Comparative study on glutathione S-transferase activity, cDNA, and gene expression between malathion susceptible and resistant strains of the tarnished plant bug, *Lygus lineolaris*

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Abstract

Control of the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), in cotton in the mid-South relies heavily on pesticides, mainly organophosphates. Continuous and dominant use of chemical sprays has facilitated resistance development in the tarnished plant bug. A natural population in Mississippi with resistance to malathion was studied to examine whether and how glutathione S-transferases (GST) played an important role in the resistance. Bioassays were first conducted to examine synergism of two GST inhibitors. Both ethacrynic acid (EA) and diethyl maleate (DM) effectively abolished resistance and increased malathion toxicity against two resistant strains by more than 2- and 3-fold, whereas incorporation of GST inhibitors did not significantly increase malathion toxicity against a susceptible strain. GST activities were compared in vitro between malathion susceptible and resistant strains by using 1-chloro-2,4-dinitrobenzene as a GST substrate. The resistant strain had significantly higher (1.5-fold) GST activity than the susceptible strain. Up to 99%, 75%, and 85% of the GST activities were inhibited by EA, sulforhodamine (SBT), and DM, respectively. The GST activities tended to increase from May to October by 1.76-fold. All three inhibitors significantly suppressed the GST activity to a constant low level over the season. Further examination of GST cDNA indicated that in the coding region only one nucleotide variation was revealed between the susceptible and resistant strain. This variation did not cause a protein sequence change, and an identical amino acid sequence was predicted for both strains. Multiple sequence alignment and phylogenetic analysis showed that the 216-residue GST from the tarnished plant bug was highly similar (up to 68% of amino acid sequence identity) to the GSTs from other insects, which conferred organophosphate resistance. GST gene expression levels were examined using real-time PCR, and the results indicated that GST gene transcripts were elevated in the resistant strain by 1.3-fold.  

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1. Introduction

Although Bt cotton that produce Cry toxins effectively control many important lepidopteran species, many sucking insects, especially the tarnished plant bug *Lygus lineolaris* (Palisot de Beauvois), have emerged in recent years as economically important pests. Chemical spray with insecticides has been the dominant measure for plant bug control on cotton, and this has resulted in widespread resistance to pyrethroid and increased resistance to organophosphate insecticides [1–3]. Zhu et al. [3] showed that more than 11-fold malathion resistance was present in a natural population of the tarnished plant bug collected in Mississippi, which was also resistant (4.5-fold) to the pyrethroid insecticide permethrin.  

Development of resistance to pyrethroid and tolerance or resistance to organophosphate insecticides in the tarnished plant bug is major factor for its increasing pest status on cotton. Understanding resistance mechanisms is...
essential for developing strategies to manage the resistance. A previous study [3] indicated that elevated esterase gene expression and esterase activity was associated with malathion resistance in a resistant strain. By mutation and/or elevated gene expression, esterases play an important role in organophosphate resistance development in many resistance cases [4–8]. Moreover, esterases can also be involved in pyrethroid resistance. Valles [9] found a significant correlation between general esterase activity and the pyrethroid resistance level in Blatella germanica. In the mid-South area, pyrethroid insecticides are no longer recommended for control of plant bugs because of resistance. Organophosphate insecticides, including malathion, are the major chemicals used for plant bug control on cotton. The natural population of tarnished plant bugs we studied had developed resistance to both pyrethroid and organophosphate insecticides. However, resistance levels to malathion were more prominent than the resistance to permethrin [3]. We assumed that factors other than elevated esterase activity might contribute to malathion resistance development in this population. It was also thought that increased esterase gene expression, rather than a mutation, was the underlying mechanism for the resistance to malathion.

