
_____Ігор КОВАЛЕНКО

ДОВІДКА

про впровадження результатів наукових досліджень у навчальному процесі

Видана Цао Чжишань (Cao Zhishan) у тому, що матеріали дисертаційної роботи «Біологічний контроль розповсюдження *Grapholitha Molesta* за допомогою імунологічних особливостей (Biological Control of Distribution *Grapholitha Molesta* Through Immune Features), які опубліковані в статтях:

Cao Zhishan, Cao Jinjun, Vlasenko Volodymyr, Wang Xinfa, Li Weihai. Transcriptome analysis of *Grapholitha molesta* (Busk) (Lepidoptera: Tortricidae) larvae in response to entomopathogenic fungi *Beauveria bassiana*. Journal of Asia-Pacific Entomology, 25 (2022) 101926. https://doi.org/10.1016/j.aspen.2022.101926

Cao Zhishan, Cao Jinjun, Zhu Hongxia, Vlasenko Volodymyr. Molecular identification of a short-type peptidoglycan recognition protein, *GmPGRP-SC* from *Grapholitha molesta*. Bulletin of the Sumy National Agricultural University of Agricultural Cultures, 3 (45), 2021. https://doi.org/10.32845/agrobio.2021.3.7

Cao Zhishan, Vlasenko Volodymyr, Li Weihai. Development of biological control of oriental fruit moth and insect immune response induced by entomopathogenic fungi. Bulletin of the Sumy National Agricultural University of Agricultural Cultures, 2 (40), 2020. https://doi.org/10.32782/agrobio.2020.2.10

Cao Zhishan, Vlasenko Volodymyr. Application and development prospect of RNA interference technology in pest control. Bulletin of the Sumy National Agricultural University of Agricultural Cultures. 2 (48), 2022. https://doi.org/10.32845/agrobio.2022.2.1

Cao Zhishan, Vlasenko Volodymyr. Laboratory Evaluation of the effect of *Beauveria bassiana* on the vital activity of *Grapholitha molesta* (Lepidoptera: Tortricidae). Bulletin of the Sumy National Agricultural University of Agricultural Cultures. 4 (50), 2022. https://doi.org/10.32845/agrobio.2022.4.1

Включені до навчальних програм (силабусів) дисциплін «Основи біологічного захисту рослин від шкідливих організмів», «Моніторинг шкідників сільськогосподарських культур та організація заходів регулювання їх чисельності», «Управління чисельністю комах-фітофагів», «Технології вирощування і використання організмів у біологічному захисті рослин» та використовуються в навчальному процесі підготовки фахівців спеціальності «Захисті карантин рослин» першого та другого рівнів вищої освіти. Також, практичні результати роботи, впроваджено у виробничу діяльність навчальної лабораторії садівництва та виноградарства.

Довідка видана для надання до спеціалізованої вченої ради.

Завідувач кафедри захисту рослин
к.с.-г.н., доцент
_____*Валентина ТАТАРИНОВА*

Завідувач навчальної лабораторії
садівництва та виноградарства
к.с.-г.н., доцент
_____*Сергій ГОРБАСЬ*

