Available online at www.sciencedirect.com
SCIENCE DIRECT
Insect Biochemistry and Molecular Biology 36 (2006) 387–395
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

Role of the transcriptional repressor *mdGfi-1* in *CYP6D1v1*-mediated insecticide resistance in the house fly, *Musca domestica*

Jianwei Gao, Jeffrey G. Scott*

Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853-0901, USA

Received 22 November 2005; received in revised form 1 February 2006; accepted 2 February 2006

Abstract

Gfi-1 is a C_2H_2-type zinc finger protein that is a transcriptional repressor in vertebrates and has been implicated in control of CYP6D1 expression in house flies (*Musca domestica*). A 15 bp insert, which disrupts a putative *mdGfi-1* binding site in the *CYP6D1v1* promoter has been implicated as a cause of increased expression of CYP6D1, and thus insecticide resistance. Using electrophoretic mobility shift assays we demonstrate that the CYP6D1 promoter from susceptible strains binds *mdGfi-1*. The 15 bp insert that interrupts the *mdGfi-1*-binding site in insecticide-resistant strains reduces the amount of *mdGfi-1* binding by 9- to 20-fold, consistent with the role of *mdGfi-1* in resistance. Partial sequences of *mdGfi-1* (spanning the first intron) from individual houseflies from 11 different strains revealed the presence of 23 alleles. There was no consistent difference in the *mdGfi-1* alleles between susceptible and CYP6D1-mediated insecticide-resistant strains, indicating that *mdGfi-1* alleles were not likely involved in resistance. Polymorphisms were used to map *mdGfi-1* to autosome 1. Quantitative real time PCR (qRT-PCR) revealed Gfi-1 expression was higher in the thorax compared to the head and abdomen, and varied between life stages and between strains. However, similar levels of *mdGfi-1* were detected in susceptible and resistant adults suggesting that altered levels of *mdGfi-1* were not likely a cause of insecticide resistance. The significance of these results to understanding insecticide resistance is discussed.

Keywords: Gfi-1; Cytochrome P450 CYP6D1; Electrophoretic mobility shift assays; Linkage analysis; Polymorphism; Quantitative real-time PCR; Insecta

1. Introduction

Gfi-1 is a C_2H_2-type zinc finger protein that is a transcriptional repressor. The proto-oncogene Gfi-1 was first cloned from rats, where it was shown to be expressed at high levels in Moloney murine leukemia virus-induced T cell lymphomas selected for growth on interleukin-2-free media (Zweidler-McKay et al., 1996). Subsequently Gfi-1 has been shown to regulate several genes involved in cell proliferation, inhibition of apoptosis, T-cell development, granulopoiesis and expression of the innate immune response (Doan et al., 2004; Duan and Horwitz, 2003; Grimes et al., 1996; Hock et al., 2004; Person et al., 2003; Yucel et al., 2004). The Gfi-1 consensus sequence has been identified in the promoter of several vertebrate genes (Zweidler-McKay et al., 1996). Recently, Gfi-1 has been shown to be a repressor of the human P450 CYP27B1 (Dwivedi et al., 2005). Sequencing of *mdGfi-1* from housefly (*Musca domestica*) revealed a high degree of sequence conservation (especially across the zinc finger region of the protein) between insects and mammals, leading to the suggestion that Gfi-1 may be an important transcriptional repressor in many taxa (Kasai and Scott, 2001). However, little is know about the functions of Gfi-1 in insects.

Cytochrome P450 CYP6D1 carries out detoxification of phenoxybenzyl pyrethroids in housefly (Scott, 1999). The LPR (pyrethroid-resistant) strain of housefly expresses a nine-fold higher level of CYP6D1 mRNA and protein relative to susceptible strains. This elevated expression is due to increased transcription of *CYP6D1v1*, and is not due to gene amplification or mRNA stabilization (Liu and Scott, 1996; Liu and Scott, 1998; Scott, 1999; Tomita and Scott, 1995). CYP6D1 was mapped to chromosome 1 and the factors responsible for increased transcription of

*Corresponding author. Tel.: +1 607 255 7340; fax: +1 607 255 0939.
E-mail address: JGS5@CORNELL.EDU (J.G. Scott).

0965-1748/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2006.02.001

CYP6D1 were mapped to chromosomes 1 and 2 (Liu and Scott, 1996; Liu and Scott, 1998; Liu et al., 1995). To identify transcription regulatory elements involved in CYP6D1 expression, the 5′-flanking region of CYP6D1 was sequenced from pyrethroid-resistant and susceptible strains of housefly (Scott et al., 1999). The most notable difference between resistant ($n = 2$) and susceptible ($n = 8$) strains was the presence of a 15 bp insert found only in the resistant strains (Scott et al., 1999; Seifert and Scott, 2002). This 15 bp insert interrupted a putative Gfi-1 binding site, leading to the suggestion that the *CYP6D1v1* promoter (from resistant strains) would bind less Gfi-1 (i.e. repressor) and this could be responsible for increased transcription of *CYP6D1v1* in resistant strains (Scott et al., 1999).

In this study, we employed electrophoretic mobility shift assays (EMSAs or gel-shifts) to demonstrate that the CYP6D1 promoters from susceptible strains bind more *mdGfi-1* than do the same promoter regions from resistant houseflies. Partial *mdGfi-1* genomic sequences were amplified from several strains of housefly and revealed numerous polymorphisms that were used to map *mdGfi-1* to autosome 1. Quantitative real time PCR (qRT-PCR) revealed Gfi-1 expression varied between life stages, strains and body regions in adults. However, similar levels of mnGfi-1 were detected in susceptible and resistant adults suggesting that altered levels of *mdGfi-1* were not likely involved in resistance. The significance of these findings to understanding insecticide resistance and the role of *Gfi-1* in insects is discussed.

2. Experimental

2.1. Electrophoretic mobility shift assays (EMSA or gel-shift)

The *mdGfi-1* protein was synthesized by a coupled transcription and translation rabbit reticulocyte lysate kit (TNT®, Promega, Madison, WI). RT-PCR amplification (Section 2.2)(forward primer: 5′-CTCGAGATGCATCA CCATCACCATCACATGTTTCAAAGACCCTTTCTA TCGCCTGCG-3′; reverse primer: 5′-TCTAGATTATAC CAACAATGAT ACCTTTTGGC-3) of *mdGfi-1* (Kasai and Scott, 2001)(accession no. AF339860) from CS house flies generated a product that was inserted into *Xho*I/*Xba*I digested pTNT® vector (Promega, Madison, WI) to produce the pTNT™-6 × His-*mdGfi-1* vector. The sequence was confirmed by direct sequencing at Cornell's Biotechnology Resource Center.

