Gene Structure and Expression of the Glutathione S-transferase, $SiGSTS1$, from the Red Imported Fire Ant, *Solenopsis invicta*

Steven M. Valles, Omatthange P. Perera, and Charles A. Strong

The structural organization and developmental expression of a previously described glutathione S-transferase cDNA from the red imported fire ant, *Solenopsis invicta*, were elucidated. The gene was previously named *Solenopsis invicta*, glutathione S-transferase, sigma class no. 1 ($SiGSTS1$). Comparison of genomic and cDNA sequences showed that the gene was comprised of 5 exons and 4 introns. All of the introns possessed the 5'GT and 3'AG splicing sites characteristic of eukaryotes. The comparative G method of quantitative-polymerase chain reaction was employed to examine the developmental expression of the $SiGSTS1$ transcript in monogyne and polygyne *S. invicta*. Polygyne queen and late instars exhibited 3.5- and 4.7-fold increased expression of $SiGSTS1$, respectively, compared with pupae. Early instars (13.1-fold) and workers (9.6-fold) exhibited the highest, and statistically significant, levels of expression of all polygyne developmental stages examined. A similar pattern of expression was observed for the monogyne social form. However, the queen showed the lowest expression level, followed by pupae (1.2-fold), late larvae (5.8-fold), early larvae (9.4-fold), and workers (10.1-fold). No differences were observed in the $SiGSTS1$ gene sequences between fire ant social forms. Although obviously developmentally expressed, the function of $SiGSTS1$ in *S. invicta* is not known. Arch. Insect Biochem. Physiol. 61:239–245, 2006.

**KEYWORDS**: *Solenopsis invicta*, glutathione S-transferase; gene expression

**INTRODUCTION**

The red imported fire ant, *Solenopsis invicta* Buren, was introduced into the United States from South America in the early 1900s and has since spread to infest more than 128 million hectares in the southeastern United States and parts of California, Arizona, and New Mexico (Williams et al., 2001). This aggressive, territorial ant adversely impacts arthropod biodiversity in infested areas (Porter and Savignano 1990; Porter et al. 1991), causes economic losses in the cattle industry (Barr et al., 1994), damages agricultural commodities (Lofgren, 1986), hampers interstate commerce of nursery stock as a result of quarantine regulations, and even represents a significant risk to human health (Williams et al., 2001). Total costs associated with fire ant control and damage have been estimated at 0.5 to 1 billion dollars annually (Thompson et al., 1995). Despite near complete reliance on insecticides for control of *S. invicta*, very little information is available concerning insecticide detoxification in this pest.

Glutathione S-transferases (GSTs) comprise an enzyme superfamily associated with a variety of functions including protection from reactive oxygen...
species, maintenance of a reductive environment for thiolated proteins, biosynthesis of prostaglandins, and glutathione conjugation of ligands (endogenous and exogenous), which facilitates excretion of these compounds by increasing their solubility (Kanaoka et al., 1997; Sheehan et al., 2001). Thus, GSTs are considered an important detoxification enzyme system (Yu, 1996) and have been shown to be involved in pesticide detoxification (Edwards et al., 2000; Abel et al., 2004), and insecticide resistance in insects (Wei et al., 2001). We recently purified the first glutathione S-transferase (GST) from adult workers of S. invicta by affinity chromatography and preparative isoelectric focusing (Valles et al., 2003). Degenerate oligonucleotide primers were designed from sequenced sections of the purified enzyme and subsequently used to identify and characterize the corresponding cDNA. BLAST analysis revealed significant identity with Sigma class GSTs. The enzyme was named, Solenopsis invicta, glutathione S-transferase, Sigma class no. 1 (SigGSTS1). Here, we continue the characterization of SigGSTS1 by elucidating the gene structure and developmental expression in S. invicta.

MATERIALS AND METHODS

Ants

Queenright polygynous or monogynous S. invicta colonies were excavated from areas in Gainesville, FL. Ants were separated from the soil and transferred to rearing trays using the floating technique described previously (Jouvenaz et al., 1977). Colony social form was determined by genotyping a sample of worker ants (20) from each nest at the Gp-9 locus (Valles and Porter, 2003). Five developmental stages were used for the expression studies, including queen, early larvae (first and second instars), late larvae (third and fourth instars), white pupae, and workers. Larval stages were determined as described by Petralia and Vinson (1979).

