Research Article

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Saltational evolution of a pesticide-metabolizing cytochrome P450 in a global crop pest

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Abstract

BACKGROUND: The cotton bollworm, *Helicoverpa armigera* (Hübner), is a damaging insect pest threatening agricultural crops worldwide as a result of its resistance to insecticides. Metabolic resistance to pyrethroid insecticides is conferred by the chimeric P450 enzyme CYP337B3, produced by unequal crossing-over between *CYP337B1* and *CYP337B2*. CYP337B3 is 99.7% similar to CYP337B1 except for the 177 N-terminal amino acids (AAs) containing the substrate recognition site 1 from CYP337B2. Here, we studied the structure–function relationship of CYP337B3 and CYP337B1 to determine the AAs that enable CYP337B3 to efficiently hydroxylate the 4′-carbon position of fenvalerate, which neither CYP337B1 nor CYP337B2 can do.

RESULTS: Site-directed mutagenesis showed that the L114F substitution in CYP337B3 reduced its 4′-hydroxylation activity by 89%, but the reciprocal F114L substitution in CYP337B1 increased its 4′-hydroxylation activity to only 49% of the level of CYP337B3. Docking models showed that AA 114 seems to have different functions in CYP337B1 and CYP337B3. Antibodies detected two- to three-fold more CYP337B1 than CYP337B3 in larval cuticle, which along with a 49% 4′-hydroxylation activity increase due to a F114L substitution *in vivo* might be expected to provide as much protection for the larva against exposure to fenvalerate as CYP337B3. However, *CYP337B3* is present at much higher frequencies than *CYP337B1-CYP337B2* in most populations, including those recently invading South America.

CONCLUSION: The metabolic resistance to pyrethroids in *H. armigera* has evolved by saltational evolution – by a single mutation, an unequal crossing-over, producing a larger selective advantage than could be attained gradually by stepwise improvement of the parental enzyme.

Supporting information may be found in the online version of this article.

Keywords: insecticide resistance; pyrethroid; fenvalerate; cotton bollworm; unequal crossing-over; site-directed mutagenesis

1 INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hübner), is one of the most damaging agricultural insect pest species worldwide owing to its highly polyphagous nature,[1] its extremely wide and expanding geographical distribution,[2, 3] and its ability to quickly evolve resistance to many chemically distinct insecticides (www.pesticideresistance.org). Studying the pesticide resistance mechanisms of *H. armigera* will help to manage this pest by reducing use of insecticides that have become ineffective due to resistance, and to develop new insecticides with a reduced risk of resistance development.

We showed previously that the chimeric cytochrome P450 monooxygenase CYP337B3, which arose by unequal crossing-over between the two parental genes, *CYP337B1* and *CYP337B2*, confers resistance to the pyrethroid insecticide fenvalerate in Australian *H. armigera*.[4] CYP337B3 shares the 315 residues at the C-terminus with CYP337B1, and the 177 residues at the N-terminus containing the first substrate recognition site, SRS1, (Fig. 1 and Supporting information, Fig. S1) with CYP337B2. Neither CYP337B1 nor CYP337B2 are capable of metabolizing fenvalerate, whereas CYP337B3 detoxifies fenvalerate by hydroxylation and has been detected in pyrethroid-resistant populations in Pakistan,[5] China,[6] Brazil,[7] and every continent except North America and Antarctica.[8] Different populations have different alleles of *CYP337B3* with subtle differences in the B2/B1 crossover point,[8] but all combine the SRS1 of CYP337B2 with the other substrate recognition sites and active site of CYP337B1.

Here, we conducted reciprocal site-directed mutagenesis to determine which residue or residues from the SRS1 of CYP337B2 conferred pyrethroid hydroxylation activity on the otherwise inactive CYP337B1. In total, we investigated the metabolic activity of

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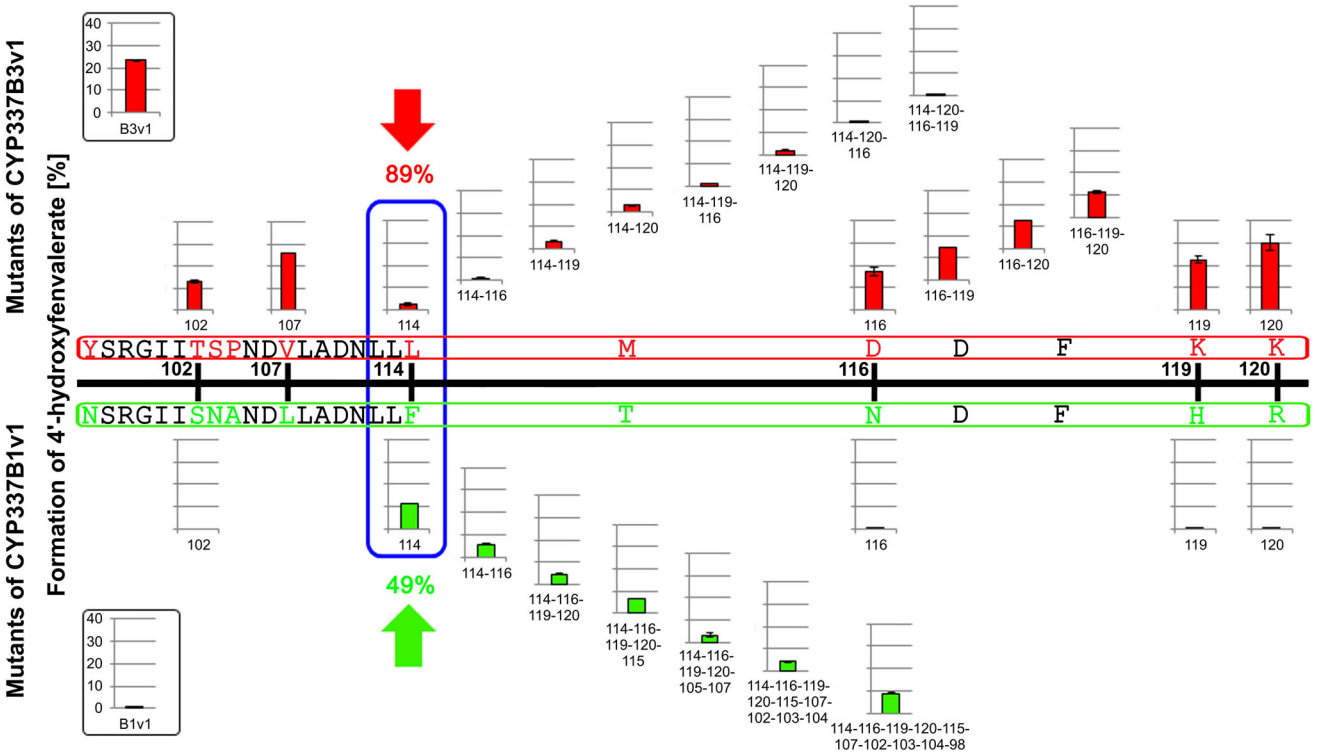

