To the Graduate Council:

I am submitting herewith a thesis written by Vita Lam entitled "*Cyp6w1* gene of *Drosophila melanogaster*: Studies on its role in DDT resistance and inducibility by different xenobiotic compounds." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry, Cellular and Molecular Biology.

_____
Ranjan Ganguly, Major Professor.

We have read this thesis
and recommend its acceptance:

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Cyp6w1 gene of *Drosophila melanogaster* : Studies on its role in DDT resistance and inducibility by different xenobiotic compounds

A Thesis

Presented for the Master of Science Degree

The University of Tennessee, Knoxville

Vita Lam

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This thesis is dedicated to my parents,

Sing Sum and Wandy Lam,

without whose endless hard work and values,

this would not have been possible

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ABSTRACT

Cytochrome P450 monooxygenases (CYP), a large family of enzymes, play an important role in insecticide resistance. Compared to the susceptible strains, the resistant strains of *Drosophila* show higher expression of one or more *Cyp* genes including *Cyp6w1*, which maps close to the resistance locus. In the present investigation I examined the *Cyp6w1* gene's role in DDT resistance and inducibility with caffeine, DDT and phenobarbital (PB). Utilizing the Gal4:UAS binary system, I overexpressed CYP6W1 RNA in either a susceptible strain alone or in combination with CYP6A2 or CYP6G1 RNA, which are known to give a low level of DDT resistance. When CYP6W1 was combined with CYP6G1 or CYP6A2, a modest increase in DDT resistance was observed. However, CYP6W1 may not be responsible for this increase because overexpression of CYP6W1 alone did not confer any DDT resistance.

In the second objective, the 0.1-, 0.5- and 0.9-kb upstream DNAs of the *Cyp6w1* gene spanning -1/-95, -1/-510 and -1/-937 bp regions were examined for caffeine, DDT and PB inducibility using the firefly luciferase reporter gene system and transient transfection of *Drosophila* S2 cells. Results showed that basal transcriptional activity of the 0.5- and 0.9-kb DNA was 2-fold greater than the activity of the 0.1-kb DNA, but none of the upstream DNA fragments was induced by DDT. While 0.1-kb DNA did not show much induction with any chemical, 0.5-kb DNA showed about 1.5-fold and 2.0-fold inductions following PB and caffeine treatment, respectively. The 0.9-kb DNA, on the other hand, showed almost a 3-fold induction with caffeine or PB. When cells transfected with 0.9-kb or 0.5-kb DNA were treated with a mixture of caffeine and PB, the level of induction was significantly greater than the induction level observed with

each chemical alone. Taken together, these results suggest that DNA between -510 and -937 contains cis-acting sequences that give high levels of caffeine and PB induction. It appears that caffeine and PB act via independent pathways because they show a synergistic effect when combined together.

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I. Introduction

History of Cytochrome P450 enzymes

Members of the Cytochrome P450 monooxygenase (CYPs) family, one of the oldest and largest multigene superfamily of enzymes, are found throughout the phylogeny ranging from bacteria to humans (Nelson, 1999; Scott, 1999). They were first discovered in the 1950s when oxidation reactions were found to take place in rat liver microsomes. Specifically, when the microsomes were treated with the reducing agent, sodium dithionite, and gassed with carbon monoxide, a unique optical absorbance peak at 450 nm was observed (Klingenberg et al., 1958; Estabrook, 1996; Omura, 1999). Later, in the 1960s, Omura and Sato (1962, 1964) discovered that this pigment contained a heme moiety, and thus aptly named it cytochrome P450 or CYP. Later, Estabrook et al. (1963) observed a CYP-mediated hydroxylation of the steroid hormone 17α-hydroxy progesterone at the C21 position in microsomal fractions of the adrenal cortex. A year later, Cooper et al. (1965) discovered CYP's role as a terminal oxidase in the metabolism of drugs, which led to its renowned function as the main detoxifying enzyme in liver microsomes.

Today, many different forms of CYPs have been found in all taxonomical groups and in all eukaryotic organisms, animals, plants, fungi, and even some prokaryotes (Omura, 1999), but the number of families and enzymes varies among the different groups. As of February 29, 2008, a total of 781 P450 families have been reported: 110 in animals, 95 in plants, 205 in bacteria (includes 10 Archaeal), 61 in protists, and 310 in fungi. To date a total of 8128 P450 sequences, which includes psuedogenes, have been reported. The amount in different species is variable. Humans have as little as 60, mice

have 103, and rice has as much as 452 sequences identified (http://drnelson.utmem.edu). Some organisms do not have any: *Salmonella typhimurium* and *Plasmodium falciparum* (Guengerich, 2003). The genome sequencing project has documented that *Drosophila melanogaster* has 90 *Cyp* genes of which seven are thought to be pseudogenes (Tijet et al., 2001).

