Pesticide Biochemistry and Physiology 104 (2012) 277–282

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

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

Overexpression of a P450 gene (*CYP6CW1*) in buprofezin-resistant *Laodelphax striatellus* (Fallén)

Yueliang Zhang, Huifang Guo, Qiong Yang, Shuo Li, Lihua Wang, Gufeng Zhang, Jichao Fang *

Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Jiangsu, Nanjing 210014, China

ARTICLE INFO

Article history:
Received 27 June 2012
Accepted 15 October 2012
Available online 24 October 2012

Keywords:
Laodelphax striatellus
Cytochrome P450 monooxygenases
Buprofezin
Insecticide resistance

ABSTRACT

Cytochrome P450 monooxygenase (P450)-mediated detoxification is an important mechanism involved in the resistance to neurotoxic pesticides. However, the molecular basis of the mechanism of P450s, as associated with the resistance to growth regulator insecticides (IGRs) remains largely unknown. In this study, a resistance strain (YN-BPF) of *Laodelphax striatellus* was developed, with 59.9-fold resistance to buprofezin, through 42 generations of discontinuous selections of the susceptible strain (YN) with buprofezin (an IGR). A synergistic study *in vivo* and a biochemical study *in vitro* indicated that an enhanced detoxification mediated by P450s to some extent contributes to the buprofezin resistance in the YN-BPF strain. A total of 38 cDNA sequences encoding tentative unique P450 genes were identified in an *L. striatellus* transcriptome database, and the mRNA expression level of these genes was examined in the YN and YN-BPF strains using quantitative real-time PCR (qPCR). A single P450 gene, *CYP6CW1*, was highly overexpressed (22.78-fold) in the YN-BPF strain compared with the YN strain. Based on the analysis of insects with similar genetic backgrounds, our results provided evidence for the role of *CYP6CW1* in the resistance of *L. striatellus* to buprofezin.

1. Introduction

The small brown planthopper, *Laodelphax striatellus* (Fallén) (Homoptera: Delphacidae), a notorious agricultural pest, causes serious damage to rice due to transmission of rice stripe virus and rice black streaked dwarf virus [1]. Buprofezin is an insect growth regulator insecticide (IGR) that is mainly used in the control of homopteran pests, such as *L. striatellus*. Buprofezin is active against larval developmental stages, causing cuticular lesions that result from the disruption of chitin synthesis. Because this insecticide has generally been considered to have a good efficacy against the target pests while being harmless to beneficial insects, it has been used widely in integrated pest management (IPM) programs [2,3]. However, the extensive applications of buprofezin inevitably results in the development of resistance in natural populations of *L. striatellus* [4]. Thus, to better control this important agricultural pest, it is important to reveal the underlying resistance mechanism of this commonly used insecticide.

Cytochrome P450 monooxygenases (P450s) can be involved in the detoxification and bioactivation of insecticides [5]. Moreover, the transcriptional overexpression of P450 genes is often thought to enhance the metabolism of insecticides and appears to be a common phenomenon in the evolution of resistance development in insects. In fact, as a reductionist's means of understanding resistance mechanisms in insects, overexpression has become a dominant criterion in identifying insecticide resistance associated with the P450 genes. The increased metabolism of neurotoxic insecticides in correlation with the overexpression of P450 genes has been well demonstrated in many insect species, including the house fly *Musca domestica* (*CYP6D1*), fruit fly *Drosophila melanogaster* (*CYP6G1*), mosquito *Culex pipiens pallens* (*CYP6F1*), brown planthopper *Nilaparvata lugens* (*CYP6ER1*), mosquito *Culex quinquefasciatus* (*CYP9M10*) and whitefly *Bemisia tabaci* (*CYP6CW1*) [6–11]. Although the molecular basis of the mechanism of the P450-associated resistance of IGRs remained largely unknown when compared with neurotoxic insecticides, a few studies have suggested that P450s are involved in the catabolism of IGRs [12,13]. In particular, Karatolos et al. (2012) found that the overexpression of *CYP4G61* was associated with pyriproxyfen (a juvenile hormone analogue) resistance [14]. However, there has been no evidence for the correlation between P450 and buprofezin resistance in *L. striatellus* reported thus far.