To confirm expression of *mdGfi-1*, reticulocyte lysate was separated by SDS/PAGE and electroblotted onto nitrocellulose using a BioRad (Hercules, CA) mini transblot apparatus. The membrane was developed using anti-His antibody:peroxidase conjugated ECL anti-mouse IgG (1:10000) using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Probes used for EMSA were based on the promoter sequences of CYP6D1 in the pyrethroid-susceptible (CS) and the pyrethroid-resistant (LPR) strain of housefly (Fig. 1). Probe 13 (5′-AATTTGTAAATAAAATTAA TGCTAGC-3′) was from a region of the CYP6D1 promoter that had no homology to the putative Gfi-1 binding site (i.e. used as a negative control). The sense and antisense probes were 5′-end labeled with the following reaction mixture: 1 ul oligonucleotide solution (20 ng/ul), 2 ul of 10 × T4-kinase buffer, 0.5 ul of T4-polynucleotide kinase (10 U/ul)(Invitrogen, Carlsbad, CA) and 2 ul of [γ-^{32}P] ATP (aqueous solution, 10 mCi/ml, Perkin-Elmer, Boston, MA). The mixture was incubated for 30 min at 37 °C. The labeled probe was purified with a QIAquick nucleotide removal kit (Qiagen, Valencia, CA). Probes were eluted from the column with 100 ul 10 mM Tris (pH 8.0). The purified sense and antisense probes (both 100 ul) were combined and incubated for 10 min at RT for annealing. Binding reactions (12 ul) contained 1 ul labeled double-stranded oligonucleotide probe (0.1 ng/ul), 20 mM HEPES, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 0.1 ul poly(dI-dC)(10 ug/ul, Roche Applied Science, Indianapolis, IN), and 0.05–1 ul reticulocyte lysate containing *mdGfi-1* protein. The mixture was incubated for 15 min at 37 °C and analyzed on a 5% polyacrylamide gel (16 × 14 cm). The gel was run for 1.5–2 h at 100 V, dried and analyzed using a Storm® gel and blot imaging system (Amersham Biosciences, Piscataway, NJ). Quantitation was performed using ImageQuant TL (Amersham Biosciences, Piscataway, NJ) software. The experiment using probes I and II (Fig. 3) was replicated three times, and the experiment using probes 13, 15 and 16 (Fig. 4) was replicated six times.

2.2. Polymorphisms of mdGfi-1

Eleven laboratory strains of housefly were used. Cornell susceptible (CS) is an insecticide-susceptible strain reared without exposure to insecticides (Hamm et al., 2005), SRS is

```
  Probe I  (oligo)                      5′-TTTACATGCATTCGAATCATTCTGTTTCACAAAATGACCGGCAACTATTCAGTTG-3′
  Probe II (oligo)                      5′-TTTACATGCATTCGAATCAT---------------GACCGGCAACTATTCAGTTG-3′
  Probe 15 (Oligo)                      5′-TTTACATGCATTCGAATCAT---------------GACCGGCAACTATTCA-3′
  Probe 16 (Oligo)                            5′-CATTCGAATCATTCTGTTTCACAAAATGACCGGCAA-3′
LPR CYP6D1 Promoter 5′-ATGATAAGAAATGTGCAAAGTTTTACATGCATTCGAATCATTCTGTTTCACAAAATGACCGGCAACTATTCAGTTGTTAATGTAACA-3′
CS  CYP6D1 Promoter 5′-ATGATAAGAAATATGCAAAGTTTTACATGCATTCGAATCAT---------------GACCGGCAACTATTCAGTTGTTAATGTAACA-3′
```

Fig. 1. Probes used in the electrophoretic mobility-shift assay and their positions in the *CYP6D1* promoter of susceptible (CS) and resistant (LPR) strains. Probes I and 16 contained the 15 bp-insert (5′-TCTGTTTCACAAAAT-3′) characteristic of resistant strains. Probes II and 15 contain the putative Gfi-1-binding site (shaded) without the 15 bp-insert.

an insecticide-susceptible strain (Keiding, 1999) obtained from the Danish Pest Infestation Laboratory (Lyngby, Denmark) in 2004, and aabys is a susceptible strain with recessive morphological markers on each autosome. LPR is a multi-resistant strain having high levels of resistance to pyrethroid insecticides due to increased oxidative metabolism mediated by overexpression of cytochrome P450 CYP6D1 (Scott, 2001; Scott and Georghiou, 1986; Wheelock and Scott, 1992). Two other mechanisms of resistance to pyrethroid insecticides in the LPR strain are insensitivity of the nervous system (*kdr*) and decreased cuticular penetration (*pen*) (Scott and Georghiou, 1986; Shono et al., 2002). YPER was collected in Japan and is a multiple resistant strain with more than 18,000-fold resistance to permethrin due to *super-kdr* and monooxygenase-mediated detoxification (Shono et al., 2002). OCR is a cyclodiene resistant (*Rdl*) strain (Kozaki and Scott, unpublished). Cornell-R is an organophosphate resistant (altered acetylcholinesterase) strain (Tripathi and O'Brien, 1973). The OCR and Cornell-R strains were obtained from F.W. Plapp Jr. in 1996 and have been maintained under biannual selection with dieldrin and tetrachlorvinphos, respectively. The 579_{kdr} (pyrethroid resistant due to *kdr*) strain (Williamson et al., 1996) was obtained from I. Denholm (Rothamsted Research, UK). NG98 is a multi-resistant strain (originally collected in Georgia in 1998) with 3660-fold resistance to permethrin due to monooxygenase-mediated detoxification and *kdr* (Kasai and Scott, 2000). NYSPIN-R is a spinosad resistant strain collected in New York (Scott, 1998; Shono and Scott, 2003). NC is a multiresistant strain collected during the summer of 2002 from a dairy in Wake County North Carolina (Hamm et al., 2005). Colonies were reared as previously described (Scott et al., 2000).

