CYP9A12 and *CYP9A17* in the cotton bollworm, *Helicoverpa armigera*: sequence similarity, expression profile and xenobiotic response

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Abstract

BACKGROUND: *Helicoverpa armigera* (Hübner) causes severe losses in many crops including cotton. Cytochrome P450s play crucial roles in the metabolism of many important compounds in various organisms. The authors attempt to identify new cytochrome P450 genes and investigate their expression profile and xenobiotic response in order to understand the nature and roles of cytochrome P450s in this important pest.

RESULTS: A novel P450 cDNA (*CYP9A17*), encoding a protein of 531 amino acid residues, was isolated from *H. armigera*. *CYP9A17* is a typical microsomal P450, showing the highest (93.9%) amino acid identity with *CYP9A12* from *H. armigera*. The high similarity is not only found between cDNAs, but also between the intron–exon organisation. *CYP9A12* is constitutively universally expressed in all four life stages and in all tested larval tissues, whereas *CYP9A17* is specifically expressed in the larval midgut and fat body. Real-time quantitative RT-PCR showed that the level of both *CYP9A12* and *CYP9A17* mRNA is affected in dose-dependent and tissue-specific manners by deltamethrin, gossypol and phenobarbital.

CONCLUSION: *CYP9A12* and *CYP9A17* showed high sequence identity, but with differential expression patterns, suggesting that *CYP9A12* and *CYP9A17* genes in *H. armigera* might diverge via subfunctionalisation after the gene duplication event.
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Keywords: *Helicoverpa armigera*; cytochrome P450; *CYP9A17*; *CYP9A12*; expression pattern; xenobiotic response

1 INTRODUCTION

The bollworm, *Helicoverpa armigera* (Hübner), is a polyphagous insect that causes severe yield losses in many different crops including cotton.[1–3] The outbreak of this pest is mainly attributed to its resistance to commonly applied insecticides and adaptability to a wide array of host plants.[2,4,5] It has been demonstrated that one of the common and major mechanisms of resistance to insecticides and plant toxins is an elevated cytochrome-P450-dependent detoxification.[2,4,6–8] The cytochrome P450 monooxygenases of insects have numerous functional roles, including growth, development, feeding, resistance to pesticide and tolerance to plant toxins.[9,10] Given the importance of insect P450 in metabolism, this versatile metabolic system has been a main topic in insect research for four decades.[10] Undoubtedly, revealing the nature and roles of the P450 monooxoygenases of *H. armigera* will facilitate the understanding of this pest and the development of reasonable control strategies.[5]

Although there have been many reports regarding the tissue distribution and developmental expression of P450 monoxygenases, their interactions with xenobiotic compounds and association with insecticide resistance in the cotton bollworm,[6,11,12] specific enzymes have proven to be very difficult to study, in part owing to the difficulties in isolating individual cytochrome P450 enzymes by protein purification.[1,13] Advances in molecular biology and its application in cytochrome P450 study have made it easier and more reliable to examine the structure and functions of specific forms of these enzymes.[1] During the past two decades, a lot of effort has been invested in the cloning of cytochrome P450 genes in this pest.[1,14–18] The first P450 gene cloned from *H. armigera* was *CYP6B2*,[1] and its sequence was corrected by sequencing a BAC clone.[19] *CYP6B6* and *CYP6B7* were subsequently isolated from Australian *H. armigera*, and the *CYP6B7* was suggested as the P450 form responsible for pyrethroid metabolism in *H. armigera*.[15] More recently, Yang *et al*.[17] isolated *CYP9A12* and *CYP9A14*, and Wee *et al*.[18] reported *CYP337B1* and *CYP4S1* from *H. armigera*. In addition, constitutive overexpression of these genes has been documented to be associated with fenvalerate resistance.[17,18] Mao *et al*.[8] identified a cytochrome P450 *CYP6AE14* which is related to

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gossypol tolerance. Additionally, a number of P450 fragments have been reported.[14,16]

The total number of P450s (excluding pseudogenes) determined from the six insect genomes ranges from 48 to 164.[10] Although there are about 47 P450 entries from *H. armigera* in the GenBank, only about ten genes (excluding allelic genes) with full coding sequence have been identified (http://www.ncbi.nlm.nih.gov/sites/entrez). Understanding of the roles that individual P450s take is hindered by the paucity of isolated genes from this pest, and by the corresponding absence of information on the substrates by functionally expressed P450s as well. Identification of cDNA is generally the initial step towards their functional characterisation. The present paper reports on the cloning and sequence analysis, expression pattern and xenobiotic response of a new cytochrome P450 cDNA (*CYP9A17*) of *H. armigera*. Further, *CYP9A17* was compared with *CYP9A12*, which has been functionally characterised. These findings lay the foundation for further studies directed towards learning the functionality and evolution of this novel cytochrome P450.

2 MATERIALS AND METHODS

2.1 Insects
The laboratory strain of *H. armigera*, originally collected from a cotton field in Shandong Province, China, in 2005, was maintained in an insectary without exposure to any insecticide. This population showed moderate resistance to fenvalerate (300-fold) and marginal resistance to phoxim (1.8-fold) and thiodicarb (4.66-fold) in 2005. The larvae were individually reared on artificial diet[3] at $25 \pm 1\,^\circ$C and 70% relative humidity (RH) with a 16:8 h light:dark photoperiod. Adults were kept under the same temperature and light conditions, and provided with a 10% honey solution.

