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GLUTATHIONE S-TRANSFERASES ARE INVOLVED IN MALATHION RESISTANCE DEVELOPMENT IN THE TARNISHED PLANT BUG

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Abstract

The tarnished plant bug, *Lygus lineolaris*, has become a serious pest because of the widespread adoption of Bt cotton and resistance development to synthetic insecticides. Previous reports indicated that the tarnished plant bug developed a metabolic resistance to permethrin and malathion through increased gene expression of a cytochrome P450 and an esterase. Glutathione S-transferase (GST) is another important metabolic enzyme for detoxification and resistance development to malathion. To understand resistance mechanisms and to develop resistance management strategies, we compared GST enzyme activity between susceptible and resistant strains, and examined if the GST inhibitors synergized malathion toxicity through suppression of the GST activity. Results showed that the resistant strain had significantly higher GST enzyme activity than a susceptible strain. GST inhibitors significantly suppressed GST activity and substantially increase susceptibility to malathion in the resistant bugs.

Introduction

Since the introduction of transgenic (Bt) cotton to the Delta region, the tarnished plant bug has become an important pest on cotton. 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. The tarnished plant bug is capable of adapting to different ecosystems due to its wide range of host plant species. In the Mid-south areas, chemical spray with pyrethroid and organophosphate insecticides has been a dominant measure for plant bug control on cotton, which in turn prompted widespread resistance development to many insecticides (Snodgrass 1996a, Snodgrass and Scott 2000). Insecticide resistance in this insect has reduced the effectiveness of chemical control and increased the cost and amount of insecticides needed to control this pest. Zhu and Snodgrass (2003) demonstrated different structures of cytochrome P450 cDNAs and corresponding gene expression differences between pyrethroid susceptible and resistant strains. This P450 gene expression was inducible by pyrethroid treatment.

Several organophosphate insecticides, including malathion, are the major chemicals used for plant bug control. Reduced effectiveness of malathion against plant bug has been noticed in many regions. Malathion resistance is usually associated with increased carboxylesterase activity (Liu and Han, 2003; Wool and Front, 2003; Perez-Mendoza et al., 2000; Smyth et al., 2000; Raghavendra et al., 1998). Single point gene mutation was also observed in many resistant insects (Campbell et al., 1998; Zhu et al., 1999; Smyth et al., 2000). Resistant insects were able to survive malathion treatment by sequestering (Karunaratne and Hemingway, 2001) or metabolizing malathion to non-toxic malathion acids and malaoxon acids (Campbell et al., 1998).

Zhu et al. (2004) showed that more than 11-fold malathion resistance had developed in a natural population collected in Mississippi, which was also resistant (4.5-fold) to the pyrethroid insecticide permethrin. Elevated esterase activity was obtained from the resistant strain, and it was likely that increased esterase gene expression rather than mutation in the malathion resistant strain was the underlying mechanism for the resistance to malathion.

Glutathione S-transferases (GSTs) are detoxification enzymes found in almost all organisms (Sheehan et al., 2001; Ketterer, 2001). 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 (Wilce and Parker, 1994). Several studies have 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 glutathione-dependent route (Oppenoorth et al., 1972). High levels of GST activity have been detected in some resistant insect strains (Motoyama and Dauterman, 1975 and 1977; Ottea and Plapp, 1984;), and the resistance development was correlated with enhanced GST activity and GST dependent insecticide metabolism (Fournier et al., 1987).

Understanding resistance mechanisms is essential to develop strategies for managing the resistance. Because GSTs are capable of catalyzing the secondary metabolism following the oxidation by the cytochrome P450s (Wilce and

Parker, 1994), it is important to understand whether and how the GSTs play a role in resistance development in the tarnished plant bug, which is resistant to both pyrethroid and organophosphate insecticides. In this study, we assessed synergism of GST inhibitors and compared GST enzyme activities between susceptible and resistant strains.

