Overexpression of cytochrome P450 *CYP6B7* mediated pyrethroid resistance in Indian strains of the cotton bollworm, *Helicoverpa armigera* (Hübner)

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Abstract: The role of cytochrome P450 monooxygenase in pyrethroid resistance was studied in different strains of the cotton bollworm, *Helicoverpa armigera*, from India. Filed collected strains of Nagpur and Delhi were compared to the laboratory reared population. The results showed a high resistance to deltamethrin, α-cypermethrin, and β-cyfluthrin. The results also showed that this resistance could be reduced by using piperonyl butoxide (PBO). The Nagpur and Delhi strains were found to have a 2.40 and 1.79 fold higher monooxygenase activity compared to a susceptible strain. A strong, positive correlation between monooxygenase activity and pyrethroid resistance was observed ($r = 0.86 – 0.98$). The relative expression of the housekeeping gene, *EF-1α*, and three P450 genes, was studied in the 5th instar larval midgut of the three strains. Out of the three P450 genes examined, expression of *CYP6B7* mRNA was not detected in the midgut of the susceptible strain though it was highly expressed in the resistant strains. The midgut of the Nagpur strain had a 2.60 fold overexpression of *CYP6B7* mRNA compared to the moderately resistant, Delhi strain. The mRNA of *CYP4G8* and *CYP6B2* were not overexpressed in either the Nagpur or Delhi strain. The results indicated that the elevated cytochrome P450 monooxygenase activity is associated with pyrethroid resistance in Indian strains of *H. armigera,* and *CYP6B7* could be the P450 form responsible for pyrethroid resistance.

Key words: cytochrome P450, *Helicoverpa armigera,* metabolic enzymes, pyrethroid resistance

Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner), is a key pest with an almost world-wide distribution. It is distributed throughout most of Asia, Australia, Africa, and the southern Mediterranean regions, including 29 cotton-producing countries such as India, China, Pakistan, and Egypt (EPPO 2006). It is recorded from 182 plant species including cotton, cereals, pulses, vegetables, and fruits. More than 75% of insecticides sprayed on cotton are targeted against *H. armigera*, and 50–70% of them are synthetic pyrethroids (Rao and Rao 2006). The cotton bollworm has developed resistance to almost all insecticides that have been used in trying to control the pest**.** The resistance of *H. armigera* to pyrethroids was first reported in Australia (Gunning *et al*. 1984) and later it was reported in many other countries. In India, pyrethroid resistance was first noticed between 1984–1985 in the southern region of India (Dhingra *et al*. 1988; McCaffery *et al*. 1988) and later it was reported in many other regions in the country.

Different mechanisms of resistance to pyrethroids have been identified in *H. armigera,* including enhanced metabolism (Ahmad and McCaffery 1991; Martin *et al*. 2002), nerve insensitivity (Gunning *et al*. 1991), and reduced penetration (Abd Elghafar and Knowles 1996). Among the known metabolic resistance mechanisms *viz*, esterase and monooxygenase have been extensively reported in *H. armigera* (Gunning *et al*. 1996; Kranthi *et al*. 1997; Gunning *et al*. 1999; Martin *et al*. 2003; Srinivas *et al*. 2004; Yang *et al*. 2005).

Yang *et al*. (2004) found that elevated cytochrome P450 monooxygenases are a major metabolic mechanism responsible for pyrethroid resistance in *H. armigera* from China, India, and Pakistan. There is also combined evidence from synergism experiments, monooxygenase activity assays with multiple substrates, and *in vitro* metabolism study.

Overexpression of certain P450 genes in resistant strains of *H. armigera* was found to be associated with pyrethroid resistance (Wang and Hobbs 1995; Ranasinghe *et al*. 1998; Brun Barale *et al*. 2010). In the current study, the activity of cytochrome P450 linked monooxygenase, of the midgut in the pyrethroid-resistant strains, was compared with that of the susceptible strain. We also studied the relative expression of three cytochrome P450 genes *viz, CYP6B7, CYP4G8*, and *CYP6B2* using real-time polymerase chain reaction (PCR) technique.

