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Transcriptome analysis of *Callosobruchus chinensis*: insight into the biological control using entomopathogenic bacteria, *Bacillus thuringiensis*

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Abstract

Bacillus thuringiensis based microbial pesticide is a potential alternative to fumigants. However, the effect of this entomopathogenic bacteria on stored-product pests, and the molecular mechanisms of insect response remain to be investigated. In this study, we showed that *B. thuringiensis* exhibited toxicity against *Callosobruchus chinensis* (L.) larvae. The 50% and 95% lethal concentrations in response to *B. thuringiensis* were 1.08×10^7 and 4.27×10^7 spores, respectively. We established a transcriptome to identify differential expressed genes. The predicted unigenes were categorized into 62 GO terms. A total of 187 DEGs were identified according to RNA-seq analysis, which 73 were downregulated and 114 were upregulated. And we preformed RT-qPCR assays to validate RNA-seq results. Both RNA-seq and RT-qPCR indicated that L-lactate dehydrogenase and glycoside hydrolase were inhibited in response to *B. thuringiensis*, while antimicrobial peptide, GST, and heat shock 90 were induced, suggesting that these genes might be involved in *B. thuringiensis* insecticidal effect for storage pests. A better understanding of molecular response to *B. thuringiensis* will facilitate us to develop a viable strategy method to control stored-product pests.

Keywords: adzuki bean weevil; biological control; *Bacillus thuringiensis*; transcriptome analysis.

Practical Application: Pathogen injection is more quantitative and straightforward than oral ingestion for some stored-product pests. The toxic effect of *B. thuringiensis* on *C. chinensis* larvae was monitored. To investigate the molecular response of *C. chinensis* exposed to *B. thuringiensis*, we injected *B. thuringiensis* spores into *C. chinensis* larvae to analyze genic changes after infection in this study. A majority of *B. thuringiensis*-response genes was identified by RNA-seq analysis and verified by RT-qPCR. This research provides us some potential targets, such as effectors, receptors, and singling pathways, for *B. thuringiensis*-based biopesticide to control stored-product pests.

1 Introduction

Approximately 10-20% stored agricultural products are lost to storage insect pests in the world each year (Weaver & Petroff, 2004). Sometimes, it can even exceed the losses during the growing period of the crop. To protect the stored products from insect damage, some disinfest technologies are applied in commodity storage, including high/low temperature, controlled atmosphere, and ionizing radiation (Fields & White, 2002; Boyer et al., 2012b; Wang et al., 2018). A limit of irradiation usage in pest control was that some individuals remain alive for weeks after treatment even with generally damage and sterility (Sang et al., 2016; Wang et al., 2019). Chemical fumigation is the most effective method to control storage pests (Fields & White, 2002). However, methyl bromide had been phased out owning to damage to the ozone layer. Phosphine was a widely used fumigation due to its easy application and high effectiveness. Increasing pest resistance and long fumigation limited the sustainable application of phosphine (Benhalima et al., 2004; Oppert et al., 2015). Other candidate fumigants, such as sulfuryl fluoride (Jagadeesan et al., 2021), ethylene oxide (Bessaire et al., 2021), and methyl benzoate (Mostafiz et al., 2022; Wang et al., 2022), may taint the storage commodity, leaving phytotoxic residues, or can not be registered by economic/health reasons.

Microbial pesticide is a kind of eco-friendly methods to control insect pests, due to its less inherently damage to environment and effective disinfestation to target pests. *Bacillus thuringiensis* (Malaikozhundan & Vinodhini, 2018), *Beauveria bassiana* (Shi et al., 2019) and *Metarhizium anisopliae* (Sabbour, 2003) are commonly used as biopesticides to control insect pests. For example, the *B. bassiana* and *M. anisopliae* can be used to control *Spodoptera littoralis* (Boisduval) under laboratory conditions. The population of *Callosobruchus maculatus* F. significantly reduced when treated with *B. bassiana* (Cherry et al., 2005). *B. thuringiensis* is gram-positive bacteria of *Bacillaece* family (Schnepf et al., 1998). It produces parasporal crystals which is toxic for insect pests in agricultural plants and products. The crystal protein is a promising biopesticide to control Lepidoptera, Diptera and Coleoptera pests, due to its highly target specificity and environmental safety (Burges, 1981; Aranda et al., 1996; Bohorova et al., 1996). It was reported that *B. thuringiensis*-abamectin diet significantly prolonged the developmental time for *Helicoverpa armigera* (Hübner) larvae (Wanna et al., 2010). In *Bracon brevicornis* Wesmael, the egg hatching, emergence rate and longevity decreased in the *B. thuringiensis* feeding insects compared to non- *B. thuringiensis* individuals (Temerak, 1982). In stored-product pests, *B. thuringiensis* was

