Microarray Analysis of Gene Regulations and Potential Association with Acephate-Resistance and Fitness Cost in *Lygus lineolaris*

Yu Cheng Zhu1*, Zibiao Guo1, Yueping He2, Randall Luttrell1

1 Southern Insect Management Research Unit, Agricultural Research Service, United States Department of Agriculture, Stoneville, Mississippi, United States of America, 2 State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control, Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Hangzhou, Zhejiang, People's Republic of China

Abstract

The tarnished plant bug has become increasingly resistant to organophosphates in recent years. To better understand acephate resistance mechanisms, biological, biochemical, and molecular experiments were systematically conducted with susceptible (LLS) and acephate-selected (LLR) strains. Selection of a field population with acephate significantly increased resistance ratio to 5.9-fold, coupled with a significant increase of esterase activities by 2-fold. Microarray analysis of 6,688 genes revealed 329 up- and 333 down-regulated (=2-fold) genes in LLR. Six esterase, three P450, and one glutathione S-transferase genes were significantly up-regulated, and no such genes were down-regulated in LLR. All vitellogenin and eggshell protein genes were significantly down-regulated in LLR. Thirteen protease genes were significantly down-regulated and only 3 were up-regulated in LLR. More than twice the number of catalysis genes and more than 3.6-fold of metabolic genes were up-regulated, respectively, as compared to those down-regulated with the same molecular and biological functions. The large portion of metabolic or catalysis genes with significant up-regulations indicated a substantial increase of metabolic detoxification in LLR. Significant increase of acephate resistance, increases of esterase activities and gene expressions, and variable esterase sequences between LLS and LLR consistently demonstrated a major esterase-mediated resistance in LLR, which was functionally provable by abolishing the resistance with esterase inhibitors. In addition, significant elevation of P450 gene expression and reduced susceptibility to imidacloprid in LLR indicated a concurrent resistance risk that may impact other classes of insecticides. This study demonstrated the first association of down-regulation of reproductive- and digestive-related genes with resistance to conventional insecticides, suggesting potential fitness costs associated with resistance development. This study shed new light on the understanding of the molecular basis of insecticide resistance, and the information is highly valuable for development of chemical control guidelines and tactics to minimize resistance and cross-resistance risks.

Introduction

During the last decade, widespread adoptions of transgenic Bt cotton and altered chemical control schemes have allowed sucking insect populations to increase. Of these pests, the tarnished plant bug (TPB), *Lygus lineolaris*, emerged as the most economically significant [1]. Management of tarnished plant bug relies almost exclusively on chemical control. Commonly used insecticides include pyrethroids, organophosphates, carbamates, and neonicotinoids. Acephate, an organophosphorous insecticide, is among the most widely used insecticides for TPB control. In order to suppress feeding damages from tarnished plant bug and bollworm/tobacco budworm, cotton is more frequently sprayed than other major crops in the South. Over the years, tarnished plant bug has become increasingly resistant to several chemical insecticides [2,3,4], including acephate [5].

Acephate (Orthene) has been widely used to control tarnished plant bug on cotton in the Delta region of Mississippi, Arkansas, and Louisiana. TPB populations have developed 3- to 5-fold resistance, and control of these field populations may become difficult once resistance ratios reached 3-fold or higher [5]. Our field surveys indicate that susceptibility to acephate decreases as the growing season progresses. During late season (Aug. to Oct.), TPBs are substantially less susceptible to insecticides than those collected in May to July. Susceptibility reaches its lowest level in October. Our field surveys also indicate that TPB populations around cotton fields are less susceptible than populations around corn or soybean fields.

Another important phenomenon in studying TPB resistance to insecticides is the difficulty of maintaining resistant colonies in the laboratory. Less susceptible strains tend to have high mortality and low egg production. Although insecticide selections may increase resistance level to 10-fold or higher, selected colonies often die or fail to reproduce enough progeny for further research. Without further selection with acephate, a resistant colony can be sustained for a few generations. Resistance gradually decreased and sustained at 2- to 3-fold higher LC50 than susceptible strain. All
of these factors limit further investigation of resistance mechanisms in this important insect pest.

With multiple generations per year, high mobility, and existence of differential activities of major detoxification enzymes [6], field populations of TPB across the Delta region have potential to evolve high levels of resistance to multiple insecticides, especially when they are under high selection pressure. Examinations of acephate resistance mechanisms have not been conducted previously, except for a few limited sequence and gene expression level comparison of one esterase and one glutathione S-transferase related to malathion-resistant TPB [7,8]. Increased economic importance and reports of pyrethroid and organophosphate resistance [4,9] prompted our research to better understand the mechanisms of acephate resistance in this cotton pest. By integrating biological and biochemical assays with novel microarray and other molecular analytic tools, this study compared more than seven thousand genes simultaneously in acephate-susceptible (LLS) and –selected (LLR) tarnished plant bugs. Results are expected to generate a general picture of acephate resistance in TPB and help explain how TPB evolves resistance to the chemical. In addition, this study provides insight into potential fitness costs associated with acephate resistance and possible development of multiple and cross resistance to other classes of insecticides.

Materials and Methods

Chemicals

The Pierce Coomassie plus protein assay kit (23238) was purchased from ThermoFisher Sci. (Pittsburgh, PA, USA). Alpha-naphthyl acetate (1-NA or α-NA) (N8505), beta-naphthyl acetate (2-NA or β-NA) (N8675), p-nitrophenyl acetate (PNPA) (N8130), 1-chloro-2,4-dinitrobenzene (CDNB) (23,732-9), fast blue salt (D9805), L-glutathione (GSH) reduced (G6529) were purchased from Sigma Chemical Co. (St. Louis, MO).

Insect laboratory colony and field collection

A laboratory colony was provided by Kathy Knighten and Fred Musser at Mississippi State University. The colony has been maintained on artificial diet for seven years without exposure to insecticide. Wild adults are introduced into this colony in the spring of each year. This colony was used as a standard susceptible strain (LLS). Resistance ratios (LC50 of field-collected population/LC50 of laboratory strain) were calculated for field and laboratory strains relative to LLS. A field population (Lula) was collected in October 2010 from pigweed around a cotton field west of Lula, Stoneville, MS. Nine individual tarnished plant bugs from each sample were briefly homogenized in sodium phosphate buffer. The homogenate was centrifuged at 10,000×g for 5 minutes at 4°C. Protein concentrations were determined using the Pierce protein assay kit which utilizes the Bradford method [11]. To determine esterase activity, micro-titer plate assays were conducted using α-NA, β-NA, and PNPA as substrates. A Bio-Tek ELx808ii plate reader (Winooski, VT) was used to monitor α-NA and β-NA reactions at 450 nm for 10 minutes with measurements taken every 15 seconds [12]. For assays with PNPA, reactions were assayed at 405 nm for 10 minutes with readings taken every 15 seconds [13]. To determine glutathione S-transferase activities, micro-titer plate assays were conducted using CDNB as substrate. The reactions were monitored at 340 nm for 10 minutes with readings taken every 15 seconds [12].

Enzyme activity assay

Esterase and glutathione S-transferase activities were comparatively examined using the protocols described by Zhu et al. [6]. In brief, nine individual tarnished plant bugs from each sample were homogenized in sodium phosphate buffer. The homogenate was centrifuged at 10,000×g for 5 minutes at 4°C. Protein concentrations were determined using the Pierce protein assay kit which utilizes the Bradford method [11]. To determine esterase activity, micro-titer plate assays were conducted using α-NA, β-NA, and PNPA as substrates. A Bio-Tek ELx808ii plate reader (Winooski, VT) was used to monitor α-NA and β-NA reactions at 450 nm for 10 minutes with measurements taken every 15 seconds [12]. For assays with PNPA, reactions were assayed at 405 nm for 10 minutes with readings taken every 15 seconds [13]. To determine glutathione S-transferase activities, micro-titer plate assays were conducted using CDNB as substrate. The reactions were monitored at 340 nm for 10 minutes with readings taken every 15 seconds [12].