The QuickPrep *micro* mRNA purification kit (Amersham Biosciences, Piscataway, NJ) was used according to the manufacturer's instructions with one male adult housefly. First-strand cDNA was synthesized by annealing mRNA (20 ng) with 2.5 mM oligo$(dT)_{20}$ and 0.5 mM dNTP mix, 1 ul SuperScript III reverse transcriptase (200 U/ul, Invitrogen, Carlsbad CA), 1 × RT buffer, 10 mM DTT, 5 mM $MgCl_2$, and 1 ul of RNaseOUT (40 U/ul). The first-strand cDNA synthesis reaction was used for PCR immediately. The full-length *mdGfi-1* cDNA was amplified (forward primer 5′-CTCGAGATGTTTCAAAGACCC TTTCTATCG-3′/ reverse primer 5′-TCTAGA TTATAC CAACAATGATACCTTTTGGC-3′), using *Taq* DNA polymerase (New England Biolab, Beverly, MA). Thermal cycler conditions were: 95 °C for 3 min, for one cycle; (95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min) for 35 cycles; and 72 °C for 10 min, for one cycle. PCR products were detected by running on 1.2% agarose gel with 1 × TAE, and were purified using QIAEX® Π Gel Extraction Kit (Qiagen, Valencia, CA). The amplified fragment was cloned into pCR® 2.1-TOPO® vector using TOPO® TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer instructions. Plasmid DNA was isolated using QIAPrep Spin Mini-Prep Kit (Qiagen, Valencia, CA). Plasmid DNA having the expected size insert was sequenced at the Cornell Biotechnology Resource Center.

Genomic DNA was extracted from a single adult male using the protocol developed by J. Rehm, Berkeley Drosophila Genome Project (http://www.fruitfly.org). An individual housefly was homogenized in 400 µl buffer A (100 mM Tris–HCl (pH 7.5), 100 mM EDTA, 100 mM NaCl, 0.5% SDS) with a disposable tissue grinder (Kontes, Vineland, NJ). The sample was incubated at 65 °C for 30 min, and then 800 µl of LiCl/KAc solution (5 M KAc: 6 M LiCl) was added and the sample was incubated on ice for 10 min. The mixture was centrifuged for 15 min at 24 °C, and 1 ml of supernatant was transferred into a new tube. After precipitation with 600 µl isopropanol, the pelleted genomic DNA was dissolved in 150 µl TE.

A *mdGfi-1* partial genomic sequence of 905 base pairs (containing two introns) was amplified by PCR using two sets of primers (set no. 1:forward primer: 5-CTCGA GATGTTTCAAAGACCCTTTCTATCG-3′; reverse primer: 5′-CCGTGATGGGTCCTTCGAGAGTGT-3′; set no. 2: forward primer 5′-GCCTCTACAGACAGCACAA AATGG-3′ and reverse primer (5′-CAATAACTGCAC GGGAATGGTCGGG-3′) using the following thermal cycler conditions: Denaturation at 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 10 min. The PCR products were purified with QIAquick PCR purification kit (Qiagen, Valencia, CA) and then directly sequenced with the forward and reverse primers. These PCR products overlapped by 128 bp. *Taq* polymerase was from New England Biolabs (Beverly, MA). A smaller *mdGfi-1* partial sequence (containing only intron 1, using primer set no. 1) was determined from individual flies using both the forward and reverse primers. If there were ambiguities between the sequencing results, additional sequence determinations were made. The alleles present in heterozygous individuals were determined by subtracting the known allele to deduce the sequence of the second allele. If two unknown alleles were present the sequences were determined by TOPO TA® cloning (Invitrogen, Carlsbad, CA).

2.3. Linkage analysis

To determine the chromosomal linkage of *mdGfi-1*, unmated aabys females (collected within 8 h of emergence) were crossed with OCR males *en mass*. F_1 males were backcrossed to unmated aabys females and 32 combinations of phenotypes were produced in the backcross (BC_1). Since crossing over is very rare in male houseflies (Hamm et al., 2005), we were able to associate polymorphism of the *mdGfi-1* sequence with the absence of a specific mutant marker to determine the linkage of this gene. The following five genotypes were isolated from the BC_1: (1) +/*ac*; *ar*/*ar*; *bwb*/*bwb*; *ye*/*ye*; *snp*/*snp*, (2) *ac*/*ac*; +/*ar*; *bwb*/*bwb*; *ye*/*ye*; *snp*/*snp*, (3) *ac*/*ac*; *ar*/*ar*; +/*bwb*; *ye*/*ye*; *snp*/*snp*, (4) *ac*/*ac*; *ar*/*ar*; *bwb*/*bwb*; +/*ye*; *snp*/*snp*, and (5) *ac*/*ac*; *ar*/*ar*; *bwb*/*bwb*; *ye*/*ye*; +/*snp*. Male flies from the F_1 and each of the BC_1 phenotypes,

were collected and stored at −80 °C. DNA was extracted and a partial *mdGfi-1* sequence was determined for individual flies using the *mdGfi-1* primer set no. 1 as described above.

