Insect Molecular Biology

Insect Molecular Biology (2011) **20**(6), 733–744 doi: 10.1111/j.1365-2583.2011.01103.x

Characterization of five *CYP4* genes from Asian citrus psyllid and their expression levels in *Candidatus* Liberibacter asiaticus-infected and uninfected psyllids

S. Tiwari*, A. D. Gondhalekar†, R. S. Mann*, M. E. Scharf‡ and L. L. Stelinski*

**Department of Entomology and Nematology, Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA;* †*Department of Entomology and Nematology, University of Florida, Gainesville, FL, USA; and* ‡*Department of Entomology, Purdue University, West Lafayette, IN, USA*

Abstract

Previously, we reported that *Candidatus* Liberibacter asiaticus (Las)-infected *Diaphorina citri* are characterized by lower levels of cytochrome P450 monooxygenases than uninfected counterparts. In the present study, we investigated expression levels of family 4 cytochrome P450 (*CYP4*) genes in Las-infected and uninfected *D. citri* adults. Five novel *CYP4* genes (*CYP4C67*, *CYP4DA1*, *CYP4C68*, *CYP4DB1* and *CYP4G70*) were identified. Four of the five *CYP4* genes were expressed at significantly higher levels in uninfected than Las-infected males, whereas only one was expressed at significantly higher levels in uninfected than Las-infected females. These results suggest that levels of cytochrome P450 monooxygenases in *D. citri* may be linked to expression levels of these *CYP4* genes. Expression of all five *CYP4* genes was induced by exposure of *D. citri* to imidacloprid, suggesting their possible involvement in metabolism of this toxin. Higher expression of the five *CYP4* genes was found in nymphs than adults, which is congruent with previous results indicating higher levels of cytochrome P450 monooxygenases in nymphs than adults. These five *CYP4* genes may be promising candidates for RNA-interference to silence overexpression of genes associated with insecticide resistance in *D. citri*. These newly identified genes may also serve as DNA-based screening markers for cytochrome P450-mediated insecticide resistance in field populations of *D. citri*.

Keywords: cytochrome P450 monooxygenases, *Diaphorina citri*, greening, huanglongbing, imidacloprid induction, Las infection.

First published online 15 September 2011.

Correspondence: Lukasz L. Stelinski, Entomology and Nematology Department, Citrus Research & Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA. Tel.: +1 863 956 8851; fax: +1 863 956 4631; e-mail: stelinski@ufl.edu

Introduction

Cytochrome P450 is an important class of enzymes that occurs in all cellular living organisms (Feyereisen, 1999, 2005). This large class of enzymes has been divided into various *CYP* families, including 4, 6, 9, 12, 15, 18 and 28, based on the degree of amino acid sequence identity (Scott, 1999). Cytochrome P450s show >40% and >55% amino acid sequence identity within a family and subfamily, respectively (Nebert *et al.*, 1991). In insects, this class of enzymes has been thoroughly investigated, and is represented by around 48–164 genes (Tijet *et al.*, 2001; Ranson *et al.*, 2002; Feyereisen, 2005). Cytochrome P450 is associated with insecticide resistance and metabolism of a wide range of endogenous and exogenous compounds that includes hormones, pheromones, insecticides and plant secondary compounds in insects (Hodgson, 1985; Feyereisen, 1999; Scott, 1999). Overtranscription of families 4, 6, 9 and 12 has been frequently linked to insecticide metabolism and resistance (Feyereisen, 2006; Li *et al.*, 2007).

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is one of the most serious pests of citrus because it vectors the putative causal agent of huanglongbing (HLB), *Candidatus* Liberibacter asiaticus (Las) (Halbert & Manjunath, 2004). HLB is one of the most economically important diseases of citrus which is present in most citrus growing regions of the world (Halbert & Manjunath, 2004; Manjunath *et al.*, 2008). Currently, the most common practice for managing HLB is aggressive use of insecticides to control the vector (Sétamou *et al.*,

2010; Tiwari *et al*., 2011a); however, the number of available modes of action is limited and in some cases repeated sequential use of the same insecticide or mode of action has led to the development of varying levels of insecticide resistance in populations of *D. citri* in Florida, USA. (Tiwari *et al*., 2011a). Increased activity of detoxifying enzymes in field populations of *D. citri* may partially explain the emerging cases of insecticide resistance observed to date (Tiwari *et al*., 2011a).

Several investigations have confirmed that the presence of a pathogen or parasite influences insecticide susceptibility and associated detoxifying enzyme levels (McCarroll *et al*., 2000; Duron *et al*., 2006; Kontsedalov *et al*., 2008; Ghanim & Kontsedalov, 2009; Tiwari *et al*., 2011b, c). For example, RNA levels of *Wuchereria bancrofti* are higher in insecticide susceptible *Culex quinquefasciatus* than in resistant counterparts. Also, susceptible *C. quinquefasciatus* were characterized by lower esterase levels than resistant ones (McCarroll *et al*., 2000). Infection by *Rickettsia* increases the susceptibility of *Bemisia tabaci* to acetamiprid, thiamethoxam, spiromesifen and pyriproxyfen (Kontsedalov *et al*., 2008). Similarly, infection of a strain of *B. tabaci* with combinations of *Wolbachia–Arsenophonus* and *Rickettsia–Arsenophonus*, increased susceptibility to thiamethoxam, imidacloprid, pyriproxyfen and spiromesifen compared with a strain infected with only *Arsenophonus* (Ghanim & Kontsedalov, 2009). Also, *Wolbachia* infection in insecticide-resistant populations of *Culex pipiens* L. results in a physiological cost to the host in terms of reduced fecundity and preimaginal survival when compared with susceptible counterparts, thereby increasing the cost of insecticide resistance in infected and resistant populations (Duron *et al*., 2006). In *D. citri*, infection of adults with the Las bacterium increases insecticide susceptibility and decreases levels of three types of detoxifying enzymes when compared with uninfected counterparts (Tiwari *et al*., 2011b, c). Although there is mounting evidence that pathogen infection can reduce host insect capacity to metabolize toxins, much less is known about the specific mechanisms underlying this phenomenon.

In the current investigation, we used degenerate primers to amplify and sequence expressed *CYP4* genes in uninfected and Las-infected *D. citri*. Genes in the *CYP4* family are known for a high degree of conserved sequence in both the i-helix and heme-binding regions, which has facilitated the use of degenerate *CYP4* primers to amplify gene fragments from cDNA templates (Snyder *et al*., 1995). This approach has been successfully applied to identify *CYP4* genes in a wide range of insects, including *Anopheles albimanus* (Scott *et al*., 1994), *Manduca sexta* (Snyder *et al*., 1995), *Drosophila melanogaster* (Dunkov *et al*., 1996), *Helicoverpa armigera* (Pittendrigh *et al*., 1997), *Diabrotica vergifera vergifera* (Scharf *et al*., 2001), *Anopheles gambiae* (Ranson *et al*., 2002) and *Reticulitermes flavipes* (Zhou *et al*., 2006). Our objectives in the present study were to identify *CYP4* genes and to compare their relative expression in uninfected and Las-infected male and female *D. citri*. In addition, we compared the relative expression of identified genes between uninfected immature and adult *D. citri* and induced expression of identified *CYP4* genes in *D. citri* adults by exposing them to varying concentrations of imidacloprid.

Results

Choice of quantitative real-time PCR reference genes

An initial test was conducted on common housekeeping genes (*alpha-tubulin, actin, NADH-dh* and *wg*), as references for quantitative real-time PCR (qPCR), and five *CYP4* genes for gene expression in all four types of *D. citri* examined: uninfected or Las-infected males and females. Amplified PCR products of five *CYP4* genes and four reference genes using male uninfected insects are shown in Fig. 1. Primer-dimer and other non-specific amplicons were not found in any of the samples. *Actin* and *NADH-dh*

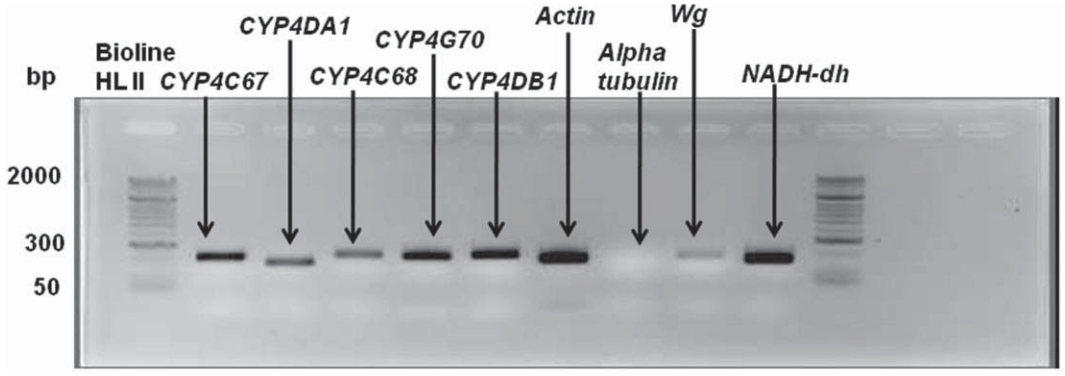

Figure 1. Representative agarose gel is shown depicting expression of five *CYP4* and four reference genes after PCR amplification using cDNA from male uninfected *Diaphorina citri*. PCR products were obtained after 35 cycles of PCR amplification, using amplification cycles described under experimental procedures.

