JOURNAL OF APPLIED ENTOMOLOGY

J. Appl. Entomol.

Cloning and expression of cytochrome P450 *CYP6B7* in fenvalerate-resistant and susceptible *Helicoverpa armigera* (Hübner) from China

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Keywords

CYP6B7, cytochrome P450s, fenvalerate, *Helicoverpa armigera*, over-expression, resistance

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Received: August 29, 2009; accepted: December 7, 2009.

doi: 10.1111/j.1439-0418.2009.01496.x

Abstract

Our previous studies have found that the levels of cytochrome P450 and oxidative activities of cytochrome P450 monooxygenases were enhanced in a fenvalerate-resistant strain (HDFR) of *Helicoverpa armigera* (Hübner) in China, comparing with those of a susceptible strain (HDS). Here, we report the molecular cloning and gene expression of a specific P450 *CYP6B7* from HDFR and HDS strain of *H. armigera*. Comparison of the deduced amino acid sequence showed that there were three substitutions of amino acids in the coding region of *CYP6B7* cDNA of HDFR strain, comparing with that of HDS strain. Meanwhile, there were three mutative nucleotides in the intron of *CYP6B7* genomic DNA in HDFR strain. Northern blotting analysis showed that the expression of *CYP6B7* mRNA in HDFR strain of *H. armigera* was more than 5-fold higher than that in HDS strain. Further studies indicated that the expression of *CYP6B7* mRNA increased as the larvae developed; however, it was rarely detected in the pupae and adult stages. The results suggested that cytochrome P450 *CYP6B7* plays an important role in the pyrethroid insecticide fenvalerate resistance of HDFR strain of *H. armigera*; over-expression of *CYP6B7* combined with relevant substitution of amino acids might be responsible for fenvalerate-resistance in *H. armigera* from China.

Cotton bollworm, *Helicoverpa amigera* (Hübner) is an important economic insect pest in a wide range of agricultural and commercial crops all over the world. Severe resistance of *H. armigera* to organochlorine, carbamate, organophosphate and pyrethroid insecticides has occurred in many countries including China (Wu and Guo 2005). The resistant insect also showed tolerance to novel insecticides such as fipronil, chlorfenapyr, spinosad and indoxacarb (Ahmad et al. 2003). Delayed penetration, nerve insensitivity and enhanced metabolic detoxification have been implicated as the major resistant mechanisms of pyrethroid resistance in *H. armigera* (Gunning et al. 1991; Wu et al. 1995; Ahmad and McCaffery 1999; Martin et al. 2002).

Cytochrome P450 monooxygenases (P450s) is a diverse and widely distributed protein super-family (Nelson and Strobel 1987; Coon et al. 1996). Insect cytochrome P450s play extremely important roles in the detoxification of exogenous compounds such as insecticides and plant toxins. Oxidative metabolism mediated by P450s has been proved to be a common resistance mechanism to different insecticides in insects (Feyereisen 2005). The constitutive overexpression of P450 genes, such as *CYP6D1* (Tomita and Scott 1995) and *CYP6A36* (Zhu et al. 2008) from house-flies and *CYP6F1* (Kasai et al. 2000) from mosquito, were proved closely associated with resistance to pyrethroid insecticides in many insects.

A fenvalerate-resistant strain of *H. armigera* (HDFR) was established by selecting with about LD_{60} of fenvalerate in lab. The HDFR strain showed significant cross-resistance to all pyrethroid insecticides tested, but not to carbamate, organophosphate and some novel insecticides (Zhang and Qiu 1998). The pyrethroid resistance of HDFR strain could be remarkably inhibited by piperonyl butoxide (PBO) (Qiu and Zhang 1999); moreover, the level of P450s and oxidative activities of cytochrome P450 monooxygenases (MROD, EROD) were significantly higher in HDFR than those in a susceptible strain (HDS), and the oxidative activities of monooxygenases could be induced by phenobarbital and fenvalerate (Qiu and Zhang 2001; Qiu et al. 2003). These studies implied that cytochrome P450s were likely to be involved in the resistance of *H. armigera* to fenvalerate.