Materials and Methods

Insect: The malathion-susceptible strain was collected from weeds near Crossett, AR (Ashley County). Cotton is not grown near Crossett and plant bugs from this location are very susceptible to most insecticides used on cotton (Snodgrass, 1996b). The resistant strain was originally collected near Mound Bayou (Bolivar County), MS. The field collected adults from Mound Bayou were selected with malathion to produce a test population for enzyme activity assays. Another malathion resistant strain was originally collected in Bruce (Calhoun County), MS. This strain was used for synergist bioassay.

Bioassay and synergist test: A glass-vial bioassay (Snodgrass 1996b) 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 (Sigma, St. Louis, MO) and diethyl maleate (Aldrich, Milwaukee, WI). Acetone was used as solvent, and was included as control. Malathion was applied by pipetting 0.5 ml of the insecticide diluted in acetone into each vial. Each vial was rolled on its side until an even layer of insecticide 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 diethyl maleate (DM) or 400 µg/vial ethacrynic acid (EA) for 24 hour. 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 ^{o}C, and humidity was not controlled. Mortality was determined after 24 h, and adults were considered dead if they were unable to right themselves or walk, or there was no movement when they were prodded. All bioassays had 3 replications for each treatment, and each replication contained 10 vials with 2 adults in each vial. In the first experiment, malathion was used at 25 µg /vial, and resistant bugs were collected from 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.

Enzyme preparation: Individual tarnished plant bugs were homogenized in 340 µl 0.1M sodium phosphate buffer, pH 8.0. Homogenates were then centrifuged at 10,000 ×*g* for 5 minutes at 4 ^{o}C, and the supernatant was used for enzyme analysis. Protein concentrations were determined using the Bradford method. Bovine serum albumin was used to obtain the standard curve.

Glutathione S-Transferase (GST) Assays: To determine glutathione S-transferase activity, microtiter plate assays were conducted using 1-chloro-2,4-dinitrobenzene (CDNB, Aldrich, Milwaukee, WI). Pre-reaction mixture (100 µl) consisted of the following: 8 mM glutathione (Sigma, St. Louis, MO), 10 µl enzyme homogenate, and 0.1 M phosphate buffer pH 8.0. Reaction was started with the addition of 200 µl 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 ^{o}C, for 10 minutes with measurements taken every 15 seconds (Ottea et al., 2000). Inhibitors for glutathione transferase activity were ethacrynic acid (final concentration 0.19 mM), sulfobromophthalein sodium hydrate (Aldrich, Milwaukee, WI) (final concentration 0.01 mM), and diethyl maleate (final concentration of 0.712 mM). Inhibitor concentrations were referred from those of Prapanthadara et al (2000). Inhibitors were added to enzyme homogenate and allowed to incubate at 37^{o}C for 10 minutes before the addition of substrate (Park, 1999).

Seasonal survey of GST activities: Through the months of May to October of 2003, tarnished plant bugs were collected from weeds in Sunflower County, Mississippi. Ten individual adults for each month were tested for levels of GST activity as previously described in duplicate.

Results

Synergism of GST inhibitors: Diethyl maleate (DM) and ethacrynic acid (EA) were selected for bioassay on malathion resistant strain of the tarnished plant bug. In the first experiment, no bugs died 24 hours after treatment with either DM alone (400 µg /vial) or EA alone (400 µg /vial). Malathion alone (25 µg /vial) killed approximate 20% of the bugs. The same concentration of the malathion with DM killed 61.7%±1.7 of treated bugs, and the malathion with EA killed 68.3%±4.4 of treated bugs. Treatment with either GST inhibitor significantly increased efficacy against the tarnished plant bug ($F_{(2)}$=25.55, P<0.05). Addition of both DM and EA increased 24-h mortality

by more than 3-fold. In the second experiment, susceptible bugs showed 41.7±14.2% mortality after 24 h treatment of malathion at 5 µg/vial. Malathion with DM and EA increased mortality to 50±4.9% and 46.7±10.0%. But, the mortality increase was not significant ($F_{(2)}$=0.16, P>0.05). In contrast to the susceptible strain, resistant bugs showed 25±4.9% mortality after treatment of malathion at 25 µg /vial. Similarly as in the first experiment, treatment with either GST inhibitor significantly increased efficacy against the resistant tarnished plant bug ($F_{(2)}$=13.63, P<0.05). Addition of both DM and EA increased 24-h mortality by more than 2-fold.