Figure 1. Metabolic activity of CYP337B3(v1) and CYP337B1v1 mutants toward fenvalerate (fraction converted to 4′-hydroxyfenvalerate in 4 h under reaction conditions). The upper half of the diagram shows the results of the CYP337B3(v1) mutants ordered by the mutations and that of the CYP337B3(v1) WT, whereas the lower half of the diagram shows the results of the CYP337B1v1 mutants ordered by the mutations and that of the CYP337B1v1 WT. The *x*-axis indicates the mutated AA. Spacing is uneven to accommodate the graphs.

27 mutants toward fenvalerate and the model substrates 7-methoxy- and 7-benzyloxyresorufin. We found that among all the substitutions examined, swapping the residue at position 114 had the largest effect on decreasing activity of CYP337B3 and increasing that of CYP337B1. However, this substitution, whether alone or in combination with others, explained only ≤49% of the activity difference in CYP337B1 compared to CYP337B3, but 89% or more in CYP337B3. Homology models of the mutants with docking by esfenvalerate were only partially consistent with the experimental results. CYP337B1 was more abundant than CYP337B3 in relevant tissues, because the unequal crossing-over event put CYP337B3 under control of the CYP337B2 promoter. These differences point to a saltational, rather than a gradual evolution of resistance to pyrethroid insecticides in worldwide populations of this insect pest.

2 MATERIALS AND METHODS

2.1 Insects

The TWB strain of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) was collected from the vicinity of Toowoomba, QLD, Australia in January 2003 and maintained in the laboratory in Jena since August 2004. A pyrethroid-susceptible line possessing the parental P450 genes *CYP337B1* and *CYP337B2* and a pyrethroid-resistant line possessing only the chimeric *CYP337B3* gene were set up already by PCR screening of suitable individuals from the polymorphic TWB strain, as described previously.[4] Larvae were reared on General Purpose Lepidoptera diet (Bio-Serv, Flemington, NJ, USA) at 26 °C and 55% humidity with a 16 h:8 h, light:dark, photoperiod. Both the TWB strain and the separated lines, *CYP337B1-CYP337B2* and *CYP337B3*, have been exclusively propagated by single-pair crosses to reduce the effects of inbreeding.

2.2 Site-directed mutagenesis

CYP337B1v1 (GenBank Accession no. JQ284023) and *CYP337B3(v1)* (GenBank Accession no. JQ284029) originating from the TWB strain were already available in the pIB/V5-His TOPO TA vector (ThermoFisher Scientific, Waltham, MA, USA) for expression in insect cell lines.[4] Different nucleotides coding for amino acids (AAs) located in the substrate recognition site 1 (AA 96 to 120; Fig. 1 and S1) were selectively mutated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Waldbronn, Germany) following the manufacturer's protocol to replace specific AAs in the encoded proteins. Adjacent AAs were replaced in one step, AAs more distant from each other in subsequent reactions. The fewest nucleotide changes necessary were employed. *CYP337B1v1* served as a template for *CYP337B3(v1)* and vice versa. Plasmids were extracted from *Escherichia coli* clones using the GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific) and mutations were verified by Sanger sequencing.

2.3 Heterologous expression in Ha2302 cells and isolation of microsomes

In order to avoid the endogenous pyrethroid-hydrolyzing activity of Sf9 cells, Ha2302 cells[4, 9] derived from hemocytes of *H. armigera* were used for P450 expression. Ha2302 cells were cultivated as adherent cells at 27 °C in Sf-900 II serum-free medium (ThermoFisher Scientific). Ha2302 cell cultures expressing wild-type (WT) *CYP337B1v1* and *CYP337B3(v1)* were already available.[4] Ha2302 cells were transfected in 6-well plates with plasmid DNA

(1.8 µg) using Insect GeneJuice Transfection Reagent (Merck Millipore, Darmstadt, Germany) following the manufacturer's protocol. After 48–72 h, stable transfected cell lines were established by selecting with blasticidin (60 µg mL^{-1}). Microsomes were prepared by differential centrifugation as described.[4] Protein was determined using the Quick Start Bradford Protein Assay (Bio-Rad, Feldkirchen, Germany).

2.4 CYP337B antibody

Using the homology model of CYP337B3[4] two regions comprising 14–16 AAs (AA 183–196, GIDTQVKSTMESPF, and AA 373–387, TKDTVLPSGIPIAKG, respectively) showing no secondary structure, being located on the surface of the protein, and in a region identical in CYP337B3 and CYP337B1 were selected as recognition sites for the specific antibody which should be produced. The peptides were synthesized at Davids Biotechnology (Regensburg, Germany), which also produced the antibodies by immunization of rats. Both antibodies were tested by Western blot using microsomes containing heterologously expressed CYP337B1v1 and CYP337B3(v1). Because the TKD antibody binding to the AAs 373–387 gave a stronger specific signal, this antibody was used for further work: TKD antibody: 1:5000 [2 h at room temperature (RT), continued overnight at 4 °C); Anti-rat-HRP antibody (Rat IgG-F(ab′)2 Fragment Antibody; Bethyl Laboratories, Montgomery, TX, USA): 1:10000 (2 h at RT); blocking in TBS with 0.1% Tween 20 and 3% BSA (2 h). This antibody also cross-reacted with CYP337B2 (corresponding region: TKDTVLPSG LPVSKD).