Table 1. Sequence summary for *Diaphorina citri CYP4* genes, identified from uninfected and Las-infected male and female *D. citri*

Gene name	No. clones	Treatment				Closest homology (identity scores, *e* values)
		UM	IM	UF	IF	
CYP4C67	37	10	10	9	8	*Diploptera punctata CYP4C1* (98%, 3*e*-63)
CYP4DA1	2	0	0	1	1	*Anopheles gambiae CYP4C27* (54%, 3*e*-45)
CYP4C68	4	0	0	3	1	*Anopheles funestus CYP4C41* (94%, 4*e*-45)
CYP4G70	1	0	0	1	0	*Carcinus maenas* (crab) *CYP4C39* (99%, 4*e*-39)
CYP4DB1	1	0	0	1	0	*Nasonia vitripennis CYP4G43* (99%, 2*e*-68)
Totals	**45**	**10**	**10**	**15**	**10**	

UM, uninfected male; IM, Las-infected male; UF, uninfected female; IF, Las-infected female.

were the only genes that were uniformly expressed. Dissociation curves generated using additional quantitative real-time PCR (qPCR) assays suggested that single amplicons were present for both *Actin* and *NADH-dh*. Expression of *Actin* and *NADH-dh* was further confirmed by conducting PCR on four types of *D. citri* samples (uninfected or Las-infected males and females) and visualizing PCR products on 2% agarose gels. Stronger and uniform expression of *Actin* across all four samples along with lower SD when compared with *NADH-dh* suggested that *Actin* was more stable than *NADH-dh*. *Actin* was therefore chosen as a standard reference gene for all subsequent qPCR assays.

Nucleotide sequences

A previous study conducted by Tiwari *et al*. (2011b) reported that levels of cytochrome P450 monooxygenases varied among uninfected male, uninfected female, Las-infected male and Las-infected female *D. citri*. The objective of the present study was to isolate expressed *CYP4* genes from four types of *D. citri*: uninfected male, uninfected female, Las-infected male and Las-infected female. In all, 96 positive clones based on the blue-white screening method ($n = 24$ positive clones from each type of *D. citri*, $n = 96$ total clones) were submitted for high-throughput DNA sequencing. Of these, 77 clones yielded a high quality sequence from which 45 were determined to represent *CYP4* sequences and the remaining 32 were considered putative. A BLASTx search of GenBank using five *CYP4* nucleotide sequences (excluding priming regions) revealed varying levels of homology with *CYP4* fragments from other organisms (Table 1). All of the sequences were significantly (identity scores: 54–99%; *e*-values $>1 \times 10^{-5}$) homologous to *CYP4* genes from either *Diploptera punctata* (*CYP4C1*), *Anopheles funestus* (*CYP4C41*), *An. gambiae* (*CYP4C27*), *Carcinus maenas* (green crab) (*CYP4C39*), or *Nasonia vitripennis* (*CYP4G43*). The 45 *CYP4* sequences were aligned into five distinct contigs that represent five unique *CYP4* genes (Table 1). Of the 45 *CYP4* sequences, 10 were obtained from male Las-infected, 10 from male uninfected, 15 from female Las-infected and 10 from female uninfected adults. Although cloning studies revealed that *CYP4C67* was the only gene expressed in males, irrespective of Las infection status (Table 1), subsequent PCR and qPCR assays (Figs 1, 5) on uninfected and Las-infected males revealed that all five *CYP4* genes exist in males. Alternatively, cloning of only one *CYP4* gene (*CYP4C67*) in males may suggest a lower proportion of high quality sequences obtained from those two treatments. The five *CYP4* contigs ranged from 437 to 458 nucleotides (Fig. 2) and encoded peptides from 145 to 152 amino acids (Fig. 3) in size. Amino acid identity and phylogenetic analysis showed that the five identified *CYP4* genes from *D. citri* share a similar lineage with *CYP4* genes from other insects (Table 1 and Fig. 4).

CYP4 expression in Las-infected and uninfected Diaphorina citri

Relative expression levels for the five *CYP4* genes were compared between uninfected and Las-infected adults within each sex (Fig. 5). Expression of *CYP4DA1* (One-way ANOVA results: $F = 10.73$; d.f. = 1, 10; $P = 0.0084$), *CYP4C68* ($F = 12.34$; d.f. = 1, 10; $P = 0.0056$), *CYP4G70* ($F = 5.19$; d.f. = 1, 10; $P = 0.0459$) and *CYP4DB1* ($F = 6.25$; d.f. = 1, 10; $P = 0.0314$) was significantly greater in uninfected than Las-infected males, while expression of *CYP4C67* ($F = 1.36$; d.f. = 1, 10; $P = 0.2704$) did not differ between uninfected and Las-infected males. Expression of *CYP4DA1* ($F = 46.10$; d.f. = 1, 10; $P < 0.0001$) was significantly greater in uninfected than Las-infected females, while expression of *CYP4C67* ($F = 0.26$; d.f. = 1, 10; $P = 0.6232$), *CYP4C68* ($F = 3.28$; d.f. = 1, 10; $P = 0.1001$), *CYP4G70* ($F = 31.06$; d.f. = 1, 10; $P = 0.0002$) and *CYP4DB1* ($F = 2.70$; d.f. = 1, 10; $P = 0.1317$) was not affected by Las infection in females.

Imidacloprid induction bioassay

The objective of this experiment was to determine whether exposure of *D. citri* to imidacloprid, a neonicotinoid insecticide to which field populations of *D. citri* exhibit varying

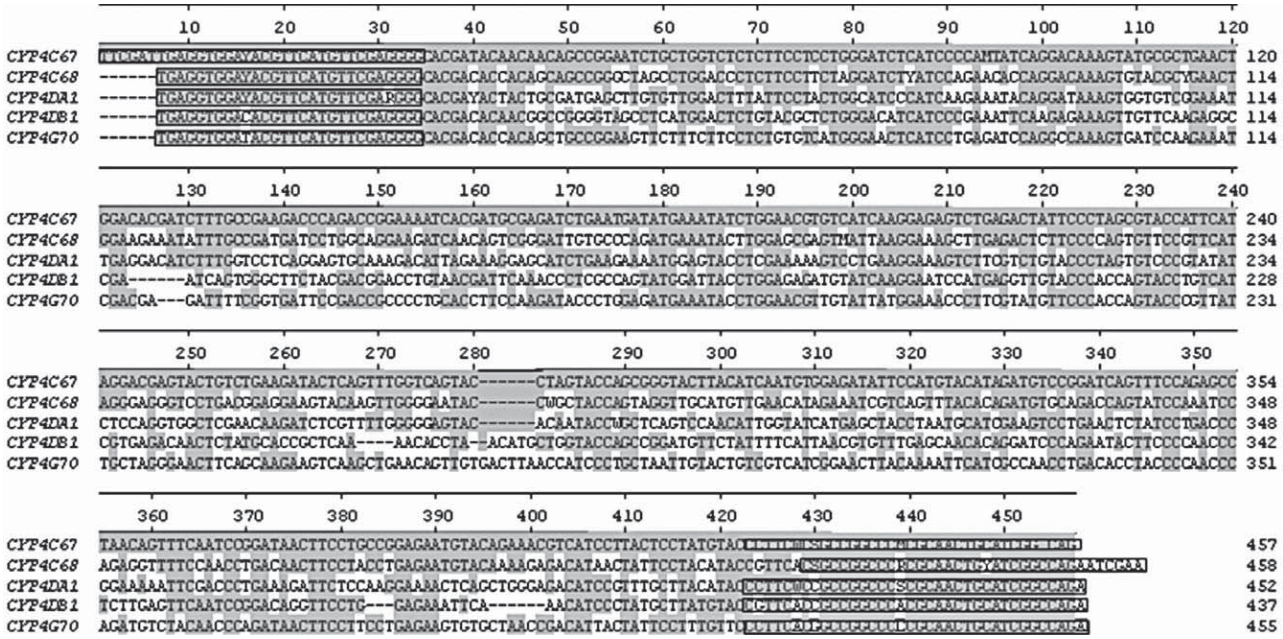

Figure 2. Clustal-V multiple alignment of five *Diaphorina citri CYP4* nucleotide fragments. Sequence positions at the right correspond to nucleotides. Invariant residues are denoted by black highlighting in the majority of the sequence. Forward and reverse primers are denoted by sequences in the boxes.