Molecular structure of P450s

In eukaryotes, cytochrome P450s are found in the endoplasmic reticulum and mitochondria, however most bacterial P450s exist in soluble form (Omura, 1999; Scott, 1999). Chemical sequencing in 1982 by Haniu et al. determined the total amino acid sequence of P450cam (in *Pseudomonas putida*), and quickly afterwards, its tertiary molecular structure was solved by x-ray crystallography (Poulos et al., 1985). The crystal structure revealed that the shape of P450cam is like an asymmetrical triangular prism made up of 12 alpha helices and five anti-parallel beta sheets (Figure 1). The entire structure is surrounded by hydrophobic amino acid residues to provide accommodation for its hydrophobic substrates. Other bacterial P450s were soon discovered to have a similar structure as P450cam. During that same time, the first eukaryotic P450 amino acid sequence, P450 2B1, in rats was being elucidated by cDNA cloning techniques and found to be analogous to that of its soluble bacterial counterparts (Omura, 1999).

Sequence and gel electrophoretic analysis showed that CYPs are approximately 500 amino acid long single polypeptides and their molecular weight ranges between 46-55 kDa. CYPs have a highly conserved C-terminal half that contains the binding site for

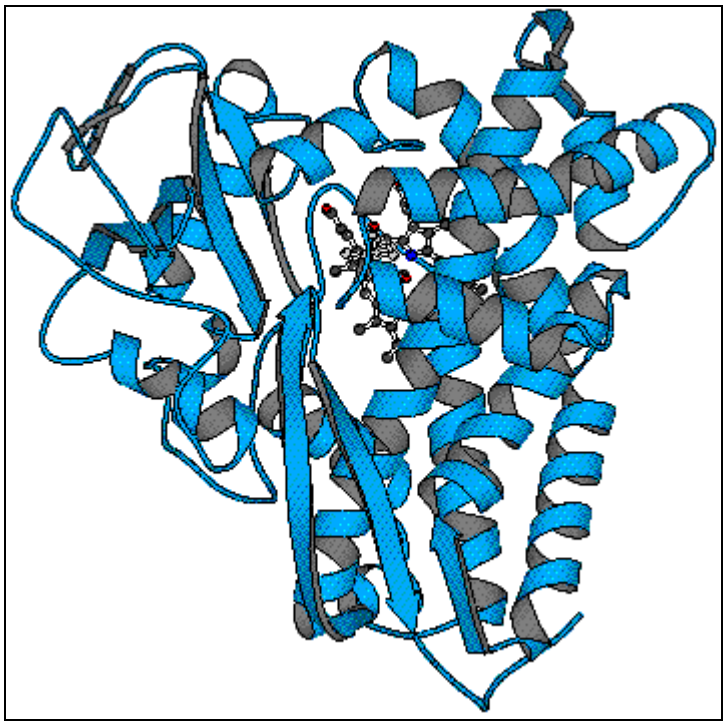


Figure 1. Ribbon representation of the *Pseudomonas putida* P450cam complex with CO and camphor structure. It is shaped like an asymmetrical triangular prism made up of 12 alpha helices and five anti-parallel beta sheets (Raag and Poulos, 1989).

a single heme prosthetic group with the axial *Cys* ligand within a conserved sequence (FXXGXXXCXG) (Lewis, 1996). The N-terminal end has a "signal anchor sequence" composed of 20-25 amino acid residues. This signal sequence targets and anchors the P450 molecules to the ER membrane (Sakaguchi et al., 1987; Omura, 1999). The N'-terminal regions of P450 enzymes show sequence diversity and are involved in substrate recognition.

Metabolic functions of P450s

P450s are involved in an array of metabolic functions that are vital to the survival of living organisms. The P450 enzymes generally catalyze a conserved monooxygenation reaction as shown:

$$2NAD(P)H + O_2 + R \xrightarrow{\text{P450 enzyme}} 2NAD(P)^+ + H_2O + RO$$

In this reaction, an oxygen molecule is split into two atoms. One of these atoms is inserted into the substrate and the other is combined with two hydrogen atoms to form water. CYP also catalyze a wide range of reactions such as aromatic and aliphatic oxidation, dealkylation, oxidative deamination, etc (Cooper et al., 1965; Agosin, 1985; Lewis, 1996).

