Supporting Information

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SI Results

***NFX-1* Transcription Factor Expression and Its Role in Deltamethrin Resistance.** The qRT-PCR analysis confirmed microarray data where a 200-fold lower expression of *NFX-1* in the QTC279 strain was detected when compared with its expression in the Lab-S strain (Table S2), suggesting that this gene may regulate the expression of *CYP6BQ9*. To determine if the reduced expression levels of *NFX-1* in the Lab-S strain could cause deltamethrin resistance, we performed bioassays to compare deltamethrin resistance levels between *NFX-1* and *malE* dsRNA-injected Lab-S beetles. The *NFX-1* dsRNA injection effectively reduced *NFX-1* mRNA levels at 5 days after injection (Fig. S3*A*). However, there was no difference in the deltamethrin susceptibility between *NFX-1* and *malE* dsRNA-injected beetles (Fig. S3*B*). The *CYP6BQ9* mRNA levels were not affected by silencing of *NFX-1* (Fig. S3*C*). We also quantified the expression of *NFX-1* in QTC279, F1 (offspring of QTC279 male and Lab-S female), and two susceptible *T. castaneum* strains, GA-2 and Lab-S (Fig. S3*D*). Comparison of these expression data with the deltamethrin bioassay data presented in Fig. 1*D* showed no positive correlation between expression of *NFX-1* and deltamethrin resistance.

Moreover, the highest level of *NFX-1* mRNA was detected in the midgut when compared with its expression in the fat body, ovary, and brain tissues dissected from the Lab-S strain (Fig. S3*E*). Taken together, these data suggest that *NFX-1* may not play an important role in the regulation of *CYP6BQ9* expression in *T. castaneum*.

SI Materials and Methods

Red Flour Beetle Strains. Three red flour beetle strains were used in this study. QTC279, originally collected from a wheat storage facility in Malu, Queensland, Australia, in 1984, was selected with pyrethroids for 10 generations until it was homozygous for the major pyrethroid resistance factor. Lab-S is an insecticide-susceptible strain. GA-2 strain was used in the whole-genome sequencing project. All four strains were obtained from Dr. R. W. Beeman (US Grain Marketing Research Laboratory of USDA, Manhattan, KS). Beetles were reared in whole wheat flour with yeast (10% by weight) and maintained in darkness at 32 °C and 55 ± 2% relative humidity.

RNA Extraction. Total RNA was extracted from adult beetles using TRI reagent (Molecular Research Center Inc.). For microarray experiments, total RNA was isolated using spin columns (RNeasy; Qiagen). The integrity of total RNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray Hybridization and Analysis. Equal quantities of total RNA isolated from three replicates of the QTC279 and Lab-S adult beetles were labeled using Low RNA Input Linear Amplification Kit with one color (Agilent) following manufacturer's instructions. Labeled cRNAs were purified using RNase mini purification columns (Qiagen) to remove unlabeled products. The yield and incorporation efficiency were measured using a spectrophotometer (NanoDrop Technologies). Fifteen picomoles of fluorescently labeled cRNAs were used for each hybridization.

