Bulletin of Entomological Research (2018) **108**, 77–83
doi:10.1017/S0007485317000517

Prokaryotic functional expression and activity comparison of three CYP9A genes from the polyphagous pest *Helicoverpa armigera*

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Abstract

Cytochrome P450s (CYPs or P450s) have been long recognized as very important enzymes in the metabolism of xenobiotic and endogenous compounds, but only a few CYPs have been functionally characterized in insects. The effort in functional characterization of insect P450s is heavily hindered by technical difficulties in preparing active, individual P450 enzymes directly from the target insect. In this paper, we describe the functional expression of two additional pyrethroid resistance-associated CYP9A genes (*CYP9A12* and *CYP9A17*) from the polyphagous pest *Helicoverpa armigera* in the facile *Escherichia coli*. The functionality of *E. coli* produced CYP9A12, CYP9A14, and CYP9A17 was investigated and activities of these CYP9As were compared against three probe substrates after reconstitution with NADPH-dependent cytochrome P450 reductase. The results showed that active forms of CYP9A12 and CYP9A17 were expressed in *E. coli* with a content of about 1.0–1.5 nmol mg^{-1} protein in membrane preparations. *In vitro* assays showed that CYP9A14 was capable of catalyzing O-dealkylation of methoxyresorufin (MROD), ethoxyresorufin (EROD), and benzyloxyresorufin (BROD), while CYP9A12 and CYP9A17 exhibited only MROD and EROD activities. Kinetic studies demonstrated that CYP9A14 had the greatest k_{cat}/K_m value for MROD, and CYP9A17 for EROD, while the lowest k_{cat}/K_m values for both MROD and EROD were observed for CYP9A12. The distinct biochemical traits suggest that the three paralogous CYP9As may play different roles in xenobiotic metabolism in this important pest.

Keywords: *Helicoverpa armigera*, cytochrome P450, CYP9A, functional expression, alkoxyresorufin O-dealkylase, *Escherichia coli*

(Accepted 8 May 2017; First published online 5 June 2017)

Introduction

Cytochrome P450s (CYPs or P450s) comprise a superfamily of heme-thiolate proteins (Feyereisen, 2012). It is well known that P450-mediated detoxification is important for insects to adapt to their host plants and to develop insecticide resistance (Schuler, 2011; Feyereisen, 2012). As one of the oldest and largest gene superfamily, P450s evolve from a common ancestral gene via multiple duplication events and subsequent divergence of duplicated genes (Feyereisen, 2012).

The cotton bollworm, *Helicoverpa armigera*, is a polyphagous pest that causes serious yield losses in many economically important crops (Wu & Guo, 2005). Insect CYPs have been long recognized as very important enzymes in the metabolism of various plant toxins and insecticides (Feyereisen, 2012), but

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Table 1. Primers used in this study.

Primer name	Usage	Sequences (reference)
CYP9A12-F	cDNA cloning	5′-ATGATACTAGTCCTGGTC-3′
CYP9A12-R	cDNA cloning	5′-CTACTGCCTAGGTCTGAA-3′
CYP9A14-F	cDNA cloning	5′-ATGATAGCCCTACTATGGCT-3′
CYP9A14-R	cDNA cloning	5′-TTACTGGCGCAGCTTGACC-3′
CYP9A17-F	cDNA cloning	5′-ATGATCCTAGCTCTGGTG-3′
CYP9A17-R	cDNA cloning	5′-TTACATCCTCAGTCTAAA-3′
CYP9A12ClaI-F	Expression plasmid construction	5′-GGATCCATCGATGCTTAGGAGGTCATATGGCTCTGTTATTA GCAGTTTTTGCGGTGCTGATCACCGTGGC-3′
CYP9A12KpnI-R	Expression plasmid construction	5′-CGGGGTACCCTACTGCCTAGGTCTGAA-3′
CYP9A14NdeI-F	Expression plasmid construction	5′-AGGAGGTCATATGGCCTGTTATTAGCAGTTTTT GTACTC GTCGCAGCTCTGACG-3′ (Liu *et al.*, 2014)
CYP9A14KpnI-R	Expression plasmid construction	5′-CGGGGTACCTTACTGGCGCAGCTTGACCCT-3′ (Liu *et al.*, 2014)
CYP9A17ClaI-F	Expression plasmid construction	5′-GGATCCATCGATGCTTAGGAGGTCATATGGCTCTGTTA TTAGCAGTTTTTGCGGTGCTGATCGCCGTGGC-3′
CYP9A17KpnI-R	Expression plasmid construction	5′-CGGGGTACCTTACATCCTCAGTCTAAA-3′