The purpose of present study is to further investigate the role of cytochrome P450s in the fenvalerate-resistance of HDFR strain of *H. armigera*. A specific cytochrome P450 *CYP6B7* from HDFR and HDS strain of *H. armigera* was cloned and the expression level was studied by Northern blotting analysis. The results demonstrated that the over-expression combined with relevant substitution of amino acids of cytochrome P450 *CYP6B7* might be associated with fenvalerate -resistance in *H. armigera* from China.

Materials and Methods

Insects

The susceptible strain of *H. armigera* (HDS) with a LD_{50} value of 9.82×10^{-2} μg/larva for fenvalerate was collected from Handan, Hebei Province of China in 1988 and since then was reared on artificial diets in the lab without exposure to insecticides. The pyrethroid-resistant strain of *H. armigera* (HDFR) was derived from the HDS strain through selection with about LD_{60} of fenvalerate for more than 30 generations. The HDFR strain showed a LD_{50} value of 24.58 μg/larva for fenvalerate and the resistance ratio was about 250-fold (Zhang and Qiu 1998; Qiu and Zhang 1999; Tang 2006).

The *H. armigera* larvae were reared on artificial diets at $26 \pm 1°C$ with a 14 : 10 (L : D) photoperiod. The adults were fed with a 10% sucrose solution. The relative humidity of the lab was 60–70%.

DNA and RNA extraction

The extraction of total RNA from pooled midgut tissues and synthesis of cDNA were carried out using SV Total RNA Isolation and Access RT-PCR System (Promega, Madison, WI) according to the manufacturer's instructions. The genomic DNA from pooled midgut tissues was isolated using standard methods (Sambrook and Russel 2001). Extracted RNA or DNA was quantified spectrophotometrically and stored at −80°C until further use.

Cloning of *CYP6B7* from HDFR and HDS strain

The *CYP6B7* cDNA (GenBank Accession No. AF031468) and *CYP6B7* genomic DNA (GenBank Accession No. DQ458470) were amplified by polymerase chain reaction (PCR) from HDFR and HDS strain of *H. armigera* with the upstream primer 5′-TCATAACAAGGTCATCAACG-3′ and downstream primer 5′-TTAAGATACAATCTTCCTAGG-3′, respectively. Purified PCR products were then ligated with pGEM-T Easy Vector (Promega) and transformed into *Escherichia coli* DH5α. The transformants were selected on LB agar plates containing 100 μg/ml ampicillin. Resultant PCR clones were sequenced by Shanghai Sangon Biotechnology, Ltd. (Shanghai, China). The resulting sequences were analyzed by DNAMAN 6.0 (Lynnon Biosoft, Quebec, Canada).

Northern blotting analysis

A *CYP6B7* cDNA fragment of 570 bp (GenBank Accession No. DQ497428) was amplified by PCR from HDFR with the upstream primer 5′-CCAAAGGAATGGCAAACC-3′ and downstream primer 5′-TTTGTCCAAGTCCGAATG-3′. This fragment was prepared as the probes in Northern blotting analysis. Northern blotting was performed with total RNA samples of 10 μg from HDS and HDFR using Formaldehyde-Based System for Northern Blots (NorthernMax Kit; Ambion, Applied Biosystems, Austin, TX). Total RNA from each sample were fractionated on 1% formaldehyde denaturing agarose gel and transferred to nylon membranes (Sambrook and Russel 2001). The probes were labelled with α-[^{32}P] dATP according to the instruction of Primer-α-Gene Labelling System (Promega) and hybridized with RNA blots. The amount of RNA loaded in each lane was standardized by comparing the density of the 18S ribosomal RNA band on agarose gel under UV light before transfer. The radiographic signal intensity was quantitatively analyzed by using Storm 820 System

(Molecular Dynamics, Sunnyvale, CA) after film scanning.