2.4. Quantitative real-time PCR

We isolated mRNA from eggs (~25 mg), four larvae (fourth instar), four pupae (2 d post pupation) and four adult males (48 h post ecdysis) of CS and LPR house flies using the QuickPrep micro mRNA purification kit according to the manufacturer's instruction (Amersham Biosciences, Piscataway, NJ) and resuspended the mRNA in 60 ul of DEPC-treated ddH_2O. First-strand complementary DNA was synthesized using reverse transcriptase SuperScript III and Oligo$(dT)_{20}$ (Invitrogen, Carlsbad, CA) from sample mRNA (8 μl) according to the manufacturer's instructions. Real-time PCR was carried out using SYBR Green PCR core reagent (Applied Biosystems, Foster City, CA) on an ABI Prism 7900 sequence detector (Applied Biosystems, Foster City, CA) in MicroAmp optical 384-well reaction plates with optical covers, according to the manufacturer's instructions. The real-time PCR reactions (final volume of 20 μl) contained 2.0 μl cDNA, 2 μl (10 × *SYBR Green PCR Buffer*), 2.4 μl (2.5 mM MgCl), 1.6 μl dNTP blend, 0.1 μl AmpliTaqGold, 0.2 μl AmpErase, 1.8 μl forward primer (10 μM) and 1.8 μl reverse primer (10 μM). Primers for *mdGfi-1* (forward primer, 5′-ACCCCACAAGTGCCAAGTGT-3′; reverse primer, 5′-GTTTGCGTGAATGGGTGA-3′) and for ribosomal protein S3 (RPS3) (forward primer, 5′-CGGGTGTTGAGGTACGTGTAAC-3′; reverse primer, 5′-GGTCTTGGTGGCCATGATG-3′) were designed using Primer Express software (Applied Biosystems, Foster City, CA). To generate standard curves, cDNA from CS egg mRNA was used as template in a 3-fold dilution series (1–81-fold). Sample cDNA was used undiluted. Relative expression was calculated using the standard curve method (*User Bulletin #2, ABI Prism 7700 Sequence Detection System*, PE Applied Biosystems, Foster City, CA). For each experiment there were six determinations per sample. The entire experiment was replicated three times.

We isolated mRNA from heads (60), thoraces (30), and abdomens (60) of CS house flies using the QuickPrep micro mRNA as described above. The relative levels of *mdGfi-1* mRNA were determined in these samples as described above, except that a *mdGfi-1* cDNA clone (in pCR® 2.1-TOPO® vector) and a partial RPS3 cDNA were used to generate standard curves. For each experiment there were six determinations per sample. The entire experiment was replicated four times.

3. Results and discussion

3.1. Electrophoretic mobility shift assays (EMSA)

A 6 × His-tagged mdGfi-1 protein was produced in vitro using the TNT® SP6 Coupled Reticulocyte Lysate system.

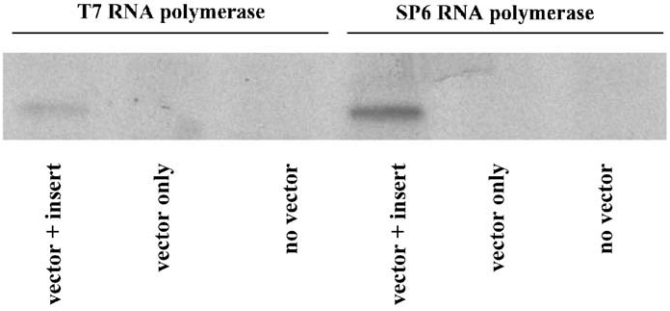

Fig. 2. Western blot detection of mdGfi-1 protein. The mdGfi-1 protein was produced with a TNT-coupled reticulocyte lysate system (Promega). Production of mdGfi-1 was driven by either T7 polymerase or SP6 polymerase.

Western blotting confirmed that mdGfi-1 protein was produced (Fig. 2). We compared SP6 and T7 RNA polymerases for production of the 6 × His-tagged mdGfi-1 protein in the TNT® Coupled Reticulocyte Lysate system. The SP6 RNA polymerase produced more mdGfi-1 protein than T7 RNA polymerase. Therefore, we used SP6 RNA polymerase for all subsequent experiments.

Initial EMSA analyses were carried out using probes based on the CYP6D1 promoter that had the putative mdGfi-1 binding site (probe II, same as sequence found in susceptible strains) or lacked the mdGfi-1 binding site. Typical results are shown in Fig. 3a. The presence of the 15 bp insert found in resistant strains caused approximately a 17- to 20-fold reduction in binding of mdGfi-1 (Fig. 3b). In order to rule out the possibility that these results were due to the differing lengths of probes I and II (rather than the 15 bp insert), we conducted a second set of experiments using probes 15 (36 bp containing the putative mdGfi-1-binding site, i.e. from the susceptible strain), 16 (36 bp containing the 15 bp deletion within the putative mdGfi-1 binding site, i.e. from the resistant strain) and 13 (26 bp region from the 5′-flanking region of CYP6D1 having no resemblance to the Gfi-1 consensus sequence (i.e. used as a negative control)). EMSA analysis with these probes indicated that the 15 bp insert found in resistant strains reduces mdGfi-1 binding by 9-fold, relative to the susceptible strain (Fig. 4). The negative control (probe 13) did not bind to mdGfi-1. This is not due to the slightly shorter length of probe 13, as probes of this same length did bind if they matched the mdGfi-1 binding sequence (data not shown). These results provide direct evidence that the 15 bp insert found in the LPR (and other resistant strains) binds less mdGfi-1 repressor protein, indicating this is likely one of the underlying mechanisms responsible for increased transcription of *CYP6D1v1* in resistant strains. The mdGfi-1 binding site within the CYP6D1 promoter of insecticide susceptible strains (5′-CGAAT CATGACC-3′, −22 to −11) was 50% identical to the mammalian Gfi-1 binding site consensus sequence overall, and 100% identical to the four non-variant nucleotides (AATC) within the consensus sequence (Zweidler-McKay