2.5 Adjustment of the protein amount of the different mutants

Different amounts (2.5–13 µg) of denatured total microsomal protein of Ha2302 cells expressing the CYP337B1v1 (Fig. S2A) and the CYP337B3(v1) mutants (Fig. S2C), respectively, were separated by SDS-PAGE (10% polyacrylamide gel) using the Criterion system with TGS buffer (Bio-Rad). Proteins were blotted on a PVDF membrane using the Criterion Blotter with TG buffer (Bio-Rad) with methanol (10%) and were detected by the TKD antibody (Davids Biotechnology) together with an Anti-rat-HRP antibody (Bethyl Laboratories) (see Section 2.4). On each blot a dilution series of CYP337B3(v1) (2.5–17.5 µg total microsomal protein) and also a sample containing CYP337B1v1 (8 µg total microsomal protein) and one from the nontransfected Ha2302 cells (10 µg total microsomal protein) were present as controls. The film was exposed to the membrane followed by film development. The intensity of the signals was estimated using Fiji software v1.47f[10] (Fig. S2B, D). The dilution series of CYP337B3 (v1) was used to generate calibration curves (Fig. S2B, D) that were used to estimate the relative amounts of the different CYP337B1v1 or CYP337B3(v1) mutants and of the WT CYP337B1v1. Samples were not compared across different individual Western blots. A correction factor (Table S1A, B) was calculated, dividing the total microsomal protein amount used by the calculated protein amount. These factors were used to adjust the amount of heterologous P450s to an equal level across all microsomes for the enzymatic assays performed. This adjustment was necessary, because the expression level of the different CYP337B mutants and the WT proteins differed in the individual cell cultures and would distort the results of the enzymatic assays because of the different individual P450 amounts.

2.6 Activity assays with 7-methoxy- and 7-benzyloxyresorufin as P450 model substrates

Assays were performed as described.[4] In short, microsomal proteins [P450 amount adjusted to 0.2 mg microsomal protein containing CYP337B3(v1)] derived from transgenic and nontransgenic Ha2302 cells were incubated with 7-methoxy- or 7-benzyloxyresorufin (1 nmol; Sigma-Aldrich, St Louis, MO, USA; dissolved in 1 µL DMSO) and an NADPH regeneration system in potassium phosphate buffer (0.1 M, pH 7.4; total volume: 200 µL) at 30 °C for 4 h. Samples were prepared[4] and measured with the Tecan Infinite 200 Reader (Tecan, Männedorf, Switzerland) at excitation wavelength 530 nm and emission wavelength 593 nm to detect the product resorufin. Samples omitting microsomes were used as background controls. Two replicates were used per assay.

2.7 *In vitro* metabolism assays with fenvalerate

Assays were performed as described[4] with slight modifications. In short, microsomal proteins [P450 amount adjusted to 0.3 mg microsomal protein containing CYP337B3(v1)] were incubated with fenvalerate (4 nmol, 1.7 µg, purity: 99.6%; PESTANAL, Sigma-Aldrich; dissolved in 4 µL DMSO) using a NADPH regeneration system in potassium phosphate buffer (0.1 M, pH 7.4; total volume: 800 µL) with sodium cholate (0.05 g L^{-1}) at 30 °C for 4 h. Samples were extracted and subjected to high-performance liquid chromatography HPLC analysis.[4] Fenvalerate and its metabolite 4′-hydroxyfenvalerate were detected at 210 nm, 230 nm and 275 nm. An external fenvalerate standard (10 pmol $µL^{-1}$) was used to calculate the total recovery of fenvalerate. All assays were performed twice.

2.8 Protein design and docking modeling

Protein design and ligand docking was carried out using MOE (Molecular Operating Environment) software v2014.09 (Chemical Computing Group, Montreal, Quebec, Canada). Models of the most important CYP337B1v1 and CYP337B3(v1) mutants were generated using the protein design function of MOE and the homology models of the WT proteins[4] (based on the crystal structure of human CYP3A4) as templates. Subsequently, the models were prepared for docking modelling (energy minimization, site finder, structure preparation). Docking was performed as described[4] with slight modifications. In short, docking was run with a dataset of all four fenvalerate isomers [esfenvalerate, (2R,αR)-fenvalerate, (2R,αS)-fenvalerate and (2S,αR)-fenvalerate] both for the models of the different mutants and of the WT proteins by using the Triangle Matcher as placement methodology, rescored by London dG, and refined by the Amber12:EHT force field. A maximum number of 50 ligand positions per isomer were kept after the rescoring stage. All ligand positions were screened manually, and the positions with the carbon ($C_{4'}$) atom of the phenoxybenzyl alcohol moiety closest to the oxygen (O) bound to the heme iron (Fe) were chosen. The ligands were included separately in the respective model and subjected to energy minimization, which assured a final energy gradient lower than 0.01 kcal mol^{-1} $Å^{-1}$ by using the Amber12:EHT force field with default settings. During energy minimization, planar systems (heme group) were treated as rigid bodies.

2.9 Detection of CYP337B proteins in larval tissues

Three fifth instar *H. armigera* larvae originating from three different families of the *CYP337B1-CYP337B2* line, of the *CYP337B3* line, and of crosses between the two lines, were snap-frozen in liquid

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Table 1. CYP337B1v1 mutants

CYP337B1v1 mutant	AAs altered
B1-m1	S102T: AGC to ACC
B1-m2	F114L: TTC to TTA
B1-m3	N116D: AAT to GAT
B1-m4	H119K: CAT to AAA
B1-m5	R120K: AGA to AAA
B1-m6	F114L and N116D
B1-m7	F114L, N116D, H119K, and R120K
B1-m8	F114L, N116D, H119K, R120K, and T115M[†]
B1-m9	F114L, N116D, H119K, R120K, T115M, and L107V[†]
B1-m10	F114L, N116D, H119K, R120K, T115M, L107V, S102T, N103S, and A104P[†]
B1-m11	F114L, N116D, H119K, R120K, T115M, L107V, S102T, N103S, A104P, and N96Y[†]

[†] Codons leading to the altered AAs: N96Y: AAC to TAC; N103S: AAC to AGC.
A104P: GCC to CCC; L107V: TTA to GTA; T115M: ACG to ATG.