2.2 Induction treatment
Three chemicals were used as xenobiotics in this study. Sodium phenobarbital (PB) (purity 99%; Sigma, USA) is a commonly used cytochrome P450 inducer. Gossypol is one of the main allelochemicals in cotton which is a main host plant of *H. armigera*. Gossypol acetic acid (GP) ($\geq$95%; China Cotton UNIS, Beijing), which is a relatively stable crystalline complex of equimolar quantities of gossypol and acetic acid, was used in this study. Deltamethrin (DM) (99%; Bayer CropScience, Germany) is a representative pyrethroid insecticide that has been extensively used for control of the cotton bollworm. Sixth-instar caterpillars newly moulted within 4 h were selected for all treatments to ensure homogeneity in developmental age. These synchronous larvae were provided with artificial diets containing different chemicals. Two concentrations were used for each chemical as follows: 1 and 10 mg g^{-1} for PB, 0.05 and 0.5 mg g^{-1} for DM, and 0.2 and 2 mg g^{-1} for GP. After 48 h, both the midgut and fat body were dissected for RNA extraction. Fifteen individuals were used for each treatment, and three biological replicates were performed for all treatments.

2.3 RNA isolation and cDNA synthesis
The midgut, fat body and integument of two-day-old sixth-instar larvae, the whole body of five-day-old pupae and adults and eggs <12 h old were used for extraction of total RNA using TRIzol (Invitrogen, CA), based on the manufacturer's instructions. The quality and concentration of RNA samples were examined by agarose gel electrophoresis and spectrophotometer analysis. RNA was digested by DNase I (Takara, Japan) in order to eliminate the genomic DNA contamination. cDNA was synthesised by reverse transcription in 20 μL reactions containing 1 μg of total RNA, 200 U PrimeScript™ reverse transcriptase (Takara, Japan), 20 U RNase inhibitor, 1 μL dNTP mixture (10 mM each) and 1 μL oligo(dT)$_{18}$ primer (50 μM) at $42\,^\circ$C for 1 h. Three independent RNA preparations representing three biological replicates were used for cDNA synthesis.

2.4 Rapid amplification of 3' cDNA ends (RACE) and 5'-RACE
Use was made of one of the eight non-degenerate forward primers (P1) (Table 1) designed on the basis of the PFG motif which is highly conserved in many cytochrome P450s. Midgut cDNA template was synthesised using the adapter primer (AP) (Table 1) in the 3'-RACE system for rapid amplification of cDNA ends (Invitrogen, CA). PCR mixture was set up with the P1 and abridged universal amplification (AUAP) primer sets according to the manufacturer's protocol (Invitrogen, CA). The reaction was heated to $94\,^\circ$C for 3 min for DNA predenaturation, followed by 35 cycles of amplification: $95\,^\circ$C for 15 s, $48\,^\circ$C for 1 min and $72\,^\circ$C for 30 s, and followed by a final extension step at $72\,^\circ$C for 10 min. The resulting PCR products were separated on a 6% polyacrylamide gel by using a Sequi-Gen GT nucleic acid electrophoresis cell (Bio-Rad, USA). The gel was stained by silver. The bands of expected sizes (200–500 bp) were cut from the gel, purified using Wizard PCR Preps DNA kit (Promega, WI) and then ligated into pGEM-T Easy Vector (Promega, WI). Positive clones were sequenced by Invitrogen (Beijing Service Centre, China). One EST with P450 characteristic sequence (FXXGXXXCXG) was identified and used for subsequent work.

To generate the 5'-end of this P450 gene, 5'-RACE strategy (Invitrogen, CA) was adopted. Based on the sequence information of the obtained P450 EST, gene-specific reverse primer (GSP-R) (Table 1) was designed. First-strand cDNA was synthesised from total RNA using GSP-R primer. A homopolymeric tail was then added to the 3'-end of purified cDNA using terminal deoxynucleotidyl transferase (TdT) and dCTP. Tailed cDNA was amplified in a 50 μL reaction consisting of the primer set GSP-R/AAP (10 μM, 1 μL each), 5 μL cDNA template and 2.5 U *Taq* polymerase and 1 μL dNTP (10 mM each). The PCR was performed at $94\,^\circ$C for 5 min for DNA predenaturation, then 35 cycles of $94\,^\circ$C for 1 min, $50\,^\circ$C for 1 min and $72\,^\circ$C for 1 min 30 s, followed by one final extension at $68\,^\circ$C for 10 min. PCR products were recovered from the gel, subcloned by TA cloning and then sequenced.

2.5 Isolation of full-length cDNA
To isolate the intact cDNA sequence for this gene, a forward primer CYPVF and a reverse primer CYPVR (Table 1) based on the preciously obtained fragments were used for PCR, using high-fidelity proof-reading *Taq* polymerase (Takara, Japan). The PCR was performed at $94\,^\circ$C for 3 min for DNA predenaturation, followed by 30 cycles of $94\,^\circ$C for 1 min, $45\,^\circ$C for 30 s and $72\,^\circ$C for 2 min, and a final extension at $72\,^\circ$C for 10 min. The PCR product was purified on gel, TA subcloned and sequenced. DNA sequencing was repeated 3 times, and each was performed by sequencing from both directions.