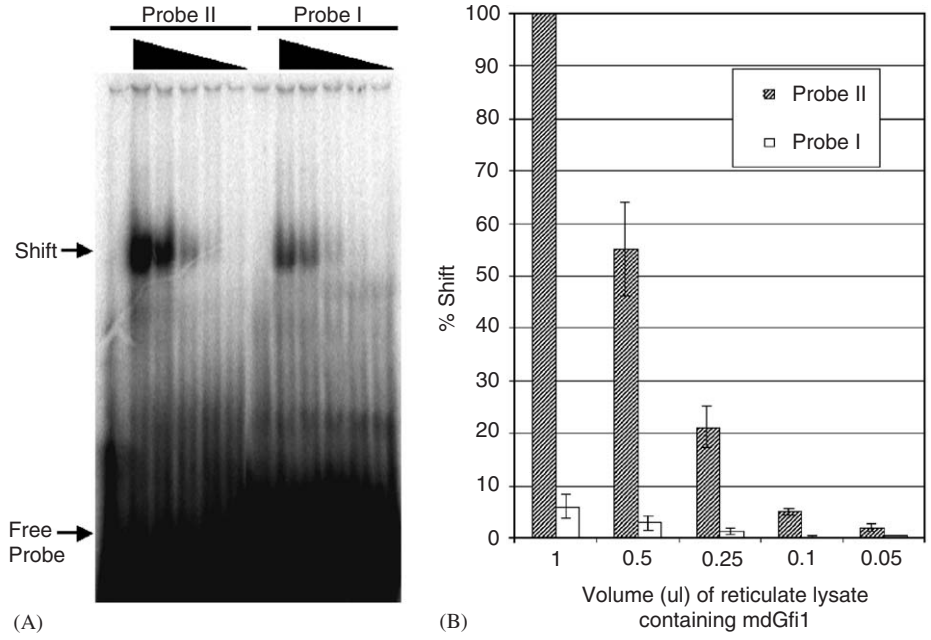

Fig. 3. Electrophoretic mobility shift assays (EMSA) reveal probe I (with the 15 bp insert interrupting the *mdGfi-1*-binding site) binds less *mdGfi-1* compared to the promoter (probe II) from the susceptible strain. (A) EMSA with probes I and II. Probe I is from the promoter of *CYP6D1* in the resistant LPR strain. Probe II is from the promoter of *CYP6D1* in the susceptible CS strain. The wedge shape represents the amount of reticulate lysate containing *mdGfi-1*. Lanes for each probe were loaded with 0, 1.0, 0.5, 0.25, 0.1, 0.05 ul (from left to right) of reticulate lysate. (B) Intensity of the shifted bands relative to 1.0 ul of reticulocyte lysate with probe II. Values represent the mean ± SD ($n = 3$).

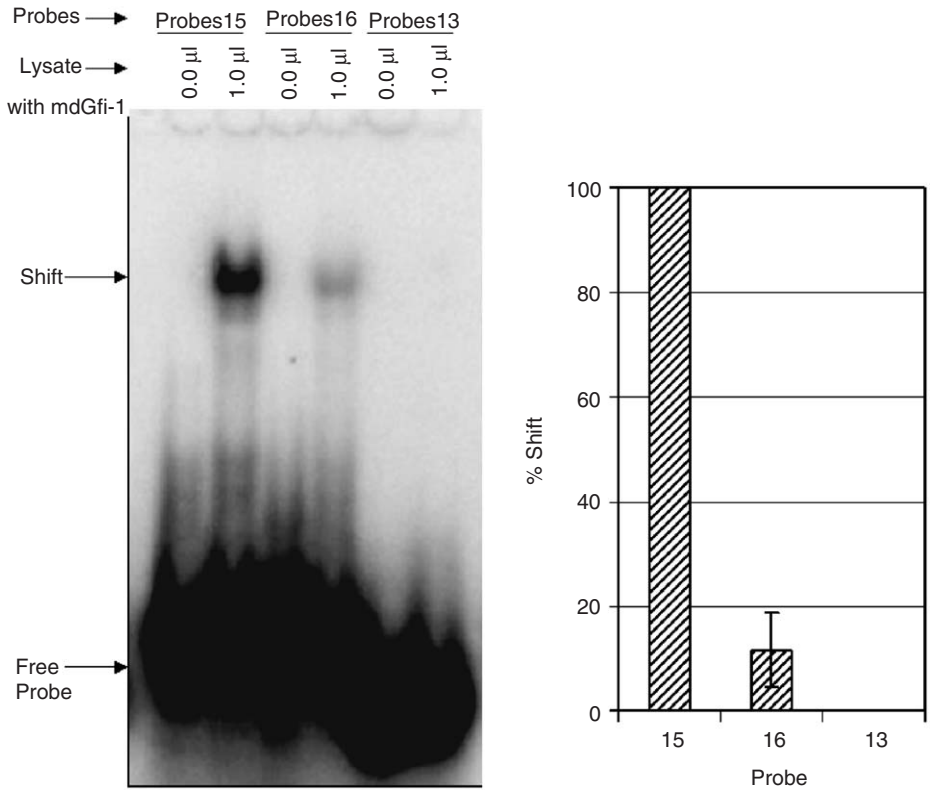

Fig. 4. Electrophoretic mobility shift assays (EMSA, gel shift) reveals that the *CYP6D1v1* promoter (probe 16)(with the 15 bp insert interrupting the *mdGfi-1* binding site) binds less *mdGfi-1* compared to the promoter from the susceptible strain (probe 15). (A) Probes 15 and 16 are of the same the same length. Probe 13 was a negative control demonstrating the specificity of binding. (B) Intensity of the shifted bands relative to 1.0 ul of reticulocyte lysate with probe 15. Mean ± SD for six experiments.

et al., 1996). The 15 bp insert found in resistant strains interrupts the mdGfi-1 binding site, but not the conserved AATC core sequence. This is likely why mdGfi-1 binding is reduced, but not abolished, by the 15 bp insert found in resistant strains.

3.2. Comparison of the mdGfi-1 cDNA sequences from resistant and susceptible houseflies

Full-length *mdGfi-1* cDNAs were sequenced from a susceptible strain (aabys) and the multi-resistant LPR strain of house fly (accession no. DQ242609–DQ242611) for comparison with the sequence reported from the susceptible CS strain (accession no. AF339860). Although there were differences observed in the nucleotide sequences, there were no differences unique to LPR. However, the existence of polymorphisms led us to compare the 5′ region of *mdGfi-1* to determine how much variation there was between strains and evaluate the use of a polymorphic region of the gene for linkage analysis (see below).

3.3. Polymorphisms of mdGfi-1

A partial genomic sequence of *mdGfi-1* was determined using CS houseflies. Two introns, of 166 and 127 nt were identified (accession no. DQ217768). Partial sequences of *mdGfi-1* (spanning the first intron) from individual house flies from 11 different strains revealed the presence of 23 alleles, which code for nine different deduced amino acid sequences (Table 1). There were 25 single nucleotide polymorphisms and two insertion/deletions. All alleles found in the CS, SRS, NYSPINR, aabys, NG98 and YPER houseflies have a 9 bp in frame insert (5′-GCGGCGGCG-3′, corresponding to three alanines) while 33.3% of LPR, 83.3% of Cornell-R and 60% of the NC house fly strains have the allele with the 9 bp insert. None of the alleles in OCR houseflies had this insert. A second insert (5′-GCGGCGACGGCG-3′, corresponding to amino acid sequence –AATA-) was found in 16.7% of Cornell-R and 12.5% of 579_{kdr} alleles. Slightly different inserts of 12 or 15 bp were found in some LPR (66.7%) and NC (8.0%) alleles, respectively. None of the insertions resulted in a frame-shift of the open reading frame.