levels of resistance (Tiwari *et al*., 2011a), induces expression of the five aforementioned *CYP4* genes (Fig. 6). There were varying levels of significant induction in all five *CYP4* genes [(*CYP4C67*: F = 8.59; d.f. = 5, 30; $P < 0.0001$) (*CYP4DA1*: F = 24.39; d.f. = 5, 30; $P < 0.0001$) (*CYP4C68*: F = 22.91; d.f. = 5, 30; $P < 0.0001$) (*CYP4DB1*: F = 4.10; d.f. = 5, 30; $P = 0.0061$) and (*CYP4G70*: F = 11.76; d.f. = 5, 30; $P < 0.0001$)]. Expression of *CYP4C67* was significantly upregulated in adults treated with 0.2, 0.4, 0.6 and 0.8 ppm of imidacloprid when compared with controls; however, exposure of adults to 1.0 ppm of imidacloprid did not affect the expression of *CYP4C67*. Expression of *CYP4DA1* and *CYP4C68* was significantly greater in adults exposed to 0.4, 0.6, 0.8 or 1.0 ppm of imidacloprid than those exposed to 0.2 ppm or the control. Expression of *CYP4G70* was significantly greater in adults exposed to 0.4, 0.6, 0.8 or 1.0 ppm of imidacloprid than in the control. Expression of *CYP4DB1* was significantly higher in adults exposed to 0.6, 0.8 and 1.0 ppm of imidacloprid than in those exposed to 0.2 or 0.4 ppm or the control.

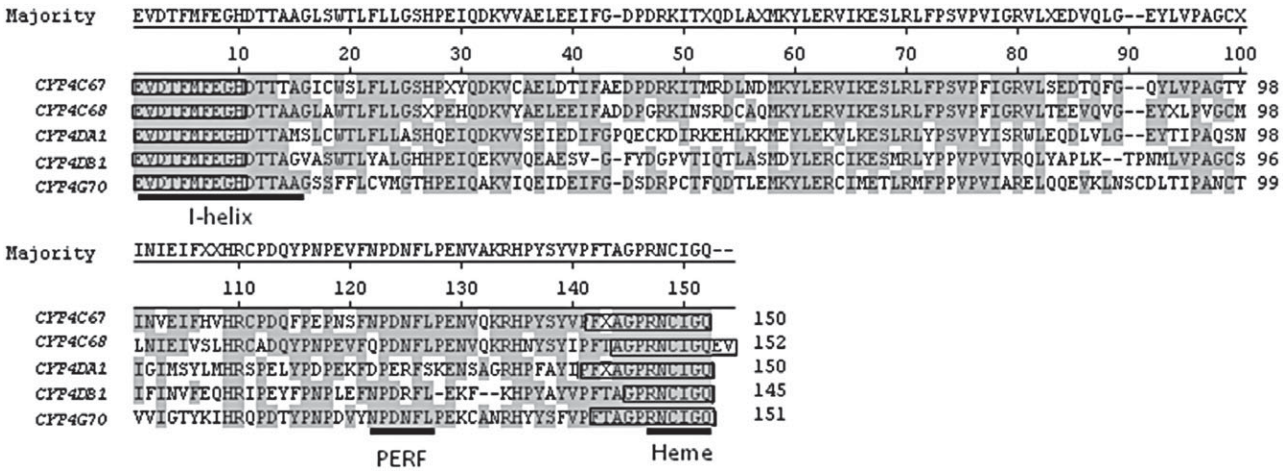

Figure 3. Clustal-V multiple alignment of five translated *Diaphorina citri CYP4* nucleotide fragments. Sequence positions at the right correspond to nucleotides. Invariant residues are denoted by black highlighting in the majority of the sequence. Forward and reverse primers are denoted by sequences in the boxes. Bold underlining denotes the highly conserved i-Helix, PERF and heme regions.

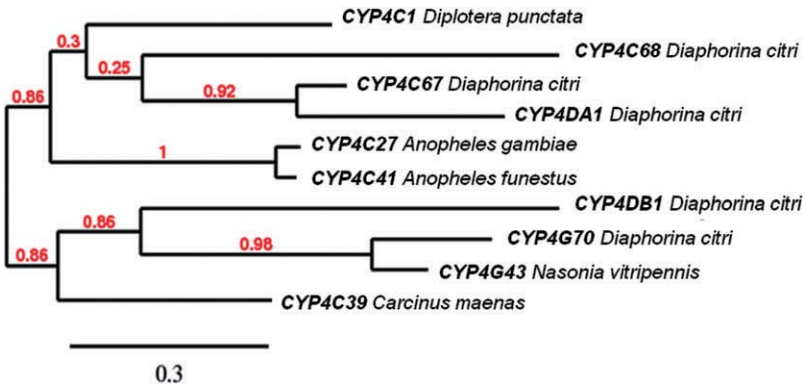

Figure 4. Phylogenetic tree depicting relationship of five *CYP4* genes fragments from *Diaphorina citri* and other *CYP4* fragments of similar lineages (see Table 1 for details and sequence accession numbers). The tree was generated using the PhyML software based on the maximum likelihood method. Bootstrap analysis values are shown above the branches. The scale bar indicates the number of amino acid residue changes per unit length of the horizontal branches.

Effect of developmental stage on CYP4 expression

Relative expression of all five *CYP4* genes was higher in nymphs than in adults [(*CYP4C67*: F = 3.63; d.f. = 2, 21; *P* = 0.0443) (*CYP4DA1*: F = 4.45; d.f. = 2, 21; *P* = 0.0245) (*CYP4C68*: F = 3.74; d.f. = 2, 21; *P* = 0.0409) (*CYP4DB1*: F = 16.12; d.f. = 2, 21; *P* < 0.0001) and (*CYP4G70*: F = 7.86; d.f. = 2, 21; *P* = 0.0028)] (Fig. 7). Expression of *CYP4C67* was significantly higher in 2nd than 4th instars or adults. Expression of *CYP4DA1* and *CYP4C68* was significantly higher in 4th instars than adults, but was not different from that in 2nd instars. Expression of *CYP4G70* was greatest in 2nd instars, followed by 4th instars and adults. Expression of *CYP4DB1* was significantly higher in 2nd and 4th instars than in adults.

Discussion

CYP4 expression in Las-infected and uninfected Diaphorina citri

Infection of *D. citri* with *Candidatus* Liberibacter asiaticus increases susceptibility of *D. citri* to insecticides used for its management (Tiwari *et al*., 2011b). Increased susceptibility appears to be related to reduction in general esterase, glutathione S-transferase and cytochrome P450 monooxygenase levels as a result of Las infection (Tiwari *et al*., 2011b, c). Higher levels of these detoxifying enzymes in insecticide resistant populations are related to elevation in associated gene expressions (Scharf *et al*., 1999, 2001; Ranson *et al*., 2001; Daborn *et al*., 2002, 2007; Gui *et al*., 2009; Wondji *et al*., 2009; Matambo *et al*., 2010). Strains of the German cockroach, *Blattella germanica*, expressing higher tolerance to an organophosphate insecticide are characterized by higher expression of P450 MA protein, an overexpressed form of cytochrome P450 monooxygenases (Scharf *et al*., 1999). Similarly, a population of western corn rootworm, *Diabrotica virgifera virgifera*, exhibiting resistance to methyl parathion and carbaryl, expresses higher mRNA levels of three *CYP4* genes than counterparts from a susceptible population (Scharf *et al*., 2001). Likewise, dichlorodiphenyltrichloroethane (DD)-resistant *An. gambiae* express higher mRNA levels of *aggst3-2*, a class III glutathione S-transferase gene, than susceptible counterparts (Ranson *et al*., 2001). In *D. melanogaster*, overexpression of eight cytochrome P450 genes is correlated with resistance to DDT, nitenpyram, dicylanil and diazinon (Daborn *et al*., 2002, 2007). In *Bombyx mori*, *BmGSTS2* (a glutathione S-transferase gene) is overexpressed in the larval midgut after 6–12 h of exposure to permethrin and glyphosate (Gui *et al*., 2009). Likewise, the cytochrome P450 genes, *CYP6P9* and *CYP6P4*, are overexpressed 25 and 51 times, respectively, in pyrethroid resistant *An. funestus* (Wondji *et al*., 2009). Also, increased activity of cytochrome P450 monooxygenase in pyrethroid resistant *An. funestus* is related to overexpression of *CYP6P9* (Matambo *et al*., 2010). The results of the present study indicate that Las infection reduced expression of certain *CYP4* genes, which may be associated with previously documented reduced levels of cytochrome P450 monooxygenases, also caused by Las infection (Tiwari *et al*., 2011c).