2.6 Real-time RT-PCR analysis
The expression levels of *CYP9A12* (EU327 673) and *CYP9A17* (AY753 201) were quantified by quantitative real-time PCR (qRT-PCR) using an Mx3000P qPCR system (Stratagene, La Jolla, CA)

Table 1. PCR primers used in this study

Name	Sequence (5′–3′)	Usage
AP	GGCCACGCGTCGACTA	Reverse transcription
P1	CCTTTCGG	3′-RACE
AUAP	GGCCACGCGTCGACTAGTAC	3′-RACE
AAP	GGCCACGCGTCGACTAGTACGGGGGGGGGGG	5′-RACE
GSP-R	TCCAATGCAATTCCTAGGTCCAAACC	5′-RACE
CYPVF	ATGATCCTAGCTCTGGTGTGG	CYP9A17 cDNA cloning
CYPVR	CTACATCCTCAGTCTAAATCTAAGCCA	CYP9A17 cDNA cloning
9A17-1F	TAAGCATGATCCTAGCTCTGGTG	Genomic CYP9A17 cloning
9A17-1R	GAACACTAGCATCTGACGGAAGT	Genomic CYP9A17 cloning
9A17-2F	GTATGCAGGGACATCCAAGAG	Genomic CYP9A17 cloning
9A17-2R	TTGGCATCGTTCTCCTTG	Genomic CYP9A17 cloning
9A17-3F	ATCTTTGCACTTTGCGTTGC	Genomic CYP9A17 cloning
9A17-3R	CTGCCTAGGTCTGAATCTAAGC	Genomic CYP9A17 cloning
9A12-1F	AAGCATGATACTAGTCCTGGTC	Genomic CYP9A12 cloning
9A12-1R	AACATGATCATCTCTCGGAATC	Genomic CYP9A12 cloning
9A12-2F	TTCCCAATACTTTGATGAAT	Genomic CYP9A12 cloning
9A12-2R	TTGGCATCATTCTCCTTG	Genomic CYP9A12 cloning
9A12-3F	TGATTATGGTTGGTCCTATG	Genomic CYP9A12 cloning
9A12-3R	AGTCCGATCAGTCGTGTCCT	Genomic CYP9A12 cloning
EF-F	GACAAACGTACCATCGAGAAG	Real-time PCR
EF-R	GATACCAGCCTCGAACTCAC	Real-time PCR
q9A12-F	ATCACCTCATAGAAGATATCC	Real-time PCR
q9A12-R	CATGTCTTTCCATTCTTGACC	Real-time PCR
q9A17-F	ATCACTTCGTAGACGATACCA	Real-time PCR

and RealMasterMix SYBR Green PCR kit (TianGen, Beijing, China). For each gene, a serial dilution from 10- to 1000-fold of each cDNA template was performed in order to assess amplification efficiency of PCR. The elongation factor-1α (EF-1α) (EF-F/EF-R primer set) was used as a reference gene to normalise the target gene expression levels among samples.[17,20] qRT-PCR of each cDNA sample and template free was performed in triplicate. All the primer sets used in this study are listed in Table 1. Owing to the high identity between *CYP9A12* and *CYP9A17* coding regions, their primer sets (q9A12-F/q9A17-R; q9A17-F/q9A12-R) shared the common reverse primer. Specificity of the PCR amplification was checked by a melting curve analysis (MxPro 4.0 program; Stratagene) and by sequencing the PCR products.

qRT-PCR was run in a 25 µL reaction containing 11.25 µL RealMasterMix/SYBR solution, 0.5 µL each of forward and reverse primer (10 µM) and 3.5 µL cDNA template using the following cycling parameters: 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 58 °C for 30 s and 68 °C for 40 s. The melting temperatures of amplicons were measured by taking continuous fluorescence reading while increasing the temperature from 58 to 95 °C in steps of 0.5 °C with 10 s at each step. The relative expression levels of target genes were calculated by the comparative C_T method as described by Livak and Schmittgen.[21] Amplification efficiency was determined from a standard curve based on C_T values against serial 10× dilutions of cDNA templates. *CYP9A17* expression abundance relative to the control was expressed as mean ± SE of three biological replicates. One-way analysis of variance (ANOVA) and Fisher's least significant difference were performed to determine the statistical difference between means (SPSS, v.13). A *P* value of <0.05 was considered significant.

2.7 Cloning of genomic DNA of *CYP9A12* and *CYP9A17*

Larval genomic DNA was extracted from the midgut as described by Sasabe *et al.*[22] with minor modifications. Briefly, midguts were homogenated in 700 µL of lysis buffer (100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 500 mM NaCl, 12.5 mg mL^{-1} SDS, 0.1 mg mL^{-1} proteinase K) incubated for 10 min at 65 °C, followed by digestion of RNase A for 5 min at 37 °C. After incubation, genomic DNAs were sequentially extracted with an equal volume of water-saturated phenol/chloroform/isoamyl alcohol (25 : 24:1 by volume) twice, then precipitated from the aqueous phase after adding one-half volume of 7.5 M ammonium acetate and two volumes of ice-cold ethanol. Finally, the pellet was washed once with 70% ethanol, resuspended in 20–50 µL sterile nuclease-free water at a final concentration of 1 µg µL^{-1} and kept at 4 °C until use.

