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Over-Expression of Cytochrome P450 CYP6B7 mRNA and Pyrethroid Resistance in Australian Populations of *Helicoverpa armigera* (Hübner)

Charani Ranasinghe,[1] Bronwyn Campbell[2] & Andrew A. Hobbs[1]*

[1] Department of Biochemistry, University of Western Australia, Nedlands, WA 6907, Australia
[2] Division of Entomology, CSIRO, Canberra, ACT 2601, Australia/Division of Botany and Zoology Australian National University, Canberra, ACT 2601, Australia

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Abstract: Three cDNA clones for cytochrome P450s, CYP6B2, CYP6B6 and CYP6B7 which have 84–87% protein sequence identity have been isolated previously from *Helicoverpa armigera*, and the CYP6B7 mRNA was found to be over-expressed in a pyrethroid-resistant field population. Subsequent analysis has shown that over-expression is observed in a majority of individuals in all populations tested. Single-pair crosses between resistant and sensitive individuals indicated that the pyrethroid resistance co-segregated with the over-expression of this mRNA. Southern analysis indicated that the over-expression, which was confined to midgut only in many insects, was not related to gene amplification. These observations add weight to the conclusion that CYP6B7 is the cytochrome P450 form involved in pyrethroid resistance, and that over-expression of cytochrome P450 CYP6B7 is a common cause of pyrethroid resistance in *H. armigera*. The results suggest that specific probes for CYP6B7 protein or mRNA could provide the basis for the development of tools for monitoring pyrethroid resistance due to mixed function oxidase activity in field populations of this insect. © 1998 Society of Chemical Industry

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Key words: cytochrome P450 CYP6B7; over-expression; pyrethroid resistance; *Helicoverpa armigera*

1 INTRODUCTION

Since the advent of widespread use of synthetic insecticides, insecticide resistance has become one of the major challenges in pest management.[1] Such resistance is a complex phenomenon often involving combinations of several different mechanisms, including, in particular, increased metabolism of the insecticides. Metabolism of insecticides can involve a number of different enzymes, depending upon the particular compound, but, depending upon the species of insect, cytochrome P450s are an important group of enzymes in this respect.[2] These latter are a group of enzymes which appear to have evolved as part of the defence mechanisms against the wide array of plant allelochemicals which are present in the diet of many insects. Increased metabolism due to cytochrome P450 activity has been implicated in many instances of resistance by the use of specific enzyme inhibitors.[3] More directly, the cytochrome P450s CYP6A1 and CYP6A2, from *Musca domestica* L. and *Drosophila melanogaster* Meig. respectively, have been shown to be involved in DDT and malathion resistance,[4,5] CYP6D1 appears to be involved in pyrethroid resistance in the lpr strain of *M. domestica*[6] and

* To whom correspondence should be addressed.
E-mail: andrewh@cyllene.uwa.edu.au
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CYP9A1 from *Heliothis virescens* F. has been shown to be involved in thiodicarb resistance.[7]

Helicoverpa armigera (Hübner) is a polyphagous insect pest in many parts of the world. It is one of the major pests affecting Australian cotton crops, and the development of insecticide resistance, particularly to pyrethroids, has become a serious problem in controlling this pest. Pyrethroid resistance was first observed in Australia in 1983[8] and in many populations 90% of the individuals are now resistant.[9] While target-site insensitivity and reduced penetration may play a part, increased metabolism appears to be the most important factor in resistance.[10,11] Metabolism of pyrethroids in lepidopterans initially appears to involve both esterase and cytochrome P450 activity.[12,13] Inhibitor studies using the cytochrome P450 inhibitor, piperonyl butoxide (PBO), have suggested that increased detoxification by one or more cytochrome P450s is involved in conferring pyrethroid resistance in *H. armigera*,[10,14] although other studies have suggested that increased esterase activity is the most important mechanism.[15] Similar observations have been made on the related species *H. zea* (Boddie) where increased metabolism was thought to be the main cause of resistance to cypermethrin.[16]