Glutathione S-transferases (GSTs) are detoxifying enzymes found in vertebrates, plants, insects, yeasts, and aerobic bacteria [10,11]. The primary function of GSTs is generally considered to be the detoxification of both endogenous and xenobiotic compounds either directly or by catalyzing the secondary metabolism of a vast array of compounds oxidized by the cytochrome P450 family [12]. GSTs catalyze the conjugation of glutathione (GSH) to hydrophobic substrates, such as herbicides and insecticides [13], which facilitates the metabolism of target compounds [10,14–16]. Several studies indicated that GSTs have an important role in the acquisition of resistance to insecticides. Some resistant insects have been found to metabolize insecticides more efficiently via a GSH-dependent route [17]. High levels of GST activity have been detected in some resistant insect strains [18–20], and the resistance development had been correlated with enhanced GST activity and GST-dependent insecticide metabolism [21]. Because GSTs are capable of catalyzing the secondary metabolism following the oxidation by the cytochrome P450s [12], it is important to understand whether and how the GSTs play a role in resistance development in the tarnished plant bug, which can be resistant to both pyrethroid and organophosphate insecticides. In this study, we assessed synergism of GST inhibitors and compared GST cDNA sequences, enzyme activities, and GST gene expression levels between susceptible and resistant strains.

2. Materials and methods

2.1. Insect

The malathion-susceptible strain was collected from weeds near Crossett (Ashley County), AR. Cotton is not grown near Crossett and plant bugs from this location were found to be very susceptible to most insecticides used on cotton [22]. The resistant strain was originally collected from weeds in or near cotton field near Mound Bayou (Bolivar County), MS. The field collected adults from Mound Bayou were selected with malathion in the laboratory to produce a test population for enzyme activity assays and molecular analyses made up of individuals more homozygous for malathion resistance. They were selected by exposing adults for 3 h in 20-ml glass vials that had been treated with 100 µg of malathion per vial. Preliminary tests showed that 100 µg of malathion would kill about 50% of the plant bugs in 3 h of exposure. The LC50 of the selected adults was not determined. Two adults were placed in each vial and survivors were held for 24 h to eliminate any adults that subsequently died. Another malathion resistant population was collected in Bruce (Calhoun County), MS. This colony was used for the synergist bioassay and was unselected. Susceptible and resistant plant bugs were tested in the bioassays within one week after they collected in the field.

2.2. Bioassay and synergist test

A glass-vial bioassay [22] was used to determine resistance levels and synergism of two GST inhibitors. Twenty-ml glass scintillation vials were first treated with malathion (≥98% technical grade, Chem Service, West Chester, PA) or GST inhibitors, ethacrynic acid (EA) (Sigma, St. Louis, MO) and diethyl maleate (DM) (Aldrich, Milwaukee, WI). Acetone was used as the solvent, and was included as a control. Malathion and GST inhibitors were applied by pipetting 0.5 ml of the insecticide or an inhibitor diluted in acetone into each vial. Each vial was rolled on its side until an even layer of insecticide or inhibitor dried on its inner surface. Malathion and GST inhibitors were applied to the vials on the same day the test was performed. A small piece of green bean pod, Phaseolus vulgaris L. (cut transversely) about 3 mM thick was added to each vial as food for the adults, and a cotton ball was used to seal each vial. Adult plant bugs were placed into treated vials (2 per vial). Synergists were tested in the glass vials by exposing adults to 400 µg/vial DM or 400 µg/vial EA for 24 h. Adults were then transferred to vials (2 per vial) treated with malathion. Vials were held during a test in an upright position at laboratory conditions of 24–26°C, and humidity was not controlled. Mortality was determined after 24 h, and adults were considered dead if they were unable to stand normally or walk, or there was no movement when they were prodded. All bioassays had three replications for each treatment, and each replication contained 10 vials with two adults in each vial. In the first experiment, malathion was used at 25 µg/vial, and resistant bugs were collected from Mound Bayou, MS. In the second experiment, malathion at 5 µg/vial was used for susceptible bugs collected from Crossett, AR and malathion at 25 µg/vial was used for resistant bug collected from Bruce, MS. The concentrations were...
determined based on LC_{50} values obtained from pretrial experiments.

2.3. Enzyme preparation

Individual tarnished plant bugs were homogenized in 340\mu l, 0.1 M sodium phosphate buffer, pH 8.0. Homogenates were then centrifuged at 10,000 g for 5 min at 4°C, and the supernatant was used for enzyme analysis. Protein concentrations were determined using the Bradford method [23]. Bovine serum albumin was used to obtain the standard curve.

