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Expression of *Cyp6g1* and *Cyp12d1* in DDT resistant and susceptible strains of *Drosophila melanogaster*

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Abstract

The *Rst(2)DDT* locus (loci) in *Drosophila* is associated with the over-expression of two cytochrome P450 genes, *Cyp6g1* and *Cyp12d1*. Using northern and western blot analysis we observed the expression pattern of these two genes in two DDT susceptible (Canton-S and 91-C) and three DDT resistant strains (Wisconsin, 91-R and Hikone-R). In Canton-S and 91-R, the CYP6G1 protein was constitutively expressed throughout development. In the Wisconsin strain, CYP6G1 was not expressed in third instar larvae unless the larvae are exposed to DDT. CYP12D1 protein was only expressed in adults. *Cyp12d1* mRNA is induced in DDT resistant strains post-exposure to DDT and the expression patterns of *Cyp12d1* mRNA varied across DDT resistant strains. Our data support the hypothesis that there is evolutionary plasticity in the expression patterns of P450s associated with metabolic pesticide resistance.

Keywords: metabolic, resistance, insecticide, pesticide.

Introduction

DDT has been extensively used for well over fifty years as a pesticide for the protection of crops and for the control of vector borne diseases such as malaria and typhus (Ahuja & Kumar, 2003); and it is still used in South-Eastern Asia and some African countries due to its (i) low cost, (ii) broad spectrum of insecticidal activity, and (iii) ease of application (Attaran & Maharaj, 2000). Several lines of evidence suggest that the voltage-gated sodium channel is the single principal molecular target site for DDT in both insects and mammals (Soderlund & Bloomquist, 1989; Dong & Scott, 1994; Williamson *et al*., 1996; Dong, 1997) causing nerve cells to repeatedly generate action potentials resulting in repetitive body tremors and eventually death (Soderlund & Bloomquist, 1989).

Two types of DDT resistance have been observed: (i) target-site sensitivity and (ii) metabolism based resistance. A major form of target-site insensitivity is *knock-down resistance* (*kdr*), which confers resistance to DDT and pyrethroids, is associated with amino acids changes in the voltage-gated sodium channel in such pest species as houseflies, *Musca domestica*, and cockroaches, *Blattella germanica* (Williamson *et al*., 1993; Dong & Scott, 1994; Dong, 1997). In *Drosophila*, the *para* gene encodes the voltage-gated sodium channel (Loughney *et al*., 1989). Amino acid changes in the *para* protein are associated with DDT and pyrethroid resistance (Pittendrigh *et al*., 1997; Martin *et al*., 2000). Another major mechanism by which insects develop resistance to insecticides is by elevated metabolism (Feyereisen, 1999; Scott, 1999). In insects, metabolic insecticide resistance is typically associated with overexpression of cytochrome P450s, glutathione-S-transferases (GSTs), esterases, or a combination of these genes (Carino *et al*., 1994; Maitra *et al*., 1996; Danielson *et al*., 1997; Liu & Scott, 1997; Dombrowski *et al*., 1998; Daborn *et al*., 2001, 2002; Brandt *et al*., 2002; Le Goff *et al*., 2003; Pedra *et al*., 2004).

In the 91-R strain of *Drosophila*, DDT resistance is associated with each of the three large chromosomes (Dapkus & Merrell, 1977), supporting previous findings that resistance is polygenic (Crow, 1954). Considerable evidence supports the hypothesis that metabolism based DDT resistance is polygenic in *Drosophila* and is at least partially associated with overexpression of cytochrome P450s (Crow, 1954; Dapkus & Merrell, 1977; Hallstrom, 1985). Many attempts have been made to map a major locus involved in polygenic DDT resistance in *Drosophila*. One of these loci, *Resistance to DDT on II* [Rst(2)DDT], has been mapped to a dominant locus, located at 65 cM on the right arm of chromosome 2

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(Kikkawa, 1961; Pittendrigh, 1999) in multiple DDT resistant *Drosophila* strains, including Wisconsin and Hikone-R (Hallstrom, 1985). Six P450 genes that are found within or close to the *Rst(2)DDT* locus are *Cyp6g1*, *Cyp6g2*, *Cyp6t3*, *Cyp12d1*, *Cyp301a1* and *Cyp9h1* (Brandt *et al*., 2002). Of these six P450s, only *Cyp6g1* and *Cyp12d1* have been observed to be overtranscribed in DDT resistant *Drosophila* strains.

Recently, Scott & Kasai (2004) demonstrated that P450s display evolutionary plasticity in their responses to insecticide selection. They observed that P450-mediated metabolic resistance evolves via the differential selection of multiple P450s and with possibly different regulatory signals, even with selection by the same insecticides. In order to determine if variability in expression patterns exist for the two P450s associated with the *Drosophila Rst(2)DDT* locus, we analysed expression patterns of *Cyp6g1* and *Cyp12d1* in both laboratory and field-selected DDT resistant strains. These analyses were performed in two susceptible (Canton-S and 91-C) and three DDT resistant (Wisconsin, 91-R and Hikone-R) strains of *Drosophila* during (i) development and (ii) with and without exposure to DDT.

