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Transcriptional profiling of *transferrin* gene from Egyptian cotton leaf worm, *Spodoptera littoralis**

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Abstract: Iron is an essential nutrient for almost all organisms, but it is also a potent toxin because it can catalyze oxidative reactions that are destructive to cells. Transferrin is an essential protein involved in iron metabolism, immunity, and vitellogenesis in insects. We have characterized a cDNA encoding a putative iron-binding transferrin (*SpliTrf*) in Egyptian cotton leaf worm, *Spodoptera littoralis* (Lepidoptera: Noctuidae), an important pest that causes extensive losses in many vegetable, fodder, and fiber crops. The structure of the putative SpliTrf showed significant homology to other insect transferrins. Gene expression of *SpliTrf* was examined from different adult tissues including the fat body, midgut, Malpighian tubules, nervous system, hemolymph, and ovaries, as well as from different host developmental stages. Transcripts for *SpliTrf* were detected in all developmental stages, but *SpliTrf* was found to be preferentially expressed in the fat body tissue. Furthermore, we analyzed *SpliTrf* expression in response to *Spodoptera littoralis* nucleopolyhedrovirus, *SpliNPV*, and *Bacillus thuringiensis* infection. Our results show that while *SpliTrf* expression is upregulated upon bacterial infection, it is downregulated upon baculoviral infection. We discuss the role of transferrin in iron metabolism as well as in host immune physiology.

Key words: *Bacillus thuringiensis*, baculovirus, *SpliNPV*, *Spodoptera littoralis*, *transferrin*

1. Introduction

Insect immune responses involved in virus defense have lately received increasing attention. While the pathways and effector molecules active in defense against bacteria and fungi are well studied (Ferrandon et al., 2007; Levitin et al., 2008), the regulation of the innate immune system against viral infections still remains to be elucidated. The baculoviruses (Baculoviridae) are double-stranded DNA viruses that infect arthropods, mainly insects and in particular Lepidoptera (Cory and Myers, 2003). Baculoviruses are not only biocontrol agents of lepidopteran pests, but also have been developed and used extensively in research, serving as expression vectors for high-level production of recombinant proteins (Bonning and Hammock, 1996; Susurluk et al., 2013). Although baculoviruses have been successfully used to control lepidopteran and hymenopteran insect pests of agriculture and forestry importance worldwide, little is known about the host immune responses towards these viral infections (Federici, 1986; Miller, 1997; Moscardi, 1999).

Transferrin is an iron-binding protein that has a role in iron transport (Nichol et al., 2002), in preventing oxidative stress, and in delivering iron to eggs for development (Yoshiga et al., 1997). In addition, it is known that transferrin synthesis is increased following exposure to bacteria, fungi, pathogens, and parasites, as well as insect parasitoids (Beernsten et al., 1994; Yoshiga et al., 1997, 1999; Kucharski et al., 2003; Thompson et al., 2003; Ampasala et al., 2004; Valles et al., 2005; Bergin et al., 2006; Lee et al., 2006; Guz et al., 2007, 2012; Magalhaes et al., 2007; Paily et al., 2007; Wang et al., 2007; Mowlds et al., 2008; Yun et al., 2009; Zhou et al., 2009; Kim et al., 2010). Interestingly, although *transferrin* expression was found to be induced by dengue virus infection of the salivary glands in yellow fever mosquito, *Aedes aegypti* (Luplertlop et al., 2011), transferrin protein levels were shown to be downregulated (Tchankouo et al., 2011). This was also the case for infection of *Aedes* mosquitoes with chikungunya virus (Tchankouo et al., 2011). Interestingly, our previous results demonstrated that *transferrin* is significantly reduced in tsetse flies (*Glossina morsitans morsitans*) carrying midgut trypanosome infections (Guz et al., 2007).

Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisduval), is a polyphagous pest that damages a wide variety of crops including cotton, tobacco, and corn in countries around the Mediterranean Basin and in Southeast Asia (Balachowsky et al., 1972; Sneh et al., 1981). The current management strategy for *S. littoralis*

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mainly relies on chemical-based control methods, such as insecticide applications. In addition to their high cost, insecticides pollute the environment and kill nontarget insects. Furthermore, resistance has developed to various types of insecticides in insects including this species, thus reducing the efficacy of these methods (Issa et al., 1984a, 1984b; El-Guindy et al., 1989; Abdallah et al., 1991; Rashwan et al., 1992). New control methods are needed to diminish reliance on insecticides for control of this serious pest. Baculoviruses and their recombinant forms constitute viable alternatives to chemical insecticides for insect control. Baculoviruses are specific to one or a few related insect species (Groner et al., 1986). *Spodoptera littoralis* nucleopolyhedrovirus (*SpliNPV*) is a member of the family Baculoviridae (Volkman et al., 1995) and is classified as a Group II NPV (Zanotto et al., 1993; Bulach et al., 1999).

In this study, we have cloned the *transferrin* cDNA from *S. littoralis* (*SpliTrf*) and analyzed the immune-related expression profile of *SpliTrf* in response to baculoviral and bacterial infection to determine whether transferrin plays a role in the immune response in *S. littoralis*. We also report on a detailed analysis of the temporal expression of *SpliTrf* mRNA, as well as the tissue- and sex-specific nature of its synthesis during development.

2. Materials and methods

2.1. Biological material

The *S. littoralis* colony maintained in the insectary at Ankara University was originally established from larvae collected from cotton fields in the Çukurova region in 2002. Insect cultures were maintained at 25 ± 1 °C with 60% relative humidity and under a constant light:dark regime of 16:8. *S. littoralis* caterpillars were reared on lettuce leaves in plastic cages (24 × 33 × 15 cm).

2.2. Tissue dissection

A total of 25 adults and larvae from the laboratory colony of *S. littoralis* were dissected using a pair of sterilized tweezers under a microscope in ice-cold phosphate buffer saline (pH 7.4). The dissected tissues included the reproductive tract containing the ovaries and the oocytes, the nervous system containing a brain in the head and ganglia in each body segment behind the head, the Malpighian tubules, the mandibles, the gut, and the fat body. All the tissues from larvae were dissected, whereas only the reproductive tract was dissected from adults.

Hemolymph was collected by bleeding sixth instar larvae and transferring it into an Eppendorf tube containing Tris buffer with 10 µL of phenylthiourea (0.02 mM) added to prevent melanization. After centrifugation at 2000 × *g* for 5 min, the supernatant was used for RNA isolation.

2.3. Cloning and partial sequencing of *transferrin*

Total RNA was isolated from whole larval bodies from sixth instar larvae using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. An additional DNase digestion was performed using RNase-free DNase (Ambion). First-strand cDNA synthesis was carried out using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. For amplification of a partial *transferrin* sequence, oligonucleotide primers (F: 5' GGG AGC TCT CCC ATT TGG TC 3'; R: 5' GCC GGG GAG CAT GCG ACG TC 3') were designed according to conserved amino acid sequences of other insect transferrins. All products were cloned into a pGem-T Easy Vector System (Promega). Ligation reactions were used for transformation of competent JM 109 cells according to standard protocols. Plasmid DNA was isolated using Wizard Plus Minipreps DNA Purification Systems (Promega). Sequencing reactions were performed with the DTCS Quick Start Kit (Beckman Coulter), cleaned with the Agencourt CleanSeq Kit (Agencourt Bioscience), and analyzed with the CEQ 8800 Genetic Analysis System (Beckman Coulter). The cDNA sequence was deposited in GenBank under accession number JX160066. The partial cDNA and deduced amino acid sequences of *SpliTrf* were compared using the BLAST tool at the National Center for Biotechnology Information (NCBI) and EXPASY. Sequence alignments were performed using the CLUSTAL W software.

The evolutionary relatedness of *SpliTrf* to other insect transferrins was inferred using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in fewer than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011).

2.4. Expression analyses

To analyze the expression of *SpliTrf* during different developmental stages, RNA was prepared from the egg, neonate, 2nd instar larvae, 3rd instar larvae, 4th instar larvae, 5th instar larvae, 6th instar larvae, pupae, female adult, and male adult using TRIzol reagent (Invitrogen).