Results

Cloning of *CYP6B7* cDNA from HDFR and HDS strain

The *CYP6B7* cDNA from HDFR and HDS strain were cloned respectively, and the sequences were analyzed by DNAMAN 6.0 (fig. 1). The results showed that *CYP6B7* cDNA from both strains had an open reading frame of 1515 nucleotides, encoding 504 amino acid residues with deduced molecular weights of about 58 kDa. The identities of nucleotides and amino acids of sequence of HDFR and HDS strain were greater than 99.2%. There were 11 nucleotides difference in the coding region of HDFR, which resulted in three mutations of the deduced amino acid (fig. 1). The sequence from HDFR strain was enrolled in GenBank with No.DQ497428 and classified as a member of subfamily of *CYP6B*, an allele of *CYP6B7* (AF031468). The *CYP6B7* cloned in this study had high amino acid sequence identity with *CYP6B7* (AF031468) in *H. armigera* from Australia (98%), *CYP6B8* (AF102263) in *Helicoverpa zea* from American (99%), but a little bit lower identity with *CYP6B6* (AY950636) and *CYP6B2* (U18085) of *H. armigera* (87% and 84%, respectively). The amino acid sequences of both strains contained the conserved domains of a membrane-anchoring signal, reductase binding sites, a haem-binding site, ETLR motif and substrate recognition sites common to all P450s genes (fig. 1). Moreover, there was one intron in the *CYP6B7* genomic DNA of HDFR and HDS strain, and the intron was 325 bp and 327 bp in length (fig. 2), respectively. Comparing with HDS

```
HDFR   MWVLYLPAVLSVLIVTLYLYFTRTFNYWKKRNVRGPEPTVFFGNLKDSTLRKKNIGIVME   60

HDS    ------------------------------------------------------------   60

HDFR   EIYNQFPDEKVVGMYRMTTPCLLVRDLDVIKHIMIKDFEAFRDRGVEFSKEGLGQNLFHA  120

HDS    ------------------------------------------------------------  120

HDFR   DGETWRALRNRFTPIFTSGKLKNMFYLMHEGADNFIDHVSKECEKKQEFEVHSLLQTYTM  180

HDS    -----------------------V------------------------------------  180

HDFR   STISSCAFGVSYNSISDKVQTLEIVDKIISEPSYAIELDYMYPKLLAKLNLSIIPTPVQH  240

HDS    ------------------------------------------------------------  240

HDFR   FFKSLVDNIISQRNGKPAGRNDFMDLILELRQMGEVTSNKYLDGVTSLEITDEVICAQAF  300

HDS    ---------------E--------------------------------------------  300

           Helix I

HDFR   VFYVAGYETSATTMTYLIYQLSLNQDVQNKLIAEVDEAIKASDGKVTYDTVKEMKYLNKV  360

HDS    ------------------C-----------------------------------------  360

         Helix K

HDFR   FDETLRMYSIVEPLQRKATRDYQIPGTDVVIEKDTMVLISPRGIHYDPKYYDNPKQFNPD  420

HDS    ------------------------------------------------------------  420

                          Heme Binding Motif

HDFR   RFDAEEVGKRHPCAYLPFGLGQRNCIGMRFGRLQSLLCITKILSKFRIEPSKNTDRNLQV  480

HDS    ------------------------------------------------------------  480

HDFR   EPRRVIIGPKGGIRVNIVPRKIVS                                      504

HDS    ------------------------                                      504
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Fig. 1 Deduced amino acid sequences of *CYP6B7* from HDFR and HDS strain of *H. armigera* (different amino acid sequences were boxed).

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HDFR    TTCGGACTTGGACAAAGAAATTGCATAGGT{AAGCAAATCCTCAACAGCAGTTAATTATCC  60

HDS     ------------------------------------------------------------  60

HDFR    TCATTAAAAAATAAGTTCATGAAGTCTGATAAAGATGTCAAGTGTCAAGTGATGTCAACT  120

HDS     ------------------------------------------------------------  120

HDFR    ACACAAACATTAGATATTAAAAACACCGACATAAATAGCCGTTTAAAATAGTTTAAAAA.  179

HDS     -----------------------------------------------------------A  180