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also a potential biological agent to control *Sitophilus granaries* (L.), *Tribolium castaneum* (Herbst), and *C. maculatus*. A dose of 4×10^8 cells/mL of *B. thuringiensis* led to 100% mortality of *C. maculatus*. The significant delay in *C. maculatus* larval, pupal, and total development period was observed after *B. thuringiensis* treatment (Malaikozhundan & Vinodhini, 2018). However, it required more *B. thuringiensis* to control *S. granaries* and *T. castaneum*, which indicated that these pests were not susceptible to *B. thuringiensis*. As the concern of environmental awareness and food safety increased, *B. thuringiensis* based biopesticide will become more and more important in the context of Integrated Pest Management (IPM), to protect stored grains from pests damage (Melo et al., 2016; Domínguez-Arrizabalaga et al., 2020). However, poor information about the molecular response of stored-product pests to *B. thuringiensis* is known, which limits the widely applications of microbial pesticide.

The *Callosobruchus chinensis* (L.) (Coleoptera: Bruchidae) is an insect pest for serious postharvest seed loss in leguminous crops, particularly in the legume growing areas, such as Brazil, India and China. Female adults can lay 50-100 eggs on the seed surface. Larvae and pupae feed and develop inside the seeds. New adults emerge, mate and a new infestation cycle begins. The total life cycle takes about a month. Insects reproduce rapid and can result in 100% infestation to leguminous seeds within few months (Wang et al., 2019). Infested seeds with weight loss and low nutrition have no market value. Most paper use *B. thuringiensis* diet to feed insect pests to evaluate its effects. However, it is difficult to assess the route of administration because adzuki bean weevil lives inside the beans, and does not survive well in artificial diets. *B. thuringiensis* injection is more quantitative and straightforward than oral ingestion for *C. chinensis*. In this paper, we injected *B. thuringiensis* spores into *C. chinensis* larvae to analyze the transcriptomic changes after infection. The morality of *C. chinensis* larvae in response to *B. thuringiensis* was monitored. By using RNA-seq technology, we identified differentially expressed *B. thuringiensis*-response genes, and verified the gene expression profiles by using real-time quantitative polymerase chain reaction (RT-qPCR). As we gradually achieve a better understanding of molecular response to *B. thuringiensis*, we may be able to develop a viable *B. thuringiensis*-based biopesticide (targeting identified genes, receptors, singling pathway et al.) to control stored-product pests.

2 Materials and methods

2.1 C. chinensis and B. thuringiensis culture

The adzuki bean weevils were maintained on cowpea seeds in 400 mL plastic cups in an environmental chamber (27 °C, 60% R.H.). To get synchronized weevils, approximately 200 adults were mixed with 150 cowpeas for egg laying (2 h). These eggs were collected and incubated for further development. Different developmental stages were determined as we previously described (Wang et al., 2019). Briefly, eggs were laid on the seed surface and easily discernible. The change of egg color (from clear to white) was considered as successfully egg hatch. The number of the larvae were determined by successfully hatched eggs. Infected seeds were broken open once time a day to trace larval development. Head capsule size was used as a standard to distinguish weevil larval stages. We used a previously method to culture and collect *B. thuringiensis* with a little modification (Cossentine et al., 2019). Briefly, *B. thuringiensis* were inoculated in 600 mL CCY medium and incubated in a rotary-shaker (Peiying, Suzhou, China) at 200 rpm at 30 °C for 3 d. The cultured *B. thuringiensis* were collected by centrifugation at 5,000 g for 5 min, washed by sterile phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and plated for colony counting under microscope (Olympus BX51, Tokyo, Japan).