only a few CYPs have been somewhat characterized in this pest. It has been documented that the expression level of *CYP9A12*, *CYP9A14*, and *CYP9A17* genes are overexpressed in pyrethroid-resistant populations of *H. armigera* (Yang *et al.*, 2006; Brun-Barale *et al.*, 2010). These CYP9A genes share 62–96% identity of amino acids, and have identical intron-exon organization (d'Alencon *et al.*, 2010; Zhou *et al.*, 2010). Recombinant CYP9A12 and CYP9A14 were expressed in yeast (*Saccharomyces cerevisiae*), and showed O-demethylation activities of *p*-nitroanisole and methoxyresorufin (MROD), and clearance activity against the pyrethroid esfenvalerate (Yang *et al.*, 2008). However, no further biochemical work on CYP9A was reported, largely due to technical difficulties in preparing active, individual P450 enzymes for detailed biochemical studies and in prediction of substrates.

In a previous study focusing on the characterization of NADPH-dependent cytochrome P450 reductase gene in the cotton bollworm (HaCPR) (Liu *et al.*, 2014), we succeeded in producing functional CYP9A14 in *Escherichia coli.* In this study, we attempted to use this facile expression system to realize recombinant functional expression of two additional pyrethroid resistance-associated genes (*CYP9A12* and *CYP9A17*) from *H. armigera* in *E. coli* in the hope of obtaining enough enzymes for pinpointing the biochemical basis of adaptability in this polyphagous pest. A preliminary biochemical *in vitro* assay was performed to confirm the functionality of these *E. coli*-produced CYP9As, and to investigate potential functional divergence among the three CYP9A paralogs before extensively substrate screening.

Materials and methods

Construction of recombinant plasmid of CYP9As

Total RNA was extracted from midguts of the fifth instar larvae of *H. armigera* from a laboratory colony (Liu *et al.*, 2014) using TRIzol according to the manufacture's protocol (Invitrogen, California, USA). First-strand cDNA was synthesized from total RNA (1 μg) by using the oligo(dT) primer and the Moloney murine leukemia virus (M-MLV) reverse transcriptase according to the manufacturer's instructions (Takara, Dalian, China). Gene-specific primers (table 1) were designed to obtain the intact open reading frames (ORFs) of CYP9A12 (GenBank ID: AY371318), CYP9A14 (EU327674), and CYP9A17 (AY753201), respectively, by Reverse transcription polymerase chain reaction (RT-PCR) using *Pfu* DNA polymerase (Takara, Dalian, China) and cDNA as templates. The PCR product was purified on gel, cloned into *p*MD19-T vector, and transformed into *E. coli*. At least three clones were sequenced from both directions to confirm the nucleotide sequence of the PCR product (Invitrogen, Beijing, China).

To construct recombinant plasmid of CYP9As for functional expression, the first eight amino acid residues in the *N*-terminus of CYP9A ORF were replaced by the *N*-terminal eight residues (MALLLAVF) of bovine 17α hydroxylase (17α strategy, Barnes *et al.*, 1991). The *N*-terminal modified CYP9A sequence was inserted into pCWori + vector (pB54) at the NdeI digestion site in order to optimize mRNA translation. The primers used for plasmid construction were listed in table 1. Each of the resulting recombinant plasmids (pCW-CYP9A) was transformed into *E. coli* cells (Rosseta DE3 strain) (Transgen, Beijing, China) for functional expression.