Results

Probe and antibody specificity

In order to analyse the specificity of our cDNA probes, we used dot blot analysis to show that CYP6G1 as well as CYP12D1 probes (cDNA) are efficient in discriminating between the closest respective homologues, CYP6G2 and CYP12A5 (Fig. 1). To perform analyses of CYP6G1 and CYP12D1 protein expression in susceptible and resistant strains, antibodies were raised against recombinant CYP6G1 and CYP12D1 proteins. Antibodies were also raised against recombinant CYP6G2 proteins. CYP6G2 protein was more highly expressed in the susceptible strains (Canton-S and 91-C) as compared to the DDT resistant strains (91-R and Wisconsin) (Fig. 1A). CYP6G1 proteins were more highly expressed in resistant strains as compared with susceptible strains (Fig. 1A). CYP6G1 and CYP12D1 cDNA probes did not cross-react with CYP6G2 and CYP12A5 cDNAs, respectively (Fig. 1B,C).

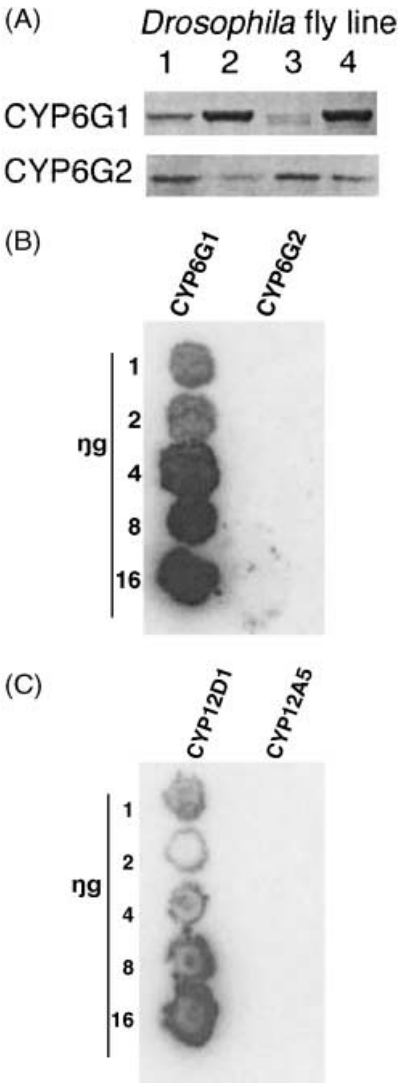

Figure 1. (A) Expression pattern of CYP6G1 and CYP6G2 in DDT susceptible and resistant genotypes. The two susceptible strains were Canton-S (lane 1) and 91-C (lane 3) and the two resistant strains were Wisconsin (lane 2) and 91-R (lane 4). Antibodies for CYP6G1 were used in the top part of 1A and antibodies for CYP6G2 were used in the bottom part of 1A. (B) Cross-reactivity of CYP6G1 and CYP6G2 cDNA probes. (C) Cross-reactivity of CYP12D1 and CYP12A5 cDNA probes.

Expression and DDT induction of Cyp6g1 *and* Cyp12d1 *in adults*

We tested the expression patterns of *Cyp6g1* and *Cyp12d1* for RNA transcript and protein in two susceptible (Canton-S and 91-C) and three DDT resistant (Wisconsin, 91-R and Hikone-R) *Drosophila* strains (Fig. 2). While the two most resistant strains, Wisconsin and 91-R, showed similar levels of *Cyp6g1* expression, they differed greatly in their resistance levels while the moderately DDT resistant Hikone-R strain showed the highest level of *Cyp6g1* expression. Although we detected basal levels of *Cyp6g1* expression in Canton-S, *Cyp6g1* expression was barely detectable in 91-C. Expression levels of mRNA and proteins, respectively, for *Cyp6g1* and CYP6G1 across all lines gave a high correlation ($r = 0.67$) and linear 1 : 1 relationship ($b = 0.75 \pm 0.30$).

There was increased constitutive expression of *Cyp12d1* mRNA in the 91-R and Wisconsin strains as compared to the susceptible strains. However, the expression pattern of *Cyp12d1* in Hikone-R, not treated with DDT, was similar to that of the susceptible strains. We found a second, less intense mRNA band in Canton-S (Fig. 2, 12D1: row B, Can-S, lanes X–Z) that corresponded to the smaller band of CYP12D1 mRNA in *91-R* (Fig. 2, 12D1: row B, 91-R, lanes

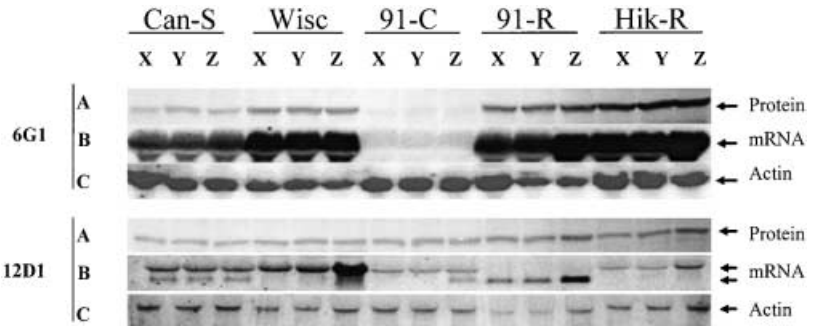

Figure 2. mRNA and protein expression of *Cyp12d1* and *Cyp6g1* in the absence and presence of DDT in adult flies. Five strains were tested: Canton-S (Can-S); Wisconsin (Wisc); 91-C; 91-R and Hikone-R (Hik-R). Protein expression is given in (A). For (B), CYP12D1 or CYP6G1 cDNA probes were used. For (C), a cDNA actin probe was used. Adults not exposed to DDT are given in X and Y. In the X lanes the adults were taken directly from the rearing bottles. In the Y lanes the adults were held in bioassay vials with no DDT for 24 h. In the Z lanes the adults were exposed to DDT for 24 h. Vials were coated with DDT on their inside surface (lanes Z), and 30 adults were held together in scintillation vials for 24 h. A concentration of DDT was used in each vial that corresponded to the LC_{25} of each strain. The surviving flies were collected and used in the experiments. The arrows indicate the position of the *Cyp6g1* and *Cyp12d1* mRNA and protein.

