Journal of Invertebrate Pathology 124 (2015) 6–14

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Early responses of silkworm midgut to microsporidium infection – A Digital Gene Expression analysis

Ya-Jie Yue [a,b,c], Xu-Dong Tang [a,b,c], Li Xu [a,b], Wei Yan [a,b], Qian-Long Li [a,b], Sheng-Yan Xiao [a,b,c], Xu-Liang Fu [a,b], Wei Wang [a,b], Nan Li [a,b,c], Zhong-Yuan Shen [a,b,c,*]

[a] Jiangsu University of Science and Technology, Zhenjiang 212018, Jiangsu, China
[b] Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, Jiangsu, China
[c] Key Laboratory of Genetic Improvement of Silkworm and Mulberry of Agricultural Ministry, Zhenjiang 212003, Jiangsu, China

ARTICLE INFO

Article history:
Received 7 June 2014
Accepted 7 October 2014
Available online 12 October 2014

Keywords:
Bombyx mori
Digital Gene Expression
Microsporidia
Midgut
Innate immune response

ABSTRACT

Host–pathogen interactions are complex processes, which have been studied extensively in recent years. In insects, the midgut is a vital organ of digestion and nutrient absorption, and also serves as the first physiological and immune barrier against invading pathogenic microorganisms. Our focus is on *Nosema bombycis*, which is a pathogen of silkworm pebrine and causes great economic losses to the silk industry. A complete understanding of the host response to infection by *N. bombycis* and the interaction between them is necessary to prevent this disease. Silkworm midgut infected with *N. bombycis* is a good model to investigate the early host responses to microsporidia infection and the interaction between the silkworm and the microsporidium. Using Digital Gene Expression analysis, we investigated the midgut transcriptome profile of P50 silkworm larvae orally inoculated with *N. bombycis*. At 6, 12, 18, 24, 48, 72, and 96 h post-infection (hpi), 247, 95, 168, 450, 89, 80, and 773 DEGs were identified, respectively. KEGG pathway analysis showed the influence of *N. bombycis* infection on many biological processes including folate biosynthesis, spliceosome, nicotinate and nicotinamide metabolism, protein export, protein processing in endoplasmic reticulum, lysosome, biosynthesis of amino acids, ribosome, and RNA degradation. In addition, a number of differentially expressed genes involved in the immune response were identified. Overall, the results of this study provide an understanding of the strategy used by silkworm as a defense against the invasion by *N. bombycis*. Similar interactions between hosts and pathogens infection may exist in other species.

1. Introduction

The silkworm is not only an important economic insect for silk production, but also an excellent fundamental research model for lepidopteran insects. Therefore, it is critical to investigate the responses of silkworm to infections particularly by microorganisms. Because the immune system in insects and vertebrates share common pathways and components including the recognition and activation of NF-κB transcription factors, and the synthesis of antimicrobial peptides (Cheng et al., 2009), our studies will provide a better understanding of the biology and immunology of insects in particular, and invertebrates and vertebrates in general.

Microsporidia are a group of fungi-like, obligate, intracellular, single-cell spore-forming pathogens, distributed worldwide and have the capacity to infect a broad spectrum of hosts from protists to mammals, including humans, causing chronic and sublethal effects on hosts (Didier et al., 2000; Tsai et al., 2009; Vivares, 2001; Yang et al., 2014). They invade hosts by rapid extrusion of a unique and highly specialized organelle, called the polar tube, which allows the injection of the infectious spore content into a target cell (Li et al., 2009; Xu and Weiss, 2005; Zhu et al., 2013). Since the microsporidian genome is extremely reduced and compacted, they have lost many genes involved in critical metabolic pathways. This makes them highly dependent on their host (Texier et al., 2010). *N. bombycis* is an important silkworm pathogen, and attracts much attention, because it causes great economic losses to the silk industry, especially in developing countries. In addition, the pathogen alters the hosts' behavior significantly (Huang et al., 2009) and changes the hosts' basal metabolism and immune responses, such as the recognition of pathogenic agent, signal transduction, and the production of antimicrobial peptides. In addition, infection also affects the development of the host

* Corresponding author at: Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, Jiangsu, China.
E-mail address: szysri@163.com (Z.-Y. Shen).

http://dx.doi.org/10.1016/j.jip.2014.10.003
0022-2011/

insect. Ma et al. (2013) showed that *N. bombycis* infection in silkworm disturbed Juvenile Hormone (JH) synthesis and metabolism, and that the serpins secreted by *N. bombycis* may affect the serine protease cascade melanization pathway in the silkworm. Similar results were reported when silkworms were infected with *Bacillus bombyseptieus* (Huang et al., 2009).