The 60-mer oligonucleotides designed based on 15,008 genes selected from the 16,000 genes predicted by *Tribolium* genome annotations and 736 control probe sets were printed onto glass slides at Agilent Technologies. The hybridization of labeled probes to arrays was performed using the Agilent Gene Expression Hybridization Kit at 65 °C for 17 h. cRNA samples were fragmented at 60 °C for 30 min. After hybridization, the microarray slides were washed with Agilent gene expression wash buffers: buffer 1 at room temperature for 1 min; and buffer 2 at 37 °C for 1 min. The slides were scanned using an Agilent microarray scanner (G2565BA) with a setting for one color using the green channel and 5-μm resolution. The microarray images were extracted using Feature Extraction software (v 9.5.1, Agilent). The raw data files (.txt) were imported into GeneSpring (GX 7.3), and the data were normalized and analyzed. GeneSpring generates an average value of the three replicates for each gene. The data were transformed to bring any negative value to 0.01. Normalization was performed using a per-chip 50^{th}-percentile method that normalizes each chip on its median, allowing comparison among chips. Then a per-gene on median normalization was performed, which normalized the expression of every gene on its median among samples. The differentially expressed genes of significance were evaluated with the aid of volcano plots (*P* value vs. fold change). Fold differences in expression were calculated by dividing the mean value of signal intensities in QTC279 strain with that in the Lab-S strain, and *P* values were obtained from *t* test. Pairwise comparison between the QTC279 and Lab-S strains used the data derived from the volcano plots. The data were also subjected to the Bonferroni and Benjamini and Hochberg false discovery rate multiple-testing corrections. The Bonferroni multiple-testing correction is one of the most stringent filters applied to microarray data. One hundred percent of the genes that pass through this filter are considered true positives. This filter limits the chance of false-positive results to be no more than the value obtained by multiplying each nominal *P* value by number of genes being tested. Benjamini and Hochberg false discovery rate procedure provides a good balance between discovery of significant genes and protection against false positives. The volcano plot was prepared using the R program (1).

qRT-PCR. qRT-PCR was performed in MyiQ single-color real-time PCR detection system (Bio-Rad Laboratories). Total RNA was isolated from 10 to 15 adult beetles for each sample using the TRI reagent (Molecular Research Center Inc.), and the RNA was treated with DNase I (Ambion, Inc.). cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). DNase I-treated total RNA was used as a template. Each qRT-PCR (20 μL final volume) contained 10 μL FastStart SYBR Green Master (Roche Diagnostics), 2 μL of cDNA, and 0.6 μL each of forward and reverse gene-specific primers (Table S1; stock 10 μM). An initial incubation of 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 30-s settings were used. A fluorescence reading determined the extension of amplification at the end of each cycle. Standard curves were obtained using a 10-fold serial dilution of pooled cDNA. The *T. castaneum* ribosomal protein rp49, endogenous control, was used to normalize expression of genes. Both the PCR efficiency and R2 (correlation coefficient) value were taken into consideration in estimating the relative quantities. Each experiment was repeated at least three times using independent biological samples. The statistical significance of the gene expressions was calculated using a Student's *t* test (two-sample comparison), and a value of $P < 0.05$ was considered statistically significant.

Northern Blot Analyses. Northern blot analyses were performed according to Sambrook *et al.* (2). Twenty micrograms of total RNA from each sample were fractionated on 1% formaldehyde denaturing agarose gel and transferred to Nytran membranes

(Schleicher and Schuell) as previously described (2). The cDNAs were labeled with [α-^{32}P]dCTP using a High Primer enzyme (Roche) and hybridized with RNA blots. The amount of RNA loaded into each lane was standardized by comparing the density of the 18S ribosomal RNA band on agarose gel under UV light before transfer. After washing, the radioactivity signals were captured in a Typhoon InstantImager (GE Healthcare). Northern blot analyses experiments were repeated three times using independent preparations of RNA samples.

RNAi. The dsRNA was synthesized using the MEGscript RNAi Kit (Ambion Inc.). Genomic DNA was isolated from *T. castaneum* adults using DNeasy Tissue Kit (Qiagen). Genomic DNA and T7 promoter-containing PCR primers [Table S1; with T7 RNA promoter sequence (TAATACGACTCACTATAGGG) appended to the 5′ ends of both sense and antisense specific primers] were used in a PCR to obtain gene-specific fragments containing T7 promoter sequence on both ends. PCR product (200-400 bp) was used as a template to synthesize dsRNA. For the dsRNA purification, phenol/chloroform extraction followed by ethanol precipitation method was applied. dsRNA was diluted in nuclease-free water to 4–5 μg/μL for injection into *T. castaneum* adults. The 1- to 2-week-old adults were anesthetized with ether vapor for 10 min and placed on a glass slide covered with double-sided tape. The dsRNA (0.8–1 μg) was injected into the side of the abdomen with an injection needle pulled out from a glass capillary tube using needle puller (Idaho Technology). Controls were injected with the dsRNA using bacterial *mal*E gene as a template. After injection, beetles were removed from the glass slide, allowed to recover for 3 h at room temperature, then returned to normal rearing conditions.