Metabolic function of CYPs is diverse also. They are known to be involved in the biosynthesis of various types of molecules in mammals and plants such as steroid hormones, vitamins, oxylipins, cholesterol, plant pigments etc. (Nebert, 1994; Coon et al., 1996; Guengerich, 2003). In insects, P450 enzymes regulate the steps in ecdysteroid and juvenile hormone biosynthesis, which are at the center stage of insect growth, development, feeding, and reproduction. In *Drosophila*, four genes, *phantom*,

disembodied, *shadow* and *shade*, are involved in ecdysone biosynthesis. Sequence analysis showed that these genes encode CYP306A1, CYP302A1, CYP315A1 and CYP314A1, respectively (Rewitz et al., 2006). Mutation of these genes causes embryonic deformity and lethality, and for these reasons these genes are called Halloween family genes. CYPs are also known to take part in various metabolic pathways in plants including biosynthesis of brassinosteroids, which are essential for normal plant growth and development. Mutation of these P450 genes, e.g., *CYP90A1* and *CYP90B1* in *Arabidopsis*, causes dwarfism (Bishop, 2006). In plants, P450s also take part in many different metabolic pathways and biosynthesis of signaling molecules such as gibberellins, auxin, abscissic acid, jasmonic acid, oxylipins and possibly, salicylic acid (Schuler and Werck-Reichhart, 2003).

P450's role in drug, insecticide, and other xenobiotic metabolism

P450s are best known for their involvement in the metabolism of various xenobiotics (synthetic foreign substances) and drugs, environmental pollutants, and different toxic chemicals including insecticides. Since they can catalyze a variety of chemicals, sometimes CYPs cause metabolic activation of harmless compounds into harmful compounds (Scott, 1999). In humans, the metabolism of xenobiotic compounds occurs in two phases. Phase I reaction is catalyzed mostly by P450 enzymes. In this reaction, an OH^- group is added to the compound, causing metabolic activation and an increase in reactivity. In phase II reaction the products of the phase I reaction are made more hydrophilic so that they can be excreted easily (Nebert et al., 1996). In humans, CYPs belonging to family 1, 2 and 3 are involved in the metabolism of various drugs. It

has been shown that coumarin, erythromycin, caffeine, and debrisoquine are metabolized by CYP2A6, CYP3A4, CYP1A2, and CYP2D6, respectively (Guengerich, 2003).

Compared to mammals, very few studies have been done on insect P450s with respect to xenobiotic metabolism. Unlike in mammals, the metabolic studies in insects have not been done using the cellular extracts obtained from the insect cells except in one study. It was shown that extract prepared from *Musca domestica* could metabolize pyrethroid and organophosphates, and the reaction could be inhibited by antibody against CYP6D1 (Wheelock and Scott, 1992 a, b; Hatano and Scott, 1993). Most metabolic studies with insect *CYP* genes have been done using a heterologous expression system such as *E. coli*, SF9 cells, S2 cells, and tobacco cells. It was shown that CYP6A1 of *Musca* could metabolize cyclodiene insecticides when it was expressed in *E. coli* (Anderson et al., 1994). When expressed in S2 cells, *Cyp6a2* of *Drosophila* was shown to metabolize chemicals such as aldrin, haptachlor, diazinon, but not DDT (Helvig et al., 2004; Pedra et al., 2004; Daborn et al., 2007). However, mutant *Cyp6a2svl* allele of a resistant strain of *Drosophila* has been shown to metabolize DDT when expressed in *E. coli* (Amichot et al., 2004). Recently, Joussen et al (2008) showed that tobacco cells expressing *Drosophila* CYP6G1 metabolized DDT and imidacloprid insecticide in vitro. Although xenobiotic metabolism is highly studied, the molecular mechanisms by which most of these CYP genes are regulated remain to be elucidated, but progress has been made on the induction of bacterial and mammalian CYP genes. These induction studies have helped scientists better understand the mechanism of regulation (Porter and Coon, 1991).

Induction of *CYP* genes

Again, major understanding about the mechanism of *CYP* induction comes from the studies in mammals. In humans, CYPs belonging to families 1, 2, 3 and 4 are induced by a wide variety of drugs and other xenobiotic compound such as barbiturate, dexamethason, rifampicin, tobacco smoke, ethanol, etc (Guengerich, 2003). The *CYP1A1* and genes belonging to *CYP2B* family of human are the most widely studied *CYP* genes with respect to the mechanism of xenobiotic induction (Ma, 2001; Sueyoshi and Negeshi, 2001). These studies discovered aryl hydrocarbon receptor and constitutive androstane receptor (CAR) which are involved in the regulation of xenobiotic induction. These studies also identified the *cis*-regulatory elements that control the transcriptional induction of these *CYP* genes.