Recombinant expression of CYP9A12 and CYP9A17 in E. coli

The recombinant expression of CYP9A14 in *E. coli* was described in Liu *et al.* (2014). A similar protocol was used to produce CYP9A12 and CYP9A17. Specifically, starter cultures were prepared by inoculating 5 ml of Luria Broth (containing 100 μg ml^{-1} ampicillin and chloromycetin) with a single colony carrying recombinant pCW-CYP9A plasmid and allowed to grow overnight at 37°C and 200 rpm. A 1 ml of overnight cultures was transferred into 100 ml Terrific Broth media. When the OD_{600} of the culture reached 0.7–1.0, 1 mM isopropyl β-D-1-thiogalactopyranoside and 0.75 mM 5-aminolevulinic acid hydrochloride were added. After a further 22–24 h growth at 28°C with 180 rpm shaking, cells were pelleted by spinning at 2800 g for 20 min at 4°C. The harvested cells were resuspended in 10 ml 1 × TSE buffer [50 mM Tris-acetate (pH 7.6), 250 mM sucrose, and 0.25 mM EDTA (ethylenediaminetetraacetic acid)]. Then the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) were added to final concentrations of 1 and 0.1 mM, respectively. The resuspended cells were sonicated (Scienta-IID, China), and centrifuged at 12,000 g for 30 min at 4°C. The 12,000 g supernatant was further centrifuged at 180,000 g for 1 h. The membrane fractions were obtained by resuspending the 180,000 g pellets in 1 × TSE buffer containing PMSF and DTT.

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Determination of CYP9A content and HaCPR activity

Protein concentration was determined according to the Bradford method (Bradford, 1976). The content of expressed CYP9A protein was estimated by the method of Omura & Sato (1964). The details for the recombinant HaCPR of the cotton bollworm in *E. coli* were given in Liu *et al.* (2014). The recombinant HaCPR activity was measured at 30°C by recording the change of absorbance at 550 nm when oxidized cytochrome *c* was reduced according to the method of Pritchard *et al.* (2006).

Alkoxyresorufin O-dealkylase (AROD) assay

Three related model substrates, 7-methoxyresorufin, 7-ethoxyresorufin, and 7-benzyloxyresorufin, were used for AROD assay. The enzyme activity was measured at excitation wavelength of 530 nm, and emission wavelength of 585 nm (Mayer *et al.*, 1977). The reconstituted enzymes were prepared by combining the membranes containing individual CYP (50 nM) and the membranes containing HaCPR (100 nM) and mildly mixing on ice for 5 min. The reaction mixture contained 25 mM $MgCl_2$, the reconstituted enzyme and each substrate [dissolved in dimethyl sulfoxide (DMSO), 0–8 µM for MROD, 0–6 µM for EROD, and 0–4.8 µM for BROD] in 50 mM Tris-HCl buffer (pH 7.5 in a total volume of 1 ml). The amount of DMSO in the reaction mixtures was 0.5%. The reaction mixtures were preincubated in a water bath at 30°C for 5 min, and followed by the addition of 0.25 mM NADPH to initiate the reactions. A standard curve was generated to quantify the amount of resorufin. Velocities were calculated based on fluorescence values within linear range and presented as the formation of resorufin per minute per nanomole of CYP. Three separate determinations, each in duplicate, were performed. A control with corresponding preparation from cells carrying the pCWori+ empty vector was set up in parallel.

Homology modeling of the structure of CYP9As

The transmembrane domain-truncated sequence (without the 21 amino acids of the *N*-terminal) of CYP9As was blasted to the Protein Data Bank (PDB) database using PSI-Blast server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). CYP3A4 (PDB code: 1tqn), with amino acid identity of 31% to CYP9A14, and 32% to CYP9A12 and CYP9A17, was chosen as the template for homology modeling. The three-dimensional structure was modeled by I-TASSER webserver (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The candidate structure with the highest *C*-score was chosen for a further molecular dynamics (MD) simulation using NAMD 2.6v (Phillips *et al.*, 2005) (energy minimization for 20,000 steps and equilibration for 1 ns). The final modeled structure has 'allowed regions' of 97.3% for CYP9A12, 97.7% for CYP9A14, and 97.3% for CYP9A17 as generated by the Ramachandran plot, respectively. The cavity of CYP9As was analyzed by MOLE 2.0 webserver (http://mole.upol.cz).

Statistical analysis

Kinetic parameters were calculated by non-linear Michaelis–Menten plots using GraphPad Prism 5 (San Diego, California, USA). Means of each kinetic parameter were subject to one-way analysis of variance analysis followed by Least Significant Difference test or Student's *t*-test.

Results

Sequence alignment of three CYP9As

The ORF of three CYP9A genes was amplified and sequenced (fig. 1). Compared with the corresponding gene sequence in GenBank, we identified five amino acid substitutions (V179M, V233I, S314T, Q321P, E352D) in CYP9A14 (compared with ABY47596.1, which was isolated from the pyrethroid-resistant YGF strain) , and one substitutions in CYP9A17 (A13T) (compared with AAY21809.1 from the YGF strain, which was named as CYP9A17v2). A high identity (95.5%) was observed between CYP9A17 and CYP9A12, while identities between CYP9A14 and CYP9A17, and between CYP9A12 and CYP9A14, were 62 and 62.6%, respectively. Notably, significant variations in the amino acids in putative substrate recognition sites (SRSs) were observed in CYP9A14 as compared with CYP9A12 and CYP9A17.