Table 1. Analysis of DDT toxicity in two DDT susceptible (Canton-S and 91-C) and three DDT resistant (Wisconsin, 91-R and Hikone-R) strains of *Drosophila*

Strain	*N*	Slope (± SE)	LC_{25} (95% CI)	LC_{50} (95% CI)	RR_{50}[a]
Canton-S	11010	1.01 (0.08)	0.15 (0.09–0.23)	0.71 (0.5–1)	–
91-C	4620	1.29 (0.04)	1.61 (1.45–1.77)	5.34 (4.93–5.79)	7.52
Hikone-R	4050	1.99 (0.09)	8.30 (7.48–9.29)	18.26 (16.94–19.67)	25.71
Wisconsin	3780	1.63 (0.11)	34.68 (27.28–42.29)	89.91 (76.42–105.27)	126.63
91-R	3780	0.7 (0.03)	142.18 (101.61–191.12)	1304 (1048–1610)	1836.61

[a]LC_{50} of resistant strain divided by LC_{50} of the most susceptible strain (Canton-S).

X–Z). The CYP12D1 protein was the same size across susceptible and resistant strains (Fig. 2, 12D1: row A).

When the adult flies were exposed to DDT, there was an increase in expression of *Cyp12d1* mRNA in all three resistant fly lines. In 91-R and Hikone-R there were marginal increases in CYP12D1 protein expression levels after treatment with DDT. Additionally, there were minor increases in expression of *Cyp6g1* mRNA in 91-R and Hikone-R flies exposed to DDT.

Toxicity of DDT to five strains

The most susceptible strain tested was Canton-S followed by 91-C (Table 1; Fig. 3). The 91-R strain was the most resistant to DDT followed by Wisconsin and Hikone-R (Table 1; Fig. 3). Although Hikone-R and 91-C were, respectively, the highest and lowest expressers of *Cyp6g1* (Fig. 2), Hikone-R was only 3.42 times more resistant to DDT than 91-C.

Developmental expression profiles of CYP6G1 and CYP12D1

We also examined the pattern of CYP6G1 and CYP12D1 expression throughout development in Canton-S and 91-R (Fig. 4). CYP6G1 protein was expressed throughout development in both strains. CYP6G1 was expressed at low levels in embryos, first- and second-instars. An increase of CYP6G1 expression began in the third-instar and continued through the adult stages. A peak of CYP6G1 expression occurred in older males and females. In the two fly lines tested (Canton-S and 91-R) CYP12D1 protein was only expressed in adults. CYP12D1 protein was expressed at low levels in newly eclosed males and females while the peak of CYP12D1 expression occurred in older males and females.

CYP6G1 is induced in third-instar larvae by DDT exposure

Analysis of CYP6G1 expression revealed that third-instar larvae of both susceptible and resistant strains showed different expression patterns (Fig. 5). Consistent with the pattern of CYP6G1 expression in adults, CYP6G1 was barely expressed in 91-C larvae. However, basal levels of CYP6G1 expression were apparent in Canton-S. In 91-R and Hikone-R, CYP6G1 protein was constitutively overexpressed. The levels of CYP6G1 constitutive overexpression were higher in Hikone-R than in 91-R. In the Hikone-R and 91-R strains CYP6G1 was also induced by DDT. Analysis of CYP6G1 expression in Wisconsin larvae revealed that CYP6G1 was not constitutively expressed in third-instar larvae; however, CYP6G1 was induced by DDT in Wisconsin third-instar larvae. We did not detect either CYP12D1 expression or induction in either unchallenged or DDT challenged larvae.

Lack of correlation between expression of CYP6G1 and LC_{50} of resistant and susceptible strains

No correlation or regression was observed between *Cyp6g1* mRNA and the LC_{50}s of the strains tested ($r = 0.23$; $b = 0.0001402 \pm 0.0003488$; $P < 0.71$). A similar lack of

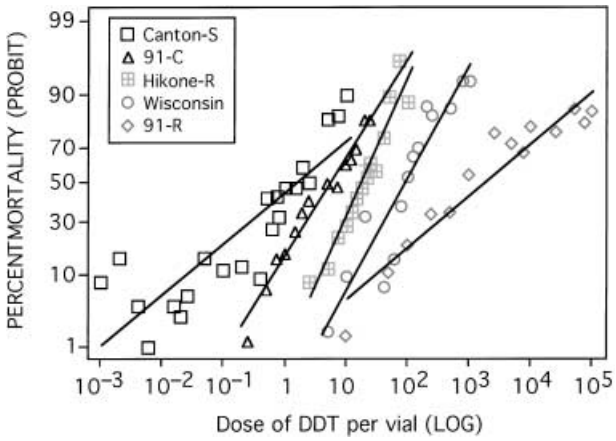

Figure 3. Dose–response curves for the strain Canton-S, 91-C, Hikone-R, Wisconsin and 91-R using DDT. On the *y*-axis is mortality in probit.