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Materials and Methods

Insect culture

The larvae of the cotton bollworm, *H. armigera* were collected from cotton and chickpea growing fields of the Delhi and Nagpur districts. The field collected larvae were transferred to the laboratory and reared on a chickpea-based, semi-artificial diet (Singh 1977). A pyrethroid susceptible laboratory strain of *H. armigera* which was maintained for several years without being exposed to any insecticides at the Central Institute for Cotton Research (CICR), Nagpur, India, was used. The field strains and the susceptible strain were maintained in the insectary under a constant temperature 25±2°C, 70±5% relative humidity (RH) and a photoperiod regime of 14 h photophase and 10 h scotophase.

Insecticides and synergist bioassay

Technical grade insecticides were obtained from various sources as follows: deltamethrin (98.5%) and *α*-cypermethrin (97.8%) from Gharda Chemicals Limited, Thane, Maharashtra and *β*-cyfluthrin (99%) from Bayer Crop Science, Thane. Piperonyl butoxide (PBO) was obtained from Sigma Chemicals Co., USA. A laboratory bioassay was undertaken on 5th instar larvae (100–120 mg) by topical application. Serial dilutions of the selected pyrethroids were prepared in acetone. An application of a 2 μl dose of the different solutions was done on the dorsal thoracic region of the larvae using a calibrated and pre-programmed micro-applicator (Stoelting Autogenic Systems, USA). Thirty larvae for each dose were treated and the control larvae were treated with acetone. The treated as well as the control larvae were held individually under the same conditions as the stock insects. All received a sufficient diet, and mortality was recorded up to three days. For each of the Delhi, Nagpur, and susceptible strains, the LD_{50} was calculated according to Finney (1971). Resistance factors were calculated as: the ratio of the LD_{50} of the resistant strain to the LD_{50} of the susceptible strain.

The synergistic activity of PBO was tested on the 5th instar larvae of different strains at a ratio of 1 : 5. This 1 : 5 (insecticide : synergist) ratio was selected based on preliminary studies. Topical application of PBO was applied as described above. The treated larvae were kept in the insectarium with sufficient food. Four hours after PBO treatment, the treated larvae were re-treated with the different doses of insecticides using the topical application method. Twenty larvae were used for each dose. The control larvae were treated with PBO only. Mortality was recorded after 72 h, and LD_{50} was calculated according to Finney (1971) for all three strains. The synergistic ratios were calculated by dividing the LD_{50} of the insecticides alone, by the LD_{50} of insecticides + + synergist.

Cytochrome P450 monooxygenase assay

A dissection of twenty, 5th instar larvae was done in cold phosphate buffer (100 mM, pH 7.0) containing 1.15% KCl. Five larvae at a time, were placed in a dissection tray containing 10 ml of the dissection buffer. The larvae were held, stretched, and fixed by using fine pins through the head and posterior region, with the dorsal side facing uppermost. Twenty midguts were dissected after removing the food bolus as completely as possible. The guts were transferred to 1 ml of freshly prepared homogenisation buffer i.e. phosphate buffer (100 mM, pH 7.0) containing 1 mM each of EDTA (ethylene diamine tetra acetic acid), PMSF (phenyl methyl sulfonyl fluride), PTU (phenyl thiourea), and 20% glycerol. The buffer containing the guts was placed in an ice bucket and homogenised using a motorised homogeniser at 1,000 rpm for 1 min. The solution was transferred to 1.5 ml polypropylene tubes and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and the volume was brought up to 6 ml using cold homogenisation buffer. Monooxygenase activity was estimated in a freshly prepared midgut homogenate. Six replications (20 midguts each) were applied for each strain using the carbon monoxide differences spectra, following reaction with sodium dithionite as described by Omura and Sato (1964) and adopted by Kranthi (2005).

Quantitative analysis of the P450 gene expression using real-time RT-PCR

The aim of this experiment was to study the possible role of any of cytochrome P450 genes in pyrethroid resistance. Primers for one housekeeping gene, *EF-1α*, and three cytochrome P450 genes, were designed as described in table 1.

Table 1. Primer sequences and expected PCR product sizes for the different primers used for real-time PCR analysis

Gene	Primer	Sequence	bp	Amplicons
*EF-1α	EF-F	GACAAACGTACCATCGAGAAG	21	279 bp
	EF-R	GATACCAGCCTCGAACTCAC	20	
CYP6B7	6B7F	TCTTGTGGACAACATTATTAGC	22	130 bp
	6B7R	AAGTGATGTTACTCCATCAAGA	22	
CYP4G8	4G8F	GAAATACTTGGAAAGGTGTC	20	234 bp
	4G8R	TAGTGACGGTTGGCAGATCG	20	
CYP6B2	qCYP6B2-F	CGCCTGGTTAGACTGGTTTC	20	~60–120 bp
	qCYP6B2-R	TGTCGCAACTCGTTTATGCT	20	