This is the first investigation to identify cytochrome P450 genes and relative gene expression levels in uninfected and Las-infected *D. citri* adults. Other *CYP* genes, in addition to those from family 4 identified here, may also be involved in regulating levels of cytochrome P450 monooxygenases for detoxification processes in *D. citri* and thus warrant further investigation. Also functional studies of the proteins encoded by these genes are needed to understand the relationship between Las infection and *CYP* gene expression.

Of the five *CYP4* genes identified, expression of only one (*CYP4DA1*) was statistically lower in Las-infected than uninfected females, whereas levels of four genes (*CYP4DA1*, *CYP4C68*, *CYP4G70* and *CYP4DB1*) were reduced in males in association with Las infection. Expression levels of most of the identified genes were comparable between uninfected males and females. Although levels of the five *CYP4* genes were not

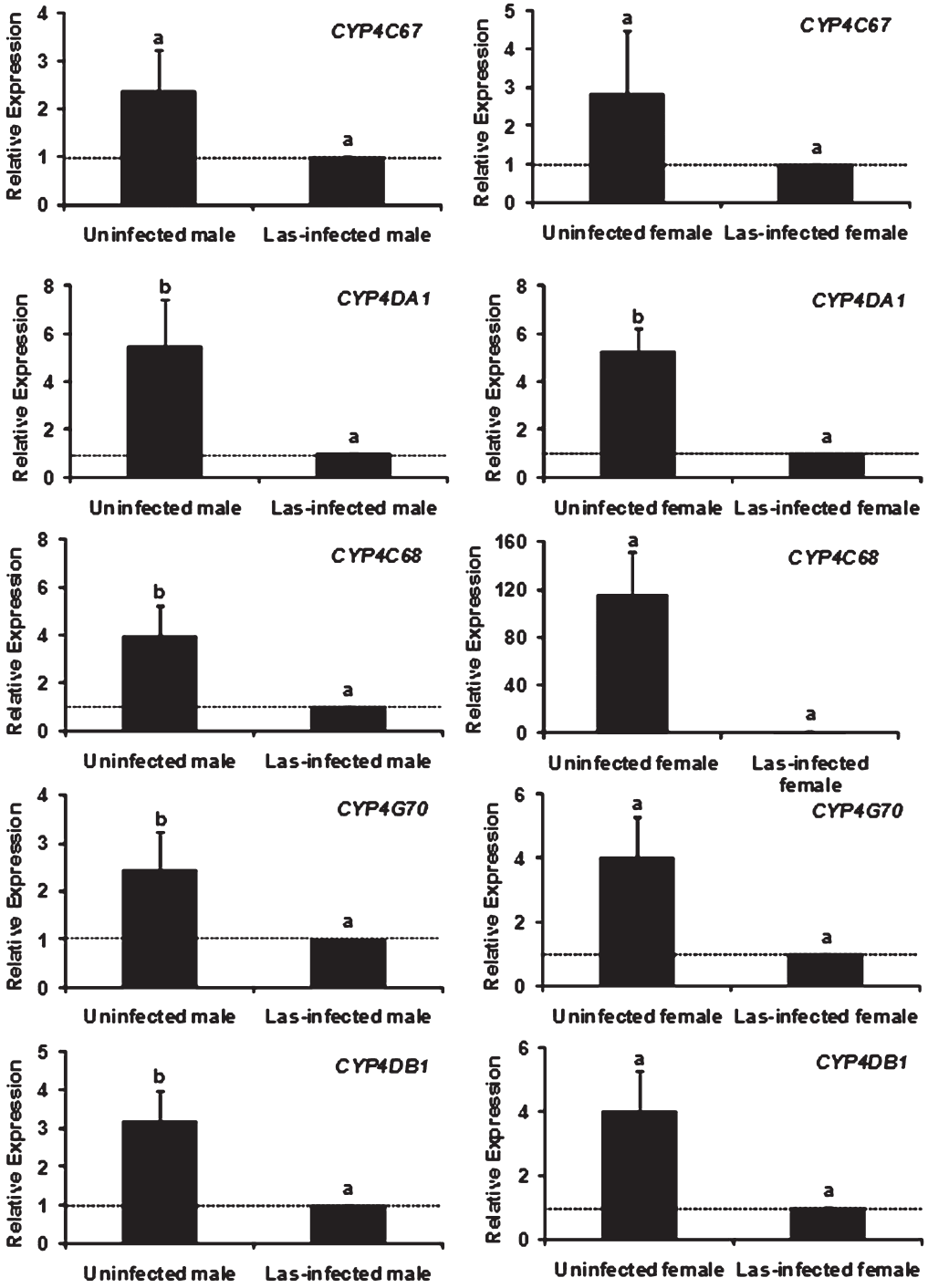

Figure 5. Relative expression levels of five *CYP4* genes in *Diaphorina citri* males and females that were either uninfected or Las-infected. Ct values were first normalized to the reference gene, *Actin*, followed by normalization to the treatment giving the lowest gene expression using the $2^{-\Delta\Delta CT}$ method. Values within graphs sharing the same letter are not significantly different ($P \geq 0.05$; Fisher's protected LSD).

directly compared between males and females, relative expression of *CYP4C68* was ≈30-fold higher in uninfected females than males. Further investigations are needed to confirm gender-based expression specificity of *CYP4* genes. Sex-specific expression patterns of *CYP4* genes have been reported previously and examples include *Ips pini* (Sandstrom *et al.*, 2006), *B. germanica* (Wen & Scott, 2001), *D. melanogaster* (Kasai & Tomita, 2003) and *Ips paraconfusus* (Huber *et al.*, 2007). Higher expression of *CYP4C68* in unin-

