Pesticide Biochemistry and Physiology 132 (2016) 53–58

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Over-expression of multiple cytochrome P450 genes in fenvalerate-resistant field strains of *Helicoverpa armigera* from north of China

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ARTICLE INFO

Article history:
Received 18 August 2015
Received in revised form 11 January 2016
Accepted 12 January 2016
Available online 14 January 2016

Keywords:
Cotton bollworm
Cytochrome P450 genes
Over-expression
Fenvalerate resistance

ABSTRACT

Pyrethroid resistance was one of the main reasons for control failure of cotton bollworm *Helicoverpa armigera* (Hübner) in China. The promotion of Bt crops decreased the application of chemical insecticides in controlling *H. armigera*. However, the cotton bollworm still kept high levels of resistance to fenvalerate. In this study, the resistance levels of 8 field-collected strains of *H. armigera* from north of China to 4 insecticides, as well as the expression levels of related P450 genes were investigated. The results of bioassay indicated that the resistance levels to fenvalerate in the field strains varied from 5.4- to 114.7-fold, while the resistance levels to lambda-cyhalothrin, phoxim and methomyl were low, which were ranged from 1.5- to 5.2-, 0.2- to 1.6-, and 2.9- to 8.3- fold, respectively, compared to a susceptible strain. Synergistic experiment showed that PBO was the most effective synergist in increasing the sensitivity of *H. armigera* to fenvalerate, suggesting that P450 enzymes were involved in the pyrethroid resistance in the field strains. The results of quantitative RT-PCR indicated that eight P450 genes (*CYP332A1*, *CYP4L11*, *CYP4L5*, *CYP4M6*, *CYP4M7*, *CYP6B7*, *CYP9A12*, *CYP9A14*) were all significantly overexpressed in Hejian1 and Xiajin1 strains of *H. armigera* collected in 2013, and *CYP4L5* was significantly overexpressed in all the 6 field strains collected in 2014. *CYP332A1*, *CYP6B7* and *CYP9A12* had very high overexpression levels in all the field strains, indicating their important roles in fenvalerate resistance. The results suggested that multiple P450 genes were involved in the high-level fenvalerate-resistance in different field strains of *H. armigera* collected from north of China.

1. Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner), is an important polyphagous agricultural pest with a wide range of distribution in Asia, Africa, Australia and Europe. *H. armigera* had developed resistance to almost all kinds of insecticides, including pyrethroids, organophosphates, carbamates, organochlorines, spinosad and Bt toxin in different areas and countries [1–4]. In China, *H. armigera* was a major pest on cotton before the commercialization of transgenic *Bacillus thuringiensis* (Bt) crops [5]; pyrethroids were introduced to control this insect pest in the early 1980s; resistance of *H. armigera* to pyrethroids was first reported in the late 1980s and heavily broke out in 1992, which led to more than ¥5 billion economic losses [6].

Increasing metabolism mediated by detoxification enzymes, such as esterases, GSTs, cytochrome P450s, have been considered to be responsible for insecticide resistance [7–9]. Among the 3 detoxification enzymes, cytochrome P450s was reported to be the major detoxification enzyme to pyrethroids and responsible for high level resistance to different pyrethroid insecticides [10–12].

