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Overexpression of a cytochrome P450 monooxygenase, *CYP6ER1*, is associated with resistance to imidacloprid in the brown planthopper, *Nilaparvata lugens*

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Abstract

The brown planthopper, *Nilaparvata lugens*, is an economically significant pest of rice throughout Asia and has evolved resistance to many insecticides including the neonicotinoid imidacloprid. The resistance of field populations of *N. lugens* to imidacloprid has been attributed to enhanced detoxification by cytochrome P450 monooxygenases (P450s), although, to date, the causative P450(s) has (have) not been identified. In the present study, biochemical assays using the model substrate 7-ethoxycoumarin showed enhanced P450 activity in several resistant *N. lugens* field strains when compared with a susceptible reference strain. Thirty three cDNA sequences encoding tentative unique P450s were identified from two recent sequencing projects and by degenerate PCR. The mRNA expression level of 32 of these was examined in susceptible, moderately resistant and highly resistant *N. lugens* strains using quantitative real-time PCR. A single P450 gene (*CYP6ER1*) was highly overexpressed in all resistant strains (up to 40-fold) and the level of expression observed in the different *N. lugens* strains was significantly correlated with the resistance phenotype. These results provide strong evidence for a role of *CYP6ER1* in the resistance of *N. lugens* to imidacloprid.

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Introduction

The brown planthopper (BPH), *Nilaparvata lugens*, is a significant pest of rice (*Oryza sativa* L.) throughout Asia. This specialist herbivore feeds on the phloem of rice plants and heavy infestations cause a series of deleterious effects known collectively as 'hopperburn' which can eventually lead to death of the plant (Gorman *et al*., 2008). As with many other sucking pests, BPH is also an efficient vector of several plant viruses, including rice grassy stunt and ragged stunt (Hogenhout *et al*., 2008). Together these can result in a dramatic reduction in yield and cause significant economic loss (Sogawa & Cheng, 1979).

The control of BPH relies heavily on the application of chemical insecticides and, as a result, this species has developed multiple resistance to many insecticide classes including organophosphates, carbamates and pyrethroids (Hemingway *et al*., 1999; Nagata *et al*., 2002). Two mechanisms, both involving the enhanced production of detoxifying enzymes, underlie resistance to these insecticides in BPH. The increased production of a carboxylesterase (Nl-EST1) confers broad-spectrum resistance to organophosphates/carbamates while elevated expression of a glutathione S-transferase (nlgst1-1) is involved in conferring resistance to pyrethroids (Small & Hemingway, 2000; Vontas *et al*., 2000, 2001, 2002). Members of the neonicotinoid class of insecticides are unaffected by these mechanisms and, consequently, became the most widely used insecticides for BPH control after the introduction of imidacloprid, the first commercialized member of the neonicotinoids, in the early 1990s. Unfortunately, resistance of BPH to imidacloprid has now been documented in several countries in Asia with more than 800-fold resistance described in China (Gorman *et al*., 2008; Wang *et al*., 2008, 2009). Although target-site resistance to neonicotinoids was described in a laboratory-selected strain of

Table 1. Concentration probit mortality data of susceptible and resistant *Nilaparvata lugens* strains against imidacloprid topically applied to adult females

Strain	LC_{50}	95% CL	SLOPE ± SE	*n*	RR
NLS	0.39	0.172–0.55	3.45 ± 0.906	244	1
NL9	97	3.4–434	0.762 ± 0.239	232	248
NL15	20.1	1.14–2440	1.1 ± 0.393	118	51.5
NL16	29.8	5.98–64.5	1.39 ± 0.361	235	76.4

LC_{50}, lethal concentration 50%; CL, confidence limit; RR, resistance ratio.

BPH before reports of resistance in the field, this mechanism has never been identified in any field-collected population (Liu *et al*., 2005a). In contrast, there is direct and indirect evidence that detoxification, and in particular enhanced cytochrome P450 monooxygenase (P450) activity, contributes to the neonicotinoid resistance of field-collected populations of BPH (Nauen & Denholm, 2005; Liu *et al*., 2005b; Wen *et al*., 2009). This detoxification mechanism was initially implicated by use of the metabolic enzyme inhibitor piperonyl butoxide (PBO) which demonstrated that pre-exposure to PBO increased the efficacy of imidacloprid against resistant BPH strains (Wen *et al*., 2009). More recently, biochemical assays using the model substrate 7-ethoxycoumarin showed that the resistance of two field-collected populations was associated with an approximate five-fold increase in mixed function oxidase activity (Puinean *et al*., 2010a). However, neither study identified the individual P450 gene(s) overexpressed in the resistant populations.

The P450s are encoded by a large supergene family with insect genomes sequenced to date containing from 46 to more than 150 P450 genes (Feyereisen, 2006). The rapid growth in genomics and post-genomic technologies has made it easier to study large complex gene families, such as those involved in detoxification. Although the genome of BPH is yet to be sequenced, a number of other genomic resources are available. These include the recently completed sequence of the pea aphid *Acyrthosiphon pisum* (International Aphid Genomics Consortium, 2010) which, like BPH, belongs to the order Hemiptera and two expressed sequence tag (EST) libraries generated by Sanger and 454 sequencing (Noda *et al*., 2008; Bass *et al*., 2011). We compared the expression of P450 genes identified by these projects and, in the present study by degenerate PCR, in imidacloprid-resistant and -susceptible strains to identify the P450s associated with the resistance phenotype.