Heterologous expression of H. armigera *CYP9A12 and CYP9A17*

The 17α *N*-terminal modification strategy was employed to express CYP9A12 and CYP9A17 genes in *E. coli*. Under the procedures described in this study, approximately 11 nmol of CYP in the membrane fraction and 1.5 nmol in the 18,000 g supernatant were obtained in 100 ml culture. The content of CYP9A12 and CYP9A17 in membrane preparations was about 1.0–1.5 nmol mg^{-1} protein. CYP9As exhibited the typical CO difference spectrum with a peak at ~450 nm (fig. 2). A trace amount of inactive form (with an absorption peak at 420 nm) was observable for CYP9A17.

Substrate specificity and enzymatic kinetics of H. armigera *CYP9As*

The membrane fractions containing expressed CYP9As were used for AROD assays. The results showed that CYP9A14 exhibited activities toward all the three tested substrates, whereas CYP9A12 and CYP9A17 displayed only MROD and EROD activities (fig. 3, table 2). Kinetic analysis of AROD activities revealed that AROD reactions of CYP9A enzymes followed Michaelis–Menten kinetics (fig. 3).

Significant differences in AROD kinetics were observed among the three CYP9A proteins. For example, CYP9A14 exhibited the greatest k_{cat}/K_m value for MROD, whereas CYP9A17 had the highest value for EROD. The lowest values for both MROD and EROD were seen for CYP9A12 (table 2). Significant differences in substrate preference for the three CYP9A proteins were observed. For CYP9A14, k_{cat}/K_m values were in the order of MROD > EROD > BROD, while this ratio was significantly higher in EROD than MROD for CYP9A12.

Discussion

Given that insect CYPs are membrane-bound and low in amount, it remains a challenge to obtain enough active individual P450 from insects for biochemical studies by conventional methods. Heterologous expression has become a practical choice to produce individual P450 protein of interest. Recombinant expression and enzyme reconstitution have greatly promoted the functional characterization of insect CYPs at the biochemical level (Feyereisen, 2012). Several heterologous expression systems such as *E. coli*, baculovirus, yeast,