2.2 Insect bioassay

To study the toxic effect of *B. thuringiensis* on adzuki bean weevils, batches of 50 larvae were plated on a plastic Petri dish (15 cm × 2 cm, Sangon Biotech, China), respectively. *B. thuringiensis* (0.5×10^7, 1×10^7, 2×10^7, 5×10^7 spores, suspended in PBS) were injected into each *C. chinensis* larvae using a Nanoject II Auto-Nanoliter injector (Drummond Scientific Co., Broomall, PA, USA) as we described before (Wang et al., 2021). Control individuals were injected with the same volume of PBS alone. All the control and treated *C. chinensis* larvae were cultured in artificial diets (80 g cowpea flour, 1.5 g sorbic acid, 40 g sucrose, 40 g casein, 20 g yeast, 4 g ascorbic acid, and 1 g vitamin per liter artificial diet) in the environmental chamber (27 °C, 60% R.H.). Larvae were considered dead if they were immobile after being stimulated by aseptic dissecting needle. The mortality rate was calculated after 12 h treatment. All the control and *B. thuringiensis* treatment larvae were frozen and stored at -80 °C refrigerator. At least three replicate assays were performed independently.

2.3 RNA extraction and transcriptome sequencing

Five larvae were collected after *B. thuringiensis* treatment (1×10^7 spores) described above. Total RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, USA). RNase-Free DNase (Qiagen, Valencia, CA, USA) was added to remove residual DNA. The quantity of RNA was measured by a NanoDrop spectrophotometer (NanoDrop Technologies). Then the samples were running on 1.5% (w/v) agarose gels to confirm the integrity of RNA. The transcriptional samples were performed according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Briefly, Ploy (A) mRNA was collected from total RNA by using biotin-oligo (dT) magnetic beads. The purified RNA was then sheared into small pieces by using the RNA fragmentation kit. The first-strand cDNA was synthesized by reverse transcriptase and random primers. Then the second-strand cDNA was synthesized by DNA Polymerase I and RNase H. These fragments were followed by an end repair process, a single A base addition, and the adapters ligations, respectively. The ligation fragments were purified and sized-selected by DNA clean beads (Vazyme, Nanjing, China). The cDNA library was enriched by PCR amplification and followed purified by DNA clean beads. The quantity of cDNA library was monitored by an Agilent Bioanalyzer (Aligent Technologies, Palo Alto, CA, USA) prior to seeding clusters on a flow cell. Transcriptome sequencing was performed on a NextSeq 500 platform (Illumina) by Personal Biotechnology Co., Ltd (Shanghai, China). Raw sequence reads were saved as FASTQ files and deposited in the NCBI Sequence Read Archive (SRA) database (accession number: PRJNA772303).

2.4 Sequence assembly and annotation

High quality reads were obtained from raw RNA-seq data after removing adapter sequences, low Q-value (< 20) bases, and < 25 bp reads. The processed reads were assembled using the Trinity software (Trinity software, Inc., Plymouth, NH, USA; http://trinitynaseq.sf.net) and clustered with TGICL Clustering tools (The Institute for Genomic Research, Rockville, MD, USA). The functions of assembled unigenes were annotated by BLAST analysis using NCBI non-redundant (Nr) database, Cluster of Orthologous Groups of proteins (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (E-value cutoff 10-5). Gene Ontology (GO) annotations was performed by Blast2GO software (http://www.geneontology.org).

2.5 Differential gene expression and RT-qPCR confirmation

Differential expressed genes (DEGs) were identified based on fragments per kilobase per million mapped reads (FPKM) value. The FPKM adjusts the number of fragments mapped to a transcript by the total number of fragments mapped to all unigenes and the length of the transcript. The DEseq software was used to analyze count data and the DEGs of the RNA-seq. A value of false discovery rate (FDR) < 0.05 and foldchange > 2 provided significance thresholds for gene expression differences. To validate the FPKM analysis, the expressional profiles of 8 selected DEGs (high foldchange with good annotation) were performed by using RT-qPCR. For each RNA sample, 2 μg of RNA was used to synthesize cDNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser following to the manufacturer's protocols (Takara, Japan). RT-qPCR was run on a CFX real-time thermal cycler (Bio-Rad, Hercules, CA, USA). Briefly, each reaction volume was 20 μL, including 1 μL of each primer (10 μM), 1 μL of cDNA (1/50 diluted), and 10 μL of SYBR Select Master Mix (Applied Biosystems, Foster City, CA, USA). The RT-qPCR reactions were proceeded at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 65 °C for 50 s. Dissociation curve analysis was used to determine amplification specificity. The primers were listed in Table S1. The *18S* rRNA of adzuki bean weevil was amplified as the internal control. Three biological independent replications were performed. Expression patterns were calculated using comparative C_T method, and gene expression levels were analyzed by $2^{-\Delta\Delta CT}$ approach.

