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# *Solenopsis invicta* transferrin: cDNA cloning, gene architecture, and up-regulation in response to *Beauveria bassiana* infection

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**Abstract**

Transferrin genes from several insects have been shown to be induced in response to bacterial or fungal infection. We were interested to know whether transferrin genes in the red imported fire ant, *Solenopsis invicta*, are similarly induced by microbial challenge. Hence, the cDNA and structure of a gene exhibiting significant homology to insect transferrins were elucidated for *S. invicta*. The cDNA was comprised of 2417 nucleotides, excluding the poly(A) tail, with a large open reading frame of 2106 nucleotides. The predicted translation product of the *S. invicta* tranferrin (SiTf) gene was a 702 amino acid polypeptide with an estimated molecular mass of 77.3 kDa and a p*I* value of 5.66, characteristics consistent with transferrin proteins. Comparative analysis of genomic and cDNA sequences revealed that the SiTf gene was comprised of 8 exons. Quantitative real-time PCR was used to examine the expression of SiTf. Expression of SiTf was induced in worker ants exposed to *Beauveria bassiana* conidia. Autoclave-killed conidia did not elicit a SiTf induction response from worker ants. Genes, like SiTf, responding to microbe attack or infection may provide a unique approach to assist in the discovery of microbial control organisms for the target insect pest.
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*Keywords:* Transferrin; *Solenopsis invicta*; Q-PCR; Gene expression

## 1. Introduction

Insects exhibit complex, interconnected, immune responses that act synergistically to provide protection against microbial infection (Tzou et al., 2002). Morphological and physiological characteristics are often considered the "first line of defense" against attack (Tanada and Kaya, 1993). Principal among these characters is the cuticle which affords a physical barrier that extends into the gut and trachea—two main routes of microbial infection. A microbe-hostile chemical environment is also created in the gut by maintenance of an alkaline or acidic pH and by the secretion of endogenous antimicrobial substances, such as lysozyme (Tzou et al., 2002). When pathogens breach these physical and chemical barriers, they elicit a systemic response comprised of cellular and humoral mechanisms (Dimarcq et al., 1998; Lamberty et al., 2001; Tzou et al., 2002). Cellular mechanisms involved in pathogen neutralization or clearance include phagocytosis and/or encapsulation of larger microorganisms or parasites (Lamberty et al., 2001). Concomitantly, a humoral response is initiated that includes the activation of proteolytic cascades leading to melanization, release of antimicrobial peptides, and, recently discovered in *Drosophila* and *Anopheles gambiae*, a complement-like cascade that may contribute to opsonization (Lagueux et al., 2000; Levashina et al., 2001; Hoffmann and Reichhart, 2002).

Recently, the iron-binding protein transferrin has been hypothesized to participate in the insect innate immune response to microbial infection by sequestering iron, an essential nutrient (Yoshiga et al., 1997; Ratledge and Dover, 2000; Nichol et al., 2002). Indeed, a number of insect transferrins have been reported to be up-regulated in response to microbial challenge (Yoshiga et al., 1997, 1999; Yun et al., 1999; Kucharski and Maleszka, 2003;

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Thompson et al., 2003; Ampasala et al., 2004). We were interested to know whether transferrin genes in the red imported fire ant, *S. invicta*, are similarly induced by fungal challenge. Thus, we report the first transferrin cDNA and gene structure from the red imported fire ant, *Solenopsis invicta* Buren. Expression of this gene was found to be up-regulated in response to challenge by the fungus, *Beauveria bassiana* (Balsamo) Vuillemin.

