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Transcriptome analysis of differentially expressed genes involved in innate immunity following *Bacillus thuringiensis* challenge in *Bombyx mori* larvae

Gongqing Wu, Yunhong Yi*

School of Chemistry and Chemical Engineering, Guangdong Pharmaceutical University, Zhongshan 528458, China

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ABSTRACT

In this study, we describe RNA-seq expression profiling of larval *Bombyx mori* response to hemocoel injection of *Bacillus thuringiensis* (Bt). Two transcriptomes were generated from the hemocytes of the PBS- and Bt-injected *B. mori* larvae. More than 49 million 100-bp paired-end reads, encompassing over 7.3 Gb of sequence data, were generated for each library. After filtering the raw reads and removing the rRNA mapped reads, more than 89% of the reads in each library could be mapped to the silkworm genome reference sequence. Comparison of gene expression levels revealed that a total of 133 unigenes were upregulated while 84 unigenes were downregulated in PBS vs Bt. To further investigate the biological functions of different expression genes (DEGs), gene ontology (GO) and functional enrichment analysis were performed to map all the DEGs to terms in the GO, euKaryotic Ortholog Groups of proteins (KOG) and Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG) database. Among these DEGs, many genes involved in immunity against Bt challenge were identified. These included genes participated in pattern recognition, antimicrobial peptides, insecticide resistance or detoxification, immune melanization, cytoskeleton reorganization and many other immune effectors. To confirm the gene expression patterns identified by the RNA-seq data, the transcript levels of 10 immune related DEGs were examined by quantitative real-time PCR (qRT-PCR). The results showed that the DEGs obtained from the deep sequencing data were accurate and gene expression profiles from RNA-Seq data were reliable. Our studies provide insights into the to immune response of *B. mori* underling the stress of Bt, which is valuable to understand how Bt affects the innate immune system of silkworm and provide new approaches to control insect pests by using Bt as a biological insecticide.

1. Introduction

Insects are the most diverse species on Earth, and they rely primarily on innate immune system to fight intruding microorganisms. The insect innate immune system consists of cellular and humoral responses. The cellular immune response refers to phagocytosis, nodule formation, and encapsulation, which are mediated by circulating hemocytes (Hillyer, 2016). The humoral immune response includes the synthesis of antimicrobial peptides (AMPs), the activation of the prophenoloxidase (proPO) system, and generation of reactive oxygen species (ROS) in the fat body, hemocytes or other tissues (Hoffmann, 1995). Humoral immunity depends on three principal signaling pathways, Toll, IMD and JAK-STAT, which are conserved across various insect species indicating they play an important role in the evolution of arthropod (Ferrandon et al., 2007). The activation of these pathways is mediated by the hosts pattern recognition receptors (PRRs), such as peptidoglycan recognition protein (PGRP), β-1,3-glucan recognition protein (βGRP), lipopolysaccharide-binding protein (LBPs) and C-type lectin (CTL), which can identify the pathogen associated molecular pattern (PAMP) on the surface of invading microorganisms (Brown and Gordon, 2005; Kurata, 2014; Warr et al., 2008; Xia et al., 2018). Specifically, Gram-negative bacteria and several Gram-positive bacteria containing meso-diaminopimelic acid-type peptidoglycan (DAP-type PGN) activate the IMD pathway, while the Toll pathway is triggered by

Abbreviations: Bt, *Bacillus thuringiensis*; DEGs, different expression genes; GO, gene ontology; KOG, euKaryotic Ortholog Groups of proteins; KEGG, Kyoto Encyclopedia of Genes and Genomes Pathway; qRT-PCR, quantitative real-time PCR; proPO, prophenoloxidase; AMPs, antimicrobial peptides; ROS, reactive oxygen species; PGRP, peptidoglycan recognition protein; PRRs, pattern recognition receptors; PAMP, pathogen associated molecular pattern; LBPs, lipopolysaccharide-binding protein; βGRP, β-1,3-glucan recognition protein; NGS, next generation sequencing; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; FPKM, fragments per kilobase million; KPI, kazal-type proteinase inhibitor; ALP, alkaline phosphatase

* Corresponding author.
E-mail address: yunhongyi@163.com (Y. Yi).

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Gram-positive bacteria, yeasts, and fungi (Lim et al., 2006; Michel et al., 2001). The JAK-STAT signaling pathways mediate diversity immune responses to virus infection (Dostert et al., 2005). Therefore, humoral response play an important role in insect immune defense systems.