RNA preparation, cDNA library construction and sequencing

Three adults (per tube) of L. lineolaris were homogenized in 1000 mL TriZol reagent (Invitrogen, Carlsbad, CA). Three to five replicates were included for each sample. mRNA was purified from total RNA using NucleoTrap mRNA purification kit (BD Bioscience Clontech, Palo Alto, CA). The Creator Smart cDNA Library Construction Kit (BD Bioscience Clontech) was used for cDNA library construction, by following manufacturer’s instructions and modified protocols described by Zhu et al. [14]. Approximately 1 µg mRNA was used for reverse transcription and cDNA library construction. cDNA was ligated into pDNR-LIB vector (Clontech). The ligation was used to transform TOP10 competent cells (Invitrogen), which then were plated on chloramphenicol-agar plates. Approximately 30,000 clones were obtained and sequenced with a M13 forward primer on an ABI 3730XL sequencer (Applied Biosystems Inc., Foster City, CA) located at the Genomics and Bioinformatics Research Unit, USDA-ARS, in Stoneville, MS.

Sequence data processing and expression gene chips

After vector trimming and assembling using DNASTar (Ver. 8, Madison, WI), sequences were subjected to a similarity search for putative identity against protein and nucleotide databases of the GenBank in the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using Blastx NR, Blastn, and tBlastX protocols of Blast2GO software (http://www.blast2go.org/) [15,16] with 10-3 for cutoff E-value.

Acquiring microarray data

Roche NimbleGen 72 K gene expression chips in 4-plex format (Roche NimbleGen, Inc., Madison, WI) were used to compare global gene expression between the acephate-selected (LLR) and non-selected (LLS) strains of TPB. A 60-bp specific oligonucleotide was designed and synthesized as a probe. Approximately 35,000

Gene Regulation in Acephate-Resistant TPB
probes (average of 5 probes per cDNA) were synthesized and printed on each gene expression chip. Microarray analysis was processed using standard NimbleGen array protocols. Total RNA was extracted from adults using TriZol reagent (Invitrogen). Double-strand cDNAs were synthesized by using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) according to the manufacturer’s protocols. Double-strand cDNA samples were labeled with One-color DNA Labeling Kit and hybridized to the microarray chips. Microarray data were acquired according to NimbleScan v.2.5 User’s Guide through Florida State University Microarray processing facility. Four arrays (4 replicates) of 72 K NimbleGen expression chips were processed for each sample.

Analysis of microarray data
After gene expression data were obtained from 4 × 72 K array processing, ArrayStar® software (DNAStar, Inc., Madison, WI) was used to analyze and compare microarray data between LLS and LLR. Expression data were log2-transformed and normalized through quantile normalization [17], and gene calls were generated using a Robust Multichip Average (RMA) algorithm [18]. Normalized data were analyzed using classical parametric statistics. P-values were calculated using Modified t-test. Clustering techniques, including the most popular “hierarchical” clustering and “k-means” clustering, were applied in the microarray data analysis. Clustering aims at dividing data points (gene or samples) into groups (clusters) using measures of similarity, such as correlation or Eucliden distance [19]. Hierarchical clustering creates a hierarchical, tree-like structure of data, and may be displayed using a “heat map”. By using hierarchical clustering in this study, the expression levels of each gene could be quantitatively compared side by side between LLS and LLR strains. Scatter plotting was also applied to generate a distribution of more than 6,688 genes tested in an effort to locate differentially expressed genes between LLS and LLR. A fold-change cutoff of 2 and p-value threshold of 0.05 were used to determine differential gene expression (www.illumina.com/documents/products/ whitepapers/whitepaper_RNASeq_to_arrays_comparision.pdf).

Cloning full-length cDNAs coding for esterases
cDNA library sequencing yielded several clones which matched esterase cDNAs in GenBank. Cloning of full-length cDNAs was achieved using procedures similar to those of Zhu et al. [7] with some modifications as described by Yang et al. [20]. Briefly, to obtain the full length cDNAs, total RNA was extracted from adults. The SuperScript First Strand cDNA Synthesis kit (Invitrogen) was used in a reverse transcriptase polymerase chain reaction (RT-PCR) with 5 μg of total RNA and an oligo-dT primer for cDNA synthesis. Forward primers were designed and used with oligo-dT primer in RT-PCR reaction to amplify 5′-end of the esterase cDNA. The 5′-end of cDNA for each of the esterases was obtained by using the 5′ rapid amplification of cDNA end (5′ RACE) system (Invitrogen). Two to three specific reverse primers for each of the three esterases were designed and used in semi-nested amplifications with a forward abridged anchor primer from 5′-RACE kit. The 5′-end of the cDNA was isolated and c-tailed, and then cloned into a pGEM-T vector (Promega). Plasmid DNAs were prepared and sequenced using an ABI 3730XL DNA analyzer to confirm full coding sequences of the esterases. To obtain error-proof full-length cDNAs, total RNAs from LLS and LLR were used for synthesizing RT-cDNA. RT–PCR amplification was repeated with a Platinum High Fidelity Taq DNA polymerase (Invitrogen). The PCR products were purified using Qiaquick PCR purification kit (Qiagen) and sequenced from both directions as described above.

Verification of esterase gene expressions using real-time PCR (qRT–PCR)
Differential expression of 4 up-regulated esterase genes (LLE1–4), detected by the microarray analysis, were validated by qRT–PCR according to methods described by Yang et al. [20]. TPB adults were collected in October 2011 from Lula, Mississippi, the same location of the original LLR colony. Collected bugs were divided into two groups. One group of bugs were treated with 1,000 mg/L acephate and maintained on treated green bean (21°C, L:D = 14:10) for 7 days (Lala1000). Another group of the bugs were untreated and maintained under the same conditions for 7 days as an aging control (Lala CK). LLS (untreated) was also included as a control.

The qRT–PCR assays were performed in a 25 μl reaction volume using iScript™ One-step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) in a thermal cycler PTC-200 with Chromo4 detector attached (Bio-Rad). The qRT–PCR thermal cycling profile was programmed to run at 50°C for 10 min of cDNA synthesis, 95°C for 5 min of iScript reverse transcriptase deactivation, followed by 40 cycles of 95°C for 10 s and 55°C for 30 s. A melting curve thermal cycling from 55°C to 95°C with an increment of 1°C for 10 s was added to check amplification specificity. Opticon Monitor 3 (Bio-Rad, Hercules, CA) was used to control all PCR reactions and data output. To obtain absolute quantities of each gene, two steps of qRT–PCR [20] were performed for each esterase gene. First qRT–PCR was conducted to amplify ribosomal 18S gene to estimate RNA concentration for each sample with 18S housekeeper gene as an internal standard. RNA samples were adjusted to the same concentration based on the first qRT–PCR with 18S as the internal standard. Second qRT–PCR was conducted to achieve an absolute estimation using standardized RNA samples and target gene (LLE1–4) cDNA as an internal standard. Three replications (samples) were used for each treatment, and 5 TPB adults were included in each RNA preparation. Gene expression quantity was automatically calcu-

**Table 1.** Biological response and enzymatic activities in acephate-treated tarnished plant bug.

| Insect | Bioassay | Esterase: α-NA | Esterase: β-NA | Esterase: PNPA | GST: CDNB |
|-------|---------|----------------|---------------|--------------|-----------|
|       | LC50 a. l. (95%FL) | Ratio | MV±SE | Ratio | MV±SE | Ratio | MV±SE | Ratio |
| LLS   | 148.3(122.9–174.8) | 1 | 21.6±2.2 | 1 | 19.4±1.5 | 1 | 29.7±3.5 | 1 | 39.2±1.6 | 1 |
| Lula* | 321.1(257.8–402.5) | 2.2 | 54.3±6.3 | 2.5 | 27.3±2.4 | 1.4 | 39.2±3.9 | 1.3 | 27.7±1.9 | 0.7 |
| Lula600** | 874.4(742.7–1042) | 5.9 | 97.7±14.3 | 4.5 | 55.8±9.1 | 2.9 | 81.7±8.2 | 2.8 | 35.9±3.1 | 0.9 |

*Field population collected in Lula, Mississippi.
**Field population collected in Lula, Mississippi and treated with 600 mg/L of acephate (90WP) before it was used for assays.

doi:10.1371/journal.pone.0037586.t001
Table 2. Identification of 107 significantly up-regulated (≥2-fold) genes in LLR using microarrays and analyzed with ArrayStar and Blat2go protocol (www.blast2go.org).