Table 2. CYP337B3(v1) mutants

CYP337B3(v1) mutant	AAs altered
B3-m1	T102S: ACC to AGC
B3-m2	V107L: GTG to TTG
B3-m3	L114F: TTA to TTC
B3-m4	D116N: GAC to AAC
B3-m5	K119H: AAA to CAT
B3-m6	K120R: AAA to AGA
B3-m7	L114F and D116N
B3-m8	L114F and K119H
B3-m9	L114F and K120R
B3-m10	D116N and K119H
B3-m11	D116N and K120R
B3-m12	L114F, K119H, and D116N
B3-m13	L114F, K119H, and K120R
B3-m14	L114F, K120R, and D116N
B3-m15	D116N, K119H, and K120R
B3-m16	L114F, K120R, D116N, and K119H

nitrogen and stored at −20 °C. Frozen larvae were dissected on ice into head, gut plus Malpighian tubules, cuticle, and rest of the body (including fat body, nervous system, and body wall muscles). Tissues from all three larvae of each line were separately pooled. Microsomes from each sample were isolated as described for cell cultures.[4] Microsomal pellets were resuspended in resuspension buffer [(30–)50 µL for heads, 125 µL for other tissues]. Protein content was determined using the Quick Start Bradford Protein Assay (Bio-Rad). Denatured microsomal proteins (2 µg for heads, 18 µg for other tissues) were separated together with positive controls consisting of microsomes of cell cultures expressing CYP337B1v1, CYP337B2v2 or CYP337B3(v1) (10 µg each), respectively, or a mixture of these microsomes (5 µg, 5 µg, and 10 µg, respectively) by SDS-PAGE (8% polyacrylamide gel) using the Criterion system with TGS buffer (Bio-Rad). Western blot was carried out as described above. The intensity of the signals was estimated using Fiji software v1.47f.[10] The membrane was stained with Coomassie brilliant blue.

3 RESULTS

3.1 Fenvalerate hydroxylation by CYP337B1 and CYP337B3 mutants

Several mutants of *CYP337B1v1* and *CYP337B3(v1)* were created by site-directed mutagenesis leading to P450 proteins with altered AA sequences in the substrate recognition site 1 (SRS1, AA 96–120).[4] Amino acids found in CYP337B1v1 were altered to AAs found in CYP337B3(v1) and *vice versa* to test for the involvement of single or several residues in the enzymatic capacity of the P450 enzymes. The altered nucleotide sequences of the mutants were verified by Sanger sequencing. In total 11 CYP337B1v1 and 16 CYP337B3(v1) mutants listed in Tables 1 and 2 were investigated in detail.

P450 mutants were stably expressed in Ha2302 cells as described previously.[4] Formation of 4′-hydroxyfenvalerate was monitored by HPLC, and corrected by the amount of protein using an antibody that detected both CYP337B1 and CYP337B3. Although activity assays with model substrates proved that all expression constructs were catalytically competent, we were unable to use the CO difference spectrum[11] to quantify the fraction of active enzyme, most likely owing to low expression levels of P450 in the insect cell cultures. Figure 1 shows the CYP337B3 (v1) mutants in the upper half and the CYP337B1v1 mutants in the lower half. Under the assay conditions, 23.3% of fenvalerate is converted to 4′-hydroxyfenvalerate by the WT enzyme CYP337B3(v1), but only 0.8% by CYP337B1v1. The substitution with the largest effect occurs at position 114. Only 2.6% of the substrate is hydroxylated by the L114F substitution of CYP337B3 (v1), a reduction of 88.8%. Double, triple, and quadruple mutants involving L114F also showed low activities (hydroxylation ranging from 0.2% in the four-fold mutant 114–120–116–119 comparable to CYP337B1v1, up to 3.3% in the double mutant 114–119). Other substitutions, singly or in combination, slightly decreased activity, or in the case of V107L and K120R, slightly increased it.

The reciprocal substitution F114L of CYP337B1v1 produced slightly more 4′-hydroxyfenvalerate, namely 11.5%, slightly less than half of the amount produced by WT CYP337B3(v1). Combining other substitutions with F114L only decreased its activity. Even the ten-fold substitution mutant in which all CYP337B1 SRS1 residues were replaced by their CYP337B3 counterparts produced only 8.9%. No other CYP337B1v1 mutants examined here showed any appreciable capacity to metabolize fenvalerate.

3.2 Activity of mutants on model substrates

All CYP337B1v1 and CYP337B3(v1) mutants also were tested in activity assays with 7-methoxyresorufin (MR) and 7-benzyloxyresorufin (BR) as model substrates for P450s to verify their enzymatic functionality and for comparison with trends shown with fenvalerate (Fig. S3). Both WT enzymes showed higher activity toward MR than BR. CYP337B3(v1) showed a higher activity overall toward MR than CYP337B1v1, and CYP337B1v1 was more active on BR than CYP337B3(v1). All substitutions of CYP337B3(v1) containing L114F were more active on BR and less active on MR and thus comparable to the activity of CYP337B1v1. The effect of CYP337B1v1 mutants was more complicated. Most showed reduced activity toward MR and also toward BR, but the mutants showing the lowest activity toward BR comparable to