To detect the immune responsive expression profile of *SpliTrf*, newly molted third instar larvae were challenged

by 10^6 *Bacillus thuringiensis* and challenged by *SpliNPV* with a dose of 3000 occlusion bodies using the droplet feeding method, respectively (Hughes et al., 1986; Toprak and Gürkan, 2004). Bacterial and viral OB stocks were prepared in a 10% sucrose solution containing 0.4% (w/v) Patent Blue V food coloring dye (Sigma-Aldrich). For each infection, 24–36 larvae were allowed to drink individually from the virus or bacteria suspension for 15 min. Control larvae were droplet fed using a virus- or bacteria-free 10% sucrose solution containing 0.4% (w/v) Patent Blue V food coloring dye. Larvae that failed to ingest the entire droplet were discarded. Challenged larvae were collected at 12 h, 24 h, 48 h, and 72 h after feeding. At each time point, 3 biological replicates were collected from the experimental and control groups and stored at –80 °C until RNA isolation.

Tissue-specific expression was accomplished using fat body, midgut, Malpighian tubules, nervous system, and ovaries dissected from various stages of *S. littoralis*. Hemolymph was collected by bleeding sixth instar larvae, and total RNA was isolated by using TRIzol reagent (Invitrogen).

2.5. qRT-PCR analysis of *SpliTrf* expression

For qRT-PCR analysis, a minimum of 6 infected and age-matched control larvae were collected at 4 different time points post-challenge (12 h, 24 h, 48 h, and 72 h). Three biological replicates were prepared for each sample, and 2 larvae were pooled for each replicate. Three technical replicates were performed for each biological replicate on the qPCR plates. Samples were treated with RNase-free DNase I (Ambion). First-strand cDNA synthesis was performed with 1 µg of total RNA by using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The reaction mixture was incubated at 55 °C for 30 min. cDNA was amplified by using the LightCycler 480 Probes Master Kit (Roche). Amplification conditions consisted of an initial preincubation at 95 °C for 10 min, followed by amplification of the target DNA for 35 cycles of 95 °C for 10 s, 53 °C for 30 s, 72 °C for 1 s, and 1 cycle of cooling at 40 °C for 10 s with the LightCycler 480. A standard curve was generated for each set of primers and the efficiency of each reaction was determined. qRT-PCR was performed using the primer pair *SpliTrf* F: 5' CGA AGG AAA ATG AGC TGA A 3' and *SpliTrf* R: 5' GTC AGG ATA GTC GCA TTT ATC A 3'. Each sample was analyzed in triplicate and normalized to the internal control, *actin* mRNA. Transcript quantification for *actin* was performed using the primer pair *SpliAct* F: 5' ATC ATG TTC GAG ACC TTC AAC 3'and *SpliAct* R: 5' GCA CGA TTT CTC TCT CGG 3'. Statistical significance was determined by using Student's t-test and Microsoft Excel software. Asterisks denote P-values less of than 0.05, which is considered to be statistically significant.

3. Results

3.1. Cloning of *Spodoptera littoralis transferrin* (*SpliTrf*) cDNA

The molecular phylogeny of insect transferrins was examined using amino acid sequences from a number of insect orders including Lepidoptera, Coleoptera, Diptera, Hemiptera, Hymenoptera, and Orthoptera (Figure 1). The phylogenetic tree based on the transferrins analyzed reflected the expected relationships of the host species and taxa. Based on the phylogenetic analysis, the partially deduced amino acid sequence of SpliTrf was clustered together with all the other Lepidoptera transferrins.

3.2. Developmental-, sex-, and tissue-specific expression profile of *SpliTrf*

We analyzed *SpliTrf* expression using qRT-PCR analysis during different developmental stages: egg, neonate, 2nd instar larvae, 3rd instar larvae, 4th instar larvae, 5th instar larvae, 6th instar larvae, pupae, female adult, and male adult (Figure 2A). Expression of *SpliTrf* was detected in all developmental stages, with the lowest levels observed in the egg and the neonate, increasing through development. On the other hand, *transferrin* is expressed in both sexes but its expression is higher in females than in males.