*housekeeping gene

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Overexpression of cytochrome P450 *CYP6B7* mediated pyrethroid resistance in Indian strains of the cotton bollworm… **289**

Isolation of DNA

The genomic DNA was isolated from the midgut of the 5th instar larvae. The midgut of the 5th instar larvae was dissected in sterile distilled water and transferred to 1.5 ml Eppendorf tubes containing 150 μl homogenisation buffer (250 mM Tris-HCl, pH 8.5, 375 mM NaCl, 25 mM EDTA, 1% sodium dodecyl sulfate (SDS), 1% *β*-mercaptophenol). After homogenisation, the pestle was washed by adding 150 μl homogenisation buffer. An addition of 100 μl of lysis buffer was then made, and the tubes were kept in a water bath at 65°C for 30 min with continuous mild shaking until the tissue was completely lysed. To each Eppendorf tube, 4 μl of RNAase was added. The tubes were kept at 37°C for 45 min. After incubation, 80 μl of sodium acetate (1 M) was added and kept at 4°C for 30 min, before centrifugation at 14,000 rpm for 20 min at 4°C. The supernatant was transferred into a fresh tube and an equal volume of PCI (25 phenol : 24 choloroform : 1 isoamyl alcohol) was added and mixed while swirling the tubes. The tubes were then centrifuged at 12,000 rpm for 15 min and the upper aqueous phase was carefully collected. Chilled isopropanol (500 μl) was added and the tubes were kept overnight at –20°C. The tubes were then centrifuged at 12,000 rpm for 15 min. The supernatant was discarded. The pellets were washed by adding 100 μl of 70% ethanol, and centrifuged for 15 min at 12,000 rpm. Ethanol was discarded and the tubes were left open at room temperature for the evaporation of any traces left of ethanol. The DNA pellets were dissolved in 40 μl of sterile double distilled water. The integrity and quality of total DNA were examined by electrophoresis on 1% agarose gel and stored at –20°C till used.

PCR

The genomic DNA of the three strains (Nagpur, Delhi, and susceptible strains) was compared for the presence of the housekeeping and cytochrome P450 selected genes. The PCR was carried out in 0.5 ml microcentrifuge tubes. The PCR reaction mixture consisted of 2 μl of DNA template (100 ng/μl), 2.5 μl of Taq buffer (10X), 1 μl of dNTP, 1 μl (1 ng/ μl) of primer (Forward and Reverse), 1 μl of MgCl₂, and 1 μl of Taq polymerase (1 U/μl). Finally, the volume was brought up to 25 μl with double distilled water. The mixture was placed in a PCR machine (Biometra thermo cycler) and the following temperature profile and cycles were performed; denaturation of DNA at 94°C for 4 min in the first cycle, 40 cycles which had three steps: (1) denaturation of DNA at 94°C for 45 sec, (2) primer annealing at 60°C for 45 sec, and (3) primer extension at 72°C for 45 sec and finally, one cycle for a final extension at 72°C for 10 min. The PCR product was examined using 2.5% agarose gel. The amplification of the different genes as well as molecular weight were compared in the three strains.

Isolation of total RNA

The total RNA was isolated from the midgut of the 5th larval instar. The midgut was crushed in liquid nitrogen using a Teflon homogeniser, and the crushed tissue was collected in an Eppendorf tube. The total RNA was isolated using RNeasy Mini Kits, Qiagen (catalogue No. 74104) according to the manufacturer's instructions.

Quantitative real-time PCR using SYBR Green Dye

The relative expression of three cytochrome P450 genes (*CYP6B7*, *CYP4G8*, and *CYP6B2*) was studied using the housekeeping gene *EF-1α*. Real-time PCR was carried out using the fluorescent dye SYBR Green as the detection chemistry. The PCR reaction mixture consisted of 12.5 μl quantitative SYBR green, 2.5 μl RT-PCR mix, 1 μl primer (Forward and Reverse), 2 μl RNA, and 8.25 μl RNAase free water.