2.6 Statistical analysis

Data on larval mortality rate, and gene expression levels after *B. thuringiensis* treatment were analyzed using SPSS 20.0 software (SPSS Inc., Chicago, IL). The survival rate and gene expression profiles were analyzed by one-way analysis of variance (ANOVA). Tukey's multiple range test was used for pairwise comparison for mean separation ($p < 0.05$).

3 Results

3.1 Effect of B. thuringiensis on the mortality of C. chinensis larvae

It was observed that *B. thuringiensis* significantly affected the mortality of *C. chinensis* larvae with the increasing concentration of spores (Figure 1). The mortality of *C. chinensis* larvae was 20.4%, 44.3%, and 71.7% when treated with 0.5×10^7, 1×10^7, and 2×10^7 spores, respectively. The LC_{50} (lethal concentration 50%) value

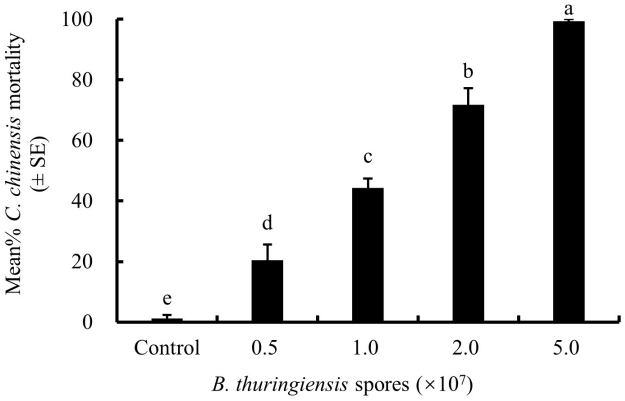

Figure 1. The mortality rate of *C. chinensis* larvae after *B. thuringiensis* injection. Batches of 50 larvae were separately placed into Petri dishes. *B. thuringiensis* spores (suspended in PBS) were injected into each *C. chinensis* larvae. All the control and treated *C. chinensis* larvae were cultured in the environmental chamber. The mortality rate was assessed after *B. thuringiensis* treatment. At least three replicate assays were performed independently. Data on larval mortality rate were analyzed by ANOVA. Tukey's multiple range test was used for pairwise comparison for mean separation ($p < 0.05$).

for individual larvae was 1.09×10^7 (0.93×10^7-1.27×10^7 with 95% confidence intervals). And the LC_{95} (lethal concentration 95%) for *B. thuringiensis* on the mortality of *C. chinensis* larvae was 4.27×10^7 (3.23×10^7-6.50×10^7 with 95% confidence intervals). A dose of 5×10^7 spores led to 100% mortality for *C. chinensis* larvae. To better understand the molecular responses of coleopteran storage pests to *B. thuringiensis*, we used the RNA-seq method to perform global transcriptome analysis of *C. chinensis* larvae.

3.2 Illumina sequencing analysis and de novo assembly

To capture the altered genes after infection, we exposed *C. chinensis* larvae to *B. thuringiensis* (treatment) and PBS (control) as samples for mRNA isolation to establish a transcriptome. High-throughput RNA-seq generated the most transcriptome for *C. chinensis*. Total of 115,098,438 and 56,574,760 paired-end raw reads were generated from the control and *B. thuringiensis* treated group, respectively. After quality checks, total 96,663,172 clean reads of control and 44,605,134 clean reads of *B. thuringiensis* group were generated after Q20 filtering. GC contents were 52.96% and 51.40%, respectively (Table S2).

3.3 Functional annotation and classification of the assembled unigenes in C. chinensis

To create RNA-seq maps, the transcriptome atlas of two groups was matched to homologs in databases with known function for cowpea bruchid. For these "non-BLASTable" transcripts, we searched them against the NCBI non-redundant (Nr) database, with an E-value threshold of 10^{-5}. A total of 41,712 unigenes had been annotated with average 839 bp length (Figure 2).