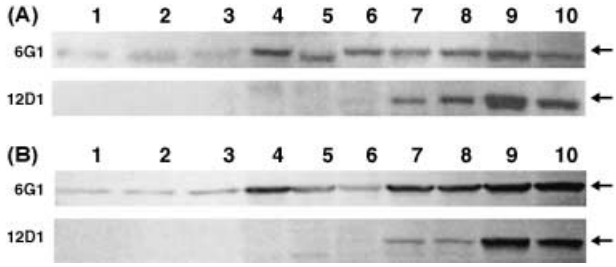

Figure 4. The P450s *Cyp6g1* and *Cyp12d1* are differently expressed during development in susceptible (Canton-S) and DDT resistant (91-R) strains. The susceptible strain in 4A was Canton-S and the resistant strain in 4B was 91-R. Analysis of expression of CYP6G1 (6G1) and CYP12D1 (12D1) during development was performed by western blots. In order to collect synchronized developmental stages, males and females, at a ratio of 1 : 3, were placed together in Petri dishes. The adults were placed on to new Petri dishes after 4 h. Eggs laid within 4 h (lane 1) were allowed to develop in order to collect: first- (lane 2), second- (lane 3) and third-instar larvae (lane 4); prepupae (lane 5); pupae (lane 6); freshly eclosed males (lane 7) and females (lane 8); and, six-day-old males (lane 9) and females (lane 10). Equal amounts of total protein (35 μg per lane) from developmental stages of susceptible and DDT resistant strains were separated by SDS-PAGE and transferred to nitrocellulose membrane, then probed with anti-CYP6G1 or CYP12D1 serum. The arrows indicate the position of the CYP6G1 and CYP12D1 proteins.

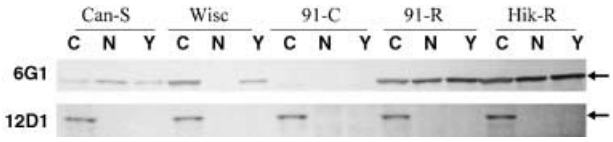

Figure 5. Third-instar larvae showed differential expression of CYP6G1 following DDT exposure. Larvae were placed together in Petri dishes without (lane N) and with (lane Y) DDT. A specific concentration of DDT was used in each Petri dish that corresponded to the LC_{25} of the adults for each strain. Eggs oviposited on the media were allowed to develop. The surviving third-instar larvae were collected and used to analyse the expression of CYP6G1 (6G1). Equal amounts of total protein (35 μg per lane) from third-instar larvae were (i) separated by SDS-PAGE, (ii) transferred to nitrocellulose membrane, and (iii) probed with anti-CYP6G1 or anti-CYP12D1 serum. As a control, protein was extracted from susceptible and resistant adults not exposed to DDT (lane C) and probed with antibodies for CYP6G1 or CYP12D1. The arrows indicate the position of the CYP6G1 and CYP12D1 proteins.

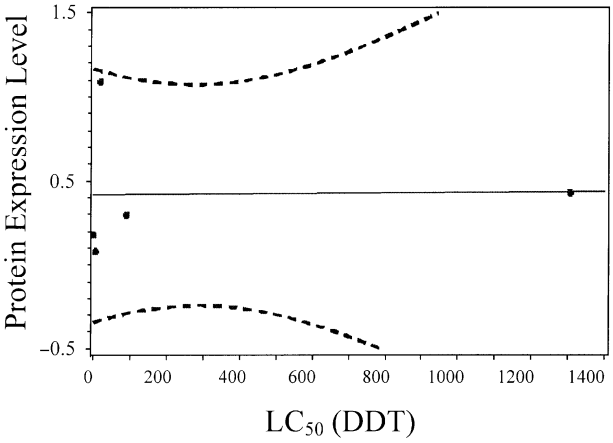

Figure 6. Lack of correlation between expression of CYP6G1 protein and DDT resistance levels (LC_{50}). The points shown in the figure respectively represent, from left to right, Canton-S, 91-C, Hikone-R, Wisconsin and 91-R. The dashed curves represent the 95% confidence interval of the regression.

correlation and regression was observed between CYP6G1 protein expression and the LC_{50}s of the strains tested (r = 0.018; b = 0.000013 ± 0.0004; $P < 0.9$) (Fig. 6).