Inserts found in the *mdGfi-1* alleles (in the exons) were always in multiples of three (5′-GCG′-3′, 5′-GCGGCG-3′, 5′-GCGGCGGCG-3′, 5′-GCGGCGGCGGCG-3′, 5′-GC GGCGAC GGCG-3′, or 5′-GCGGCGGCGGCGG CG-3′), which do not result in a frame shift. The inserted bases correspond to amino acids-A-, -AA-, -AAA-, -AAAA-, AATA-, or -AAAAA-. Alignment of the *mdGfi-1* deduced amino acid sequences with *Drosophila melanogaster* shows that the poly Ala region (amino acids 61–69, -AAAAAAAAA-) of *mdGfi-1* isoform H corresponds exactly to amino acids 453–461 (-AAAAAAAAA-) of *Drosophila* Gfi-1 (Protein ID, NP-723223). In addition, house fly and *D. melanogaster* have a second poly Ala region (house fly, -AAASAAAAAAAA-; *D. melanogaster*, -AAAAAAASAELSAAAAAAAAA-, accession no. NP

Table 1
Alleles of *mdGfi-1* identified from 11 strains of housefly

Allele	Accession number	Isoform	Aabys (25)	LPR (10)	OCR (9)	Cornell-R (6)	NYSPINR (5)	NC (16)	579_{kdr} (8)	CS (7)	SRS (10)	YPER (15)	NG98 (6)
v1	DG192550	C			38.9			29.0					
v2	DG192551	C			61.1				87.5				
v3	DG192568	E				16.7			12.5				
v4	DQ219299	F		70.0									
v5	DG192552	A	24.0									10.0	
v6	DG192553	A					30.0						
v7	DG192554	A								14.3	30.0		
v8	DG192555	A					70.0						
v9	DG192556	G										10.0	
v10	DG192557	D									25.0	20.0	
v11	DG192558	A	40.0			66.8				28.6	30.0	60.0	
v12	DG192559	A	24.0										
v13	DG192560	A				16.7							
v14	DG192561	D	12.0										
v15	DG192562	B		30.0						57.1	15.0		
V16	DG192563	A						16.1					
V17	DG192564	A						9.7					
V18	DG192565	I						6.5					
V19	DG192568	H						6.5					
V20	DG192569	H						29.0					
V21	DG192566	A						3.2					
V22	DQ219300	J											91.7
V23	DQ219301	A											8.3

Values indicate the frequency of the allele in each strain. Alleles that varied in deduced amino acid sequences are designated by different isoforms. Values in parentheses indicate number of individual flies genotyped.

723223) in the N-terminal region of the Gfi-1 protein. This poly Ala region is not found in *Drosophila pseudoobscura* (accession EAL32822), human, mouse, or rat (accession no. U67369, U78312, L06986). Understanding the function of these poly Ala regions will require further study.

It does not appear that the different *mdGfi-1* alleles have any role in pyrethroid resistance. There were multiple alleles found in all strains and there was no association of a particular allele with resistant or susceptible strains. For example, the LPR strain had two alleles (4 and 15) one of which (15) was found in two susceptible (CS and SRS) strains. If an allele offered a selective advantage in the presence of pyrethroids we would have expected the resistant strains to be homozygous for a unique allele (or at least have unique alleles).

3.4. Linkage analysis

There was a 9 bp deletion in *mdGfi-1* in all OCR alleles relative to aabys (Fig. 5). Therefore, this deletion was used to determine the linkage of *mdGfi-1* relative to the recessive morphological markers in the aabys strain. It was expected that the absence of one specific mutant marker (indicating a heterozygote) would associate with individuals that were polymorphic for the *mdGfi-1* sequence. Houseflies that were +;*ar*;*bwb*;*ye*;*snp* were heterozygous for *mdGfi-1*. Houseflies of all other phenotypes had the same *mdGfi-1* allele as aabys (and were homozygous). There was one exception: 5 *ac*;*ar*;+;*ye*;*sn*p flies were homozygous for the aabys *mdGfi-1* sequence while one was a heterozygote. This is presumably due to a cross-over event which, although rare, do occur in male house flies (Hamm et al., 2005). These results indicate *mdGfi-1* is on autosome 1 in the housefly.

3.5. Expression of mdGfi-1

All life stages (eggs, larvae, pupae and adults) express *mdGfi-1* (Fig. 6). Highest levels were found in the egg and pupal stages, relative to larvae or adults. The level of expression varied between strains, with more *mdGfi-1* detected in the CS strain than in LPR for all life stages, except for adults. Given that CYP6D1 is expressed only in adult houseflies, and given the similar level of *mdGfi-1* found in LPR and CS adult flies it appears that the relative abundance of *mdGfi-1* is not a factor in resistance.

All the three body regions of houseflies express *mdGfi-1*, although expression was significantly higher in thoraces, compared to heads and abdomens (Fig. 7). Whether this higher level of *mdGfi-1* expression is due to a higher abundance in muscle (muscle is a major constituent of the housefly thorax), midgut or other tissues will require further investigation.

In summary, our results indicate that the putative Gfi-1 binding region within the CYP6D1 promoter found in susceptible strains does indeed bind this transcriptional repressor. The 15 bp insert that interrupts this binding