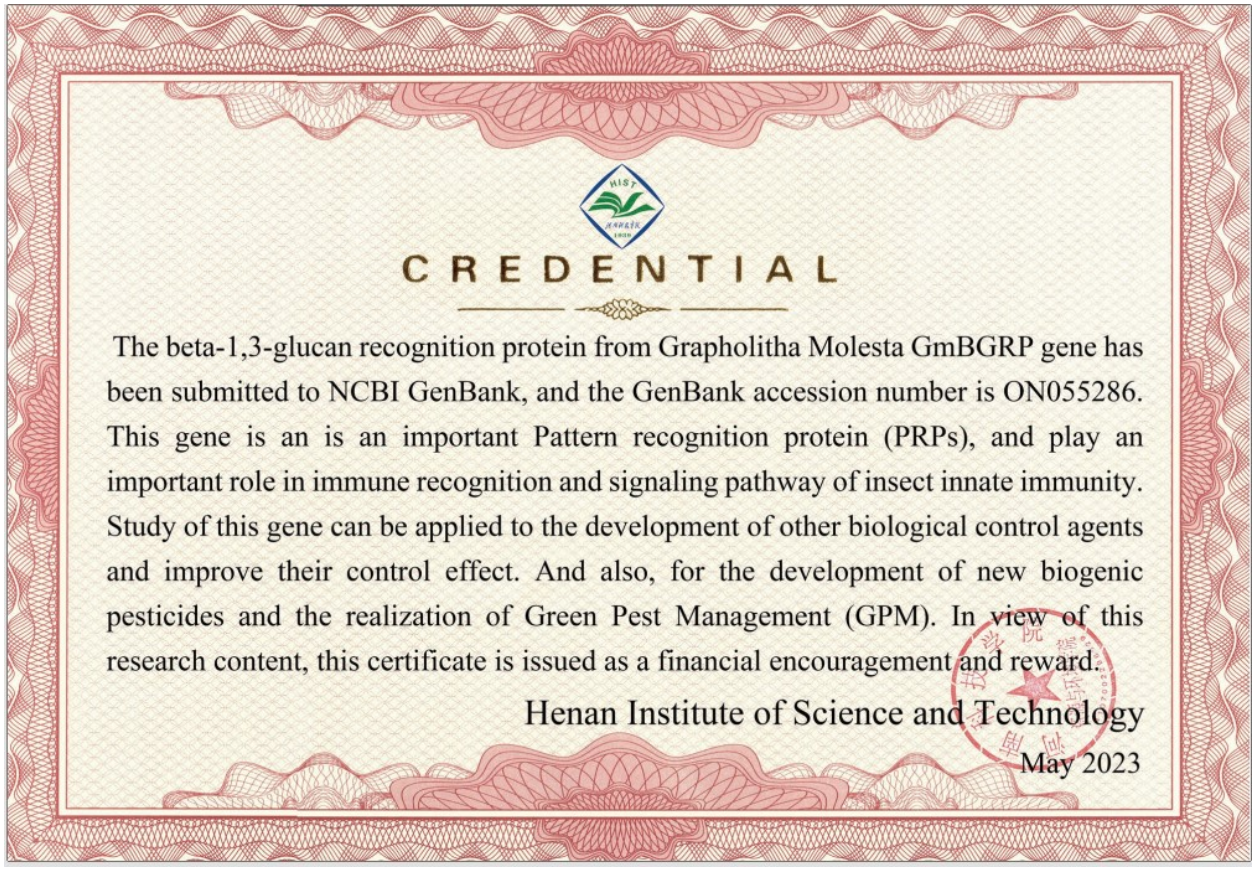
Appendix B

CREDENTIAL

The Peptidoglycan recognition protein from Grapholitha Molesta GmPGRP-SC gene has been submitted to NCBI GenBank, and the GenBank accession number is MW773839. This gene is an important pattern recognition receptor, this gene can as a potential target molecule, and provide scientific basis for the development of new biogenic pesticides and the realization of Green Pest Management (GPM). In view of this research content, this certificate is issued as a financial encouragement and reward.

