SHORT COMMUNICATION

Hymenolepis diminuta (Cestoda) induces changes in expression of select genes of Tribolium confusum (Coleoptera)

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Abstract The flour beetle Tribolium confusum is a common experimental intermediate host for the tapeworm Hymenolepis diminuta, but while many aspects of their host-parasite interactions have been determined to have genetic basis, the genes involved have not been identified. Here we report on expression of several predicted metabolic and defense-related genes using quantitative PCR, 2 weeks after initial infection of the beetle. The expression of heat shock protein 68, a predicted sugar transporter, a pheromone binding protein and endoglin were up-regulated in infected beetles. The expression of thaumatin-like protein and prophenoloxidase 2/3 was down-regulated in infected beetles, while the mRNA levels of Toll-like receptor 3, Toll-like receptor 4, and lysozyme 4, were not affected by infection with H. diminuta.

Introduction

Beetles of the genus Tribolium (Coleoptera: Tenebrionidae) are important pests of storage grains and, more recently, have been recognized as an important insect genetic model following the sequencing of the Tribolium castaneum genome (Tribolium Genome Sequencing Consortium 2008). Tribolium spp. vary in their response to pathogens. For example, *T. castaneum* are particularly resistant to infection by Wolbachia spp., but these bacteria are capable of killing some other species of Tribolium (Brown et al. 2003). While resistant to some pathogens, these beetles are susceptible to infection by the tapeworm Hymenolepis diminuta (Cestoda: Cyclophyllidea). While H. diminuta is only a minor human parasite, the H. diminuta – Tribolium confusum relationship is an important laboratory model for host – parasite relationships (Voge and Heyneman 1957; Keymer 1980; Evans et al. 1998; Shostak et al. 2008).

Infection of beetles with H. diminuta involves ingestion of eggs, hatching of a hexacanth embryo in the intestine, and penetration of the intestine as the hexacanth enters the hemocoel, primarily at the level of the thorax (Lethbridge 1971). The parasite does not multiply within the host, but following infection there is a 1-2 week period of growth and differentiation of the parasite before it reaches a cysticercoid stage (Shostak et al. 2008) that is transmissible to the next host. During growth, each parasite increases in size about 800 fold. The parasite causes physical damage upon initial entry and becomes an increasing physical and metabolic presence within the hemocoel of the beetle.

Infection by H. diminuta may seriously impact the host. Infected T. confusum have reduced survival and fecundity (Keymer 1980), reduced mating vigor (Pai and Yan 2003), and altered pheromone responses (Robb and Reid 1996) and carbohydrate metabolism (Novak et al. 1993). While genetics influences the infection of Tribolium spp. with this tapeworm (Yan and

Norman 1995; Zhong et al. 2003) the genetic basis of resistance/susceptibility, or indeed other aspects of the host-parasite relationship, is unknown.

The recent demonstration of increased expression of immune genes of T. castaneum following bacterial lipopolysaccharide (LPS) stimulation (Altincicek et al. 2008), suggests that beetles may be capable of mounting a directed response against microbial pathogens. Changes in gene expression by Tribolium spp. in response to invasion by a much larger and complex metazoan parasite have not been studied. This prompted us to ask whether infection of T. confusum by H. diminuta affected expression of the host genes examined by Altincicek et al. (2008). In this report we show using quantitative PCR, that infection of beetles with H. diminuta induces a change in expression of a number of immune-related and metabolic genes.

Materials and Methods

Hymenolepis diminuta and T. confusum were maintained in our laboratory as described elsewhere (Shostak et al. 2006). Two rats used to provide eggs to infect beetles for gene expression studies were each infected via gastric intubation with 10, 2-week-old cysticercoids and infections in the rats were 10-11 months old at time of fecal collection and egg separation by sucrose gradient centrifugation. A third rat was infected with 15, 2-week-old cysticercoids and killed 18 days PI to obtain a large quantity of H. diminuta tissue free of beetle contamination; the intestine of that rat was slit longitudinally, and worms were removed and washed four times in phosphate buffered saline (PBS), quickly rinsed with 70% ethanol to kill potential surface contaminating bacteria and placed back in PBS.

