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Multiple resistance to pirimiphos-methyl and bifenthrin in *Tribolium castaneum* involves the activity of lipases, esterases, and laccase2☆

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ABSTRACT

Several recent studies have elucidated the molecular mechanisms that confer insecticide resistance on insect pests. However, little is known about multiple resistance in red flour beetle (*Tribolium castaneum*) at molecular level. The multiple resistance is characterized as resistance to different classes of insecticides that have different target sites, and is mediated by several enzymatic systems. In this study, we investigated the biochemical and molecular mechanisms involved in multiple resistance of *T. castaneum* to bifenthrin (pyrethroid [Pyr]) and pirimiphos-methyl (organophosphate [Org]). We used artificial selection, biochemical and *in silico* approaches including structural computational biology. After five generations of artificial selection in the presence of bifenthrin (F5Pyr) or pirimiphos-methyl (F5Org), we found high levels of multiple resistance. The hierarchical enzymatic cluster revealed a pool of esterases (E), lipases (LIPs) and laccase2 (LAC2) potentially contributing to the resistance in different ways throughout development, after one or more generations in the presence of insecticides. The enzyme-insecticide interaction network indicated that E2, E3, LIP3, and LAC2 are enzymes potentially required for multiple resistance phenotype. Kinetic analysis of esterases from F5Pyr and F5Org showed that pirimiphos-methyl and specially bifenthrin promote enzyme inhibition, indicating that esterases mediate resistance by sequestering bifenthrin and pirimiphos-methyl. Our computational data were in accordance with kinetic results, indicating that bifenthrin has higher affinity at the active site of esterase than pirimiphos-methyl. We also report the capability of these insecticides to modify the development in *T. castaneum*. Our study provide insights into the biochemical mechanisms employed by *T. castaneum* to acquire multiple resistance.

1. Introduction

Insecticide resistance is considered a model of abbreviated natural selection process because the extensive usage of chemicals has accelerated the accumulation of factors needed for the survival of insects (Zhu et al., 2013). Insects can develop resistance to more than one insecticide simultaneously. In cross resistance, an insect can become resistant to two insecticides from different classes, since they have the same target site and can be metabolized by the same enzyme. For instance, the cytochrome P450 6M2 (CYP6M2) promotes cross resistance to DDT and pyrethroid in *Anopheles gambiae sensu stricto* (Mitchell et al., 2012). In contrast, multiple resistance to different classes of insecticides that have different target sites is mediated by several enzymatic systems including esterases, CYPs and glutathione-S-transferases (GSTs) as reported in the case of *Anopheles culifacies* populations in Sri Lanka, which are resistant to DDT, organophosphate (malathion) among others (Perera et al., 2008).

Abbreviations: E1, esterase1; LIPs, lipases; LACs, laccases; Pyr, pyrethroid; Org, organophosphate; F1Pyr and F5Pyr, beetles selected throughout one or five generations in the presence of bifenthrin (pyrethroid), respectively; F1Org and F5Org, beetles selected throughout one or five generations in the presence of pirimiphos-methyl (organosphosphate) respectively; Maf, muscle aponeurosis fibromatosis; CncC, cap 'n' collar C; XRE, xenobiotic response elements; EcRe, Ecdysone Response Element; NC2, Nuclear receptor subfamily 2 factors; JHE, juvenile hormone esterase; TcJHE, juvenile hormone esterase of *Tribolium castaneum*; CYP, cytochrome P_{450}; GST, glutathione-S-transferase; LC, lethal concentration; LcE7, α-esterase7 of *Lucilia cuprina*; CPB, Colorado potato beetle; JH, juvenile hormone; SEM, scanning electron microscope; SPE, soluble peptide expression; RP, ribosomal protein.

