



# The epsilon glutathione S-transferases contribute to the malathion resistance in the oriental fruit fly, *Bactrocera dorsalis* (Hendel)

Xue-Ping Lu, Luo-Luo Wang, Yong Huang, Wei Dou, Chang-Tong Chen, Dong Wei, Jin-Jun Wang \*

Key Laboratory of Entomology and Pest Control Engineering, College of Plant Protection, Southwest University, Chongqing 400716, PR China

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## ABSTRACT

Epsilon glutathione S-transferases (eGSTs) play important roles in xenobiotics detoxification and insecticides resistance in insects. However, the molecular mechanisms of eGSTs-mediated insecticide resistance remain largely unknown in the *Bactrocera dorsalis* (Hendel), one of the most notorious pests in the world. Here, we investigated the roles of eight GST genes which belonged to epsilon class (*BdGSTe1*, *BdGSTe2*, *BdGSTe3*, *BdGSTe4*, *BdGSTe5*, *BdGSTe6*, *BdGSTe7* and *BdGSTe9*) in conferring malathion resistance in *B. dorsalis*. Adult developmental stage-, sex- and tissue-specific expression patterns of the eight eGST genes were analyzed via quantitative reverse transcription PCR. The results showed that *BdGSTe2*, *BdGSTe3*, *BdGSTe4* and *BdGSTe9* were abundant in the midgut, fat body and Malpighian tubules. Notably, *BdGSTe2*, *BdGSTe4* and *BdGSTe9* were significantly overexpressed in a malathion-resistant (MR) strain of *B. dorsalis* compared to the malathion-susceptible (MS) strain. Functional expression and cytotoxicity assays showed significantly higher malathion detoxification capabilities in *BdGSTe2*-, *BdGSTe3*-, *BdGSTe4*- and *BdGSTe9*-expressing Sf9 cells compared to the parental and green fluorescent protein (GFP)-expressing Sf9 cells. Moreover, malathion susceptibility in MS adults was increased 30%, 14%, and 33% when *BdGSTe2*, *BdGSTe3* and *BdGSTe4* mRNA levels were repressed by RNA interference (RNAi)-mediated knock-down, respectively. Taken together, overexpression of the isoforms of eGSTs, including *BdGSTe2*, *BdGSTe4*, and particularly, *BdGSTe9* plays an important role in the malathion resistant development in *B. dorsalis*.

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## 1. Introduction

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a superfamily of multifunctional enzymes commonly found in both prokaryotes and eukaryotes. They occur as cytosolic, microsomal and mitochondrial GSTs based on their distribution within the cell, but mitochondrial GSTs have not been found in insects (Li et al., 2009). In insects, cytosolic GSTs have been classified as sigma, theta, omega, zeta, and insect specific delta and epsilon, on the basis of whether the GSTs share  $\geq 40\%$  amino acid sequence similarity. Among these, delta and epsilon are the two largest classes of insect GSTs, and they are involved in the detoxification of insecticides (Ranson and Hemingway, 2010). The omega, sigma, theta and zeta classes are widely distributed and may play cellular housekeeping roles, but their physiological functions are unclear (Ranson and Hemingway, 2010).

GSTs play important roles in phase II detoxification of both xenobiotics and endogenous compounds by inactivation of the toxic products of oxygen metabolism. For example, GSTs have effects, at several different stages, in protection against oxidative stress generated by reactive oxygen species (ROS), including hydrogen peroxide, superoxide anions

and hydroxyl radicals, from the aerobic respiration (Ranson and Hemingway, 2010). GSTs are also involved in the direct metabolism of xenobiotic compounds such as organophosphorous insecticides (OPs) and DDT (Wei et al., 2001; Riveron et al., 2014). Specifically, GSTs can catalyze the conjugation of reduced glutathione (GSH) to electrophilic centers of toxic compounds, including insecticides, arene oxides, quinones, and  $\alpha$ ,  $\beta$ -unsaturated carbonyl compounds, making them more water-soluble and thus easier to rapidly excrete from the body (Enayati et al., 2005; Hayes et al., 2005; Riveron et al., 2014).