Silkworm, *Bombyx mori*, is agriculturally very important for silk production in many countries and now as a typical model of modern genetics and immunology research. Silkworms have several experimental advantages, including bio-safety, low cost, suitable size for performance of experiment and the availability of rich genetic resources (Matsumoto et al., 2015; Xia et al., 2004). Additionally, they are easy to culture and they are not subject to animal ethics regulations. *Bacillus thuringiensis* (Bt) is widespread in nature, being present in the gut of caterpillars of various types of moths, insect-rich environments, and in microflora on the surface of leaves (Aptosoglou et al., 1997; Maduell et al., 2002). Bt is a Gram-positive, soil-dwelling bacterium, commonly used as a biological pesticide. These pesticides are regarded as environmentally friendly, with little or no effect on humans. With the long-time and large-scale use of Bt insecticides and the enlargement of the area of transgenic Bt toxin crop, more and more pests develop resistance to Bt (Bravo and Soberon, 2008; Tabashnik et al., 2008). Previous study using flour moth *Ephestia kuehniella* as a host model demonstrated that there was a positive correlation between systemic immune induction and the ability to evolve resistance to Bt (Rahman et al., 2004). In another word, Bt tolerance can be induced by a low concentrations of the Bt formulation, which acts as an immune elicitor. To better understanding the molecular mechanisms of host-pathogen interaction in insects, many immune-related genes have been identified and analyzed using silkworm as a model over the past decade. Using suppression subtractive hybridization and transcriptome analysis, many genes encoding AMPs and PRRs have been found to be expressed differentially during *Beauveria bassiana* infection (Hou et al., 2011). In the silkworm midgut, many immune-related genes were up-regulated when infected with BmNPV and BmBDV (Liu et al., 2015; Wang et al., 2016). Furthermore, many other studies reported that infection with appropriate dose of Bt induces the cellular (e.g. encapsulation rate, coagulation index and phagocytic activity of hemocytes) and humoral (e.g. phenoloxidase activity and antibacterial activity in total hemolymph) responses in *Galleria mellonella*, *Aedes caspius* and *B. mori* larvae (Ahmed, 2013; Dubovskiy et al., 2008; Grizanova et al., 2014; Taszlow et al., 2017; Wu et al., 2015). However, the molecular regulatory mechanisms underlying host immune response to Bt infection is still unknown. There remains a need for further studies to better understand the humoral immune response to Bt in insect that can be helpful to develop effective strategies for biological control.

Recently, the high-throughput nature of next generation sequencing (NGS), using platforms such as Illumina HiSeqTM 2500 has become a very powerful and useful tool to analyze the function of insect genes. This technology provides both the sequence and frequency of RNA molecules that are present in distinct tissues or cells, during development, or upon activation of immune responses, and could aid synergistic, relative, and compositive genomics research (Malone and Oliver, 2011). The transcriptome analysis of immune-related gene expression profiles upon immune challenges has been conducted in many insect species such as *Helicoverpa armigera* (Xiong et al., 2015), *Manduca sexta* (Cao et al., 2015), *G. mellonella* (Vogel et al., 2011), *Locusta migratoria* (Zhang et al., 2015), *Plutella xylostella* (Lin et al., 2018) and also fungal and viral infection of *B. mori* (Hou et al., 2014b; Swevers et al., 2013). The complete genome sequence data from *B. mori* is now available. However, the global view of the molecular changes in silkworms during Bt infection remains largely unknown.

In this study, we employed '*B. mori* + Bt' as a model to investigate the regulation of immune responses to Bt infection of the invertebrate from transcriptomic views using NGS technology. We systematically analyzed the gene expressional profiles in the control and infected silkworm larvae and characterized previously undiscovered Bt-induced molecular events. Our study will be of benefit to the development of new approaches for preventing resistance of insect pests against biopesticides and epidemic of infectious disease in economic invertebrates mass rearing industries.

2. Materials and methods

2.1. Silkworm strain

The silkworm strain, Dazao, provided by the Guangdong academy of agricultural sciences, was used in the study. The newly exuviated larvae of the five instar were used for the experiments.

2.2. Treatment with B. thuringiensis and hemolymph collection

The bacteria *B. thuringiensis* HD-1 were cultured on Luria–Bertani (LB) broth at 28 °C on a shaker at 200 rpm to an OD_{600} of approximately 1.5. The bacterial cultures were heat-blocked for 20 min at 100 °C and diluted to a concentration of 10^7 cells/ml with phosphate buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 1000 ml distilled water, pH 7.2). The larvae were immune primed by a hemocoel injection of 10 µl of PBS containing 1×10^5 heat killed cells of HD-1 per larva. The control ones were injected with 10 µl of PBS. Then all the larvae were reared on fresh mulberry leaves at 25 °C. Forty-eight hours after injection, about 50 µl of hemolymph was collected from each larva (20 larvae per group) each one biological replicate and preserved in Trizol in liquid nitrogen for RNA isolation.

2.3. Isolation of total RNA

The total RNA was extracted from the hemocytes of both Bt-challenged and control larvae by using Trizol reagent (Invitrogen). Total RNA quality was assessed using a Agilent Technologies 2100 Bioanalyzer System, and the RNA integrity was determined via RNase free agarose gel electrophoresis. Only extractions showing distinctive 28S and 18S bands and 260 nm/280 nm ratio was between 1.8 and 2.0 were prepped for RNA sequencing.

2.4. cDNA library preparation and sequencing

In brief, poly-dT oligo on magnetic beads (Qiagen) were used to isolated mRNA from total RNA samples, and then broken into short fragments (~300 nt) by adding fragmentation buffer. First-strand cDNA was synthesized using random hexamer-primed reverse transcription, Second-strand cDNA was generated by DNA polymerase I, and the RNA template was digested using RNase H. The cDNA fragments were purified and washed for end reparation poly (A) addition. After that, the short fragments were ligated with sequencing adapters. Following agarose gel electrophoresis, the cDNA fragments (~300 bp) were purified and enriched by PCR to construct the final cDNA library. The cDNA library was sequenced on the Illumina sequencing platform (Illumina HiSeq™ 2500) using the paired-end technology in a single run, by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.5. Illumina reads processing and annotation of gene expression levels

Low quality reads were filtered by excluding those reads containing adaptors, reads with > 5% unknown nucleotides, and low-quality reads with > 50% of bases with a quality value ≤5. The resulting high quality reads were mapped to ribosome RNA (rRNA) database by Bowtie2 software and the mapped reads were removed. The remaining reads were mapped to the reference sequence by TopHat2 software (Kim et al., 2013).

Gene abundances were quantified by software RSEM. The fragments per kilobase million (FPKM) value can be directly used for comparing the differences in gene expression among samples and the formula was