We have previously isolated cDNA clones for three cytochrome P450s from *H. armigera* that are members of the CYP6B subfamily, CYP6B2, CYP6B6 and CYP6B7.[17] Our studies have shown that CYP6B7 mRNA (the largest mRNA) is inducible by the major monoterpene components of mint,[18] a plant which can cause induced pyrethroid resistance, as well as by a pyrethroid itself.[19] In addition, it is over-expressed in pyrethroid-resistant individuals.[17] In this report we describe the over-expression of CYP6B7 mRNA in pyrethroid-resistant larvae of *H. armigera*, and its co-segregation with resistance in genetic crosses of individual insects, giving further evidence that over-expression of this cytochrome P450 is involved in pyrethroid resistance in *H. armigera*. We have found that CYP6B7 mRNA is over-expressed in all of the populations tested and is possibly a common mechanism of resistance in this species.

2 MATERIALS AND METHODS

2.1 Insects

The sensitive larvae from a laboratory population were maintained as described previously.[20] Initially, in 1993, larvae from the Narrabri valley were tested with fenvalerate (0·2 μg) and PBO (50 μg), the survivors raised to the fifth instar and then midguts removed for preparation of RNA. Subsequently, in 1995–1996, *H. armigera* larvae were collected from cotton crops in the Narrabri valley, Gooloogong, Bathurst and Bega (New South Wales), reared through to late third- or early fourth-instar larvae weighing 30–40 mg, and then tested with a discriminating dose of fenvalerate (0·2 μg per larva) as described previously.[17] Surviving larvae were used to set up separate populations from each location. The percentage of resistant individuals in each population was determined by exposure of adults in a vial-test assay or by applying a discriminating dose of pyrethroid to larvae.[9]

2.2 Genetic crosses

Single resistant individuals from the 1996 Narrabri valley population were crossed with single sensitive male or female moths. Nothing is known of the genotypes of the insects used except that most of the insects in the population over-expressed the CYP6B7 mRNA. Two crosses gave rise to fertile eggs which then hatched. The majority of the larvae from the F1 generation were tested with a discriminating dose of fenvalerate (6 μg^{-1}) while some were kept untreated. Fourteen survivors from the discriminating dose from the F1 generation were individually back-crossed to the sensitive larvae. Two crosses were fertile, giving F2 progeny, which were again treated with fenvalerate as before.

2.3 RNA and DNA analysis

The mid-gut tissues from fifth-instar resistant larvae were collected individually, washed in saline to remove gut contents, placed in liquid nitrogen and the tissues stored at $-80°C$ until used. From one resistant population the fat body and integument were also collected individually. The RNA was extracted using the guanidine thiocyanate procedure.[21] Where indicated, both the RNA and DNA were extracted from midgut tissue from individual larvae using a modified procedure. The tissue was homogenized in ten volumes of buffer (NaCl 75 mM, Tris-Cl pH 8·0 100 mM, EDTA 10 mM) then made up to 5 g $litre^{-1}$ SDS, and an equal volume of buffer-saturated phenol/chloroform was added. The sample was centrifuged, the aqueous phase collected and LiCl (4 M) was added to give a final concentration of 3 M, and stored at $-20°C$ for 2–3 h. The sample was centrifuged for 10 min and the RNA was collected as a pellet, dissolved in guanidine thiocyanate (4 M), and the RNA was extracted as before. The supernatant was used for the DNA extraction. DNA was precipitated with ethanol, collected by centrifugation and the DNA pellet dissolved in TE (Tris-Cl, EDTA pH 8·0). The DNA solution was treated with RNase A (100 μg ml^{-1} at 37°C for 30 min), extracted using phenol/chloroform[22] and precipitated with ethanol. The DNA pellet was dissolved in water and the concentration of DNA determined from the absorbance at 260 nm.