To obtain the full-length genomic DNA sequences of the two genes, three sets of oligonucleotide primers for each gene were designed to amplify three reliable overlapping fragments (Table 1) based on the cDNA sequences of *CYP9A12* and *CYP9A17*. All the PCR products were sequenced directly by Invitrogen (Beijing Service Centre, China).

2.8 Sequence analysis

The molecular mass and isoelectric point (*pI*) were calculated (ProtParam, http://www.expasy.ch/tools/protparam.html), and the signal peptide (SignalP, http://www.cbs.dtu.dk/services/SignalP/), the subcellular location (WoLF PSORT, http://wolfpsort.org/), the transmembrane regions (TMpred, http://www.ch.embnet.org/software/TMPRED_form.html) and the secondary structure (PSIPRED, http://bioinf.cs.ucl.ac.uk/psipred/) were predicted for *CYP9A17* (GenBank Accession No. ACB30272). Moreover, the amino acid sequences of six CYP9A subfamily members from

Helicoverpa species were aligned, and the characteristic motif and substrate recognition sites (SRSs) of P450s were analysed on the basis of their secondary structure. TBLASTN search against the non-redundant database of GenBank was performed with the amino acid sequence of *CYP9A17* as a query under the default parameters. The retrieved sequences with above 55% amino acid similarity and related P450 protein (*CYP9A1*) sequences were aligned using ClustalX v.2.[23]

3 RESULTS

3.1 Sequencing and identification of a novel cytochrome P450 gene (*CYP9A17*)

From eight RT-PCR products sequenced, a 324 bp sequence containing the P450 fingerprint was identified. This sequence was used to design the gene-specific reverse primer GSP-R for 5′-RACE in order to obtain the 5′-end of its cDNA. A 1605 bp amplicon was amplified by 5′-RACE. A full-length cDNA of 1893 bp was compiled by assembling the 3′-fragment and 5′-fragment. Using gene-specific primer sets designed on the basis of the sequence information of previously mentioned fragments, an intact PCR product was amplified. Sequencing results showed that this PCR product was identical to the corresponding region in the assembled sequence. This sequence (GenBank Accession No. AY753201) represented a new cytochrome P450, and was designated as *CYP9A17* by the Cytochrome P450 Nomenclature Committee.

CYP9A17 cDNA contains a longest open reading frame of 1593 bp coding for a protein of 531 amino acids. Based on the translated amino acid sequence, *CYP9A17* (ACB30272) has a theoretical p*I* value of 8.47 and molecular mass of 61 200. Pairwise comparison showed highest amino acid identity (93.9%) between *CYP9A17* and *CYP9A12* from both *Helicoverpa armigera* (ABY47595) and *H. zea* (ABH09252). *CYP9A17* shows 58.4–64.8% identity with members from *Bombyx mori* (*CYP9A19*, 64.8%, ABQ08709; *CYP9A20*, 67.1%, NP_001077079; *CYP9A21*, 64.6%, NP_001103394; *CYP9A22*, 58.4%, ABQ08707). Amino acid identities of *CYP9A17* are 62.2% with *CYP9A14* (AAR37015) and 65.6% with *CYP9A18* (ABB69055) from *H. armigera*. In addition, *CYP9A17* showed identity of predicted protein sequence to different extents with *CYP9A1* (53.3%, AAC25787) from *H. virescens*, *CYP9A22* (58.0%, ABQ08708) from *B. mandarina* and two unannotated P450s from *Spodoptera litura* (73.0%, AAP80766) and *Mamestra brassicae* (83.4%, AAR26518).

Using neural networks and hidden Markov models trained on eukaryotes, the boundary of the hydrophobic signal peptide of *CYP9A17* was found to occur between amino acid 24 and 25. Three strong inside-to-outside transmembrane helices from amino acid 1 to 19, 215 to 237 and 319 to 338 in the protein sequence were predicted, suggesting that *CYP9A17* is an endoplasmic reticulum membrane-bound protein. All of the mentioned characters are in good agreement with currently known P450s. By aligning *CYP9A17* with five other CYP9A subfamily members from *Helicoverpa* (Fig. 1), it was found that they shared several conserved motifs, such as the P450 heme-binding signature (FXXGXXXCXG), the typical aromatic motif FXPXRF (meander) coinciding with the K′-L loop, the EXXR in the K helix and the WXXXR motif in the C helix. The region around the cysteine of the heme-binding domain is almost identical in all aligned cytochrome P450 sequences. Glu and Arg of the EXXR motif and Arg of the PXRF motif may form an E–R–R triad salt bridge that locks the cysteine pocket into the right position and assures heme association with the protein.[24] The Arg in the WXXXR motif may form a charge pair with the propionate of the heme.[25]

Table 2. Genomic sequence identity between *CYP9A12* and *CYP9A17*[a]

Exon No.	Length (bp) of *CYP9A17*/ *CYP9A12*	Identity (%)	Intron No.	Length (bp) of *CYP9A17*/ *CYP9A12*	Intron phase
1	203	88.2	1	263/254	1
2	161	90.7	2	92/90	2
3	138	93.5	3	161/161	2
4	203	94.6	4	1494/726	0
5	136	98.5	5	79/81	1
6	96	97.9	6	535/321	2
7	222	98.6	7	122/166	0
8	101	99.0	8	185/180	1
9	169	96.4	9	256/352	1
10	167	99.4			

[a] Accession number for *CYP9A17* is EU541247, and for *CYP9A12* it is EU541248.