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is an important agricultural pest throughout India, East Asia and the Pacific (Drew and Hancock, 1994; Clarke et al., 2005). It causes damage by ovipositing inside  $> 250$  host plant species, especially economically important fruit and vegetable crops. Damage from oviposition and larval feeding increases the rate of fruit abscission and decreases fruit quality (Fletcher, 1987; Stephens et al., 2007). The use of chemical insecticides remains a primary control method because physical, biological and agricultural controls are less effective. OPs have been used for *B. dorsalis* control since 1952 and continue to play a major role in this pest control (Vontas et al., 2011; Casida and Durkin, 2013). However, *B. dorsalis* populations have evolved high levels of OP resistance (Hsu and Feng, 2000). A better understanding of the molecular mechanisms of insecticide resistance can help us to develop novel potential practical strategies for resistance management.

\* Corresponding author at: College of Plant Protection, Southwest University, Chongqing 400716, PR China. Tel.: +86 23 68250255; fax: +86 23 68251269.

E-mail addresses: [jjwang7008@yahoo.com](mailto:jjwang7008@yahoo.com), [wangjinjun@swu.edu.cn](mailto:wangjinjun@swu.edu.cn) (J.-J. Wang).

Elevated GSTs activity was considered to be involved in OP resistance (Lewis, 1969; Ku et al., 1994). The mode of detoxification of OPs, verified in the diamondback moth (*Plutella xylostella* L.), and housefly (*Musca domestica* L.) by recombinant GST enzymes, is mainly via an O-dealkylation or O-dearylation reaction (Huang et al., 1998; Wei et al., 2001). GSTs also play an important role in phase II detoxification of OPs. In insects, cytochrome P450s can detoxify OPs to the non-toxic phosphorothionate form and the insecticidal form. GSTs can sequester OPs and/or detoxify the secondary metabolism of lipid peroxidation products induced by OPs (Li et al., 2009). However, studies on the biochemical mechanisms of insecticide resistance showed GSTs were not involved in malathion resistance in *B. dorsalis* based on the studies of synergists on insecticide toxicity and variation of the overall GST activity (Hsu et al., 2004a; Hsu et al., 2004b). GSTs, as one of three major detoxifying enzymes, were generally considered to be important for OPs resistance in many other insect species, so whether GSTs were related to malathion resistance in *B. dorsalis* deserves our investigation.

In our previous study, we identified 17 GST genes from the transcriptomic databases of *B. dorsalis*, and analyzed their developmental and tissue-specific expression patterns (Hu et al., 2014). In this study, to better understand the molecular mechanism of GSTs-mediated malathion resistance in *B. dorsalis*, a series of experiments employing biochemical and molecular approaches were conducted. Developmental and tissue-specific expression patterns were analyzed more detailed. Then, the expression of eight epsilon GSTs (eGSTs) was compared between malathion-susceptible (MS) and -resistant (MR) strains of *B. dorsalis*. Malathion sensitivity of Sf9 cells expressing recombinant eight eGSTs was determined. RNA interference (RNAi) was used to evaluate the metabolic roles of GSTs to malathion sensitivity. Taken together, we clarified the function of the eight eGSTs in *B. dorsalis* malathion resistance.

## 2. Materials and methods

### 2.1. Insect

The susceptible strain of *B. dorsalis* (MS) was originally collected from Guangdong province in 2008 and subsequently maintained in the Key Laboratory of Entomology and Pest Control Engineering, Southwest University, Chongqing, China. A malathion-resistant strain (MR) was obtained through repeated malathion exposure of *B. dorsalis* for 27 generations. Adult bioassays were carried out using standard methods as previously described (Wang et al., 2015). The resistance ratio of the MR strain to malathion compared to the MS strain was approximately 21-fold. Both of the strains were reared in a temperature controlled room at  $27 \pm 1$  °C,  $70 \pm 5\%$  relative humidity, and a 14:10 (L: D) photoperiod. Larvae were fed on an artificial diet consisting of sucrose, yeast power, wheat and corn flour. Adults were provided with artificial diets containing yeast power, honey, sucrose, vitamin C, and water (Shen et al., 2010).