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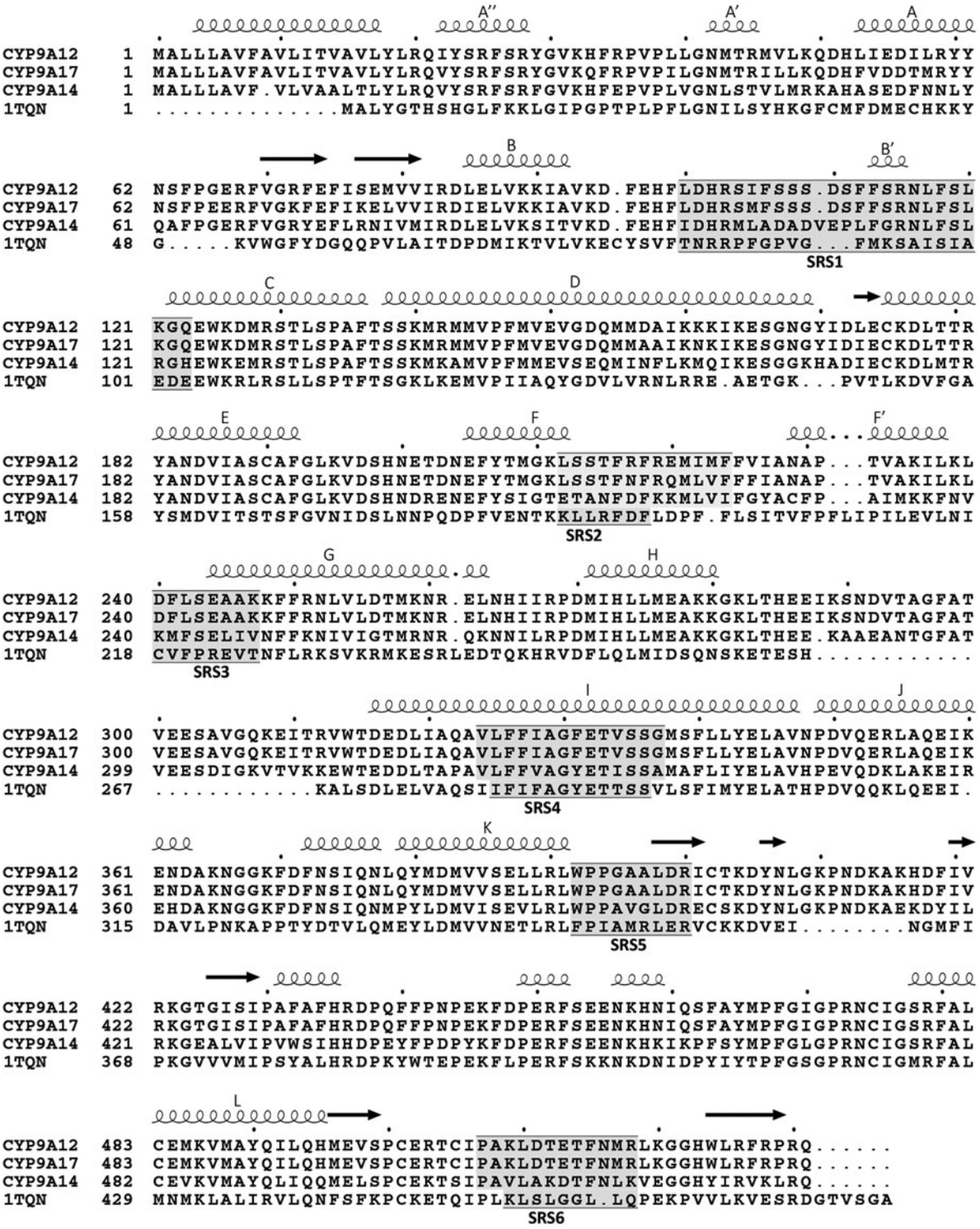

Fig. 1. Structure-based sequence alignment of three CYP9A proteins and human CYP3A4 (1TQN). The secondary structure elements were marked above the sequence (with helix for helices, arrow for sheets). The putative substrate recognition sites (SRS) were indicated.

insect or plant (e. g. *Nicotiana tabacum*) cells have been introduced for expressing insect P450 genes (Andersen *et al.*, 1994; Saner *et al.*, 1996; Dunkov *et al.*, 1997; Smith & Scott, 1997; Amichot *et al.*, 2004; Rupasinghe *et al.*, 2007; Joußen *et al.*, 2008, 2012; Mao *et al.*, 2009; Cheesman *et al.*, 2013; Liu *et al.*, 2014). We attempted to realize functional expression of CYP9As in *E. coli* due to its ease of use, high protein production, and especially lack of endogenous P450s. By using this

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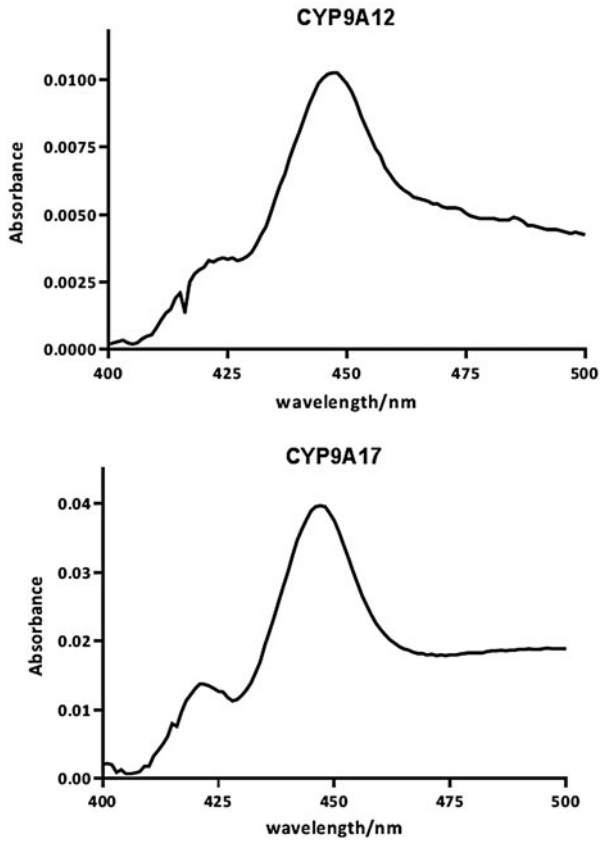