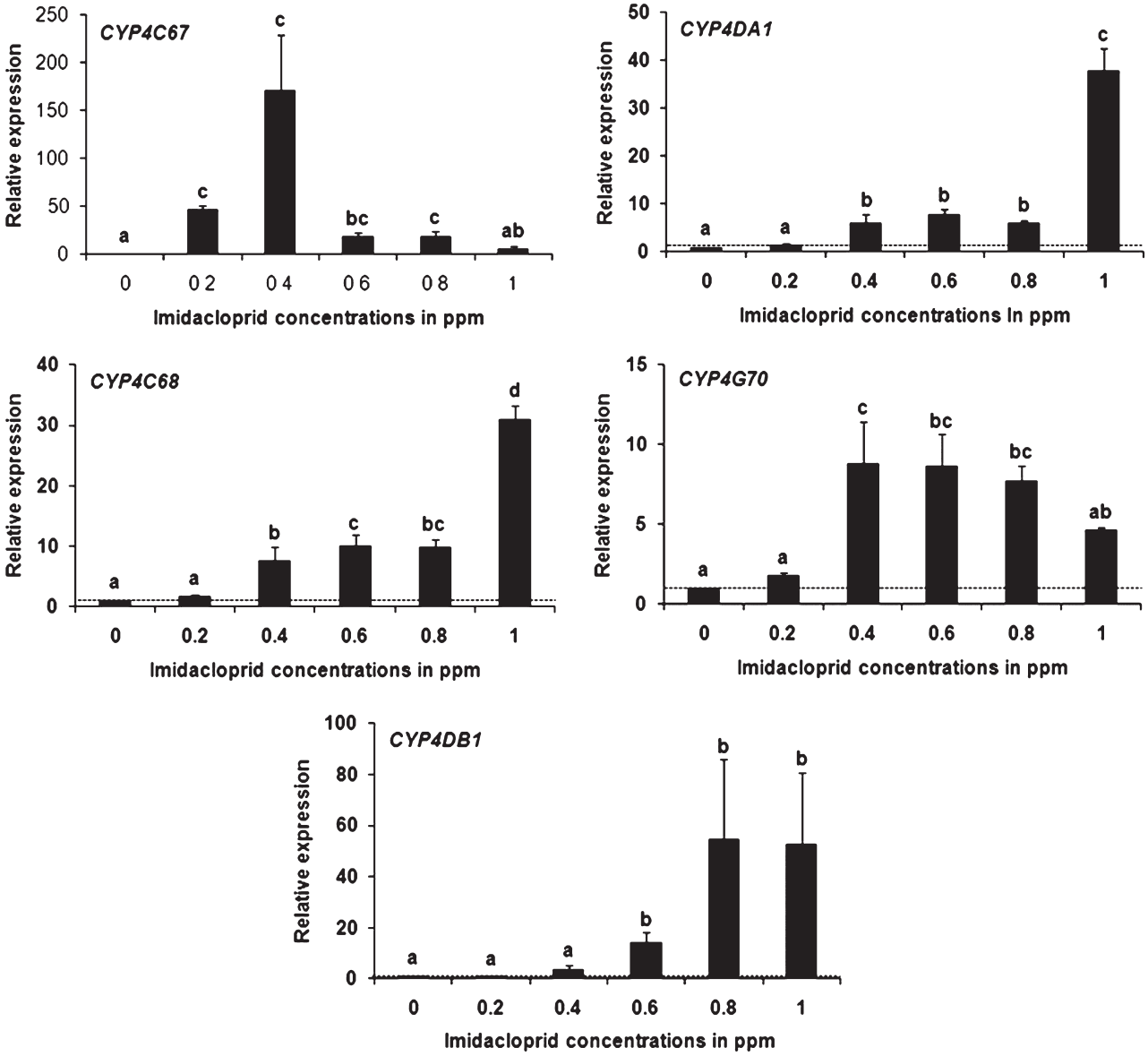

Figure 6. Relative expression levels of five *CYP4* genes in *Diaphorina citri* adults (uninfected and mixed gender) exposed to varying concentrations of imidacloprid. Ct values were first normalized to the reference gene, *Actin*, followed by normalization to the treatment giving the lowest gene expression using the $2^{-\Delta\Delta CT}$ method. Values within graphs sharing the same letter are not significantly different ($P \geq 0.05$; Fisher's protected LSD).

fected female than male *D. citri* suggests their involvement in female-specific physiological activities, such as pheromone production, juvenile hormone biosynthesis and/or reproduction.

Imidacloprid induction bioassay

The leaf dip method has been reported to result in greater levels of P450 induction than the topical application method (Baek *et al.*, 2010), therefore, the leaf dip method was used here to investigate induction of *CYP4* genes as a result of varying concentrations of imidacloprid in uninfected adult *D. citri*. Induction observed after exposure to the 0.6 ppm concentration for each *CYP4* gene was significantly higher than in controls suggesting that the LC_{50} (0.47 ppm; Tiwari *et al.*, 2011b) is sufficient to induce gene expression. Reduced expression of *CYP4C67* after exposure of *D. citri* to 1.0 ppm of imidacloprid suggests that a lethal concentration of imidacloprid or its metabolites may have inhibited expression of this gene. Expression of *CYP4C67* was 22, 28, 20 and 43-fold higher than that of *CYP4DA1*, *CYP4C68*, *CYP4G70* and *CYP4DB1* at the 0.4 ppm dosage. Differences in *CYP4* gene induction suggest that imidacloprid may cause selective induction rather than

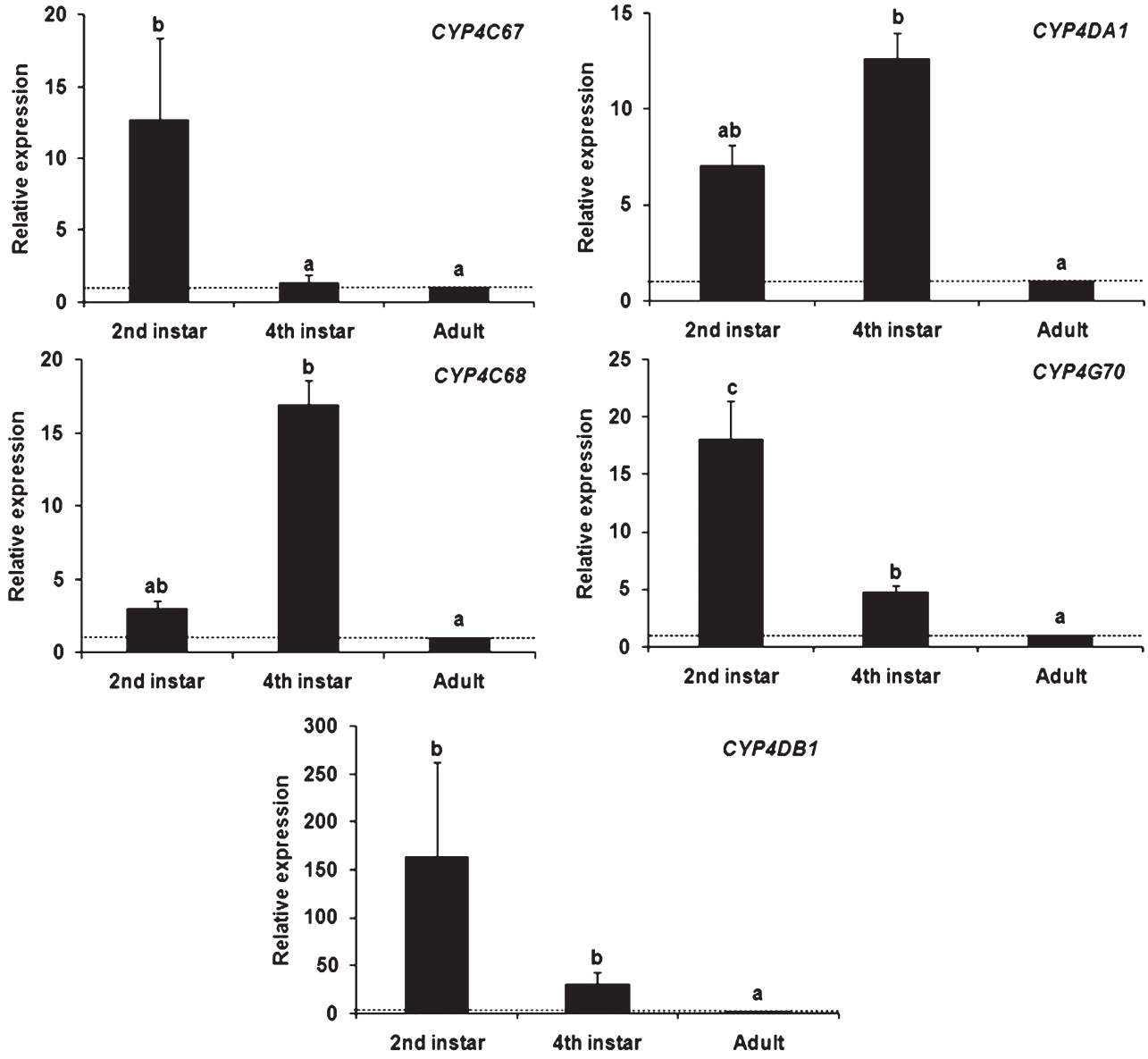

Figure 7. Relative expression levels of five *CYP4* genes in three development stages of *Diaphorina citri*. Ct values were first normalized to the reference gene, *Actin*, followed by normalization to the treatment giving the lowest gene expression using the $2^{-\Delta\Delta CT}$ method. Values within graphs sharing the same letter are not significantly different ($P \geq 0.05$; Fisher's protected LSD).

constitutive overexpression of P450 genes in *D. citri*. However, further identification of P450 genes and optimization of exposure times and insecticide concentrations are needed for a better understanding of specific induction pathways. Reducing the duration of exposure and concentration of insecticide should reduce physiological stress and possible inhibitory effects on certain P450 genes. In *Plutella xylostella* larvae, peak levels of P450 induction were observed within 3 h of exposure at sublethal concentrations of cypermethrin (Baek *et al*., 2010).

The *CYP4* genes reported here may provide a molecular tool for surveying field populations of *D. citri* for potential development of insecticide resistance. Given that imidacloprid is one of the most commonly used insecticides for *D. citri* management currently and that levels of resistance to this insecticide have reached up to 34-fold in 2009 (Tiwari *et al*., 2011a), investigating the expression levels of *CYP4* genes may be particularly useful for surveying resistance in field populations of *D. citri*. Induction of one or more P450 genes has been reported in *H. armigera* after exposure to permethrin (Wang & Hobbs, 1995); in *D. melanogaster* after exposure to DDT (Brandt *et al*., 2002; Willoughby *et al*., 2006); and in *P. xylostella* after exposure to cypermethrin (Baek *et al*., 2010). It is

also possible that sublethal or lethal concentrations of imidacloprid may indiscriminately induce other unidentified P450 genes. Therefore, identification of other P450 genes and description of their induction pathways in *D. citri* is warranted. In addition, determining whether other xenobiotics or insecticides of different modes of action induce these genes will be useful with respect to resistance monitoring for other modes of action.