| Seq ID | Sequence annotation | Fold change | P value |
|--------|---------------------|-------------|---------|
| L1103  | 26s ribosomal rna boehmeria macrophylla scabrella | 2.02 | 0.0245 |
| L6649  | acid phosphatase-1 | 2.90 | 0.0000845 |
| L869   | aggrecan core | 2.09 | 0.000243 |
| L3143  | aldo-keto reductase | 3.45 | 0.000687 |
| L2988  | alpha-amylase | 2.58 | 3.24 × 10⁻⁶ |
| L542   | alpha-amylase | 2.44 | 0.000186 |
| L3683  | alpha-amylase 1 | 3.83 | 1.40 × 10⁻⁶ |
| L4739  | alpha-amylase 1 | 3.70 | 3.07 × 10⁻⁶ |
| L6184  | alpha-amylase 1 | 2.36 | 5.31 × 10⁻⁶ |
| L881   | antigen 5 precursor | 2.10 | 0.00124 |
| L6486  | antigen 5 scp domain-containing | 2.59 | 7.90 × 10⁻⁶ |
| L186   | antigen-5-like protein precursor | 2.42 | 0.00155 |
| L4347  | carbonyl reductase | 4.04 | 0.0000102 |
| L5131  | carbonyl reductase | 5.17 | 3.16 × 10⁻⁶ |
| L5648  | carbonyl reductase | 2.68 | 0.0000357 |
| L305   | carboxypeptidase b | 2.31 | 0.000215 |
| L1451  | carboxypeptidase b-like | 2.34 | 0.000213 |
| LL_390 | carboxypeptidase cpvl precursor | 3.12 | 7.07 × 10⁻⁶ |
| L2051  | cell surface sd repeat protein precursor | 254.87 | 0.0000377 |
| L4783  | chemosensory protein a 7a | 2.34 | 0.000104 |
| L2075  | circumsporozoite protein | 2.06 | 0.000731 |
| L861   | circumsporozoite protein | 2.50 | 0.000165 |
| L5167  | counting factor associated protein d-like | 2.46 | 0.0000303 |
| L6543  | cuticular protein lcp family 2 mna nasonia vitripennis | 3.19 | 0.00265 |
| L194   | cysteine proteinase 2-like digestive enzyme | 2.75 | 0.000159 |
| L3359  | cytochrome p450 | 3.00 | 0.00168 |
| LL_39  | cytochrome p450 | 2.81 | 0.000373 |
| L4510  | cytochrome p450 6a8 | 2.70 | 0.000229 |
| L2414  | elongation of long chain fatty acids protein aae008004 | 2.58 | 0.000146 |
| LL_214 | enth domain-containing protein | 3.33 | 0.000125 |
| L1233  | esterase [Lygus lineolaris]-1 | 2.60 | 0.000102 |
| L2508  | esterase-2 | 2.14 | 0.00018 |
| L2520  | esterase [Lygus lineolaris]-3 | 2.57 | 6.67 × 10⁻⁶ |
| L5104  | esterase-4 | 7.57 | 5.76 × 10⁻⁷ |
| LL_227 | carboxylesterase-6 | 2.70 | 0.000343 |
| L6522  | esterase fe4-7 | 2.17 | 0.000128 |
| L1833  | GK17235 [Drosophila willistoni] | 7.23 | 5.14 × 10⁻⁶ |
| L4183  | GK17235 [Drosophila willistoni] | 8.69 | 0.000104 |
| L6147  | glutathione s-transferase | 2.14 | 0.000217 |
| L5529  | glycoside hydrolases | 3.03 | 3.03 × 10⁻⁶ |
| L2609  | hemagglutinin family protein | 2.79 | 0.0645 |
| L3629  | hypothetical protein | 16.29 | 0.000112 |
| L216   | hypothetical protein LOC100678289 (N. vitripennis) | 2.11 | 0.00078 |
| L5474  | hypothetical protein LOC100679379 [N. vitripennis] | 3.03 | 8.32 × 10⁻⁶ |
| LL_225 | hypothetical protein LOC100679379 [N. vitripennis] | 2.39 | 0.00242 |
| L1323  | immunodominant interspersed repeat antigen | 2.22 | 0.000199 |
| LL_322 | immunodominant interspersed repeat antigen | 2.53 | 0.000425 |
| L5610  | isoform a | 2.16 | 0.000488 |
| Seq ID  | Sequence annotation                                      | Fold change | P value    |
|--------|----------------------------------------------------------|-------------|------------|
| L2469  | isoform b                                                | 2.46        | 0.0000358  |
| L501   | kynurenine-oxoglutarate transaminase 3-like              | 2.08        | 9.31 × 10^-6 |
| LL_632 | liprin-beta-2- partial                                    | 2.72        | 0.0000129  |
| L2869  | luciferin-regenerating enzyme                             | 2.26        | 0.0000277  |
| L4778  | microsatellite II-5 sequence apolys g lucorum           | 2.14        | 0.0012     |
| L1939  | mitochondrial import receptor subunit tom20 homolog      | 2.05        | 0.0122     |
| LL_248 | mus musculus bac clone rp24-358i7 from chromosome       | 2.25        | 0.0000311  |
| L3451  | mus musculus chromosome clone rp23-                      | 3.54        | 2.74 × 10^-6 |
| L4498  | mus musculus chromosome clone rp23-                      | 2.81        | 0.000247   |
| L1449  | mus musculus chromosome clone rp23-                      | 2.26        | 0.00342    |
| LL_135 | mycobacterium complete genome                            | 2.18        | 0.00135    |
| L753   | neuroparsin 1 precursor                                   | 3.87        | 3.87 × 10^-6 |
| L4114  | nocardia farcinica ifm 10152 complete genome             | 2.32        | 0.00041    |
| LL_574 | nucleolar protein family 2 (h aca small nucleolar mps)   | 2.40        | 0.000937   |
| L2338  | omega-amidase nitz2-like                                  | 42.92       | 9.60 × 10^-7 |
| L4563  | pancreatic lipase-related protein 2-like                 | 2.40        | 0.0000264  |
| L2487  | pancreatic triacylglycerol lipase                        | 3.38        | 0.000234   |
| L380   | partial genome (lygus lineolaris)                        | 2.54        | 0.386      |
| L964   | partial genome (lygus lineolaris)                        | 2.15        | 0.527      |
| L4630  | peptidyl-prolyl cis-trans isomerase 10                    | 2.80        | 4.80 × 10^-6 |
| L5883  | phospholipid scramblase 1                                | 2.06        | 0.00206    |
| L4906  | plasmid falciparum 3d7 chromosome 8                       | 4.07        | 0.00218    |
| L1904  | endo-polygalacturonase b                                 | 2.16        | 0.000925   |
| L1124  | polygalacturonase 4                                      | 2.27        | 3.32 × 10^-6 |
| L6079  | polygalacturonase                                        | 3.65        | 8.55 × 10^-6 |
| L3578  | endopolygalacturonase                                    | 5.61        | 0.000182   |
| L3333  | polygalacturonase                                        | 4.11        | 0.000803   |
| L518   | polygalacturonase pg1                                    | 2.02        | 0.000231   |
| L4832  | endopolygalacturonase                                    | 13.92       | 8.72 × 10^-6 |
| L2316  | endo-polygalacturonase                                   | 2.64        | 0.000782   |
| L4070  | predicted protein [Nematostella vectensis]               | 2.17        | 0.000601   |
| L1882  | Predicted: C09D4.2 [Hydra magnipapillata]               | 2.32        | 0.000533   |
| L600   | Predicted: ENSANGP00000004103 [S. purpuratus]            | 3.11        | 0.000214   |
| L28    | protein CLONEX_01831 ZP_03289628                         | 2.40        | 0.434      |
| L6646  | pyridoxamine 5'-phosphate oxidase                        | 2.08        | 0.00456    |
| L2427  | regucalcin                                              | 2.26        | 0.000378   |
| L2931  | regucalcin                                              | 2.21        | 0.000119   |
| L4962  | secreted salivary gland                                  | 3.80        | 0.000262   |
| L5724  | serine 3-dehydrogenase                                   | 2.13        | 0.000213   |
| LL_55  | serine protease                                         | 36.71       | 0.000308   |
| L4164  | serine protease inhibitor serpin-4                       | 2.99        | 0.000112   |
| L3882  | serine proteinase stubble                                | 3.92        | 0.000638   |
| L5938  | sp185 333                                               | 2.28        | 0.000225   |
| L5880  | spore coat assembly protein                              | 3.37        | 0.000209   |
| LL_773 | spore coat assembly protein exsa                         | 2.92        | 6.57 × 10^-6 |
| L1658  | st14a protein                                           | 2.29        | 0.0645     |
| L5575  | tetraodon nigroviridis full-length cdna                  | 2.10        | 0.0032     |
| L5521  | thaumatin-like protein                                   | 2.31        | 0.000111   |
| L6464  | thaumatin-like protein                                   | 2.01        | 0.000163   |
than the LC50 in Table 1, because both bugs and food were sprayed with spray distance of 30.5 cm to ensure a uniform deposition of insecticide mist on inner surface of the container, green beans, and TPBs were collected in September 2011 from northwest Mississippi. Approximately 25 bugs were used for each replicate and 3 replicates were used for each population. Bugs along with 4 green bean pods were placed in a plastic container (D×H: 10.5×7 cm). A 9-cm hole was cut on lid and covered with a fine net cloth (10 grids/cm). Five hundred μl of insecticide solution was delivered into the container using a modified Potter Spray Tower. The sprayer was set at 7.5 psi and dissolving inhibitor in ethanol, thereafter, an equal volume of dd-H2O was added to the solution. The bugs were sprayed with inhibitor solution one hour before they treated with acephate. The bugs in control were treated with 50% ethanol in water. Spray tower settings and spray volume were the same as described above. Mortality was recorded 48 h after treatment.

