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An independent occurrence of the chimeric P450 enzyme CYP337B3 of *Helicoverpa armigera* confers cypermethrin resistance in Pakistan

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ABSTRACT

The increasing resistance level of insect pest species is a major concern to agriculture worldwide. The cotton bollworm, *Helicoverpa armigera*, is one of the most important pest species due to being highly polyphagous, geographically widespread, and resistant towards many chemical classes of insecticides. We previously described the mechanism of fenvalerate resistance in Australian populations conferred by the chimeric cytochrome P450 monooxygenase *CYP337B3*, which arose by unequal crossing-over between *CYP337B1* and *CYP337B2*. Here, we show that this mechanism is also present in the cypermethrin-resistant FSD strain from Pakistan. The Pakistani and the Australian *CYP337B3* alleles differ by 18 synonymous and three nonsynonymous SNPs and additionally in the length and sequence of the intron. Nevertheless, the activity of both CYP337B3 proteins is comparable. We demonstrate that CYP337B3 is capable of metabolizing cypermethrin (*trans*- and especially *cis*-isomers) to the main metabolite 4'-hydroxycypermethrin, which exhibits no intrinsic toxicity towards susceptible larvae. In a bioassay, CYP337B3 confers a 7-fold resistance towards cypermethrin in FSD larvae compared to susceptible larvae from the Australian TWB strain lacking CYP337B3. Linkage analysis shows that presence of *CYP337B3* accounts for most of the cypermethrin resistance in the FSD strain; up-regulation of other P450s in FSD plays no detectable role in resistance. The presence or absence of *CYP337B3* can be easily detected by a simple PCR screen, providing a powerful tool to rapidly distinguish resistant from susceptible individuals in the field and to determine the geographical distribution of this resistance gene. Our results suggest that *CYP337B3* evolved twice independently by unequal crossing-over between *CYP337B2* and two different *CYP337B1* alleles.

1. Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner), is one of the most significant pests of agriculture worldwide. This is due to its extremely wide geographical distribution, its highly polyphagous life style, and its ability to quickly evolve resistance against control agents from different chemical classes. *H. armigera* can be found in most countries of Africa, Asia, Oceania, and Europe, and even in regions where no overwintering is possible because of its ability to migrate long distances (Nibouche et al., 1998). Recently, likely due to

Abbreviations: AFLP, amplified fragment length polymorphism; BC, backcross; EPIC, exon-primed intron-crossing primer; FSD, *Helicoverpa armigera* strain from Faisalabad; Punjab, Pakistan; P450, cytochrome P450 monooxygenase; PBO, piperonyl butoxide; RF, resistance factor; R_t, retention time; RT-qPCR, reverse transcription-quantitative real-time polymerase chain reaction; SR, synergism ratio; TWBR, resistant line of the *Helicoverpa armigera* strain TWB from Toowoomba; Queensland, Australia; TWBS, susceptible line of the *Helicoverpa armigera* strain TWB.

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human activity, *H. armigera* invaded the state of Bahia and other north-eastern states of Brazil, and thus also occurs now on the American continent (Tay et al., 2013). In Bahia, the infestation will lead to estimated costs of about $1 billion in soybean, cotton, and maize fields due to increased insecticide applications and yield losses (Stewart, 2013). *H. armigera* is highly polyphagous with more than 200 host plants, including important crop plants like cotton, tobacco, potato, soybean, and maize (Brun-Barale et al., 2010). Furthermore, its control is hampered by its resistance level that is by far the highest known from all noctuid species with evolved resistance against pyrethroids, organophosphates, carbamates, organochlorines (www.pesticideresistance.org), and recently against the macrocyclic lactones spinosad (Aheer et al., 2009; Alvi et al., 2012) and abamectin (Alvi et al., 2012), as well as *Bacillus thuringiensis*-derived toxins (Alvi et al., 2012; Zhang et al., 2011).

In Pakistan, resistance of *H. armigera* against the pyrethroid cypermethrin was observed first by Ahmad et al. (1995) in 1991. Since then, cypermethrin resistance has been reported several times from different populations (Aheer et al., 2009; Ahmad, 2004; Ahmad et al., 1997; Alvi et al., 2012; Yang et al., 2004), but the resistance mechanism and the underlying genes remained unidentified until now, even though Yang et al. (2004) proposed a resistance mediated by cytochrome P450 monooxygenases (P450s).

Recently, we characterized a novel P450 enzyme that is responsible for the resistance of Australian *H. armigera* towards the pyrethroid fenvalerate (Joußen et al., 2012). *CYP337B3* is a chimeric gene that arose by unequal crossing-over between the two parental P450 genes, *CYP337B1* and *CYP337B2*. The chimeric gene is allelic to its parents, i.e. either *CYP337B3* occurs alone or *CYP337B1* and *CYP337B2* occur in a tandem array on Chromosome 15. We showed that only CYP337B3 is capable of metabolizing fenvalerate to the nontoxic 4'-hydroxyfenvalerate, while CYP337B1 and CYP337B2 exhibit no detectable fenvalerate metabolism. The polymorphic Australian *H. armigera* strain TWB was bred into a susceptible line (TWBS) possessing only the parental genes *CYP337B1* and *CYP337B2* and a resistant line (TWBR) possessing only *CYP337B3*. In bioassays, we demonstrated that the presence of CYP337B3 confers a 42-fold resistance towards fenvalerate, which is comparable to the resistance factor of 49 reported by Forrester et al. (1993) for an Australian field-collected population.