The I helix with conserved Thr was thought to be involved in proton delivery and catalysis.[26] Six approximate SRS regions (Fig. 1) were predicted on the basis of the secondary structure elements and the schematic topology of P450.[27–29]

3.2 Genomic organisation of *CYP9A17* and *CYP9A12* genes from *Helicoverpa armigera*

The deduced amino acid sequence of *CYP9A17* (ACB30272) had the highest identity (93.9%) with *CYP9A12* from *H. armigera* (Fig. 1). To reveal the evolutionary relationship of these closely related genes, the full genomic sequences of *CYP9A12* and *CYP9A17* genes in *H. armigera* were sequenced by a PCR method. The intron–exon boundaries of the genomic sequence were determined by alignment of the compiled sequence with their corresponding cDNA and the canonical GT/AG rule. Both *CYP9A12* and *CYP9A17* genes were split into ten exons. The intron–exon organisation was highly conserved. Among the nine intron insertions in each sequence, six are phase 1, two phase 0 and one phase 2 (Table 2). The overall nucleotide identity between the *CYP9A17* and *CYP9A12* gene sequences is shown in Table 2. Pairwise comparison showed that the identity between each pair of exons was 88.2–99.4%. Sequence identity between introns of *CYP917* and *CYP9A12* was generally lower than that of exons, ranging from 42.6 to 95.7% (Table 3).

3.3 Constitutive expression profile of *CYP9A17* and *CYP9A12*

To explore whether evolutionary differences between *CYP9A12* and *CYP9A17* reflect their gene expression patterns, qRT-PCR was employed to determine their expression abundance. Use was made of gene-specific forward primers specific for *CYP9A17* and *CYP9A12*, and a common reverse primer to distinguish *CYP9A17* from *CYP9A12* (Table 1). *CYP9A12* transcript was detected in all four life stages, lowest in eggs and highest in larvae. Expression in male adults and pupae was slightly higher than in the female counterparts. During the larval stage, highest expression of *CYP9A12* was observed in the larval midgut, followed by the malpighian tubule, fat body, integument and head. In contrast, *CYP9A17* was detectable only in the larval midgut and fat body. The level of *CYP9A17* in the midgut was 3.6-fold higher than in the fat body (Table 4). Notably, *CYP9A12* was expressed significantly more (71–95-fold) than *CYP9A17* in larvae (Table 4).
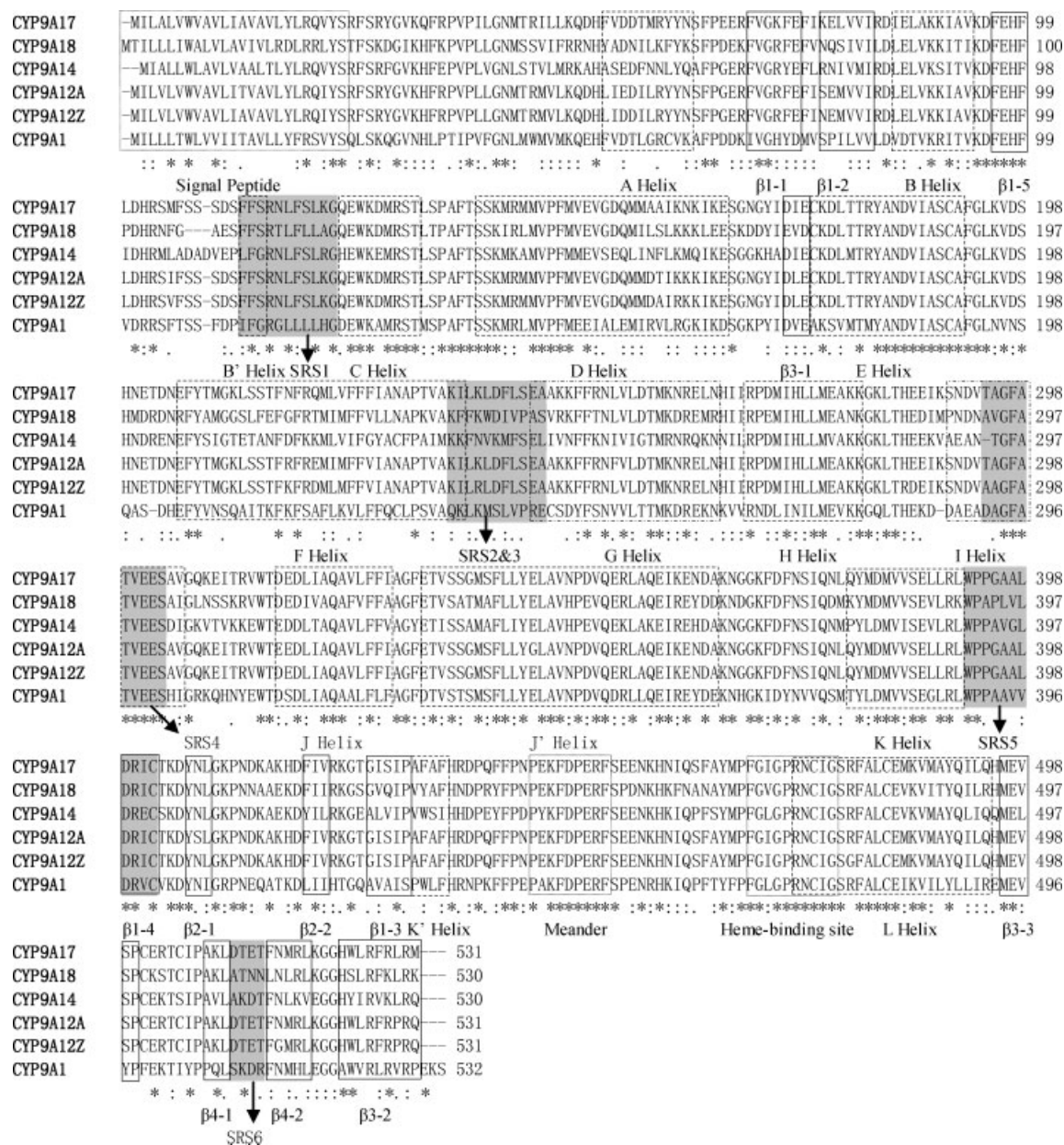
Figure 1. Alignment of amino acid sequences of *CYP9A17* and five other CYP9A subfamily members. *CYP9A12A, CYP9A12* from *Helicoverpa armigera*. *CYP9A12Z, CYP9A12* from *Helicoverpa. zea*. Asterisks (*) indicate identical sites among the six sequences; colons (:) represent sites with conserved substitutions; black dots (·) stand for sites with weakly conserved sites in the six-sequence alignment. Signal peptide, meander and heme-binding region are boxed with dots. α-helices and β-sheets are boxed with dashed and solid lines respectively. Shaded regions represent six substrate recognition sites (SRSs).