Examination of synergistic effect of esterase inhibitors on acephate toxicity against TPB

A field population was collected in April 2012 from Leland, MS, and treated with 72 mg a.i./L acephate. A laboratory colony (LLX) was collected in 2010 from many locations in Delta regions of Mississippi and Arkansas, and was selected with 225 mg/L acephate. In this experiment, LLX colony was treated with 144 mg/L acephate. DEF or TPP solution at 1% was prepared by dissolving inhibitor in ethanol, thereafter, an equal volume of dd-H2O was added to the solution. The bugs were sprayed with inhibitor solution one hour before they treated with acephate. The bugs in control were treated with 50% ethanol in water. Spray tower settings and spray volume were the same as described above. Mortality was recorded 48 h after treatment.

Results

Comparison of acephate-susceptibility and enzyme activities

The acephate LC50 for the susceptible strain (LLS) was 148.3 mg a.i./L (Table 1). The LC50 for the Lula field population was 2.2-fold higher than LLS. After selection with 600 mg/L Orthene 90WP, the observed LC50 reached 5.9-fold higher than LLS and 2.7-fold higher than the unslected Lula field population. Esterase activity determined with β-NA was 2.5-fold higher in Lula field population and 4.5-fold higher in the acephate selected bugs (Lula600) than LLS. The B-NA and PNPA activities also significantly increased by 1.3–1.4-fold in Lula, and by 2.8- to 2.9-fold in Lula600, respectively (Table 1). Unlike the esterases, glutathione S-transferase activity in both Lula and Lula600 decreased by 1.4- and 1.1-fold (Table 1).

Microarray analysis of gene regulation in LLR

Identification of differentially expressed genes in acephate-resistant bug (LLR).

A total of 7,446 unique contigs and singletons were obtained from cDNA library sequencing, and 6,688 genes had valid expression values from microarray analysis. Hybridization signals were analyzed and gene expression changes in LLR were presented as mean of fold changes when compared to the LLS. Of 6,688 genes examined in the LLS and LLR strains, approximately 9.9% genes were up- or down-regulated in the LLR. Expressions of the remaining 90% of genes were not significantly different between LLS and LLR. Based on P values (P<0.05) and fold change (≥2), significant differences in mRNA levels were detected in 662 genes between the LLS and LLR, which included 329 up-regulated and 333 down-regulated genes in LLR. Among the 662 differentially expressed genes, only 225 genes were putatively identified using Blast2go search of GenBank, including 107 up-regulated (Table 2) and 118 down-regulated (Table 3) genes in the LLR. Identities of the remaining 437 genes have not been determined in similarity search of GenBank using Blast2go.

Comparison of gene expression levels by hierarchical clustering.

The 329 up-regulated genes and 333 down-regulated genes (identified with microarray and Blast2go similarity search) in the LLR strain, selected based on the P value (P<0.05) and fold change (≥2), were analyzed by using hierarchical clustering analysis and plotted as a heat map with the ArrayStar software (Fig. 1A). Each column represented a sample (LLS or LLR) and each row represented a gene. The gray scale depicted relative levels of gene expressions from low to high with corresponding grayscale from light (for lower expression) to dark (for higher levels). The gene clustering at the left of the heatmap indicated the presence of three groups of genes with distinct expression profiles of 662 genes (≥2-fold change) across samples. Group 1 (G1) (413 genes) was separated into subgroups of G1a

| Seq ID | Sequence annotation | Fold change | P value |
|-------|---------------------|-------------|---------|
| L3539 | transmembrane protease | 30.46 | 0.000386 |
| L2022 | trypsin: salivary | 3.52 | 2.19×10^-6 |
| L3508 | venom serine carboxypeptidase-like | 5.36 | 2.48×10^-6 |
| L4429 | venom serine carboxypeptidase-like | 2.14 | 0.000131 |
| L2932 | ves g 5 allergen | 2.39 | 0.000061 |
| L4213 | ves g 5 allergen | 2.85 | 0.000014 |
| L_466 | ves g 5 allergen | 2.24 | 0.000212 |
| L868 | vitellogenic carboxypeptidase | 2.26 | 0.000508 |
| L2069 | xenopus tropicalis clone ch216- complete sequence | 3.38 | 0.0153 |
| L4505 | zinc metalloproteasen c | 2.98 | 0.0000355 |

DOI:10.1371/journal.pone.0037586.t002

Table 2. Cont.
Table 3. Identification of 118 significantly down-regulated gene ($\geq$2-fold) in LLR using microarrays and analyzed with ArrayStar and Blat2go protocol (www.blast2go.org).