Results

Bioassays

The known susceptible reference strain NLS yielded an lethal concentration 50% (LC_{50}) value for imidacloprid of 0.39 with a relatively steep response slope of 3.45, similar to data previously reported for this strain (Gorman *et al*., 2008). Three field collected strains from Asia showed significant levels of resistance, with resistance ratios of 51.5 (NL15), 76.4 (NL16) and 248 (NL9) and significantly shallower response slopes of 1.1, 1.39 and 0.762, respectively (Table 1).

Biochemical measurement of cytochrome P450 monooxygenase activity in imidacloprid-resistant and -susceptible strains of the brown plant hopper

Biochemical assessment of P450 activity using 7-ethoxycoumarin as an artificial substrate showed that all three resistant strains had significantly higher levels of activity than the susceptible strain (Table 2). Surprisingly, P450 activity was higher in the moderately resistant BPH strains NL15 and NL16 (≈seven-fold higher than the laboratory susceptible strain NLS) than NL9, the most resistant strain (≈three-fold higher than NLS).

Identification of brown planthopper P450 genes by data mining and degenerate PCR

A total of 30 full-length or partial P450 gene sequences were identified from the combined assembly of two recent sequencing projects (Noda *et al*., 2008; Bass *et al*., 2011) and from GenBank. Since these projects full-length or almost full-length cDNA sequences have been obtained for three of these partial EST sequences (NL7816, FUKV2O402FQVT4 and NL7686) using rapid amplification of cDNA ends (RACE; own unpublished data) and the full-length sequences derived from NL7816 and FUKV2O402FQVT4 have been officially named *CYP418A1* and *CYP417A1*, respectively (Table 3). In addition, a total of eight different partial P450 sequences were identified in this study by degenerate PCR (three using CYP4 family-specific primers and five using CYP6 family-specific primers). Of these, five sequences correspond to previously identified P450 genes, two were members of the CYP6 family (*CYP6ER1* and *CYP6-like 2*) and one belonged to the CYP4 family (*CYP4-like 1*).

Table 2. Cytochrome P450-dependent monooxygenase activity of susceptible (Bayer-S) and resistant (NL9, NL15 and NL16) *Nilaparvata lugens* strains as determined by *O*-deethylation of 7-ethoxycoumarin

Strain	ECOD pmol/min protein/mg	Relative activity
NLS	2.35 ± 0.98	1
NL9	7.10 ± 1.92	3.0
NL15	18.2 ± 2.73	7.7
NL16	16.2 ± 3.24	6.9

Data are mean values ± SD ($n = 3$); ECOD, 7-ethoxycoumarin-O-deethylation.

Table 3. Full-length and partial tentative unique P450 genes identified from GenBank, two recent sequencing projects and by degenerate PCR. Gene names/expressed sequence tag identifiers, GenBank accession numbers, sequence length and description based on the closest BLAST hit is detailed along with any protein domain identified in the Pfam database