The mixture was placed in a real-time-PCR machine (Stratagen, MX3005). One step RT-PCR was performed as follows; cDNA synthesis at 50°C for 30 min in the first cycle, initial denaturation at 94°C for 10 min for one cycle, and 40 cycles having three steps of (1) denaturation of DNA at 94°C for 30 sec, (2) primer annealing temperature at 55°C for 1 min, and (3) primer extension at 72°C for 1 min. The purity of the amplified products was checked by the observation of a single melting peak. The threshold cycle (C_T) values and dissociation curves were produced by Maxpro (Applied Biosystems). Triplicate measurements were performed for each RNA sample. A no template control (NTC, does not contain RNA) was also run. The relative expression levels of target genes were calculated with the comparative C_T method as described by Winner *et al.* (1999) and Yang *et al.* (2006). The amount of a target gene, normalised to the endogenous housekeeping gene *EF-1α* and relative to a calibrator, was given by $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ of the target sample (resistant) – ΔC_T of the calibrator sample (susceptible), and $\Delta C_T = C_T$ (sample) – C_T (*EF-1α*).

Results

Bioassay results of the dose-response relationships of the 5th instar larvae to different pyrethroids and the synergist PBO, are presented in table 2. The 5th instar larvae displayed high resistance ratios against all the pyrethroids used. The 5th instar larvae are less susceptible to deltamethrin with LD₅₀ values of 0.00706, 13.00880, and 20.12174 μg/larva against susceptible, Delhi and Nagpur strains, respectively. The active ingredient, *β*-cyfluthrin, was the most effective of the three pyrethroids against the 5th instar larvae with very low LD₅₀ values (0.06431, 0.34432 and 4.71041 μg/larva against susceptible, Delhi and Nagpur strains, respectively).

There were significant differences in the LD₅₀ values of the different strains. As the predicted values of the probit model did not differ significantly from the observed values in all pyrethroid-strain combinations, the probit model was suitable for the dose-response analysis. The laboratory observation showed that in the case of deltamethrin there was almost no larval mortality within the first 24 h after treatment.

The 5th instar larvae exhibited the highest resistance ratio against deltamethrin (2,850.10 and 1,842.61 fold in Nagpur and Delhi strains, respectively), followed by

Table 2. Effect of synergists on the toxicity of synthetic pyrethroids against 5th instar larvae of different strains of *H. armigera*

Pyrethroids	Strains	df	χ^2	Regression equation (Y)	LD_{50} [µg/ /larvae]	Fudical limits	Synergistic ratio	Resistance ratio
Deltamethrin	Nagpur	6	6.70843	Y = 0.50676 + 3.44662X	20.12174	16.76085–24.15705	–	2,850.10
	Delhi	4	6.36328	Y = 1.40404 + 3.22728X	13.00880	11.61567–14.59957	–	1,842.61
	Susceptible	5	0.79438	Y = 7.33757 + 1.08666X	0.00706	0.00478–0.01042	–	1.00
Deltamethrin + PBO	Nagpur	5	0.52723	Y = 5.59259 + 1.18132X	0.31504	0.21667–0.45809	63.87	44.62
	Delhi	6	5.59856	Y = 5.98670 + 0.94204X	0.08966	0.06900–0.11650	145.09	12.74
α-Cypermethrin	Nagpur	5	5.73764	Y = 3.56790 + 1.46117X	9.55178	7.79601–1.70294	–	1,906.54
	Delhi	4	1.68805	Y = 2.54352 + 1.23875X	0.96168	0.73596–1.25662	–	191.95
	Susceptible	5	0.50655	Y = 6.72658 + 0.75057X	0.00501	0.00253–0.00991	–	1.00
α-Cypermethrin + PBO	Nagpur	5	0.62606	Y = 5.78050 + 0.93163X	0.14529	0.09494–0.22233	65.74	29.00
	Delhi	6	11.9453	Y = 6.08556 + 1.13620X	0.11081	0.06673–0.18399	8.68	22.12
β-Cyfluthrin	Nagpur	4	2.37911	Y = 4.17763 + 1.22184X	4.71041	3.40620–6.51403	–	73.25
	Delhi	5	1.03652	Y = 5.47401 + 1.02369X	0.34432	0.18955–0.62545	–	5.35
	Susceptible	4	0.20022	Y = 6.32030 + 1.10793X	0.06431	0.04430–0.09374	–	1.00
β-Cyfluthrin + PBO	Nagpur	5	1.59436	Y = 5.69842 + 1.37224X	0.30977	0.23911–0.40132	15.21	4.82
	Delhi	4	0.98759	Y = 6.71406 + 1.26285X	0.04392	0.02639–0.07312	7.66	0.68

In none of the cases was the data found to be significantly heterogeneous at p = 0.05; Y – probit kill, X – log dose;

final instar = 100–120 mg body weight

α-cypermethrin (1,906.54 and 191.95 fold in Nagpur and Delhi strains, respectively). The resistance ratios were relatively less against β-cyfluthrin (73.35 and 5.35 fold in Nagpur and Delhi strains, respectively).