| Seq ID  | Sequence annotation                                | Fold change | P value   |
|---------|-----------------------------------------------------|-------------|-----------|
| L2460   | acyrthosiphon pisum protein takeout-like mmna       | 2.04        | 0.00091   |
| L4322   | alkylated dna repair protein alkb-like protein 8    | 3.38        | 0.000356  |
| LL_476  | apolygus lucorum microsatellite II-5 sequence       | 2.30        | 0.00369   |
| L6435   | atp-binding cassette superfamily                    | 4.49        | 0.00267   |
| L6338   | ax4 myb domain-containing protein cds               | 4.00        | 0.0000124 |
| L4040   | brachyspira intermedia pws complete genome          | 2.18        | 0.0116    |
| L2273   | callithrix jacchus sonic hedgehog mmna              | 3.06        | 0.00976   |
| L5702   | candidatus pelagibacter complete genome             | 2.02        | 0.0184    |
| L1023   | cathepsin a                                         | 3.46        | 3.07 x 10^-6 |
| L3275   | cathepsin a                                         | 2.35        | 3.04 x 10^-6 |
| L3761   | cathepsin a                                         | 2.61        | 0.0000398 |
| L4973   | cathepsin a                                         | 2.82        | 0.0000325 |
| L6192   | cathepsin a                                         | 2.72        | 3.49 x 10^-6 |
| L6305   | cathepsin b precursor                               | 2.32        | 0.000194  |
| L6308   | cathepsin d                                         | 2.48        | 0.000595  |
| L6180   | cathepsin l                                         | 15.51       | 4.35 x 10^-7 |
| LL_76   | cathepsin l                                         | 2.18        | 6.58 x 10^-6 |
| L3298   | cathepsin l precursor (major excreted protein) mmna | 2.57        | 0.0000781 |
| L4110   | cathepsin r-like                                    | 2.15        | 0.000068  |
| L496    | receptor for egg jelly 2 protein precursor          | 17.67       | 6.25 x 10^-7 |
| L2462   | hypothetical protein                                | 71.20       | 2.69 x 10^-6 |
| L2548   | chromosome 3 clone RP11-556G18 map 3p (H. sapiens)  | 8.81        | 4.11 x 10^-7 |
| L5687   | chromosome clone rp11- (homo sapiens)               | 4.62        | 0.0000177 |
| L1965   | chromosome clone rp23- (mus musculus)               | 2.07        | 0.000113  |
| L3117   | chromosome clone rp23- (mus musculus)               | 100.39      | 0.0000318 |
| L5568   | chromosome clone rp23- (mus musculus)               | 15.46       | 5.17 x 10^-6 |
| L1035   | conserved protein                                   | 38.71       | 1.60 x 10^-7 |
| L5626   | conserved protein                                   | 29.51       | 6.99 x 10^-7 |
| LL_395  | conserved protein                                   | 48.71       | 6.21 x 10^-7 |
| L3444   | counting factor associated protein d-like           | 2.49        | 0.0000287 |
| L2459   | cuticle protein 6                                   | 2.08        | 0.0244    |
| L787    | cuticular protein 62bc                              | 4.62        | 0.00108   |
| L3905   | cysteine protease cp5                               | 2.60        | 6.57 x 10^-6 |
| L561    | cytoplasmic polyadenylation element-binding protein 2 | 27.52      | 1.72 x 10^-6 |
| LL_46   | cytoplasmic polyadenylation element-binding protein 2 | 28.05    | 1.06 x 10^-6 |
| L583    | defensin a                                          | 4.22        | 1.08 x 10^-6 |
| L562    | drosophila erecta gg13471 (dere'gg13471) mmna       | 2.56        | 0.00976   |
| L1412   | drosophila mojavensis gi24712 (dmo'gi24712) mmna     | 12.38       | 1.66 x 10^-7 |
| L1974   | drosophila mojavensis gi24712 (dmo'gi24712) mmna     | 16.90       | 5.64 x 10^-7 |
| L5888   | eggshell RP45 [Rhnodius prolixus]                   | 19.78       | 1.89 x 10^-7 |
| L930    | eggshell rhodnius prolixus rp45 partial cds         | 7.56        | 1.60 x 10^-7 |
| L4775   | eggshell RP45 [Rhnodius prolixus]                   | 147.45      | 0.0000252 |
| L3689   | endopolygalacturonase                               | 5.88        | 5.21 x 10^-6 |
| L410    | eukaryotic aspartyl protease family protein         | 2.66        | 0.000479  |
| L4254   | follicle cell protein 3c-1                          | 10.72       | 0.000177  |
| LL_358  | foot protein 1 variant 4                            | 7.11        | 1.86 x 10^-7 |
| L755    | ga24146 (dpse#ga24146) mmna                         | 25.37       | 5.29 x 10^-6 |
| L6045   | gametocyte-specific factor 1                        | 2.26        | 0.00597   |
### Table 3. Cont.

| Seq ID | Sequence annotation | Fold change | P value |
|--------|---------------------|-------------|---------|
| LL_304 | gamma-interferon-inducible lysosomal thiol reductase | 5.41 | 0.000419 |
| L4147 | genomic chromosome clone: (lotus japonicus) | 19.29 | 0.000114 |
| L552 | granzyme h-like | 3.62 | 0.0062 |
| L1938 | gryllus bimaculatus gbcontig30355 | 5.31 | 0.000247 |
| L6235 | gtp-binding protein alpha gna | 2.57 | 0.00257 |
| LL_573 | heinz 1706 chromosome 1 clone hba-57j16 map sequence | 4.13 | 0.000213 |
| L4603 | histone h1 | 2.42 | 0.000541 |
| L369 | homeobox protein pknox2 isoform 2 | 2.27 | 0.00135 |
| L5329 | hypothetical protein [C. tropicalis MYA-3404] | 2.02 | 0.00295 |
| L5570 | isoform a | 2.07 | 0.000726 |
| L1141 | keratin-associated protein 10-4-like mmn | 5.33 | 3.00 × 10⁻⁶ |
| L5833 | laminin subunit alpha | 2.08 | 0.000552 |
| L1954 | lipase 1 precursor | 2.48 | 0.000117 |
| L6274 | lipase 3 | 3.10 | 0.00138 |
| L2994 | lysosomal acid | 2.81 | 0.000292 |
| L3946 | merozoite surface protein | 2.50 | 0.041 |
| L3911 | mitotic spindle assembly checkpoint protein mad2 | 2.07 | 0.00304 |
| L1931 | multidrug resistance protein 1 | 2.15 | 0.000514 |
| L261 | nasonia vitripennis protein piwi-like mmn | 2.32 | 0.00215 |
| L6396 | nematostella vectensis protein partial mmn | 7.02 | 3.24 × 10⁻⁷ |
| L3125 | odorant-binding protein 5 | 2.07 | 0.259 |
| L3878 | oryza sativa japonica group os04g0107700 complete cds | 2.49 | 4.64 × 10⁻⁶ |
| L3316 | ov9090 dna polymerase delta subunit 3 complete cds | 4.22 | 0.000434 |
| LL_617 | pao retrotransposon peptidase family protein | 2.09 | 0.000151 |
| LL_645 | pediculus humanus corporis protein takeout mmn | 2.87 | 0.000225 |
| L4935 | peptidase c1a papain | 5.77 | 6.36 × 10⁻⁷ |
| L871 | peptide-n4-(n-acetyl-[d-glucosaminyl]-asparaginase amidase | 65.26 | 0.000227 |
| L1761 | predicted protein [Nematostella vectensis] | 4.62 | 0.00192 |
| L3170 | prolaxxin antimicrobial peptide | 3.28 | 1.50 × 10⁻⁶ |
| L1206 | pseudonocardia dioxanivorans complete genome | 3.44 | 3.54 × 10⁻⁶ |
| L5409 | pseudonocardia dioxanivorans complete genome | 3.57 | 3.63 × 10⁻⁶ |
| L6148 | pseudonocardia dioxanivorans complete genome | 3.82 | 3.04 × 10⁻⁶ |
| LL_744 | reverse ribonuclease integrase | 2.12 | 0.000185 |
| L2434 | ribosomal protein l27e | 2.18 | 0.000763 |
| L6457 | schistosoma mansoni strain puerto rico chromosome | 2.68 | 0.00563 |
| L4730 | secreted salivary gland | 2.80 | 4.17 × 10⁻⁶ |
| LL_19 | secreted salivary gland mmn (ixodes scapularis) | 33.51 | 0.000169 |
| LL_20 | secreted salivary gland mmn (ixodes scapularis) | 27.27 | 6.85 × 10⁻⁷ |
| L605 | seminal fluid protein hacp037 | 2.01 | 0.0985 |
| LL_54 | seminal fluid protein hacp037 | 2.18 | 0.00268 |
| L3098 | serine carboxypeptidase-like enzyme | 11.01 | 0.000138 |
| L1017 | serine protease | 34.41 | 6.20 × 10⁻⁷ |
| L5977 | serine protease | 146.15 | 0.000199 |
| L6207 | serine protease | 10.29 | 0.000794 |
| LL_104 | serine protease | 3.44 | 7.25 × 10⁻⁶ |
| LL_119 | serine protease | 30.08 | 6.11 × 10⁻⁷ |
| LL_672 | serine protease | 2.42 | 7.21 × 10⁻⁶ |
| L3307 | serine protease [Creontiades dilutus] | 2.12 | 0.000554 |
and G1b. All genes in G1a (23 genes) were down-regulated in LLR strain. G1b was separated into G1b1 (202 genes) and G1b2 (188 genes). The genes in G1b1 appeared to be up-regulated, while the genes in G1b2 were down-regulated in LLR (Fig. 1A). Group 2 had subgroups G2a (122 genes) and G2b (125 genes). Genes in G2a appeared to be down-regulated. Genes in G2b were up-regulated in LLR. Group 3 (G3) was a small group (2 genes), and the genes in G3 were highly up-regulated in the LLR strain.