Fig. 2. Reduced CO difference spectra of *E. coli* membrane containing expressed CYP9As of *H. armigera*.

system, we successfully produced three CYP9As in *E. coli* at a satisfactory level. To our experience, success in expressing these insect CYP enzymes is greatly benefited from the adoption of 'ATG' of the NdeI digestion site in the polylinker of the expression vector pCWori+ as the initiation codon (Pritchard *et al*., 2006; Yang et al, 2013). *N*-terminal modification (Barnes *et al*., 1991) is helpful in most cases, and co-expression with the groES and groEL chaperones (Nishihara *et al*., 1998) may increase CYP expression in the membranes by around 30%.

Successful heterologous expression of CYP9A was confirmed by AROD assays. Our results demonstrated that CYP9A12, CYP9A14, and CYP9A17 had both MROD and EROD activities (table 2). Similarly, MROD activity was detected with yeast-expressed CYP9A12 and CYP9A14 (Yang *et al*., 2008). These observations suggest that these paralogous P450s share some substrates. Notably, only CYP9A14 among the three P450s is able to catalyze the BROD reaction, indicating that divergence in substrate specificity evolves among these paralogs. Functional difference among these P450s is also reflected by enzymatic kinetics (table 2, fig. 3). For example, CYP9A14 has the greatest k_{cat}/K_m ratio for MROD, indicating its preference to the relatively small substrate MROD; no significant difference in k_{cat}/K_m value was observed for MROD and EROD activities for CYP9A17, implying that CYP9A17 has similar preference to both MROD and EROD. A higher k_{cat}/K_m value of EROD than that of MROD indicates that CYP9A12 prefers EROD over MROD. The distinct biochemical traits suggest that the three paralogous CYP9As may play varying roles in this important pest.

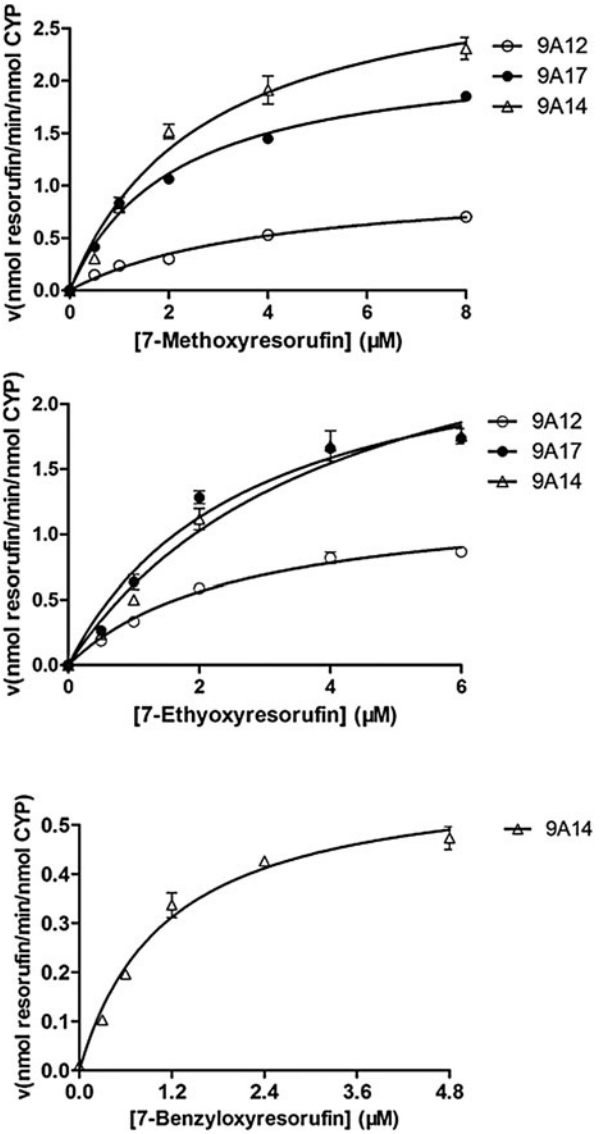

Fig. 3. Kinetic analysis of *E. coli* expressed *H. armigera* CYP9As for MROD, EROD, and BROD activities. Results are presented as mean ± SE of three independent determinations with each in duplicate.