Northern analysis was performed as described previously.[18] The control RNA and DNA samples were obtained from a pool of 10 insects. Genomic DNA was digested with restriction enzyme EcoRI, electrophoresed through an agarose gel and transferred by capillarity to a nylon membrane after denaturing the DNA.[22] Both DNA and RNA filters were hybridized with either the cDNA clone pHACYP14, which hybridizes to all three mRNAs, or the 3′ non-coding region of CYP6B7, as indicated. The DNA probes were labelled with $\alpha[^{32}P]$dCTP by random priming.[23] After hybridization, the filters were washed twice at 68°C (60°C when using the 3′ non-coding region of CYP6B7, due to the high A/T content in this region) for 15 min each in NaCl (30 mM), sodium citrate (3 mM) and sodium dodecyl sulfate (1·0 g litre^{-1}), and then exposed to an imaging plate and scanned using a phosphoimager (BAS 1000, Fuji). After scanning, the membranes were reprobed with a cDNA clone for *H. armigera* β-actin using the method above, and finally stained with methylene blue. Both actin binding and methylene blue staining were used to confirm equal loading of RNA in each lane. The fold increases were again calculated as described previously.[18]

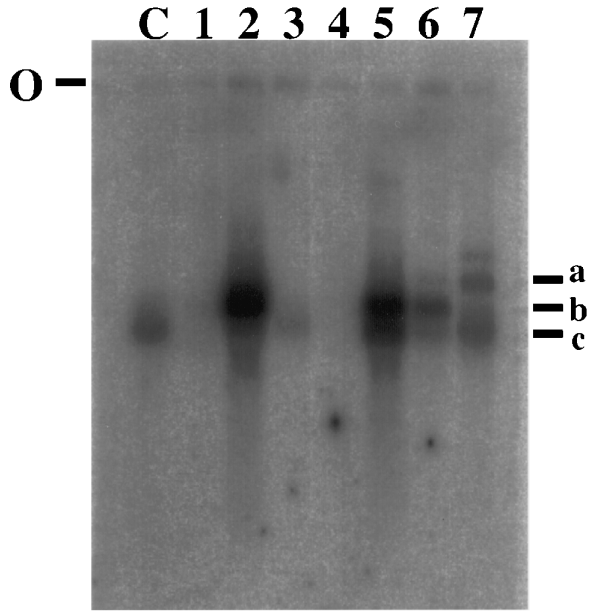

Fig. 1. Northern analysis of selected pyrethroid-resistant individuals. Total RNA was extracted from (lanes 1 to 7) midguts of field-collected resistant individuals; (lane C) pooled midguts of 10 sensitive larvae. The membrane was probed with the cDNA clone pHACYP14 as probe. The O indicates the origin and the three lines indicate (a) Possible polymorphic form of CYP6B7; (b) CYP6B7 mRNA and (c) CYP6B2 and CYP6B6 mRNAs.

3 RESULTS

Initially a group of larvae raised from field-collected specimens which had been selected as resistant in a discriminating dose test in the presence of piperonyl butoxide were tested for expression of the CYP6B mRNAs. Approximately 5–10% of larvae exhibited such resistance, which would be unrelated to cytochrome P450, while 50–80% of larvae exhibited mixed function oxidase-based resistance. With such high levels of resistance, one would expect that many larvae would in fact exhibit multiple resistance mechanisms, the genes for which would probably segregate independently. This is in fact what was found. Northern analysis of midguts of individual pyrethroid-resistant larvae with CYP6B2 cDNA clone as probe indicated that many individuals in the population over-expressed the large mRNA, corresponding to CYP6B7, compared to the control (Fig. 1). Quantitative analysis of the intensities of the bands indicated that this mRNA was increased approximately 20-fold in individual 2 and 5 and to a lesser extent in individual 6. CYP6B7 mRNA was not detectable in RNA from individuals 1,3 and 4. A band corresponding to the normal size of CYP6B7 was also not detectable in the RNA of individual 7, but, in this case, there was another larger homologous mRNA around 3·0 kb. Later analysis showed binding of the CYP6B7-specific probe to this band, indicating that the larger band also corresponded to CYP6B7 mRNA, and may have been due to utilization of alternative polyadenylation sites. By contrast, the smaller mRNAs (CYP6B2 and CYP6B6) were not elevated in any of the individuals compared to the control and in individuals 1 and 4 these mRNAs were not detectable, as was found for CYP6B7 mRNA. None of these differences was due to unequal loading of RNA onto the gel, since the methylene blue staining of rRNA showed equal amounts in each lane. Similar results were obtained with further individuals from the same group (results not shown) giving a total of nine out of 17 individuals in this group of survivors overexpressing CYP6B7 mRNA.