### Scattered-plot comparison of gene expression levels between LLS and LLR

A total of 6,688 valid gene expression data (log2) points (dots) of LLR were pairwise plotted against the same genes of LLS (Fig. 1B). The two sets of gene expression data showed linear correlation with R² 0.89. When the Student’s t-test was selected, there were 1,638 and 2,534 genes that showed significantly different between LLS and LLR at 99% and 95% confidence interval levels, respectively. Dots of the scatter plot in the upper left corner represented up-regulated genes, and dots in low right corner represented down-regulated genes (Fig. 1B). Distance between a dot and the regression line (R) indicated a gene expression level with longer distance for higher up- or down-regulation. A total of 662 genes (dots above line 2a and below line 2b) showed 2-fold changes, 191 genes (dots above line 4a and below line 4b) showed 4-fold changes, and 113 genes (dots above line 8a and below line 8b) exhibited 8-fold changes (Fig. 1B) between LLS and LLR.

### Annotation and functional analysis of up-and down-regulated genes

Three hundred and twenty-nine up-regulated and 333 down-regulated (≥2-fold) gene cDNAs were subjected separately to blast2go mapping and annotation to determine each putative protein’s role in biological process and molecular function. Annotation with blast2go showed that 66 genes of the 662 differentially expressed genes were involved in biological processes at GO level 2, including 41 up-regulated and 25 down-regulated genes in LLR. Among the 41 up-regulated genes, 18 (22.7% of the 66 biological process-related genes) genes were associated with metabolic processes (Fig. 2A), while only 3 of the 25 (7.6% of the 66 genes) down-regulated genes were associated with the same function of metabolic process (Fig. 2A). Among the 662 differentially expressed genes, 76 genes were involved in molecular functions, including 49 up-regulated and 27 down-regulated genes at GO level 2 in LLR. Among the 49 up-regulated genes, 37 (48.7% of 76 molecular function-related genes) genes were associated with catalytic activities (Fig. 2B), while only 17 of the 27 (22.4% of the 76 genes) down-regulated genes were associated with catalytic activities. Results indicated that more than 3.6-fold of the metabolic-related genes were up-regulated compared to those down-regulated genes with the same biological function. The results also indicated a substantial increase of metabolic activities in LLR. Similarly, analysis of molecular function revealed a large portion of catalytic-related genes with significant up-regulations, suggesting an increase of catalytic activities in LLR compared to LLS.

### Potential association of up-regulated genes with altered acephate-resistance in *L. lineolaris*

Up-regulation of esterase genes and metabolic resistance development. Increase of esterase activities in LLR prompted further examination and comparison of esterase gene expressions and cDNA sequences between LLS and LLR. Six esterase genes in LLR showed significantly higher gene expression levels (2.14–7.57-fold increase) than those in LLS (Table 2). No esterase genes were down-regulated in LLR (Table 3). By using cDNA library
sequencing and RT-PCR, two different full length esterase cDNAs were obtained from both LLS (designated as LLSE1 and LLSE4) and LLR (designated as LLRE1 and LLRE4). LLRE1 represented the most abundant esterase gene transcripts of 4 highly similar variants (data not shown) among the 6 up-regulated esterase genes in LLR. The others had one or two variants. The LLSE1 showed 2.6-fold increase of the esterase gene expressions in LLLR. LLSE4 showed the highest increase of esterase gene expressions (7.57-fold) in LLLR. Sequence alignment of deduced proteins, including a previously published esterase AAT09370 [7], revealed three conserved catalytic center residues, S313, E332, and H164 (Fig. 3). Searching with InterPro Scan and MyHits (http://www.expasy.ch/tools/) indicated that all putative proteins belonged to type-B carboxylesterase with conserved sequence pattern (F-[GR]-G-x(4)-[LIVM]-x-[LIV]-x-G-x-[STAG]-G) around serine active site at positions 200–215 (Fig. 3). The 1171-bp LLSE1 and LLRE1 had 9 nucleotide substitutions, resulting in 5 amino acid differences in the deduced 570-residue proteins between LLS and LLR. The second 1801-bp esterase cDNA, LLSE4 from LLS and LLRE4 from LLR, encoded 546-residue protein. There were 11 nucleotide and 4 amino acid substitutions between LLSE4 and LLRE4. Pair-wise sequence alignment showed that esterase sequence identities were approximately 99.1%, 52%, and 52% between AAT09370 and LLSE1, AAT09370 and LLRE4, and LLSE1 and LLRE4, respectively. The LLSE1 is very similar to AAT09370, but they differ at 8 amino acid positions (Fig. 3).

Validation of up-regulated esterase genes using real-time PCR (qRT-PCR). Real-time PCR was conducted to verify reproducibility of microarray data. Four representative genes (LLE1–4) were selected and subjected to real-time qRT-PCR validation in acephate-resistant TPB adults (Lula1000) collected from the same location in 2011 as was done for LLR in 2010. Expression levels of LLE1 in Lula-CK and Lula1000 were significantly up-regulated by 24- and 57.9-fold compared to the gene expression in LLS (Fig. 4A). LLE2 gene expressions in Lula-CK and Lula1000 were also up-regulated by 3.4- and 4-fold over that of LLS (Fig. 4B). Lula-CK and Lula1000 showed 6.3- and 15-fold higher LLE3 gene expressions than LLS (Fig. 4C). LLE4 gene expressions in Lula-CK and Lula1000 were also up-regulated by 2.9- and 6.3-fold over that of LLS (Fig. 4D). Lula1000 and Lula-CK were collected from the same location and were the same age. After acephate (1,000 mg/L) selection and removal of susceptible bugs, all four esterase gene (LLLE1–4) expressions increased 2.4-, 1.2-, 2.4-, and 2.2-fold, respectively. These results support up-regulated esterase genes are closely associated with reduced susceptibility in acephate-selected TPB (Lula600, Table 1).

Synergistic effect of esterase inhibitors on acephate toxicity against two TPB colonies. By applying esterase inhibitor, the toxicity of acephate was increased against both field and acephate-selected colonies of TPB (Fig. 5). Approximately 35.90% of Leland TPBs were killed by 72 mg/L acephate-only treatment. DEF-only killed 7.97% of the bugs. Acephate+DEF killed 40.31% of the bugs, which was numerically higher but not significantly different from acephate-only treatment. Acephate+TPP killed 49.04% of Leland field bugs, which was significantly higher than that of acephate-only treatment. After corrections with each inhibitor’s mortality, synergistic ratios of DEF and TPP were 0.98 and 1.34, respectively. Similarly, approximately 63.330% of acephate-selected TPBs (LLX) were killed by 144 mg/L acephate-only treatment. DEF-only killed 13.33% of the bugs. Acephate+DEF killed 88.33% of the bugs, which was significantly different from acephate-only treatment. Acephate+TPP killed 85.24% of Leland field bugs, which was also significantly higher than that of acephate-only treatment. After corrections with each inhibitor’s mortality, synergistic ratios of DEF and TPP against LLX colony were 1.37 and 1.34, respectively. The correlation was significant with R2 value 0.81 (Fig. 6).
Figure 2. Annotation and functional analysis of 329 up-regulated and 333 down-regulated genes (≥2-fold) in the LLR. A: Proportion of up-regulated genes (left side) and down-regulated genes (right side) categorized based on their involvement in biological process at GO level 2 (Blast2go). B: Proportion of up-regulated genes (left side) and down-regulated genes (right side) categorized based on different molecular function at GO level 2 (Blast2go).

doi:10.1371/journal.pone.0037586.g002
that three eggshell protein genes were down-regulated by 7.56- to 147.45-fold in LLR. In addition, three vitellogenin genes were also down-regulated by 3.34- to 6.26-fold in LLR (Table 3). Consistently, no eggshell or vitellogenin genes expressed significant increases in their gene transcript abundances in LLR (Table 2). Vitellogenin coding region of L4564 and LL_168 was overlapped with that of L140, but their cDNA sequence had only 33% and 45% identity, respectively, with L140. Therefore, L4564 and LL_168 are different vitellogenin cDNAs from L140. Because L4564 and LL_168 were not overlapping, whether they are from the same vitellogenin gene needs to be determined in a future study.