GO assignments were used to classify the function of predicted unigenes. The assembled sequences were categorized

into 62 GO terms, which contains three domains: biological process, cellular component and molecular functions (Table 1). The most abundantly expressed genes in "biological process" were involved in cellular nitrogen compound metabolic process (5.52%), biosynthetic process (5.58%), small molecule metabolic process (4.90%). The genes most abundantly expressed in the "cellular component" were those related to external encapsulating structure and "molecular functions", those involved in molecular functions (Table 1). In this study, DEGs were defined as genes exhibiting a fold transcriptional change ≥ 2 with FDR ≤ 0.05. Of the 187 identified DEGs, 73 were downregulated and 114 were upregulated (Figure 3 & Table S3).

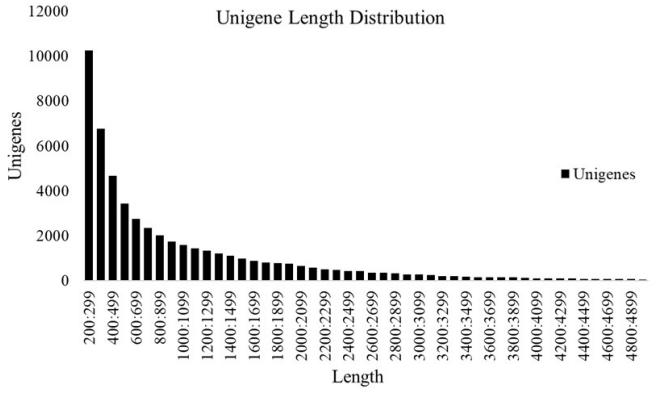

Figure 2. Overview of assembled unigenes for *C. chinensis* larvae in response to *B. thuringiensis*. The transcriptome atlas of control and *B. thuringiensis* treatment groups were matched to the NCBI non-redundant (Nr) database, with an E-value threshold of 10^{-5}. A total of 41,712 unigenes had been annotated with average 839 bp length.

3.4 Validation of differential transcript expression using RT-qPCR

To verify gene expression profiles identified from RNA-seq, RT-qPCR was performed to measure the expression of 8 selected genes (Figure 4). These genes encoded salivary gland antimicrobial peptide (*CcAMP*), lipase 3-like protein (*CcLipase*), juvenile hormone binding protein (*CcJHP*), heat shock 90 kDa protein (*CcHSP90*), L-lactate dehydrogenase isoform X2 (*CcLDH*), glycoside hydrolase family protein 28 (*CcGH*), glutathione S-transferase (*CcGST*), gut cathepsin B-like cysteine protease (*CcCatB*). In general, the RT-qPCR data matched the RNA-seq results well. *CcAMP*, *CcJHP*, *CcGST*, *CcCatB* and *CcHSP90* were upregulated, whereas *CcLDH*, *CcGH*, and *CcLipase* were downregulated more than two times, as shown by both RNA-seq and RT-qPCR.

4 Discussion

An alternative substitute for chemical insecticide to control insect pests is microbial insecticide. The global market for biocontrol agents was about 3.5 billion USD in the world with more than 10% annual increase, of which the share of microbial pesticides was about 807 million USD with 8% annual growth

Table 1. GO enrichment of differentially expressed genes in *C. chinensis*.

	Description	GO	DEGs Number	Adjusted *P*-value
Biological Process	biological process	GO:0008150	1158	1.54E-17
	biosynthetic process	GO:0009058	595	2.80E-11
	carbohydrate metabolic process	GO:0005975	139	9.90E-08
	catabolic process	GO:0009056	255	3.07E-02
	cell wall organization or biogenesis	GO:0071554	6	3.55E-02
	cellular nitrogen compound metabolic process	GO:0034641	589	3.09E-02
	cofactor metabolic process	GO:0051186	96	8.17E-16
	generation of precursor metabolites and energy	GO:0006091	87	6.31E-11
	hydrolase activity, acting on carbon-nitrogen	GO:0016810	33	2.39E-03
	isomerase activity	GO:0016853	90	3.50E-10
	ligase activity	GO:0016874	94	4.71E-03
	lipid metabolic process	GO:0006629	99	1.20E-06
	lyase activity	GO:0016829	133	3.05E-17
	methyltransferase activity	GO:0008168	64	1.33E-04
	nitrogen cycle metabolic process	GO:0071941	5	1.99E-02
	oxidoreductase activity	GO:0016491	368	1.95E-22
	small molecule metabolic process	GO:0044281	523	2.94E-32
	sulfur compound metabolic process	GO:0006790	48	2.38E-08
	transferase activity, transferring acyl groups	GO:0016746	70	1.32E-06
	transferase activity, transferring alkyl or aryl	GO:0016765	24	1.38E-04
	transferase activity, transferring glycosyl groups	GO:0016757	46	2.40E-02
Cellular Component	external encapsulating structure	GO:0030312	18	1.76E-07
Molecular Function	molecular function	GO:0003674	1149	2.09E-02
	protein binding, bridging	GO:0030674	4	3.51E-02