Cytochrome P450s is encoded by a superfamily genes and capable of metabolizing endogenous and exogenous substance in virtually all organisms, and contribute to the adaptability of animals to a wide range of host plants and insecticides resistance of pest [10,13–15]. In *H. armigera*, multiple cytochrome P450 genes have been reported to be overexpressed and related to insecticide resistance, such as *CYP6B7*, *CYP9A12*, *CYP9A14*, *CYP337B3* [3,16–24]. However, the expression levels of specific genes are different in different strains and the contribution of a CYP gene varied among different strains, e.g. *CYP337B3*, which was arisen from unequal crossing-over of *CYP337B1* and *CYP337B2*, was reported to be involved in fenvalerate resistance of *H. armigera* in an Australia strain and cypermethrin resistance in a Pakistan strain, and could metabolize fenvalerate to 4′-hydroxyfenvalerate [3] and cypermethrin to 4′-hydroxycypermethrin [24], respectively. While in several field-derived resistant populations in China, fenvalerate resistance was validated to be not correlated with *CYP337B3* [25], suggesting the complexity of resistance mediated by cytochrome P450 in *H. armigera*. Such phenomenon also occurred in other insect species. In *Drosophila melanogaster*, DDT resistance resulted from the over-transcription of a single P450 gene *CYP6G1*, which can metabolize a wide range of insecticides, including imidacloprid and lufenuron [26]. Overexpression of *CYP12A4* was also reported conferring lufenuron resistance in another field population of

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http://dx.doi.org/10.1016/j.pestbp.2016.01.003
0048-3575/

D. melanogaster [27]. *CYP6G1* and *CYP12D1* were found both over-expressed in a field strain of *D. melanogaster* selected by DDT. When *CYP6G1* was knocked down, *CYP6A8* was selected instead [28]. In housefly, over-expression of *CYP6D1* was the major reason for permethrin resistance in a permethrin-selected strain LPR [29], while *CYP6D3v2*, *CYP6A24*, and *CYP12A1* were reported to be overexpressed in some other permethrin-resistant strains of housefly [30–32].

High levels of fenvalerate resistance in *H. armigera* had been reported after 15 years of cotton planting in China [33].This can be explained by the inevitable application of insecticides for the control of mirid bugs and late-season cotton bollworms. Investigation on the resistance levels of *H. armigera* to commonly used insecticides and the resistance mechanisms could provide information for the effective control of pyrethroid resistance of *H. armigera* in field. In the present study, we detected the resistance levels of 8 field-collected strains of *H. armigera* to 4 insecticides, investigated the expression levels of eleven cytochrome P450 genes in order to understand the roles of different P450 genes in pyrethroids resistance in field strains of *H. armigera* from north of China.

2. Materials and methods

2.1. Insects

HDS strain of *H. armigera*, was first collected from Handan, Hebei province in 1988, maintained in laboratory on artificial diet without insecticide exposure and served as a susceptible strain. Field strains of *H. armigera* were collected from six locations (see Table 1) from north of China in 2013 and 2014. Most *H. armigera* were collected from corns except for that of Weifang strain, which was collected from non-Bt cotton. The larvae were all reared on artificial diet at 27 ± 1 °C, 70 ± 10% humidity and 14:10 h of light:dark photoperiod in the laboratory. Moths were supplied with 5% honey solution to provide nutrients.

2.2. Chemicals

The insecticides used were of technical grade: fenvalerate (93.4%, Changzhou Pesticide Plant, Changzhou, Jiangsu, China), phoxim (90%, Huarong Pesticide Plant, Beijing, China), methomyl (98%), lambda cyhalothrin (96.9%, ShenYang Research Institute of Chemical Industry, China), piperonyl butoxide (PBO) (90%, J&K, China), and S,S,S-tributylphosphorothioate (DEF) (99%, Accustandard, United Kingdom), diethyl maleate (DEM) (97%, J&K, China).

2.3. Bioassay

Topical application was used to determine the resistance levels of field strains. Bioassay was applied during F1 to F4 generations. One microliter of serial dilutions of insecticide in acetone was applied on the thoracic dorsum of the third instar larvae weighing 15–20 mg by Hamilton syringe. Each insecticide was diluted to 5–7 concentrations and 30 larvae were treated in each concentration. Synergist experiments were performed with PBO (an inhibitor of cytochrome P450), DEM (an inhibitor of GST), DEF (an inhibitor of esterase) at 10 μg larva^{-1}, which was applied 1 h before the insecticides treatment. Control groups were treated with acetone or synergist alone. Larvae were held individually in a 12-well tissue culture plates with artificial diet. Mortality was checked 48 h after treatment.