Construction of Transgenic Fly Strains. *CYP6BQ9* cloned from the QTC279 strain was inserted into pCaSpeR-hs (3) and pUAST (4) to prepare. pCa-*CYP6BQ9* and UAS-*CYP6BQ9* that were transformed into the germline of *D. melanogaster* yw^{1118} strain using standard P-element-mediated transformation techniques (5). The inserted DNA construct in two independent transformed lines was mapped on chromosome and balanced. Two independent transformed lines homozygous for UAS-*CYP6BQ9* expression were chosen for further analysis. For tissue-specific expression experiments, transformed lines homozygous for UAS-*CYP6BQ9* expression were crossed with CNS-GAL4 (P{GawB} 60IIA, Bloomington Stock #7029, GAL4 expressed in the nervous system) and Act5C-GAL4 (P{Act5C-GAL4}17bFO1, Bloomington Stock #3954 ubiquitous expression of GAL4). The female offspring were chosen for further analysis.

***Drosophila* Contact Bioassays.** Females at 1–3 days posteclosion were used in the contact bioassays. Deltamethrin was coated on the inside of 35-mL plastic scintillation vials (Research Products International Corp.) by applying 200 μL acetone containing varying concentrations of deltamethrin and rolling the vial until the acetone had evaporated. After preliminary tests, a diagnostic dose of 10 μg deltamethrin per vial was chosen. Vials were plugged with cotton balls soaked in 5% sucrose. Ten flies were placed in each vial and the mortality was scored after 24 h exposure to insecticide. For each assay, at least five replicates were performed. For transformed lines with pCa-*CYP6BQ9*, two independent lines with homozygous *CYP6BQ9* expression were maintained at 25 °C or heat shocked in a 37 °C water bath for 1.5 h twice a day for two consecutive days before performing the contact bioassays.

Baculovirus-Mediated CYP6BQ9 Expression. Full-length *CYP6BQ9* cDNA spanning the start codon and the stop codon was PCR amplified with sense primer (Sf7318F-PG, 5′ CCGGAATTCACCACCATGACTCTAATAACAAACAACC 3′) and antisense primer (Sf7318R, 5′ CTAGTCTAGAGTCCAGCTTTTCTACATCC 3′; Table S1). The *CYP6BQ9* PCR product was subcloned into the pFastBac donor plasmid and then transformed into DH10Bac *E. coli* cells. The recombinant baculovirus DNA was produced and transfected to the Sf9 insect cells (Invitrogen) using Bac-to-Bac baculovirus expression system (Invitrogen). The titer of the recombinant virus was determined following manufacturer's instructions. *D. melanogaster* NADPH CPR sequence (GenBank accession no. Q27597) was obtained by gene synthesis and inserted into pDEST8 expression vector (Invitrogen) for baculoviral expression. Sf9 cells (Invitrogen) were maintained under serum-free conditions at 27 °C with Sf-900 II SFM. Sf9 cells were coinfected with recombinant baculoviruses expressing *CYP6BQ9* and NADPH CPR with a MOI of 1 and 0.1, respectively. Hemin chloride (2.5 μg/mL) was added to the culture media to compensate for the low levels of endogenous heme in the insect cells. After 48 h, cells were harvested and washed with PBS, and the microsomes of the membrane fraction were prepared according to standard procedures (6) and stored at −80 °C.