No	Name	Accession No.	Length	NCBI NR top hit	EVAL	Score	Pfam Domain
1	CYP3A25	GQ911996	1937	gi|261157177|gb|GQ911996.1| Nilaparvata lugens cytochrome P450 CYP3A25 (CYP3A25) mRNA, complete cds	na	na	p450
2	NL7686	JF928995	1380	gb|EFN67752.1| Probable cytochrome P450 49a1 [Camponotus floridanus]	9.00E-160	568	p450
3	CYP418A1	JF928996	1921	ref|XP_003250070.1| PREDICTED: cytochrome P450 9e2-like [Apis mellifera]	5.00E-56	223	p450
4	CYP6CW1	FN421126	2063	gi|241659420|emb|FN421126.1| Nilaparvata lugens mRNA for cytochrome P450 (cyp6CW1 gene)	na	na	p450
5	CYP6CS1	FM994118	2700	gi|239935044|emb|FM994118.3| Nilaparvata lugens mRNA for CYP6CS1 protein	na	na	p450
6	CYP4CE1	FN356974	2160	gi|239934802|emb|FN356974.2| Nilaparvata lugens mRNA for CYP4CE1 protein	na	na	p450
7	CYP303A1	FJ907954	1824	gi|233770156|gb|FJ907954.1| Nilaparvata lugens cytochrome P450 (CYP303A1) mRNA, complete cds	na	na	p450
8	CYP4C62	FM163385	2146	gi|189178725|emb|FM163385.1| N. lugens mRNA for cytochrome P450 (cyp4C62 gene)	na	na	p450
9	CYP4C61	FM163384	2581	gi|189178723|emb|FM163384.1| N. lugens mRNA for cytochrome P450 (cyp4C61 gene)	na	na	p450
10	CYP6AX1	AJ852422	2395	gi|56756173|emb|AJ852422.2| N. lugens CYP6AX1 gene for cyrochrome P450 CYP6AX1 protein	na	na	p450
11	CYP6AY1	AJ852423	2720	gi|56756175|emb|AJ852423.2| N. lugens CYP6AY1 gene for cytochrome P450 CYP6AY1 protein	na	na	p450
12	C_NLTH8451	DB840517.1	1178	AAV84214.1 cytochrome B5 [Culicoides sonorensis]	5.00E-39	166	Cyt-b5
13	C_NLNB2799	DB828531.1	511	ref|XP_532219.2| PREDICTED: similar to NADPH cytochrome B5 oxidoreductase [Canis familiaris]	2.00E-23	112	NAD_binding_1
14	S_NLTH2887	DB838220.1	742	ref|XP_001951829.2| PREDICTED: cytochrome P450 4C1-like [Acyrthosiphon pisum]	7.00E-37	179	p450
15	S_NLNB8867	DB830779.1	713	ref|NP_495052.1| CYtochrome P450 family member (cyp-44A1) [Caenorhabditis elegans]	2.00E-26	124	p450
16	S_NLNA3020	DB853232.1	631	ref|NP_001107860.1| cytochrome P450 monooxigenase CYP4G7 [Tribolium castaneum]	2.00E-15	107	p450
17	S_NLEA7091	DB844112.1	620	>gb|EFN60908.1| Cytochrome P450 315a1, mitochondrial [Camponotus floridanus]	1.00E-28	130	p450
18	S_NLEA5884	DB843531.1	798	ref|XP_001600798.1| PREDICTED: similar to cytochrome P450 Cyp18a1, partial [Nasonia vitripennis]	5.00E-57	225	p450
19	S_NLEA2063	DB841996.1	819	ref|NP_495052.1| CYtochrome P450 family member (cyp-44A1) [Caenorhabditis elegans]	3.00E-42	176	p450
20	C_NLEA7312	DB844235.1	649	ref|XP_974252.1| PREDICTED: similar to CYP302a1 [Tribolium castaneum]	8.00E-23	111	p450
21	C_NLEA2397	DB842170.1	1107	ref|NP_001035347.1| cytochrome P450 314A1 [Apis mellifera]	1.00E-53	214	p450
22	FUKV2O402FXVW5	SRX018177	241	ref|NP_001165992.1| cytochrome P450 4G43 [Nasonia vitripennis]	6.00E-25	117	p450
23	contig06052	SRX018177	621	ref|XP_001649539.1| hypothetical protein AaeL_AAEL014754 [Aedes aegypti]	2.00E-20	103	Cyt-b5
24	contig02701	SRX018177	2264	ref|XP_001809620.1| PREDICTED: similar to cytochrome P450 [Tribolium castaneum]	1.00E-61	264	p450
25	FUKV2O402JGCLL	SRX018177	240	ref|XP_001944205.2| PREDICTED: cytochrome P450 4g15-like [Acyrthosiphon pisum]	2.00E-21	126	p450
26	FUKV2O402HFA0Q	SRX018177	242	ref|XP_001945361.2| PREDICTED: cytochrome P450 4C1-like [Acyrthosiphon pisum]	8.00E-22	107	p450
27	gi|166384703	DB821573.1	586	ref|XP_975578.2| PREDICTED: similar to cytochrome P450 [Tribolium castaneum]	8.00E-24	135	p450
28	gi|166399656	DB835514.1	666	ref|NP_001089965.1| tRNA wybutosine-synthesizing protein 1 homolog [Xenopus laevis]	2.00E-20	124	Flavodoxin_1
29	CYP417A1	JF928997	1931	sp|P29981.1|CP4C1_BLADI RecName: Full = Cytochrome P450 4C1; AltName: Full = CYPIVC1 gb|AAA27819.1| cytochrome P450 [Blaberus discoidalis]	6.00E-70	270	p450
30	FUKV2O402IEQH1	SRX018177	241	ref|XP_002423499.1| cytochrome P450, putative [Pediculus humanus corporis]	4.00E-04	48.1	None detected
31	CYP6ER1	JF928994	2264	emb|CAX33138.2| CYP6CS1 protein [N. lugens]	2.00E-123	447	p450
32	CYP4-like 1	JF928998	443	ref|NP_001071070.1| cytochrome P450, family 4, subfamily V, polypeptide 2 [Danio rerio]	2.00E-43	178	p450
33	CYP6-like 2	JF928999	384	gb|ACF17813.2| DIMBOA-induced cytochrome P450 [Ostrinia furnacalis]	1.00E-49	199	p450

EVAL, E-Value.

Table 4. Fold change in expression of 32 P450 genes in three resistant *Nilaparvata lugens* strains (NL9, NL15 and NL16) compared with the susceptible reference clone NLS as determined by quantitative real-time PCR

Gene/EST ID	NL15	NL15 95% CL	NL16	NL16 95% CL	NL9	NL9 95% CL
CYP6CS1	5.36	2.75	8.19	1.22	2.01	0.94
CYP6CW1	2.84	1.01	2.23	0.70	−1.49	0.29
CYP417A1	−1.25	0.23	8.22	4.60	11.24	6.72
CYP6AX1	2.95	1.16	1.81	1.06	1.16	0.13
CYP418A1	2.48	0.76	2.03	0.24	3.61	0.67
7686	−2.44	0.07	−1.61	0.10	−1.61	0.22
2397	−1.75	0.24	−3.45	0.12	−2.17	0.14
7312	−1.75	0.55	−3.85	0.03	−1.12	0.34
2799	−2.94	0.12	−4.0	0.03	−2.0	0.12
3020	−1.67	0.15	−2.44	0.08	−1.20	0.42
7091	−1.01	0.10	−1.67	0.14	2.07	0.55
5884	−3.03	0.04	−1.67	0.12	−1.18	0.41
2063	−1.64	0.15	−1.92	0.12	−1.15	0.43
CYP4CE1	1.26	0.33	2.60	0.59	−1.18	0.16
CYP6AY1	−3.70	0.29	−10.0	0.07	1.05	0.47
2887	1.71	0.16	1.71	0.55	3.71	0.96
3A25	1.59	0.25	1.57	0.27	3.69	0.96
8867	2.37	0.19	2.03	0.11	2.00	0.08
8451	1.18	0.08	−1.20	0.10	1.90	0.37
CYP4CS2	−1.85	0.27	−10.0	0.03	−1.92	0.26
CYP4CS1	−3.57	0.01	−10.0	0.03	−2.13	0.25
XVW5	−2.70	0.14	−3.57	0.02	−2.22	0.08
6052	−2.27	0.17	−2.86	0.06	1.38	0.28
2701	−1.15	0.16	−1.28	0.23	2.52	0.47
JGCLL	−2.50	0.14	−4.16	0.05	−1.64	0.18
HFAOQ	1.46	0.21	1.04	0.44	2.35	0.44
84703	−1.28	0.55	−1.85	0.18	1.51	0.85
9656	−3.70	0.15	−8.33	0.05	−1.92	0.08
IEQH1	−2.44	0.15	−2.94	0.07	3.39	0.04
cyp4-like 1	−4.55	0.16	−2.63	0.08	1.63	0.49
CYP6ER1	8.17	2.65	7.97	3.24	40.32	2.87
CYP303A1	−50.0	0.00	−50.0	0.00	−11.1	0.06