Comparison of GST activities between S and R strains: Glutathione S-transferase activities were examined *in vitro* using 1-chloro-2,4-dinitrobenzene as GST substrate. 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 resistant strain (57.65) than that in the susceptible strain (31.69) ($F_{(1)}$=39.27, P<0.0001). Inhibitor treatments also significantly suppressed GST activities in both strains ($F_{(3)}$=128.61, 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.

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 substrate. Results demonstrated that without inhibitor the GST activity increased as the season progressed. 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 revealed from the insects collected in October, when the last samples were collected in 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 ($F_{(5)}$=3.52, 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 ($F_{(3)}$=235.49, P<0.0001). EA almost completely suppressed GST activity over whole season. The GSTs treated with SBT showed irregular fluctuation over the season, whereas the GST treated with DM showed decreasing trend over the season, but no significant linear relationship was detected (F=1.68, P>0.05).

Discussion

Glutathione transferases are involved in detoxification of many endogenous and xenobiotic compounds (Fournier et al., 1992). Detoxification of insecticides occurs via a dealkylation in which glutathione is conjugated with the alkyl portion of the insecticide (Oppenoorth et al., 1979), or via a dearylation reaction which is the reaction of glutathione with the leaving group (Chiang and Sun, 1993). This catalytic action can be suppressed by many agents. GST inhibitors, such as ethacrynic acid and diethyl maleate, covalently bind and modify GSTs at N-terminal domain that results in inactivation of the GSTs (Phillips and Mantle, 1993; Ploemen et al., 1993). This phenomenon was confirmed in our bioassay experiments. EA and DM significantly increased susceptibility of the bugs to malathion. Both malathion resistant strains showed two- to three-fold higher mortalities after treatment with EA and DM. Whereas the susceptible strain only received marginal increase in mortality, which was not statistically different from the control. We further examined GST activities *in vitro* using 1-chloro-2,4-dinitrobenzene as substrate. The malathion resistant strain had 1.5-fold higher activity than the susceptible strain. Pre-incubation of GST enzymes with inhibitors significantly suppressed activities by up to 99%. The treatment of live insects with GST inhibitors substantially suppressed GSTs. The reduced GST activity in the bugs then slowed down detoxification of malathion which resulted in higher mortality compared to the malathion only treatment. All these results indicated that GSTs in the tarnished plant bug are involved in the 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 seen in the resistant strains. It is likely that inhibition is concentration-dependent on both inhibitor reaching the target and the abundance of the target. Live insect bioassay and *in vitro* enzyme activity assay are two different systems. The enzyme activity assay only measureed GST activity with a specific substrate. If susceptible and resistant bugs have identical GST proteins, the inhibitors would non-preferentially suppress the GSTs in both susceptible and resistant strains. Because cloned GST cDNAs encode identical GST proteins in susceptible and resistant strains (data not shown), increased enzyme activity in the resistant strain might come from higher concentration of the GST proteins, which is likely to be generated by elevated GST gene expression or increased GST mRNA stability conferred from nucleotide substitution. These hypotheses must be verified in future studies.

In the bioassay system, the situation was more complicated compared to the 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 has higher GST activity, presumably conferred from elevated GST gene expression or mRNA stability, GST inhibitors could have decreased the GSTs to a level which was no longer important for detoxifying malathion. This inhibition should have heavier impact on the resistant strain than on the susceptible strain because relatively greater amount of GSTs were suppressed in the resistant strain, and more of the resistant insects died after treatment with a GST inhibitor and malathion. In the susceptible insects, relatively lower amount of GSTs were suppressed by GST inhibitors, and only marginal increases of mortality were obtained in our bioassay. In addition to the role of GSTs, esterases may play another key role in the malathion detoxification and resistance development as documented by Zhu et al. (2004). In our results, we found that treatment 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, because only marginal increases in mortality were 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 toxicity of malathion and (2) resistant insects could tolerate a five-fold higher dose of malathion (25 vs. 5 µg/vial) and still maintained approximately similar survival rates after GST inhibitor/malathion treatment as susceptible insects.

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