## 2. Materials and methods

### 2.1. Cloning and sequencing of the S. invicta transferrin cDNA

Examination of expressed sequence tags (ESTs) of a monogyne *S. invicta* colony (Valles et al., unpublished data) revealed a single clone (1D5) exhibiting significant E value ($10^{-41}$) by BLAST with insect tranferrins. An expression library was prepared previously from all stages and castes of a monogyne *S. invicta* colony using the ZAP-cDNA synthesis/Gigapack III Gold Cloning kit (Stratagene, La Jolla, CA). ESTs were generated by directionally sequencing approximately 2000 randomly-picked clones. The 1D5 clone served as starting point for the design of oligonucleotide primers which were used to conduct 5′ and 3′ rapid amplification of cDNA ends (RACE) of the putative fire ant transferrin transcript. 5′ and 3′ RACE were completed with the GeneRacer kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Total RNA was extracted from adult worker ants (~40 individuals) taken from a monogyne colony (started from a newly-mated queen collected from the field) using the Agilent Technologies (Wilmington, DE) total RNA isolation mini kit. The total RNA (2.5 μg) was decapped, and ligated to the GeneRacer RNA 5′ oligonucleotide provided with the kit. The decapped, ligated RNA was reverse transcribed to produce cDNA with the gene-specific oligonucleotide primer, p285 (Table 1), at 42 °C for 50 min in a thermal cycler. PCR was subsequently conducted with the cDNA as template using the GeneRacer 5′ oligonucleotide primer and a nested gene-specific primer, p286 (Table 1), at 94 °C for 2 min, 35 cycles at 94 °C for 15 s, 68 °C for 2 min.

cDNA synthesis for the 3′ RACE reaction was completed with the GeneRacer oligo dT primer and 2.5 μg of total RNA at 42 °C for 50 min in a thermal cycler. PCR was subsequently conducted using the cDNA as template with the GeneRacer 3′ primer and gene-specific primer, p287 (Table 1), at 94 °C for 2 min, 35 cycles at 94 °C for 15 s, 68 °C for 1 min.

The sequence gap between the 5′ and 3′ reactions was amplified from worker ant total RNA using the One-Step Reverse Transcription PCR kit (Invitrogen) with oligonucleotide primers p298 and p285 (Table 1).

In all instances, amplicons were separated on a 1.2% agarose gel, ligated into pCR-4 TOPO vector and transformed into One Shot Top10 competent cells (Invitrogen). Insert-positive clones were sequenced by the Interdisciplinary Center for Biotechnology Research, University of Florida. Three clones were sequenced for each cDNA region.

### 2.2. Cloning and sequencing the genomic DNA of the S. invicta transferrin gene

To determine the architecture of the *S. invicta* transferrin (SiTf) gene, PCR was conducted with genomic DNA using a series of overlapping oligonucleotide primers (p296/p295, p294/p286, p338/p337, p298/p297, p316/p297, p336/p335, p358/p357) designed from the transcript (Table 1; Fig. 1). Genomic DNA was isolated from *S. invicta* workers from a

Table 1
List of oligonucleotide primers used for cDNA synthesis and amplification of the SiTf gene

| Primer | Nucleotide position | | Sequence (5′ to 3′ orientation) |
|---|---|---|---|
| | cDNA | Gene | |
| p85 | NA[a] | 1064–1088 | GCTAGCTGGCATCGTTTATGGTTAG |
| p285 | 1915–1941 | IJ[b] | AGCGCCGTCCTTCTCGTTATCGAGTTG |
| p286 | 1309–1341 | 2160–2192 | GGAGAACGCGGCTCTAGCGAGATTACGACACTT |
| p287 | 1780–1808 | 3070–3098 | GATGACGATCAGGCGAAAGAAGCGACGAA |
| p294 | 422–453 | 422–453 | CTGGATACAATGTCATCGAACAGGTGAGGACG |
| p295 | 717–744 | 717–744 | CCTCTGATTGATGGCAGGATCGGGTGAC |
| p296 | 1–35 | 1–35 | CATGGACTGAAGGAGTAGAAAGGACAGTCTCGACA |
| p297 | 2212–2242 | 4130–4160 | CCCAATCATTCCATTTGTCCCATGTGTTGTC |
| p298 | 1261–1285 | 2112–2136 | CCGGAGAGAGACGCTCGTTGGTGTG |
| p316 | 1544–1578 | 2593–2627 | CTGTTATCAAGAGTGGCTCCTCCCTAAATGGATTG |
| p335 | 1921–1948 | 3479–3506 | GTTTGCCAGCGCCGTCCTTCTCGTTATC |
| p336 | 1713–1736 | 3003–3026 | GTCTTGTGCGCCTGGCGCACCTCT |
| p337 | 1261–1285 | 2112–2136 | CACACCAACGAGCGTCTCTCTCCGG |
| p338 | 717–744 | 717–744 | GTCACCCGATCCTGCCATCAATCAGAGG |
| p357 | 2383–2417 | 4301–4335 | ATCGACAGTACTTTATTTATCTGACAGTTATCTCG |
| p358 | 2215–2240 | 4133–4158 | AACACATGGGACAAATGGAATGATTG |

[a] Not applicable.
[b] Primer spans an intron junction.