2.4. Glutathione S-transferase (GST) assays

To determine glutathione transferase activity, microtiter plate assays were conducted using 1-chloro-2,4-dinitrobenzene (CDNB, Aldrich, Milwaukee, WI). The pre-reaction mixture (100\mu l) consisted of the following: 8mM glutathione (Sigma, St. Louis, MO), 10\mu l enzyme homogenate, and 0.1 M phosphate buffer pH 8.0. Reaction was started with the addition of 200\mu l CDNB in 0.1 M phosphate buffer pH 8.0 containing 15% glycerol with a final concentration of 0.5 mM. A Bio-Tek plate reader ELx808IU equipped with a 340 nm filter was used to monitor the activity at 25°C, for 10 min with measurements taken every 15 s [24]. Inhibitors for glutathione transferase activity were EA (final concentration 0.19 mM), sulfobromophthalein sodium hydrate (Aldrich, Milwaukee, WI) (final concentration 0.01 mM), and DM (final concentration 0.712mM). Inhibitor concentrations were referred to enzyme homogenate, and allowed to incubate at 37°C for 10 min before the addition of substrate [26].

2.5. Seasonal survey of GST activities

Through the months of May to October of 2003, tarnished plant bugs were collected from weeds in or near a cotton field in Sunflower County, Mississippi. Ten individual adults for each month were tested for levels of GST activity as previously described in duplicate.

2.6. RNA extraction and cDNA synthesis

Five tarnished plant bug adults (approximately 30mg) were homogenized in 500\mu l Trizol solution (Invitrogen, Carlsbad, CA). Total RNA was precipitated with isopropanol and resuspended in double distilled-H_{2}O. Five\mu g of RNA was used as a template for cDNA synthesis using an oligo(dT) primer in the reverse transcription (RT) reaction (SuperScript First Strand cDNA Synthesis System, Invitrogen). The procedures were performed using insects from the susceptible strain and repeated using insects from the malathion-selected strain.

2.7. Cloning glutathione S-transferase cDNA

To clone GST cDNA fragments, two degenerate forward primers GST1F: GARWSNMNGNCNATHGACNTAYYT and GST2F: CARMGNYNTNTAYTTYGAY ATGGGNAC, were designed. These two primers were expected to anneal to corresponding sequences coding for two conserved regions (ESRAMTYL, QRLYFDMG) of a GST from the brown planthopper, Nilaparvata lugens [27]. These two regions are highly conserved in other insect GSTs, including those from Drosophila simulans (GenBank: AAK66764), Drosophila melanogaster (AA52032), Drosophila mauritiana (P30105), Musca domestica (P28338), Lucilia cuprina (P42860), Bactrocera papayae (AAO19738), Anopheles gambiae (Q53113), Anopheles dirus (AAB41104), Culicoides variipennis (AAB94639), and Galleria mellonella (AAK64362). RT-cDNA was used as the template for the first PCR amplification with GST1F primer and an oligo(dT) primer (no band was obtained). The second semi-nested PCR was conducted to amplify the first PCR product (GST1F + dT) with GST2F + dT primers. A distinct fragment, approximately 600 bp, was revealed from the second PCR amplification. The cDNA fragment band was sliced from a low-melting point gel and extracted using the Promega (Madison, WI) Wizard PCR Prep DNA purification system. Purified DNA was cloned into a pGEM-T vector (Promega) and then it was sequenced. The fragment was confirmed as a part of the GST cDNA by similarity searching [28] the GenBank database (BlastX, NR) of the National Center for Biotechnology Information (NCBI).