Determination of Gene Structure

To determine the gene sequence of SigGSTS1, PCR was conducted with genomic DNA using a series of overlapping oligonucleotide primers (p27A, p34; p33, p28A; p300, p301) designed from the transcript (Table 1). Genomic DNA was used as the template for PCR and isolated from monogyn and polygyne S. invicta with the PIREGENE DNA isolation kit (Cenfrea, Minneapolis, MN). Construction of the gene was accomplished by creating three overlapping fragments by PCR with genomic DNA using 3 pairs of oligonucleotide primers (within exons) that were designed from the cDNA sequence. PCR was conducted in an MJ Research PTP-100 thermal cycler using the following temperature regime: 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 15 sec, 53°C for 15 sec, 68°C for 0.5–2 min, and one cycle at 68°C for 5 min. Amplicons produced were subsequently ligated into pGEM-T Easy, transformed into JM109-compent cells (Promega, Madison, WI), and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida). To determine the structural organization of the SigGSTS1 gene, genomic sequence (accession number DQ191801) was compared with the cDNA sequence (accession number AY255670). Comparative analysis of the genomic and cDNA sequences was accomplished with the SPIDEY application of the NIT Vector software (Invitrogen).

SigGSTS1 Expression by Quantitative PCR

SigGSTS1 expression was quantified using real-time quantitative PCR by the comparative Ct

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide position</th>
<th>Sequence (5' to 3' orientation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q5/GST1F</td>
<td>388-352</td>
<td>CACAGGAGTTAGCCACATTTTG</td>
</tr>
<tr>
<td>Q5/GST1R</td>
<td>401-421</td>
<td>TTGAACGCCAGTTCCTGTCCACAT</td>
</tr>
<tr>
<td>Q5/GST1PProbeFAM</td>
<td>371-392</td>
<td>AGCAATAAGCAAGGCTTATTC</td>
</tr>
<tr>
<td>Q185F</td>
<td>501-525</td>
<td>CCCCTATGAGAATGTACACCTTTT</td>
</tr>
<tr>
<td>Q185R</td>
<td>577-599</td>
<td>AGCCTTGGCCGTTGAAGTTACC</td>
</tr>
<tr>
<td>Q185ProbeFAM</td>
<td>549-565</td>
<td>CACAGCTGCTCCCTGCC</td>
</tr>
<tr>
<td>p33</td>
<td>85-111</td>
<td>AGTCGACGTAAAGCAAGGCTTATTC</td>
</tr>
<tr>
<td>p34</td>
<td>667-689</td>
<td>CATGCTGCTTGGCTCTCTTTCT</td>
</tr>
<tr>
<td>p35</td>
<td>1084-1183</td>
<td>CAGGCTGAGCCTGTCTCTTGTAG</td>
</tr>
<tr>
<td>p27A</td>
<td>1-21</td>
<td>AGTGGTCCAGATCCAGAACCAC</td>
</tr>
<tr>
<td>p28A</td>
<td>876-900</td>
<td>GGTATTGCTGTAGAAGGAACTC</td>
</tr>
<tr>
<td>p300</td>
<td>931-963</td>
<td>TACGATTTTTAAGGTTTTTTCTTTCTTAT</td>
</tr>
<tr>
<td>p301</td>
<td>827-853</td>
<td>CGTCAACTACCTAGTACTTTATTACATACC</td>
</tr>
</tbody>
</table>

* Positions correspond to the transcript.
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. We chose the 18S ribosomal RNA gene (Valles and Pereira, 2003; GenBank accession number: AY334566) as our endogenous reference. Nucleotide sequences for the SiGSTS1 and 18S ribosomal genes from S. invicta were submitted to the Assays-by-Design Service at Applied Biosystems Inc. (Foster City, CA) 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 1. 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, SiGSTS1, was designed to span intron 3 as an additional measure to avoid amplification of genomic DNA (Fig. 1).