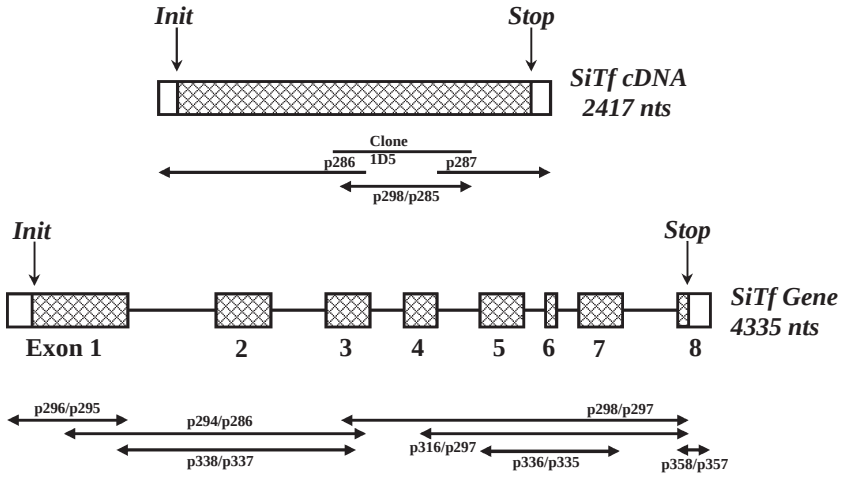


Fig. 1. Schematic representation of the SiTf cDNA (top). Init and Stop denote the approximate locations of the initiation and stop codons, respectively. Genomic architecture of the region encoding SiTf (bottom). Exons are indicated by boxes. Cross-hatched areas represent SiTf coding region. Open areas represent the 5′ and 3′ untranslated regions. In both instances, arrows represent the approximate positions of amplicons that were cloned and sequenced to construct the gene. Clone 1D5 represents the approximate position of the EST clone used to develop primers for 5′ and 3′ RACE.

monogyne colony with the PUREGENE DNA isolation kit (Gentra, Minneapolis, MN).

### 2.3. SiTf Q-PCR validation

Expression of the SiTf gene was quantified using real-time quantitative PCR (Q-PCR) by the comparative $C_T$ method (Livak and Schmittgen, 2001). This method utilizes an endogenous reference and treatment calibrator to determine the relative quantity of a target transcript. One crucial requirement for use of this method is a validation experiment to demonstrate that reverse transcription efficiencies of the target and reference genes are approximately equal over a usable range of RNA concentrations. We chose the 18 S ribosomal RNA gene (Valles and Pereira, 2003, GenBank accession number: AY334566) as our endogenous reference. Nucleotide sequences for the SiTf and 18 S ribosomal genes from *S. invicta* were submitted to the Assays-by-Design Service at Applied Biosystems for synthesis of TaqMan MGB probes and primers for each respective gene. Nucleotide sequences and gene positions for each of the TaqMan probes and oligonucleotide primers are provided in Table 2. Both probes utilized the reporter dye, FAM (6-carboxyfluorescein), attached to the 5′ end and a dark quencher, DABCYL (4-(4′-dimethylaminophenylazo)benzoic acid) attached to the 3′ end. The probe for the target gene, SiTf, was designed to span intron 3 (Fig. 1).