3.4 Xenobiotic response of *CYP9A17* and *CYP9A12*

To determine the extent to which *CYP9A12* and *CYP9A17* genes were expressed in response to xenobiotics, phenobarbital (PB), deltamethrin (DM) and gossypol (GP) were used. The three chemicals influence the expression of *CYP9A17* and *CYP9A12*, ranging from induction to repression. In the midgut (Table 5), PB at 10 mg g^{-1} and DM at 0.05 mg g^{-1} increased gene expression of *CYP9A17* by 2.7- and 4.5-fold ($P < 0.05$) respectively. In contrast, PB and DM at both tested concentrations increased the expression of *CYP9A12*. In the fat body, significant increases (8–14-fold for *CYP9A17*; 4–16-fold for CYP9A12) were observed when insects were treated with PB at both concentrations, whereas DM repressed the expression of both *CYP9A17* and *CYP9A12* by 64–89% (Table 5). GP slightly induced (1.78) *CYP9A12* in the fat body at low concentration or suppressed (by about 50%) the expression of both genes in the midgut at the high concentration.

4 DISCUSSION

It is well known that cytochrome P450s play crucial roles in the metabolism of many important compounds in various organisms, and each living organism contains a number of P450 isoforms.[10] Therefore, identification and characterisation of new cytochrome P450 genes have become a very attractive research area. PCR-based strategies (RT-PCR, RACE) have been widely and effectively used in sequencing P450 genes.[17,30–32] One central considera-

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Table 3. Pairwise comparison of the introns of *CYP9A12* and *CYP9A17* sequences from *Helicoverpa armigera* (percentage nucleotide identity)

		CYP9A12								
		Intron 1	Intron 2	Intron 3	Intron 4	Intron 5	Intron 6	Intron 7	Intron 8	Intron 9
CYP9A17	Intron 1	63.5	19.9	33.9	21.2	21.1	46.9	33.5	41.3	43.5
	Intron 2		79.3	31.0	8.2	58.0	15.8	33.7	28.0	16.8
	Intron 3			95.7	13.6	33.5	29.7	48.4	52.1	23.6
	Intron 4				42.6	3.5	12.6	6.8	6.8	13.3
	Intron 5					95.1	16.7	25.4	26.6	13.8
	Intron 6						48.7	18.3	18.8	37.8
	Intron 7							66.5	37.1	21.9
	Intron 8								87.6	30.1
	Intron 9									60.4

Table 4. Spatiotemporal expression of *CYP9A12* and *CYP9A17* genes in *Helicoverpa armigera*[a]