☆ Data deposition: The model of juvenile hormone esterase of *Tribolium castaneum* has been deposited in Protein Model DataBase (PMDB), (PMDB ID: PM0080733)

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The red flour beetle (*Tribolium castaneum*) (Herbst, 1797) (Coleoptera: Tenebrionidae), has shown resistance to 33 different active ingredients, with detected resistance to all main classes of insecticides used in its control (http://www.pesticideresistance.org/). *T. castaneum* infests stored grain, flour, cereal products, and putrefying wood among other products (Brown et al., 2009; Park et al., 2008) causing substantial loss annually; hence, it is considered one of the most destructive insect pests of stored grain (Chen et al., 2015). This beetle has been widely studied because of its short generation time, ease of culturing, global distribution and its critical phylogenetic position (Denell, 2008; Zou et al., 2007). It is also the first beetle whose whole genome has been sequenced (Richards et al., 2008). In beetles such as *T. castaneum,* as well as in other insects, esterases (EC 3.1.1.1) are among the main isoenzymes involved in the mechanism underlying insecticide resistance to organophosphates, carbamates and pyrethroids (Haubruge et al., 2002; Saavedra-Rodriguez et al., 2014) *via* genetic amplifications, mutations in coding sequences and over expression. They can act by hydrolyzing or by sequestering the insecticides (ffrench-Constant et al., 2004; X. Li et al., 2007; Silva and Lapenta, 2011). In addition to the esterases, lipases (LIPs) (EC 3.1.1.3) have the capability to catalyze many hydrolytic and synthetic reactions involving ester bond in the synthetic and natural substrates (Song et al., 2008). They have been associated with pyrethroid resistance (Araújo et al., 2008).

The machinery of gene regulation underlying xenobiotic detoxification in invertebrates and vertebrates can be linked to transcription factors that respond to xenobiotic stress (Kalsi and Palli, 2015; Xie et al., 2000). The transcription factors, Xenobiotic Response Elements (XRE) and Ecdysone Response Element (EcRE), are implicated in regulation of cytochrome P_{450} (CYP) *6Z8* and of juvenile hormone esterase (*CCEjhe1F*) genes. These genes are involved in pyrethroid and organophosphate detoxification in *Aedes aegypti* (Poupardin et al., 2008). The transcription factors, Muscle aponeurosis fibromatosis (Maf) and cap 'n' collar C (CncC), promote overexpression of CYP6BQ9 and other CYPs in the CYP6BQ cluster associated with deltamethrin resistance in *T. castaneum* (Kalsi and Palli, 2015).

In addition to enzymatic systems involved in metabolic resistance, molecular mechanisms that reinforce the cuticle, thus preventing the penetration of insecticides into resistant insects, have also been reported (Pan et al., 2009; Puinean et al., 2010). The most important gene in cuticle sclerotization process in *T. castaneum* is *Laccase2* (*Lac2*) (Arakane et al., 2005). *Lac2* was found to be overexpressed in *Culex pipiens pallens* resistant to fenvalerate (pyrethroid) (Pan et al., 2009). The authors suggested that LAC2 (EC 1.10.3.2) could reinforce the cuticle, thus decreasing the insecticide penetration in the organism.

Recent studies have revealed the molecular mechanisms conferring resistance on *T. castaneum* to deltamethrin (Kalsi and Palli, 2015; Zhu et al., 2010), phosphine gas (Chen et al., 2015; Schlipalius et al., 2012) and pirimiphos-methyl (Mujeeb and Shakoori, 2012). However, there is little information at molecular level about multiple resistance in *T. castaneum*, specifically to pirimiphos-methyl, an irreversible inhibitor of acetylcholinesterase (Colović et al., 2013), as well as to bifenthrin, a modulator of sodium channel activity (Cao et al., 2014). Identifying the presence and functionality of transcription factors and their binding sites that render the insects resistant to different classes of insecticides can help to understand the mechanisms underlying the regulation of genes coding for proteins involved in the detoxification of insecticides (Kalsi and Palli, 2015).