First, an experiment was conducted to determine the suitability of the 18 S ribosomal gene as a reference for the SiTf gene. RNA (0.1, 1, 10, and 50 ng) extracted from fire ant workers (~20) was incubated with 1 unit of amplification grade DNAse I (Invitrogen) for 15 min at room temperature. The DNAse activity was killed by addition of EDTA to a final concentration of 2.27 mM and heating to 65 °C for 10 min. cDNA synthesis was subsequently completed with Super-Script II reverse transcriptase (Invitrogen) and oligonucleotide primers, p285 and p85, for SiTf and 18 S, respectively (Table 1). Reverse transcription was completed in a thermal-cycler at 45 °C for 30 min and terminated by heating to 70 °C for 15 min. Q-PCR was subsequently completed in a 25 μl reaction volume with the corresponding probe/primer combination (Table 1) using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) following the temperature regime: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Successful DNAse I treatments were confirmed by lack of amplification in Q-PCR among identical samples without reverse transcription.

Table 2
Oligonucleotide probes and primers used in quantitative PCR analysis

| Primer name | Nucleotide position[a] | Sequence (5′ to 3′ orientation) |
|---|---|---|
| QSiTfF | 1300–1323 | GCGCTGAAAAAGTGTCGTAATCTC |
| QSiTfR | 1362–1386 | CTCGTCTTTCTCCAGTATGCAATCA |
| QSiTfProbeFAM | 1331–1347 | CCGCGTTCTCCAGAGAC |
| Q18SF | 501–525 | CCCGTAATCGGAATGAGTACACTTT |
| Q18SR | 577–599 | ACGCTATTGGAGCTGGAATTACC |
| Q18SProbeFAM | 549–565 | CACCAGACTTGCCCTCC |

[a] cDNA position.

### 2.4. SiTf expression in response to B. bassiana infection

Expression of the SiTf transcript was evaluated in response to challenge with the entomopathogenic fungus, *B. bassiana*. Preliminary experiments had indicated an up-regulation of the SiTf gene in worker ants challenged with this fungus. Therefore, we conducted two experiments to confirm and characterize this response. Fungal challenge was achieved by a modified method described by Pereira et al. (1993). Induction of the SiTf gene was monitored after exposure of ants to varying doses of *B. bassiana* conidia (dose response) and varying time periods after exposure to a

Table 3
SiTf comparative sequence identities with other insect transferrins

| Insect species | Accession number | Amino acid identity (%) | Citation |
|---|---|---|---|
| *Apis mellifera* | AAQ02340, AAO39761 | 67.0 | Nascimento et al., 2004; Kucharski and Maleszka, 2003 |
| *Blaberous discoidalis* | Q02942 | 43.7 | Jamroz et al., 1993 |
| *Manduca sexta* | P22297 | 39.9 | Bartfield and Law, 1990 |
| *Aedes aegypti* | AF019117 | 32.5 | Yoshiga et al., 1997 |
| *Sarcophaga peregrina* | Q26643 | 31.2 | Kurama et al., 1995 |
| *Drosophila melanogaster* | AAC77913 | 28.9 | Yoshiga et al., 1999 |

fixed quantity of conidia (time course). For the dose response, 60 large workers from a monogyne colony were placed into a 60 ml soufflé cup containing 0.5 ml of a *B. bassiana* suspension of conidia (0, $10^4$, $10^5$, $10^6$, and $10^7$ conidia/ml). Conidia viability ranged from 94 to 100% for all experiments as determined by plating spores on SDAY and quantifying germination after 18–24 h. The cups were swirled for 10 s to wet all of the ants, after which, the ants were transferred to a 120 ml soufflé cup containing a single paper towel (21 × 11.5 cm) to absorb the excess liquid. The ants were held in an incubator at 27 °C without food. Thirty ants were removed at 3 days and RNA extraction and Q-PCR were conducted as described above. Mortality was monitored on the remaining ants for 10 days. Dead ants were removed daily, surface sterilized in 95% ethanol, and placed individually into microplate wells and incubated under moist conditions to verify infection by monitoring for fungal sporulation. Controls were treated identically with deionized water in place of the spore suspension. The time course experiment was completed in a similar manner except a larger number of ants (250–300) were treated in a single cup with 1.5 ml of $10^7$ conidia/ml. Groups of 30 ants were periodically removed (0, 7, 12, 24, 48, 72 h) from the cup and analyzed. In addition, a control group was included that was treated with deionized water only. The dose response and time course experiments were replicated 4 and 3 times, respectively, each time with ants from a different laboratory colony. Change in critical threshold ($\Delta C_T$) was determined by subtracting the SiTf $C_T$ from the 18 S $C_T$ value. $\Delta\Delta C_T$ was then determined by subtracting all $\Delta C_T$ values from the $\Delta C_T$ of the calibrator. The calibrator was identified as the treatment with the lowest expression level (i.e., highest $\Delta C_T$). The relative expression ($2^{-\Delta\Delta CT}$) of SiTf was calculated and non-overlapping confidence intervals were used to indicate significant differences between treatments (Livak and Schmittgen, 2001).