Mixed-age beetles were removed from a stock colony, held off food for 4 days, then randomly assigned for parasite-exposed or control treatments. Parasite-exposed beetles were

placed for 24 hr in arenas containing oatmeal flakes coated with freshly collected parasite eggs. Control beetles were treated similarly, but the oatmeal flakes had no eggs. Beetles were killed for RNA isolation (pool of n = 100 infected beetles; pool of n = 100 control beetles) at 14 days post infection. Different beetles (n = 100 infected; n = 20 control) were dissected at this time to assess infection levels (100% of beetles in the parasite-exposed group were infected with 2-21 parasites each, mean = 8.3, while none of the control group beetles were infected.) An additional group of 100 unexposed beetles from the stock colony reared at 27^{o}C was placed at 37^{o}C for 1 hour prior to RNA isolation from the pooled sample, and used as a positive control for expression of heat shock protein.

For RNA isolation, beetles and adult parasites were homogenized in liquid nitrogen using a mortar and pestle for total RNA isolation with Trizol reagent (Invitrogen Corp., Carlsbad, California). cDNA synthesis was carried out with oligo-dT primers using the First-strand Superscript II cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. cDNA purity was determined using spectrophotometry and absorbance ratios (Nanodrop ND-1000, Thermo Fisher Scientific Inc., Waltham, Massachusetts).

We selected heat shock protein (HSP68), a predicted sugar transporter (ST), a member of the A10/OS-D pheromone binding protein family (PBP), endoglin, Toll-like receptors (TLR3/4; TLR3 and TLR4 were likely amplified by the same primer set), lysozyme (Lz4), thaumatin-like protein (TLP), and prophenoloxidase enzyme (proPO2/3). Heat shock proteins are important for dealing with heat stress, but they also play roles in other situations including infection related stresses (Altincicek et al. 2008). A predicted sugar transporter (Altincicek et al. 2008) was chosen because of its role in host carbohydrate metabolism. Pheromone binding proteins, which act in chemoreception and host behavior, can be induced by infection (Sabatier et al. 2003).

104 Endoglin (CD105) has only been characterized in mammals, where it mediates the signaling of
105 the cytokine transforming growth factor beta (TGF-ß) that is responsible for mediating
106 inflammation and development (Torsney et al. 2002; Schmidt-Weber et al. 2005). Toll-like
107 receptors 3 and 4, while not proven immune genes in Tribolium spp., have a potential to play a
108 role in development and innate immunity (Zou et al. 2007). Lysozyme molecules mediate
109 antimicrobial activity by cleaving the peptidoglycan layer, mostly in gram positive bacteria (Yu
110 et al. 2002). We arbitrarily chose Lz4, one of 4 lysozyme genes in Tribolium spp. (Zou et al.
111 2007). Thaumatin-like proteins are known anti-fungal peptides originally discovered in plants
112 and found recently in several insects (Altincicek et al. 2008). Prophenoloxidases are enzymes
113 important in recognition of non-self, clot formation, melanin formation, production of reactive
114 oxygen and nitrogen species and microbe killing (Gregório and Ratcliffe 1991; Zou et al. 2007).
115 We labeled prophenoloxidases as proPO2/3 because gene duplication in Tribolium has resulted
116 in 2 nearly identical genes (Zou et al. 2007). A common endogenous control used in relative
117 quantification studies is 18S rRNA due to its consistent constitutive expression, and we initially
118 attempted to use this control based on primers from Altincicek et al. (2008). However, H.
119 diminuta 18s rRNA had 69.4% identity with the Tribolium 18S rRNA partial sequence.
120 Attempting to remove cysticercoids before isolating Tribolium RNA caused loss and/or
121 degradation of beetle tissue and reduced the effectiveness of the RNA isolation. Therefore, a
122 protein with NADH dehydrogenase (NADHd) activity, and believed to be insect-specific (Zhang
123 et al. 2007), was employed as an endogenous control.
124 All primers (Table 1), except those for TLP, were based on sequences from T. castaneum.
125 Primers for HSP68 were from Altincicek et al. (2008). Primers for ST, PBP, endoglin, TLR3/4,
126 Lz4, and NADHd were designed based on T. castaneum genome sequences from BeetleBase

