Pesticide Biochemistry and Physiology 132 (2016) 29–37

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Survey of organophosphate resistance and an Ala216Ser substitution of acetylcholinesterase-1 gene associated with chlorpyrifos resistance in *Apolygus lucorum* (Meyer-Dür) collected from the transgenic Bt cotton fields in China

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ARTICLE INFO

Article history:
Received 4 February 2016
Received in revised form 19 April 2016
Accepted 22 April 2016
Available online 23 April 2016

Keywords:
Apolygus lucorum
Organophosphorus insecticide resistance
Acetylcholinesterase
Ala216Ser

ABSTRACT

The mirid bug is frequently controlled by the application of organophosphorus insecticides in the transgenic Bt cotton field of China. A topical bioassay method was performed to evaluate the toxicities of chlorpyrifos and malathion towards field-collected Chinese populations of *Apolygus lucorum* from transgenic Bt cotton fields. For chlorpyrifos, the resistance ratios ranged from 0.8 to 9.4-fold compared to a susceptible strain. For malathion, the resistance levels relative to the susceptible strain ranged from 1.2 to 14.4-fold. Compared to a susceptible strain, the Cangzhou population from Hebei province showed the highest resistance ratios towards these insecticides. A comparison of the detoxifying and target enzyme activities between the Cangzhou population and a susceptible strain revealed that altered acetylcholinesterase possibly account for the chlorpyrifos and malathion resistance in the Cangzhou population. Two acetylcholinesterase (AChE-encoding) genes (designated *Alace1* and *Alace2*) from the green mirid bug (*A. lucorum*) were identified. The *Alace1* and *Alace2* genes encoded 597 and 645 amino acids, respectively. Both AChE proteins had conserved motifs including a catalytic triad, a choline-binding site, and an acyl pocket. Quantitative real-time PCR analysis showed that *Alace1* had a much higher transcriptional level than *Alace2*, for the expression profiles of both spatial and time distributions. One amino acid substitution, Ala216Ser in Alace1, was found in the Cangzhou population. These results suggest that the mutation Ala216Ser should be most likely involved in organophosphorus resistance in *A. lucorum*.

1. Introduction

In the nervous system of both vertebrates and invertebrates, acetylcholinesterase (AChE, EC 3.1.1.7), encoded by the *ace* gene, regulates the amounts of the neurotransmitter acetylcholine and terminates nerve impulses [1]. As the primary target for organophosphate (OP) insecticides, AChE activity can be inhibited via the phosphorylation of the serine residue in the active site gorge, leading to the repetitive firing of the postsynaptic nerve and the desensitization of the nervous system and finally causing the death of insects [2]. The organophosphates (OPs) are analogous to the substrate acetylcholine, and account for about 13% of total global insecticide sales in year 2010 [3]. The extensive use of OPs has led to the development of resistance in a number of insect pest species. Insensitive AChE caused by mutation(s) is most commonly associated with the resistance towards OPs [4].

To date, two ace loci (*ace1*, encoding AChE1, paralogous to *Drosophila ace*; *ace2*, encoding AChE2, orthologous to *D. ace*) have been identified in many insect species [5], especially in Hemiptera [6–13]. AChE1 had a central catalytic function in most investigated insect species of Hemiptera order [5].

Before the 1990s, Mirid bug has historically been as minor pests of cotton. After the large-scale adoption of transgenic Bt cotton in1997, mirids have gradually become destructive economic pests in cotton production systems [14]. *Apolygus lucorum* appeared as one of the dominant mirid species within the Changjiang River and Yellow River regions. Many chemical insecticides, including OPs and pyrethroid insecticides, have been extensively applied in efforts to control *A. lucorum* in China [14], and this has set the stage for the emergence of OP resistance traits in some *A. lucorum* populations. In Shandong province, the Huimin field population showed 5.6-fold for chlorpyrifos in comparison to a susceptible Juye strain [15]. In the same province, the Binzhou field population revealed 6.1-fold for chlorpyrifos compared to susceptible reference strain SLF [13]. A point mutation in the acetylcholinesterase-1 gene (A216S) is determined to be associated with chlorpyrifos resistance in the chlorpyrifos-selected *A. lucorum* strain [13].