Discussion

It has been suggested that *Cyp6g1* is a single resistance allele that is globally associated with DDT resistance, and that overtranscription of *Cyp6g1* alone is both 'necessary and sufficient' for DDT resistance (Daborn *et al.*, 2002). However, Tang *et al.* (2003) found that transgenic over-expression of *Cyp6g1* conferred only low levels of DDT resistance. Recombinant strains derived from a highly DDT resistant strain, which lost overexpression of *Cyp6g1*, were still resistant (Tang *et al.*, 2003). This does not rule out the possibility that *Cyp6g1* may play a role in DDT resistance, but it does suggest that the DDT resistance phenotype is more complex than just the over-expression of this single gene. Low doses of DDT were previously used to map one locus (or tightly linked loci), which may be associated with DDT resistance (Pittendrigh, 1999; Daborn *et al.*, 2001). The low doses of DDT used in mapping were, in many cases, far less than the LC_{50}s of resistant strains. Thus, it is likely that when low doses were used to map a single locus, other loci involved with DDT resistance were simply not be detected. In contrast to the hypothesis that DDT resistance is monogenic, we have found that not only one P450 *Cyp6g1*, but at least two P450s, *Cyp6g1* and *Cyp12d1*, are associated with the *Rst(2)DDT* locus (loci) in laboratory and field selected DDT resistant strains in *Drosophila* (Brandt *et al.*, 2002). In additional agreement, recent microarray

analyses support the concept that DDT resistance is polygenic in *Drosophila melanogaster* (Pedra *et al*., 2004).

Consistent with Daborn *et al*. (2001), in Hikone-R the expression of *Cyp6g1* mRNA was higher than in the Wisconsin strain. However, Wisconsin was more resistant to DDT than Hikone-R. In contrast to Daborn *et al*. (2002), we observed that *Cyp6g1* mRNA was expressed in the susceptible strain Canton-S. *Cyp6g1* was constitutively overexpressed at both transcriptional and translational levels in resistant strains Wisconsin, 91-R and Hikone-R under basal conditions. After DDT exposure, low levels of *Cyp6g1* induction were observed in 91-R and Hikone-R; however, in Wisconsin we did not detect *Cyp6g1* induction. Analysis of CYP6G1 protein expression in third-instar larvae of Wisconsin showed considerable induction by DDT, while CYP6G1 protein was constitutively over-expressed in the third-instar larvae of 91-R and Hikone-R. These observations support the hypothesis that *Cyp6g1* may be differentially regulated across DDT resistant *Drosophila* strains.

It has been reported that laboratory (WIS1lab) and field selected (WIS1 and Hikone-R) DDT resistant strains show differential expression of multiple P450 genes such as *Cyp12d1* and *Cyp6a8* and that only *Cyp6g1* is apparently overexpressed in both laboratory and field selected DDT resistant strains (Le Goff *et al*., 2003). In contrast, we have observed that *Cyp12d1* was inducible by DDT in the isochromosomal field-isolated Wisconsin strain, the field selected Hikone-R strain, and the laboratory selected 91-R strains. However, in Hikone-R, we found the lowest levels of *Cyp12d1* induction at the mRNA level. Therefore, it seems that not only one P450 (*Cyp6g1*), but at least two P450s (*Cyp6g1* and *Cyp12d1*) are associated with DDT resistance in these three *Drosophila* strains of diverse origins.

Cyp12d1 mRNA was induced in resistant strains by treatment with DDT. CYP12D1 protein expression was induced, albeit at a very low level, by treatment with DDT in 91-R and Hikone-R, but no induction was observed in the Wisconsin strain. Several factors could explain the discrepancy between higher level mRNA induction and the low level, or no protein induction: (i) there was minimal to no induction at the protein level; (ii) our experiments were not sensitive enough to detect differences at the protein level; (iii) there was cross-reactivity with epitopes from another protein that was constitutively expressed and this masked differences in CYP12D1 protein levels that may have occurred; (iv) there were post-transcriptional modifications influencing expression of CYP12D1 proteins; or (v) several of these phenomena occurred in combination. In the most basic terms, it is clear that there are increases in expression levels of *Cyp12d1* mRNA in both Hikone-R and Wisconsin post-treatment with DDT.

The 91-R *Cyp12d1* mRNA was smaller than the *Cyp12d1* mRNA of Wisconsin and Hikone-R, but CYP12D1 protein was the same size across all susceptible and resistant strains. Whether or not alternative splicing or different start sites are involved in the regulation of *Cyp12d1* expression in resistant strains is still unknown. Additionally, we do not know if the different sized bands are due to differences between expression of *Cyp12d1* and the recently reported *Cyp12d2* (Le Goff *et al*., 2003). This is beyond the scope of the present study.

The lower mRNA band that was labelled by the *Cyp12d1* probe was induced by DDT in 91-C. However, the 91-R strain constitutively expressed only the lower band, and this lower band was further induced by DDT. It is important to note that 91-C and 91-R come from a common genetic origin. 91-C has not been selected with DDT, but 91-R has been under heavy DDT selection in the laboratory for decades. One could hypothesize that this lower band may have been selected for constitutive expression through the many years of DDT selection. Alternatively, expression of the smaller band may be an artifact of the genetic bottlenecking of the 91-R population during DDT selection and it may play no role in resistance. At this point it is not clear what this lower band codes for, in terms of a protein, because it is too small to code for a full length P450.

If resistance to DDT were due to only a single P450, *Cyp6g1*, one would expect a direct correlation between mRNA/protein expression and the LC_{50} of resistant and susceptible strains. Toxicological analysis of susceptible and resistant *Drosophila* strains clearly show that *Cyp6g1* mRNA and protein expression levels do not correlate directly with LC_{50}s of the strains tested (Fig. 6). The highest levels of *Cyp6g1* expression were found in Hikone-R; a strain that has only intermediate DDT resistance levels. Of the three resistant strains analysed, the 91-R was the most resistant strain followed by Wisconsin (an approximate fourteen-fold difference in resistance levels). However, 91-R and Wisconsin did not show large differences in *Cyp6g1* expression levels. The susceptible strain 91-C was more tolerant to DDT than Canton-S, but the expression level of *Cyp6g1* was higher in Canton-S than 91-C. Consequently, we conclude that there is no direct correlation between *Cyp6g1* expression and DDT resistance. These data are in keeping with recent work by Schlenke & Begun (2004) showing that *Cyp6g1*'s association with resistance in *Drosophila simulans* may be population specific and not a universal phenomenon across all DDT resistant strains.