First, an experiment was conducted to determine the suitability of the 18S ribosomal gene as a reference for the SiGSTS1 gene. RNA (0.01, 0.1, 1, 10, and 100 ng) extracted from workers was incubated with 1 U of amplification grade DNAse I (Invitrogen, Carlsbad, CA) for 15 min at room temperature. The DNAse activity was terminated 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 SuperScript II reverse transcriptase (Invitrogen) and oligonucleotide primers, p34 and p85, for SiGSTS1 and 18S, respectively (Table 1). These primers were downstream of the area amplified during the subsequent quantitative PCR. Reverse transcription was completed in a thermalcycler at 45°C for 30 min and terminated by heating to 70°C for 15 min. Quantitative PCR was subsequently completed in an Applied Biosystems, Inc., Prism Sequence Detection System 7000 in a 25-μl reaction volume with the corresponding probe/primer combination (Table 1) using the TaqMan Universal PCR Master Mix (Applied Biosystems) following the temperature regime: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 1 min. Successful DNase I treatments were confirmed by lack of

![Fig. 1. Schematic representation of the SiGSTS1 gene and tabulated locations of relevant characters. Genomic architecture of the regions encoding SiGSTS1. Init, Stop, and pA denote the approximate locations of the initiation codon, stop codon, and polyadenylation signal, respectively. Exons are indicated by boxes. Cross-hatched areas represent SiGSTS1 coding regions; stippled areas represent the 5' and 3' untranslated regions.](image-url)
amplification in quantitative PCR among identical samples without reverse transcription.

Once the suitability of the 18S gene as a reference was established (Fig. 2), the relative quantity of the SiGSTS1 transcript was determined in polygyne and monogyne queens, early and late larvae, workers and pupae by this same method. Total RNA was extracted using the Agilent Technologies (Wilmington, DE) RNA isolation protocol. Ten to 20 mg of ants or brood, or a single queen, was used as the RNA source. The RNA was quantified spectrophotometrically and diluted to 10 ng/µl for analysis.

Three replications of each experiment were conducted. Change in critical threshold (ΔC_T) was determined by subtracting the SiGSTS1 C_T from the 18S C_T value. ΔΔC_T was then determined by subtracting ΔC_T from the ΔC_T of the calibrator (treatment [developmental stage] with the lowest expression level [highest ΔC_T]). The calibrator was the treatment with the lowest expression level (i.e., queen stage for monogyne fire ants and pupal stage for the polygyne fire ants). The relative expression (2^−ΔΔCT) of SiGSTS1 was calculated and non-overlapping confidence intervals were used to indicate significant differences between treatments (Livak and Schmittgen, 2001).

**SiGSTS1 Cloning and Sequencing From Polygyne and Monogyne S. invicta**

_S. invicta_ exists in two distinct social forms, monogyne and polygyne. Valles et al. (2003) had previously cloned and sequenced the SiGSTS1 gene exclusively from polygyne fire ant colonies. To determine if there were any differences between monogyne and polygyne SiGSTS1, individual worker ants (about 45) from verified monogyne and polygyne colonies (Valles and Porter, 2003) were used to amplify the SiGSTS1 gene by One-Step RT-PCR with oligonucleotide primers p27A and p28A (Table 1). The 900-bp amplicon was cloned into the pCR4 vector, transformed into TOP10 competent cells (Invitrogen), and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida). Three clones derived from each social form were sequenced in entirety.

**Glutathione S-Transferase Activity**

Glutathione S-transferase (GST) activity was measured in the different developmental stages (queen, early larvae [first and second instars], late larvae [third and fourth instars], white pupae, and workers) from a monogyne colony with 1-chloro-2,4-dinitrobenzene (CDNB) as described previously (Habig et al., 1974). The different fire ant developmental stages were homogenized in 0.1 M sodium phosphate buffer, pH 6.5, filtered through 2 layers of cheesecloth, and centrifuged at 105,000g for 1 h. The supernatant was used as the enzyme source. The 3-ml reaction mixture contained 1 ml of 15 mM glutathione and 0.025–0.05 mg of soluble fraction protein in 2 ml of homogenization buffer. The mixture was incubated at 25°C for 3 min, then 0.02 ml of 150 mM CDNB in ethylene glycol monomethyl ether was added and mixed. The change in absorbance at 340 nm was measured on a Varian Model 3 Bio uv/vis spectrophotometer equipped with a diffuse reflectance accessory against a reference containing buffer, CDNB, and glutathione. En-
zyme activity was calculated with the molar extinction coefficient of 9.6 mM$^{-1}$cm$^{-1}$ for the product, S-(2,4-dinitrophenyl)glutathione.