(www.bioinformatics.ksu.edu/BeetleBase). Primers for proPO2/3 and GLEAN accession numbers for TLR3, TLR4, proPO2/3 and Lz4 were based on sequences from Zou et al. (2007). GLEAN accession numbers for the NADHd, PBP, and endoglin were obtained from Zhang et al. (2007). TLP real-time primers were designed (see below) using Primer Express 3.0 (Applied Biosystems, Foster City, California) from a partial sequence in T. confusum. Multiple primer sets were designed for each gene and the primers with the best amplification efficiencies and consistent melt curves (data not shown) were chosen. Primers were ordered from Integrated DNA Technologies (Coralville, Iowa).

Consensus sequences from T. castaneum thaumatin-like protein 1 (XM_963631) and 5 (XM_970082), and a thaumatin-like protein (DQ_023319) from another beetle, Dendroides canadensis, were used to develop the primers (forward 5'-CGGCCCAAGCCGTTGAATTC-3' and reverse 5'-TCACATTTTTTGCAACTGTA-3') for T. confusum thaumatin-like protein. A 406bp PCR product was then cloned into the pCR® 2.1-TOPO® vector (Invitrogen) transformed into chemically competent TOP10 Escherichia coli (Invitrogen). Successful cloning was verified by colony PCR. Successfully transformed colonies were grown overnight, while shaking at 37°C, in 1mL of LB broth with 100 µg/mL ampicillin. Plasmids were isolated using the QIAprep® Spin Miniprep Kit (Qiagen Inc., Mississauga, Ontario). All DNA sequencing was performed using the DYEnamic ET-Terminator Cycle Sequencing Kit (Amersham Biosciences, Pittsburgh, Pennsylvania) and Applied Biosystems 377 DNA sequencers (Applied Biosystems) according to standard manufacturer-supplied protocols. Searches for sequence comparison and identification of the T. confusum thaumatin-like protein were performed using the BLAST search tool of the National Center for Biotechnology (NCBI), Bethesda, Maryland.

Real-time PCR was used to generate accurate gene expression data by mapping the amplification of cDNA in real time. Relative quantification was performed using the 7500 Fast Real-time system (Applied Biosystems). Samples were run at least in triplicate. Primer specificity was verified by dissociation curve. Because the infected beetle sample contained cysticercoid genetic material, primers were tested against cDNA from adult worms to verify that results were not skewed by the amplification of parasite material. Uniformity of endogenous control expression was verified by observing the cycle threshold for identical concentrations of cDNA across the uninfected, infected and heat shocked samples. A 1/40 dilution of cDNA was used for relative quantification (~44ng/µL). Primer concentration was 0.2 nM. 5µL SYBR Green PCR Master Mix (Applied Biosystems) was used with 2.5µl primers and 2.5µL cDNA to create 10µL reactions. Statistical analyses were done using the 7500 Software V2.0.1. Results represent the average of 3 test plates. Reactions were run with the following parameters: 95 C for 10 min, and 40 cycles of 95 C for 15 sec, 55 C for 1 min.

Results and Discussion

Initially we used the primers developed by Altincicek et al. (2008) for T. castaneum to determine gene expression levels of an array of immune related genes in its congener T. confusum. Unfortunately, sequence variation between species reduced functionality of a number of these primer sets for T. confusum. For example, the partial sequence of T. confusum TLP had only 84% identity with its most closely related T. castaneum protein. The relationship was close enough however, to argue that primers based on T. castaneum sequences would elicit the sought after gene in T. confusum. To confirm this we examined the expression of HSP68 in heat-shocked beetles to verify that we were measuring expression of the correct gene. If that was the

case, one would expect that the mRNA levels of HSP68 to substantially increase following heat shock. The expression of HSP68 was 32 times higher after heat shock (Fig. 1). The high level of sequence identity between TLPs and the behavior of HSP68 following heat shock convinced us that we could accurately measure a variety of other T. confusum genes, using primers based on the T. castaneum genome. Expression of HSP68, ST, PBP, and endoglin increased in infected beetles, although the mRNA level of HSP68 in infected, non heat-shocked beetles was much lower than that induced by heat shock (Fig. 1 and Table 2). In contrast the mRNA levels of TLP and proPO2/3 were reduced in infected beetles, while there were no changes in the mRNA levels of TLR3/4 or Lz4 (Table 2).