HDFR    TATATCTGTAACTAATTGTCGCCCACGACTTTGTGTCTTTGAATATCAGAGGTTTT.GTT  238

HDS     --------------------------------------------------------T---  240

HDFR    AAGTTTTCTATCTGTGATGCGTAATTACGCGTAACAAATAAACACAGTCACTTGTAGCTT  298

HDS     --------------C---------------------------------------------  300

HDFR    TTTAATACTTATTATTCTTTCTTTTTCTTTTAACACGTCAAATTTTTCGTTTCAGGC}ATG  358

HDS     ------------------------------------------------------------  360
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Fig. 2 Nucleotide sequences of the intron of *CYP6B7* from HDFR and HDS strain of *H. armigera* (different amino acid sequences were boxed).

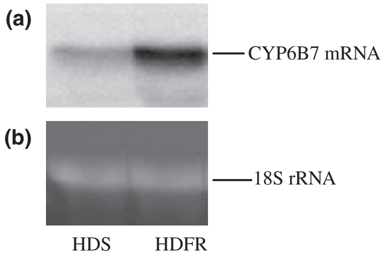

Fig. 3 Northern blot analysis of expression of *CYP6B7* mRNA from HDS and HDFR strain of *H. armigera* in 6th instar of larvae. (a) *CYP6B7* mRNA; (b) Relative amounts of 18S rRNA on the 1% agarose gel following electrophoresis and visualized with ethidium bromide (10 μg/lane).

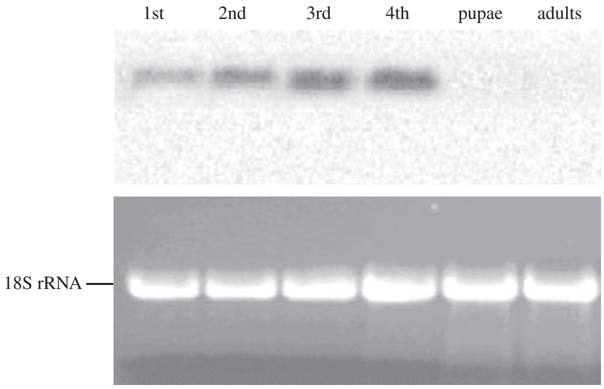

Fig. 4 Northern blot analysis of *CYP6B7* mRNA from HDFR strain of *H. armigera* at different life stages.

strain, there were three mutative sites including two nucleotides missing in the intron of HDFR strain.

Northern blotting analysis

Northern blotting analysis was carried out to study the expression level of *CYP6B7* mRNA in the midgut of 6th instar larvae of HDS and HDFR strain. The results showed that comparing with HDS strain, the *CYP6B7* mRNA was over-expressed in the resistant HDFR strain of *H. armigera*, which was more than 5-fold higher in intensities of the hybridization band (fig. 3). This result indicated that the over-expression of *CYP6B7* might play an important role in the resistance of *H. armigera* to fenvalerate in China.

The expression characteristics of *CYP6B7* mRNA at different life stages of HDFR strain were further analyzed. As shown in fig. 4, the expression level of *CYP6B7* mRNA varied at different life stages of HDFR. The expression of *CYP6B7* mRNA was detected from first to fourth instar larvae but could rarely be detected in the pupae and adult. Moreover, the expression level increased as the larvae developed and the level in the 4th instar larvae was about 4-fold higher than that in the 1st instar larvae.

Discussion

Due to intensive use of insecticides on cotton and other crops, severe resistance of *H. armigera* to pyrethroids has occurred around the world. Cytochrome P450s are important enzymes involved in catalyzing the metabolism of insecticide and leading to metabolic resistance in *H. armigera* and other insects. Enhanced P450s activities have been found in pyrethroid-resistant insects such as cotton bollworm, mosquito, diamondback moth and sea lice, and were considered as a major resistance mechanism to pyrethroids. Moreover, some P450s have been shown to metabolize specific insecticides. For example, *CYP6A2* from *D. melanogaster* metabolized DDT (Amichot et al. 2004), *CYP6A1* from *Musca domestica* metabolized aldrin, heptachlor and diazinon (Andersen et al. 1994; Sabourault et al. 2001), and *CYP12A1* from *M. domestica* metabolized aldrin, heptachlor, diazinon and azinphosmethyl (Guzov et al. 1998). However, there was no direct evidence at present to prove that pyrethroids could be metabolized by P450s from pyrethroid-resistant *H. armigera*.