We evaluated the transcript abundance of *SpliTrf* using qRT-PCR analysis from different larval tissues including the fat body, midgut, Malpighian tubules, nervous system, hemolymph, and adult ovaries. Results shown in Figure 2B indicate that the *SpliTrf* is preferentially expressed in the fat body tissue. Fewer *SpliTrf* transcripts are detectable in the ovaries, nervous system, Malpighian tubules, hemolymph, and midgut, respectively.

3.3. *SpliTrf* expression in response to *B. thuringiensis* and *SpliNPV* infection

To characterize the expression profile of *SpliTrf* in response to bacterial challenge, 3rd instar larvae were infected with *B. thuringiensis* and the transcript levels for *SpliTrf* were analyzed using qRT-PCR analysis (Figure 3A). The results showed that *SpliTrf* was upregulated upon bacterial infection relative to control larvae. The expression of *SpliTrf* was significantly higher in the larvae 48 h after bacterial challenge and remained high at the 72-h time-point. Expression of the *SpliTrf* transcript levels was similarly evaluated in response to infection with *SpliNPV* (Figure 3B). In contrast, *SpliTrf* expression decreased by 3-fold at 12 h and remained significantly lower even at the 24-h time-point after viral challenge relative to uninfected larvae. The expression of *SpliTrf* returned to control levels by 48 h after viral challenge. Thus, it appears that *transferrin* expression is differentially regulated by pathogenic agents, with the levels increasing in response to bacteria and decreasing in response to viral introduction.