In this study, a significantly buprofezin-resistant strain of *L. striatellus* was found in our laboratory. Compared with the susceptible (YN) strain, the buprofezin-selected (YN-BPF) strain developed a 59.9-fold resistance to buprofezin. The establishment of this buprofezin-resistant strain in addition to a transcriptome database of *L. striatellus* provided an excellent platform and opportunity to systematically identify P450 genes and assess their roles in

* Corresponding author. Fax: +86 25 84390891.
E-mail address: fangjc@jaas.ac.cn (J. Fang).

0048-3575/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pestbp.2012.10.002

buprofezin resistance in *L. striatellus*. Here, we report the following: (1) a synergistic study *in vivo* and a biochemical analysis *in vitro* to evaluate the role of P450s in buprofezin resistance in the YN-BPF strain and (2) the expression patterns of 38 P450 genes in an effort to identify candidate genes associated with the buprofezin-resistant phenotype.

2. Materials and methods

2.1. Insects

The susceptible (YN) strain of *L. striatellus* was collected from Yunnan, China, in July 2001 and has been reared since then without any contact with insecticides. The buprofezin-resistant (YN-BPF) strain was derived from the YN strain by a discontinuous selection with buprofezin in the laboratory. In each selection generation, approximately 3000 3rd-instar nymphs were bioassayed using the rice seedling dip method [15]. The average mortality was approximately 65%. After 42 generations, with 32 generations exposed to buprofezin, a resistant strain (YN-BPF) was obtained. The insects were reared on rice seedlings at 27 (±1) °C under a 14: 10 h light: dark regime.

2.2. Bioassays

The resistance levels of the YN-BPF and YN strains were assayed by the rice-stem dipping method, as based on a previous work and with minor modifications [16]. Rice stems, including the roots, were collected and washed thoroughly. The basal 8 cm long stems were cut and air dried to remove the excess water. The formulated insecticides were diluted with distilled water to generate five serial dilutions. Three rice stems were grouped and dipped into the appropriate insecticide test solution for 30 s. After the rice stems had been air dried for approximately 1 h, moistened cotton was used to wrap the basal end of the rice roots. Treated rice stems were then placed into a 500 mL plastic cup. A total of fifteen 3rd-instar nymphs were introduced into each plastic cup using a suction device. Rice stems dipped in distilled water were used as the control. For each dilution, 45 insects in all were treated. All of the test cups were maintained at 27 (±1) °C under a 14: 10 h light: dark regime, and the mortality was recorded after 144 h. For the analysis of the synergistic effect of the enzyme inhibitors on buprofezin in the YN and YN-BPF strains, 15 mg L^{-1} PBO was added to each dilution. The bioassay data were analysed using POLOPLUS software (LeOra Software, Petaluma, CA).