1. R Development Core Team (2005) R: A Language and Environment for Statistical Computing, reference index version 2.2.1. R Foundation for Statistical Computing (Vienna), http://www.R-project.org.
2. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
3. Thummel CS, Pirrotta V (1991) New pCaSpeR P-element vectors. *Dros Inf Serv* 71:150.
4. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401–415.
5. Spradling A (1986) In *Drosophila: A Practical Approach*, ed Roberts D (IRL, Oxford), pp 175–197.
6. Phillips IR, Shephard EA, eds (2005) , ed (2005) *Cytochrome P450 Protocols* (Humana, Totowa, NJ).

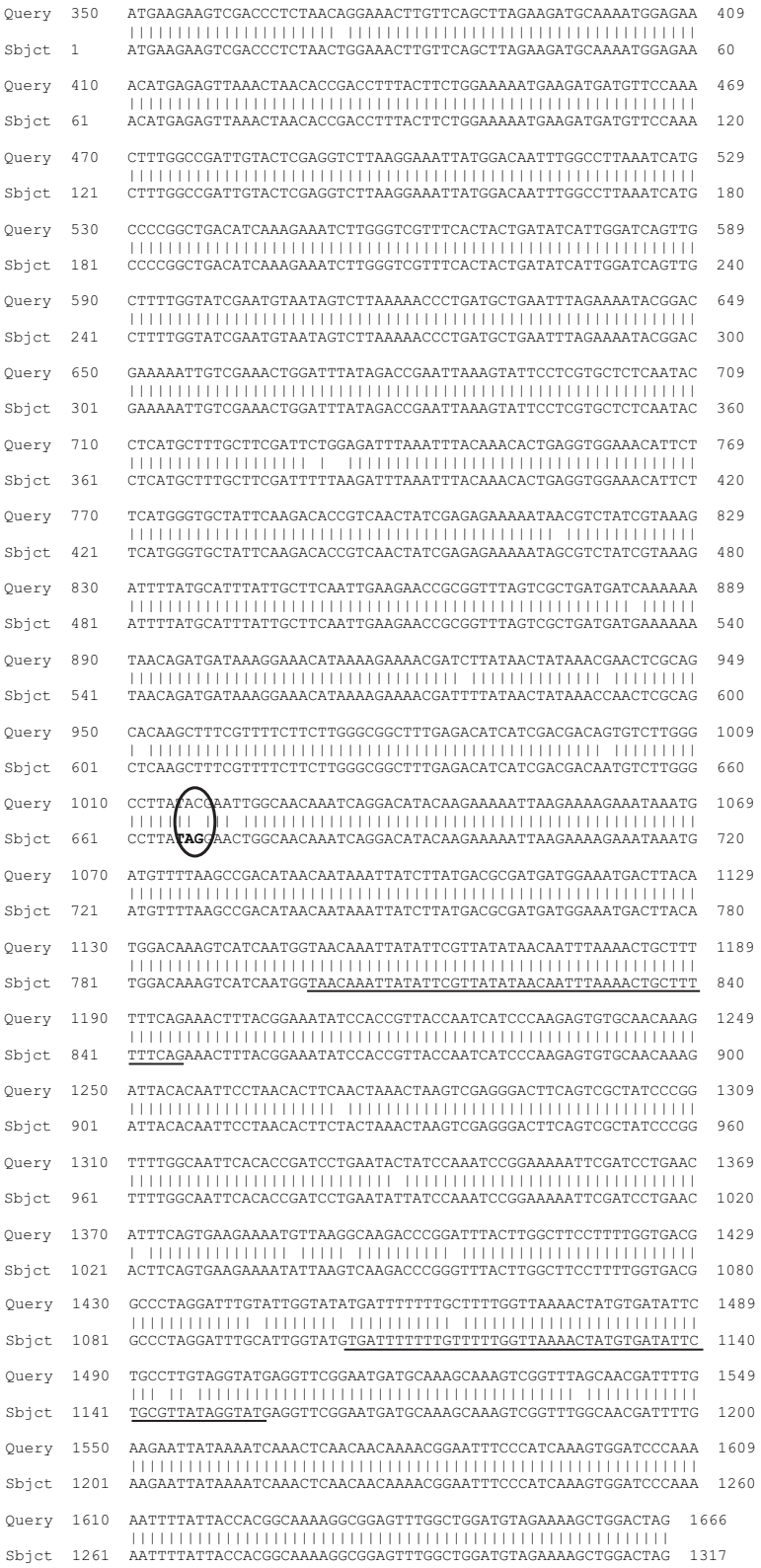

Fig. S1. Alignment of sequences of cloned gene fragment (genomic DNA) from the GA-2 strain with the genome sequences of GLEAN_07318 and GLEAN_07319 (ClustalW2). The result showed that the premature termination codon reported in genome sequence is an error because the new sequence from all three strains (QTC279, Lab-S, and GA-2) showed TAC but not TAG (labeled in black circle) at this position. The upper line