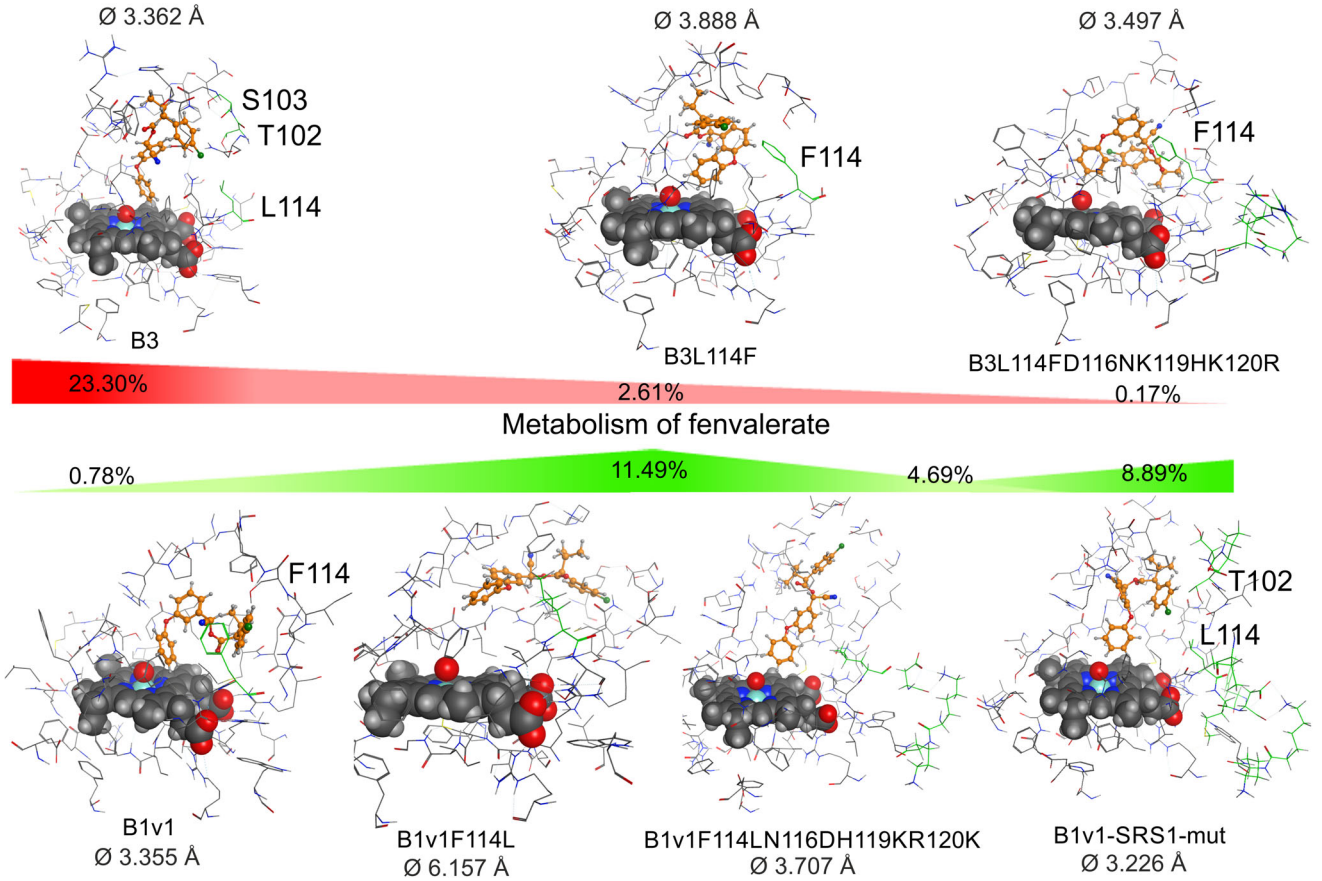

Figure 2. Docking models of CYP337B3(v1) and CYP337B1v1 mutants with esfenvalerate in comparison to the WT enzymes. The upper half of the diagram shows the docking models of CYP337B3(v1) WT and of two of the most important CYP337B3(v1) mutants, whereas the lower half shows these of CYP337B1v1 WT and of three of the most important CYP337B1 mutants. The mutated AAs are indicated in the name of the mutant. SRS1-mut means that all AAs in the SRS1 region were mutated to that of CYP337B3(v1). The heme group is shown in space-filling mode and esfenvalerate in ball and stick mode, with the carbon atoms in orange. Amino acids of the SRS1 region that are different between CYP337B3(v1) and CYP337B1v1 allozymes and that are in a distance of 4.5 Å from esfenvalerate are labeled and shown in stick mode, with their carbon atoms in green. These AAs of the SRS1 region that are >4.5 Å distance are only labeled by their carbon atoms shown in green. The average distance between $C_{4'}$ of the four different fenvalerate isomers and O coordinated to the Fe of the heme group are given. The metabolic activity of the different WT and mutant enzymes toward fenvalerate are given.

CYP337B3(v1) exhibited overall lower activities. Results with the model substrates showed that all mutants examined here were catalytically competent and that the effect of substitutions was generally substrate-dependent.

3.3 Docking models are insufficient to explain the capacity of the mutants

The most important CYP337B1v1 and CYP337B3(v1) mutants were modeled starting from the respective WT enzyme[4] using the protein design function of MOE (Molecular Operating Environment) software v2014.09. Figure 2 shows the active centers of these mutants and the WT enzymes with esfenvalerate, the most insecticidal fenvalerate isomer. For CYP337B3(v1), the L114F substitution and the four-fold mutant L114F, D116N, K119H, and K120R are shown, and for CYP337B1v1 the corresponding reciprocal mutants plus the mutant that possesses the entirely changed SRS1 region. The models supplemented with the average distance between the $C_{4'}$ atom of the four fenvalerate isomers [(2*S*, *αS*), (2*R*, *αR*), (2*R*, *αS*), (2*S*, *αR*)] and the O atom coordinated to the Fe atom of the heme group are compared with the fenvalerate metabolism observed. The distance across CYP337B3 (v1) and the CYP337B3(v1) mutants ranged from 3.4 to 3.9 Å, and residue 114 was ≈4.5 Å from the esfenvalerate substrate for all models, but the capacity to metabolize fenvalerate decreased from 23.3% to 0.2%. By contrast, the distance across CYP337B1v1 and the CYP337B1v1 mutants varied between 3.2 to 6.2 Å. The CYP337B1v1–114 mutant with the longest distance showed the highest activity toward fenvalerate of 11.5%. Furthermore, residue 114 was >4.5 Å away from the esfenvalerate molecule. For the CYP337B1v1 mutant with the SRS1 region completely altered to that of CYP337B3(v1), the binding of the fenvalerate molecule was similar to the CYP337B3(v1) WT as well as close to residue 114, but the metabolic activity of 8.9% did not attain the capacity of the WT CYP337B3(v1).