To obtain the 5'-end of the GST cDNA, three reverse primers, LLGSTR1, LLGSTR2, and LLGSTR3 (Fig. 4), were synthesized. Reverse transcription was performed using the reverse primer LLGSTR1, and the 5'-end of GST cDNA was isolated and C-tailed using the 5'-RACE (rapid amplification of cDNA end) system (Invitrogen). Subsequent semi-nested amplifications were performed using a forward abridged anchor primer (5'-RACE kit) and the specific reverse primers LLGSTR2 and LLGSTR3. The DNA fragment resolved from the 5'-RACE amplification was cloned into a pGEM-T vector and sequenced from both directions. GST cDNA was assembled, and it was confirmed that it contained the full coding sequence for the GST-like protein. To obtain full GST-like cDNA from the susceptible and malathion-selected strains of the tarnished plant bug, total RNA was extracted from these strains and used for RT-cDNA synthesis. A forward primer LLGSTF0 and a reverse primer LLGSTR1 were used in PCR to flank the 5' and 3' non-coding regions, respectively (Fig. 4). To eliminate potential error created by Taq DNA polymerase, a thermal-stable proof-reading Pfu DNA polymerase (Promega, Madison, WI) was used to reamplify a full length GST coding region cDNA from the RT-cDNAs of the susceptible and resistant strains. The resolved cDNA fragments (700 bp) were purified from low-melting point agarose gel, and then A-tailed for 30 min at 72°C using the nucleotide adenine and Taq DNA polymerase. They were then cloned into a pGEM-T vector (Promega), and the resolved clones were sequenced from both directions on an ABI 3700 sequencer.
The BlastX program was used to search the sequence database of the National Center for Biotechnology Information Internet server for proteins with amino acid sequence similarity to the GSTs [28]. The ExPASy Proteomics tools of the ExPASy Molecular Biology Server of Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/) were used to process data of the deduced protein sequences. DNAStar software (version 6; ClustalW method; [29]), parameters were set as default: gap penalty = 10, gap length penalty = 0.2, delay divergent seqs(%) = 30, and Gonnet series was used for protein weight matrix.) or an Internet Server (http://pbil.ibcp.fr) was used to conduct multiple sequence alignment.

2.8. Quantitative reverse transcription (QRT) PCR analysis of GST mRNA expression

The expression levels of GST mRNAs were determined quantitatively in the susceptible and resistant strains using a real-time PCR system (ABI 7500, Applied Biosystems, Foster City, CA) and iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA). Two primers, LLGSTF3 and LLGSTR6 (Fig. 4), were designed to amplify a 93 bp GST cDNA fragment. A clear single band was obtained from a pre-trial gradient PCR amplification of GST cDNA within \( T_m \) ranging from 55 to 68 °C. RNA was individually extracted from adults of the susceptible and resistant strains of the tarnished plant bug. The RNA was reverse transcribed using a SuperScript First Strand cDNA Synthesis System (Invitrogen). The RT cDNA was spectrophotometrically quantified and equal amount of the cDNA was added to each real-time PCR. An absolute quantification protocol was adopted and a series of concentrations of the cloned GST cDNA were used as standards. Two step PCR (95 °C for 10 s and 60 °C for 1 min) was conducted for 40 cycles and data were collected at 60 °C.

2.9. Data analysis

Means for each treatment were compared using ANOVA and separated using Fisher’s protected least significant difference procedure at the \( \alpha = 0.05 \) level (PROC GLM, SAS version 9.1, SAS Institute Inc., Cary, NC). Regression analysis was conducted with the SAS Proc Reg procedure.

3. Results

3.1. Synergism of GST inhibitors

In the first experiment, no plant bug died 24 h after treatment with either DM alone (400 µg/vial) or EA alone (400 µg/vial) (Fig. 1). Malathion alone (25 µg/vial) killed approximate 20% of the plant bugs. The same concentration of the malathion with DM killed 61.7 ± 1.7% of treated plant bugs, and the malathion with EA killed 68.3 ± 4.4% of treated plant bugs. Treatment with either GST inhibitor significantly increased toxicity against the tarnished plant bug (\( F = 25.55, df = 2, P < 0.05 \)). Addition of both DM and EA increased 24-h mortality by more than 3-fold. In the second experiment (Fig. 2), susceptible bugs showed 41.7 ± 14.2% mortality after 24 h treatment with malathion at 5 µg/vial (Fig. 2A). Malathion with DM and EA increased mortality to 50 ± 4.9% and 46.7 ± 10.0%. But, the mortality increase was not significant (\( F = 0.16, df = 2, P > 0.05 \)). In contrast to the susceptible strain, resistant bugs showed 25 ± 4.9% mortality after treatment with malathion at 25 µg/vial (Fig. 2B). Similarly, treatment with either GST inhibitor significantly increased efficacy against the resistant tarnished plant bug (\( F = 13.63, df = 2, P < 0.05 \)). Addition of both DM and EA increased 24-h mortality by more than 2-fold.