Experiments were also conducted to examine the influence of killed conidia on SiTf expression. A suspension of *B. bassiana* conidia ($10^7$conidia/ml) was prepared and divided into two vials. One vial was autoclaved for 15 min at 121 °C at 1 kg/cm$^2$ to kill the conidia. Confirmation of inviability was determined by failure of the conidia to germinate when placed on SDAY growth medium as described above. Worker ants were treated with killed or live conidia and expression of SiTf was evaluated by Q-PCR after a 3-day exposure. Controls were again treated with deionized water only. Three replicates were conducted, each with ants from a different laboratory colony.

## 3. Results

### 3.1. S. invicta transferrin cDNA and gene architecture

The SiTf cDNA was constructed by compiling sequences from 5′ and 3′ RACE reactions, and a single subclone joining the RACE-derived sequences (Fig. 1, Accession number AY940116). The EST clone, 1D5, which was used as a starting point for oligonucleotide primer design, matched the SiTf cDNA sequence. The cDNA was found to be comprised of 2417 nucleotides, excluding the poly(A) tail. Analysis revealed a single large open reading frame (ORF) with an untranslated region (UTR) at each end. The ORF commenced at the first start AUG codon present at nucleotide 145 and terminated at a stop codon (UAG) at nucleotide position 2251. The predicted translation product of the SiTf gene was a 702 amino acid polypeptide with an estimated molecular mass of 77.3 kDa and a p*I* value of 5.66. BLASTp analysis of the SiTf deduced polypeptide yielded significant identity to several insect transferrins (Table 3).

The SiTf gene was constructed from 7 overlapping fragments generated by PCR with genomic DNA as

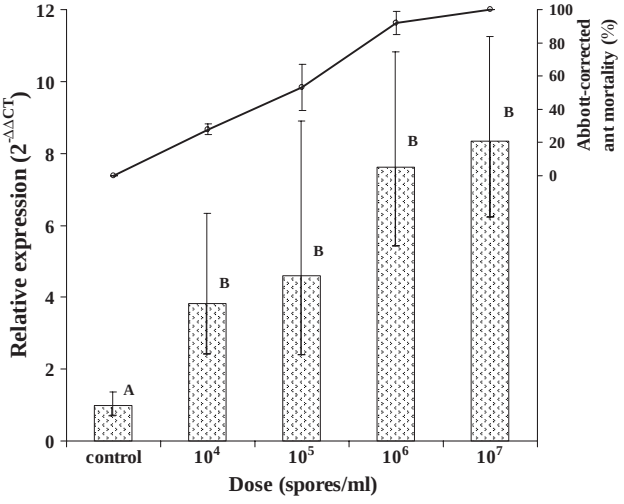


Fig. 2. Relative expression ($2^{-\Delta\Delta CT}$) of SiTf in worker ants after a 3-day exposure to different concentrations of *B. bassiana* conidia as determined by quantitative PCR. (lower graph). Corresponding cumulative ant mortality measured on day 10 (upper graph). Means with the same letters do not differ significantly by comparison of confidence intervals as calculated by the comparative $C_T$ method (Livak and Schmittgen, 2001).

template (Fig. 1, Accession number AY940115). Compilation of these overlapping fragments yielded a 4335 nucleotide sequence. The structural organization of the SiTf gene was determined by comparing cDNA and genomic sequences using the SPIDEY application of the NTI Vector software (Invitrogen). The comparative analysis of genomic and cDNA sequences revealed that the SiTf gene was comprised of 8 exons (Fig. 1). All introns possessed the 5′GT and 3′AG splicing sites characteristic of eukaryotes (Breathnach and Chambon, 1981).

