Title
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Authors
Zhong, D
Wang, MH
Pai, A
et al.

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

Daibin Zhong, Mei-Hui Wang, Aditi Pai, Guiyun Yan

**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.

**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...
also an intermediate host to *Hymenolepis diminuta* (Cestoda: Cyclophyllidae) 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 cysticeroid 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 & 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 phenomr 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 cysticeroids 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-gram 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 cysticeroids 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 cysticeroids. 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 (PRCP-LE, CTL7, GALE1, TEP-C, CSP66, lysozyme 2–4, cecropin 3, defensin 3 and defensin 4) 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 (PRCP-LE, GALE2, TEP-C, CSP66 and lysozyme 2–4) (Table 1), but could not amplify 5 other genes (CTL7, GALE1, cecropin 3, defensin 3 and defensin 4). 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 χ² 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


ADct values between target gene and reference S3 gene. To determine tapeworm infection induced gene expression, we compared gene transcription levels between beetles challenged and not challenged with tapeworm parasites for each strain and each gene. The statistical significance of p-value was adjusted for multiple comparisons. Fold changes of gene expression were calculated by the relative differences in $2^{-\Delta \Delta CT}$ values between the two strains that were not exposed to tapeworm eggs (Livak and Schmittgen, 2001), whereas fold changes of exposure-induced gene expression were calculated in a similar manner for beetles challenged and not challenged with tapeworm parasites. JMP ver. 9.0. (SAS Institute Inc.) was used to perform all statistical analyses.

### Table 1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene function</th>
<th>Locus IDs</th>
<th>LG</th>
<th>Forward primer ('5-3')</th>
<th>Reverse primer ('5-3')</th>
<th>Length (bp)</th>
</tr>
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<tr>
<td>PGRP-LE</td>
<td>Peptidoglycan recognition protein</td>
<td>LOC657369</td>
<td>3</td>
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<td>β-Galactoside recognition protein</td>
<td>LOC10014126</td>
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<tr>
<td>TEP-C</td>
<td>Thioester-containing protein</td>
<td>LOC663570</td>
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<td>ATGCAGCAATACATCAATTAA</td>
<td>134</td>
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<tr>
<td>cSP66</td>
<td>Clip-domain serine protease</td>
<td>LOC663186</td>
<td>8</td>
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### Table 2

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<th>Strain</th>
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<th>Prevalence</th>
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<tbody>
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<td>cSM</td>
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<td>80</td>
<td>98.1</td>
<td>8.7 ± 0.4</td>
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<tr>
<td>TIW1</td>
<td>Female</td>
<td>80</td>
<td>94.4</td>
<td>8.5 ± 0.4</td>
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<tr>
<td></td>
<td>Male</td>
<td>80</td>
<td>35.0</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>80</td>
<td>32.5</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

### 3. Results

#### 3.1. Infection intensity between resistant (TIW1) and susceptible (cSM) strains

The cSM strain exhibited about 3-fold higher infection prevalence than the TIW1 strain ($p < 0.001$) (Table 2). Among those infected beetle individuals, mean infection intensity in cSM was about 4–5 times higher than the TIW1 strain ($p < 0.001$). There was no statistical difference between males and females for either strain. This confirms that TIW1 strain was resistant to tapeworm infection, and cSM strain was far more susceptible than TIW1.

#### 3.2. Sex difference in gene expression

We did not detect differences in the expression of the 29 genes examined for cSM strain regardless of whether the beetles were exposed to tapeworm or not between males and females. However, TIW1 exhibited statistically significant differences ($p < 0.001$) in transcription for five genes (lysozyme2, PGRP-LE, serpin30 and Toll1) between males and females (Fig. 1).

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**Fig. 1.** Gene expression profile between male and female beetles. The quantitative real-time PCR data (ADct values) of 29 selected essential immunity-related genes are labeled in different colors. The average (Mean ± SD) values of ADct were grouped according to strain (resistant: TIW1 or susceptible: cSM) and parasite status (before or after exposure to parasites). Statistical analyses were used to determine the significance of transcript accumulation based on sex. The p-values are shown in each comparison group. (For interpretation of color in this figure, the reader is referred to the web version of this article.)
3.3. Constitutively differentially expressed genes and transcription profiles of immune genes before and after parasite exposure

Comparison of the two strains found that 13 out of the 29 immune genes exhibited significantly different expression (Fig. 2), suggesting that a significant number of immunity genes were constitutively different in expression between the resistant and susceptible strains. Ten genes (attacin2, GALE2, GNBP3, lysozyme2, PGRP-LE, PGRP-SB, proPO1, serpin30, TEP-C and Toll2) showed significantly higher levels of transcription in TIW1 compared with cSM strains (Fig. 2). Conversely, cSP66, cSPH2 and lysozyme3 genes showed significantly lower transcription in TIW1 compared with cSM strain. The remaining 16 genes showed no statistical differences in expression between TIW1 and cSM strain.