2.3. P450 preparation and assay

The preparation of microsomal P450 and measurement of the 7-ethoxycoumarin O-deethylase (ECOD)-metabolising activity were performed as described by Wang and Rauch and Nauen [15,17]. A total of 80 3rd-instar nymphs were homogenised in 1 mL 0.1 M phosphate buffer (pH 7.8, containing 1 mM EDTA, 1 mM α-phenyl-2-thiourea, 1 mM phenylmethanesulphonyl fluoride and 0.1 mM dithiothreitol DTT). The homogenates were centrifuged at 4 °C and 5000×*g* for 5 min, the resulting supernatant was filtered through cotton wool, the filtrate was centrifuged at 10,000×*g* for 20 min, and the filtration step was repeated. After centrifugation at 100,000×*g* for 60 min, the microsomal pellet was collected and resuspended in 250 μL 0.1 M phosphate buffer (pH 7.8, containing 1 mM EDTA, 1 mM α-phenyl-2-thiourea and 0.1 mM DTT) and then used as the enzyme source. Quantities of 70 μL of the microsomal fraction and 40 μL of 7-ethoxycoumarin (2 mM in 0.1 M, pH 7.8 phosphate buffer) were added to each well of a 96-well microplate. The reaction was started by the addition of 10 μL aqueous NADPH (9.6 mM in 0.1 M, pH 7.8 phosphate buffer) to the well, and the plate was incubated while shaking for 30 min at 30 °C. The background fluorescence was removed by the addition of 10 μL 30 mM oxidised glutathione and glutathione reductase (10 μL, 0.5 U). After 10 min at 25 °C, the reaction was stopped with 140 μL 50% acetonitrile in Tris-base buffer (0.05 M, pH 10.0). The amount of 7-hydroxycoumarin produced during incubation was quantified using a spectrofluorometer (Spectramax Geminixs; Molecular Devices) at an emission wavelength of 465 nm and an excitation wavelength of 390 nm. The assay was replicated at least 3 times with each strain of *L. striatellus*. Wells without any microsomal pellet served as the controls.

2.4. Screening of the L. striatellus P450 genes associated with buprofezin resistance

The total RNA was extracted using the SV Total RNA Isolation system (Promega) according to the manufacturer's protocol. To obtain the complete transcriptome database information, a pooled RNA sample including different developmental stages was used for the transcriptome database analysis. The complete transcriptome database of *L. striatellus* was created using a short-read sequencing technology (Illumina), as previously described [18]. The P450 genes were searched for systematically based on the five conserved motifs of the P450 genes present in insects. These conserved motifs were the heme-binding (PFxxGxRxCxG), heme-interacting (helix-C, WxxxR), oxygen-binding (helix-I, AGxxT), critical hydrogen-bonding (helix-K, ExxR), and "PERF" motifs (PxxFxPxRF), and most of these motif sequences were conserved in the insect and vertebrate P450 genes [19]. Furthermore, in anticipation of homozygosity problems for the P450 genes in the transcriptome database, we resequenced these P450 genes via PCR amplification. After the candidate P450 genes were obtained, a BlastP search was performed in NCBI for further support of the annotation predictions.

The total RNA was extracted from the YN-BPF and YN 3rd-instar nymphs using the SV Total RNA Isolation system. The first-strand cDNA was synthesised using 2 μg of total RNA with Superscript III reverse transcriptase (Promega). The relative expression levels of 38 *L. striatellus* P450 genes were determined for the YN-BPF and YN strains using real-time quantitative PCR (qPCR) with ADP ribosylation factor (*ARF*) as a reference gene (GenBank number: JF728807). This reference gene has been proven to have a high stability via geNorm and NormFinder analyses [20,21]. The primer sequences and the expected size of each PCR product are shown in Table S1. The qPCR was performed using three biological samples for each *L. striatellus* strain, and each sample was analysed in three technical replications using an ABI Prism 7300 system (Applied Biosystems Inc., Foster, CA). The 20 μl PCR mixture consisted of 10 μl SYBR® *Premix Ex Taq*™ (Takara, Japan), 1 μl cDNA, 0.4 μl 10 μM sense and antisense primers, 0.4 μl ROX Reference Dye and distilled water to 20 μl. The optimised cycling program was 1 cycle of 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 31 s, and a final disassociation that was automatically added by the 7300 System SDS software. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method [22]. Student's *t*-test was performed to determine the significance of the difference in the relative expression of the 38 P450 genes between the YN-BPF and YN strains.

2.5. Rapid amplification of cDNA ends (RACE)

The 5′ and 3′ ends of the newly obtained partial cDNAs of *CYP6CW1* were amplified by RACE–PCR. The first-strand cDNA was synthesised using the SMART™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, US) according to the manufacturer's protocol. The PCR amplification reaction was as follows: 94 °C for