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Variation in P450-mediated fenvalerate resistance levels is not correlated with *CYP337B3* genotype in Chinese populations of *Helicoverpa armigera*

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ABSTRACT

Metabolic resistance to synthetic pyrethroids in *Helicoverpa armigera* has recently been associated with the chimeric cytochrome P450 enyzme CYP337B3. One variant of the latter, CYP337B3v1, accounts for 40–50 fold resistance to fenvalerate in an Australian population while a second variant, CYP337B3v2, has been associated with ~7 fold resistance to cypermethrin in a Pakistani population. Here we show that ~250–1200 fold resistance to fenvalerate in populations of the species from northern and north-western China is largely due to P450-based metabolism, and that CYP337B3v2 is also at high frequency in these populations but absent in a susceptible control strain. However we find little correlation between the level of resistance and CYP337B3v2 frequency, either across the resistant populations studied, or over time within them. While there is variation between populations in the levels of *CYP337B3v2* expression, this is not correlated with the level of resistance either. These data suggest that much of the variation in the level of fenvalerate resistance in China is explained by P450s other than CYP337B3. We also find that both the level of resistance and *CYP337B3v2* frequency decline in field populations transferred to the laboratory and remained there without fenvalerate exposure, suggesting a fitness cost to both characters in the absence of the pesticide pressure. However the declines in the two characters are not well correlated across populations, again consistent with a large contribution to the variation in resistance levels from factors other than CYP337B3.

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1. Introduction

The cotton bollworm *Helicoverpa armigera* is one of the most serious pests of agriculture in the world and has evolved resistance to many classes of insecticides, including organophosphates, synthetic pyrethroids and, most recently, the Cry1Ac toxin of Bt (*Bacillus thuringiensis*) [1,2]. Its resistance to the chemical insecticides led to major outbreaks of the species in China up to 1997, when transgenic cotton was released there [3]. However, insecticide sprays were still required to control increasing numbers of mirid bugs [4] and high levels of fenvalerate resistance have re-emerged since [5].

Enhanced metabolism of pyrethroids by detoxification enzymes including cytochrome P450s and esterases plays an important part in pyrethroid resistance in many insect pests, although target site insensitivity caused by mutations in voltage-gated sodium channels is often also involved. In the case of *H. armigera,* the resistance is generally due to enhanced metabolism, and bioassays with the P450 inhibitor piperonyl butoxide (PBO) and the esterase inhibitor S,S,S-tributyl phosphorotrithioate (DEF) implicate both in the resistance but find that the relative contributions of the two classes of enzyme to resistance vary among populations [6–11]. Several individual P450s [12–17] and esterases [10,18,19] have been shown to metabolize pyrethroids and some, specifically CYP337B3, CYP6B7, CYP9A12 and CYP9A14 [20–23], and CCE001a, CCE001c, CCE001d and CCE001i [10,18,19], have also been found to be more highly expressed in resistant strains in at least one study. However quantitative trait loci (QTL) mapping has only so far directly associated one enzyme, CYP337B3, with resistance [14]. This enzyme maps to linkage group (LG) 13, and is encoded by a chimeric P450 gene that has apparently arisen from unequal crossing-over between the closely linked *CYP337B1* and *CYP337B2* genes. QTL mapping between a fenvalerate susceptible and resistant Australian strain (TWB) found the *CYP337B3v1* allele to be absent from susceptible backcross individuals but present in those showing ~50 fold resistance. Subsequently Rasool et al. [24] have associated a different

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allele, *CYP337B3v2* (which has a slightly different cross over site), with ~7 fold resistance to cypermethrin in a Pakistani population.

In the present study, twelve Chinese field populations with fenvalerate resistance levels ranging up to ~1200 fold were investigated. Bioassays with PBO and DEF and enzyme activity assays indicated a major role for P450s in resistance, and a much smaller effect due to esterases. A strong positive correlation between P450 activity and resistance level was found across the seven populations collected in 2013. PCR of genomic DNAs showed *CYP337B3* alleles to be absent from a susceptible control strain whereas the CYP337B3v2 allele occurred at high frequencies in all the field derived resistant strains. However there was little correlation between *CYP337B3* frequency and level of resistance, either among the seven 2013 collections or in comparisons with five populations collected in previous years. Moreover, quantitative RT-PCR revealed high levels of variation in *CYP337B3* expression among strains but these levels were not correlated with resistance levels. Finally, both resistance and *CYP337B3* frequency declined when field-collected populations were maintained in the laboratory without fenvalerate exposure but the declines in the two measures were poorly correlated across populations. Our data are thus consistent with a role for *CYP337B3* in fenvalerate resistance in Chinese *H. armigera* but also suggest that one or more other P450s explain much of the variation in resistance levels observed.

2. Materials and methods

2.1. Insects

Three sets of populations were used in this study (Table S1). The first set was used for a study of geographic variation in resistance parameters and comprised seven field populations collected from different sites in northern (six) and northwestern (one) China in 2013. These populations, plus the fenvalerate susceptible laboratory strain SCD (collected in Cote d'Ivoire over 30 years ago) as a control, were subject to a range of resistance bioassays, biochemical assays and PCR analyses of *CYP337* variants and their expression. A second susceptible control strain, GR, from Australia [25] was also used in the bioassays. The second set was used in a time series study of field populations and involved one strain from the first set and five others collected in previous years. These lines were just compared in resistance bioassays and PCR analyses of CYP337 variant frequencies (carried out within a generation of collection), again using SCD as a control. The final set was used to study changes in resistance parameters in the absence of insecticide pressure and involved laboratory strains established from four of the collections in the second set which were subsequently maintained without exposure to any chemical insecticides. These were also just tested in resistance bioassays and PCR analyses of CYP337 variant frequencies, with SCD as a control.