In this study, we focused on the resistance monitoring and mechanisms of *A. lucorum* in China to chlorpyrifos and malathion. Bioassays exhibited that the Cangzhou population possessed the highest resistance ratios compared with a susceptible strain. The possible causes

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http://dx.doi.org/10.1016/j.pestbp.2016.04.008
0048-3575/

for the OP resistance in Cangzhou population were determined by the comparison of detoxifying metabolism-enzymes and molecular target AChEs, indicating that the chlorpyrifos resistance basis of Cangzhou population was correlated with the substitution (Ala216Ser) in acetylcholinesterase-1 (AChE1). It suggests that the point mutation (Ala216Ser) of AChE is universal as the chlorpyrifos resistance mechanism in *A. lucorum* from the transgenic Bt cotton field of China. Our results increase the understanding of the molecular properties of AlAChEs and provide crucial information that can be used for more effective management of mirids in the future.

2. Material and methods

2.1. Insects

A. lucorum was fed with fresh beans (*Phaseolus vulgaris* L.) and 10% honey in aerated plastic boxes (20 × 15 × 10 cm) at 26 ± 1 °C, 60 ± 5% (relative humidity), with a 16:8 h (L:D) photoperiod. The strain designated SS has been reared for many generations without exposure to any insecticides.

Field populations of *A. lucorum* were separately collected during July to September of 2014 from transgenic Bt cotton fields at the following locations: Binzhou city of Shandong province, Qiuxian county of Hebei province, Cangzhou county of Hebei province, Xihua county of Henan province, Wuwei county of Anhui province, and Wangjiang county of Anhui province (for detailed information, see Table 1). These *A. lucorum* populations were designated as SD-BZ, HB-QX, HB-CZ, HN-XH, AH-WW, and AH-WJ, respectively.

2.2. Bioassays

Technical grade malathion and chlorpyrifos (96 and 95.3% purity, respectively) were dissolved in acetone in a series of concentrations. The susceptibility of the SS strain and field populations to malathion and chlorpyrifos was determined with a topical application method [16]. 0.6 μL of malathion or chlorpyrifos solution was dropped underneath the pronotum of the green mirids with a semi-automatic dropper (PB-600 PAT, 3161323, Hamilton Company of America, Reno, Nevada, USA). Each assay was carried out with at least five separate concentrations and a solvent control. Mortality was recorded 24 h after treatment. Control mortality never exceeded 10%. All of the bioassays were conducted with three replicates. LD_{50} values were estimated by probit analysis.

2.3. Detoxifying enzyme activities and AChE inhibition assays

Total protein content was determined with Bradford assays using bovine serum albumin as the standard [17]. Carboxylesterase activities were determined using α-naphtyl acetate (α-NA) as the substrate with previously described methods [18]. 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 α-NA solution (0.3 mM). The reaction was terminated by adding 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 of the hydrolysis product, α-naphthol, was measured at 600 nm with a UV/VIS Spectrometer (T6 New Century, China).

Glutathione S-transferase (GST) activity was determined with the substrate 1-chloro-2, 4-dinitrobenzene (CDNB) following methods described elsewhere [19,20]. The reaction was initiated by adding 50 μL of enzyme preparation to a mixture containing 30 μL of CDNB (30 mM) and 30 μL GSH (30 mM) in 790 μL of 0.1 M phosphate buffer (pH 6.5). The change of absorbance for 2 min at 340 nm was measured with a UV/VIS Spectrometer Lambda Bio-40 (Perkin Elmer, USA).