Effect of developmental stage on CYP4 expression

Greater expression of *CYP4* genes in nymphs than adults is congruent with previous results obtained with *H. armigera* where expression of two homologous cytochrome P450 mRNAs peaked during the larval stage (Ranasinghe *et al*., 1997). Likewise, midgut *CYP4* mRNA levels are higher during the active feeding stage of *M. sexta* than during non-feeding (prepupal and pupal) stages (Snyder *et al*., 1995). In addition, expression of P450s occurs during specific developmental stages. For example, *CYP6B2* is expressed in larval *H. arimigera* (Ranasinghe *et al*., 1997), while *CYP6D1* is expressed in adult *Musca domestica* (Scott *et al*., 1996). The quantity of moulting hormone produced during specific developmental stages is known to influence expression of P450 enzymes. For example, expression of *CYP6A2* was elevated in 20-hydroxyecdysone (moulting hormone)-treated *D. melanogaster* (Spiegelman *et al*., 1997). Collectively, the above investigations suggest that active feeding and moulting are related to increased expression of specific P450s in immature insects as compared with adults. With respect to *D. citri*, greater expression of *CYP4* genes in immature stages than adults is congruent with greater levels of cytochrome P450 monooxygenases (Tiwari *et al*., 2011c).

In conclusion, this is the first report of *CYP4* gene sequences from *D. citri*. Identification of these genes should allow development of nucleic acid-based screening methods for cytochrome P450-mediated insecticide resistance in field populations of *D. citri*. In addition, these *CYP4* genes could be targeted for RNA interference-based silencing in highly resistant field populations of *D. citri*. Induction of these *CYP4* genes as result of imidacloprid exposure supports the hypotheses that they are involved in metabolism of this insecticide. Greater expression of *CYP4* genes during immature stages than the adult stage suggests they may be involved in other physiological processes in addition to detoxification.

Experimental procedures

Asian citrus psyllid culture

Uninfected and Las-infected *D. citri* were drawn from cultures continuously reared at the Citrus Research and Education Center (CREC), University of Florida, Lake Alfred, FL, USA. The uninfected culture was established in 2000 using field populations collected in Polk Co., FL, USA (28.0′ N, 81.9′ W) and maintained on sweet orange (*Citrus sinensis* (L.) Osbeck) without exposure to insecticides in a greenhouse at 27–28°C, 60–65% relative humidity and a 14:10 (light:dark) photocycle. The Las-infected *D. citri* culture was established in 2009 from the uninfected laboratory population by rearing *D. citri* on Las-infected citrus seedlings in a separate greenhouse approved for rearing Las-infected citrus plants and *D. citri* under the environmental conditions described above.

RNA isolation and cDNA synthesis from Las-infected and uninfected Diaphorina citri

Diaphorina citri adults reared on Las-infected host plants were analysed using qPCR to confirm Las infection. DNA extractions and qPCR were performed on specific body sections of *D. citri*: head + thorax or abdomen sections of uninfected or Las-infected males and females using methods described by Tiwari *et al*. (2010, 2011b). Sections of *D. citri* found positive for Las were retained and matched with their corresponding sections to be used for subsequent RNA isolation, cDNA synthesis and qPCR, as described below.

After an individual *D. citri* was confirmed for Las infection, it was subjected to RNA isolation and cDNA synthesis. RNA isolations were performed on pooled head + thorax and abdominal sections from Las-infected and uninfected males and females, using the SV total RNA isolation kit (Promega, Madison, WI, USA). RNA isolation was performed on groups of 40–50 psyllids. Each treatment was replicated three times. Quality and quantity of RNA from each sample was measured on a NanoDrop 1000 Spectrophotometer using A_{260} and A_{260}/A_{280} ratio, respectively (Scharf *et al*., 2008), to ensure uniform quality and quantity (100 ng/μl) among all treatments for subsequent cDNA synthesis. Additionally, quality of RNA was assessed by visualizing the RNA samples on 1% agarose gel. All RNA samples showed strong 18S and 28S ribosomal RNA bands and no evident degradation. cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) using pooled RNA. Briefly, cDNA was synthesized in a 20 μl reaction volume containing 4 μl of 5× iScript reaction mix, 1 μl of iScript reverse transcriptase, 8 μl of nuclease free water and 7 μl of total RNA (100 ng/μl). Amplification cycles consisted of 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and were held at 4°C. The PCR products were verified by 1% agarose electrophoresis in (tris-acetate-EDTA) TAE buffer with ethidium bromide staining.

Degenerate PCR, cloning and sequencing

cDNA templates from each treatment were used for degenerate PCR using 'set B' degenerate *CYP4* primers (Snyder *et al*., 1995) following methods described by Zhou *et al*. (2006). The forward and reverse primers, corresponding to the i-helix and heme-binding regions of family 4 P450 genes, respectively, were as follows: Forward = GAGGTIGAYACITTCATGTTCGARGGI-CACGAYAC; Reverse = CTGICCGATRCAGTTICGBGGICCIGC-SIWGAABG. PCR reactions were conducted using iQ SYBR Green Supermix (Bio-Rad) and a temperature cycle consisting of 94°C for 4 min, 5 cycles of 94° for 30 s, 45° for 30 s and 72° for 30 s, followed by 30 cycles of 94° for 30 s, 55° for 30 s and 72° for

Table 2. Quantitative real-time PCR primer details

Gene	Accession No.	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
CYP4 Genes			
CYP4C67	JF934716	TGGAACGTGTCATCAAGGAG	CCGGATTGAAACTGTTAGGC
CYP4DA1	JF934718	AGTGGTGTCGGAAATTGAGG	GTTCGAGCCACCTGGAGATA
CYP4C68	JF934717	CTAGCCTGGACCCTCTTCCT	ACCCTCCCTATGAACGGAAC
CYP4G70	JF934720	GCCGGAAGTTCTTTCTTCCT	TAACGGGTACTGGTGGGAAC
CYP4DB1	JF934719	CTGTACGCTCTGGGACATCA	TTGAGCGGTGCATAGAGTTG
Reference genes			
Alpha-tubulin	DQ675542	CAGGTCTTGTGTGGGACGTA	GGCCACAGTTTGTTTCTTGC
Actin	DQ675553	CCCTGGACTTTGAACAGGAA	CTCGTGGATACCGCAAGATT
NADH-dh	DQ673395	GAAACACACTGCTCTGATTCCA	TACTGCTTGCCATCCCTGA
wg	AF231365	GCTGAATCCGTACAATCCTGA	CCCACAGCACATCAGATCAC

30 s, then 72° for 8 min. PCR products were verified by 1% agarose electrophoresis in TAE buffer with ethidium bromide staining. PCR products of expected size (450 bp) were excised and purified using the Qiagen gel extraction kit (Qiagen, Valencia, CA, USA).

Cloning and sequencing of each treatment followed methods described by Zhou *et al.* (2006). Briefly, 3 μl of purified PCR products were cloned using a pGEM-T vector (pGEM-T Easy Vector system kit; Promega). This was followed by heat-shock transformation of JM-109 competent cells (Promega) along with ligation reaction. Transformed cells were allowed to grow on Luria-Bertoni-agar plates containing ampicillin, x-gal and IPTG. Plates wereLuria-Bertoni incubated overnight at 37°C for colonies to develop. Positive white colonies were picked and allowed to grow overnight in individual wells of 96-well culture plates containing 0.5 ml LB media prepared with 8% glycerol and 0.1 mg/ml ampicillin. High-throughput plasmid isolation and sequencing were performed by the University of Florida Genomics Core Facility, Gainesville, FL.

Sequence analysis was performed using parameters described by Zhou *et al.* (2006).

High-quality sequences obtained from cloning were trimmed of vector and primer sequences, and searched in Blastx for the non-redundant arthropod database. All sequences with significant identity to cytochrome P450 genes were assembled into contigs using SeqMan software (DNA Star, Madison, WI, USA). Sequence identities, amino acid translations and alignments were generated using MegAlign (DNA Star). After the selected sequences were aligned, phylogeny was determined using PhyML software based on the maximum likelihood method (Dereeper *et al.*, 2008) and Bootstrap values determined from 100 replicates. Unique contigs were sent to Dr David Nelson (University of Tennessee, Memphis, TN, USA) for classification and nomenclature assignment.