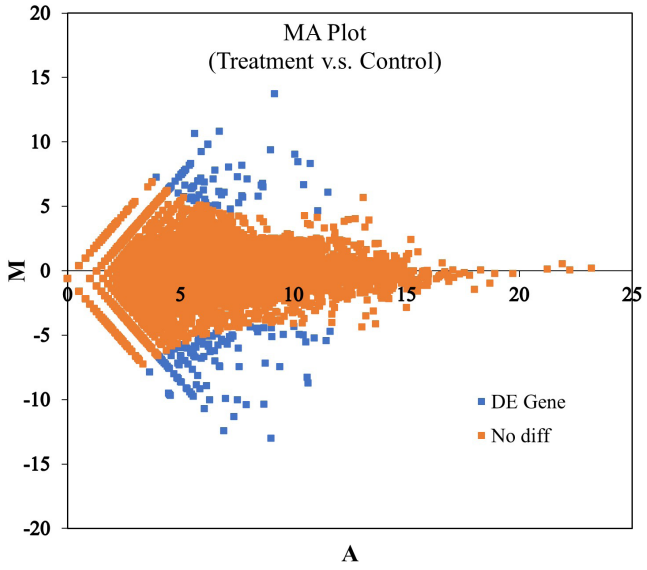

Figure 3. The "MA plot" picture of differentially expressed genes (DEGs) in *C. chinensis*. DEGs were defined as genes exhibiting a fold transcriptional change ≥ 2 with FDR ≤ 0.05. Of the 187 identified DEGs, 73 were downregulated and 114 were upregulated. Blue spot, differentially expressed gene; yellow spot, no difference in expression.

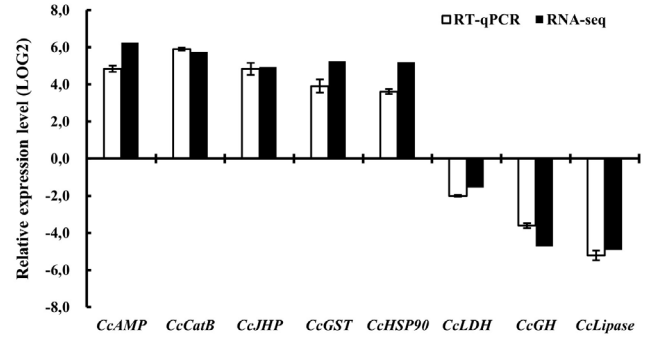

Figure 4. RT-qPCR analysis of selected transcripts to confirm expression profiles identified by RNA-seq. *CcAMP*, salivary gland antimicrobial peptide; *CcLipase*, lipase 3-like protein; *CcJHP*, juvenile hormone binding protein; *CcHSP90*, heat shock 90 kDa protein; *CcLDH*, L-lactate dehydrogenase isoform X2; *CcGH*, glycoside hydrolase family protein 28; *CcGST*, glutathione S-transferase; *CcCatB*, gut cathepsin B-like cysteine protease. Value represents mean ± SE of three independent PCR amplifications and quantifications.

(Lacey et al., 2015; Jouzani et al., 2017). Microorganisms could cause diseases for insect pest population and eventually led to death, protecting agricultural commodities from insect damage in an environmental-friendly manner. *B. thuringiensis* has been considered as the most successful bioinsecticide during 20th century (Lacey et al., 2015). It produces parasporal crystal protein or δ-endotoxins, which are toxic to many insect species including Diptera, Lepidoptera and Coleoptera. *B. thuringiensis* based bioinsecticide is efficient and host-specific, so limited adverse effects occurs in non-target organisms. With the growing demand to replace chemical pesticide for food free, the application of *B. thuringiensis* is taken into consideration to control stored-product pests (Arthurs & Dara, 2019).