In the current study, we have used an artificial selection approaches to explore the capability of *T. castaneum* to develop high levels of multiple resistance. Particularly after five generations under selection in the presence of pirimiphos-methyl (organophosphate) or bifenthrin (pyrethroid), which belong to two major classes of insecticides employed worldwide (Barr et al., 2010; Ding et al., 2015; Oulhote and Bouchard, 2013; Quirós-Alcalá et al., 2014). Next, we employed biochemical and *in silico* approaches to elucidate the involvement of esterases, LIPs, and LAC2 in multiple resistance to pirimiphos-methyl and bifenthrin. We focused on these enzymatic families because they have been associated with high levels of resistance previously in pest insects including *T. castaneum* (Araújo et al., 2008; ffrench-Constant et al., 2004; Mujeeb and Shakoori, 2012; Pan et al., 2009; Silva and Lapenta, 2011).

We identified a pool of esterases, LIPs, and LAC2 that potentially contribute to resistance in different ways throughout the ontogenetic development after one and five generations under selection in the presence of insecticides. The enzyme-insecticide interaction network based on the results of relative activity in gel indicated E2 (esterase2), E3, LIP3, and LAC2 as enzymes potentially required for multiple resistance phenotype. *In silico* analysis of 1-kb upstream of *Lips*, *Esterases*, and *Lacs* genes suggested that they may be regulated by transcription factors that respond to xenobiotic stress, indicating a possible reciprocal response to pirimiphos-methyl and bifenthrin. These insecticides were also capable of changing the expression of soluble peptides throughout development, as well as morphological features. Finally, kinetic analysis of total esterases from adult F5Pyr and F5Org revealed that the esterases may act by sequestering pirimiphos-methyl and especially bifenthrin. We paid special attention to esterases and conducted phylogenetic and structural analysis, followed by molecular docking simulations. Our simulations indicated that organophosphates (pirimiphos-methyl and malathion) and specially pyrethroids (bifenthrin and deltamethrin) show high affinity at the active site of juvenile hormone esterase of *T. castaneum* (TcJHE), providing further evidences of the potential role of esterases within development of multiple resistance by sequestering insecticides.

2. Material and methods

2.1. Establishment of susceptible beetles and artificial selection using pirimiphos-methyl or bifenthrin

For establishing resistant and susceptible strains of *T. castaneum*, a wild strain (n = ~6000) was collected from Iguatemi city, Brazil, in July 2014. These insects were maintained for two generations in the laboratory without exposure to any insecticide, fed with white flour, and were considered the susceptible population after perform preliminary experiments using bifenthrin and pirimiphos-methyl to determine the status of the beetles collected. Next, this susceptible population was separated into three groups. The first group remained on white flour feed without exposure to any insecticide (Lab-S). The second group was artificially selected by feeding throughout the entire life cycle for one or five generations, white flour containing commercial insecticide Talstar EC 100® (FMC Corporation, Philadelphia, PA, USA) (bifenthrin 100 μL active ingredient/mL [a.i./mL]) at a concentration of 30 μL a.i./100 g flour. The third group was selected artificially by continuous feeding throughout the whole life cycle for one or five generations with white flour containing commercial insecticide Actellic 500 EC® (Syngenta International, Basel, GA, Switzerland) (pirimiphos-methyl 480 μL a.i./mL), in the following concentrations: F1Org 0.2 μL a.i./100 g flour, F2Org 0.1 μL a.i./100 g flour; and from F3 to F5Org 0.05 μL a.i./100 g flour. For some generations of selection with pirimiphos-methyl, lower concentrations were used because of the severe damage this insecticide caused to the offspring (low emergence ratio and high level of mortality).

After one or five generations under selection in the presence of pirimiphos-methyl or bifenthrin, 3° instar larvae, 4-day old pupae and adults older than 3-days were used in biochemical experiments. F1Pyr, F1Org, and Lab-S were also prepared for scanning electron microscopy (SEM). To expedite the life cycle of the beetles and preventing cannibalism, 100–150 beetles per 100 mL jar were maintained in the dark, at 36 °C, 65 ± 3% relative humidity and flour *ad libitum* (The Beetle Book: http://wwwuser.gwdg.de/~gbucher1/tribolium-castaneum-beetle-book1.pdf). The bioassays were conducted directly on F1 and F5. The time allowed to beetles recover was only the time necessary to prepare them to the experiments. To evaluate whether the