3.2. Comparison of GST activities between S and R strains

Glutathione S-transferase activities were examined \textit{in vitro} using 1-chloro-2,4-dinitrobenzene as the GST substrate (Fig. 3). Protein was also treated with three GST inhibitors, EA, sulfobromophthalein (SBT), and DM, to examine suppression of GST activities. The baseline of GST activity in the susceptible strain was 90.05 ± 8.8. The resistant strain had 134.4 ± 8.27 baseline activity, which was significantly (1.5-fold) higher than that of the susceptible strain. Pooled data also showed significant greater activity in the resistant strain (57.65) than that in the susceptible strain (31.69) (\( F = 39.27, df = 1, P < 0.0001 \)). Inhibitor treatments also significantly suppressed GST activities in both strains (\( F = 128.61, df = 3, P < 0.0001 \)). EA treatment significantly suppressed GST activity by 99% and 96% in the susceptible and resistant strains, respectively. SBT inhibited 75% GST activity in the susceptible strain and 65% GST activity in the resistant strains. Similarly, DM suppressed 85% GST activity in the susceptible strain and 67% GST activity in the resistant strains.

3.3. Seasonal change of the GST activity

The tarnished plant bug adults were collected once a month from May to October in 2003. GST activities were measured using 1-chloro-2,4-dinitrobenzene as the substrate in the presence or absence of inhibitors. Results demonstrated that without an inhibitor the GST activity increased as the season progressed (Fig. 4). The lowest GST activity (31.11 ± 4.58) was obtained in May, the earliest month that samples were collected from the field. The highest GST activity (54.85 ± 3.93) was found in the insects collected in October, the last samples for the year. The GST activities increased 1.76-fold over the season. The GST activities obtained in September and October were significantly higher than those obtained in May and June (\( F = 3.52, df = 5, P < 0.05 \)). Regression analysis indicated that the linear relationship between GST activity and season was significant (\( F = 16.95, P < 0.01 \)). Unlike the control, the GST treated with any of three inhibitors showed significantly lower activity (\( F = 235.49, df = 3, P < 0.0001 \)).
EA almost completely suppressed GST activity over whole season. The GST treated with SBT showed irregular fluctuations over the season, whereas the GST treated with DM showed a decreasing trend over the season, but no significant linear relationship was detected (F = 1.68, P > 0.05).

3.4. GST cDNA sequence

A GST cDNA fragment was successfully amplified by using two degenerate primers with oligo(dT) reverse primer. Five clones were sequenced and they contained identical sequences. After the 5′-end of the GST cDNA was obtained, a full GST coding region (700 bp) was amplified from both susceptible and resistant RT-cDNAs using forward primers LLGSTF0 and LLGSTR1 and proof-reading Pfu DNA polymerase. Combined full length cDNA from the susceptible strain contained 889 nucleotides (LLSGST, Fig. 5). BlastX search of the GenBank indicated that the LLSGST was matched to insect GST and its open reading frame (76–723 bp, Fig. 5) encoded a full GST protein sequence (216 amino acid residues). A GST fragment was also sequenced from the resistant strain, and sequence alignment indicated that the cDNA fragment amplified from the resistant strain had one nucleotide difference from the cDNA of the susceptible strain at position 477 (Fig. 5). A cytosine was obtained from the susceptible strain and a thymine from the resistant strain. However, the cDNAs from both strains coded for an identical protein sequence.

3.5. GST genomic DNA sequence

A 700-bp fragment was amplified from genomic DNA of the susceptible strain using primers LLGSTF0 + LLGSTR1. Pair-wise alignment with corresponding cDNA of the same strain showed exactly identical sequences, indicating no intron exists in the corresponding region of the GST gene (Fig. 5).