3.4 CYP337B1 is more abundant in *Helicoverpa armigera* larvae than CYP337B3

Because the three P450 proteins CYP337B1, CYP337B2, and CYP337B3 showed slightly differing mobilities in the SDS-PAGE, and because they could be detected by the same antibody, it was possible to estimate the relative proportion of the proteins in the tissues of *H. armigera* larvae. Separate strains of *H. armigera*[4] were used: one fixed for the *B1–B2* haplotype expressing only CYP337B1 and CYP337B2 proteins, one fixed for the *B3* haplotype expressing only the CYP337B3 protein, and hybrids expressing all three P450 proteins. Figure 3 shows the Western

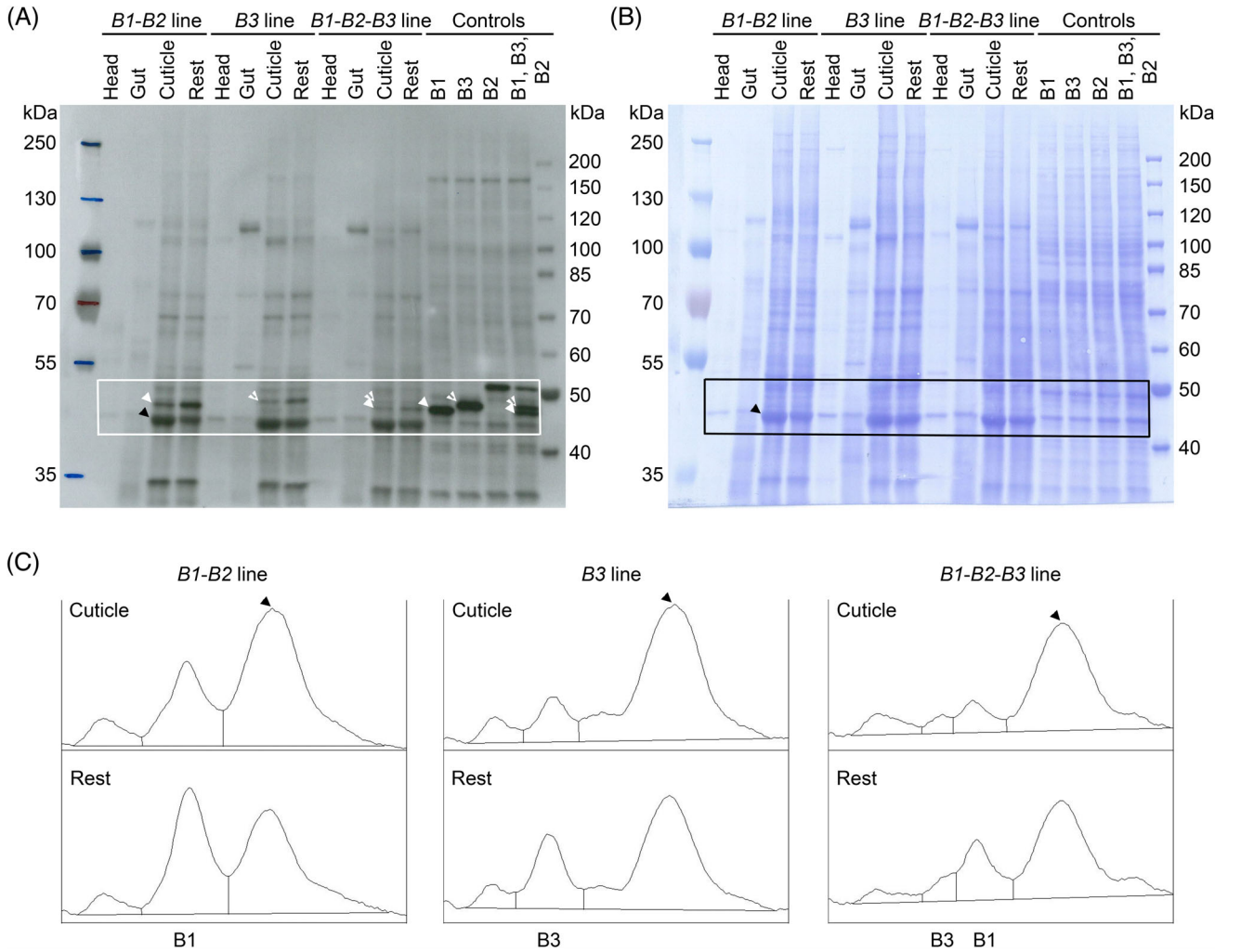

Figure 3. Relative quantification of CYP337B1 and CYP337B3 in different larval tissues of *Helicoverpa armigera*. (A) Western blot of microsomal proteins from several larval tissues of different lines of *H. armigera* in comparison to CYP337B1, CYP337B3, and CYP337B2 from heterologous cell cultures. White triangles, CYP337B1 band; white notched triangles, CYP337B3 band; black triangle, reference band. (B) Coomassie-stained membrane of the Western blot. Black triangle, reference band. (C) Diagram of the protein bands plotted with the Fiji software.[10]

blot (A), the Coomassie-stained membrane (B), and plots of the bands representing the CYP337B enzymes in the different *H. armigera* lines quantified using Fiji software[10] (C). As the antibody also binds to other proteins, many more bands are visible in Fig. 3(A), but with the help of the positive controls from the cell cultures and the Coomassie-stained membrane [Fig. 3(B)] it is possible to determine CYP337B1 and CYP337B3. The cross-reactivity of the antibody to CYP337B2 was too weak to be able to detect the low amounts in the larval tissues. Both CYP337B1 and CYP337B3 could be detected in the cuticle and in the rest of the body (including fat body, nervous system, body wall muscles) but not in the head and the gut plus Malpighian tubules. The amount of CYP337B1 and CYP337B3 was ≈1.6-fold higher in the rest of the body compared to the cuticle samples. From Fig. 3 (A) and (C) it also is obvious that the amount of CYP337B1 is higher than that of CYP337B3, when the signals from the *B1–B2* line and that from the *B3* line are compared to each other as well as a direct comparison of the signals from the *B1–B2–B3* line. Thus, CYP337B1 protein is two- to three-fold more abundant in the larval tissue of *H. armigera* than CYP337B3.