Stage/tissue	CYP9A12 $\triangle C_T$	CYP9A12 RE	CYP9A17 $\triangle C_T$	CYP9A17 RE	Ratio of *CYP9A12* to *CYP9A17*
Egg	14.59 ($\pm$0.34)	1.0 a	–	ND	–
Larval midgut	2.90 ($\pm$0.30)	3329.3 ($\pm$185.0) h	9.47 ($\pm$0.48)	35.2 ($\pm$3.4) a	94.6*
Larval fat body	5.17 ($\pm$0.47)	692.4 ($\pm$63.0) g	11.35 ($\pm$0.50)	9.7 ($\pm$1.4) b	71.4*
Larval integument	9.02 ($\pm$0.29)	48.0 ($\pm$4.1) d	–	ND	–
Larval head	9.20 ($\pm$0.32)	42.2 ($\pm$2.3) d	–	ND	–
Larval malpighian tube	4.89 ($\pm$0.37)	836.4 ($\pm$25.7) g	–	ND	–
Male pupa	11.18 ($\pm$0.18)	11.9 ($\pm$3.9) c	–	ND	–
Female pupa	12.66 ($\pm$0.25)	3.9 ($\pm$0.6) b	–	ND	–
Male adult	6.40 ($\pm$0.40)	294.0 ($\pm$18.9) f	–	ND	–
Female adult	7.25 ($\pm$0.24)	163.8 ($\pm$16.8) e	–	ND	–

[a] Data are means ($\pm$ SE) of three biological replicates by qRT-PCR. RE, gene expression level relative to that of *CYP9A12* in eggs; $\triangle C_T$, difference in C_T value of target gene minus that of reference gene; ND, not detectable. In each column, means followed by different letters differ significantly ($P < 0.05$, one-way ANOVA for *CYP9A12* or Student's *t*-test for *CYP9A17*). An asterisk (*) indicates significant difference between *CYP9A17* and *CYP9A12* ($P < 0.05$, Student's *t*-test).

tion to this approach is the design of primers for PCR. Usually, researchers design degenerate primer sets for cloning the P450s based on the conserved characteristic motifs of P450 such as the I helix and heme-binding region. The PFG motif of the heme-binding region was selected for this purpose, mainly based on the authors' survey results showing that this PFG motif in the heme-binding domain is very well conserved among insect P450 genes. Taking insects whose genomes are sequenced as examples, 41 of 85 P450 sequences in *Drosophila melanogaster* Meig., 36 of 46 in *Apis mellifera* L. and 53 of 100 in *Anopheles gambiae* Giles contain this motif. Notably, there are 43 of 45 CYP6 family entries (http://p450.sophia.inra.fr/) that contain the PFG motif. Moreover, the corresponding codons [CC(T/A/G/C)TT(C/T)GG] for PFG contain only two degenerate positions; thus, a set of only eight non-degenerate primers is enough to cover all sequence possibilities for codon usages at the PFG site.[33] More importantly, other conserved residues following the PFG motif in the heme-binding region, including the strictly conserved cysteine, allow rapid confirmation of the P450 fingerprint from all PCR products.[34] The high conservation of PFG in cytochrome P450 sequences and the low number of degenerate positions of codons for PFG suggest that this approach may facilitate the identification of P450 ESTs from various insects. Similar strategies have been successfully used in plants.[33,34]

Analysis of protein sequences using computational biology methods showed that characters such as molecular weight, p*I* and secondary structure elements of *CYP9A17* were in good

agreement with those of the known CYP9A members (Fig. 1). The presence of signal anchor peptide with high hydrophobicity and three transmembrane helices strongly suggests that *CYP9A17* is an endoplasmic reticulum membrane-bound protein. Sequence alignment and secondary structure prediction revealed that *CYP9A17* shares typical features of insect P450s[35] with five other CYP9A subfamily members, such as the five consensus motifs, 13 α-helices and four β-sheets (Fig. 1).

Differences in spatial and temporal expression of P450 genes have been suggested to be indicative of their functional divergence.[9,36] It is well known that P450 expression profiles are diverse in insects.[37] For example, *Cyp4d1* in *Drosophila*, *CYP6A1* in the house fly and *CYP6BB1* in *A. sollicitans* are expressed in all developmental stages,[31,38,39] whereas *CYP6Z1* in *A. gambiae* and *CYP6D1* in *Musca domestica* L. are adult specific.[40,41] *CYP6L1* is specifically expressed in the reproductive tissues of adult male cockroaches,[42] whereas CYP4G15 is predominantly expressed in the central nervous system of third-instar larvae of *Drosophila*.[43] The midgut and fat body are generally considered to be the primary detoxification organs[44] where most insect detoxification P450s are expressed.[45] Constitutive expression analysis reveals different expression patterns between *CYP9A12* and *CYP9A17*. *CYP9A12* mRNA was detected in all four developmental stages and all the tested tissue of larvae, whereas *CYP9A17* was only detectable in the midgut and fat body of larvae (Table 4). In addition, the level of *CYP9A12* mRNA in the midgut and fat body

Table 5. Xenobiotic response of CYP9A12 and CYP9A17 in Helicoverpa armigera[a]