The observed differences in the substrate specificity and kinetics among these P450s are theoretically determined by dissimilarity in their amino acid sequences. Sequence alignment reveals higher identity between CYP9A12 and CYP9A17 (95.5%) than between CYP9A12 and CYP9A14 or between CYP9A14 and CYP9A17 (around 62%). Specific comparisons between the SRS regions indicate that CYP9A12 and CYP9A17 share extremely high amino acid identity (fig. 1), with only one and three residue variations in SRS1 and SRS2, respectively and no difference in SRS3–SRS6. In contrast, much more divergence is observed in SRS of CYP9A14 when compared with CYP9A12 or CYP9A17. The cavity of CYP9A14 (1796 Å^3) is larger than CYP9A12 (1644 Å^3) and CYP9A17 (1375 Å^3) as indicated by the structure models. Based on the data of our AROD assays (fig. 3 and table 2),

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Table 2. Kinetic parameters of AROD activities in *E. coli* membranes containing expressed CYP9As of *H. Armigera*.

Activities	Parameters	CYP9A12	CYP9A17	CYP9A14
MROD	k_{cat}	1.06 ± 0.03a	2.30 ± 0.02b	3.16 ± 0.11c
	K_m	4.08 ± 0.32a	2.12 ± 0.10b	2.69 ± 0.06b
	k_{cat}/K_m	0.26 ± 0.01a	1.09 ± 0.04b	1.17 ± 0.01b
EROD	k_{cat}	1.15 ± 0.02a*	2.37 ± 0.02b	2.58 ± 0.06c*
	K_m	2.11 ± 0.04a*	2.20 ± 0.11a	2.99 ± 0.04b*
	k_{cat}/K_m	0.55 ± 0.02a*	1.08 ± 0.05b	0.86 ± 0.03c*
BROD	k_{cat}	n.d.	n.d.	0.61 ± 0.02+
	K_m	n.d.	n.d.	1.13 ± 0.05+
	k_{cat}/K_m	n.d.	n.d.	0.54 ± 0.01+

Values present mean ± SE from three independent experiments with each determination made in duplicate. The units for k_{cat}, K_m and k_{cat}/K_m are nmol resorufin formed per nmol CYP per minute, μM, and μM^{-1} min^{-1}, respectively. n.d. means not detectable. Significant difference for each substrate among different CYP9As (line comparison) was marked by different letters. Significant difference between different substrates for each CYP9A (column comparison) was marked by a symbol * or + ($P < 0.05$).

the substrate specificity appears to be related to the degree of sequence difference among the three P450s: the most divergent CYP9A14, which has the largest cavity, specifically exhibits AROD activity toward the largest substrate 7-benzyloxyresorufin (fig. 1). However, our current data could not allow a clear answer to which amino acid(s) define the substrate range. In fact, the structure–function relationship of CYPs is very complicated. There have been reported cases showing that phylogenetically distinct P450s may metabolize similar substrates, similar P450s may have distinct substrates (Schuler & Berenbaum, 2013). Similarly, we currently are unable to figure out the exact amino acid(s) that determine the AROD kinetics without site-directed mutagenesis studies. Numerous previous studies have provided evidence that kinetic properties of CYPs are often influenced in complex ways by residues in active site, and/or substrate access channel, even in proximal surface (Schuler & Berenbaum, 2013).

In conclusion, three CYP9As of the cotton bollworm were functionally produced in *E. coli*. Functional divergence was observed among these paralogous CYP9As by AROD assays, suggesting that they may play different roles in xenobiotic metabolism. The establishment of functional expression system for CYP9As and their partner HaCPR (this study and Liu *et al*., 2014) allows us to reconstitute a completely species-specific CYP monooxygenase to screen potential substrates of individual P450s, thus will facilitate further elucidation of molecular mechanisms of the chemical adaptability in this important pest. Extensive substrate screening is underway in our group. We hope that our experience in the use of the simple and highly efficient *E. coli* system is helpful for functional studies of other insect P450s.

Acknowledgements

The authors thank Dr Thomas Friedberg for the gift of the pB54 plasmid. This work was supported by grants from the National Natural Science Foundation of China (no. 31471796) to X. Qiu and the State Key Laboratory of Integrated Management of Pest Insects and Rodents (ChineseIPM1408) to M. Zheng and X. Qiu.

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