Henan Institute of Science and Technology

May 2023



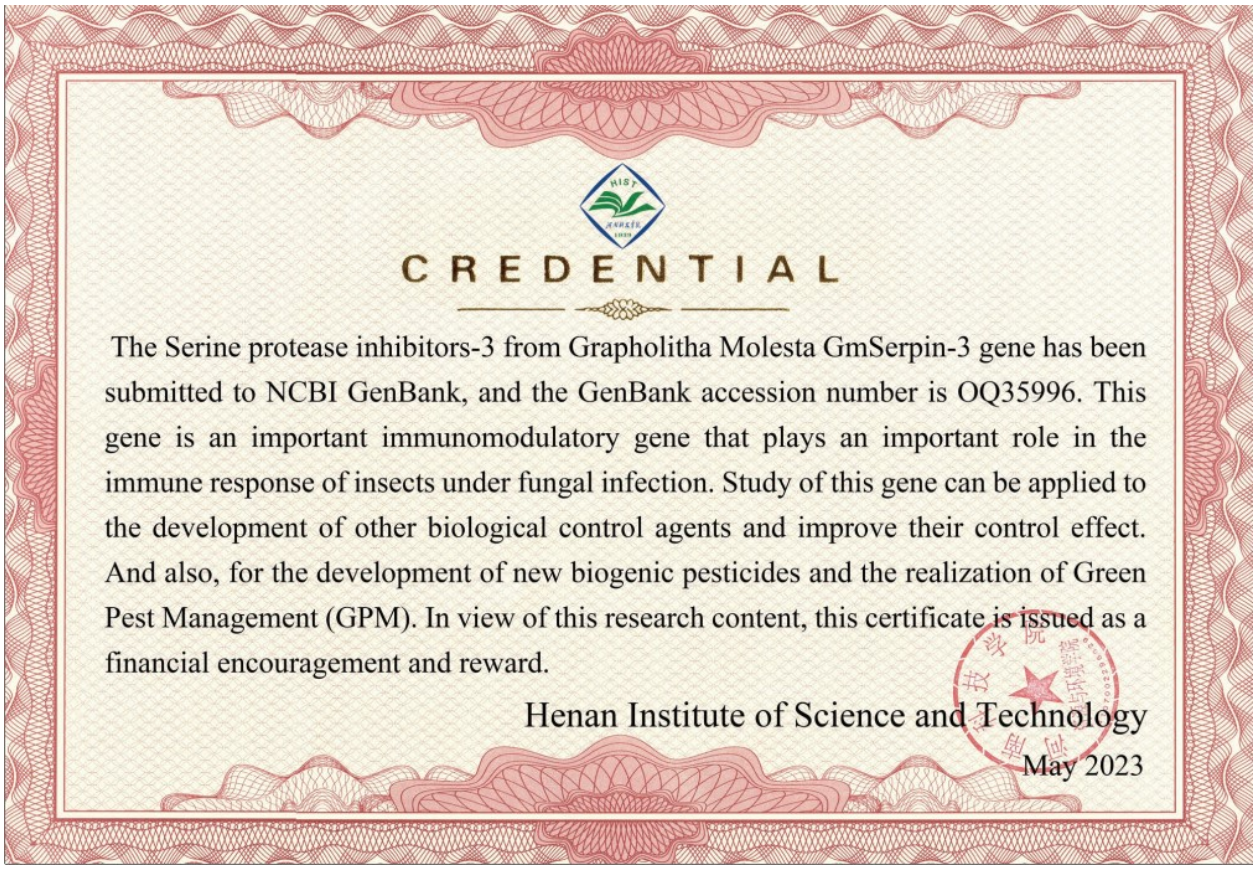
CREDENTIAL

The beta-1,3-glucan recognition protein from Grapholitha Molesta GmBGRP gene has been submitted to NCBI GenBank, and the GenBank accession number is ON055286. This gene is an is an important Pattern recognition protein (PRPs), and play an important role in immune recognition and signaling pathway of insect innate immunity. Study of this gene can be applied to the development of other biological control agents and improve their control effect. And also, for the development of new biogenic pesticides and the realization of Green Pest Management (GPM). In view of this research content, this certificate is issued as a financial encouragement and reward.