is the new sequence, and the lower line is the previous genome sequence in which the sequence of GLEAN_07318 is from nucleotide 1–668, and the sequence of GLEAN_07319 is from nucleotide 762–1317. The sequence similarities between the new sequences and the genome sequences of GLEAN_07318 and GLEAN_07319 are 98% and 97%, respectively. Two introns (intron 1, 48 bp; and intron 2, 52 bp) in the sequence are underlined. The sequences have been deposited into GenBank (accession no. GU727869 for the QTC279 strain and GU727868 for the GA-2 strain have been assigned).

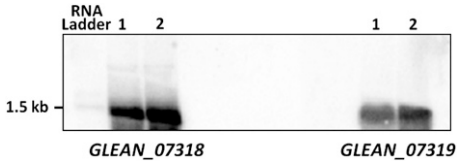

Fig. S2. Northern blot hybridization analysis to identify mRNA sizes of GLEAN_07318- and GLEAN_07319-predicted genes. Regions of GLEAN_07318- and GLEAN_07319-predicted genes were PCR amplified and labeled with ^{32}P and hybridized to membranes. The size of hybridizing mRNA was determined by comparing against sizes of RNAs in a concurrently run RNA ladder (Invitrogen Life Technologies). RNAs from two independent experiments were used in lines 1 and 2.