Six of eight T. confusum genes examined in this study had altered expression 2 weeks after infection with H. diminuta, a time when acute effects on the host resulting from the rapid growth of the parasite (Shostak et al. 2008) had likely recently peaked. While there are numerous reports of altered gene expression in insects infected with various pathogens (Gregório and Ratcliffe 1991; Reineke and Löbman 2005; Sabatier et al. 2003), studies involving a helminth parasite in an insect host are rare: Warr et al. (2006) reported that H. diminuta secretes a molecule that reduces vitellogenin expression in T. molitor. Two genes of T. confusum (ST and PBP) with increased expression in our study appear unrelated to host defense, and other genes with predicted defensive roles varied from increased expression (HSP68, endoglin), no change (TLR3/4, Lz), or decreased expression (TLP, proPO2/3) in the presence of H. diminuta. The parasite appears to modulate metabolism as well as the defense system of its intermediate host. The genes we examined have not been fully characterized in Tribolium spp., but our observations are consistent with general expectations for the H. diminuta – T. confusum relationship.

Increased expression of a predicted sugar transporter was not surprising given the effects of H. diminuta on hemolymph carbohydrates in T. confusum (Novak et al. 1993) and the ability of this parasite to grow even in fasted hosts (Shostak et al. 2008). Up-regulation of pheromone binding proteins occurs in virus-infected Drosophila melanogaster, but their antiviral role is unclear (Sabatier et al. 2003). Pheromones modulate many behaviors in T. confusum (Ryan and O'Ceallachain 1976; Yan and Phillips 1996). Further functional characterization of the PBP that was up-regulated in our study is needed to determine whether it is host-protective or perhaps increases susceptibility of infected hosts to predation.

As a stress-induced protein the up-regulation of HSP68 might be expected, and has been linked to various microbial elicitors (Altincicek et al. 2008). Although our observation might simply reflect introduction of microbes into the hemocoel during penetration of the parasite at initial infection, there was no change in expression of TLR3/4 or Lz4. Interestingly, HSP68 in mammals has also been reported during severe inhibition of protein synthesis (Ovelgönne et al. 1995) and may therefore be a marker of the severe nutritional stress that this parasite may induce in its host.

Our study is the first to report up-regulation of endoglin in an insect. The role of endoglin in inflammation and wound healing in mammals (Torsney et al. 2002) suggests that endoglin may be up-regulated in response to the physical damage done by the parasite. Similarly, a TLP expressed in T. castaneum has antifungal properties and is strongly induced by septic injury (Altincicek et al. 2008). The decreased expression of TLP in beetles infected with H. diminuta is opposite to what one would expect if the response was solely due to damage during initial parasite penetration, and may suggest a novel role for TLP in helminth infections.

The effector activities of prophenoloxidases, including melanin formation (Altincicek et al. 2008), are long lasting (Haine et al. 2008) and could harm a helminth parasite in the hemocoel (Lemaitre and Hoffmann 2007). The lack of melanization following infection with H. diminuta (Voge and Heyneman 1971) could be explained by reduced expression of proPO2/3. Ubelaker et al. (1970) observed destruction of hemocytes in the general vicinity of cysticercoids. Hemocytes such as lamellocytes initiate encapsulation and contribute to melanization with the help of prophenoloxidases released from crystal cells (Lemaitre and Hoffmann 2007). Heyneman and Voge (1971) observed the beginning of encapsulation in early infections but by day 5 PI encapsulating cells were no longer present. Simple destruction of crystal cells could explain the reduced expression of proPO2/3.

We have demonstrated that cysticercoid presence in the beetle host is associated with altered expression of several host genes. The expression of the genes we selected, with some up-regulated and others down-regulated, differs from the uniform up-regulation of genes following injection of beetles with bacterial LPS (Altincicek et al. 2008), suggesting that the response to H. diminuta is not simply a general response to infection. Although the changes on the mRNA levels of certain genes may be protective for the host, the observed changes in specific gene expression appear to favor the persistence of the parasite.