4 DISCUSSION AND CONCLUSIONS

Cytochrome P450 monooxygenases belong to the most important xenobiotic detoxifying enzymes. Because of their genetic diversity, broad substrate specificity and catalytic versatility, P450s and their associated NADPH-dependent cytochrome P450 reductase represent the only metabolic system that is capable of mediating resistance to all chemical classes of insecticides.[12] Most P450s are capable of metabolizing different substrates; one substrate may be metabolized by different P450 enzymes. Nevertheless, it is very difficult to predict which AAs are critical for the binding of a specific substrate and its turnover or play an important role in the binding of the reductase and the electron transfer or participate in the forming of the substrate access channel, the product release channel or the binding chamber. Critical AAs in all of these positions will alter the specificity of the P450. There are several studies investigating the structure–function relationship of specific insect P450s,[13] but for most P450s the critical AAs are still unknown. However, the identification of structural components of P450s that facilitate their activities in the detoxification of exogenous compounds and in insecticide resistance is of

great interest, as this could lead to new effective insecticides to control insect pests.

Here, we report on critical AAs of the P450 CYP337B3, conferring fenvalerate resistance in *H. armigera*, in comparison to its parent enzyme CYP337B1. As CYP337B3 is a chimeric P450 most likely arisen by an unequal crossing-over between CYP337B1 and CYP337B2,[4] it shares most of its functional regions with its parent CYP337B1, but its SRS1 region with its more distant parent CYP337B2. The AA occupying position 114 is the single most important residue affecting activity towards fenvalerate. With the L114F substitution, CYP337B3 lost 89% of its capability to metabolize fenvalerate. The reciprocal substitution F114L in CYP337B1 increased its capability to metabolize fenvalerate but could not reach the level of CYP337B3, even when nine additional sites were mutated, rendering the SRS1 of the two proteins identical. We did not investigate residues outside SRS1, which also might contribute to fenvalerate metabolism, such as residue M144 in CYP6B7.[13]

The crystal structure of CYP102A1 from the bacterium *Bacillus megaterium* (P450 BM3) is regarded as a useful scaffold for model production.[13] Analyzing the list of critical residues predicted from different insect P450s shown in table 1 of Hlavica,[13] reveals that SRS1 possessed 28% of the most critical residues among all six SRS regions. The phenylalanine residue F87 of CYP102A1 has been shown previously to be in close van der Waals' contact with the heme and undergoes motion from the substrate free-state to the substrate-complexed form.[13] Interestingly, this residue F87 of CYP102A1 aligned with the AA F116 of CYP6B1 from the black swallowtail, *Papilio polyxenes*, the AA F118 of CYP6B8 from the corn earworm, *Helicoverpa zea*, and the AA F118 of CYP6AB3 from the parsnip webworm, *Depressaria pastinacella*, all located in the SRS1 region and assumed to be critical in the detoxification of exogenous compounds.[13] Aligning CYP102A1 with CYP337B3, we found T88 of CYP102A1 aligning with L114 of CYP337B3 (Fig. S4); thus a neighboring residue influences the catalytic activity of CYP337B3 comparable with the P450s mentioned above.

The bulkier side-chain of phenylalanine might lead to a change in the size of the binding chamber or the access channel or the correct positioning of the substrate to the heme group in the active side, relative to the smaller side chain of leucine. Furthermore, leucine might be directly involved in the binding of esfenvalerate. Analogously, in addition to two more AA substitutions between CYP321A1 and CYP6B8v1 of *H. zea*, the replacement of T119 in CYP321A1 with the bulky aromatic F118 in CYP6B8v1 slows down and sometimes halts metabolism of larger natural toxins by CYP6B8v1 compared to CYP321A1.[14]

Pan *et al.*[15] showed that AAs of the SRS1 region of the CYP6B subfamily exert dramatic effects on the range of the plant toxins furanocoumarins metabolized, even when they occur potentially distal from the substrate. This effect is possibly explained by the rearrangement of the local hydrogen bond network surrounding the heme controlling its spin state and by this the catalytic activity of the P450.[15] This phenomenon was studied by means of I115 and A113 mutations of CYP6B1 from the black swallowtail, *P. polyxenes*, that are highly variable among the different members of the CYP6B subfamily. In particular, the I115L mutation significantly increased the spin state of the heme and thus led to a two- to four-fold increase in the turnover of linear furanocoumarins.[15]

By contrast, Wen *et al.*[16] reported that the I115L mutation of CYP6B1 reduces the turnover of furanocoumarins in studies conducted under conditions of high P450 reductase compared to the WT enzyme, because the mutant utilizes NADPH more slowly at high P450 reductase levels.[16] Amino acid 115 is predicted to be involved in a product release channel of CYP6B1 resulting in a more constricted channel extending from the catalytic site to the P450 surface limiting the rate of product release under conditions not limited by the rate of electron transfer from NADPH in the I115L mutant.[16]

Despite the evidence from other systems cited above, the importance of position 114 of CYP337B3 was poorly supported by the modelling and docking simulations. The L114F substitution, whether alone or with three other substitutions, had little effect on the distances between the $C_{4'}$ atom and the O atom coordinated to the Fe atom (3.4–3.9 Å). Both L114 of CYP337B3 and F114 of the mutant models are close to the esfenvalerate molecule (~4.5 Å). Conversely, the reciprocal substitution F114L in CYP337B1 producing its highest observed conversion produced a model with the $C_{4'}$ atom and the O atom coordinated to the Fe atom separated by 6.2 Å, with the leucine outside the 4.5 Å range. Converting the entire SRS1 to the CYP337B3 version decreased both distances but reduced the catalytic activity. These inconsistencies suggest that replacement of the entire 177 residues at the amino-terminus affects the conformation in a way that is not well-captured by the models. Like most investigators of insect P450s, we used human CYP3A4 as the template for homology modelling, because so far, no P450 from an insect has been crystallized. Moreover, no single model can capture the complexity of conformational changes during the reaction cycle, which could be very important.