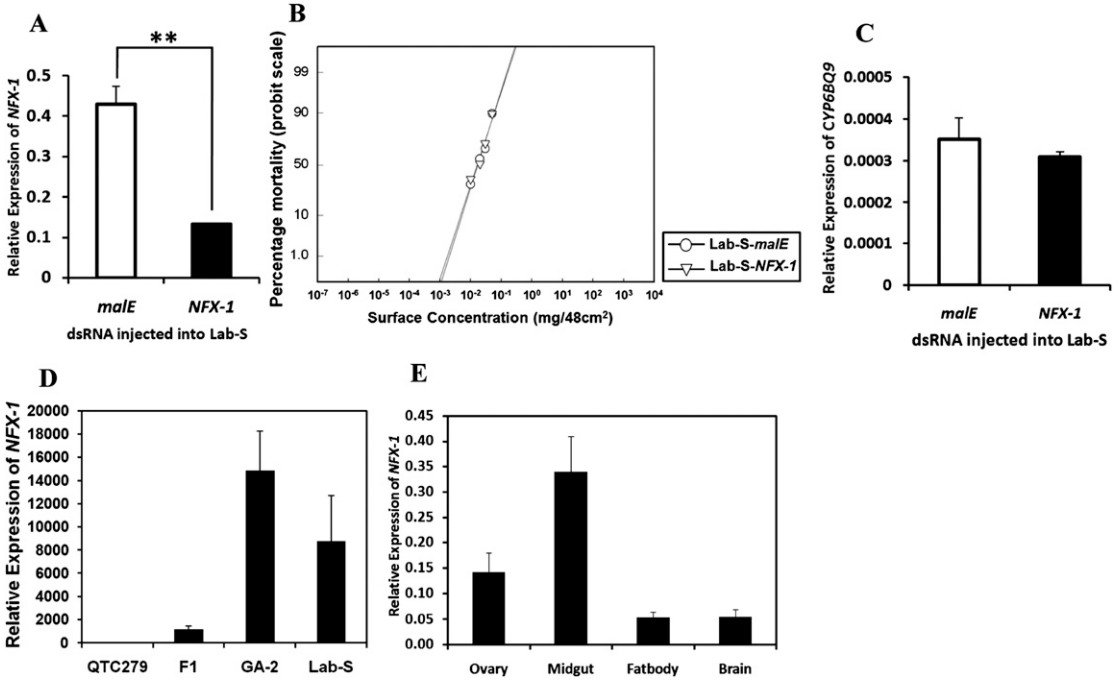

Fig. S3. The expression of *NFX-1* in *T. castaneum*. (*A*) Injection of *NFX-1* dsRNA knocked down its expression. The mRNA levels of *NFX-1* were quantified by qRT-PCR at 5 days after dsRNA injection into the Lab-S beetles. The relative mRNA levels were shown as a ratio in comparison with the levels of *rp49* mRNA. The results were represented as the mean + SEM ($n = 3$). Statistical significance of the gene expression between two samples was calculated using Student's *t* test. $^{**}P < 0.01$. (*B*) Dose-response curves for *T. castaneum* adults exposed to deltamethrin. Lab-S beetles injected with either *malE* or *NFX-1* dsRNA were exposed to various doses of deltamethrin, and the mortality was recorded and graphed. (*C*) Injection of *NFX-1* dsRNA did not affect the expression of *CYP6BQ9* at 5 days after dsRNA injection. The mRNA levels of *CYP6BQ9* were quantified by qRT-PCR at 5 days after dsRNA injection into the Lab-S strain. The relative mRNA levels were shown as a ratio in comparison with the levels of *rp49* mRNA. (*D*) Comparative expression of *NFX-1* in QTC279, F1 (offspring of QTC279 and Lab-S), and GA-2 compared with that of the Lab-S strain. The results were represented as mean + SEM ($n = 5$). (*E*) Tissue-specific expression of *NFX-1* in the ovary, midgut, fat body, and brain of the Lab-S strain. Different tissues were dissected from live beetles and put in ice-cold 1% PBS separately. The *NFX-1* mRNA levels in these tissues were determined by qRT-PCR as described in *Materials and Methods*. Relative expression levels were normalized by *rp49* mRNA levels. Data shown is the mean + SEM ($n = 5$).

Table S1. Primers used for qRT-PCR, dsRNA synthesis, Northern blotting, recombinant protein synthesis, and transgenic expression