### 3.2. Validation of the comparative $C_T$ method

The reverse transcription efficiencies of the *S. invicta* transferrin and 18 S genes were relatively equal when the starting quantity of RNA was between 0.1 and 50 ng, inclusive. According to Livak and Schmittgen (2001), relatively equal reverse transcription efficiencies are demonstrated when a plot of $\Delta C_T$ (target gene $C_T$ − reference gene $C_T$) versus log total RNA yields a slope of <0.1. Thus, the slope of 0.028 observed across the RNA quantities used, validates use of the comparative $C_T$ method for the SiTf gene when using the *S. invicta* 18 S gene as reference.

### 3.3. SiTf expression in response to B. bassiana infection

The SiTf relative expression was significantly greater in worker ants exposed to all concentrations of *B. bassiana* conidia as compared with the untreated control (Fig. 2). Although a dose response trend was obvious, the differences in relative expression levels in ants treated with $10^4$ through $10^7$ conidia/ml were not significantly different from each other. Relative SiTf expression, at all treatment concentrations, was significantly greater than the untreated control. A corresponding dose response was observed for ant mortality. Ten-day cumulative Abbott-corrected mortality ranged from 28% at $10^4$ conidia/ml to 100% at $10^7$ conidia/ml. Autoclave-killed conidia did not elicit an induction response; the SiTf transcript level in ants treated with killed conidia was not significantly different from the control (data not shown). Cumulative ant mortality (10 days) was 1.3 ± 1.3% for ants treated with autoclave-killed conidia and 100% for ants treated with live conidia. Significant increases in SiTf transcript level did not occur until 48 h after challenge with conidia (Fig. 3). SiTf relative expression at 48 and 72 h was not significantly different.

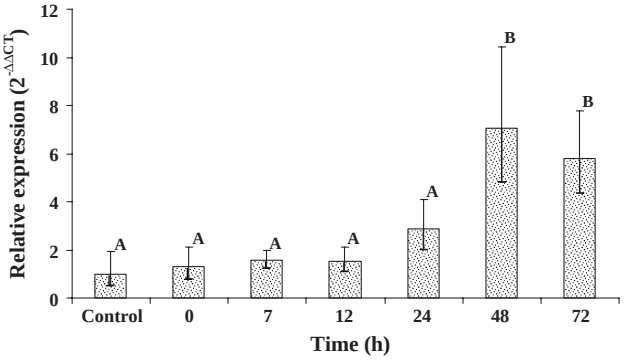


Fig. 3. Relative expression ($2^{-\Delta\Delta CT}$) of SiTf in worker ants at different times after exposure to $10^7$ *B. bassiana* conidia/ml as determined by quantitative PCR. Means with the same letters do not differ significantly by comparison of confidence intervals as calculated by the comparative $C_T$ method (Livak and Schmittgen, 2001).

## 4. Discussion

Clone 1D5 from our *S. invicta* EST library exhibited significant homology to Metazoan transferrins. From this EST, we developed oligonucleotide primers for 5′ and 3′ RACE reactions and RT-PCR which were used to elucidate the entire 2417 nucleotide cDNA. The cDNA sequence presented a single large ORF encoding a polypeptide comprised of 702 amino acids with a predicted molecular mass of 77.3 kDa consistent with transferrin proteins (Baker et al., 2002). Moreover, the *S. invicta* sequence exhibited significant identity with insect transferrins; the highest level of identity (67%) was with a transferrin from the honey bee, *Apis mellifera* (Table 3; Kucharski and Maleszka, 2003; Nascimento et al., 2004). The genomic architecture of SiTf was also similar to a transferrin from *A. mellifera* (Nascimento et al., 2004); both genes were comprised of a large number of exons (8 for SiTf and 9 for *A. mellifera* transferrin).