This does not rule out the possibility that *Cyp6g1* may play a role in resistance, but it does suggest that there is more to resistance than this single gene and the protein it encodes. These data are in keeping with microarray data from our laboratory, which shows overexpression of numerous detoxification enzyme genes, including *Cyp12d1*, in field collected and laboratory selected DDT resistant strains (Pedra *et al*., 2004). Pedra *et al*. (2004) also demonstrated that there are other P450s, GSTs, ion transport, and lipid and carbohydrate metabolism genes that are overtranscribed

in DDT resistant *Drosophila* strains. Data presented here demonstrate that the expression patterns of two P450s associated with the *Rst(2)DDT* locus (loci) vary between DDT resistant *Drosophila* strains. These findings support the hypothesis that there is evolutionary plasticity within the expression patterns of genes associated with P450-mediated metabolic pesticide resistance (Scott & Kasai, 2004).

Experimental procedures

Drosophila melanogaster

Drosophila strains were maintained in plastic bottles on standard medium (Applied Scientific, San Francisco, CA). Flies were transferred to new bottles every three weeks. For all five strains, Canton-S, Wisconsin, 91-C, 91-R and Hikone-R, the insects were reared at a constant room temperature of approximately 25 °C with a photophase of 16 h and a scotophase of 8 h. The susceptible strain, Canton-S, was obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). The resistant strain Wisconsin was collected from the field (Door County, WI) and was described previously by Pittendrigh (1999) and Brandt *et al.* (2002). The DDT resistant strain Hikone-R was originally collected in the field in Japan, and was obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). The 91-R strain was kindly provided by Professor Dr Ranjan Ganguly (University of Tennessee-Knoxville). To select highly resistant flies, in the 91-R strain, we performed bioassays with 4000 μg per vial; survivors were collected, and the population was re-established. Progeny from these flies were used in our experiments. Resistance to DDT in both 91-R and Wisconsin are only partially suppressible with piperonyl butoxide (PBO) (Pittendrigh *et al.*, 1997; Brandt *et al.*, 2002).

Manipulation of insects

Insects at a ratio of 3 females to 1 male were placed together in Petri dishes filled with the standard medium. Once the females began to lay eggs, the adults were transferred to new Petri dishes at 4-h intervals in order to obtain eggs of similar age. This allowed us to obtain synchronized developmental stages. Eggs laid within a 4 h interval were allowed to develop in order to collect the following groups: (i) first, (ii) second and (iii) third instar larvae; (iv) prepupae; (v) pupae; freshly eclosed (vi) females and (vii) males; and six day old (viii) males and (ix) females. To collect freshly eclosed flies, for the developmental experiments, males and females were collected 5 h after they emerged from the pupal stage. Also, males and females were kept together for 6 days after they emerged from pupae allowing them to mate and age. All samples were flash frozen at −80 °C, and total RNA and protein were extracted for further analysis.

Bioassays

Adult bioassays and data analysis were performed as described by Brandt *et al.* (2002). Larval bioassays were performed as follows. Acetone solutions containing specific amounts of DDT at the correspondent dose to the LC_{25} for the adult flies of each strain (Table 1) were pipetted into Petri dishes previously filled with the standard medium. These represented the doses at which the larvae from the respective strains were exposed. To provide uniform application of the pesticide inside the Petri dishes, the dishes were constantly shaken until the acetone evaporated. Petri dishes were left to sit for at least 1 h after the acetone had evaporated to ensure that there was no residual solvent in the Petri dishes. Experimental flies were collected using CO_2 as an anaesthetic, and flies at the ratio of 3 females to 1 male were placed together inside each Petri dish. The flies were transferred to new Petri dishes at 4-h intervals in order to obtain eggs of a similar age. Eggs laid in the Petri dishes were allowed to develop in order to collect the third-instar larvae that survived an exposure to DDT. The third-instar larvae which survived treatment with DDT were stored at −80 °C.

Whole insect protein isolation

About 400 first- and second-instar larvae, 100 third-instar larvae or 100 flies were crushed in microcentrifuge tubes using micropestles. The biological materials were homogenized in protein extraction buffer PEB [100 mM K_2HPO_4-KH_2PO_4, pH 7.4; 10% (v/v) glycerol; 1 mM PMSF; 0.1 mM DTT; 10 mM EDTA; Triton X-100 0.1% (v/v) and 10 mM β-mercaptoethanol]. Debris was precipitated by centrifugation at 4 °C for 10 min at 15 000 × *g*, and the total protein was recovered by filtering the supernatants. The microsomal fraction was separated from the soluble fraction using centrifugation at 4 °C for 60 min at 100 000 × *g*. The microsomal pellet was washed two times with PEB and was subsequently resuspended in PEB. Protein concentration was determined by the Bradford (1976) method using BSA as a standard. All steps were performed on ice or at 4 °C.

