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Transcriptomic insight into the immune defenses in the ghost moth, *Hepialus xiaojinensis*, during an *Ophiocordyceps sinensis* fungal infection

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ABSTRACT

Hepialus xiaojinensis is an economically important species of Lepidopteran insect. The fungus *Ophiocordyceps sinensis* can infect its larvae, which leads to mummification after 5–12 months, providing a valuable system with which to study interactions between the insect hosts and pathogenic fungi. However, little sequence information is available for this insect. A time-course analysis of the fat body transcriptome was performed to explore the host immune response to *O. sinensis* infection. In total, 50,164 unigenes were obtained by assembling the reads from two high-throughput approaches: 454 pyrosequencing and Illumina Hiseq2000. Hierarchical clustering and functional examination revealed four major gene clusters. Clusters 1–3 included transcripts markedly induced by the fungal infection within 72 h. Cluster 4, with a lower number of transcripts, was suppressed during the early phase of infection but returned to normal expression levels sometime before 1 year. Based on sequence similarity to orthologs known to participate in immune defenses, 258 candidate immunity-related transcripts were identified, and their functions were hypothesized. The genes were more primitive than those in other Lepidopteran insects. In addition, lineage-specific family expansion of the clip-domain serine proteases and C-type lectins were apparent and likely caused by selection pressures. Global expression profiles of immunity-related genes indicated that *H. xiaojinensis* was capable of a rapid response to an *O. sinensis* challenge; however, the larvae developed tolerance to the fungus after prolonged infection, probably due to immune suppression. Specifically, antimicrobial peptide mRNAs could not be detected after chronic infection, because key components of the Toll pathway (MyD88, Pelle and Cactus) were downregulated. Taken together, this study provides insights into the defense system of *H. xiaojinensis*, and a basis for understanding the molecular aspects of the interaction between the host and the entomopathogen.

Abbreviations: AMP, antimicrobial peptide; cSP, clip domain serine protease; CTL, C-type lectin; DEG, Differentially expressed gene; FPKM, fragments per kilobase per million mapped reads; βGRP, β-glucan recognition protein; IMD, immune deficiency; PGRP, peptidoglycan recognition protein; PPO, prophenoloxidase; PPAE, prophenoloxidase-activating enzyme; PAP, prophenoloxidase-activating proteinase; qPCR, Quantitative real-time PCR; RCL, reactive center loop; RNA-seq, RNA sequencing; rpS3, ribosomal protein S3; semiRT-PCR, semi-quantitative reverse transcription-PCR; SPZ, Spätzle; SCR, scavenger receptor; TEP, thioester-containing protein.

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1. Introduction

Insects inhabit microbe-rich environments and have evolved an innate immune system as protection against pathogenic invaders over millions of years, despite the fact that their immune system lacks a large repertoire of elegant receptors (immunoglobulins and T cell receptors) encoded by gene rearrangement to detect a large variety of microbial antigens (Hoffmann and Reichhart, 2002). In mammals, innate reactions are an indispensable component of the immune response against microbial infections (Vilmos and Kurucz, 1998), and similarities do exist in the evolving picture of mammalian innate immunity and insect immune defenses (Gunaratna and Jiang, 2013; Hergannan and Rechhart, 1997; Hultmark, 1993). Thus, the molecular aspects of the interactions between microbes and insect hosts warrant further investigation.

Insect immunity is composed of humoral plasma-borne factors and cellular- or hemocyte-associated molecules, which is dynamic in response to parasitic or pathogenic infections (Lemaitre and Hoffmann, 2007). The hemocytes secrete defense molecules and engage in phagocytosis, encapsulation, and nodulation of pathogens (Christophides et al., 2002; Fauvarque and Williams, 2011; Jiang et al., 2010). Humoral immunity, composed of the immune signaling pathways and melanization, is more complex. The fat body (comparable to vertebrate liver and adipose tissues) is the main source of circulating immune-related components, such as pattern recognition receptors (PRRs), which are responsible for microbial detection, and antimicrobial peptides (cytotoxic to microorganisms). Immune signal modulation and transduction pathways include Toll (Valanne et al., 2011), IMD (Immune deficiency) (Silverman and Maniatis, 2001), JNK (Ramet et al., 2002), and JAK/STAT, (Baeg et al., 2005), which are triggered by recognition of pathogen-associated molecular patterns (PAMPs) on the microbial surface. The production of anti-microbial peptides (AMPs) is mainly determined by Toll and IMD pathways. The Toll pathway is induced by Gram-positive bacteria and fungi; its activation is mediated by an extracellular serine protease (SP) cascade. In contrast, the IMD pathway is induced primarily in response to Gram-negative bacterial infections. The activation of both pathways results in the expression of AMP via NF-κB-like transcription factors. The recognition of these PAMPs also provokes melanization. Activation of prophenoloxidase (PPO), the key enzyme of melanization, requires sequential reactions of a SP cascade, similar to the Toll pathway. Proteolytic cleavage of PPO leads to melanin synthesis, which sequesters and kills microbes.

