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Elevated expression of esterase and cytochrome P450 are related with lambda–cyhalothrin resistance and lead to cross resistance in *Aphis glycines* Matsumura

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ABSTRACT

A resistant strain of the *Aphis glycines* Matsumura (CRR) has developed 76.67-fold resistance to lambda–cyhalothrin compared with the susceptible (CSS) strain. Synergists piperonyl butoxide (PBO), S,S,S-Tributyltrithiophosphate (DEF) and triphenyl phosphate (TPP) dramatically increased the toxicity of lambda–cyhalothrin to the resistant strain. Bioassay results indicated that the CRR strain had developed high levels of cross-resistance to chlorpyrifos (11.66-fold), acephate (8.20-fold), cypermethrin (53.24-fold), esfenvalerate (13.83-fold), cyfluthrin (9.64-fold), carbofuran (14.60-fold), methomyl (9.32-fold) and bifenthrin (4.81-fold), but did not have cross-resistance to chlorfenapyr, imidacloprid, diafenthiuron, abamectin. The transcriptional levels of *CYP6A2-like*, *CYP6A14-like* and *cytochrome b-c1* complex subunit 9-like increased significantly in the resistant strain than that in the susceptible. Similar trend were observed in the transcripts and DNA copy number of *CarE* and *E4* esterase. Overall, these results demonstrate that increased esterase hydrolysis activity, combined with elevated cytochrome P450 monooxygenase detoxicatication, plays an important role in the high levels of lambda–cyhalothrin resistance and can cause cross-resistance to other insecticides in the CRR strain.

1. Introduction

The soybean aphid (*Aphis glycines* Matsumura) is a predominant insect pest of soybean in Asia and North America. The soybean aphid colonizes fields during early vegetative stages of soybean and increases population densities through the reproductive stages of the crop, leading to up to 40% yield losses in outbreak years [1]. Heavy infestations of soybean aphid can cause severe damage to plant health and reduce seed yield through direct feeding, leaf distortion, reduced pod set, plant virus transmission and the buildup of black sooty mold on the secreted honeydew [2,3]. Furthermore, the cost of soybean production increases because of extensive use of insecticide. In China, broad-spectrum insecticides such as pyrethroids were widely used for the control of soybean aphid. However, the risk of development of resistance and possible resistance mechanisms is still unknown in soybean aphid.

Field soybean aphid populations have developed resistance to organophosphates, and some field populations were not very sensitive to deltamethrin in resistance monitoring during 2009–2011 in Heilongjiang province of China [4,5]. After being maintained in laboratory for over 25 generations without exposure to any insecticides, a field originated soybean aphid population is still less sensitive to fenvalerate [6]. Due to the extensive use of pyrethroids, soybean aphid may develop resistance rapidly similar to the *Aphis gossypii* that has developed resistance to cyfluthrin, cyhalothrin and cypermethrin with LC_{50} over 3000 mg L^{-1} for cyhalothrin in both omethoate susceptible and resistant aphids [7].

In insects, alteration of detoxification enzymes presents a main mechanism for insecticide resistance [8–10]. Increased esterase detoxification, as an evolutionary response to selection pressure by pyrethroid insecticides, has also been found in many insects. In the housefly, *Musca domestica*, overexpression of carboxylesterase accounts for beta-cypermethrin resistance [11,12]. Overexpressed esterase was found in a fenvalerate resistant strain of the cotton bollworm, *Helicoverpa armigera* [13].

The cytochrome P450 monooxygenases (P450s) form an important metabolic system involved in the detoxification of xenobiotics, including insecticides [14]. Overexpression of a brain-specific cytochrome P450 (*CYP6BQ9*) was responsible for the majority of deltamethrin resistance in the *Tribolium castaneum* [15]. More than

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that, overexpression of *CYP6CM2* in the malaria vector *Anopheles gambiae* confers metabolic resistance to deltamethrin [16]. The *CYP337B3* in a resistant strain of *Helicoverpa armigera* could metabolite fenvalerate and confers a 42-fold resistance [17]. Similarly, the elevated transcription of *CYP6BQ23* confers resistance to deltamethrin in European populations of pollen beetle, *Meligethes aeneus* [18]. *CYP6P3* and especially *CYP6M2* originally derived from *A. gambiae* introduce bendiocarb resistance via *in vitro* expression in *Drosophila* that was resistant to pyrethroid [19]. Cytochrome P450-*CYP6P9b* in the major malaria vector *A. funestus* can metabolize both type I (permethrin and bifenthrin) and type II (deltamethrin and Lambda–cyhalothrin) pyrethroids [20].

In the present study, we established a lambda–cyhalothrin-resistant strain of *A. glycines* by continuous selection with lambda–cyhalothrin. The synergism effects of PBO, TPP and DEF against this strain, kinetics and specific activity of CarE, cross resistance, and transcriptional changes of *CarE, E4* esterase and P450 genes in this strain were investigated. These results will help promote efficient control of this pest and have major implications for successful implementation of resistance management strategies for *A. glycines*.