Table 1
Information about field-collected strains of *H. armigera*.

Province	Strain/ collection site	Time of collection	Developmental stage	Host plant
Henan	Anyang	2014.6	Adult	–
	Zhengzhou	2014.8	Larvae	corn
Shandong	Weifang	2014.6	Larvae	cotton
	Xiajin1	2013.8	Larvae	corn
	Xiajin2	2014.8	Larvae	corn
Hebei	Hejian1	2013.8	Larvae	corn
	Hejian2	2014.8	Larvae	corn
Beijing	Beijing	2014.7	Larvae	corn

2.4. Quantitative RT-PCR

Total RNA was extracted from the pooled midgut of 6th instar larvae using SV Total RNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions. First strand cDNA was synthesized from 1 μg total RNA using FastQuant RT Kit (With gDNase) (TIANGEN, Beijing, China) according to the manufacturer's protocol. Primers were synthesized based on reference [19] (Table S1). Quantitative RT-PCR was conducted in a 20 μl system with 2 × Super real SYBR Mix(10 μl), cDNA (1 μl), 50 × ROX(0.4 μl) and each primer (0.6 μL, 10 μM) using the SuperReal PreMix Plus (SYBR Green, TIANGEN, Beijing, China) by ABI Prism7500 Real-Time PCR System (Applied Biosystems by Life Technologies, Foster, CA, USA). Thermal cycling was run at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 32 s. A melting curve was added as a final step to make sure the PCR product was unique and specific. Each test was repeated with 3 independent mRNA samples and each reaction was carried out in triplicate.

2.5. Statistical analyses

The data of bioassay was analyzed with SPSS (version 16.0). An overlapping of the 95% fiducial limits of LD_{50} value was considered not significantly different.

The data of RT-qPCR was calculated with the Ct value on 7500 software v2.3 (ABI) according to the $2^{-\triangle\triangle Ct}$ method [34]. The values were expressed as mean ± standard deviation. Student t-test was performed to analyze the statistical difference between means. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Insecticide resistance

The results of bioassay (Table 2, Table 3) showed that the field strains of *H. armigera* collected in 2014, including Hejian2, Xiajin2, Weifang, Beijing and Anyang, showed quite high levels of resistance to fenvalerate, with resistance ratio (RR) of 55.8-, 96.1-, 68.9-, 114.7- and 66.3-fold respectively, comparing with the susceptible HDS strain. Since PBO, DEF and DEM are well known inhibitors of cytochrome P450s, esterases and GSTs, respectively, a synergist experiment was performed to detect the involvement of detoxification enzymes in the resistance of *H. armigera* to fenvalerate. The results showed that PBO had significant synergistic effects on fenvalerate, with synergism ratio (SR) ranging from 91.5- to 4497.2-fold (Table 3). However, the SR value of DEF on fenvalerate were only 3.4- and 5.2-fold for Anyang and Hejian2 strains, respectively; and the SR value of DEM on fenvalerate were only 0.5- and 0.9-fold for Anyang and Beijing strains, respectively. The results suggested that cytochrome P450s is the main detoxification enzyme responsible for fenvalerate resistance in field strains of *H. armigera* from north of China, while the contribution of esterases and GSTs to the resistance is very small.

The resistance levels of *H. armigera* to phoxim, lambda-cyhalothrin and methomyl were much lower than that to fenvalerate in all the 8 field-collected strains (Table 2, Table 4). It was worth to note that all the strains of *H. armigera* showed no resistance to phoxim, with resistance ratio (RR) of only 0.2–1.6- fold.

3.2. Transcription analysis of cytochrome P450 genes in field-collected strains of H. armigera

The results of quantitative RT-PCR in 2013 (Fig. 1) indicated that eight P450 genes were significantly overexpressed in both Hejian1