The synergistic activity of PBO was less against β-cyfluthrin. Synergism tended to be greater for the Nagpur strain than for the Delhi strain, except in the case of deltamethrin.

Table 3 presents the monooxygenase activity in the susceptible and pyrethroid-resistant strains. The resistant strains, i.e. the Nagpur and the Delhi, were found to have elevated monooxygenase activity compared to the susceptible strain. The highest P450 content was recorded in the Nagpur strain (130.83±1.99 pmol/mg protein) followed by the Delhi strain (97.5±2.86 pmol/mg protein) while monooxygenase activity was only 54.5±2.3 pmol/mg protein in the susceptible strain. The Nagpur and Delhi strains had a 2.40 and 1.79 fold higher monooxygenase activity compared to the susceptible strain. Correlation analysis revealed a strong positive correlation between monooxygenase activity and pyrethroid resistance. The correlation coefficient (r) was 0.98, 0.88, and 0.86 for deltamethrin, α-cypermethrin, and β-cyfluthrin, respectively. The highest monooxygenase activity synchronised with

the highest LD_{50} values and hence the highest resistance ratio. The purity of a reconstituted monooxygenase system can often be determined from a scan of cytochrome P450. Problems with preparations result in a large amount of a degradation product in the form of cytochrome P420. In this experiment, monooxygenase preparations were of a high purity because only minimal amounts of P420 were detected in these assays.

PCR amplification of the genomic DNA of the three strains selected with the gene-specific primers was performed and PCR products were electrophoresed on 1.0% agarose gel. All four genes tested were present in the genomic DNA in all three strains (Nagpur, Delhi, and susceptible strains). All the four primers used, had generated a single band in all the strains tested that revealed the gene-specificity of the primers. The approximate band sizes generated were: 250, 150, 300, and 250 bp for *EF-1α*, *CYP6B7*, *CYP4G8*, and *CYP6B2*, respectively. The band size obtained was the same in all three of the tested strains.

Table 4 represents the relative expression of P450 genes selected in the midgut of the 5th instar larvae of different strains of *H. armigera*. The data indicated significant variation in the expression of different P450 genes

Table 3. Activity of cytochrome P450 linked monooxygenase from the midgut of the 5th instar larvae of different strains of *H. armigera*

Strains	Monooxygenase activity [pM/mg protein]	*Relative activity
Nagpur	130.83±1.99	2.40
Delhi	97.5±2.86	1.79
Susceptible	54.5±2.3	1.00

*monooxygenase activity in resistant strain/monooxygnease activity in susceptible strain

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Overexpression of cytochrome P450 *CYP6B7* mediated pyrethroid resistance in Indian strains of the cotton bollworm… **291**

Table 4. Relative expression of P450 genes in the midgut of the 5th instar larvae of different strains of *H. armigera*

Strains	Gene	C_T Value	ΔC_T	*Relative expression $2^{-\Delta\Delta CT}$
Nagpur	*EF1-α*	19.94	–	–
	CYP6B7	23.26	3.32	2.60**
	CYP4G8	37.19	17.25	0.1191
	CYP6B2	27.56	7.62	0.1948
Delhi	*EF1-α*	18.99	–	–
	CYP6B7	23.69	4.7	1.00**
	CYP4G8	36.1	17.11	0.1312
	CYP6B2	30.06	11.07	0.0178
Susceptible	*EF1-α*	19.53	–	1.00
	CYP6B7	not detected	–	–
	CYP4G8	33.71	14.18	1.00
	CYP6B2	24.79	5.26	1.00

*relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Winer *et al.* 1999), expression of the particular gene in the susceptible strain was used as the calibrator

**as *CYP6B7* was not expressed in the susceptible strain the expression level of *CYP6B7* mRNA in the Delhi strain was used as a calibrator to calculate the relative expression

between different pyrethroid-resistant and susceptible strains. Out of the three P450 genes, the expression of *CYP6B7* mRNA was not detected in the susceptible strain, though it was highly expressed in the two resistant strains (the Nagpur and Delhi strains) with less C_T values of 23.26 and 23.69 for the Nagpur and Delhi strains, respectively. Compared to the Delhi strain, the Nagpur strain had a 2.60 fold overexpression of *CYP6B7* mRNA.