**RESULTS AND DISCUSSION**

Comparative analysis of the genomic and cDNA sequences of SiGSTS1 revealed 5 exons spanning 2 kb (Fig. 1). All of the introns possessed the 5'GT and 3'AG splicing sites characteristic of eukaryotes (Breathnach and Chambon, 1981). Exon 1 was comprised entirely of an untranslated region, while exons 2, 3, 4, and 5 comprised the majority of the translated nucleotide sequence of SiGSTS1. Exon 2 contained the translation initiation site and exon 5 contained the stop site and a polyadenylation signal.

Efficiency of reverse transcription for the *S. invicta* SiGSTS1 and 18S genes was determined to be similar within the range of RNA quantities used in our experiments (Fig. 2): the absolute value of the slope (0.007) of log RNA versus ΔC$_T$ was less than 0.1 as required for comparative C$_T$ method validity. Reference and target genes must exhibit relatively similar reverse transcription efficiencies for the comparative C$_T$ method to be valid (Livak and Schmittgen, 2001). Thus, utilization of the comparative C$_T$ method to determine relative gene expression of the target gene, SiGSTS1, with the 18S gene provided a suitable reference based on the defined requirements.

In polygyne *S. invicta*, SiGSTS1 expression was lowest in the pupae, and so this developmental stage was used to calibrate expression in other stages (Livak and Schmittgen, 2001). Polygyne queen and late larvae exhibited 3.5- and 4.7-fold higher expression of SiGSTS1, respectively, compared with pupae, although no significant difference was noted between queens and late larvae (Fig. 3). Expression in early larvae (13.1-fold) and workers (9.6-fold) exhibited the highest, and statistically significant, levels of expression of all polygyne developmental stages. A similar pattern of expression was observed for the monogyne social form. However, the queen showed the lowest expression level, followed by pupae (1.2-fold), late larvae (5.8-fold), workers (10.1-fold), and early larvae (9.4-fold).

Interestingly, a general decreasing trend in SiGSTS1 transcript corresponded to developmental

![Fig. 3. Relative expression ($2^{-\Delta\Delta C_T}$) of SiGSTS1 in different developmental stages of monogyne and polygyne fire ant colonies. Error bars represent confidence intervals as calculated by the comparative C$_T$ method (Livak and Schmittgen, 2001). Means with overlapping confidence intervals are not significantly different.](image-url)
progression. The SiGSTS1 transcript level decreased from early larvae through late larvae to pupae (Fig. 4), while a corresponding increase in general GST enzyme activity (CDNB conjugation) occurred for the same developmental stages. This result supports our earlier conclusion that a number of GST isozymes other than SiGSTS1 are likely present in S. invicta because despite a decrease in transcript level, general GST activity increases. The relationship between transcript level, GST activity, and division of labor was somewhat unexpected. Because GSTs are known to play crucial roles in xenobiotic detoxification, as well as provide important antioxidant protective effects, we expected the late larvae and workers to possess the highest activities and transcript levels. Fourth instar larvae (included in our late larvae category) do all of the digestion of solid food for the entire colony (Petralia and Vinson, 1978) and workers must leave the relative protection of the nest to collect food (solid and liquid) for the colony. Thus, these two stages would, ostensibly, need more protection from xenobiotics and oxidative stress due to higher environmental exposure. Workers did possess the highest SiGSTS1 transcript level and general GST activity. However, late larval GST activity and SiGSTS1 transcript levels were intermediate. Papadopoulos et al. (2004) reported that GST activity (CDNB-conjugation) was highest in adult Apis mellifera macedonica compared with other developmental stages examined. They also found dramatic shifts in the expression of GST genes among development stages. These data further support our hypothesis that S. invicta possesses different GST genes that are differentially expressed during development.

Finally, because a genetic difference between polygynous and monogynous fire ants has been identified at the Gp-9 locus (Krieger and Ross, 2002), we wanted to determine if there were any corresponding differences in the SiGSTS1 gene between social forms. Valles et al. (2003) had reported the cDNA sequence for SiGSTS1 from the polygynous social form only. No differences were observed in the SiGSTS1 gene sequences between fire ant social forms. Indeed, the monogynous sequence was identical to the polygynous sequence we reported earlier (Valles et al., 2003; accession number AY255670).

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