The near-identity of the carboxy-terminus enabled use of the same antibody to control for expression differences among the different mutant cell lines, as well as quantifying both CYP337B1 and CYP337B3 in tissues of larvae of all three genotypes. Both are expressed in the cuticle and major detoxicative organ, the fat body, appropriate sites for defense against contact insecticides. CYP337B1 is two- to three-fold more abundant in the larval tissues than CYP337B3; the unequal crossing-over event put CYP337B3 behind the promoter for CYP337B2. One can readily envisage a stepwise evolution of pyrethroid resistance, with a 49% activity increase due to a F114L substitution in CYP337B1 *in vivo* and a higher expression level, which might be expected to provide as much protection of the larva against exposure to fenvalerate as CYP337B3. However, *CYP337B3* is present at much higher frequencies than *CYP337B1-CYP337B2* in most populations, including those recently invading South America. Despite the expected very low frequency of unequal crossing-over, the available evidence suggests that novel CYP337B3 alleles have been created *de novo* by this process in several localities worldwide.[8] We conclude that metabolic resistance to pyrethroids in *H. armigera* has evolved repeatedly by saltational evolution – namely, by a single mutation, an unequal crossing-over, producing a larger selective advantage than could be attained gradually by stepwise improvement of the parental enzyme.

Helicoverpa armigera is now firmly established in Brazil, where it is difficult to control with insecticides.[3, 7] Yet in the absence of a coordinated resistance management plan, higher levels of resistance are likely to evolve. The invader is hybridizing with its native sister species, *H. zea*,[17] which has traditionally been easier to control with pyrethroids. The ability to detect CYP337B3 with a simple PCR test[4] or a LAMP assay[18] offers the opportunity to monitor introgression of a resistance gene and avoid use of ineffective insecticides as the invader spreads into North America, where according to climate modeling it should occupy a wide range.[19]

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Brun-Barale A, Héma O, Martin T, Suraporn S, Audant P, Sezutsu H *et al.*, Multiple P450 genes overexpressed in deltamethrin-resistant strains of *Helicoverpa armigera*. *Pest Manag Sci* **66**:900–909 (2010).
2 Fitt GP, The ecology of *Heliothis* species in relation to agroecosystems. *Annu Rev Entomol* **34**:17–52 (1989).
3 Tay WT, Soria MF, Walsh T, Thomazoni D, Silvie P, Behere GT *et al.*, A brave new world for an old world pest: *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Brazil. *PLoS One* **8**:e80134 (2013).
4 Joußen N, Agnolet S, Lorenz S, Schöne SE, Ellinger R, Schneider B *et al.*, Resistance of Australian *Helicoverpa armigera* to fenvalerate is due to the chimeric P450 enzyme CYP337B3. *Proc Natl Acad Sci U S A* **109**: 15206–15211 (2012).
5 Rasool A, Joußen N, Lorenz S, Ellinger R, Schneider B, Khan SA *et al.*, An independent occurrence of the chimeric P450 enzyme CYP337B3 of *Helicoverpa armigera* confers cypermethrin resistance in Pakistan. *Insect Biochem Mol Biol* **53**:54–65 (2014).
6 Han Y, Yu W, Zhang W, Yang Y, Walsh T, Oakeshott JG *et al.*, Variation in P450-mediated fenvalerate resistance levels is not correlated with *CYP337B3* genotype in Chinese populations of *Helicoverpa armigera*. *Pestic Biochem Physiol* **121**:129–135 (2015).
7 Durigan MR, Corrêa AS, Pereira RM, Leite NA, Amado D, de Sousa DR *et al.*, High frequency of *CYP337B3* gene associated with control failures of *Helicoverpa armigera* with pyrethroid insecticides in Brazil. *Pestic Biochem Physiol* **143**:73–80 (2017).
8 Walsh TK, Joussen N, Tian K, McGaughran A, Anderson CJ, Qiu X *et al.*, Multiple recombination events between two cytochrome P450 loci contribute to global pyrethroid resistance in *Helicoverpa armigera*. *PLoS One* **18**:e0197760 (2018).
9 Marteijn RCL, Oude-Elferink MMA, Martens DE, de Gooijer CD and Tramper J, Effect of low inoculation density in the scale-up of insect cell cultures. *Biotechnol Prog* **16**:795–799 (2000).
10 Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T *et al.*, Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**:676–682 (2012).
11 Omura T and Sato R, A new cytochrome in liver microsomes. *J Biol Chem* **237**:1375–1376 (1962).
12 Li X, Schuler MA and Berenbaum MR, Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52**:231–253 (2007).
13 Hlavica P, Insect cytochromes P450: topology of structural elements predicted to govern catalytic versatility. *J Inorg Biochem* **105**:1354–1364 (2011).
14 Rupasinghe SG, Wen Z, Chiu T-L and Schuler MA, *Helicoverpa zea* CYP6B8 and CYP321A1: different molecular solutions to the problem of metabolizing plant toxins and insecticides. *Protein Eng Des Sel* **20**: 615–624 (2007).
15 Pan L, Wen Z, Baudry J, Berenbaum MR and Schuler MA, Identification of variable amino acids in the SRS1 region of CYP6B1 modulating furanocoumarin metabolism. *Arch Biochem Biophys* **422**:31–41 (2004).
16 Wen ZM, Baudry J, Berenbaum MR and Schuler MA, Ile115Leu mutation in the SRS1 region of an insect cytochrome P450 (CYP6B1) compromises substrate turnover via changes in a predicted product release channel. *Protein Eng Des Sel* **18**:191–199 (2005).
17 Anderson CJ, Oakeshott JG, Tay WT, Gordon KHJ, Zwick A and Walsh TK, Hybridization and gene flow in the mega-pest lineage of moth, Helicoverpa. *Proc Natl Acad Sci USA* **115**:5034–5039 (2018).
18 Choi BH, Hur JH, Heckel DG, Kim J and Koh YH, Development of a highly accurate and sensitive diagnostic tool for pyrethroid-resistant chimeric P450 CYP337B3 of *Helicoverpa armigera* using loop-mediated isothermal amplification. *Arch Insect Biochem Physiol* **99**: e21504 (2018).
19 Kriticos DJ, Ota N, Hutchison WD, Beddow J, Walsh T, Tay WT *et al.*, The potential distribution of invading *Helicoverpa armigera* in North America: is it just a matter of time? *PLos One* **10**:e0119618 (2015).