EST, expressed sequence tag; CL, confidence limit.

A list of all genes is detailed in Table 3 along with gene accession numbers and a description based on the closest BLAST hit in the nonredundant database of GenBank.

Expression of P450 genes in imidacloprid-resistant and -susceptible strains of the brown planthopper

Quantitative real-time (RT)-PCR was used to compare the expression level of each P450 gene for the three resistant BPH strains and the susceptible strain. Results were obtained for all except one of the P450 genes (*CYP6-like 2*) for which a specific product could not be amplified, despite repeated attempts with multiple primer combinations. The results for each strain are shown in Table 4 and Fig. 1. For each of the resistant strains a number of P450 genes were significantly ($P \leq 0.05$) differentially expressed compared with NLS, however, of these, only a limited number were significantly up/downregulated by at least two-fold in all three resistant strains. Three genes were overexpressed, *CYP418A1* (2.0 to 3.6-fold), 8867 (2.0 to 2.4-fold) and *CYP6ER1* (8.0 to 43-fold), and one was underexpressed, *CYP303a1* (−11.5 to −62.5-fold). The relationship between the expression of these genes and the level of phenotypic resistance (LC_{50}) for the four BPH strains was examined by linear regression. Only *CYP6ER1* showed a significant linear relationship ($R^2 = 0.98$, $P = 0.0088$) in the present analysis.

CYP6ER1 cDNA characterization

The ≈400-bp partial sequence of *CYP6ER1* obtained by degenerate PCR was completed by 3′ and 5′ RACE and the mRNA sequence of 2264 bp is shown in Fig. 2. This includes a 5′ untranslated region (UTR) of 265 bp and a 3′ UTR of 481 bp. The cDNA has an open reading frame (ORF) of 1518 bp encoding 506 amino acid residues. The predicted isoelectric point of the protein is 8.5 and the theoretical molecular weight is 57.97 kDa. *CYP6ER1* shares similarity with other microsomal proteins with a strongly hydrophobic N-terminus acting as a transmembrane anchor. As shown in Fig. 2, the encoded protein contains conserved domains characteristic of P450s, such as the oxygen-binding motif (helix I) ([A/G]GX[E/D]T[T/S], position 314), the helix K motif (EXXRXXP, position 368), the heme-binding 'signature' motif (PFXXGXXXCXG, position 441) and a sequence motif