Quantitative real-time PCR

Quantitative real-time PCR was performed using iQ SYBR Green Supermix with an iCycler iQ real-time PCR detection system (Bio-Rad). Primers for the five *CYP4* genes and the reference gene, *Actin*, are reported in Table 2. Primers for five *CYP4* genes as well as the reference genes were designed with the Primer 3 program. Primers were designed to provide products of 200 ± 25 base-pairs (bp) within putative open reading frames, 45–55% guanine-cytosine content and $T_m = 57–62°C$. Forward and reverse primers were checked for non-complementarity and pyrimidine nucleotides on their 5′ and 3′ ends. For all qPCR experiments, the production of gene-specific products and absence of 'primer-dimers' was verified by 1% agarose electrophoresis in TAE buffer with ethidium bromide staining. Briefly, qPCR was performed in a 20-μl reaction volume containing 10-μl of SYBR mix, 1-μl of cDNA, 1-μl of each forward and reverse primers and 7-μl of nuclease-free water. Amplification cycles consisted of an initial denaturing step at 95°C for 10 min, followed by 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, an extension step at 72°C for 10 min, and the final melting-curve step (90 cycles of temperature reduction from 90°C to 50°C at a rate of 0.5°C/10 s). Six biological replicates were performed per gene per experiment.

To compare the relative expression of each gene among treatments, we used the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) by normalizing to *Actin* (reference gene) gene expression, followed by normalization to the treatment giving the lowest gene expression. Separate ANOVA tests were performed to compare the relative expression of each gene between uninfected and Las-infected males and females. One-way ANOVA was performed to compare the relative expression between concentrations of imidacloprid tested for each gene. Likewise, one-way ANOVA was also performed to compare differences in relative expression between developmental stages for each gene. All ANOVAs were followed by Fisher's protected LSD tests for mean separation (PROC GLM) (SAS Institute, 2004).

CYP4 expression in Las-infected and uninfected D. citri

CYP4 expression was quantified in four types of *D. citri* samples: uninfected male, uninfected female, Las-infected male and Las-infected female. Each type of *D. citri* was considered as a treatment. Las infection in *D. citri* was confirmed using methods described earlier. Each type consisted of ≈40–50 individuals of similar age. Individuals from each type were subsequently processed for RNA isolation, cDNA synthesis and qPCR, using the methods described above. Before cDNA synthesis, quality and quantity of RNA from each type was measured using methods described earlier.

Imidacloprid induction bioassay

Imidacloprid (Provado 1.6F, Bayer CropScience LP, Research Triangle Park, NC, USA) was serially diluted in water to prepare various concentrations. Induction of cytochrome P450 monooxygenases at the protein level was conducted previously using a similar Petri dish bioassay (Prabhaker *et al.*, 2006; Tiwari *et al.*,

2011a). Fresh citrus leaves collected from Valencia orange trees maintained in a CREC greenhouse were used in bioassays. Leaf discs (60 mm diameter) were excised, dipped in test insecticide dilutions for 30 s and allowed to air dry in a fume hood for 1 h prior to bioassays. For the control treatment, leaf discs were dipped in distilled water alone. After 1 h, leaf discs were placed on agar beds within 60 mm diameter plastic disposable Petri dishes (Fisherbrand; Thermo Fisher Scientific, Waltham, MA, USA), and 20–25 uninfected adult *D. citri* of mixed gender and similar age were transferred into each dish using a camel hair brush. Petri dishes were wrapped with parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) to prevent escape of adults. Sealed Petri dishes with adults were transferred into a growth chamber (Percival Scientific, Inc., Perry, IA, USA) set at 25 ± 1°C, 50 ± 5% RH and a 14:10 h light:dark photoperiod. Each concentration of an insecticide was replicated 3 times ($n = 60$–75 adults per concentration). Mortality of *D. citri* was assessed 48 h after transfer into the growth chamber. Live *D. citri* ($n = 20$ adults per concentration) from each concentration (treatment) were collected for RNA isolation, cDNA synthesis and qPCR, using the methods described above. Before cDNA synthesis, quality and quantity of RNA from each treatment was measured as described earlier.

Effect of developmental stage on CYP4 expression

CYP4 expression was quantified for three developmental stages of uninfected *D. citri*: 2nd and 4th instar nymphs, and adults. Each stage was considered as a treatment. Approximately 40–50 individuals of similar age from each developmental stage were obtained from the culture and adults were of mixed gender. Each treatment was subsequently processed for RNA isolation, cDNA synthesis and qPCR, using the methods described above in three replicates. Before cDNA synthesis, quality and quantity of RNA from each treatment was measured as described earlier.

Acknowledgements

This project was supported by a grant from the Citrus Research and Development Foundation to LLS. We thank Dr David Nelson (University of Tennessee-Memphis) for naming genes. We also thank Wendy Meyer, Nandlal Chaoudhary and Amit Jhala for reviewing a previous version the manuscript. We acknowledge Y. Cruz-Plemons, D. Diaz and A. Hoyte for *D. citri* collections, processing and other technical assistance.

References

Baek, J.H., Clark, J.M. and Lee, S.H. (2010) Cross-strain comparison of cypermethrin-induced cytochrome P450 transcription under different induction conditions in diamondback moth. *Pestic Biochem Physiol* **96**: 43–50.

Brandt, A., Scharf, M., Pedra, J.H.F., Holmes, G., Dean, A., Kreitman, M. *et al*. (2002) Differential expression and induction of two *Drosophila* cytochrome P450 genes near the *Rst(2)DDt* locus. *Insect Mol Biol* **11**: 337–341.

Daborn, P.J., Yen, J.L., Bogwitz, M.R., Goff, G.L., Feil, E., Jeffers, S. *et al*. (2002) A single P450 allele associated with insecticide resistance in *Drosophila*. *Science* **297**: 2253–2256.

Daborn, P.J., Lumb, C., Boey, A., Wong, W., ffrench-Constant, R.H. and Batterham, P. (2007) Evaluating the insecticide resistance potential of eight *Drosophila melanogaster* cytochrome P450 genes by transgenic over-expression. *Insect Biochem Mol Biol* **37**: 512–519.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F. *et al*. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* **36**: W465–W469.

Dunkov, B.C., Rodriguez-Arnaiz, R., Pittendrigh, B., ffrench-Constant, R.H. and Feyereisen, R. (1996) Cytochrome P450 gene clusters in *Drosophila melanogaster*. *Mol Gen Genet* **251**: 290–197.

Duron, O., Labbe, P., Berticat, C., Rousset, F., Guillot, S., Raymond, M. *et al*. (2006) High *Wolbachia* density correlates with cost of infection for insecticide resistant *Culex pipiens* mosquitoes. *Evolution* **60**: 303–314.

Feyereisen, R. (1999) Insect P450 enzymes. *Annu Rev Entomol* **44**: 507–533.

Feyereisen, R. (2005) Insect cytochrome *P450*. In *Comprehensive Molecular Insect Science [Vol, 4]: Biochemistry and Molecular Biology* (Gilbert, L.I., Iatrou, K. and Gill, S.S., eds), pp. 1–77. Elsevier, Amsterdam.

Feyereisen, R. (2006) Evolution of insect P450. *Biochem Soc Trans* **34**: 1252–1255.

Ghanim, M. and Kontsedalov, S. (2009) Susceptibility to insecticides in the Q biotype of *Bemisia tabaci* is correlated with bacterial symbiont densities. *Pest Manag Sci* **65**: 939–942.

Gui, Z., Hou, C., Liu, T., Qin, G., Li, M. and Jin, B. (2009) Effects of insect viruses and pesticides on glutathione *S*-transferase activity and gene expression in *Bombyx mori*. *J Econ Entomol* **102**: 1591–1598.

Halbert, S.E. and Manjunath, K.L. (2004) Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. *Fla Entomol* **87**: 330–353.

Hodgson, E. (1985) Microsomal monooxygenases. In *Comprehensive Insect Physiology, Biochemistry, and Pharmocology, Vol 11 Pharmacology* (Kerkut, G.A. and Gilbert, L.I., eds), pp. 225–322. Pergammon, Elmsford, NY.

Huber, D.P.W., Erickson, M.L., Leutenegger, C.M., Bohimann, J. and Seybold, S.J. (2007) Isolation and extreme sex-specific expression of cytochrome P450 genes in the bark beetle, *Ips paraconfusus*, following feeding on the phloem of host ponderosa pine, *Pinus ponderosa*. *Insect Mol Biol* **16**: 335–349.

