Experimental Parasitology 134 (2013) 61–67

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

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

Transcription profiling of immune genes during parasite infection in susceptible and resistant strains of the flour beetles (*Tribolium castaneum*)

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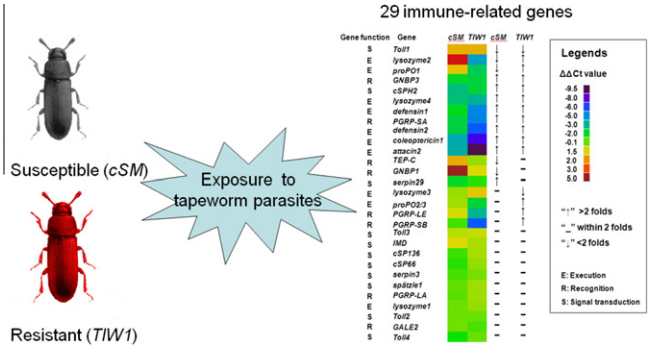
HIGHLIGHTS

- We examine transcription profile of immune genes before and after parasite exposure.
- Differential transcriptional response patterns of immune genes were detected.
- Host genetic background has significant effects on the expression of immune genes.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 June 2012
Received in revised form 10 January 2013
Accepted 14 January 2013
Available online 1 February 2013

Keywords:
Parasite resistance
Transcriptional profiles
Immunostimulant
Hymenolepis diminuta
Tribolium castaneum

ABSTRACT

The flour beetle, *Tribolium castaneum*, is an intermediate host for the tapeworm *Hymenolepis diminuta* and has become an important genetic model to explore immune responses to parasite infection in insect hosts. The present study examined the immune responses to tapeworm infection in resistant (*TIW1*) and susceptible (*cSM*) strains of the red flour beetle, *T. castaneum*, using real-time quantitative reverse transcription PCR on 29 immunity-related genes that exhibit antimicrobial properties. Thirteen of the 29 genes showed constitutive differences in expression between the two strains. Fourteen to fifteen of the 29 genes exhibited significant differences in transcription levels when beetles were challenged with tapeworm parasite in the resistant and susceptible strains. Nine genes (*GNBP3*, *cSPH2*, *lysozyme4*, *defensin1*, *PGRP-SA*, *defensin2*, *coleoptericin1*, *attacin2* and *serpin29*) in *cSM* and 13 genes (*lysozyme2*, *proPO1*, *GNBP3*, *cSPH2*, *lysozyme4*, *defensin1*, *PGRP-SA*, *defensin2*, *coleoptericin1*, *attacin2*, *proPO2/3*, *PGRP-LE* and *PGRP-SB*) in *TIW1* were up-regulated by infections or showed parasite infection-induced expression. Seven genes (*attacin2*, *coleoptericin1*, *defensin1*, *defensin2*, *lysozyme2*, *PGRP-SA* and *PGRP-SB*) were more than 10 folds higher in the resistant *TIW1* strain than in the susceptible *cSM* strain after exposure to tapeworm parasites. This study demonstrated the effects of genetic background, the transcription profile to parasite infection, and identified the immunity-related genes that were significantly regulated by the infection of tapeworms in *Tribolium* beetles.

1. Introduction

The fundamental aspects of the immune response to pathogens have revealed a high degree of conservation across various taxa (Beck and Habicht, 1996); for that reason, the insect model is a valuable system to determine the fundamental processes of immunity. The study of insect immunity is also important in its own right, with managed infection at the core of new approaches to the biological control of agricultural pests and human disease vectors. The red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), is an important stored-grain pest worldwide (Granousky, 1997; Sinha and Watters, 1985; Small, 2007), and is

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http://dx.doi.org/10.1016/j.exppara.2013.01.014

also an intermediate host to *Hymenolepis diminuta* (Cestoda: Cyclophyllidea) known as rat tapeworm, commonly found in rat feces. Eggs of *H. diminuta* are passed in the feces of the infected definitive hosts, and the mature eggs can be ingested by *Tribolium* beetles; when oncospheres are released from the eggs, they penetrate the intestinal wall of the host and subsequently develop into cysticercoid larvae (Schantz, 1996).

The availability of the complete *T. castaneum* genome makes *Tribolium* beetles an excellent model to study molecular processes of innate immune responses (Richards et al., 2008). *Tribolium* beetles harbor a wide range of natural pathogens, including bacteria, fungi, microsporidians and cestoda (Blaser and Schmid-Hempel, 2005; Wade and Chang, 1995; Younas et al., 2008; Yokoi et al., 2012a,b). Tapeworm infections can induce a wide range of physiological, ecological, and behavioral responses in *Tribolium*, including reduced survival and fecundity (Keymer, 1980), reduced mating vigor (Pai and Yan, 2009), altered pheromone responses (Robb and Reid, 1996), and changes in carbohydrate metabolism (Novak et al., 1993). However, the molecular aspects of immune response of the beetle to tapeworm infection are unknown. The interactions between flour beetles and parasites help to elucidate basic principles in genetic variation, adaptive immune system, and life history evolution (Schulenburg et al., 2009).