Some studies suggested that the substitution of amino acids in P450s was relevant to resistance. Heterologous expression verified that three mutations in amino acids (R355S, L336V, V476L) of CYP6A2 from resistant *Drosophila melanogaster* led to enhanced activity in metabolizing DDT (Berge et al. 1998; Amichot et al. 2004). In the present study, *CYP6B7* from fenvalerate-resistant HDFR and susceptible HDS strain of *H. armigera* was cloned, and three substitutions of amino acids in sequence of *CYP6B7* of HDFR strain were found comparing with that of HDS strain. Meanwhile, there were three mutative sites in the introns of *CYP6B7* genomic DNA. Since HDFR was derived from HDS strain and both strains were reared in the same conditions, the only difference between the two strains is that the HDFR strain has been selected by pyrethroid fenvalerate and acquired 250-fold resistance to fenvalerate while HDS strain has not, thus we suggested the substitutions and mutation of *CYP6B7* might be involved in the fenvalerate-resistance of *H. armigera*. However, further investigations are still needed to confirm this suggestion.

Many studies have showed that overexpression or elevated levels of cytochrome P450 were observed in pyrethroid-resistant insects. Rongnoparut et al. (2003) reported that increased mRNA level of *CYP6A2* was observed in *Anopheles minimus* deltamethrin-resistant strain; and the over-expression of *CYP6F1* (Gong et al. 2005), *CYP4H1*, *4H22v1*, *4J6v1* and *4J6v2* (Shen et al. 2003) of *Culex pipiens pallens* in deltamethrin-resistant strain, *CYP4G8* (Pittendrigh et al. 1997) in pyrethroid-resistant strain of *H. armigera*, *CYP6X1* and *CYP6X2* (Zhu and Snodgrass 2003) in permethrin-resistant strain of *Lygus lineolaris* and *CYP6Z1* (Nikou et al. 2003) in permethrin-resistant strain of *Anopheles gambiae* have also been observed. The study of Ranasinghe and Hobbs (1998) showed that the level of *CYP6B7* mRNA in a pyrethroid-resistant field population of *H. armigera* in Australia was over-expressed. Yang et al. (2006) reported that constitutive overexpression of multiple cytochrome P450 genes (*CYP9A12*, *CYP9A14* and *CYP6B7*) was found associated with pyrethroid resistance in *H. armigera* in Asia. Enhanced oxidative detoxification of P450s has also been reported to be the major resistance mechanism to pyrethroids in *H. armigera* in China (Shen and Wu 1995; Qiu and Zhang 2001; Yang et al. 2005); however most of these studies were carried out at the biochemical level. In this study, Northern blotting analysis indicated that the *CYP6B7* mRNA in HDFR strain of *H. armigera* was more strongly expressed than that in HDS strain (more than 5-fold higher). The result was in consistent with our previous findings that the activities of monooxygenases in midguts and fatbodies in HDFR strain were much higher than those in susceptible HDS strain (Qiu and Zhang 2001), and further confirmed the role of *CYP6B7* in the fenvalerate-resistance of *H. armigera* from China.

Introns with different size have been found in many P450 genes of different insects (Li et al. 2002; Grubor and Heckel 2007). Of the 83 *D. melanogaster* P450 genes, 78 P450 genes have between one and eight introns each (the average length is between 50 and 70 nucleotides) and all of them follow the canonical GT/AG rule except the first intron of *CYP*9C1 (Tijet et al. 2001). A report on the intron size in *D. melanogaster* genes showed that large and very small introns tend to occur in regions of low recombination and were selected against during the evolution (Bernardo Carvalho and Clark 1999). Gotoh (1998) / link> demonstrated that frequent loss and gain of introns has occurred during the evolution of *CYP* genes by parsimonious analysis of the divergent structures of *Caenorhabditis elegans* P450 genes. However, there were no reports so far about intron(s)'s role in the resistance of insects. Grubor and Heckel (2007) reported a single phase-1 intron was found in the encoding region of *CYP6B2*, *CYP6B6* and *CYP6B7* of fenvalerate-resistant *H. armigera* AN02. In our study, there was also one intron in the encoding region of *CYP6B7* of HDFR and HDS strain (325 bp and 327 bp, respectively), and there were three mutative nucleotides in the intron of *CYP6B7* in HDFR strain comparing with the original susceptible HDS strain; moreover, the intron size was different from that of the intron (291 bp) of *CYP6B7* from AN02 (Grubor and Heckel 2007). Since HDFR strain was derived from HDS strain through selection with fenvalerate and acquired high level resistance to fenvalerate, we thus speculated that the mutation of the intron might be related with the role of *CYP6B7* in the fenvalerate resistance.

Cytochrome P450 genes of insects are variable in their expression, and this variety is in agreement with the functions of P450s. *CYP6A1* mRNA was expressed both in larvae and adults of house fly, but the expression level was very low in pupae and egg (Carino et al. 1994). *CYP6D1* mRNA relevant to the pyrethroid resistance was only expressed in adults of house fly (Scott et al. 1996). The level of midgut *CYP4* mRNA of *Manduca sexta* was higher during the active feeding, midwandering, prepupal, and pupal stages (Snyder et al. 1995). In the present study, it was found that *CYP6B7* mRNA was expressed in the larval stage of *H. armigera* but rarely detected in the pupae and adults. Moreover, the mRNA expression level increased as the larvae developed. These results suggested that cytochrome P450 *CYP6B7* plays much more important role in the detoxification of insecticides in the larval stage than that in the pupae and adults.

There were three *CYP6B* genes (*CYP6B2*, *CYP6B6* and *CYP6B7*) have been cloned from *H. armigera* in Australia (Wang and Hobbs 1995; Ranasinghe and Hobbs 1998); and *CYP6B7* was later suggested being involved in the resistance of field-collected *H. armigera* that resistant to pyrethroid, for its mRNA was over-expressed in a majority of individuals in the field population, relative to a susceptible strain (Ranasinghe et al. 1998). However, in the AN02 strain of *H. armigera* that resistant to fenvalerate from eastern Australia, Grubor and Heckel (2007) found that any of the three *CYP6B* genes was unlikely to be responsible for the fenvalerate resistance, but they suggested this did not exclude its possible role in other strains of *H. armigera*. In the present study, the results demonstrated that *CYP6B7* plays an important role in the fenvalerate resistance of *H. armigera*, constitutive over-expression of *CYP6B7* mRNA combined with relevant substitution of amino acids might be responsible for fenvalerate-resistance in *H. armigera* from China. The studies mentioned above implied that the role of *CYP6B7* in the pyrethroid resistance of different strains of *H. armigera* from different areas may not be the same.

Heterologous expression of *CYP6B7* and studying on its ability to metabolize fenvalerate and other pyrethroids would provide direct evidence to prove *CYP6B7*'s role in the resistance. The research on the degradation of some pyrethroids by recombinant CYP6B7 protein expressed in yeast is now being undertaken in the authors' laboratory, and the results will be reported in another paper.

Acknowledgements

This research was financially supported by National Natural Science Foundation (Grant No. 30400293) and the National Basic Research Programme (973 Programme, Grant No. 2010CB126104) of China.

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