In the above experiment the individuals had been exposed five days previously to pyrethroid during the resistance test. While these high levels may be due to induction, previously induction of CYP6B7 mRNA had been rapid and returned to normal within a day.[18] Subsequently, studies were performed to confirm that the over-expression was heritable and not induced by the insecticide. RNA was extracted from midguts of 18 individuals from the F1 generation derived from the Narrabri valley population in 1996, and Northern analysis was performed using the 3′ non-coding region of CYP6B7 as probe (Fig. 2). It is important to note that this F1 generation had never encountered insecticide prior to removal of tissue and was maintained on control diet. Out of the 18 individuals tested, 15 overexpressed CYP6B7 mRNA, while this mRNA was barely detectable in sensitive individuals (lane C). Individuals in lanes 4, 6, 10 and 16 (from the left) expressed the larger P450 mRNA (3·0 kb) that was observed in

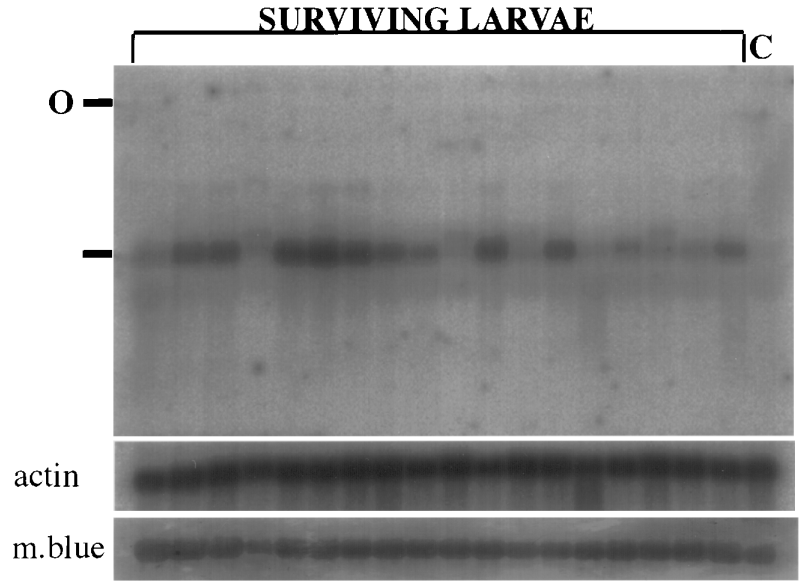

Fig. 2. Total RNA was extracted from ('surviving larvae') midguts of individual larvae from the F1 generation derived from the Narrabri valley population (lane C) pooled midguts of 10 sensitive larvae, and subjected to Northern analysis using the cDNA clone specific for the 3′ non-coding region of CYP6B7 mRNA as probe. The O indicates the origin and the other line indicates the CYP6B7 mRNA band. Note that in lane 4 from the left only 2·5 μg of RNA was loaded onto the gel. The membrane was reprobed with the cDNA clone for *Helicoverpa armigera* β-actin (actin) and finally stained with methylene blue to visualize ribosomal RNA (m.blue).

individual 7 in Fig. 1 Probing this membrane with *H. armigera* actin as probe and staining the membrane with methylene blue after the hybridization indicated that the differences in expression were not due to unequal loading of RNA onto the gel.