In comparison to the susceptible strain, the mRNA of *CYP4G8*, and *CYP6B2* were not overexpressed in either the Nagpur strain or the Delhi strain. The relative expression of *CYP4G8* mRNA was less in the Nagpur strain (0.1191) compared to the Delhi strain (0.1312). The relative expression of *CYP6B2* mRNA was almost 10 times more in the Nagpur strain (0.1948) compared to the Delhi strain (0.0178).

The dissociation curves of the three P450 genes and the housekeeping gene *EF-1α* indicated that there is only one peak in each dissociation curve which verified the specificity of the primers used in this study.

Discussion

Detoxification by cytochrome P450 monooxygenase is one of the most important mechanisms of insecticide resistance. Due to the broad substrate spectra of P450s, this mechanism may potentially affect several classes of insecticides and thereby confer cross-resistance to unrelated compounds (Agosin 1985). Scott (1999) proposed two criteria for demonstrating that P450 is involved in resistance: (1) the P450 must be shown to detoxify the compound to which the strain has monooxygenase-mediated resistance, and (2) the resistant strain should have a greater amount of this P450, or the protein coded by the resistant strain allele should be shown to have a greater catalytic activity compared to the protein coded by the susceptible strain allele.

In the current study, the resistant strains, i.e. the Nagpur strain and the Delhi strain that displayed a high resistance ratio to the tested pyrethroids were found to have an elevated 2.40 and 1.79 fold higher monooxygenase activity compared to the susceptible strain. The high synergistic activity obtained by PBO and the strong positive correlation observed between monooxygenase activity and pyrethroid resistance indicated that the detoxification by P450 monooxygenase is one of the important mechanisms of pyrethroid resistance in the selected strain. However, the role played by esterase was also reported in the same strains (Abd El-Latif and Subrahmanyam 2010a, b).

Elevated monooxygenase activity in pyrethroid-resistant *H. armigera* was reported by many authors. Barden *et al.* (1992) found a 1.7 fold increases in cytochrome P450 activity in the larvae of the pyrethroid-resistant strain of *H. armigera*, compared to the susceptible strain. Rose *et al.* (1995) found up to a 44-fold higher quantity of cytochrome P450 in the gut, fat body, and carcass of the resistant strain of *H. armigera*, in comparison to the susceptible strain. Martin *et al.* (2002) reported that the deltamethrin resistance in a selected resistant field strain was correlated to an increase of mixed function oxidase. Enhanced monooxygenase levels were then confirmed in several *H. armigera* field strains collected in cotton areas of West Africa from 1999 to 2000. A resistant strain of *H. armigera* that showed a 1,850–7,140 fold resistance to four ester-bonded phenoxybenzyl alcohol pyrethroids (fenvalerate, deltamethrin, cypermethrin, and cyhalothrin) was found to have a 3.7, 4.7, and 10 fold increase on the activity of cytochrome P450 towards the substrates ρ-nitroanisole, ethoxycumarin, and methxoycumarin, respectively, compared to the susceptible strain (Yang *et al.* 2005). Chen *et al.* (2005) found a strong positive correlation ($r = 0.98$) between the activity of P450 monooxygenase and fenvalerate resistance in *H. armigera.* The four resistant strains

that had a 6, 71, 2540, and 11,800 fold resistance to fenvalerate, showed a 4, 10, 24, and 60 fold increase in P450 activity, respectively, compared to the susceptible strain. Bues *et al.* (2005) reported a 2–3 fold increase in P450 activity of the deltamethrin-resistant strain of *H. armigera* compared to the susceptible strain. Kranthi *et al.* (1997, 2001) reported the importance of monooxygenase in pyrethroid resistance in Indian populations of *H. armigera*. Brun-Barale *et al.* (2010) reported that *CYP4L5, CYP4L11, CYP6AE11, CYP332A1,* and *CYP9A14* were significantly overexpressed in the Burkina Faso and Spain strains of *H. armigera* when compared with Heliar, a susceptible strain. Significant overexpression of multiple *CYP* genes (*CYP4M6, CYP4M7, CYP6AE11, CYP9A12, CYP332A1,* and *CYP337B1*) was also found in six field strains with different levels of resistance from Benin, Burkina Faso, and Mali.