Transgenic plants, such as maize, potato and cotton, which contained *B. thuringiensis* genes encoding the pesticide proteins, were raised for providing protection against pests without need for spraying. However, very little information is available on the use of microbial pesticides in stored-grains pest control, especially *C. chinensis*. And the mechanisms of *B. thuringiensis* action have not been fully elucidated in storage pests (Melo et al., 2016). Therefore, the present study was aimed to find and develop the effective control measures against the *C. chinensis* in the stored grains, possibly based on the identified genes. In this paper, we use *B. thuringiensis* injection instead of oral ingestion, mostly due to its quantifiability (Yokoi et al., 2012; Koyama et al., 2015). Our previous study also used bacteria injection, rather than oral ingestion, to analyze the transcriptome for adzuki bean weevil larvae in response to *Escherichia coli* (Wang et al., 2021). Pathogen injection may not occur in nature, but we can trace the gene expression immediately by using RNA-seq technology, instead of oral ingestion for several days which we can not quantify the bacteria ingestion. Besides, the mechanisms of *B. thuringiensis* injection may be different from oral ingestion. Pathogen injection induced insect innate immunity, while oral ingestion can only affect the digestion and intestinal immunity.

In storage pests, the efficacy of *B. thuringiensis* Cry3Aa was evaluated in *T. molitor*, *T. castaneum*, and *R. dominica* (Oppert et al., 2011). Results showed that *T. molitor* was most sensitive to Cry3Aa, followed by *R. dominica* and *T. castaneum*. A dose of 42 mg/kg Cry3Aa was fatal for *T. molitor*, and LC_{50} for *R. dominica* was 1177 mg/kg (Oppert et al., 2011). While in cowpea bruchid, the mortality of *C. maculatus* was 28% when treated with *B. thuringiensis* (3×10^5 cells), and it significantly increased to 100% when concentration increased to 4×10^8 cells (Malaikozhundan & Vinodhini, 2018). In the present study, our results showed that the LC_{50} for *C. chinensis* larvae was 1.09×10^7 spores. It required more than 5×10^7 spores to eliminate *C. chinensis* larvae (Figure 1). And immature *C. maculatus* or *C. chinensis* developed inside the beans, it probably required more *B. thuringiensis* to control weevil larvae.

It should be noted that *B. thuringiensis* significantly prolongs developmental time for insect pests. The larval and pupal period of *C. maculatus* prolonged to 24 days (4 days delay) and 12 days (4 days delay) after *B. thuringiensis* treatment, respectively (Malaikozhundan & Vinodhini, 2018). The decreasing larval weight and increasing developmental time were also observed in *T. castaneum* larvae after Cry3Aa treatment. A diet containing 2,100 mg/kg Cry3Aa extended about 50% longer larval development time for red flour beetles (Oppert et al., 2011). Also, this *B. thuringiensis*-based diet delayed developmental time to 40 days (50% longer) for *R. dominica* compared to 20 days control group (Oppert et al., 2011). Wanna et al. (2010) reported that the larval period of *H. armigera* was delayed 2.8-3.4 days after *B. thuringiensis*-abamectin treatment compared to the control. Similar results showed that the *B. thuringiensis* diet decreased

egg hatching, egg-larval duration, emergence rate, and lifespan in *B. brevicornis* (Temerak, 1982).

Next generation sequencing technology has allowed us to investigate the molecular mechanisms of *B. thuringiensis* response for storage pests. It should be noted that we did not identified a majority of DEGs as we assumed, probably due to lower than 2 fold expression change and poor genomic information. However, expression profiles of some DGEs are largely in agreement with the results in other storage pest species.

The parasporal crystals, produced by *B. thuringiensis*, damage the epithelial cells in midgut for insect peats, and may decrease the activity of digestive enzymes. It was reported that α-amylase was sharply inhibited after *B. thuringiensis* treatment in cowpea bruchid (Malaikozhundan & Vinodhini, 2018) and elm leaf beetle (Shekari et al., 2008). The activity of carbohydrate hydrolyzing enzymes, including invertase, trehalase, and amylase, were reduced by *B. thuringiensis*. In *C. maculatus*, α and β-glucosidase activity were significantly inhibited after *B. thuringiensis* treatment (Malaikozhundan & Vinodhini, 2018), which was consistent with the results in *H. cunea* larvae (Zibaee et al., 2010). The downregulation of glycoside hydrolase also suggested that *B. thuringiensis* may have a negative effect on carbohydrate metabolism in *C. chinensis* larvae. We performed RT-qPCR to estimate the expression profiles of cysteine protease (Cathepsin B-like and L-like). Interestingly, the cathepsin L was not downregulated, while cathepsin B was induced significantly after *B. thuringiensis* challenge. *B. thuringiensis* influences larval digestion by damaging gut epithelial cells. It suggested that storage pests might induce cathepsin B gene expression to compensate for digestion inhibition. The activity of α-amylase in elm leaf beetle after *Artimisia annua* extract treatment decreased at first, but it sharply increased after 48 h (Shekari et al., 2008). These data suggested that storage pests, may regulate digestive genes expression in transcriptional level in response to *B. thuringiensis*-induced digestion suppression. The survival strategy for insect pests may be based on digestion compensation, rather than digestion conservation. Moreover, Zhao et al., (2020) illuminated that Cry41 toxin enhanced the cathepsin B activity and facilitated caspase-initiated apoptosis in aphid cells. Cathepsin B has been shown to cleave antiapoptotic proteins and regulate apoptotic caspase. However, the competitive relationship between *B. thuringiensis* and cathepsin B was rarely known and need to be investigate in future.