Here, we focus on the cypermethrin-resistant Pakistani *H. armigera* strain FSD collected from a chickpea field in Faisalabad, Punjab. Bioassays with a synergist indicated involvement of P450s, and the chimeric P450 *CYP337B3* was identified as a candidate gene. We describe sequence differences between the Pakistani and the Australian *CYP337B3* alleles that are relevant to the question of whether this chimeric gene has a single origin or evolved twice through independent unequal crossing-over events. Heterologous expression and functional analysis of CYP337B3 with model substrates and the pyrethroids cypermethrin and α-cypermethrin were conducted, including larval toxicity bioassays of the FSD strain in comparison with the susceptible Australian TWBS line lacking *CYP337B3* to determine the resistance level conferred by CYP337B3. Through linkage analysis using AFLPs and expression level analysis of 51 P450 genes, the involvement of other genes in the cypermethrin resistance was assessed. CYP337B3 is the major cypermethrin resistance mechanism in the FSD strain, and its presence in the disjunct regions of Pakistan and Australia hints at a more extensive worldwide distribution.

2. Materials and methods

2.1. *H. armigera* strains

For the Pakistani pyrethroid-resistant strain FSD of *H. armigera* (Hübner) (Lepidoptera: Noctuidae) about 250 larvae were collected in March 2011 from a chickpea field in Faisalabad, Punjab, Pakistan, where cotton and chickpea are used in rotation and multiple rounds of pyrethroid insecticides are applied for pest control. The larvae were reared on pinto bean diet (Joyner and Gould, 1985) at 26 °C and 55% humidity with a 16:8 (light:dark) photoperiod. The Australian pyrethroid-resistant line TWBR (*CYP337B3* line) and the susceptible line TWBS (*CYP337B1-CYP337B2* line) described in Joußen et al. (2012) were maintained on Bio-Serv diet (General Purpose Lepidoptera) under the same conditions. The FSD strain was maintained through mass rearing (10 pairs in a single cross), while the TWBR and TWBS lines were maintained through single-pair crosses.

2.2. Toxicity bioassays with cypermethrin and its main metabolite

Resistance levels of *H. armigera* strains were determined by screening with technical grade cypermethrin (mixture of isomers, purity 95.1%, PESTANAL, Sigma–Aldrich). Six different doses of cypermethrin in a range from 10 ng to 3162.3 ng were prepared in acetone. Thirty to forty healthy third-instar larvae of the FSD strain, the TWBR line, the TWBS line, and of crosses between the FSD strain and the TWBS line received topical application on the thorax of 1 μL of the specific dose using a Hamilton syringe fixed in a Hamilton PB600-1 Repeating Dispenser (Hamilton Messtechnik). For the FSD strain, the toxicity bioassay was repeated with a pretreatment of the larvae with piperonyl butoxide (PBO; Endura Fine Chemicals; 1 μL of 30 mM PBO in acetone), a known P450 and esterase inhibitor (synergist), 2 h prior to the application of cypermethrin. Larvae were kept on their normal diet under normal rearing conditions. Larval mortality was recorded after 48 h on the basis of criteria proposed by Joußen et al. (2012). Observed mortalities were transformed by probit analysis using SPSS Statistics version 17.0 to determine regression lines including slope and LD_{50} values.

A toxicity bioassay was also performed with the main metabolite of α-cypermethrin using the HPLC-purified metabolite from the upscaled *in vitro* assay. Fourteen third-instar larvae of the susceptible TWBS line were treated with approximately 0.17 μg of metabolite (dissolved in 1 μl of acetone; determined by HPLC).

2.3. Screening for the presence or absence of *CYP337B1, CYP337B2, and CYP337B3*

Genomic DNA was extracted as described in Joußen et al. (2012) with slight modifications. Genomic DNA was resuspended in 100 μL of nuclease-free water and stored at −20 °C. DNA from 36 FSD adults were screened by PCR (for primers, see Table S1) for the presence or absence of *CYP337B1*, *CYP337B2*, and *CYP337B3*.

2.4. Amplified fragment length polymorphism (AFLP) analysis

To generate informative families for genetic mapping, ten males of the resistant FSD strain were crossed with ten females of the susceptible TWBS line in a mass mating. F_1 males and females were backcrossed to TWBS in single-pair matings. To determine the resistance dominance in the F_1 population, F_1 larvae were treated with different doses of cypermethrin as described above. Additionally, 100 third-instar larvae of one male-informative backcross family derived from a F_1 father and 50 larvae of one female-informative backcross family from a F_1 mother were treated with the discriminating dose of 400 ng of cypermethrin. All treated larvae, dead or survived, and 50 untreated third-instar larvae were frozen at −80 °C for subsequent analysis.

For AFLP analysis, we followed the method published by Vos et al. (1995) with a few modifications described by Kaiser and