No.	Gene name	GLEAN no.	GeneBank accession no.		Primer (5′ to 3′)	
1	*CYP6BQ3P*	GLEAN_07312	XM_970481	qRT-PCR	qTc07312F	ACACCATCATGGATGAAGCA
					qTc07312R	TTTCGGGGAAAAATCAGTTG
2	*CYP6BQ8*	GLEAN_07317	XM_970474	qRT-PCR	qTc07317F	GGGCCGATACCCTTCTACAT
					qTc07317R	AGTGGTACAATCGGCCAAAG
				dsRNA	dsTc07317F	TAATACGACTCACTATAGGGGTCGCA-ACATTCTTCATGGAC
					dsTc07317R	TAATACGACTCACTATAGGGAAGTCA-TTGCCGTGGATGAC
3	*CYP6BQ9*	GLEAN_07318	———	qRT-PCR	qTc07318F	TCCTCGTGCTCTCAATACCTCATG
					qTc07318R	TGACGGTGTCTTGAATAGCACC
				dsRNA	dsTc07318F	TAATACGACTCACTATAGGGAACACCG-ACCTTTACTTCTGGA
					dsTc07318R	TAATACGACTCACTATAGGGATCGAAG-CAAAGCATGAGGT
				Northern blotting	NTc07318F	TTTGGCCGATTGTACTCGAGGTC
					NTc07318R	AACGAAAGCTTGAGCTGCGAGTTG
				Protein expression	Sf7318F-PG	CCGGAATTCACCACCATGACTCTAATA-ACAAACAACC
					Sf7318R	CTAGTCTAGAGTCCAGCTTTTCTACATCC
				Transgenic expression	Dr7318F-P	CCGGAATTCCAAAATGACTCTAATAAC-AAACAACC
					Sf7318R	CTAGTCTAGAGTCCAGCTTTTCTACATCC
		GLEAN_07319	———	Northern blotting	NTc07319F	CACCGTTACCAATCATCCCAAGAG
					NTc07319R	ATACCAATGCAAATCCTAGGGCCG
4	*CYP6BQ10*	GLEAN_07320	XM_970471	qRT-PCR	qTc07320F	CGGAAAAATTCGATCCTGAA
					qTc07320R	AAAATTGCCGTCAAACCAAC
				dsRNA	dsTc07320F	TAATACGACTCACTATAGGGTGC-GAAAATATCCTCCGC
					dsTc07320R	TAATACGACTCACTATAGGGAT-CCGGGTCTTGACTTAAC
5	*CYP346B1*	GLEAN_14359	XM_963658	qRT-PCR	qTc14359F	AATTCTGACCACGGATTTCG
					qTc14359R	TTTTGAGTTTTCCGGACGAG
6	*CYP346B2*	GLEAN_14360	XM_963730	qRT-PCR	qTc14360F	TTCCGGGGACCAATGTAGTA
					qTc14360R	AACGGCATAAACGTGAAAGG
7	*CYP6BK2*	GLEAN_10255	XM_965388	qRT-PCR	qTc10255F	AAGGTCCACGAATTTGCATC
					qTc10255R	CGAGTCCACTTGCATTCTCA
8	*GSTΩ1*	GLEAN_00054	XM_966154	qRT-PCR	qTc00054F	CGATGCAGTTTTGCCCTTAT
					qTc00054R	TTGGTGTACCATTCGGGTTT
9	Oxidoreductase	GLEAN_03807	XM_961621	qRT-PCR	qTc03807F	GTTGGCAAAGTGGCCATAGT
					qTc03807R	GCTTTGATTGCGTGGAGTTT
10	Unknown	GLEAN_13434	XM_963585	qRT-PCR	qTc13434F	TGGTGCAAGTAAGCGACAAG
					qTc13434R	CGTTGGTGCTCCATAGGTTT
11	*NFX-1*	GLEAN_02938	XM_969686	qRT-PCR	qTc02938F	ATTACGACGTCCGTTGCTGTGAAG
					qTc02938R	AGTCACCTTGATTTGGCACTCTTGG
12	Unknown	GLEAN_11271	XM_001808138	qRT-PCR	qTc11271F	TCGACGGCGTCTTAGAGAAT
					qTc11271R	CCCAATGCTGCCTTCATAAT

Table S2. Differential expression of genes between QTC279 and Lab-S beetles in microarray and qRT-PCR analysis

No.	Gene name	GLEAN no.	Microarray (QTC279/Lab-S)	qRT-PCR (QTC279/Lab-S)
1	*CYP6BQ3P*	GLEAN_07312	11.5	0.181
2	*CYP6BQ8*	GLEAN_07317	4.6	2.52
3	*CYP6BQ9*	GLEAN_07318	217	302
4	*CYP6BQ10*	GLEAN_07320	7.9	12.09
5	*CYP346B1*	GLEAN_14359	45	60.8
6	*CYP346B2*	GLEAN_14360	8.9	20.5
7	*CYP6BK2*	GLEAN_10255	6.5	13.99
8	*GSTΩ1*	GLEAN_00054	232	1.193
9	Oxidoreductase	GLEAN_03807	52	143.58
10	Unknown	GLEAN_13434	63	36.69
11	*NFX-1*	GLEAN_02938	0.0048	0.00036
12	Unknown	GLEAN_11271	100	100.21