Isolation and sequencing of CYP6G1, CYP6G2, CYP12A5 and CYP12D1 clones

First-strand cDNA was synthesized from 1 μg total RNA from the Wisconsin strain using the Reverse Transcription System Kit (Promega Corp., Madison, WI) according to the manufacturer's instructions. A typical reverse transcription reaction consisted of 5 mM $MgCl_2$; 10 mM Tris-HCl (pH 9.0); 50 mM KCl; 0.1% (v/v) Triton X-100; 1 mM each dNTP; 20 U recombinant RNasin ribonuclease inhibitor; 15 U AMV Reverse Transcriptase; 0.5 μg Oligo$(dT)_{15}$ primer and 1 μg total RNA in a total volume of 20 μl. The reaction was incubated at 42 °C for 60 min, which provided a high cDNA yield. To inactivate the AMV Reverse Transcriptase, the reaction was incubated at 95 °C for 5 min and then 0 °C for 5 min. The cDNA of *Cyp6g1* or *Cyp12d1* was cloned into TOPO-2.1 and the cDNA of *Cyp6g2* or *Cyp12a5* was cloned into TOPO-4 according to the recommendations of the manufacturer (Invitrogen, Carlsbad, CA). To clone *Cyp6g1* and *Cyp12d1*, *Not*I sites immediately adjacent to the ATG and stop codon were created by PCR using the *Pfu* DNA polymerase (Stratagene, La Jolla, CA). PCR was performed for 35 cycles (45 s at 95 °C, 1 min at 62 °C, and 2 min and 30 s at 75 °C) with a final extension at 75 °C for 10 min. A PCR reaction consisted of: 20 mM Tris-HCl (pH 8.8); 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 0.1% (v/v) Triton X-100; 0.1 mg/ml nuclease-free BSA; 2.5 U *Pfu* Polymerase; 100 μM each dNTPs; 50–100 μg total first strand cDNA; 0.2 μM cyp6g1NR and cyp6g1NF, 0.2 μM cyp12d1NR and cyp12d1NF, 0.2 μM cyp12a5 +1F and cyp12a5 +1824 or 0.2 μM cyp6g2 +1F and cyp6g2 +1591R primers. The 3′ A-overhang reaction was performed for 15 min at 72 °C by adding 0.5 U *Taq* polymerase and 50 μM dATP following the TOPO TA Cloning Manual (Invitrogen, Carlsbad, CA). Primers with and without *Not*I restriction sites were designed according to CYP6G1, CYP6G2, CYP12A5 or CYP12D1 sequences deposited in Flybase (*http://flybase.bio.indiana.edu*),

and were obtained from MWG Biotech (High Point, NC). The following sets of forward and reverse primer combinations were used in the PCR reaction: cyp6g1NF (5′-GCG GCC GCA TGG TGT TGA CCG AGG TCC TCT TTG-3′) (coordinates 1–25, *Not*I site underlined) and cyp6g1NR (5′-GCG GCC GCT CAT TGG AGC GAT GGA GCG CTC TG-3′) (coordinates 1552–1575, *Not*I site underlined); cyp6g2 +1F (5′ATG GAA CTG GTA CTG CTG ATC CTC G-3′) (coordinates 1–25) and cyp6g2 +1591R (5′-TTA GAC GCC CAA AGC ATC GCG-3′) (coordinates 1591–1611); cyp12a5 +1F (5′ATG TTG AAA GGG CGT ATC GCA C-3′) (coordinates 1–22) and cyp12a5 +1824 (5′-TTA GTT GGG CAC ATC TGT AAA CTT A-3′) (coordinates 1824–1848) and cyp12d1NF (5′-GCG GCC GCA TGA ATA CAT TGA GCA GTG CGC G-3′) (coordinates 1–23, *Not*I site underlined) and cyp12d1NR (5′-GCG GCC GCT TAT TGT TCG ATA TCC GTG AAT TTG-3′) (coordinates 1542–1566, *Not*I site underlined) (Sambrook & Russell, 2001). The PCR products and DNA extracted from putative positives clones were separated by electrophoresis on 1% (w/v) agarose in TAE gels and visualized using ethidium bromide-staining under UV transillumination. The identity of CYP6G1, CYP6G2, CYP12A5 and CYP12D1 cDNAs was checked by complete sequencing of each clone at the Purdue Genomics Center. A set of internal primers for CYP6G1, CYP6G2, CYP12A5 and CYP12D1 was used to sequence the complete cDNAs of these genes. Primer walking was performed; at least three independent readings were carried out, and the CAP software (*http://bio.ifom-firc.it/ASSEMBLY/assemble.html*) was used to contig all the sequences. The CYP6G1, CYP6G2, CYP12A5 and CYP12D1 sequences were deposited in GenBank (*http://www.ncbi.nlm.nih.gov/*), respectively, under accession numbers AY081960, AY459353, AY459354 and AY081961.