The primary mechanism of *N. bombycis* infection in silkworm is by ingestion of infective spores. The insect midgut serves as the first physiological and immune barrier to incoming pathogen in addition to being a vital organ for digestion and nutrient absorption. Therefore, the silkworm midgut is an appropriate tissue for the investigation of the early responses of a host to *N. bombycis* oral infection.

Recent advances in the development of high-throughput deep sequencing technologies, for example, RNA-Seq or Digital Gene Expression (DGE), have greatly promoted genome research (Cloonan and Grimmond, 2008; Morozova and Marra, 2008). Sequencing-based methods generate absolute rather than relative gene expression measurements and avoid many of the inherent limitations of microarray analysis (Gao et al., 2014), which has been the most commonly used technology for transcriptome profiling over the last decade. Digital Gene Expression has been used to investigate genome-wide transcription analysis in many species (Qi et al., 2012; Qin et al., 2011; Yan et al., 2014).

In this study, we used the Illumina Genome Analyzer platform to investigate the midgut transcriptome profile of the P50 silkworm strain orally infected with *N. bombycis* at 6, 12, 18, 24, 48, 72, and 96 h post-infection (hpi). The gene expression profiles between the control and infected silkworm larvae were then systematically analyzed by differential gene expression, Gene Ontology categories and KEGG pathways. We identified many differentially expressed genes (DEGs) involved in the immune response of silkworm and its defense against the invasion of *N. bombycis*. The results of this study may contribute to understanding the molecular mechanisms involved in the silkworm's early response to the infection by *N. bombycis*, and interaction between microsporidia and its host.

2. Materials and methods

2.1. Insect rearing and preparation of parasites

Nosema bombycis isolates were maintained at the Sericultural Research Institute of Chinese Academy of Agricultural Sciences, China. Silkworm larvae of the P50 strain were reared at 25 °C and a 12 h:12 h light:dark photoperiod to fifth stadium. Larvae were orally inoculated with *N. bombycis* to simulate the natural infection process.

2.2. Preparation of spores, experimental infection and tissue sampling

Spores of *N. bombycis* were prepared by washing three times in distilled water and then suspending in distilled water to achieve a concentration of 10^8 spores per ml. Mulberry leaves (500 g) were immersed in 100 ml of the spore solution and air dried. Before inoculation, newly molted fifth instar silkworm larvae were placed in a petri dish without food for 4 h. Spores-coated mulberry leaves were then fed to the silkworms for 4 h followed by feeding untreated mulberry leaves. The silkworms in the control group were fed mulberry leaves immersed in distilled water. Midgut tissues were randomly dissected from four larvae at 6, 12, 18, 24, 48, 72, and 96 hpi respectively, snap frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Three duplicate samples were obtained for each time point.

2.3. RNA extraction

Total RNA was extracted using RNAiso Reagent (TaKaRa), quantified using a BioPhotometer plus 6132 spectrophotometer (Eppendorf, Hamburg, Germany) and verified on a 1.0% formaldehyde-denatured agarose gel electrophoresis. All samples were confirmed to have good integrity, and absorbance ratios (260/280) between 1.8 and 2.1. The samples were stored at −80 °C until further analysis.

2.4. Library preparation for DGE sequencing

About 3 μg total RNA per sample was used for sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. mRNA was then fragmented in NEBNext First Strand Synthesis Reaction Buffer (5×) using divalent cations at an elevated temperature. First strand cDNA was synthesized using random hexamer primers and MMLV Reverse Transcriptase (RNase H). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends using exonucleases/polymerases. After adenylation of 3′ ends, NEBNext Adaptor with hairpin loop structure was ligated prior to hybridization. In order to select cDNA fragments that were 150–200 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, MA, USA). Then 30 μl USER Enzyme (NEB) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and the library quality was assessed on the Agilent Bioanalyzer 2100 system.

2.5. Clustering and sequencing

Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2000/2500 platform to generate 100 bp/50 bp single-end reads.

2.6. Analysis of differential gene expression

The differential expression of genes across samples was detected using the DGESeq R package. *P*-values were adjusted using the Benjamini & Hochberg method, and a *P*-value of 0.05 and log2 (Fold change) of 1 were set as the threshold to determine significant differential expression.

2.7. GO and KEGG analyses

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented using the GO seqR package where the bias in gene length was corrected. GO terms with corrected *P*-values less than 0.05 were considered significantly enriched. In the KEGG database, we used KOBAS software to test the statistical enrichment of differential gene expression in KEGG pathways.