ELEVATED METABOLIC DETOXIFICATION ASSOCIATED WITH MULTIPLE/CROSS RESISTANCE TO DIFFERENT INSECTICIDE CLASSES IN TARNISHED PLANT BUG

Yu Cheng Zhu
Randall Luttrell
USDA-ARS
Stoneville, MS

Abstract

Tarnished plant bugs (TPB, *Lygus lineolaris*) were collected from multiple locations in the Delta regions of Mississippi, Arkansas, and Louisiana (covering 200 miles in E-W and N-S directions), and were subjected to bioassays to acephate, imidacloprid, dicrotophos, thiamethoxam, and sulfoxaflor (representing four insecticide classes: pyrethroids, organophosphates, neonicotinoids, and sulfoximine). Results showed that survival rates increased by 2-fold from May to September. Field populations exhibited up to a 10-fold difference in their susceptibility, and survival rates ranged from 7 to 76%. Populations collected around cotton fields were usually less susceptible than populations collected around soybean and corn fields. Regression analysis indicated that populations with higher survival rates from organophosphate insecticide treatments also had higher survival rates from neonicotinoid insecticide treatments with moderate correlation (R^2 was up to 0.63; $P<0.001$). Examination of detoxification enzymes, synergism with different inhibitors, and gene expression profiles consistently indicated that many TPB populations have developed certain levels of multiple/cross resistances to different insecticide classes. The resistance is mainly conferred by elevated esterase and P450 oxidase gene expressions, hence increased detoxification in resistant bugs. To slow down the resistance evolution, precautions must be taken to reduce selection pressure by properly rotating insecticides with different modes of action and avoiding use of insecticides that may induce common resistance mechanisms in target populations.

Introduction

Widespread adoption of transgenic Bt cotton and altered chemical control schemes have allowed sucking insect populations to increase (USDA-ARS, 1999). Of these pests, the tarnished plant bug (TPB), *Lygus lineolaris*, has emerged as the most economically significant (USDA-ARS, 1999). Management of tarnished plant bug relies almost exclusively on chemical control. Commonly used insecticides include pyrethroids, organophosphates, carbamates, and neonicotinoids. In order to suppress feeding damage from TPB and bollworm/tobacco budworm, cotton is more frequently sprayed than other major crops in the South. Over the years, TPB has become increasingly resistant to several chemical insecticides (Hollingsworth et al., 1997; Snodgrass, 1996).

With multiple generations per year, high mobility, and existence of differential activities of major detoxification enzymes (Zhu et al., 2011), field populations of TPB across the Delta region have the potential to evolve high levels of resistance to multiple insecticides, especially when they are under high selection pressure. Increased economic importance of TPB and insecticide resistance development prompted our research to better understand the mechanisms of insecticide resistance in this cotton pest. Results were expected to generate a general picture of insecticide resistance in TPB and help explain how TPB evolves resistance to commonly used insecticides.

Materials and Methods

Chemicals

The Pierce Coomassie plus protein assay kit (23238) was purchased from ThermoFisher Sci. (Pittsburgh, PA, USA). S,S,S-tributylphosphorotrithioate (DEF) was purchased from Supelco (Bellefonte, PA). Alpha-naphthyl acetate (1-NA or α-NA) (N8505), beta-naphthyl acetate (2-NA or β-NA) (N6875), ρ-nitrophenyl acetate (PNPA) (N8130), 1-chloro-2,4-dinitrobenzene (CDNB) (23,732-9), fast blue salt (D9805), L-glutathione (GSH) reduced (G6529), triphenyl phosphate (TPP) (105856), diethyl maleate (DEM) were purchased from Sigma Chemical Co. (St. Louis, MO). Piperonyl butoxide (PBO) was purchased from Aldrich (Milwaukee, WI).