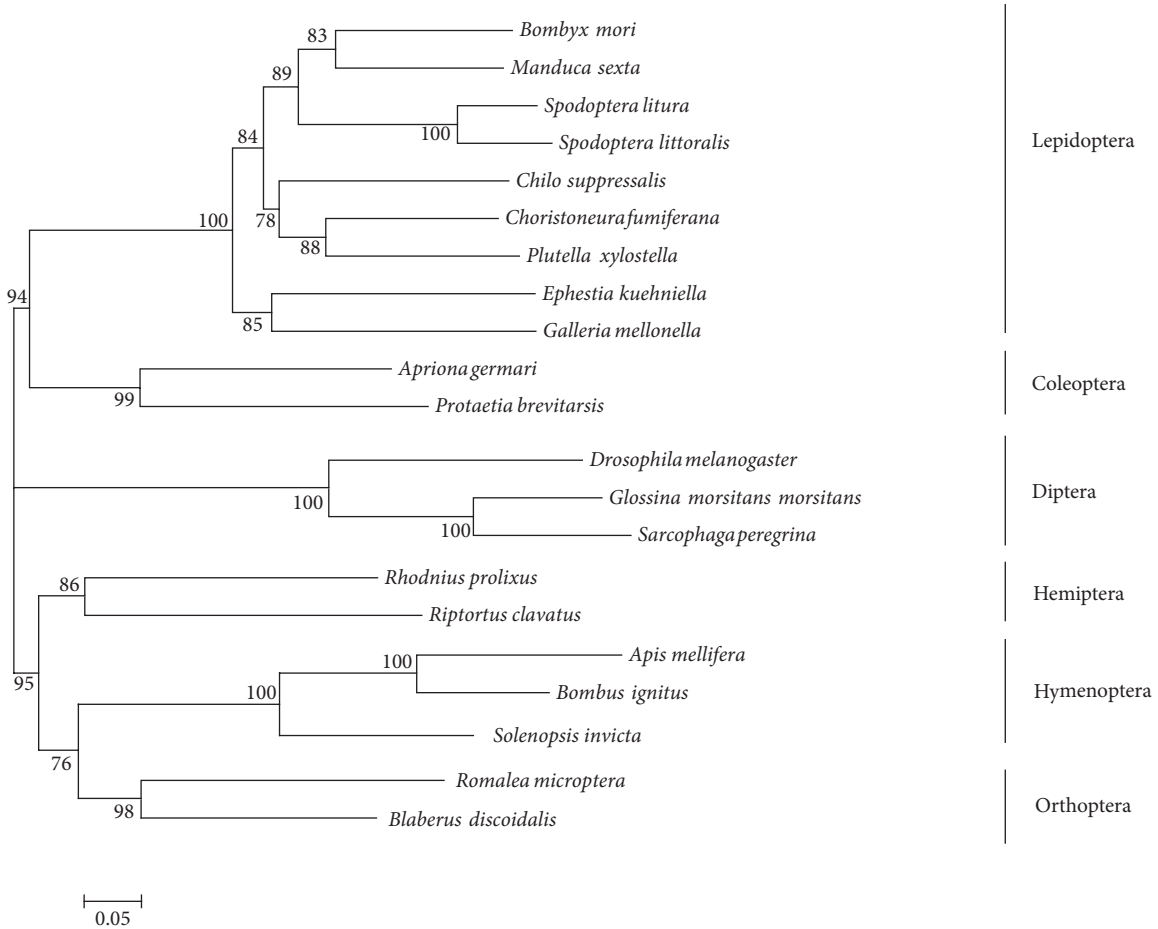

Figure 1. Molecular phylogeny of insect transferrins. Pairwise alignment and tree generation was performed using MEGA5 after 1000 bootstraps with the neighbor-joining algorithm. Bootstrap values above 75% are shown in the nodes. The species and GenBank accession numbers of the transferrin sequences used to construct the phylogenetic tree are as follows: *Bombyx mori*, NP_001037014; *Manduca sexta*, AAA29338; *Spodoptera litura*, ABF21123; *Spodoptera littoralis*, AFP89365; *Chilo suppressalis*, BAD27263; *Choristoneura fumiferana*, AAT08022; *Plutella xylostella*, BAF36818; *Ephestia kuehniella*, ADF35768; *Galleria mellonella*, AAQ63970; *Apriona germari*, AAW70172; *Protaetia brevitarsis*, ABI31834; *Drosophila melanogaster*, AAC67389; *Glossina morsitans morsitans*, AAM46784; *Sarcophaga peregrina*, BAA06067; *Rhodnius prolixus*, ABU96701; *Riptortus clavatus*, AAD02419; *Apis mellifera*, NP_001011572; *Bombus ignitus*, ABV68876; *Solenopsis invicta*, AAY21643; *Romalea microptera*, AAQ62963; *Blaberus discoidalis*, AAA27820.

4. Discussion

We have cloned a putative iron-binding transferrin (*SpliTrf*) from the lepidopteran Egyptian cotton leaf worm, *S. littoralis*. The deduced amino acid sequence of SpliTrf showed significant homology with other known lepidopteran transferrins from *Spodoptera litura* (92%), *Danaus plexippus* (79%), *Chilo suppressalis* (78%), *Bombyx mori* (77%), *Galleria mellonella* (75%), and *Ephestia kuehniella* (71%). To investigate the evolutionary relationship of SpliTrf, phylogenetic analysis was carried out using the neighbor-joining method based on 21 transferrin sequences from different insects. The dendrogram that was obtained places the SpliTrf as a sister taxa to the transferrin from the species *S. litura* in a separate lineage clustering with other lepidopterans.

In insects, the fat body is important for a number of physiological processes, including immunity, reproduction, flight, and energy, as well as iron metabolism (Arrese and Soulages, 2010). Fat body cells not only control the synthesis and utilization of energy reserves, but are also the site where most of the hemolymph proteins and circulating metabolites are synthesized (Kanost, 1990).

In this study, we found that the fat body has the highest level of *SpliTrf* mRNA compared to the other analyzed tissues, which is similar to results from other insects where *transferrin* is expressed primarily in the fat body (Yoshiga et al., 1999; Hirai et al., 2000; Ampasala et al., 2004; Harizanova et al., 2005; Strickler-Dinglasan et al., 2006; Guz et al., 2007, 2012; Kim et al., 2008). Like other storage or lipid transport proteins, transferrin is a protein