Among vertebrate transferrins, the amino- and carboxyl-terminal halves of the polypeptide exhibit significant sequence identity (~40%, MacGillivray et al., 1983) and each lobe reversibly binds an iron atom (as $Fe^{+3}$). These characteristics suggest that vertebrate transferrins have evolved by duplication of an ancestral gene of approximately 40 kDa (Metz-Boutigue et al., 1984). To date, only two insect transferrins appear to fit the vertebrate diferric paradigm, the cockroach *Blaberus discoidalis* (Jamroz et al., 1993; Gasdaska et al., 1996) and termite *Mastotermes darwiniensis* (Thompson et al., 2003), both hemimetabolous insects. Evidence from the remaining known insect transferrins (all holometabolous) suggests that they are all monoferric (specifically at the N-lobe). SiTf appears to fall within this group because it exhibits low sequence identity between its N-and C-lobes (16%) and the putative iron binding sites are present only in the N-lobe. Of the 5 polypeptide ligands thought to be involved in iron binding in vertebrate transferrins (Anderson et al., 1989), 4 are present in the N-lobe of SiTf (D77, Y113, R143, and Y227) and none are present in the C-lobe. The fifth polypeptide ligand, a histidine, is conspicuously absent from SiTf and replaced with a glutamine (Q299 in SiTf) as in other insects (Yun et al., 1999). Yoshiga et al. (1997) hypothesized that insects lost the ability to bind iron by the C-lobe as an adaptation to evade iron piracy (Cornelissen and Sparling, 1996) by pathogens requiring iron. Their hypothesis stems

from data indicating that bacterial receptors interact principally with the tranferrin C-lobe (Alcantara et al., 1993). However, subsequent reports show that certain bacteria can acquire iron from both the N-and C-lobes of human tranferrin, in direct fashion (Yu and Schryvers, 1993) and via siderophores (Modun et al., 1998; Ratledge and Dover, 2000).

Consistent with previously characterized insect transferrins, SiTf was induced in response to pathogen challenge (Figs. 2 and 3). Maximum induction (~7-fold relative increase) occurred 48 h after exposure to *B. bassiana* conidia (Fig. 3). The only other temporal induction experiment conducted for an insect transferrin gene was in *Bombyx mori* (Yun et al., 1999). Yun et al. (1999) showed that *B. mori* transferrin induction, in response to *E. coli* challenge, reached a maximum 24 h after initial exposure. However, they injected *E. coli* which bypassed the physical barrier afforded by the cuticle. In our case, worker ants were treated topically with *B. bassiana* conidia which would have taken time (approximately 24 h [Moino et al., 2002]) to penetrate the cuticle and, presumably, elicit a response. This conclusion is supported by a lack of SiTf induction by killed *B. bassiana* conidia, and, while in cell culture, heat-killed bacteria were able to induce transferrin in *Aedes aegypti* and *Aedes albopictus* cells (Yoshiga et al., 1997). Therefore, if a delay in cuticular penetration is considered for *B. bassiana* conidia, the temporal pattern of induction is nearly identical between *S. invicta* and *B. mori* transferrin. As pointed out by Kucharski and Maleszka (2003) in *A. mellifera*, transferrin induction is low compared with antimicrobial peptides (AMPs) such as hymenoptaecin and drosomycin, and may only be peripherally related to pathogen defensive pathways. Indeed, such AMPs exhibit a more rapid response, to a significantly higher level, and have been shown to be pathogen specific (Lemaitre et al., 1997).

Based primarily on the vertebrate lactoferrin paradigm, it is widely concluded that insect transferrins may play a role in insect immune defense by sequestering iron away from invading pathogens. However, this conclusion lacks direct empirical evidence. In our experiments, ants exposed to *B. bassiana* conidia suffered high levels of mortality despite significant increases in transferrin transcript levels. However, ants used in our experiments were exposed to an extreme number of conidia that, most likely, would not have been experienced under natural conditions. Further studies will be necessary to demonstrate protection afforded by transferrin induction and to link the transferrin protein titer with transcript levels. This is especially relevant when we consider the possibility that pathogen metabolites may interfere with transferrin gene expression, protein production or stability. The use of RNA interference to selectively knock down the transferrin transcript levels may be the most likely method to achieve this objective. An alternative hypothesis has been posited by Kucharski and Maleszka (2003) who suggest that transferrin may only be peripherally related to immunity. As multi-gene analyses become more prevalent, additional understanding between the relationship of pathogen challenge and tranferrin induction in insects will likely occur.

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