As the over-expression was heritable, it was of interest to determine whether the over-expression co-segregated with resistance. Therefore individual pair crosses were set up between resistant individuals of the Narrabri valley population and sensitive individuals. In the F1 generation of the single-pair crosses, 64 larvae were tested with a discriminating dose of fenvalerate and 39 larvae (60%) survived the fenvalerate treatment (Table 1). When eight of these F1 survivors were tested for over-expression of CYP6B7 mRNA, with CYP6B7 3′ non-coding region as probe, all eight individuals (100%) over-expressed this mRNA. In the untreated group from the F1 generation, only seven out of 11 larvae over-expressed the CYP6B7 mRNA. Similar results were obtained with individuals of the F2 generation which resulted from an individual pair cross between an F1 survivor of the fenvalerate test and a sensitive insect (Table 1). In the F2 untreated group, six out of 18 over-expressed the CYP6B7 mRNA. Of the 96 F2 individuals tested, 46 (48%) survived the discriminating dose of fenvalerate. When 18 of these survivors were tested, 17 (95%) over-expressed the CYP6B7 mRNA. The single survivor which did not overexpress the CYP6B7 mRNA may have been a normal sensitive survivor since there was overlap in the dose-mortality curves.[9] This meant that it was not possible to choose an ideal discriminatory dose, and the chosen dose allowed a small percentage survival of sensitive individuals.

TABLE 1
Co-segregation of Over-Expression of CYP6B7 mRNA with Pyrethroid Resistance

Generation	*Number surviving discriminating dose*	*Number over-expressing CYP6B7 mRNA*	
		Treated	*Untreated*
F1	39/64 (60%)[a]	8/8 (100%)	7/11 (63%)[a]
F2	46/96 (48%)[a]	17/18 (95%)	6/18 (33%)[a]

[a] These results are not significantly different from 50% (Chi-square test) as expected if resistance and over-expression of CYP6B7 mRNA were determined by a dominant or codominant mutation in single gene.

Over-expression of a mRNA may be due to a number of causes, including gene amplification. To determine whether this may be the case here, Southern analysis was carried out after careful quantitation to ensure equal loading of DNA in each lane prior to gel electrophoresis (Fig. 3). DNA from individuals 1 to 6, for which earlier Northern analysis had indicated variations in expression of the cytochrome P450 mRNAs (Fig. 1), as well as a pooled sample of DNA from the control strain was analysed. The Southern analysis showed a similar banding pattern in all seven lanes, although the DNA from the control strain showed a weak extra band which was not found in the resistant individuals. This could be due to a restriction fragment length polymorphism in one individual of the

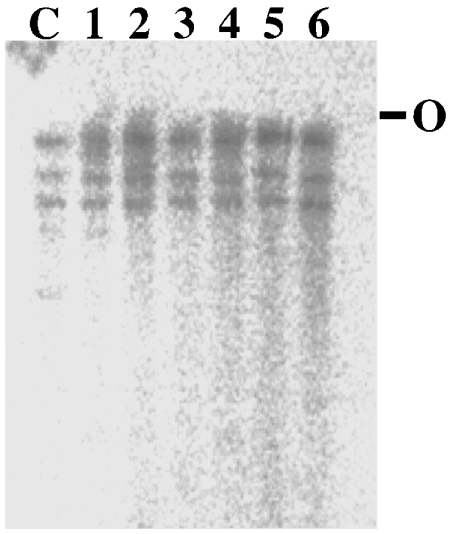

Fig. 3. DNA was extracted from (lane C) pooled sensitive insects (10); (lanes 1 to 6) field-collected resistant individuals as described in Section 2. The DNA was digested with restriction enzyme EcoR1, subjected to electrophoresis, and, after transfer, the membrane was probed with cDNA clone pHACYP14. The O indicates the origin of the gel. The individuals 1–6 correspond to individuals 1–6 in Fig. 1.

pooled sample of the sensitive strain. However, no major differences were found between the intensities of the corresponding bands of the resistant individuals. These results suggest that the 20-fold increase of the larger mRNA in individuals 2 and 5 could not be accounted for by a corresponding degree of gene amplification.

