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# Functional characterization of an α-esterase gene involving malathion detoxification in *Bactrocera dorsalis* (Hendel)

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ABSTRACT

Extensive use of insecticides in many orchards has prompted resistance development in the oriental fruit fly, *Bactrocera dorsalis* (Hendel). In this study, a laboratory selected strain of *B. dorsalis* (MR) with a 21-fold higher resistance to malathion was used to examine the resistance mechanisms to this organophosphate insecticide. Carboxylesterase (CarE) was found to be involved in malathion resistance in *B. dorsalis* from the synergism bioassay by CarE-specific inhibitor triphenylphosphate (TPP). Molecular studies further identified a previously uncharacterized α-esterase gene, *BdCarE2*, that may function in the development of malathion resistance in *B. dorsalis* via gene upregulation. This gene is predominantly expressed in the Malpighian tubules, a key insect tissue for detoxification. The transcript levels of *BdCarE2* were also compared between the MR and a malathion-susceptible (MS) strain of *B. dorsalis*, and it was significantly more abundant in the MR strain. No sequence mutation or gene copy changes were detected between the two strains. Functional studies using RNA interference (RNAi)-mediated knockdown of *BdCarE2* significantly increased the malathion susceptibility in the adult files. Furthermore, heterologous expression of *BdCarE2* combined with cytotoxicity assay in Sf9 cells demonstrated that BdCarE2 could probably detoxify malathion. Taken together, the current study bring new molecular evidence supporting the involvement of CarE-mediated metabolism in resistance development against malathion in *B. dorsalis* and also provide bases on functional analysis of insect α-esterase associated with insecticide resistance.



## 1. Introduction

Insecticide resistance has evolved in an increasing numbers of insects, which is gradually becoming a major limiting factor for effective pest management, therefore results in huge economical losses and ecological problems. At the biochemical levels, resistance to insecticides typically involves an increased metabolic capability by detoxification enzymes along with a decreased target site sensitivity [1]. In the last two decades, there has been a dramatic progress in investigating the underlying molecular mechanisms of insecticide resistance [2]. Studies on many species of insects have frequently suggested the role of carboxylesterases (CarEs) in the metabolic resistance to organophosphate (OP) insecticides [3]. Nevertheless, much in contrast to the generally important role of CarEs, research on the molecular basis of CarEs-mediated resistance, including identifying the function of particular resistance-related genes, is quite limited [4].

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), is one of the most economically important and widespread pests in the world. For more than 50 years, extensive applications of insecticides, particularly OP-based toxicant lures, are the main method to control this pest [5]. In recent years, resistance monitoring of this pest found that *B. dorsalis* has evolved resistance to many OP insecticides commonly used for its control, such as malathion, diazinon, and naled, which therefore resulted in destructive outbreaks of *B. dorsalis* in southern China including Taiwan as well as Hawaii of USA [6–8]. Given the ongoing extensive use of OP insecticides in controlling *B. dorsalis*, the trend for developing more severe OP resistance in *B. dorsalis* is very likely to continue. Furthermore, with the whole genome sequencing completed recently, *B. dorsalis* has gradually become an ideal model organism for research on insecticide resistance [9]. Thus, investigating the underlying mechanisms of resistance is of great significance for maintaining insecticide efficacy, developing new insecticides and novel resistance management strategies.

In most cases reported so far, the existence of two molecular mechanisms for OP resistance was identified in *B. dorsalis*, including decreased target site sensitivity and CarE-mediated hydrolysis/sequestration [8,10–12]. The first mechanism involves modification of target site sensitivity. Alternations in the *ace* gene encoding acetylcholinesterase (AChE)

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including three point mutation (I214V, G488S, and Q643R) were found in the OP-resistant lines of *B. dorsalis* [12–14]. The second mechanism was investigated in our previous study showing that overexpression of two α-esterase genes could effectively detoxify or sequester the insecticide away from its target site [10]. In our current study, the resistance to malathion of a laboratory selected strain (MR) of *B. dorsalis* is confirmed and gradually elevated after 37 generations of resistance selection, and now it is at 21-fold resistance when compared to the relative susceptible (MS) strain. It is interesting to further investigate the differences in the resistance mechanisms imposed by different levels of resistance in *B. dorsalis*.

In this study, the synergism bioassays with CarE specific inhibitor triphenylphosphate (TPP) in both MR and MS strains were firstly conducted to identify the role of CarE during the resistance development in *B. dorsalis*. Subsequently, to better understand the underlying molecular mechanism of CarE-mediated malathion resistance in *B. dorsalis*, a series of experiments employing molecular approaches was conducted. The transcriptional profiles, deduced amino acid sequences and gene copy number of a previously unidentified CarE gene, *BdCarE2*, were compared between the MR and MS strains. We further analyzed and characterized the developmental- and tissue-dependent expression patterns of *BdCarE2*. To evaluate its possible roles in malathion metabolic resistance, heterologous expression combined with cytotoxicity assays in Sf9 cells overexpressing *BdCarE2*, along with RNAi bioassays downknocking this gene in adult flies, were performed. This study presents the detailed characterizations of this CarE gene as well as some evidence bearing on its possible involvement in malathion resistance, which could expand our understanding of the important role of esterase-mediated resistance in *B. dorsalis* and provide information to annotate similar proteins whose functions are still unknown.