Kasai, S. and Tomita, T. (2003) Male specific expression of a cytochrome P450 (Cyp312A1) in *Drosophila melanogaster*. *Biochem Biophys Res Commun* **300**: 894–900.

Kontsedalov, S., Zchori-Fein, E., Chiel, E., Gottlieb, Y., Inbar, M. and Ghanim, M. (2008) The presence of *Rickettsia* is associated with increased susceptibility of *Bemisia tabaci* (Homoptera: Aleyrodidae) to insecticides. *Pest Manag Sci* **64**: 789–792.

Li, X., Schuler, M.A. and Berenbaum, M.R. (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52**: 231–253.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**: 402–408.

Manjunath, K.L., Halbert, S.E., Ramadugu, C., Webb, S. and Lee, R.F. (2008) Detection of '*Candidatus* Liberibacter asiaticus' in

Diaphorina citri and its importance in the management of citrus Huanglongbing in Florida. *Phytopathology* **98**: 387–396.

Matambo, T.S., Paine, M.J.I., Coetzee, M. and Koekemoer, L.L. (2010) Sequence characterization of cytochrome P450 CYP6P9 in pyrethroid resistant and susceptible *Anopheles funestus* (Diptera: Culicidae). *Genet Mol Res* **9**: 554–564.

McCarroll, L., Paton, M.G., Karunaratne, S.H.P.P., Jayasuryia, H.T.R., Kalpage, K.S.P. and Hemingway, J. (2000) Insecticides and mosquito-borne disease. *Nature* **407**: 961–962.

Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., Fujii-Kuriyama, Y. *et al*. (1991) The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol* **10**: 1–14.

Pittendrigh, B.R., Aronstein, K., Zinkovski, E., Andreev, O., Campbell, B., Daly, J. *et al*. (1997) Cytochrome *P450* genes from *Helicoverpa armigera*: expression in a pyrethroid-susceptible and -resistant strain. *Insect Biochem Mol Biol* **27**: 507–512.

Prabhaker, N., Castle, S.J., Byrne, F.J., Henneberry, T.J. and Toscano, N.C. (2006) Establishment of baseline susceptibility data to various insecticides for glassy-winged sharpshooter, *Homalodisca coagulata* Say (Homoptera: Cicadellidae) by comparative bioassay techniques. *J Econ Entomol* **99**: 141–154.

Ranasinghe, C., Headlam, M. and Hobbs, A.A. (1997) Induction of the mRNA for CYP6B2, a pyrethroid inducible cytochrome P450, in *Helicoverpa armigera* (Hubner) by dietary monoterpenes. *Arch Insect Biochem Physiol* **34**: 99–109.

Ranson, H., Rossiter, L., Ortelli, F., Jensen, B., Wang, X., Roth, C.W. *et al*. (2001) Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochem J* **359**: 259–304.

Ranson, H., Nikou, D., Hutchinson, M., Wang, X., Roth, C.W., Hemingway, J. *et al*. (2002) Molecular analysis of multiple cytochrome P450 genes from the malaria vector, *Anopheles gambiae*. *Insect Mol Biol* **11**: 409–418.

Sandstrom, P., Welch, W.H., Blomquist, G.J. and Tittiger, C. (2006) Functional expression of a bark beetle cytochrome P450 that hydrooxylates myrcene to ispdienol. *Insect Biochem Mol Biol* **36**: 835–845.

SAS Institute (2004) *SAS Users Guide*. SAS Institute, Cary, NC.

Scharf, M.E., Lee, C.Y., Neal, J.J. and Bennett, G.W. (1999) Cytochrome P450 MA expression in insecticide-resistant German cockroaches (Dictyoptera: Blattellidae). *J Econ Entomol* **92**: 788–793.

Scharf, M.E., Parimi, S., Meinke, L.J., Chandler, L.D. and Siegfried, B.D. (2001) Expression and induction of three family 4 cytochrome *P450* (CYP4) genes identified from insecticide-resistant and susceptible western corn rootworms, *Diabrotica v. virgifera*. *Insect Mol Biol* **10**: 139–146.

Scharf, M.E., Zhou, X. and Schwinghammer, M.A. (2008) Application of RNA interference in functional genomics studies of a social insect. In *Methods in Molecular Biology [Vol, 442]: Sirna, Shrna and Mirna Protocols* (Barik, S., ed.), pp. 205–229. ed., Humana Press, Totowa, NJ.

Scott, J.G. (1999) Cytochromes P450 and insecticide resistance. *Insect Biochem Mol Biol* **29**: 757–777.

Scott, J.G., Collins, F.A. and Feyereisen, R. (1994) Diversity of cytochrome *P450* genes in the mosquito, *Anopheles albimanus*. *Biochem Biophys Res Commun* **205**: 1452–1459.

Scott, J.G., Sridhar, P. and Liu, N. (1996) Adult specific expression and induction of cytochrome P450lpr in house flies. *Arch Insect Biochem Physiol* **31**: 313–323.

Sétamou, M., Rodriguez, D., Saldana, R., Schwarzlose, G., Palrang, D. and Nelson, S.D. (2010) Efficacy and uptake of soil-applied imidacloprid in the control of Asian citrus psyllid and a citrus leafminer, two foliar-feeding citrus pests. *J Econ Entomol* **103**: 1711–1719.

Snyder, M.J., Stevens, J.L., Andersen, J.F. and Feyereisen, R. (1995) Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, *Manduca sexta*. *Arch Biochem Biophys* **321**: 13–20.

Spiegelman, V.S., Fuchs, S.Y. and Belitsky, G.A. (1997) The expression of insecticide resistance-related cytochrome P450 forms is regulated by molting hormone in *Drosophila melanogaster*. *Biochem Biophys Res Commun* **232**: 304–307.

Tijet, N., Helvig, C. and Feyereisen, R. (2001) The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intro-exon organization and phylogeny. *Gene* **262**: 189–198.

Tiwari, S., Lewis-Rosenblum, H., Pelz-Stelinski, K. and Stelinski, L.L. (2010) Incidence of *Candidatus* Liberibacter asiaticus infection in abandoned citrus occurring in proximity to commercially managed orchards. *J Econ Entomol* **103**: 1972–1978.

Tiwari, S., Mann, R.S., Rogers, M.E. and Stelinski, L.L. (2011a) Insecticide resistance in field populations of Asian citrus psyllid in Florida. *Pest Manag Sci* doi: 10.1002/ps.2181.

Tiwari, S., Pelz-Stelinski, K. and Stelinski, L.L. (2011b) Effect of *Candidatus* Liberibacter asiaticus infection on susceptibility of Asian citrus psyllid, *Diaphorina citri*, to selected insecticides. *Pest Manag Sci* **67**: 94–99.

Tiwari, S., Pelz-Stelinski, K., Mann, R.S. and Stelinski, L.L. (2011c) Glutathione S-transferase and cytochrome P_{450} activity levels in *Candidatus* Liberibacter asiaticus-infected and uninfected Asian citrus psyllid, *Diaphorina citri*. *Ann Entomol Soc Am* **104**: 297–305.

Wang, X.P. and Hobbs, A.A. (1995) Isolation and sequence analysis of cDNA clone for a pyrethroid inducible cytochrome P450 from *Helicoverpa armigera*. *Insect Biochem Mol Biol* **25**: 1001–1009.

Wen, Z. and Scott, J.G. (2001) Cytochrome P450 CYP6L1 is specifically expressed in the reproductive tissues of adult male German cockroaches, *Blattella germanica* (L.). *Insect Biochem Mol Biol* **31**: 179–187.

Willoughby, L., Chung, H., Lumb, C., Robin, C., Batterham, P. and Daborn, P.J. (2006) A comparison of *Drosophila melanogaster* detoxification gene induction responses for six insecticides, caffeine and Phenobarbital. *Insect Biochem Mol Biol* **36**: 934–942.

Wondji, C.S., Irving, H., Morgan, J., Lobo, N.F., Collins, F.H., Hunt, R.H. *et al*. (2009) Two duplicated P450 genes are associated with pyrethroid resistance in *Anopheles funestus*, a major malaria vector. *Genome Res* **19**: 452–459.

Zhou, X., Song, C., Grzymala, T.L., Oi, F.M. and Scharf, M.E. (2006) Juvenile hormone and colony conditions differentially influence cytochrome *P450* gene expression in the termite *Reticulitermes flavipes*. *Insect Mol Biol* **15**: 749–761.