Some of the field collections were made by harvesting adults from light traps and some were based on eggs collected from Bt cotton plants. Laboratory cultures were maintained by rearing larvae on an artificial diet based on wheat germ and soybean powder [1] at 27 ± 1 °C with a photoperiod of 14:10 (L:D) h. Adults were held under the same temperature and light conditions at a RH of 60% and supplied with a 10% sugar solution.

2.2. Chemicals

Of the three bioassay reagents, fenvalerate (93%) was purchased from Changzhou Pesticide Plant (Changzhou, China), piperonyl butoxide (PBO, 95%) from Endura (Ravenna, Italy) and S,S,S-tributylphosphorothioate (DEF) (98%) from Sigma (USA). Suppliers of the reagents for the biochemical assays were as follows: Sigma (USA) for the Fast blue RR salt, 1-chloro-2, 4-dinitrobenzene (CDNB), p-nitroanisole (p-NA), 7-ethoxycoumarin (EC) and 1-naphthyl acetate (α-NA); Fluka (USA) for the 1,2-dichloro-4-nitrobenzene (DCNB) and methoxyresorufin (MR); and Amresco (USA) for the reduced glutathione (GSH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). All chemicals were AR grade.

2.3. Bioassay and synergism tests

Bioassays of resistance to topically applied fenvalerate were carried out on individual third instar larvae as per Yang et al. [11]. An 0.25 µl drop of fenvalerate in acetone was applied to the dorsal prothorax of each third instar larva tested and mortality recorded after 48 hrs under otherwise normal rearing conditions. Synergism tests involved applying DEF or PBO to the dorsal prothorax of individual larvae 1 h before the fenvalerate treatment, at a dose of 8 µg/larva. All resistance/synergism tests were replicated three times for each strain, with 10 larvae per replicate. Control larvae were treated with acetone or the synergists alone.

2.4. Enzyme assays

Forty-eight late fourth instar larvae (100 ± 10 mg) from each of the seven 2013 populations and SCD were homogenized individually on ice in 600 µl of 0.1 M phosphate buffer, pH 7.8, containing 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylthiourea, 1 mM PMSF and 20% glycerol. These homogenates were then centrifuged at 16,000 *g* for 30 min at 4 °C and the resultant supernatants filtered through glass wool and recentrifuged at 16,000 *g* for 20 min. The supernatants from the second centrifugation were then transferred to a cold microplate (on ice) and assayed for monooxygenase (*O*-dealkylase), esterase and GST activities, and protein concentration. The monooxygenase assays were carried out within 30 min and the other assays within 2 h. All enzyme activities were calculated with SOFTmax software (Molecular Devices, USA).

O-dealkylase assays with p-NA, MR and EC as substrates (PNOD, MROD and ECOD activities respectively) were carried out in 96-well microplates following the methods of Rose et al. [26] and Mayer et al. [27], with minor modifications. For the PNOD assays, 90 µl of enzyme solution and 100 µl of 2 mM p-NA were mixed and incubated at 30 °C for 3 min and then 10 µl of 9.6 mM NADPH was added. The reactions were allowed to proceed at 30 °C and absorbance measured by a microplate reader (Versa Max) at 405 nm for 15 min. For the MROD assays, 50 µl of enzyme solution and 100 µl of 0.01 mM MR were mixed and incubated at 30 °C for 3 min, then 10 µl of 9.6 mM NADPH was added. The assays were conducted at 30 °C and monitored by a luminescence microplate reader (Spectra Max Gemini XS, USA) at excitation/emission wavelengths of 530/585 nm for 12 min. ECOD activity was assayed as per the MR assays except that 80 µl of 0.5 mM EC was used as substrate and fluorescence intensity was measured at excitation/emission wavelengths of 380/460 nm for 15 min.

GST activities with CDNB or DCNB as substrates were measured as follows [11]. For CDNB, the 210 µl reaction mixture consisted of 10 µl of enzyme solution (diluted 10-fold in 0.1 M pH 7.6 sodium phosphate buffer), 100 µl of 1.2 mM CDNB, and 100 µl of 6 mM GSH. For DCNB, the 225 µl reaction mixture consisted of 25 µl of (undiluted) enzyme solution, 100 µl of 1.2 mM DCNB, and 100 µl of 6 mM GSH. The assays were again conducted in 96-well microplates and absorbance measured by a microplate reader (Versa Max) at 340 nm and 30 °C for 20 min.

The assay for non-specific esterase activity was based on Van Asperen [28], with the following modifications. The assay mixture contained 205 µl of substrate solution (0.2 M sodium phosphate buffer containing 100 mM α-NA and 1 mM Fast Blue RR salt, pH 6.0) and 20 µl of enzyme solution (diluted 10-fold in 0.1 M sodium

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