When beetles were exposed to tapeworm parasites, 14 genes in cSM and 15 genes in TIW1 showed significant changes (>2-fold) in gene expression, respectively (Fig. 3). In cSM, 5 genes (TollI, lysozyme2, proPO1, TEP-C and GNBP1) were down-regulated and 9 genes up-regulated in gene expression, whereas in TIW1, only 2 genes (TollI and lysozyme3) were down-regulated and 13 genes up-regulated in gene expression. The TollI gene showed down-regulation in both strains, while 8 genes (GNBP3, cSPH2, lysozyme4, defensin1, PGRP-SA, defensin2, coleoptericin1 and attacin2) were up-regulated in both strains. Two genes (lysozyme2 and proPO1) exhibited significantly decreased expression in cSM, but increased expression in TIW1 strain. Seven genes (TEP-C, GNBP1, serpin29, lysozyme3, proPO2/3, PGRP-LE, PGRP-SB) exhibited significant changes in one of the two strains. Levels of transcripts in 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 parasites (Fig. 4).

4. Discussion

Hymenolepis diminuta causes hymenolepiosis when mammals intentionally or unintentionally eat material contaminated by infected insects. T. castaneum–H. diminuta is a valuable system to study the interaction between insect hosts and microparasites and molecular mechanisms of resistance due to the ease of ecological manipulation of infection and the availability of the T. castaneum genome sequence (Richards et al., 2008; Zhong et al., 2003, 2005). In our study, we infected beetles through direct exposure of beetles to tapeworm-infected rat feces. While this is the natural infection route for the beetles, it should also be noted that infected rat feces also contain bacteria derived from rat intestines, which may affect beetle immune response to some extent. It is unknown whether the bacteria will facilitate or inhibit tapeworm infection. Since we compared immune gene expression between susceptible and resistant beetle strains under the same conditions, the effect of bacteria on the constitutive expression of immune genes and infection-induced expression should be minimal.

To determine the effects of genetic background on the expression of immune genes, the present study examined the transcription profile of 29 previously identified immune genes in two beetle strains with contrasting differences in susceptibility to tapeworm infection. Prior to exposure to tapeworm parasites, we found 13 genes showed constitutive difference in expression between the cSM and TIW1 strains. Among these, 10 genes (attacin2, GALE2, GNBP3, lysozyme2, PGRP-LE, PGRP-SB, proPO1, serpin29, TEP-C, Toll2) showed significantly higher levels of transcription in TIW1 compared with cSM strains. When beetles were exposed to tapeworm parasite, the two strain exhibit significantly different number of genes showing parasite infection-induced expression. 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 showed parasite infection-induced expression (>2-fold change). Among 29 genes, mRNA abundance in 7 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. These results suggest that these genes might be playing an important role in beetle tapeworm infection resistance.

The results indicated that transcript levels of certain genes were quite diverse under different genetic backgrounds. Prior to exposure to the parasite, 5 genes (coleoptericin1, defensin1, defensin2, lysozyme4 and PGRP-SA) showed no difference between the two strains, but significantly increased gene expression after infection in both susceptible and resistant strains. Five genes (proPO1, PGRP-LE, PGRP-SB, lysozyme2 and proPO2/3) showed TIW1 strain specific parasite infection-induced gene expression, while only one gene serpin29, showed cSM strain specific parasite infection-induced gene expression. Zou et al. (2007) identified no changes in transcript levels for PGRP-LA, GALE2, PGRP-LE and TEP-C, some elevations in cSP66, serpin29 and serpin30 transcripts, and a significant increase for peptidoglycan recognition protein (PGRP-SA and PGRP-SB) and antimicrobial peptides (AMPs) (attacin2, coleoptericin1, defensin1 and defensin2) after exposure to bacterial and fungal pathogens in T. castaneum. Similar results were observed in the study after exposure to tapeworm parasites, the mRNA transcription level of PGRP-SA and PGRP-SB became more abundant after the parasite infection, whereas the increase was not
significant for PGRP-LA, GALE2, PGRP-LE and TEP-C after exposure to parasites. However, no significant changes of mRNA level were observed in the three genes cSP66, serpin29 and serpin30. For the five AMP group genes (attacin2, coleoptericin1, lysozyme2, defensin1

Fig. 3. Parasite-induced gene expression in *Tribolium castaneum* beetles. \( \Delta \Delta Ct \) values were calculated by comparing the mRNA abundance between exposed and un-exposed beetles for each gene. Arrows indicate increased or decreased gene expression after exposure to parasites in the resistant TIW1 strain and in the susceptible cSM strain. Fold changes in gene expression were calculated between the two strains.