3.6. Deduced GST

Predicted GST-like protein consisted of 26 positively charged residues (Arg + Lys) and 26 negatively charged residues (Asp + Glu). Deduced GST-like protein had a molecular mass of 23.95 kDa and a theoretical pI of 6.72. It had...
no signal peptide when it was examined using SignalP 3.0 program [30]. Homology search of GenBank using BlaxtP revealed that the deduced 216-residue protein was similar to several insect GSTs which were believed to be responsible for detoxification and insecticide resistance development. Ten GST sequences were downloaded from the GenBank, including the GSTs from the rice brown plant hopper *N. lugens* [27] (GenBank: AAM21563), from the African malaria mosquito *A. gambiae* [31] (GenBank: CAB03593; GenBank: EAA44713), from the greater wax moth *G. mellonella* [32] (GenBank: AAK64362), from the marmalade hoverfly *Episyris palateau* (Vanhaelen, unpublished; GenBank: CAH58743), from *A. dirus* [33] (GenBank: AAG38507), from *D. simulans* (Le Goff et al., unpublished; GenBank: AAK66764), from the housefly *M. domestica* (Syvanen, unpublished; GenBank: CAA43599), from *A. dirus* [34] (GenBank: AAB41104), and from the domestic silkworm *Bombyx mori* [35] (GenBank: BAD60789). Multiple sequence alignment of these ten GSTs with the GST from the tarnished plant bug using the ClustalW method (GapOpen = 10, GapLength = 0.2) exhibited over 34% sequence identity (Fig. 6). Seventy-four residues, including many residues for protein folding and GSH binding, were conserved among all 11 insect GSTs.

Pair-wise sequence comparison showed that the GST from the tarnished plant bug shared 57.4% to 68.1% identity with these ten insect GSTs (Fig. 7A). The phylogenetic tree generated by using DNAStar software (Fig. 7B) showed that the GSTs from Hemiptera and Diptera were clearly separated from the GSTs from Lepidoptera. The GST from the tarnished plant bug was closely related to the GST from the planthopper, both of which were clearly separated from GSTs of flies and mosquitoes (Fig. 7B).

3.7. GST mRNA expression levels

GST mRNA expression levels were compared using real-time PCR. Results showed that the malathion susceptible strain had $2.83 \pm 0.28$, and the resistant strain had $3.60 \pm 1.02$ pg per 25 μl reaction. The resistant strain had 1.3-fold higher GST expression levels than the susceptible strain.

4. Discussion

Glutathione transferases are enzymes involved in detoxification mechanisms of many endogenous and xenobiotic compounds [36]. They act by catalyzing the conjugation of glutathione with numerous potentially toxic compounds to form a S-substituted glutathione. Detoxification of insecticides occurs via a dealkylation in which glutathione is conjugated with the alkyl portion of the insecticide [37], or via a dearylation reaction which is the reaction of glutathione with the leaving group [38].
This catalytic action can be suppressed by many agents. GST inhibitors, such as ethacrynic acid and diethyl maleate, covalently bind and modify GSTs at the N-terminal domain which results in inactivation of the GSTs [39,40]. This phenomenon was confirmed in our bioassay experiments. First, we found that EA and DM significantly increased susceptibility to malathion. Further examination indicated that malathion resistant strain had significantly higher activity than the susceptible strain, and the activity was suppressible. It is possible that the treatment of live insects with GST inhibitors substantially suppressed GSTs. Reduced GST activity in the bugs suppressed or slowed down the detoxification of malathion, which resulted in higher mortality as compared to treatment with malathion only. All these results indicated that GSTs in the tarnished plant bug are involved in malathion detoxification and resistance development.