Henan Institute of Science and Technology

May 2023

Continuation of Appendix B

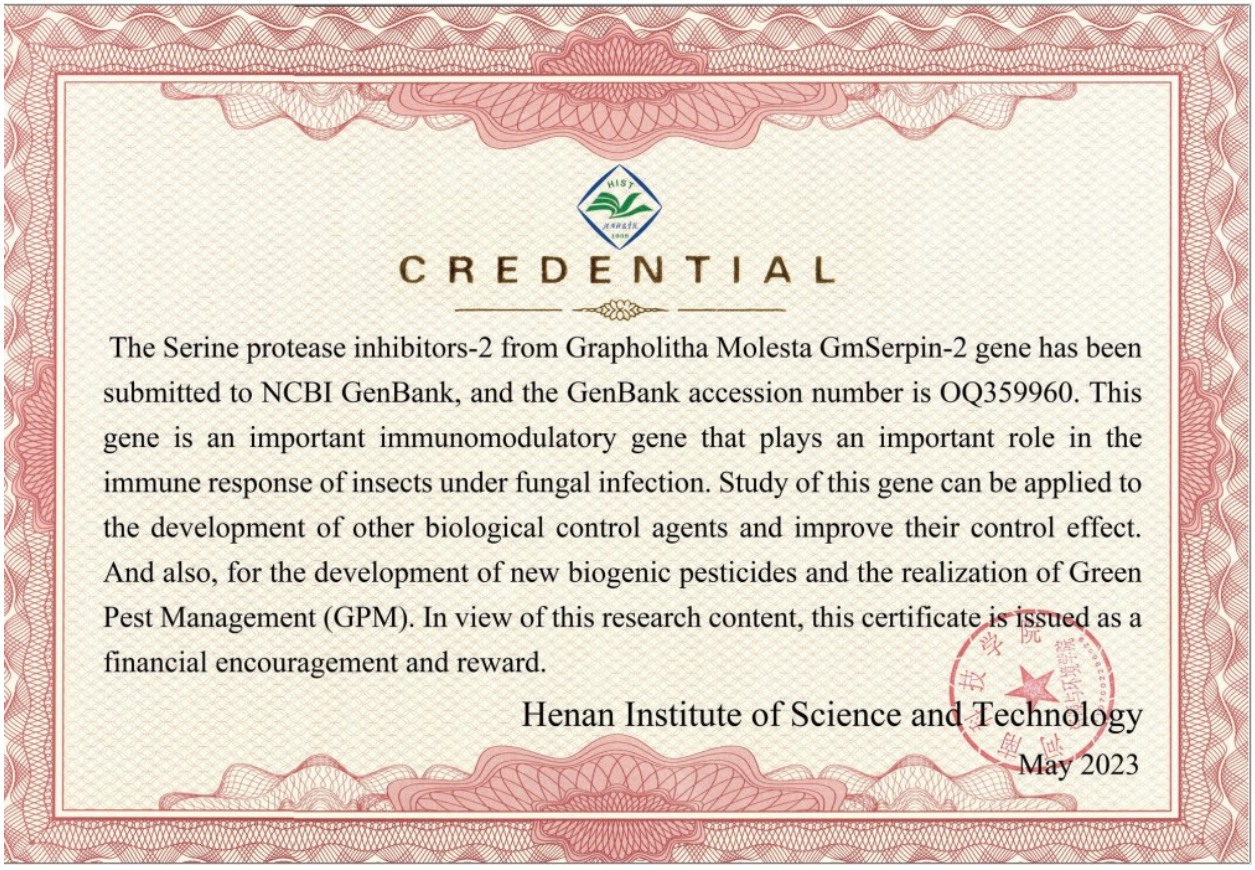


CREDENTIAL

The Serine protease inhibitors-3 from Grapholitha Molesta GmSerpin-3 gene has been submitted to NCBI GenBank, and the GenBank accession number is OQ35996. This gene is an important immunomodulatory gene that plays an important role in the immune response of insects under fungal infection. Study of this gene can be applied to the development of other biological control agents and improve their control effect. And also, for the development of new biogenic pesticides and the realization of Green Pest Management (GPM). In view of this research content, this certificate is issued as a financial encouragement and reward.

Henan Institute of Science and Technology

May 2023



CREDENTIAL

The Serine protease inhibitors-2 from Grapholitha Molesta GmSerpin-2 gene has been submitted to NCBI GenBank, and the GenBank accession number is OQ359960. This gene is an important immunomodulatory gene that plays an important role in the immune response of insects under fungal infection. Study of this gene can be applied to the development of other biological control agents and improve their control effect. And also, for the development of new biogenic pesticides and the realization of Green Pest Management (GPM). In view of this research content, this certificate is issued as a financial encouragement and reward.

Henan Institute of Science and Technology

May 2023

Appendix C

Investigation on the occurrence and dynamics of *G. molesta* in peach orchard and evaluation on the control of five different biological pesticides