Cytochrome P450 monooxygenase activity (ECOD) was determined with the substrate O-deethylation of 7-ethoxycoumarin (7-EC) according to the method detailed by Aitio [21] with slight modifications. Adult green mirid bugs were homogenized in ice-cold 0.1 M sodium phosphate buffer (pH 7.5, containing 1.0 mM EDTA, 1.0 mM PMSF, 1.0 mM PTU, 0.1 mM DTT, and 10% glycerol), and centrifuged at 10,800 g for 20 min at 4 °C. The reaction was started by adding 230 μL of enzyme preparation to a mixture containing 5 μL of NADPH (10 mM) and 25 μL of 7-EC (0.05 mM) in 650 μL of 0.1 M Tris-HCl buffer (pH 7.8). After incubation at 34 °C with 200 rpm for 15 min, 300 μL of stop solution containing 15% TCA was added to the samples to terminate the reactions. The mixture was centrifuged at 10,800 g for 2 min at 4 °C, and the supernatant was transferred to a new tub. After adding 450 μL of 1.6 M Gly-NaOH buffer (pH 10.5), the 7-OH EC content was measured immediately with a Spectrofluorometer LS-55 (Perkin Elmer, USA) using 356-nm excitation and 465-nm emission filters.

The AChE activity was measured based on the method of Ellman et al. [22] as modified by Zhu et al. [23] with acetylthiocholine iodide (ATChI) as the substrate and 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) as the chromogenic reagent, in micro-titer plate assays. 50 μL of enzyme supernatant was incubated at 30 °C for 10 min, with the inhibitor chlorpyrifos-oxon at a final concentration of 0.5 nM. The two remaining reaction mixture ingredients were then added, 0.4 mM DTNB and 0.5 mM ATChI, for a final volume of 200 μL for each sample. The residual AChE activity was then measured kinetically over for 15 min at 405 nm with a measurement taken at 15 s intervals in a microplate reader (Spectra Max PLUS384, USA).

2.4. Extraction of the total RNA and cDNA synthesis

Total RNA was extracted from 50 mg ground green mirid bugs with the RNAiso Reagent (Takara Co., Japan), according to the manufacturer's instructions. The quality and the quantity of the RNA were assessed with a Nanodrop 2000 spectrophometer (Thermo, USA) and electrophoresis on a 1.5% agarose gel. After the extraction of RNA, first strand cDNA were immediately synthesized following the instructions included with a PrimeScript™ RT-PCR Kit (Takara Co., Japan).

First strand cDNA for the 3′ RACE and 5′ RACE procedures was synthesized using SMART RACE cDNA Amplification Kits (Clontech, USA) according to the manufacturer's instructions.

2.5. Cloning and sequencing of Alace1 and Alace2 genes

The primers were designed according to transcriptome data (unpublished) (Table 2). PCR was performed with cDNA as the template. The thermal cycling program was as follows: 94 °C for 3 min, 35 cycles, each with 94 °C for 30 s; 55 °C (50–60 °C depending on Tm) for 30 s; and 72 °C for 0.5–2 min (depending on the length of the PCR product), and final extension for 10 min at 72 °C. PCR products were cloned into the pMD18-T vector (Takara Co., Japan). The inserted fragment was sequenced by Shanghai Songon Biologic Technology Co., Ltd.

The 3′ and 5′ end fragments of the *aces* were amplified with gene specific primers (Table 2). The thermal cycling program was as follows: 95 °C for 3 min followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and one additional cycle at 72 °C for 10 min.

Table 1
Locations in China where green mirid bugs were collected from transgenic Bt cotton fields.

County	Collection date	Latitude/longitude
Binzhou	7/2014	N 37.36° E 118.03°
Qiuxian	8/2014	N 36.82° E 115.17°
Cangzhou	7/2014	N 38.3° E 116.83°
Xihua	8/2014	N 33.79° E 114.5°
Wuwei	9/2014	N 31.3° E 117.92°
Wangjiang	9/2014	N 30.12° E 116.69°