To determine whether the over-expression was tissue-specific, RNA from midgut, fat body and integument of individual resistant larvae previously shown to over-express the CYP6B7 mRNA, was subjected to Northern analysis using the 3′ non-coding region of CYP6B7 as probe. All resistant individuals showed over-expression of the CYP6B7 mRNA in midgut (Fig. 4, panel A, lanes 1 to 5) as expected. By contrast only individuals 2 and 4 over-expressed this mRNA in fat body and integument (Fig. 4, panels B and C) and in these individuals the over-expression was much greater in fat body. In this experiment CYP6B7 mRNA is barely visible in all three tissues of the sensitive individuals (Fig. 4, lane C). Methylene blue staining of these three panels indicated that the differences in expression were not due to unequal loading of RNA onto the gel.

It was also of interest to determine the expression profile for the CYP6B7 mRNA during larval development. Therefore the level of CYP6B7 mRNA was determined after extracting RNA from pools of whole larvae at each instar from both the control strain and the F1 generation of the single-pair cross. A minimum of 10 larvae were used in each pool. Northern analysis of the RNA samples showed that the level of the CYP6B7 mRNA was increased about four-fold in the early instars, decreasing to two-fold in the fifth instars com-

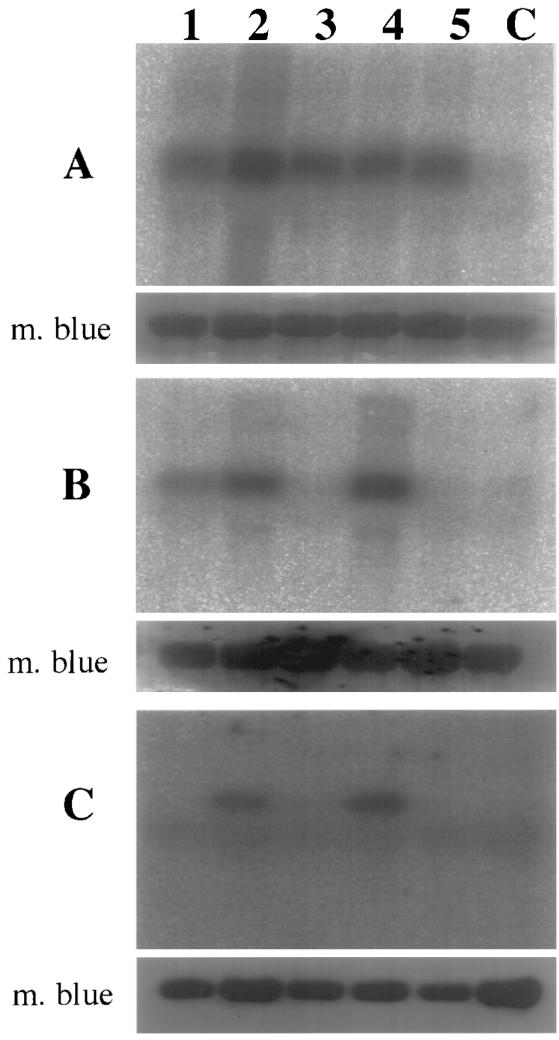

Fig. 4. The RNA was extracted from A, midgut, B, fat body and C, integument of individual resistant larvae from (lanes 1 to 5) the F1 Narrabri valley population; (lane C) pooled sensitive larvae (10). The panels 'm.blue' indicate the methylene blue staining of the ribosomal RNA to indicate RNA loading.

pared to the sensitive strain (results not shown). This low level of over-expression in the resistant strain compared to the other results given here probably reflects the fact that midgut represents a small proportion of the overall larval mass which dilutes the increase in midgut alone.