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319 **Figure Legend**

320 Figure 1. Quantitative expression level of HSP68 in beetles. HSP68 expression was induced by
321 heat shock (1 hr at 37^{o}C). An NADH dehydrogenase gene (NADHd) was used as a reference.
322 The bars represent the collective results of 3 replicate plates. Individual variation was accounted
323 for by the use of a large population of beetles to make cDNA. Bars are 95% confidence intervals
324 (* indicates significance, $P < 0.05$).

325

Table 1. Real-time PCR genes and primer sequences.

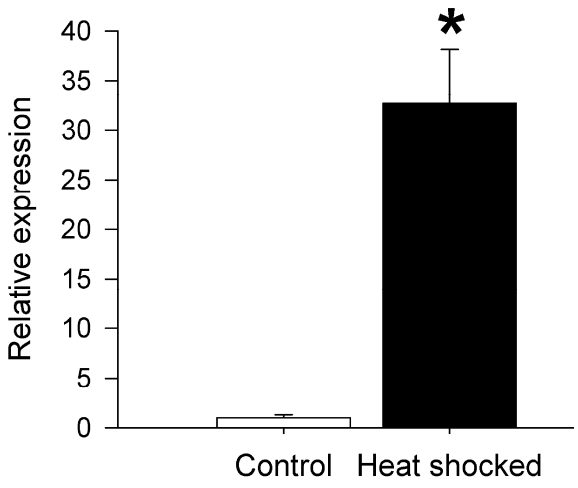
Gene (abbreviation) [accession number]	Forward primer (5' to 3')	Reverse Primer (5' to 3')
Heat shock protein 68 (HSP68) [XM_969349]	ATCGCCGGTTTGAACGTCAT	ACGTTTCGTTCGCCCTTCAA
Sugar transporter (ST) [XM_965271]	GCTGTGGAGTCGGCGAAA	CCTCACCATCATCCCCTTCA
Pheromone binding protein (PBP) [GLEAN_08682]	AACGTTCGTGCTTGTTGCCTTTGC	CGATGTTGTCGTACTTGGTGGTGT
Endoglin (CD105) [GLEAN_09552]	AATGGCAACGAATCGGAGCGAATG	TGAGTTCGTCGAGTGAATCGGCTT
Toll-like receptor 3/4 (TLR3/4) [GLEAN_04438/GLEAN_04439]	AAACCTCCTCCCGGTCCTT	GCGGTAGTGGAGACAGAGTTTGT
Lysozyme 4 (Lz4) [GLEAN_10352]	GTTCCCTGCAGAGCATATTTCC	CGGTGTTAAAAGCGGATTCG
Thaumatin-like protein (TLP) [FJ348342]	CAGCGGCTTGAAGTGTGATACT	TCCCGGCCATGAACATCTTA
Prophenoloxidase 2/3 (proPO2/3) [GLEAN_14907]	TCCCCAACGACTACCTCAAC	TCCAATTCGAGGATTTCACC
NADH dehydrogenase (NADHd) [GLEAN_12859]	AACGAGCGTTCCGTAAACAATGGC	TGGCTTTGTACATTTCGGGAACCG

Table 2. Relative expression (mean ± 95% CI) of genes in uninfected control and Hymenolepis diminuta-infected Tribolium confusum.

Gene	Control	Infected
HSP68	1.0 ± 0.28	1.49 ± 0.08 *
ST	1.0 ± 0.27	3.30 ± 0.39 *
PBP	1.0 ± 0.27	1.63 ± 0.14 *
Endoglin	1.0 ± 0.14	1.36 ± 0.06 *
TLR3/4	1.0 ± 0.16	1.15 ± 0.08
Lz4	1.0 ± 0.22	1.21 ± 0.11
TLP	1.0 ± 0.15	0.67 ±0.03 *
proPO2/3	1.0 ± 0.15	0.38 ± 0.03 *

* $P < 0.05$

Figure 1


*
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15
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0
Relative expression
Control
Heat shocked