Down-regulation of protease and cathepsin genes associated with protein hydrolyzation. In LLR, only two protease and one salivary trypsin were up-regulated by 30.46–36.71- and 3.52-fold (Table 2), respectively. Thirteen serine protease genes showed 2.09- to 146.15-fold decrease in expression in LLR (Table 3). Another 11 cathepsin genes were also down-regulated by 2.15- to 15.51-fold in LLR (Table 3).

Discussion

Up-regulation of esterase genes associated with increased detoxification

Possessing ester bonds is a common feature in organophosphate insecticides, including acephate. Esterases detoxify organophosphate insecticides directly by splitting esters into an acid and an alcohol [22]. In addition, esterases might be involved in pyrethroid resistance development [23]. In this study, we consistently demonstrated that esterases are major detoxification enzymes and are responsible for the metabolic resistance closely associated with increased acephate resistance in the tarnished plant bug. By using acephate selection, we first demonstrated that elevated resistance level was closely correlated with increased esterase activities (Table 1). A previous study showed that fluctuation of esterase activities was well synchronized with seasonal increases of resistance levels in TPB [7]. Increased esterase activity in the late season likely resulted from pesticide applications on cotton. Without further exposure to insecticides in winter, the populations become less resistant. Therefore, insecticide resistance levels are closely associated with the intensity of chemical sprays. Seasonal variability in insecticide resistance are well documented [24,25,26]. Insecticides kill susceptible individuals and resistance levels evolve as resistant individuals increase over the growing season [27,28].

Higher esterase activities might result from altered gene sequence and hence the protein sequence difference between LLS and LLR. Careful examination of amino acid substitutions between LLSE1 and LLRE1 revealed 5 amino acid substitutions, but these substituted amino acids were strongly similar. Besides 2 strongly similar residue substitutions, LLRE4 had one weakly similar amino acid substitution (G to A) and a different amino acid substitution (S to R), suggesting a quality change of the LLRE4 esterase. Esterases are often found in multigene families [29,30] and it is very likely that a complex of esterases is present in the tarnished plant bug. cDNA library sequencing and microarray
analysis in this study revealed multiple esterase genes and differential expressed esterase genes in LLR. Overproduction of gene transcripts might have resulted from the transcription of a single copy of the esterase gene [31] or multiple copies of the esterase genes [32,33]. It is possible that resistant TPBs may have additional copies of esterase genes. A total of 14 esterase cDNA fragments were obtained in this study. Further assembling of these esterase cDNAs obtained at least 7 highly different esterase cDNAs or genes in the tarnished plant bug. Of the esterase genes examined, six of them were significantly up-regulated and none were down-regulated in LLR.

Synergists are often used as a diagnostic tool to characterize resistance mechanisms in pest populations. S,S,S-Tributyl phosphorotrithioate (DEF) inhibits hydrolysis of insecticides with ester linkages [34,35]. Triphenyl phosphate (TPP) was recognized as a specific inhibitor of carboxylesterase, which synergized the effect of malathion by blocking the production of malathion mono-acid in a resistant strain of Tribolium castaneum [36]. Based on our 2010 data, Leland population had relatively lower resistance ratio (1.6) to acephate. In this study, DEF was unable to synergize acephate toxicity, indicating that the population had no elevated DEF-suppressible esterases. The acephate-selected LLX colony is a mixture of several populations collected from Delta regions of Mississippi and Arkansas. It maintains 2–3-fold resistance ratio relatively to the LLS colony. Significant DEF synergism to acephate in acephate-selected LLX colony indicated that elevated esterase gene expressions exist in LLX for detoxifying acephate. Unlike DEF, TPP synergized acephate toxicity in both Leland field population and acephate-selected LLX colony, suggesting that both colonies had elevated carboxylesterases. These synergistic data functionally proved that esterase-mediated metabolic detoxification is potential resistance mechanism in acephate-resistant TPB. To knock down detoxification genes, RNAi will be conducted in future study to identify specific esterase genes that are the most responsible for acephate resistance in TPB.

Up-regulation of a glutathione S-transferase gene and potential association with metabolic detoxification in LLR

Glutathione S-transferases (GSTs) catalyze transformations of a wide range of endogenous and xenobiotic compounds, including carcinogens, therapeutic drugs, products of oxidative stress, herbicides, and insecticides [37,38,39]. They have the capacity
to conjugate reduced glutathione on the thiol of cysteine to various electrophiles and to bind with high affinity to various hydrophobic compounds [40]. Elevated GST activity has been associated with resistance to all the major classes of insecticides, commonly through increases in transcriptional rate, rather than qualitative changes in individual enzymes [41,42]. Of the 19 GSTs analyzed using microarray in this study, only one GST gene showed significantly higher transcripts in LLR. However, no GST was significantly down-regulated in LLR. GST enzyme activity data were also consistent with GST gene expression data. Significantly lower GST activity was found in the Lula natural population, suggesting a lower GST activity baseline than LLS (Table 1). Selection with Orthene 600 mg/L removed approximately 80% susceptible bugs from the sample causing GST activity to increase to a level similar to that of LLS. Considering GST enzyme activity, expression fold changes, and the number of genes involved, we postulate that GSTs may play a less significant role than esterases in reduced susceptibility of *L. lineolaris* to acephate.

**P450 oxidation and potential multiple resistance and cross resistance to different classes of insecticides in LLR**

Cytochrome P450 enzymes (mixed function oxidases, cytochrome P450 monoxygenases, CYP) are a large and diverse class of enzymes found in virtually all insect tissues. The function of most P450 enzymes includes catalyzing the oxidation of organic substances to fulfill many important tasks, from the synthesis, degradation, and metabolic intermediations of lipids, ecdysteroids and juvenile hormones to the metabolism of xenobiotics substances of natural or synthetic origin [43]. P450-mediated resistance is probably the most frequent type of metabolism based insecticide resistance [44,45]. This mechanism may potentially affect several classes of insecticides and thereby confer cross-resistance to unrelated compounds due to their broad substrate spectra [46]. Most cases of P450-mediated resistance result from an increase in detoxification. Resistance can occur by increased transcription of a P450 leading to both increased expression of the protein and increased detoxification of the insecticide [47]. In addition to over-expressions of several esterase genes, acephate-selected bugs (LLR) showed increased gene expressions of at least 3 different P450 genes. In the Mississippi Delta area, a variety of insecticides have been used for cotton insect control. Tarnished plant bug has a history of exposure to organophosphates and pyrethroids, and resistances to these pesticides have been reported [9,5]. In recent years, neonicotinoids have become a popular alternative for plant bug control. P450s are associated with resistance to both pyrethroids [44] and neonicotinoids [48]. Elevated expressions of at least three P450 genes may confer multiple and/or cross resistance to the three commonly used insecticide classes. Our dose-response assays on field populations of TPB (Fig. 6) highly supported this statement of multiple/cross resistance in LLR. Dose-response assays on multiple populations (including Lula) indicated a close correlation between survival rates of TPB treated separately with LC_{50} of acephate and imidacloprid. We are currently trying to develop a P450 enzyme activity assay method to link enzyme activity and elevated P450 gene expression with biological data.

**Down-regulation of eggshell, vitellogenin, protease genes associated with fitness cost**

Insect eggshell is composed of a set of proteins (rich in proline and alanine) synthesized by the follicular epithelium during the oogenesis and organized into an inner zone (vitelline membrane) and an outer zone (chorion) [49]. Vitellogenin is a unique group of proteins that are synthesized extraovariably and become the major egg yolk protein, vitellin [50]. Vitellogenin production by females is a prerequisite for successful egg production, and is directly linked to survival and reproductive success, thereby affecting both individual and colony-level fitness [51]. Serine proteases are enzymes that cleave peptide bonds in proteins. They are responsible for coordinating various physiological functions, including digestion, immune response, blood coagulation, and reproduction [52]. Very little research has been conducted to link eggshell, vitellogenin, and protease production with fitness cost and resistance to conventional insecticides. We observed a substantial decrease of egg production in survivors after treatment with Orthene 90WP at 240 mg/L. In this study, we report an association of organophosphate resistance with down-regulations of eggshell, vitellogenin, and protease genes in *L. lineolaris*.