## 2. Materials and methods

### 2.1. Insect

Two strains of *B. dorsalis*, malathion resistant (MR) and susceptible (MS) strains, were used in this study. The MS strain was originally collected from fields in Guangzhou, Guangdong Province, China, in 2009, and this strain was reared with artificial diet without any exposure to malathion or any other insecticides. The MR strain of *B. dorsalis* was originally collected in 2008 from fields in Guangzhou, Guangdong Province, China. This MR strain has been selected for malathion resistance in the laboratory by topical application as previously described [10]. These two geographically very close populations (nearly 40 miles away from each other) were considered to have similar genetic backgrounds. MR strain has developed a 21-fold resistance to malathion after 37 generations of selection when this study was carried out. Insects were reared in a temperature-controlled incubator at 27 ± 0.5 °C, 70 ± 5% relative humidity with a photoperiod of 14 h light:10 h dark, and with artificial diet as previously described [10].

### 2.2. Insecticide bioassay and synergistic experiment

The susceptibilities of *B. dorsalis* to malathion were evaluated using the micro-drop method as previously described [11]. Malathion was dissolved and diluted to six different concentrations with acetone based on our preliminary tests. The test insects were anesthetized by exposure to a low temperature (−20 °C) for less than 1 min. Subsequently, a 0.5 μl droplet of malathion solution was applied topically onto the thoracic tergum of the fly with a hand micro-applicator (Hamilton, Reno, NV). In the controls, flies were treated with a 0.5 μl droplet of acetone alone. The treated insects were kept individually in a Petri dish (d = 15 cm), and about 30 flies were used per replicate, and three replicates were run per dosage. Mortality was assessed 24 h after exposure to malathion and insects were considered dead if they did not move after stimulation with a camel hairbrush. For the synergism bioassays, the synergist TPP was dissolved in acetone and then was mixed with malathion at a ratio of 3:1 in volume. The mixtures were diluted to six different concentrations with acetone. A droplet of 0.5 μl of the mixture was applied onto the thoracic tergum of the MR and MS strain flies using a hand micro-applicator as mentioned above.

### 2.3. RNA/DNA extraction

Total RNA was isolated from the MR and MS adults, from different developmental stages (eggs; 3rd instar larvae; late pupae; and adults on days 1, 3, 5 and 7), from various body parts or tissues (head, thorax, abdomen, midgut, fat body, and Malpighian tubules) of adults, as well as from treated adults (ds*BdCarE2*-, $H_2O$- and dsGFP-injection), using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol, and genomic DNA was eliminated by treating with DNase I (Promega, Madison, WI) when isolating the RNA. In addition, genomic DNA was also isolated from the MS and MR adults with the Genomic DNA Purification Kit (Promega, Madison, WI) following the manufacturer's protocol. The quality and quantity of DNA/RNA were ascertained with a Nanovue UV–Vis spectrophotometer (GE Healthcare, Fairfield, CT).

### 2.4. Identification and phylogenetic analysis

The open reading frame (ORF) of *BdCarE2* cDNA was amplified separately from the MR and MS strains. PCR was carried out using gene-specific primers (Table 1) and PrimeSTAR® HS DNA Polymerase (TaKaRa Bio, Dalian, China) with the following procedure: 98 °C of initial incubation for 2 min; followed by 35 cycles of 98 °C for 15 s, 61 °C of 15 s, and 72 °C for 1–2 min; and 72 °C of final extension for 10 min. The purified amplicons were cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and transformed into *Escherichia coli* DH5α (TransGen Biotech, Beijing, China). The transformants were selected with Luria–Bertani (LB) agar plates containing 0.1% ampicillin, and positive clones were sequenced (Invitrogen, Shanghai, China). Blastp in the non-redundant protein sequences (nr) database of NCBI (http://www.ncbi.nlm.nih.gov) was conducted in the homology analysis. Sequence alignments were performed with DNAMAN v.6.03 (Lynnon Biosoft, USA). Conserved domains were predicted using the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/cdd). The signal peptide was predicted by the SignalP 4.1 server program (http://www.cbs.dtu.dk/services/SignalP).

A phylogenetic tree was constructed with all the 35 identified CarE genes from *Drosophila melanogaster* and two published CarE genes from *B. dorsalis* to classify *BdCarE2* based on the classification scheme for insect CarEs as previously described [10,15]. ClustalW [16] and MEGA5.0 [17] were used to construct the phylogenetic tree with the neighbor-joining method. Branch support was estimated by bootstrap analysis with 1000 replicates. All the 35 *D. melanogaster* CarE sequences for phylogenetic tree construction were downloaded from FlyBase (http://flybase.org/).

### 2.5. Reverse transcription quantitative PCR (RT-qPCR)

The RT-qPCR was performed in a final volume of 10 μl containing 400 ng of cDNA templates, 10 μl of SYBR Green Supermix, 10 pM of each gene specific primers (Table 1), and double-distilled water. The reaction was conducted on a Stratagene Mx3000P System (Bio-Rad, Hercules, CA) using iQ SYBR Green Supermix (Promega, Madison, WI) under the following cycling regime: 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A dissociation curve from 60 to 95 °C was included at the end of each RT-qPCR run to verify the specificity of the amplicon for each primer pair. *α-Tubulin* (GU269902) was used as an internal reference gene as evaluated by our previous study [18]. Gene copy number was determined using the same experimental design with genomic DNA as the template prepared individually from 5-d-old adult fly from each of the MR and MS strains and *ace* was used as a