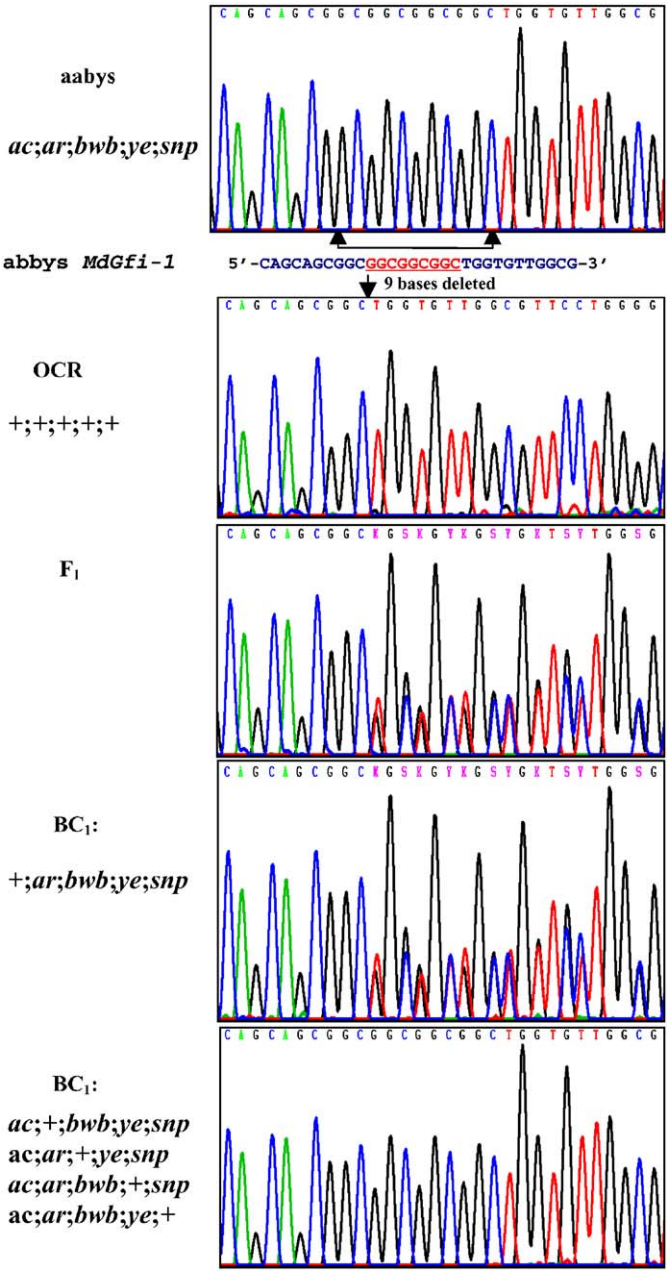

Fig. 5. Linkage analysis of *mdGfi-1*. Electropherograms represent the partial *mdGfi-1* sequences derived from aabys ($n = 25$), OCR ($n = 9$), F_1 (aabys × OCR)($n = 10$), and the five genotypes isolated from the back-cross ($n = 3$ for each genotype, except $n = 6$ for *ac*;*ar*;+;*ye*;*snp*).

sequence in resistant strains results in a CYP6D1 promoter that is able to bind 5- to 10-fold less *mdGfi-1*. These results are entirely consistent with the original hypothesis that reduced binding of *mdGfi-1* in resistant strains is an underlying cause of the increased transcription of *CYP6D1v1*, and thus resistance. In future studies it will be important to examine the function of *mdGfi-1* using in vitro promoter assays to confirm its role as a transcriptional repressor. Sequencing of individual houseflies revealed numerous polymorphisms of *mdGfi-1*. It does not appear that these different alleles have a role in

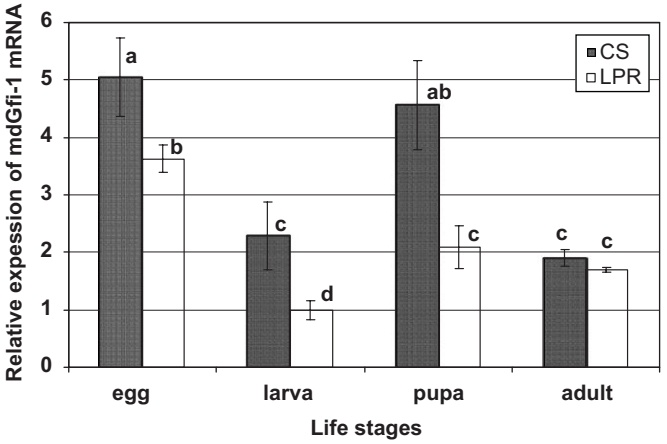

Fig. 6. Expression of *mdGfi-1* in CS and LPR house flies at four different life stages. Values are relative to expression in LPR larvae and represent the mean ± SD ($n = 3$). Bars with different letters are significantly different ($P < 0.05$).

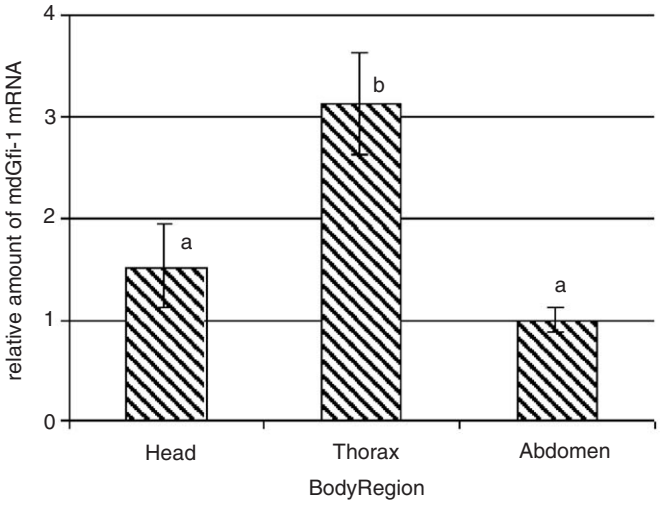

Fig. 7. Expression of *mdGfi-1* in housefly heads, thoraces and abdomens. Values are relative to expression in abdomens and represent the mean ± SD ($n = 4$). Bars with different letters are significantly different ($P < 0.05$).

insecticide resistance. *mdGfi-1* is on autosome 1 and its expression is developmentally regulated.

Acknowledgments

We thank J. Deacutis for sequencing the *mdGfi-1* alleles from the NC strain, M. Kristensen for the SRS strain, K. Liu for valuable discussions and C. Leichter for technical assistance. This work was supported by a grant from the National Institutes of Health (GM47835) and the Daljit S. and Elaine Sarkaria Professorship.

References

Doan, L.L., Porter, S.D., Duan, Z., Flubacher, M.M., Montoya, D., Tsichlis, P.N., Horwitz, M., Gilks, C.B., Grimes, H.L., 2004. Targeted transcriptional repression of *Gfi1* by GFI1 and GFI1B in lymphoid cells. Nucl. Acids Res. 32, 2508–2519.