Both the RNA-seq and RT-qPCR results indicated that GST in *C. chinensis* was upregulated significantly after *B. thuringiensis* treatment. GST is an essential antioxidant enzyme, and the upregulation of GST suggested that *C. chinensis* larvae have great antioxidant defense in response to *B. thuringiensis*. It was reported that the presence of detoxifying enzymes enabled insect pests to resist *B. thuringiensis*. There was a positive correlation between a lower mortality rate of *Aedes rusticus* larvae and GST activity (Boyer et al., 2012a). However, a difference result in *C. maculatus* suggested that the decreased GST activity may occur in response to *B. thuringiensis* (Kolawole et al., 2009; Kolawole et al., 2011; Boyer et al., 2012a). Lactate dehydrogenase (LDH) plays an important role in carbohydrate metabolism and is considered as an indicator of chemical stress (Diamantino et al., 2001). When amount of additional energy is required, LDH is involved in energy production regulation. In present study, *B. thuringiensis* significantly reduced LDH expression in *C. chinensis* larvae, which agreed with the decreased LDH activity in *C. maculatus* and *Hyphantria cunea* (Drury) (Zibaee et al., 2010; Malaikozhundan & Vinodhini, 2018). The decreased LDH activity may have a positive correlation with decreased rate of metabolism, metabolite transport, and energy synthesis. Lipases catalyze the hydrolysis of fatty acid ester bonds, and are involved in many physiological processes, including growth, reproduction, and pathogens defense (Khosravi & Sendi, 2013). Both RNA-seq and RT-qPCR results showed that lipase was downregulated after *B. thuringiensis* treatment. It was reported that *B. thuringiensis* significantly decreased the lipase activity in midgut for *Cnaphalocrocis medinali* (Guenée). Similar results also showed that the activity of lipase was inhibited when exposed to *B. thuringiensis* and plant extracts in *H. cunea* larvae (Zibaee et al., 2010). Malaikozhundan & Vinodhini (2018) found that a sharply decrease of lipase activity occurred when exposed to *B. thuringiensis* (4×10^8 cells) in *C. maculatu*s midgut.

5 Conclusion

B. thuringiensis is a promising biopesticide to control storage pests. It led to 100% mortality at 5×10^7 spores with an estimated LC_{50} of 1.09×10^7 spores for *C. chinensis* larvae. RNA-seq technology has allowed us to investigate the molecular response of storage pests exposed to *B. thuringiensis*. In this study, we presented the transcriptome profiles of *C. chinensis* larvae and identified key genes involved in *B. thuringiensis* response. We used RT-qPCR to measure the transcriptional activities of identified genes. As microbial pesticides become increasing important in the context of IPM strategies, we may be able to develop more efficient strategies to control stored-product pests.

Ethical approval

Informed consent was obtained from all individual participants included in this study. The article does not contain any studies with human participants performed by any of the authors.

Conflict of interest

The authors have declared that no conflict of interest.

Availability of data and material

Raw sequence reads were saved as FASTQ files and deposited in the NCBI Sequence Read Archive (SRA) database (accession number: PRJNA772303).

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Supplementary material

Table S1. The primers of analyzed genes by RT-qPCR.
Table S2. Overview of the obtained RNA-seq reads for *C. chinensis* larvae in response to *B. thuringiensis*.
Table S3. Differentially expressed genes indicated by the FPKM statistical analysis.

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