Insects can evolve monooxygenase-mediated resistance through over-expression of either a single, or more commonly, a set of P450 genes, depending on factors such as insect strains, insecticide type, and selection pressure (Yang *et al.* 2006). It is necessary to examine several alternative hypotheses in discussing the observed strain-to-strain variation in P450 gene expression: (1) *H. armigera* P450 genes show overlapping substrate activities and thus, different P450 genes may be overexpressed in different resistant strains while still achieving the same net pyrethroid resistance; (2) both constitutive overexpression and inducible expression may be important in field-based resistance (Pittendrigh *et al.* 1997).

In the present study, the real-time PCR data indicate significant variation in the expression of different P450 genes between different pyrethroid-resistant and susceptible strains. Out of the three P450 genes examined, expression of *CYP6B7* mRNA was not detected in the midgut of the susceptible strain, though it was highly expressed in the midgut of the resistant strains. The midgut of the most resistant Nagpur strain, had a 2.60 fold overexpression of *CYP6B7* mRNA compared to the moderately resistant, Delhi strain. In comparison to the susceptible strain, the mRNA of *CYP4G8* and *CYP6B2* were not over-expressed in either the Nagpur strain or the Delhi strain. The relative expression of *CYP4G8* mRNA was less in the Nagpur strain (0.1191) compared to the Delhi strain (0.1312). The relative expression of *CYP6B2* mRNA was almost 10 times more in the Nagpur strain (0.1948) compared to the Delhi strain (0.0178).

The molecular basis of oxidative metabolism for pyrethroid resistance in *H. armigera* has been studied by many authors. *CYP6B2, CYP6B6,* and *CYP6B7* were cloned from Australian *H. armigera,* and *CYP6B7* was suggested as the form responsible for pyrethroid resistance in *H. armigera* based on *CYP6B7* mRNA overexpression on the pyrethroid-resistant strain (Wang and Hobbs 1995). Ranasinghe *et al.* (1998) reported that *CYP6B7* is the cytochrome P450 form involved in pyrethroid resistance and that overexpression of *CYP6B7* is a common cause of pyrethroid resistance in *H. armigera*. It was reported by Pittendrigh *et al.* (1997) that out of eight P450 genes: seven from the *CYP4* family (*CYP4S1, CYP4S2, CYP4G8, CYP4G9, CYP4G10, CYP4M4,* and *CYP4L3*) and one from

CYP9 family (*CYP9A3*), one of these genes, *CYP4G8,* over-expressed two-fold in the resistant strain. The other *CYP4s* had either similar or undetectable levels of expression.

In Chinese pyrethroid-resistant strains of *H. armigera,* Yang *et al.* (2006) studied the relative expression of *CYP9A12, CYP9A14, CYP6B7,* and *CYP4G8,* using quantitative real-time PCR in the final instars of a field derived YG strain (with a seven-fold resistance) and the YGF strain (selected from the YG strain with fenvalerate in the laboratory, with a 1,690-fold resistance to fenvalerate). These authors reported that the constitutive overexpression of multiple cytochrome P450 genes (*CYP9A12, CYP9A14,* and *CYP6B7*) is associated with pyrethroid resistance in *H. armigera.* But the overexpression of those particular genes in the resistant strains was not compared with a susceptible strain.

Based on the results of this study, it can be concluded that elevated cytochrome P450 monooxygenase activity is associated with pyrethroid resistance in Indian strains of *H. armigera,* and that *CYP6B7* is the P450 form associated with pyrethroid resistance. Hence, expression of *CYP6B7* in heterologous systems would then be a more efficient means of testing its competency in metabolising the pyrethroid. For example, the functional expression of the *CYP9A12* and *CYP9A14* in the W(R) strain of yeast (*Saccharomyces cerevisiae*) using a plasmid shuttle vector pYES2, provided important evidence about the role of *CYP9A12* and *CYP9A14* in conferring pyrethroid resistance in *H. armigera* (Yang *et al.* 2008). Altering the expression of *CYP6B7* using RNA interference (RNAi) techniques could also prove its role in pyrethroid resistance and provide a new possibility of controlling pyrethroid resistance in *H. armigera.*

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Overexpression of cytochrome P450 *CYP6B7* mediated pyrethroid resistance in Indian strains of the cotton bollworm…   **293**

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