Duan, Z., Horwitz, M., 2003. Targets of the transcriptional repressor oncoprotein Gfi-1. PNAS 100, 5832–5937.

Dwivedi, P.P., Anderson, P.H., Omdahl, J.L., Grimes, H.L., Morris, H.A., May, B.K., 2005. Identification of the growth factor independent-1 (GFI1) as a repressor of 25-hydroxyvitamin D 1-alpha hydroxylase (CYP27B1) gene expression in human prostate cancer cells. Endocr. Relat. Cancer 12, 351–365.

Grimes, H.L., Gilks, C.B., Chan, T.O., Porter, S., Tsichlis, P.N., 1996. The Gfi-1 protooncoprotein represses Bax expression and inhibits T-cell death. Proc. Natl. Acad. Sci. USA 93, 14569–14573.

Hamm, R., Shono, T., Scott, J.G., 2005. A cline in frequency of autosomal males is not associated with insecticide resistance in house fly, (Diptera: Muscidae). J. Econ. Entomol. 98, 171–176.

Hock, H., Hamblen, M.J., Rooke, H.M., Schindler, J.W., Saleque, S., Fujiwara, Y., Orkin, S.H., 2004. Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. Nature 431, 1002–1007.

Kasai, S., Scott, J.G., 2000. Overexpression of cytochrome P450 CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia. Pestic. Biochem. Physiol. 68, 34–41.

Kasai, S., Scott, J.G., 2001. A house fly gene homologous to the zinc finger proto-oncogene *Gfi*-1. Biochem. Biophys. Res. Commun. 283, 644–647.

Keiding, J., 1999. Review of the global status and recent development of insecticide resistance in field populations of the housefly, *Musca domestica* (Diptera: Muscidae). Bull. Entomol. Res. 89, S7–S67.

Liu, N., Scott, J.G., 1996. Genetic analysis of factors controlling elevated cytochrome P450, CYP6D1, cytochrome b_5, P450 reductase and monooxygenase activities in LPR house flies, *Musca domestica*. Biochem. Genet. 34, 133–148.

Liu, N., Scott, J.G., 1998. Increased transcription of CYP6D1 causes cytochrome P450-mediated insecticide resistance in house fly. Insect Biochem. Mol. Biol. 28, 531–535.

Liu, N., Tomita, T., Scott, J.G., 1995. Allele-specific PCR reveals that the cytochrome $P450_{lpr}$ gene is on chromosome 1 in the house fly, *Musca domestica*. Experientia 51, 164–167.

Person, R.E., Li, F., Duan, Z., Benson, K.F., Wechsler, J., Papadaki, H.A., Eliopoulos, G., Kaufman, C., Bertolone, S.J., Nakamoto, B., Papayannopoulou, T., Grimes, H.L., Horwitz, M., 2003. Mutations in proto-oncogene *GFI1* cause human neutropenia and target *ELA2*. Nat. Genet. 34, 308–312.

Scott, J.G., 1998. Toxicity of spinosad to susceptible and resistant strains of house flies, *Musca domestica*. Pestic. Sci. 54, 131–133.

Scott, J.G., 1999. Molecular basis of insecticide resistance: cytochromes P450. Insect Biochem. Mol. Biol. 29, 757–777.

Scott, J.G., 2001. Cytochrome P450 monooxygenases and insecticide resistance: lessons from *CYP6D1*. In: Ishaaya, I. (Ed.), Biochemical Sites of Insecticide Action and Resistance. Springer, New York, pp. 255–267.

Scott, J.G., Georghiou, G.P., 1986. Mechanisms responsible for high levels of permethrin resistance in the house fly. Pestic. Sci. 17, 195–206.

Scott, J.G., Liu, N., Wen, Z., Smith, F.F., Kasai, S., Horak, C.E., 1999. House fly cytochrome P450 *CYP6D1*: 5 prime flanking sequences and comparison of alleles. Gene 226, 347–353.

Scott, J.G., Alefantis, T.G., Kaufman, P.E., Rutz, D.A., 2000. Insecticide resistance in house flies from caged-layer poultry facilities. Pest Manage. Sci. 56, 147–153.

Seifert, J., Scott, J.G., 2002. The *CYP6D1v1* allele is associated with pyrethroid resistance in the house fly, *Musca domestica*. Pestic. Biochem. Physiol. 72, 40–44.

Shono, T., Scott, J.G., 2003. Spinosad resistance in the house fly, *Musca domestica*, is due to a recessive factor on autosome 1. Pestic. Biochem. Physiol. 75, 1–7.

Shono, T., Kasai, S., Kamiya, E., Kono, Y., Scott, J.G., 2002. Genetics and mechanisms of permethrin resistance in the YPER strain of house fly. Pestic. Biochem. Physiol. 73, 27–36.

Tomita, T., Scott, J.G., 1995. cDNA and deduced protein sequence of *CYP6D1*: the putative gene for a cytochrome P450 responsible for pyrethroid resistance in house fly. Insect Biochem. Mol. Biol. 25, 275–283.

Tripathi, R.K., O'Brien, R.D., 1973. Insensitivity of acetylcholinesterase as a factor in resistance of houseflies to the organophosphate Rabon. Pestic. Biochem. Physiol. 3, 495–498.

Wheelock, G.D., Scott, J.G., 1992. Anti-$P450_{lpr}$ antiserum inhibits specific monooxygenase activities in LPR house fly microsomes. J. Exp. Zool. 264, 153–158.

Williamson, M., Martinez-Torres, D., Hick, C., Devonshire, A., 1996. Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (*kdr*) to pyrethroid insecticides. Mol. Gen. Genet. 252, 51–60.

Yucel, R., Kosan, C., Heyd, F., Moroy, T., 2004. Gfi1: green fluorescent protein knock-in mutant reveals differential expression and autoregulation of the growth factor independence 1 (*Gfi1*) gene during lymphocyte development. J. Biol. Chem. 279, 40906–40917.

Zweidler-McKay, P.A., Grimes, H.L., Flubacher, M.M., Tsichlis, P.N., 1996. *Gfi*-1 encodes a nuclear zinc finger protein that binds DNA and functions as a transcriptional repressor. Mol. Cell. Biol. 16, 4024–4034.