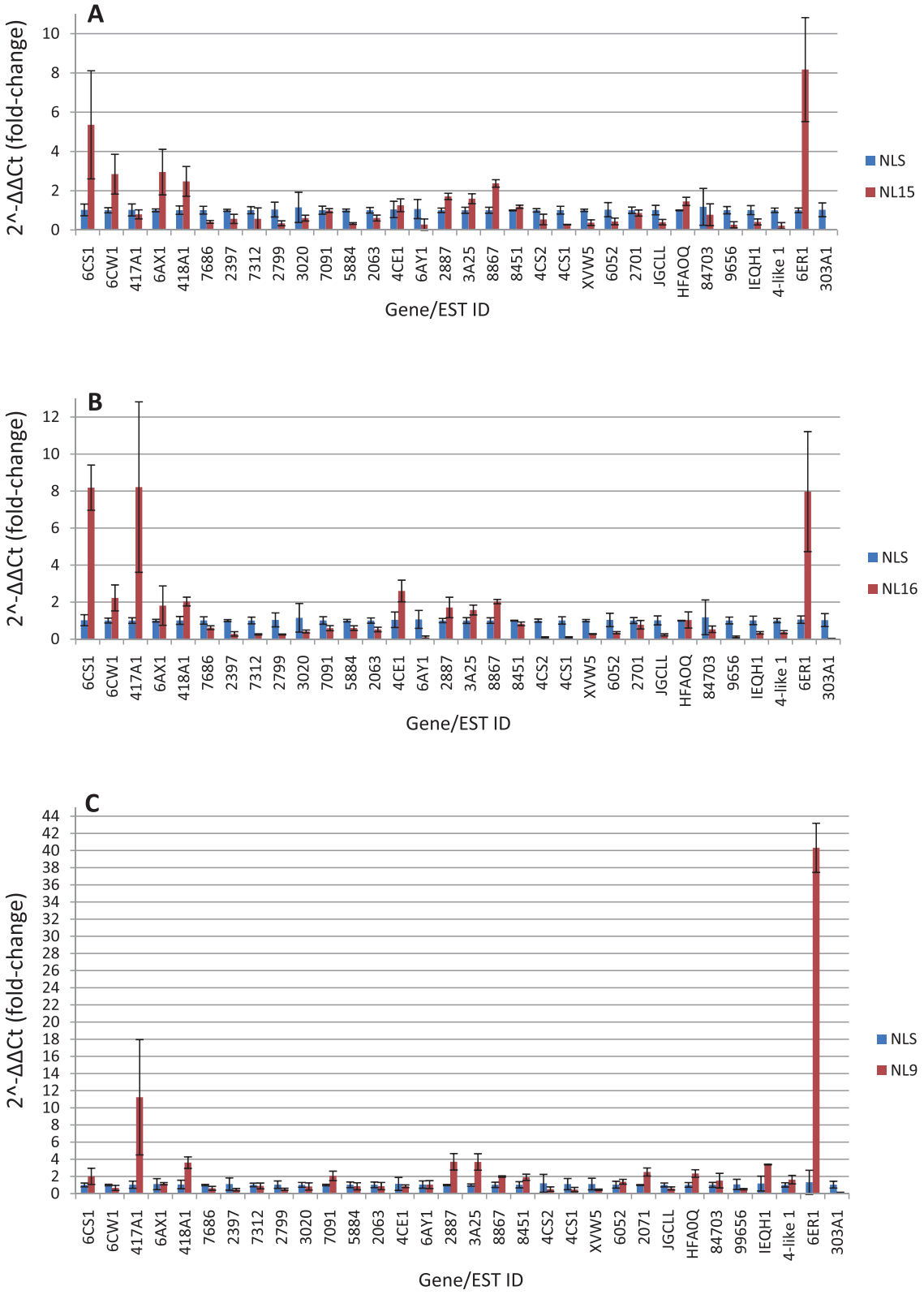

Figure 1. Fold change in expression of 32 P450 genes in three resistant *Nilaparvata lugens* strains compared with the susceptible reference clone NLS as determined by quantitative real-time PCR. (A) NL15 vs. NLS, (B) NL16 vs. NLS and (C) NL9 vs. NLS. Error bars display 95% confidence intervals. EST, expressed sequence tag.

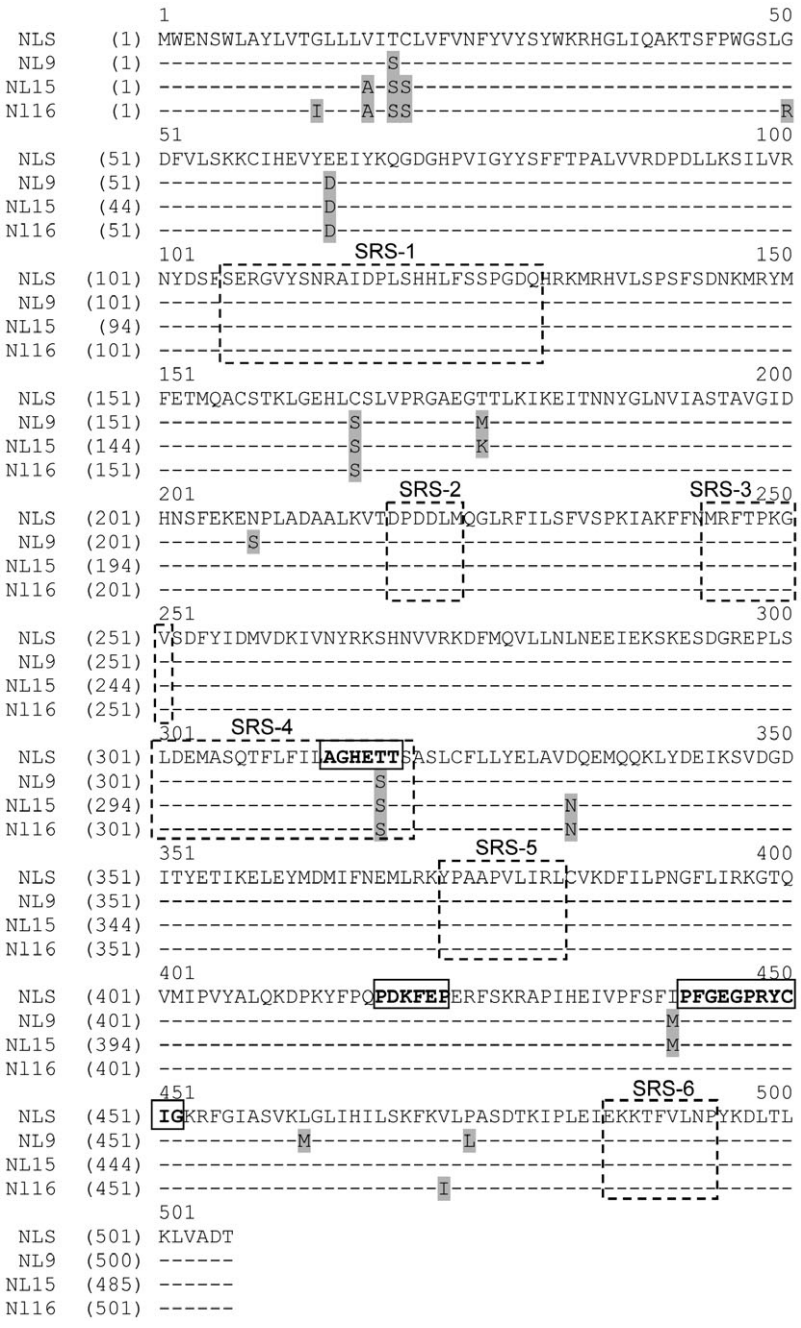

Figure 2. Amino acid alignment of the *CYP6ER1* gene sequence of imidacloprid resistant and susceptible *Nilaparvata lugens* strains. Identical residues are designated by dashes. Amino acid substitutions and deletions are highlighted. Conserved domains common to cytochrome P450 monooxyenases or to CYP6 members such as the oxygen-binding motif (position 314), the heme-binding motif (position 441), the helix K motif (position 386) and a sequence motif (position 417) specific to CYP6 members are boxed. Proposed substrate recognition sites (SRSs) are boxed with broken lines.