The ghost moth, genus *Hepialus*, belongs to the family Hepialoidea, which is one of the most phylogenetically primitive lineages of extant Lepidoptera (Nielsen et al., 2000). Members of the *Hepialus* are hosts for the ascomycete *Ophiocordyceps sinensis*, commonly known as the Chinese caterpillar fungus (Cao et al., 2012). The parasitic complex, composed of the remains of the caterpillar and fungal sexual stroma, has a long history of use in traditional Chinese medicine (TCM) for its anti-inflammatory, immunomodulatory, and other pharmacological effects (Hu et al., 2013; Ng and Wang, 2005; Zhou et al., 2009). *O. sinensis* is a psychrophilic fungus with optimum growth at 18 °C. During the early phase of infection, the fungus is hardly observed *in insecta*. Only after several months of colonization is the sexual structure finally produced in the cadaver of the ghost moth. This is very different from other entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*, which kill the host within a few days. Therefore, a model of the ghost moth challenge by *O. sinensis* is valuable for studying molecular interactions between insect hosts and entomopathogentic fungi. The outcomes would be also useful for the artificial cultivation of the Chinese cordyceps (literally “winter worm, summer grass”). However, due to the difficulty of rearing them in a laboratory, research on *Hepialus* has focused mostly on their physiology and ecology, and on the *O. sinensis* parasite.

Recent advances in molecular genetics technologies, as well as high-throughput sequencing have provided in-depth insights into the immune systems of many insects, including the Hymenopteran bee, *Apis mellifera* (Evans et al., 2006); three Dipteran insects, *Drosophila melanogaster* (Hoffmann and Reichhart, 2002), *Anopheles gambiae* (Christophides et al., 2002), and *Aedes aegypti* (Waterhouse et al., 2007); three Coleopteran beetles, *Tribolium castaneum* (Zou et al., 2007), *Harmonia axyridis* (Schmidtberg et al., 2013), and *Meligethes aeneus* (Vogel et al., 2014); and three Lepidopteran moths, *Bombyx mori* (Tanaka et al., 2008), *Manduca sexta* (Gunaratna and Jiang, 2013), and *Ostrinia furnacalis* (Liu et al., 2014). Immunity-related genes have enabled the identification of orthologues in these insect species; the expansion and loss of genes and gene families may reflect natural selection, enabling adaptation to ecological and physiological conditions. A comprehensive study of the mechanism and evolution of these defenses is needed, based on immunity-related genes from phylogenetically diverse insects.

The mechanisms of the immune system in *Hepialus* remain unknown; however, they have important implications for its economic importance and are critical in understanding the phylogenetic position of this Lepidopteran species. *H. xiaojinensis*, a species of *Hepialus*, reproduces one generation every 4 years, and inhabits the alpine meadows of Xiaojin County (Zhang et al., 2011). *H. xiaojinensis* larvae have been successfully reared under laboratory conditions, and a model of larval parasitization with *O. sinensis* (unpublished results) has been established. In this study, high-throughput RNA sequencing (RNA-seq) was used to analyze time-course transcriptomic profiles of *H. xiaojinensis* larvae infected with *O. sinensis*, which were also used for screening of immunity-related genes. We identified potential immunity-related genes and pathways in the primitive Lepidopteran species, *H. xiaojinensis*, and analyzed the phylogenetic relationship with homologous members from other insect species. Expression patterns of key components of the Toll pathway were also investigated to understand the immune interactions between *H. xiaojinensis* and *O. sinensis*.

2. Materials and methods

2.1. Experimental insects

Eggs from *H. xiaojinensis* were collected from alpine meadows in Xiaojin County, China, and reared in the laboratory at 16 °C and 80% humidity for more than two generations. The *O. sinensis* fungus was kindly gifted by Dr. Yi-Jian Yao from the Institute of Microbiology, Chinese Academy of Sciences. Seventh instar larvae were pricked using a glass capillary loaded with 2 μl diluted fungal suspension (3×10^6 blastospores/μl).

2.2. RNA preparation and sequencing

At 12 h, 48 h, 72 h, and 1 year post-infection, the groups of three larvae with *O. sinensis* infections were collected, and total RNA from the fat body was extracted using the RNeasy Plus Universal Mini Kit (Qiagen, Venlo, Limburg, Netherlands), according to the manufacturer's instructions. As a control, RNA was isolated from the fat body of uninfected larvae (MOCK). A Nanodrop ND-2000 spectrophotometer was used to determine the quantity of RNA in the samples, and sample integrity was analyzed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). A cDNA library was constructed using 6 μg RNA from the infected and control groups, and sequenced on a Hiseq™ 2000 platform (Illumina, San Diego, CA,