Although GST in vitro activities were significantly suppressed in both susceptible and resistant strains by inhibitor treatments, it is not clear why treatment of the susceptible strain with GST inhibitors did not significantly increase mortality as was found in the resistant strains. It is likely that inhibition was concentration-dependent on both the inhibitor reaching the target and the abundance of the target. A live insect bioassay and an in vitro enzyme activity assay are two different systems. The enzyme activity assay only measured GST activity with a specific substrate. If susceptible and resistant bugs have identical GST proteins, the inhibitors would have been equally effective in both systems. The discrepancy suggests that additional factors, such as the abundance of GSTs in the live insect, may play a role in the resistance phenotype. Further studies are needed to elucidate the mechanisms underlying GST-mediated resistance in the tarnished plant bug.
non-preferentially suppress the GSTs in both susceptible and resistant strains. Because cloned GST cDNAs encode identical GST proteins in susceptible and resistant strains (Fig. 5), increased enzyme activity in the resistant strain might result from a higher concentration of the GST proteins. A higher concentration of GST proteins could have been generated by elevated GST gene expression, increased GST mRNA stability conferred from nucleotide substitution (Fig. 5), or modification of the upstream regulatory region. These hypotheses must be tested in future studies.

In the bioassay system, the situation was more complicated as compared to an enzyme activity assay using a specific substrate. Many enzymes may be involved in the detoxification of malathion, including GSTs, esterases, and other potential proteins. Although the resistant strain had higher GST activity, presumably conferred from elevated GST gene expression or mRNA stability, GST inhibitors could have depressed the GSTs to a level which was no longer important in detoxifying malathion. This inhibition should have had a larger impact on the resistant strain than on the susceptible strain because relatively greater amounts of GSTs were suppressed in the resistant strain, and subsequently more of the resistant insects died after treatment with a GST inhibitor and malathion. In the susceptible insects, relatively lower amounts of GSTs were suppressed by GST inhibitors, and only a marginal increase in mortality was obtained in our bioassay (Fig. 2). In addition to the role of GSTs, esterases may play another key role in the malathion detoxification and resistance development [3].

From the result in Fig. 2, we can see that treatments of susceptible bugs with GST inhibitors did not increase mortality substantially. It is possible that in the susceptible insects esterases and other detoxification enzymes play a key role and GSTs play a minor role in malathion detoxification, since only a marginal increase of mortality was obtained after the GSTs were suppressed. However, in the resistant insects, both GSTs and esterases may play an important role, because (1) inhibition of GSTs significantly increased non-specifically.
toxicity of malathion and (2) resistant insects tolerated a 5-fold higher dose of malathion (25 vs. 5 μg/vial) and still maintained survival rates similar to susceptible insects after GST inhibitor/malathion treatment.

The malathion resistant strain tended to have higher GST gene expression levels than the susceptible strain. By using real-time PCR, we detected approximately 1.3-fold higher GST gene expression levels in the resistant strain. The resistant strain also had a relative large amount of variation in its GST expression levels than the susceptible strain. This might have been caused by large variation among resistant individuals. Strain impurity and heterozygous genotypes might also have contributed to the large variation among resistant individuals. The ratio would be higher than 1.3-fold if a homogeneous resistant strain could be obtained (though it is difficult) and used for real-time PCR analysis. Another possibility is that several GSTs may exist in the tarnished plant bug. Elevated GST gene expression needs to be further studied by cloning and characterization of other GST members in the tarnished plant bug. Increased transcription in the resistant strain may be an underlying cause of GST-mediated insecticide resistance in the tarnished plant bug. The consistency between bioassay data and enzyme activity assay data supports this hypothesis. This phenomenon was also observed by other researchers who found that higher enzyme activity was usually due to an increase in the amount of one or more GST enzymes, either as a result of gene amplification or more commonly through increases in the transcriptional rate, rather than by qualitative changes in the individual enzymes [41,42]. They further concluded that elevated GST activity was implicated in resistance to at least four classes of insecticides. Other evidence also suggested that the level of expression of GST was a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals [15].

In summary, this study provides information on resistance gene regulation in malathion-resistant strains of the tarnished plant bug. GST inhibitors effectively abolished the resistance and significantly increased susceptibility in resistant strains. In addition, we found that the GST activity increased over the season. This phenomenon was well synchronized with the movement of the bugs into cotton, where they were exposed to organophosphate insecticides. The consistency of reduced sensitivity to malathion and increased GST activity, may have resulted from elevated gene expression, and demonstrated that GSTs were involved in malathion detoxification and resistance development.

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