Protein purification and production of CYP6G1, CYP6G2 and CYP12D1 antiserum

The pET system (Novagen Inc., Madison, WI) was used to clone and express recombinant CYP6G1, CYP6G2 and CYP12D1 proteins in *E. coli* bacteria. The full cDNAs of CYP12D1 and CYP6G1 were excised by digestion with *Not*I and the full cDNA of CYP6G2 were excised by digestion with *Eco*RI, and ligated into pET30a(+) at the *Not*I or *Eco*RI site. The recombinant vectors pET30a(+)-CYP12D1, pET30a(+)-CYP6G2, and pET30a(+)-CYP6G1 were used to transform *E. coli* [strain BL21(DE3)] (Sambrook & Russell, 2001). The pET30a(+) vector carries an N-terminal His-Tag and an optional C-terminal His-Tag sequence that allowed us to purify the CYP6G1, CYP6G2 and CYP12D1 proteins by Ni-affinity chromatography. The full length cDNAs of CYP6G1, CYP6G2 and CYP12D1 were synthesized under the control of the T7 RNA polymerase promoter in the *E. coli* strain BL21(DE3), which carries a T7 RNA polymerase gene regulated by the lac promoter. The synthesis of the recombinant proteins was induced by IPTG (Sigma-Aldrich Co, St Louis, MO). To perform the induction, clones were inoculated separately in LB medium supplemented with 30 μg/ml kanamycin and 1% (w/v) glucose, then incubated at 37 °C with shaking until an OD_{600} μm between 0.6 and 1.0 was reached. IPTG was added to the medium at a final concentration of 0.4 mM. After 3 h, the cells were harvested via centrifugation at 6500 × *g* for 10 min at 4 °C, and the cellular pellet was kept frozen until the next step. Protein extraction and purification were performed according to the pET System Manual (Novagen Inc., Madison, WI). The induced protein was visualized by electrophoresis on SDS-PAGE gels (Laemmli, 1970) with comassie staining. The purification of CYP6G1, CYP6G2 or CYP12D1 protein was performed using the His-Bind® Purification Kit (Novagen Inc., Madison, WI) following recommendations of the manufacturer. A second methodology was also used to purify the CYP6G1, CYP6G2 or CYP12D1 protein. The CYP6G1, CYP6G2 or CYP12D1 protein bands were excised from SDS-PAGE gels. Excised gel pieces containing the desired protein bands were placed inside a dialysis bag with running buffer (25 mM Tris-HCl, 200 mM glycine, 1 mM EDTA, and 3.5 mM SDS) and were electrophoresed with the same buffer. After electrophoresis, gels pieces were dialysed against running buffer without glycine. The solution that contained the CYP6G1, CYP6G2 or CYP12D1 protein was aliquoted and stored at –80 °C. Approximately 150 μg purified CYP6G1, CYP6G2 or CYP12D1 protein was used per injection per rabbit. Four injections were made with a space interval in between each injection of 1 week. The rabbits were bled once a week after the second injection and resultant serum was stored at –80 °C.

Immunoblot analysis

Equivalent amounts of microsomal proteins (35 μg) were separated by 10% SDS-PAGE (Laemmli, 1970). The resolved proteins were electrotransferred to a nitrocellulose membrane using a blot apparatus (Bio-Rad, Hercules, CA) following the recommendations of the manufacturer. The membrane was blocked with 5% (w/v) non-fat dry milk in TBST [100 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% (v/v) Tween-20]. The membrane was incubated with the polyclonal anti-CYP6G1, anti-CYP6G2 or anti-CYP12D1 sera at a 1 : 1000 dilution for 1 h. This was followed by incubation for 1 h with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma-Aldrich Co, St Louis, MO) at a 1 : 5000 dilution. Sigma Fast™. tablets (Sigma-Aldrich Co, St Louis, MO), containing BCIP and NBT, were used to visualize CYP6G1, CYP6G2 or CYP12D1 protein bands based on alkaline phosphatase activity of the second antibody.

RNA isolation, dot blots and Northern blots

Total RNA was extracted using TRI reagent or RNeasy kit (Qiagen, Valencia, CA). About 100 third-instar larvae or 100 adult flies were crushed in microtubes using micropestles. The contents of the microtubes were vortex homogenized for 20 s, followed by RNA extraction. Total RNA for gel blot analysis was isolated from third-instar larvae, males or females. Total RNA was quantified by spectrophotometry (Smartspec™ 3000, Bio-Rad, Hercules, CA), and equivalent amounts of total RNA were denatured by formamide/formaldehyde, and then visualized on 0.8% (w/v) agarose gels containing 0.75 M formaldehyde. The RNA was transferred to a Duralon-UV nylon membrane (Stratagene, La Jolla, CA) by capillary transfer and immobilized by UV cross-linking. The prehybridization and hybridization steps were performed using Washing and Pre-Hyb Solution and High Efficiency Hybridization System Solution, respectively (Molecular Research Center Inc., Cincinnati, OH). The nylon membrane was prehybridized for 20 min at 65 °C, followed by hybridization for 2 h at 60 °C. The CYP6G1 and CYP12D1 clones were digested with *Not*I. The released CYP6G1 or CYP12D1 cDNA was gel-purified and labelled using the Primer-IT Fluor Fluorescence Labeling Kit (Stratagene, La Jolla, CA). Probe detection was accomplished using the Illuminator™. Chemiluminescent Detection System (Stratagene, La Jolla, CA). Autoradiography was performed at room temperature using a Lightning-Plus intensifying screen (Sigma-Aldrich Co., St Louis, MO). The quantity and the integrity of the RNA were determined

by re-hybridizing the membranes with an actin cDNA probe. The northern blots were repeated three times (minimally) and quantified using a densitometer; the P450 expression levels were standardized using the actin controls.

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