Microarray data revealed significant down-regulations of eggshell, vitellogenin, and protease genes in LLR. The finding could be valuable in addressing resistance issues in *L. lineolaris*. When selection pressure increases, either through laboratory selections or field sprays, resistant bugs with elevated esterase and P450 levels could survive and relatively increase resistance gene frequency after removal of susceptible bugs. But, reproductive incompetency substantially limits resistant population growth. When selection pressure is low and suitable host are abundantly available, susceptible bugs, with higher quantities of proteases and eggshell/volk proteins, take reproductive advantage and increase population density quickly. Considering seasonal fluctuation and exposure-driven natures, we suggest that the acephate resistance in TPB is controlled by multiple genes. We also suggest that the resistance is associated with certain fitness cost.

In summary, microarray gene expression, Blast2go annotation, and other molecular comparisons, in concert with bioassays and enzyme activity data, revealed a significant increase of metabolic processes in the acephate-selected strain, suggesting metabolic detoxification as a major resistance mechanism. The resistance is controlled by many genes. Esterases are critical in detoxification of acephate, and suppression of esterases with inhibitors could significantly abolish acephate resistance. P450s also played an important role, along with less significant influence of GSTs. Down-regulations of many reproductively- and digestive-related genes indicated a potential fitness cost, which might dynamically keep the resistance from becoming fixed in the population. However, up-regulation of P450s and GSTs may increase the risk of multiple and/or cross resistance to other insecticide classes. Precautions must be taken to reduce selection pressure on target insect. Genetic composition of field populations should be constantly monitored to prevent potential genetic shift.

**Acknowledgments**

The authors are grateful to Dr. Fred Musser (Mississippi State University, Mississippi State, MS), Dr. Haobo Jiang (Oklahoma State University, Stillwater OK), and Dr. Ming-Shun Chen (USDA-ARS, Manhattan KS) for valuable comments and suggestions that improved an early version of this manuscript. The authors also appreciate Xiaofen Fanny Liu, Sandy West, Lily Luo, Tashanika Knight, Linda Ballard, and Brian Scheffler for their assistance in this study. Mention of a proprietary product does not constitute a recommendation or endorsement by the USDA.

**Author Contributions**

Conceived and designed the experiments: YCZ. Performed the experiments: YCZ ZG YH. Analyzed the data: YCZ ZG. Contributed reagents/materials/analysis tools: YCZ RL. Wrote the paper: YCZ.
References

1. USDA-ARS (1999) Banishing Tarnished Plant Bugs From Cotton. Agric Res 47: 12–14.
2. Hollingsworth RG, Steinkraus DC, Tugwell NP (1997) Responses of Arkansas populations of tarnished plant bugs (Heteroptera: Miridae) to insecticides, and tolerance differences among nymphs and adults. J Econ Entomol 90: 21–26.
3. Snodgrass GL, Elzen GW (1998) Insecticide resistance in a tarnished plant bug population in cotton in the Mississippi Delta. Proc Beltwide Cotton Conference. Memphis, TN: National Cotton Council. pp 975–977.
4. Snodgrass GL (1996) Insecticide resistance in field populations of the tarnished plant bug (Heteroptera: Miridae) in cotton in the Mississippi Delta. J Econ Entomol 89: 783–790.
5. Snodgrass GL, Gore J, Abel CA, Jackson R (2009) Acephate resistance in plant bug, Lygus lineolaris. Insect Biochem Mol Biol 34: 1175–1186.
6. Zhu YC, West S, Snodgrass G, Luttrell R (2011) Variability in resistance-related enzyme activities in field populations of the tarnished plant bug, Lygus lineolaris. Pest Biochem Physiol 99: 263–273.
7. Zhu YC, Snodgrass GL, Chen MS (2004) Enhanced esterase gene expression and activity in a malathion resistant strain of the tarnished plant bug, Lygus lineolaris. Insect Biochem Mol Biol 34: 1175–1186.
8. Zhu YC, Snodgrass GL, Chen MS (2006) Comparative study on glutathione S-transferase activity, cDNA, and gene expression between malathion susceptible and resistant strains of the tarnished plant bug, Lygus lineolaris. Pest Biochem Physiol 87: 62–72.
9. Snodgrass GL, Scott WP (2000) Seasonal changes in pyrethroid resistance in tarnished plant bug (Heteroptera: Miridae) populations during a three-year period in the Delta Area of Arkansas, Louisiana, and Mississippi. J Econ Entomol 93: 441–446.
10. SAS Institute (2003) SAS® System (Version 9.1) for Windows®, Cary, NC: SAS Institute.
11. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.
12. Otte JA, Ibrahim SA, Younis AM, Young R (2000) Mechanisms of pyrethroid resistance in larvae and adults from a cypermethrin-selected strain of Heliothis zea. Pest Biochem Physiol 66: 20–32.
13. Rose RL, Barbhaiya L, Roe RM, Rock GC, Hodgson E (1995) Cytochrome P450-associated resistance and the development of biochemical assay protocols in Heliotis zea. Pest Biochem Physiol 51: 178–191.
14. Zhu YC, Guo Z, Chen MS, Zhu KY, Liu XF, et al. (2011) Major putative pesticide receptors, detoxification enzymes, and transcriptional profile of the midgut of the tobacco budworm, Heliothis zea (Lepidoptera: Noctuidae). J Invertebr Pathol 106: 296–307.
15. Alschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
16. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, et al. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674–3676.
17. Blostad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185–193.
18. Irizarry RA, Blostad BM, Collin F, Cope LM, Hobbs B, et al. (2003) Summaries of affymetrix GeneChip probe level data. Nucleic Acids Res 31: 15.
19. Enayati A, Ranson H, Hemingway J (2005) Insect glutathione S-transferases and resistance to organophosphate insecticides. Pest Biochem Physiol 87: 62–72.
20. Haubruge E, Amichot M, Guany A, Berge J-B, Arnaud L (2002) Purification and characterization of a carboxyl esterase involved in malathion-specific resistance from Tribolium castaneum (Coleoptera: Tenebrionidae). Insect Biochem Mol Biol 32: 1181–1190.
21. Douglas KT (1987) Mechanism of action of glutathione-dependent enzymes. Adv Enzymol Relat Areas Mol Bio 59: 103–167.
22. Armstrong RN (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem Rev Toxicol 10: 2–18.
23. Allocati N, Federici L, Macaluso M, Di Ilio C (2009) Glutathione transferases in insects: a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector, Anopheles gambiae. Biochem J 359: 295–304.
24. Feyereisen R (1999) Insect P450 enzymes. Adv Enzymol Relat Areas Molec Bio 59: 103–167.
25. Ranson H, Rossier L, Ottelli F, Jensen B, Wang X, et al. (2003) Identification of a novel class of insect glutathione S-transferases involved in resistance to DDIT in the malaria vector, Anopheles gambiae. Biochem J 359: 295–304.
26. Scott GJ (1999) Cytochrome P450 and insecticide resistance. Insect Biochem Mol Biol 29: 737–777.
27. Hodgson E, Kulkarni AP (1983) Characterization of cytochrome P450 in studies of insecticide resistance. In: Georghiou GP, Saito Y, eds. Pest Resistance Management. New York: Plenum Press. pp 295–304.
28. Liu N, Scott GJ (1998) Insecticidal activity of CYP6M1: a key enzyme of the pyrethroid resistance mechanism in house fly. Insect Biochem Mol Biol 28: 531–535.
29. Liu W, Zhao H, Wang Y, Zhang L, Zhang H, et al. (2003) Selection for malathion resistance in bollworm: cross-resistance patterns and possible mechanisms. Pest Manag Sci 59: 1355–1359.
30. Aronson WP, Arlene RW, Fotsi CK (1976) Specific protein synthesis in cellular differentiation: III. The eggshell proteins of Drosophila melanogaster and their program of synthesis. Dev Biol 49: 183–199.
31. Hagedorn HH, Kunzel JG (1979) Vitellogenin and vitellogenin in insects. Annu Rev Entomol 24: 475–505.
32. Clement FK, Auner I, Alexandra CB, Amro Z (2011) Adaptive evolution of a key gene affecting queen and worker traits in the honey bee, Apis mellifera. Mol Ecol 20: 5226–5235.
33. Hedstrom L (2002) Serine protease mechanism and specificity. Chem Rev 102: 4501–24.