Approximately 300 immune-related proteins have been identified in *T. castaneum* based on homology to the honeybee, mosquito, and fruit-fly (Christophides et al., 2002; Zou et al., 2007). Expression of some *T. castaneum* immune genes can be induced by bacterial lipopolysaccharide stimulation (Altincicek et al., 2008), which suggests that it can mount a direct response against microbial pathogens. Hitchen et al. (2009) demonstrated that the presence of cysticercoids in the beetle host can alter the expression of several host genes. Investigations of the immune processes during parasite infection in *Tribolium* have, however, been hindered by a lack of species-specific antibodies (Watthanasurorot et al., 2011). The molecular mechanisms for *Tribolium* immunity to tapeworm infection are poorly understood.

In this study, we examined constitutive and tapeworm infection-induced expression profile of immunity-related genes in two *T. castaneum* strains that exhibit contrasting differences in susceptibility to infection by tapeworm parasite *H. diminuta*. The reverse transcription quantitative real-time PCR (qRT-PCR) is one of the most important technologies for quantification of mRNA abundance (Bustin, 2000; Bustin et al., 2005; VanGuilder et al., 2008). Our approach to study gene transcription patterns under different genetic backgrounds is the first step towards identifying molecular pathways involved in a process of insect innate immunity against parasite infection. We identified the changes in transcript abundance, including genes responsible for pathogen recognition of invading organisms by plasma proteins or cell surface receptors, extra- and intracellular signal transduction and modulation, and controlled release of defense molecules.

2. Materials and methods

2.1. Beetle strains and tapeworm infection

The susceptible and resistant *T. castaneum* strains used in this study were *cSM* and *TIW1*, respectively. Beetles used in study were reared in 8-dram shell vials with 5 g standard food containing 95% whole wheat flour and 5% yeast. They were kept in a dark incubator regulated at 28 °C and 70% relative humidity. Pupae were sexed and reared in separated vials. The newly emerged male and female beetles were collected and randomly assigned to the control or experimental infection group. Prior to infection, adult beetles were fasted to promote ingestion of parasite eggs (Dunkley and Mettrick, 1971), and subsequently exposed to a fresh mixture of rat feces (control) or tapeworm infected rat feces (treatment) for 48 h. Under the aforementioned environmental conditions, the cysticercoids can reach maximum growth rate at 7 days post-exposure (PE), and parasite growth ceases after 14 days PE (Shostak et al., 2008). Tapeworm-infected rat feces were acquired from Carolina Biological Supplies (Burlington, North Carolina, USA). The infection procedures were conducted following the methods of Yan (1997).

2.2. Beetle dissection and RNA extraction

We examined expression pattern of immune genes 14 days after the beetles were exposed to tapeworm eggs. Therefore, the gene expression pattern from the present study reflects the time point when tapeworm eggs developed into mature cysticercoids. Total RNA was isolated from 20 beetles per treatment using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The RNA was treated with RNase-free DNase to remove genomic DNA contamination prior to cDNA production via reverse transcription.

2.3. Immunity-related genes selection and qRT-PCR primers

Zou et al. (2007) identified 34 immune-related genes that exhibit antimicrobial properties in *T. castaneum*. We conducted quantitative RT-PCR (qRT-PCR) with the PCR primer pairs reported by Zou et al. (2007), but amplification for 12 genes (*PGRP-LE*, *CTL7*, *GALE1*, *TEP-C*, *cSP66*, *lysozyme 2–4*, *cecropin3*, *defensin3* and *defensin4*) was either unsuccessful or inconsistent among our beetles, probably due to among-strain variation in nucleotide sequences. We redesigned PCR primers based on more conserved regions across a variety of insect species for each of these 12 genes, and conducted RT-PCR. The new PCR primers yielded excellent amplifications for 7 genes (*PRGP-LE*, *GALE2*, *TEP-C*, *cSP66* and *lysozyme 2–4*) (Table 1), but could not amplify 5 other genes (*CTL7*, *GALE1*, *cecropin3, defensin3* and *defensin4*). Therefore, this study focused on 29 immune genes that were reliably amplified by qRT-PCR (Table 1).

2.4. Quantitative real-time PCR assay

About 1 μg of RNA was used as a template in reverse transcription to produce cDNA in 20-μL reactions using the iScript™ cDNA synthesis Kit (Bio-RAD Hercules, CA). Real-time PCR was conducted according to Chen et al. (2004) with slight modifications. The qRT-PCR was performed in triplicate using 5 μl of cDNA (1: 10 dilution) and 10 pmol of each primer in iQTM SYBR green Supermix (Bio-RAD, Hercules, CA, USA) on a DNA Engine Opticon™ 2 real-time PCR system (MJ Research). Thermal cycling was performed at 50 °C for 2 min, 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Melting curve analysis was performed from 55 to 95 °C. *Tribolium* ribosomal protein S3 was used as the internal control for PCR product normalization (Zou et al., 2007).

2.5. Statistical analysis

Infection prevalence and mean infection intensity were calculated for each beetle strain and compared with χ^2 test and non-parametric Mann–Whitney *U* test. Mean infection intensity is calculated as the average number of tapeworm parasites in the infected beetles. To determine the constitutive gene expression differences between resistant and susceptible beetle strains, the transcription of the 29 genes in beetle populations not challenged with tapeworm eggs was compared. Transcription level of the genes studied was expressed by the difference in Ct values and