![Gene expression (fold changes) after exposure](image)

**Fig. 4.** Gene expression (in fold changes) of the immune-related genes between resistant TIW1 and susceptible cSM beetles after exposure to parasites. * Indicates that genes were expressed more than 10 folds higher in the resistant TIW1 strain than in the susceptible cSM strain.
and defensin2), a dramatic increase in mRNA levels was observed in the resistant TIW1 strain after the parasite infection.

Among the genes analyzed in our study that produce plasma proteins directly involved in microbe immobilization or killing (proPOs and lysozymes), there was no significant increase of transcripts when compared to the controls in the cSM strain (the sole exception was lysozyme4). By contrast, transcripts of proPO1, proPO2, lysozyme2 and lysozyme2 increased remarkably in the TIW1 strain. Among the 7 genes (attacin2, coloceptorin1, defensin1, defensin2, lysozyme2, PGRP-SA and PGRP-SB) for which levels of transcripts were more than 10 folds higher in the resistant TIW1 strain than in the susceptible cSM strain after exposure to parasites, three of them (attacin2, lysozyme2 and PGRP-SB) were constitutively differentially expressed genes while the other 4 genes (coloceptorin1, defensin1, defensin2 and PGRP-SA), are expressed due to parasite infection-induced. Defensin, which is active against bacteria, fungi and viruses in mammals, functions by binding to the microbial cell membrane and forming pore-like membrane defects that critically disrupt ion balance (Tieu et al., 2009). No significant inter-strain differences in transcript levels were observed for genes defensin1 and defensin2 before ingestion of tapeworm eggs (Fig. 2). However, both these genes showed increased transcript levels after parasite exposure, and a greater increase was observed in the resistant strain (Fig. 3). Rather than directly affecting the efficiency of parasite transmission, defensin1 is known to indirectly reduce infection efficiency by limiting Plasmodium parasite development in Anopheles gambiae (Blandin and Levashina, 2004). Therefore, it can be suggested that defensin1 and defensin2 may play a significant role in the Tribolium immune response to H. diminuta challenge. Still, the induction of lysozyme and defensin genes is not fully understood in Tribolium, and the need for gene silencing studies to elucidate these potentially critical components of Tribolium immunity must be acknowledged.

Similar abundance of transcripts for genes coloceptorin1, cSP136, defensin1 and PGRP-SA were observed between cSM and TIW1 before exposure to tapeworm eggs. Likewise, both strains exhibited similar changes in the transcript levels of these genes in response to exposure to tapeworm eggs, thus indicating that these genes may not be involved in parasite resistance selection. In the resistant strain, TIW1, all these genes were highly up-regulated after infection. Coloceptorin1 and defensin1 play important roles in responses to parasite infection (Zou et al., 2007; Elahi et al., 2006). Consequently, they were highly up-regulated in our treatments. PGRP-SA, a member of the peptidoglycan recognition proteins (PGRPs), serves as an important surveillance mechanism for microbial infection by binding to Lys- and diaminopimelate-type peptidoglycans of bacteria (Steiner, 2004; Yu et al., 2010). Moreover, the Toll-dependent defense against Gram-positive bacterial infections is mediated through PGRP-SA (Gobert et al., 2003), which supports the role of gene PGRP-SA in response to parasite infection in the beetle.

Genes related to the immune deficiency (IMD) pathway should be highly regulated in response to parasite infection, as the IMD is critical for fighting microbial attacks (Zou et al., 2007). Upon recognition of diaminopimelate-peptidoglycan by PGRPs, the ‘danger’ signal is transported into the cell through IMD. In this study, we observed no difference in IMD gene expression between cSM and TIW1, neither before nor after tapeworm infection. The IMD and toll pathways are involved in the production of different sets of antimicrobial peptides (AMPs) in response to specific pathogens as a component of the humoral immune response in Drosophila (De Gregorio et al., 2002). Both the Toll and the IMD pathways work synergistically to limit infectivity, such that cellular immunity plays a more critical role in overcoming parasite infection (Meister et al., 2005). We did not observe significant responses of genes Toll1–4 to parasite infection, with genes from both pathways showing similar transcription patterns across all our treatments. However, our observations of Toll and IMD gene expression in Tribolium were similar to those from studies of Toll and IMD mediated immunity in Drosophila (Tani et al., 2007).

In summary, the results of this study examined the immunity-related gene response to tapeworm infection, and expanded our knowledge of the variation of different genetic backgrounds in Tribolium beetles acting to infectivity. Our data show that 13 of the 29 immunity-related genes demonstrated significant constitutive differences in transcript abundance between resistant and susceptible strains before exposure to the H. diminuta parasites. After exposure, 14–15 genes showed parasite infection-induced gene expression in susceptible and resistant strains, with seven of these genes lacking differential expression prior to exposure in both strains. Collectively, our study indicated that Tribolium resistance to H. diminuta is primarily due to the up-regulation of critical immune genes. These results should aid in the identification of genes or Quantitative Trait Loci (QTL) related to immune response to parasite infection.

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