Chemical[b] (mg g^{-1})		Midgut		Fat body	
		CYP9A12	CYP9A17	CYP9A12	CYP9A17
PB	0	1 a	1 a	1 a	1 a
	1	2.87 ($\pm$0.42) b	1.32 ($\pm$0.11) a	4.87 ($\pm$0.45) b	8.17 ($\pm$0.45)b
	10	5.09 ($\pm$0.47) c	2.66 ($\pm$0.27) b	16.52 ($\pm$0.76) c	14.53 ($\pm$0.76)c
GP	0	1 a	1 a	1 a	1 a
	0.2	1.11 ($\pm$0.17) a	1.16 ($\pm$0.12) a	1.90 ($\pm$0.66) a	1.79 ($\pm$0.18) b
	2	0.47 ($\pm$0.12) b	0.52 ($\pm$0.11) b	0.75 ($\pm$0.22) a	1.00 ($\pm$0.20) a
DM	0	1 a	1a	1a	1a
	0.05	2.40 ($\pm$0.30) b	4.49 ($\pm$0.61) b	0.11 ($\pm$0.04) b	0.37 ($\pm$0.07) b
	0.5	2.60 ($\pm$0.17) b	0.87 ($\pm$0.21) a	0.19 ($\pm$0.04) b	0.14 ($\pm$0.04) c

[a] Data are means ($\pm$ SE) of expression level relative to control. The relative levels are shown as a ratio in comparison with that for 0 mg g^{-1} (control) of each chemical treatment. Means followed by different letters differ significantly in the same column and chemical treatment, $P < 0.05$, one-way ANOVA.
[b] PB, phenobarbital; GP, gossypol; DM, deltamethrin.

of sixth-instar larvae was much higher (71–95-fold) than that of *CYP9A17* (Table 4). The ubiquitous expression of *CYP9A12* suggests that it probably has roles beyond xenobiotics metabolism. Given that *CYP9A17* is specifically expressed in the larval midgut and fat body, it may be involved mainly in detoxification of xenobiotics.

P450s are inducible by xenobiotic compounds such as insecticides and plant allelochemicals. These chemicals themselves may act as the substrates of the corresponding P450s.[9] Phenobarbital induction of P450s has been observed across insect species, including *D. melanogaster*,[46,47] *H. zea*,[48] *H. armigera*[49] and *Blatella germanica* (L.).[50] Therefore, the induced expression of *CYP9A17* and *CYP9A12* expression by phenobarbital in *Helicoverpa armigera*, as observed in this study, is not unexpected. Gossypol, a polyphenolic aldehyde that cotton bollworms often encounter in their host plants, does not obviously influence the expression of *CYP9A17* and *CYP9A12*, implying that these two cytochrome P450s are not the major proteins involved in gossypol metabolism. Recently, Mao *et al.*[8] reported a gossypol-inducible P450 *CYP6AE14* and illustrated that this protein confers the ability to tolerate gossypol on the cotton bollworm. The present data showed that a low concentration of deltamethrin caused more than a fourfold increase of *CYP9A17* and 2.5-fold increase of CYP9A12 in the midgut. Induction of P450 in this pest by pyrethroids was also reported by Qiu *et al.*[51] and Ranasinghe and Hobbs.[15] However, a high concentration of DM induced *CYP9A12* but repressed *CYP9A17* expression. Taken together, although constitutive expression of *CYP9A12* is ubiquitous whereas *CYP9A17* is tissue-specific, overall the two genes respond to xenobiotics similarly but still in a tissue- and concentration-specific manner.

CYP9A12 and *CYP9A17* not only share high identities in nucleotide sequences of their ORFs (94.6%) and in amino acid sequences (93.9%) but also have completely conserved intron–exon organisations (Table 2). Their apparent overall sequence similarity and conserved genomic organisations strongly suggest that these two genes have evolved from an ancestral gene-by-gene duplication. *CYP9A17* and *CYP9A12* show relatively extensive variation both in their exon and intron sequences (Tables 2 and 3), and the *CYP9A12* gene (DQ788839) has also been identified in *H. zea*. These observations suggest that the gene duplication is relatively ancient. The expression patterns of *CYP9A12* and *CYP9A17* support the classical view that gene duplication enables duplicates to be-

come specialised in different tissues or developmental stages,[52] implying that subfunctionalisation has occurred in the evolution of *CYP9A12* and *CYP9A17*.

Gene duplication provides material for functional divergence of proteins and hence allows organisms to adapt to changing environments.[53] Duplication of P450 genes involved in metabolic resistance or host adaptation has been observed in *Drosophila melanogaster*,[54,55] *Anopheles funestus* Giles[56] and *Papilio polyxenes* Stoll.[53] One model of gene evolution following duplication events is that duplicates may retain the original functions of their single ancestor.[57] Given that *CYP9A12* functions as a xenobiotic metabolising enzyme and confers pyrethroid resistance,[17,58] it is suggested that *CYP9A17* probably functions as a xenobiotic metabolising enzyme and may be involved in metabolic resistance in this pest. This conclusion is mainly based on the sequence information, expression profile and induction pattern of *CYP9A17*. However, further analysis is needed to determine whether *CYP9A17* is able to metabolise pyrethroids *in vitro*, and this work is under way in the authors' laboratory.

ACKNOWLEDGEMENTS

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