(PXXFXP, position 417) specific to CYP6 members. The *CYP6ER1* gene shows greatest sequence similarity (43%) to another BPH P450 gene *CYP6CS1*. Variation in the coding sequence of *CYP6CER1* copies in the BPH strains was examined by both direct nucleotide sequencing and by cloning and sequencing the full cDNA ORF. A total of 78 polymorphic sites were identified between the four strains that result in 15 amino acid substitutions (Fig. 2). Of these, four substitutions are conserved in all three resistant strains compared with the susceptible strain. The 5′UTR (a ≈260-bp region upstream of the start codon) was also amplified by PCR and sequenced for three of the strains (this region could not be amplified for NL15 despite repeated attempts). A number of single nucleotide polymorphisms (SNPs) were observed between the resistant and susceptible strains and, in the case of NL9, several insertions/deletions (indels) were also present (Fig. 3).

Gene copy number

Quantitative RT-PCR was used to compare *CYP6ER1* gene copy number in the NL9 strain (which exhibits the

Figure 3. Alignment of the 5′ untranslated region of the CYP6ER1 mRNA sequence in different *Nilaparvata lugens* strains. The start codon is boxed. Single nucleotide polymorphisms are highlighted. Dashes indicate deletions.

highest level of imidacloprid resistance and *CYP6ER1* expression) and NLS strain using genomic DNA as a template. Data were normalized to *para* which is present in two copies in diploid insect genomes. A slight difference in copy number was observed between NL9 (1.0 ± 0.037) and NLS (1.48 ± 0.22), which probably represents technical variation in the assay rather than a significant biological difference.

Discussion

To date, the resistance of field populations of BPH to neonicotinoids has been exclusively attributed to enhanced detoxification by P450s (Liu *et al*., 2005b; Nauen & Denholm, 2005; Wen *et al*., 2009). In the present study, the involvement of P450s in the imidacloprid resistance of three field-collected BPH strains was initially implicated by biochemical assay using the model substrate 7-ethoxycoumarin (7-EC). Although enhanced P450 activity was observed in all three resistant strains compared with a susceptible reference strain, monooxygenase activity was higher in the moderately resistant BPH strains NL15 and NL16 (~seven-fold higher than the laboratory susceptible strain NLS) than NL9 the most resistant strain (~three-fold higher than NLS). Three possible explanations for this observation are: (1) a greater number of P450s that are capable of deethylating 7-EC are overexpressed in NL15/NL16 than in NL9 but not all of these are involved in conferring resistance to imidacloprid; (2) the overexpressed P450 that confers resistance may be incapable of oxidizing 7-EC as has been described for other insect species (Philippou *et al*., 2011); and (3) qualitative changes (mutations) to the resistance conferring P450, in addition to quantitative changes in expression, have altered the ability of this enzyme to oxidize 7EC. A recent study of four field populations of BPH collected from China also reported that the P450 activity of each strain did not perfectly correlate with the resistance phenotype (Wen *et al*., 2009).

To investigate which P450(s) were overexpressed in these strains, quantitative RT-PCR was used to examine the expression of 32 P450 genes, identified from two EST databases and by degenerate PCR. This analysis provided two lines of evidence to support the involvement of a single P450, encoded by *CYP6ER1*, in resistance to imidacloprid. Firstly this gene was the only P450 that was highly overexpressed in all three resistant BPH strains (up to ~40-fold in the most resistant strain). Secondly a significant linear relationship was observed between the level of expression of this gene and the level of phenotypic resistance (LC_{50}). Although two additional P450 genes were significantly overexpressed (*CYP418A1* and 8867) and one was highly downregulated (*CYP303a1*) in all three resistant strains, their expression was not significantly correlated with the level of phenotypic resistance. Further investigation is required to determine if the P450s these genes encode contribute to resistance in any way.

The full-length sequence of the *CYP6ER1* gene is most similar in sequence to another BPH P450 gene, *CYP6CS1*, and belongs to the CYP6 family. This family has been implicated in insecticide resistance more often than any other and in other insect species, such as *Bemisia tabaci*, *Myzus persicae* and *Drosophila*

melanogaster, the selective upregulation of a single P450 gene belonging to the *CYP6* family (*CYP6CM1, CYP6CY3* and *CYP6G1* respectively) contributes to resistance to imidacloprid (Daborn *et al*., 2002; Karunker *et al*., 2008; Puinean *et al*., 2010b). The enhanced expression of P450 genes in these and other resistant insects has been shown to arise through mutations in *cis*-acting promoter sequences and/or *trans*-acting regulatory loci, or more recently, via gene amplification (Li *et al*., 2007; Puinean *et al*., 2010b). Quantitative RT-PCR analysis of *CYP6ER1* gene copy number provided little evidence of a role for gene amplification in the increased transcription of *CYP6ER1*; however, in the immediate 5′ putative promoter region of the resistant strains (–260 bp of the start codon) a number of SNPs occurred between the resistant and susceptible strains and, in the case of NL9, several indels were also present (Fig. 3). In addition, when the coding sequence of the *CYP6ER1* gene was compared between strains several polymorphic sites were observed that result in four conserved amino acid substitutions between all three resistant strains and the susceptible strain (see Fig. 2). Further investigation is needed to determine the functional significance of these genetic changes and it would be interesting, in future, to examine their effect on the substrate specificity or efficiency of this P450 and/or their effect on gene expression attributable to the potential loss or alteration of regulatory loci (especially in the case of NL9). Previous studies have shown that high levels of imidacloprid resistance in field populations of BPH are unstable and that this is attributable to a loss of fitness associated with resistance (Liu & Han, 2006). The studies proposed above may also shed light on possible molecular mechanisms underlying this fitness cost. Based on the results of this study, *CYP6ER1*, represents a strong candidate for the development of molecular diagnostics that can be used to screen field populations of BPH for this mechanism and inform resistance management strategies. In this regard, identification of the genetic changes that underlie enhanced P450 expression may also facilitate the development of DNA-based methods that can be used to screen insect material preserved without the need for a cold chain (Bass *et al*., 2010).