### 2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and treated by RQ1 RNase-Free DNase (Promega, Madison, WI, USA) to digest the genomic DNA based on manufacturer protocol. Concentration, quality, and purity of RNA were measured at 260 nm and 280 nm using a NanoVue UV-Vis (GE Healthcare Bio-sciences, Uppsala, Sweden), and RNA integrity was checked by 1.0% agarose gel electrophoresis. Complementary DNA was synthesized using 1 µg of total RNA with the PrimeScript<sup>TM</sup> First-strand synthesis system (Takara, Dalian, China) for RT-qPCR following manufacturer protocol.

### 2.3. Quantitative reverse transcription PCR (RT-qPCR)

For adult stage-specific gene expression studies, total RNA was isolated from whole bodies of *B. dorsalis* daily for 8 days following adult emergence, using TRIzol RNA isolation kit (Invitrogen Life Technologies, Carlsbad, CA, USA). Three independent replicates were run for each day-old adult stage. For tissue-specific gene expression studies, total RNA was also isolated using TRIzol total RNA isolation kit (Invitrogen Life Technologies, Carlsbad, CA, USA) from each of seven different body parts or tissue samples, including head, thorax, abdomen, midgut, Malpighian tubules, fat body, and reproductive organ, dissected from 3- to 5-day-old adult females or males. Gene expression profile analysis was performed on a Stratagene Mx3000P system (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Promega, Madison, WI, USA). The total reaction volume was 20 µl including 10 µl SYBR Green Supermix, 7 µl nuclease free water, 1 µl reverse primer, 1 µl forward primer (primers were the same as Hu et al. (2014)) and 1 µl cDNA. The PCR method was 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The melting curve was recorded at the end of procedure in 60–95 °C to ensure the specificity of each pair of primers.  $\alpha$ -Tubulin (GU269902) was used as an internal reference gene based on previous evaluations (Shen et al., 2010).

### 2.4. Functional expression

All recombinant virus expressions in *Spodoptera frugiperda* Sf9 cells were performed using the Bac-to-Bac baculovirus expression system (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, gene-specific primers (Table S1) were used to amplify the open reading frames (ORF) of the epsilon GSTs cDNA. PCR was carried out as follows: 98 °C of initial incubation for 2 min, subsequently followed by 35 cycles of 98 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; finally 72 °C of final extension for 10 min. The PCR products were sub-cloned in pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5 $\alpha$  chemically competent cells (TransGen Biotech, Beijing, China). Positive clones were confirmed by nucleotide sequencing (Invitrogen, Shanghai, China). The confirmed PCR products were inserted into the pFastbac<sup>TM</sup> HT A vector (Invitrogen Life Technologies, Carlsbad, CA, USA), and the vector containing the GFP gene was used to produce a virus control. After the recombinant baculovirus DNA was constructed, the transfection was carried out. Sf9 cells ( $2 \times 10^6$  cells/ml) in 25 ml of serum-free conditions (SF-900 II SFM, Invitrogen, Carlsbad, CA, USA) were infected in suspension at 27 °C and 100 rpm. The culture medium of baculovirus-infected cells was harvested 72 h after infection. Subsequently, the recombinant baculoviruses containing either eGSTs or GFP cDNA were collected, respectively. Cells were obtained by centrifugation at  $2000 \times g$  for 10 min and were resuspended in 5 ml 0.05 M PBS (pH 8.0) containing 0.1% Triton X-100, 0.5 M NaCl and 0.05% Tween 20. The homogenate was centrifuged at  $10,000 \times g$  for 10 min and the supernatant was stored at  $-80$  °C for further study.

### 2.5. Enzymatic assay

GSTs enzyme activity was assayed using 1-chloro-2, 4-dinitrobenzene (CDNB, Shanghai Chemicals, Shanghai, China) and reduced glutathione (GSH, Sigma-Aldrich, St. Louis, MO, USA) as standard substrates according to Habig et al. (1974) with slight modifications. The assays were conducted in 96-well microtiter plates with 100 µl CDNB (0.6 mM) and 100 µl GSH (6 mM) and were incubated for 20 min at 28 °C. Subsequently 100 µl enzyme solutions were added to individual wells and absorbance was determined by a microplate reader (Bio-Rad, Hercules, CA, USA) at 340 nm and 28 °C for 5 min. The protein content of samples was measured using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard.

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