2. Materials and methods

2.1. Insects

Two soybean aphid (*A. glycines*) strains were used in this study: one strain (CRR) was resistant to lambda–cyhalothrin and the other strain (CSS) was susceptible. The CSS strain was collected in 2009 in Jilin province of China, where limited insecticides were applied. This strain has been maintained without any insecticide exposure since its collection. The CRR strain was established from the CSS population. The CRR strain was continually selected with lambda–cyhalothrin by the leaf-dipping method [21]. Both resistant and susceptible strains were reared on soybean plants, *Glycine max* (L.), in the laboratory at 20–23 °C with a photoperiod of 16:8 (L:D) h.

2.2. Chemicals

α-Naphthyl acetate (α-NA), β-naphthyl acetate (β-NA), fast blue B salt, Piperonyl butoxide (PBO), triphenyl phosphate (TPP), and S,S,S-Tributyltrithiophosphate (DEF) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Technical insecticides were obtained from Shanghai Chemical Reagent Company with greater than 98% purity. All other chemicals and solvents used were reagent grade.

2.3. Bioassays

Insecticide toxicity for different strains of *A. glycines* was determined by the leaf-dipping method described by Moores et al. [21]. A stock solution of insecticides was prepared in acetone and diluted to a series of concentrations with distilled water containing 0.05% (v/v) Triton X-100 and 1% acetone for bioassays. Soybean leaf discs (15-mm diameter) were dipped into insecticide solutions for 5 s, placed in shade to air dry and then placed upside down on an agar bed (25 mm in depth) in wells of 12-well tissue-culture plates. Bioassays were carried out by inoculation of 45 apterous adults (15 per well, three replicates for each concentration) to insecticide treated leaves for each concentration. The aphids were confined by the application of a ring of fluon to the lip of each well. Control soybean aphids were treated with leaves dipped in distilled water containing 0.05% (v/v) Triton X-100 and 1% acetone. Mortality was assessed after 24 h. LC_{50} values were calculated by probit analysis using POLO-PC software (LeOra Software Inc., Berkeley, CA). The resistance factors (RF) were estimated by the formula of RF = LC_{50} of CRR strain/LC_{50} of CSS strain, and the 95% CLs for the RFs were calculated according to Robertson and Preisler [22].

2.4. Synergism bioassays

The maximum dose that led to zero mortality in the susceptible strain was assigned as the maximum sublethal concentration in our study. The maximum sublethal doses for PBO, DEF and TPP, using the susceptible strain, were determined using the bioassay method described in Section 2.3. At least six concentrations including a control (acetone-only) were used for each synergist. Soybean aphids were exposed to cotton leaf discs (15-mm diameter) treated with a PBO, DEF or TPP (at the maximum sublethal dose; final concentrations were 100 mg L^{-1}) and lambda–cyhalothrin mixture. Mortality was recorded after 24 h. The synergistic ratio was calculated using the conventional approach that divides the LC_{50} without synergist by the LC_{50} with synergist. Probit analysis was conducted using POLO software (LeOra Software Inc.).

2.5. Detoxification enzymes assay

CarE activity was determined using α-NA and β-NA as substrate according to the method of Pan et al. [23]. The working concentration of α-NA or β-NA was 0.3 mM (containing 0.3 mM eserine); diluted from their respective 0.03 M stock solution in acetone. The assay mixture contained 100 μL of enzyme preparation, 900 μL of phosphate buffer (0.04 M, pH 7.0), and 3.6 mL of substrate solution (0.3 mM). The reaction was stopped by the addition of 0.9 mL of stop solution (two parts of 1% fast blue B and five parts of 5% sodium dodecyl sulfate) after incubation at 30 °C for 15 min. The color was allowed to develop for 15 min at room temperature, and the absorbance was measured at 600 nm for α-NA with UV/VIS Spectrometer Lambda Bio40 (Perkin-Elmer, USA). Mean levels of esterase activity were calculated based on protein content and α-NA or β-NA standard curves. Protein content was determined using the method of Bradford (1976), using bovine serum albumin as the standard. Significant differences between CSS and CRR strains were identified by Student's t-test ($P < 0.05$), using the GraphPad InStat3 statistical software (GraphPad Software, Inc., 2000).

2.6. RNA, DNA extraction and cDNA synthesis

Total RNA was isolated from apterous adults using Trizol (Invitrogen, USA) according to the manufacturer's instruction. Total RNA was treated with DNase (RNase free) to digest DNA contamination. First-strand cDNA was synthesized with 4 μg total RNA using Reverse Transcriptase M-MLV (RNase H-) (Takara, Dalian), following the manufacturer's instruction. Genomic DNA was extracted from apterous adults using DNAzol (Sangon, Shanghai, China). The RNA and DNA were quantified by measuring the absorbance at 260 nm, and the quality was checked by agarose gel electrophoresis.

2.7. Quantitative real time PCR and data analysis

Quantitative real time PCR was performed on ABI 7500 (Applied Biosystems) using an SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara). Gene specific primers (Table 1) were designed using Primer Premier 5.0 and synthesized by Sangon Biotech Co., Ltd. The standard plasmid was constructed by cloning the complete open reading frames of *CarE* and *E4* esterase of cotton aphid into the pGEM-T Easy vector (Promega). The mass of the plasmid was then converted into copy concentration using the formula copies/μL = $C \times 10^{-6} \times 6.02 \times 10^{23}/(660 \times L)$, where C is the plasmid concentration (μg/μL) and L is the plasmid length (bp). The