Although the results of this study provide strong evidence of a role for *CYP6ER1* in imidacloprid resistance, functional characterization of this P450 to confirm its ability to detoxify imidacloprid is now required. Previous studies have shown that the metabolic enzyme inhibitor PBO synergizes the effect of neonicotinoids on resistant field populations of BPH (Wen *et al*., 2009). It will also be interesting therefore to examine the effect of this and other inhibitors on recombinant *CYP6ER1* as a means of identifying mechanisms to overcome resistance mediated via this mechanism.

Experimental procedures

Insect strains

The three imidacloprid-resistant BPH field strains analysed in this study were collected by Bayer CropScience (Monheim, Germany) from Thailand (strain designated NL9), Guangxi Province, China (NL15) and Jiangsu Province, China (NL16) in August–September 2009. The susceptible reference strain (NLS) was provided by Bayer CropScience and had been reared on untreated rice plants as a laboratory colony for over five years. Insects were reared as age-structured colonies on rice under controlled environment conditions (26°C/16 h photoperiod).

Resistance bioassays

Adult female *N. lugens*, <10 days old, were used for topical application bioassays. These consisted of three replicates per dose, each with 10–20 individuals. Insects for testing were removed from rearing cages, lightly anaesthetized using carbon dioxide and dosed with 0.25 μl of technical imidacloprid dissolved in acetone [analytical research (AR) grade] on the pronotum using a microapplicator (Burkard Manufacturing Ltd, Rickmansworth, UK). Control insects were dosed with 0.25 μl acetone only. Treated individuals were transferred to ventilated plastic tubes containing untreated, rooted rice stems and maintained under controlled environmental conditions (26°C/16 h photoperiod). Mortality was assessed 48 h after treatment. Bioassay data were analysed using Genstat® 13th Edition software (VSN International) to generate statistically validated (conforming to 95% confidence limits) estimates of LC_{50} values. Resistance ratios were calculated by dividing the LC_{50} values of field strains by those of the susceptible reference strain. Bioassay data is presented in parts per million (ppm) and to three significant figures unless otherwise stated.

Biochemical measurement of cytochrome P450 monooxygenase activity

Ten adult BPH were homogenized on ice in 1 ml 0.1 M sodium phosphate buffer, pH 7.6, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM 1-phenyl 2-thiourea (PTU), 1 mM phenylmethylsulfonyl fluoride (PMSF) in 1.46 M sucrose and centrifuged at 20 000 × *g* for 5 min. The supernatant was taken and centrifuged at 100 000 × *g* at 4°C for 1 h. The resulting microsomal fraction was resuspended in 250 ul of the homogenization buffer lacking sucrose. This was then used immediately as the enzyme source.

O-deethylation of 7-EC was measured according to Ullrich and Weber and adapted to the microplate format as described by DeSousa (Ullrich & Weber, 1972; De Sousa *et al*., 1995). Briefly, 7-EC was dissolved in ethanol (100%, AR) to make a 20 mM stock solution and diluted by the addition of 0.1 M sodium phosphate buffer, pH 7.8, to give a concentration of 0.5 mM. Enzyme (50 μl, containing approx. 200 μg protein) was added to separate wells of a microplate (Optiplate™, Perkin Elmer, Boston, MA, USA) followed by the addition of 80 μl 0.5 mM 7-EC. The microplate was incubated for 5 min at 30°C and the reaction initiated by the addition of 10 μl 9.6 mM nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) in 0.1 M sodium phosphate, pH 7.8. Enzyme activity was read in a Molecular Devices Spectramax Gemini XPS fluorescence microplate reader (Molecular Devices

Corp., Menlo Park, CA, USA) for 30 min with readings taken every 30 s, using an excitation wavelength of 370 nm and an emission wavelength of 460 nm, with a 435 nm cut-off. The rate, fluorometric units over time (FU/min), was calculated by fitting a linear regression through the readings following an initial 'lag phase' (GRAFIT 3.0, Erithacus Software). The specific activity of 7-EC-*O*-deethylation was determined using a 7-hydroxycoumarin standard curve. Protein was determined using bovine serum albumin as a standard according to Bradford's method (Bradford, 1976). All assays were performed in triplicate.