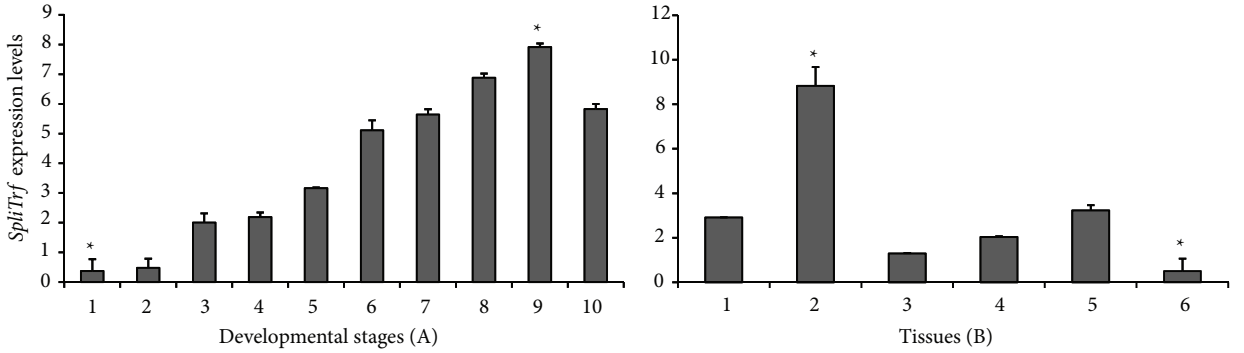

Figure 2. qRT-PCR analysis of developmental-, sex-, and tissue-specific expression profile of *SpliTrf*. A) Lanes: 1: egg, 2: neonate, 3: 2nd instar larvae, 4: 3rd instar larvae, 5: 4th instar larvae, 6: 5th instar larvae, 7: 6th instar larvae, 8: pupae, 9: female adult, 10: male adult. B) Lanes: 1: nervous system, 2: fat body, 3: hemolymph, 4: Malpighian tubules, 5: ovaries, 6: midgut (t-test; *: $P < 0.05$).

that shuttles iron among various tissues that are involved in iron storage. We detected lower levels of *transferrin* in various host tissues, and the role of *transferrin* in these tissues remains to be studied. It is clear that transferrin plays a role in the growth and normal function of the human central nervous system (Monteros et al., 1989). Although it is not known whether a transferrin receptor pathway exists in insects, studies suggest that a transferrin receptor is necessary for development of the mouse nervous system (Arrese and Soulages, 2010). We also detected *transferrin* expression in the nervous system of *Spodoptera*, which might be due to juvenile hormone regulation or a protection mechanism against reactive oxygen intermediates, as has been previously suggested in the honeybee central nervous system (Jamroz et al., 1993; Hirai et al., 2000; Kucharski and Maleszka, 2003; Nascimento et al., 2004). *Transferrin* mRNA is also detected in the ovaries of *Spodoptera*, supporting its classification as a vitellogenic protein (Kurama et al., 1995). Expression of *SpliTrf* has also been detected from the hemolymph of larvae, suggesting that *transferrin* may also be expressed by hemocytes, unless this has resulted from fat body contamination of the isolated hemolymph. Zinc, iron, and copper were first reported in insects in certain types of storage vacuoles located in the Malpighian tubules of houseflies (Sohal et al., 1976). Iron must be absorbed from the diet into gut cells, shuttled from the apical to the basal membrane of the gut epithelium, and transferred to the hemolymph (Nichol et al., 2002). Since excess iron is destructive to many cells, the *SpliTrf* mRNA found in *Spodoptera* Malpighian tubules and the midgut might function to stabilize the availability of iron between cells.

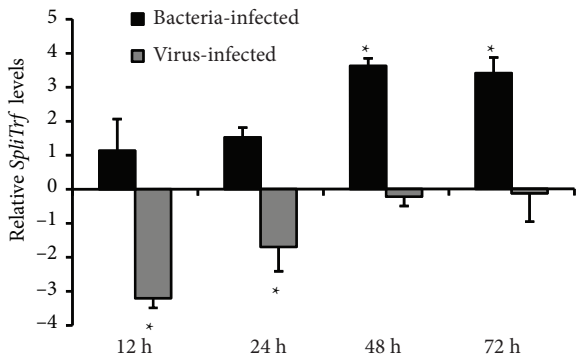

Figure 3. Expression of *SpliTrf* in larvae challenged with A) *Bacillus thuringiensis* and B) *SpliNPV* relative to the unchallenged controls analyzed during the same time periods. Asterisk represents fold difference between the challenged and unchallenged larvae as significant with a t-test score of $P < 0.05$.