As there was a marked increase of CYP6B7 in some resistant individuals, it was of interest to study the expression of the CYP6B7 mRNA in different pyrethroid-resistant populations of *H. armigera*. Individuals from four populations established from specimens collected from the Narrabri valley, Gooloogong, Bathurst and Bega areas were tested with CYP6B7-specific probe. A high proportion of the larvae from each population over-expressed CYP6B7 mRNA (Table 2). The percentages of pyrethroid-resistant larvae in each of these populations as determined by direct application of a discriminating dose of pyrethroids to larvae were of similar high levels (Table 2). Note that the

TABLE 2
Over-Expression of the CYP6B7 mRNA in Different Pyrethroid-Resistant Populations of *Helicoverpa armigera*

Region	*No. of field-collected larvae tested*	*No. over-expressing CYP6B7*	*Resistant larvae in population (%)*[a]
Narrabi valley	18	16 (88%)	85–90%
Gooloogong	18	11 (61%)	60%[b]
Bathurst	18	15 (83%)	61%[b]
Bega	15	10 (66%)	77%[b]

[a] The percentage of pyrethroid-resistant larvae in these four populations is not significantly different from the number that over-express the CYP6B7 mRNA (Chi-square test).
[b] The resistance frequencies were determined by adult vial testing of 50 females and 50 males with an error rate of $\pm$ 10%.

numbers showing resistance were not significantly different (using Chi-square test) from the number over-expressing the CYP6B7 mRNA.

4 DISCUSSION

At present cDNA clones for three members of the CYP6B subfamily of cytochrome P450 have been isolated and sequenced from *H. armigera*.[17,19] The three cytochrome P450s show 84–87% amino acid sequence identity between them. This high degree of identity is reflected in the observation that cDNA clones cross-hybridized with each other and with all three mRNAs upon Northern analysis of larval RNA, giving two major bands, since the sizes of CYP6B2 and CYP6B6 mRNAs are similar. Hybridization studies have shown that the larger mRNA corresponds to the CYP6B7 form of cytochrome P450 and we concluded that CYP6B7 is probably the form involved in pyrethroid resistance.[17] This was based upon several observations. Firstly the larger mRNA band was shown to be inducible to a small degree by the pyrethroid, permethrin.[19] Secondly the larger mRNA was inducible by the major monoterpene components of mint[18] while mint leaves had been shown to cause induced resistance to pyrethroids.[24] Thirdly, CYP6B7 mRNA has been shown to be over-expressed in one population of pyrethroid-resistant individuals.[17] The analysis of the genetic crosses provided here indicates that pyrethroid resistance co-segregates with over-expression of the CYP6B7 mRNA which provides further evidence for the involvement of cytochrome P450 CYP6B7 in pyrethroid resistance.

Previous studies have concluded that a number of resistance mechanisms are present but that, in Australian populations of *H. armigera*, mixed function oxidase was the major resistance mechanism, based upon studies using the enzyme inhibitor, piperonyl butoxide.[9,10] However, these conclusions were somewhat tentative since there has been some question about the specificity of the inhibitor.[3] The direct measurement of the CYP6B7 mRNA in the present study overcomes these reservations. The results show that the percentages of resistant larvae in all four 1995–1997 populations were not statistically different from the number that over-expressed the CYP6B7 mRNA, and that almost all of the insects that survived the discriminating dose over-expressed the CYP6B7 mRNA. This confirms the important role of this cytochrome P450 in pyrethroid resistance in field populations. However, despite the importance of the cytochrome P450 for resistance, the results obtained initially from the larvae collected and tested in 1993 (Fig. 1) clearly indicate that some insects are resistant in the absence of over-expression of CYP6B7 gene. Selection in the presence of piperonyl butoxide presumably selects for insects exhibiting cytochrome P450-independent resistance. Such resistance was found in about 10% of the population in this region at this time.[9] The observation that half of these individuals over-expressed CYP6B7 mRNA reflects the expected independent genetic segregation of these independent resistance mechanisms. This is consistent with other observations that two pyrethroid-resistant strains showed no evidence of over-expression of cytochrome P450 mRNAs[25] and that esterases may also play a role in pyrethroid resistance.[15]