Sample storage RNA extraction and cDNA synthesis

Adult BPH, <10 days old, were collected, flash frozen in liquid nitrogen and stored at –80°C before extraction of RNA. Total RNA was extracted from three pools of 10 adult BPH using Trizol following the manufacturer's instructions. Genomic DNA was removed by DNaseI digestion using DNA-free DNase Treatment and Removal Reagent (Ambion, Austin, TX, USA). The quality and quantity of the RNAs were assessed by spectrophotometery (Nanodrop Technologies, Wilmington, DE, USA) and by running an aliquot on a 1.5% agarose gel. For the latter RNA was mixed with 1x loading buffer (95% formamide; 0.025% xylene cyanol; 0.025% bromophenol blue; 18 mM EDTA; 0.025% sodium dodecyl sulphate), heated for 5 min at 65°C and briefly chilled on ice before loading. Four micrograms of RNA was used for cDNA synthesis using Superscript III and random hexamers (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Identification of tentative unique P450 genes by degenerate PCR

Degenerate PCR used primers designed by Karunker *et al*. which are based on conserved amino acid sequences specific to the CYP4 and CYP6 families of P450 genes (Karunker *et al*., 2008). PCR reactions (20 μl) contained 4 μl of cDNA (10 ng), 10 μl of Dream Taq Master Mix 2× (Fermentas, Cambridge, Cambridgeshire, UK), and 0.5 μM of each primer. Thermocycling conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 46°C for 45 s, 72°C for 1 min) and a final extension at 72°C for 5 min. PCR products of the expected size (~440 and ~390 bp for the CYP4 and CYP6 primer pair, respectively) were recovered from agarose gels, cloned using the Strataclone PCR Cloning kit (Stratagene, La Jolla, CA, USA) and sequenced using the ABI BigDye Terminator Cycle Sequencing kit and T3/T7 primers. Degenerate PCR was carried out on cDNA extracted from both resistant and susceptible BPH strains.

Quantitative real-time PCR

For quantitative RT-PCR, primers were designed to amplify a fragment of 90–150 bp in size for each P450 gene. All primers had theoretical melting temperatures of ≈60°C, were designed using Primer3 (Untergasser *et al*., 2007), and are listed in Supporting Table S1. PCR reactions (20 μl) contained 4 μl of cDNA (10 ng), 10 μl of SensiMix SYBR Kit (Bioline, London, UK), and 0.25 μM of each primer. Samples were run on a Rotor-Gene 6000 (Corbett Research, ST Neots, Cambridgeshire, UK) using the temperature cycling conditions of: 10 min at 95°C followed by 40 cycles of 95°C for 15 s, 57°C for 15 s and 72°C for 20 s. A final melt-curve step was included post-PCR (ramping from 72°C–95°C by 1°C every 5 s) to check for nonspecific amplification. The efficiency of PCR for each primer pair was assessed using a serial dilution of 100 ng to 0.01 ng of cDNA. Each quantitative RT-PCR experiment consisted of three independent biological replicates with two technical replicates for each. Technical replication was limited to two replicates as (1) PCR reactions were set up using a liquid handling robot (CAS 1200, Corbett Research) which provided exquisite technical reproducibility and (2) to allow us to employ a sample maximization strategy (i.e. running as many samples as possible in the same run in order to minimize technical run-to-run variation). Data were analysed according to the $\Delta\Delta C_T$ method [36]. For normalization, three reference genes were validated experimentally for each strain, elongation factor 1-α, glyceraldehyde-3-phosphate dehydrogenase and *para* which encodes the voltage-gated sodium channel. The two most stably expressed reference genes (lowest *M*-values) were determined using the GENORM Software [37]. The geometric mean of the selected genes was then used for normalization according to the strategy described previously [37]. Two genes were used for normalization as (1) they had acceptable *M*-values (Hellemans *et al*., 2007), ranging from 0.253 [coefficient of variation (CV) 0.1] to 0.974 (CV 0.43) for the different strains and (2) the cost of using further genes for normalization was prohibitively expensive as each run would consist of mainly samples for reference genes and this, in turn, would prevent the sample maximization strategy we employed.

Linear regression and one way analysis of variance was used to examine the relationship between the expression of each P450 gene with the level of phenotypic resistance (LC_{50}) for all BPH strains using the data analysis add-in of Excel 2007. Statistical significance of *F*-values was assumed at $P < 0.05$.

Rapid amplification of cDNA ends and amplification of full-length cDNA from CYP6ER1

Five and three prime RACE was carried out using the RLM-RACE kit (Ambion) following the manufacturer's instructions. The gene-specific primers for this purpose are detailed in Supporting Table S1. PCR products were recovered from agarose gels, cloned using the Strataclone PCR Cloning kit (Stratagene) and sequenced using the ABI BigDye Terminator Cycle Sequencing kit and T3/T7 primers. To verify the assembly the full-length coding sequence of *CYP6ER1* was amplified by nested PCR using primers detailed in Supporting Table S1). PCR reactions (20 μl) contained 4 μl of cDNA (10 ng), 1.5 units of Pfu DNA polymerase (Promega, Madison, WI, USA) and 0.5 μM of each primer and were subjected to cycling conditions of: 2 min at 94°C followed by 35 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 4 min. PCR products were purified and cloned and sequenced as above or sequenced directly using primers as detailed in Supporting Table S1.

Sequence analysis

Molecular mass and isoelectric point were predicted by Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html). The N-terminal transmembrane anchor of deduced proteins was predicted by the TMHMM Server v. 1.0 (http://www.cbs.dtu.dk/services/TMHMM/). DNA and predicted protein sequences were

assembled, analysed and aligned using the Vector NTI Advance 10 package (Invitrogen). The full P450 gene sequence identified in the present study was named by Dr David Nelson (Department of Molecular Science, University of Tennessee, Memphis) in accordance with the P450 nomenclature committee convention (Nelson, 2009).

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2583.2011.01105.x

Table S1. Table of the primers used in this study. Primer sequences are listed along with the purpose for which they were used.

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