Insect Laboratory Colony and Field Collection

A laboratory colony (originally provided by Mississippi State University) was used as a standard susceptible strain (LLS: without exposure to insecticide for nine years). Resistance ratios (LC_{50} of field-collected population/ LC_{50} of laboratory strain) were calculated for field and laboratory strains relative to LLS. By using the sweeping net, field

populations were collected mainly from pigweed around cotton, corn, or soybean fields in Mississippi and Arkansas. To develop a resistant colony, approximately 45,000 bugs were collected from Lula, Mississippi and selected with acephate (Orthene 90WP at 600 mg/L). Both unselected field population and acephate-selected populations were subjected to dose-response bioassays to obtain LC_{50} values. Survivors (LLR with resistance ratio >25-fold) from acephate treatment (Orthene 90WP at 2,000 mg/L) were used for comparison of gene regulation using microarray analysis.

Monitoring Susceptibility to Different Insecticides in Field Populations of the Tarnished Plant Bug

To detect potential resistance to multiple insecticides in TPB, susceptibility assays to acephate (Orthene 90WP at 80 mg/L), imidacloprid (Advise 2FL 21.4% at 85 mg/L), dicrotophos (Bidrin 8EC at 60 mg/L), thiamethoxam (Centric 40WG at 35 mg/L), permethrin (Arctic 3.2EC 36.8% at 145 mg/L), and sulfoxaflor (Transform WG 50% at 29 mg/L) were evaluated for different field populations by using a modified Potter Spray Tower. The insects, green bean, and container were simultaneously sprayed with 500 µl of insecticide solution. Mortality was recorded 48 h after treatment.

Enzyme Activity Assay

Esterase and glutathione S-transferase activities were comparatively examined using the protocols described by Zhu et al. (2011). To determine esterase activity, micro-titer plate assays were conducted using α-NA, β-NA, and PNPA as substrates. A Bio-Tek ELx808iu plate reader (Winooski, VT) was used to monitor α-NA and β-NA reactions at 450/405 nm for 10 minutes with measurements taken every 15 seconds. To determine glutathione S-transferase activities, micro-titer plate assays were conducted using CDNB as the substrate. The reactions were monitored at 340 nm for 10 minutes with readings taken every 15 seconds.

Gene Expression Analysis Using Microarray

Roche NimbleGen 72K 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. 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). 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.25 User's Guide through Florida State University Microarray processing facility. ArrayStar® software (DNAStar, Inc., Madison, WI) was used to analyze and compare microarray data between LLS and LLR. Normalized data were analyzed using classical parametric statistics. *P*-values were calculated using Modified *t*-test. Scatter plotting was also applied to generate a distribution of more than 6,688 genes tested between LLS and LLR.

Results and Discussion

Comparison of Insecticide Resistance Levels in Different Populations Using Bioassay

Bioassay results indicated that TPB survival rates increased by 2-fold from May to September. Different field populations showed differences in susceptibility. Survival rates from acephate and imidacloprid treatments exhibited up to 8-fold difference among 29 populations. TPBs collected around cotton fields, including those in northwest Mississippi and Tillar, Arkansas, usually had higher survival rate than those collected around soybean and corn fields. Similarly, survival rates from dicrotophos (at LC_{90}) and thiamethoxam (at LC_{50}) treatments ranged from 7% to 76%. The higher survival rates were also associated with cotton growing areas. Regression analysis indicated that populations with higher survival rates from organophosphate insecticide treatments also had higher survival rates from neonicotinoid insecticide treatments with moderate correlation (R^2 was up to 0.63; $P<0.001$). In addition, selection with acephate could elevate resistance levels by 2.7-fold.

Detoxification Enzyme Activities and Association with Resistance Development

Reduced susceptibility to acephate was highly correlated with elevated esterase activities. The acephate-resistant populations from cotton growing areas (northwest Mississippi and Tillar, Arkansas) consistently had higher (up to 5.3-fold) esterase activities than susceptible populations. Laboratory selection with acephate increased esterase activities by up to 2.9-fold. Regression analysis of LC_{50}s with kinetic esterase activities revealed a significant polynomial quadratic relationship with R^2 up to 0.89 ($P<0.001$). Glutathione S-transferase (GST) also had elevated activity in most populations, but variations in GST activities were not significantly correlated with changes of acephate susceptibility.