Expression of *SpliTrf* was detected in all developmental stages, indicating an important role throughout the Egyptian cotton leaf worm life cycle. Although there are differences in insect developmental stages (Kurama et al., 1995; Yoshiga et al., 1999; Strickler-Dinglasan et al., 2006), *transferrin* transcript levels tended to increase through larval development, reaching a maximum in either the last instar or pupal stage (Ampasala et al., 2004; Valles et al., 2005; Guz et al., 2012).

Multiple functions have been attributed to transferrin, such as iron metabolism, immunity, and reproduction (Nichol et al., 2002). Transcription and translation of the transferrin protein are upregulated by bacterial infection in several species of insects, including Lepidoptera (Nichol et al., 2002; Seitz et al., 2003; Ampasala et al., 2004; Yun et al., 2009; Guz et al., 2012). Here we demonstrate that *SpliTrf* is also upregulated in response to challenge with a gram-positive bacterium, *B. thuringiensis*. Although the antimicrobial responses of insects largely involve the Toll pathway for combating gram-positive bacteria and fungi, and IMD pathway functions for gram-negative bacteria (Lemaitre et al., 1997; Hoffmann, 2003), expression of *transferrin* was also reported to be upregulated by both gram-positive and gram-negative bacteria in *Drosophila* and *Choristoneura* (De Gregorio et al., 2001; Ampasala et

al., 2004). However, the scenario for *transferrin* expression in *SpliNPV*-infected *Spodoptera* larvae differs from the host response to bacterial challenge, as we detected lower levels of *transferrin* following baculovirus challenge relative to uninfected controls. The decrease in host *transferrin* expression mediated by *SpliNPV* infection could be explained by different mechanisms. *SpliNPV* could repress the expression of host *transferrin* due to a shut-off mechanism of host protein synthesis similar to that shown in *Spodoptera frugiperda* (Salem et al., 2011). Baculovirus-infected host protein synthesis is shut down at approximately 10–12 h after infection when the virus starts producing new virions (Carstens et al., 1979; Maruniak et al., 1981). After 24 h post baculovirus infection, the majority of newly synthesized proteins are either virus-encoded or virus-induced. The shut-off mechanism associated with host protein synthesis appears to be correlated with suppression of host gene transcription (Ooi et al., 1988; van Oers et al., 2001, 2003). An alternate mechanism could be the host downregulation of *SpliTrf* to reduce virus survival, replication, and/or transmission. Since iron is an essential nutrient for most pathogens, one effective host immune response is to reduce iron availability to pathogens. Thus, reduced expression of *transferrin* could represent a host immune response for reducing *SpliNPV* infection success. A similar pathogen iron-scavenging mechanism has been documented in protozoan infections (Wilson and Britigan, 1998; Marquis and Gros, 2007). Results obtained for the interaction between *Trypanosoma brucei* (African trypanosomiasis/nagana) and its hosts (humans/animals) show that *T. brucei* obtains iron through receptor-mediated endocytosis of host transferrin (Gerrits et al., 2002; Taylor and Kelly, 2010). Since all organisms require iron, baculoviruses may similarly have yet uncharacterized iron-acquiring mechanisms.

Iron concentration also regulates microbial symbiotic interactions in insects that acquire iron from host transferrin (Collins, 2003). In the case of the *Wolbachia* endosymbiont and its hosts, varying iron levels have been suggested to play a pivotal role in the interaction (Kremer et al., 2009). Furthermore a reduction in the expression of *transferrin* has been detected in *Wolbachia*-infected flies (Rances et al., 2012). Although potential endosymbionts of *Spodoptera* species remain as yet unknown, it will be interesting to know how iron metabolism occurs in these symbionts.

It is not clear that baculoviruses are capable of actively suppressing the immune responses of their hosts; however, Sim and Dimopoulos (2010) indicated that DENV is capable of inhibiting immune pathway activation in mosquito cell lines. On the other hand, since *Heliothis virescens* (Fabricius) (tobacco budworm) larvae have altered iron tissue distribution and movement following baculoviral infection, it is proposed that larval iron homeostasis may be substantially disrupted by baculoviral infection (Popham et al., 2012). For functional analysis of the effects of the reduced *SpliTrf* transcripts on protein levels and infection outcome between *S. littoralis* and the *SpliNPV* system, future functional studies through application of RNAi may be important.

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