The results presented here shed some light on the nature of the over-expression of CYP6B7 mRNA in these insects. There have been several instances where insecticide resistance caused by over-expression of esterase activity has been found to be due to gene amplification.[26,27] In addition, several cases of over-expression of a cytochrome P450 in humans have been found to be caused by gene amplification.[28,29] However the present results suggest that this is not a likely mechanism in these insects. More likely is that the mutation is a point mutation, either in the controlling region of the CYP6B7 gene or in a gene involved in the control of CYP6B7 expression. The genetic cross showing 50% resistance suggests that resistance in this cross is, in fact,

probably due to a single gene effect with the mutation being dominant or co-dominant. In this case the original individual would have been a heterozygote. Since all resistant individuals also over-expressed CYP6B7 mRNA, the mutation probably involves control of expression of the CYP6B7 gene. Similar observations have been made for over-expression of CYP6A1 and CYP6A2 in *M. domestica* and *D. melanogaster* respectively.[30] Also, over-expression of CYP6D1, which causes pyrethroid resistance in the lpr strain of *M. domestica*, is controlled by a single mutation.[6] In all three instances the mutated loci involved in the control of expression of these cytochrome P450s are on different chromosomes to the cytochrome P450 genes themselves, and it was concluded that these loci produced a protein involved in the regulation of expression of the respective cytochrome P450 genes.

The results for the genetic crosses presented here are derived from a single original pair mating. A second cross showed a more complex inheritance pattern of resistance, which could be explained partly by the presence of several mechanisms of resistance, one of which did not involve the CYP6B7 gene. More extensive genetic analysis was hindered by two problems. The first was the inherent difficulty of handling and crossing *H. armigera*. However, of more importance was the difficulty of obtaining a good sensitive control strain. The only sensitive strain available was constructed by selection for sensitive individuals from field specimens. While not showing resistance, the strain may carry mutations involved in the expression of resistance by other genes, which would complicate analysis and interpretation of results.

In insects, midgut is generally thought to be the primary detoxification organ.[3] The pattern of over-expression of CYP6B7 mRNA in the midguts of *H. armigera* in this study is consistent with this conclusion. However, the finding that CYP6B7 mRNA is also over-expressed in the fat body and integument of some resistant larvae is also compatible with other studies showing that the detoxification site in some insects could involve other tissues.[31,32] What is not known is whether the enzyme in the fat body and integument is normally involved in pyrethroid metabolism and whether a higher level of enzyme in these organs results in a higher level of resistance to the insecticide. It would be of interest to analyse these populations further to determine whether over-expression of CYP6B7 mRNA in fat body and integument results in higher levels of pyrethroid resistance. It would also be of interest to examine the pattern of expression in other, more highly resistant populations which often show several hundred-fold increases in resistance.[14]

Daly *et al.*[33] have shown that there was no difference in the sensitivity of first and second instars of resistant and control strains towards pyrethroids. Resistance was manifested only in the third and later instars as well as the adult forms of *H. armigera*. These results are in contrast to the present study, which indicates that the CYP6B7 mRNA is expressed in resistant larvae at several-fold higher levels compared to the sensitive strain for all larval stages (from newly hatched first to fifth instars).

Clearly the evidence for the involvement of CYP6B7 in pyrethroid resistance is circumstantial and requires more direct evidence that CYP6B7 can bind or metabolize pyrethroids. However, given that this evidence can be obtained, the use of specific probes for CYP6B7 mRNA or protein may be valuable as a tool in detecting pyrethroid resistance in the field populations of *H. armigera*.

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