Synergistic Effect of Esterase, GST, and P450 Enzyme Inhibitors on the Toxicity of Different Insecticides

By applying an esterase inhibitor, the toxicity of acephate (Orthene) was increased against both field and acephate-selected colonies of TPB. DEF and TPP showed approximately 1.3-fold synergistic ratios (mortality from combined insecticide+inhibitor treatment / additive mortality from individual insecticide and inhibitor treatment). In 2012, the esterase inhibitor TPP, GST inhibitor DEM, and the P450 inhibitor PBO were used to synergize the toxicity of dicrotophos, permethrin, and thiamethoxm against a dicrotophos-selected and a thiamethoxm-selected colony. Results indicated that TPP and PBO significantly synergized insecticide toxicity, while DEM showed only an additive effect. These data demonstrated that some field populations have developed certain levels of multiple/cross resistance through elevation of esterases and P450 oxidases, which reduce efficacy of all three classes of commonly used insecticides, e.g. pyrethroids, organophosphates, and neonicotinoids.

Microarray Analysis of Gene Regulation in 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. 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. A total of 662 genes showed 2-fold changes, 191 genes showed 4-fold changes, and 113 genes exhibited 8-fold changes between LLS and LLR. Annotation with Blast2go showed that 66 genes of the 662 differentially expressed genes were involved in biological processes and 76 genes were involved in molecular functions in LLR. 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 in LLR. 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 (detoxification) in LLR compared to LLS. Increases in 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. No esterase genes were down-regulated in LLR. Microarray data showed that only one of the 19 GST genes was up-regulated by 2.14-fold in LLR. However, no GST genes were down-regulated in LLR. A total of 13 partial cDNAs of P450s were obtained from cDNA library sequencing. Microarray data showed that three P450 genes were up-regulated by 2.7- to 3-fold in LLR. No P450 genes were down-regulated in LLR. The cytochrome P450 enzymes 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 to the metabolism of xeno-biotic substances of natural or synthetic origin (Feyereisen, 1999). P450 genes are under complex regulation, with induction playing a central role in the host plant adaptation and insecticide resistance. It is well established that many cases of metabolic resistance to insecticides are the result of elevated levels of P450 (Agosin, 1985). P450 cytochrome monooxygenases are well documented to be associated with resistance development to pyrethroid and neonicotinoid insecticides (Scott, 1999; Liu et al., 2003). To test whether over-expression of P450 genes confer multiple resistances to different insecticide classes, dose responses to acephate and imidacloprid were evaluated in 9 field populations collected from northwest Mississippi. Results showed variable survival rates in different populations. Responses to both insecticides appeared to be correlated. The population showing low susceptibility to acephate also had low susceptibility to imidacloprid. The correlation was significant with an R^2 value 0.82 ($P<0.01$). Therefore, many field populations may develop certain levels of multiple/cross resistance to pyrethroid, organophosphate, and neonicotinoid insecticides, which are commonly used for TPB control.

Acknowledgements

The authors are grateful to Dr. Ryan Jackson and Dr. Clint Allen (USDA-ARS, Stoneville, MS) 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.

References

Agosin, M., 1985. Role of microsomal oxidations in insecticide degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Physiology, vol. 12. Pergamon, Oxford, UK, pp. 647–712.

Feyereisen R (1999) Insect P450 enzymes. Annu Rev Entomol 44: 507-533.

Hollingsworth RG, Steinkraus DC, Tugwell NP (1997) Responses of Arkansas populations of tarnished plant bugs (Heteroptera: Miridae) to insecticides, and tolerance differences between nymphs and adults. J Econ Entomol 90: 21-26.

Liu Z, Han Z, Wang Y, Zhang L, Zhang H, et al. (2003) Selection for imidacloprid resistance in Nilaparvata lugens: cross-resistance patterns and possible mechanisms. Pest Manag Sci 59: 1355–1359.

Scott JG (1999) Cytochrome P450 and insecticide resistance. Insect Biochem Molec Biol 29: 757–777.

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.

USDA-ARS (1999) Banishing Tarnished Plant Bugs From Cotton. Agricul Res 47: 12-14.

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*. Pestic Biochem Phys 99: 265-273.