Drosophila and housefly P450 genes are also induced by an array of xenobiotic compounds such as phenobarbital, barbital, atrazine, ecdysone, caffeine, and a variety of pesticides including diazinon, lufenuron, and DDT, to name a few (Maitra et al., 1996, 2002; Dombrowski et al., 1998; Kasai and Scott, 2001; Bhaskara et al., 2006, 2008; Willoughby et al., 2006). Although the mechanism of the induction process is not well understood, Bhaskara et al (2008) demonstrated that *Drosophila Cyp6a8* gene is regulated by cAMP and activator proteins (AP1) transcription factors.

P450's and insecticide resistance

Resistance is the ability of an organism to survive doses of insecticide that are generally toxic to normal individuals. Cytochrome P450s has long been implicated in insecticide resistance. First evidence of this phenomenon came about when resistant

insects were treated with CYP inhibitors such as piperonyl butoxide (PBO) or sesamex, the treated insects became susceptible (Feyereisen, 1999). Another observation that the resistant insects have higher expression of one or more P450 genes than the susceptible ones also made P450s the main enzymes involved in insecticide resistance. Higher expression of a *CYP* gene in the resistant strain compared to the susceptible ones have been observed for *CYP6A1*, *CYP6D1* and *CYP6D3* of *Musca* (Carino et al., 1994; Feyereisen et al., 1995; Tomita et al., 1995; Kasai and Scott, 2001), *CYP6F1* of *Culex* (Kasai et al., 2000) and *P450 MA* of German cockroach (Scharf et al., 1999). In adult *Drosophila* at least four *Cyp* genes show higher expression in the resistant strains than in the susceptible ones. These are *Cyp6a2, Cyp6g1, Cyp12d1 and Cyp6a8* (Waters et al., 1992; Maitra et al., 1996; Dombrowski et al., 1998; Daborn et al., 2002; Pedra et al., 2004). CYPs have the capacity to confer metabolic resistance to different insecticides, which explains the observations that resistant insects have higher levels of one or more CYPs than their susceptible counterpart. A higher level of P450 enzymes is expected to catalyze faster metabolism of DDT and other insecticides. Cytochrome P450 enzymes are known to metabolize DDT and imidacloprid in vitro (Kitamura et al., 2002; Joussen et al., 2008). Now, it is widely believed that this upregulation of *CYP* genes may be responsible for insecticide resistance in many insects (Scott and Wen, 2001).

Dichloro-diphenyl-trichloroethane (DDT), originally created to combat mosquitoes spreading malaria, typhus, and other insect-borne human diseases, has become the most commonly used organic pesticide today in many developing nations. DDT kills insects by opening the voltage gated sodium ion channels in the neurons, causing the neurons to fire spontaneously. This leads to spasms and eventual death. In

Drosophila, the voltage gated sodium channel protein is encoded by the X-linked *para* gene. Although DDT is the most effective insecticide to control insects, problems associated with extensive use of DDT began to appear in the late 1940s. Insects began to develop resistance to DDT. In some cases, resistance is a result of mutations in the sodium channel gene. This is called target site mutation. The mutated channel protein remains functionally active but it cannot bind with DDT. As a result, the mutant flies do not experience the toxic effect of DDT. However, as mentioned above, in most cases the resistance is mediated by CYPs.

Because of the evolution of resistance in insects, doses of DDT and other insecticides had to be doubled or tripled. This created an environmental problem. Evidence began to grow that DDT has a tendency to become more concentrated at higher levels in the food chain. DDT has also been cited as a major cause for the decline of the bald eagle in the 1950s and 1960s (USEPA, 2006). DDT disrupts calcium absorption and thereby impairs the egg-shell quality (Cooke, 1973). In addition to acute toxic effects, the overuse of DDT may cause a significant bioaccumulation in fish and other aquatic species, leading to long-term exposure to high concentrations (U.S. FWS, 2006). DDT and its metabolite DDE are called environmental estrogens and have been linked to cancer and a decline in male fertility (Stone et al., 1994; Kelce et al., 1995; Swan et al., 1997).

These observations make it clear that not only do resistant insects cause a loss of billions of dollars every year in agricultural industry, but the chemicals used to combat the resistant insects also pose a severe environmental threat. Therefore, the need to find means to control resistant insects in an environmentally friendly manner has become a

necessity. Understanding the molecular basis by which insects become resistant may help in this matter (Scott, 1999). Although *Drosophila* is not a pest, it has been used as a model organism to understand various biological phenomena that are operating in higher organisms including humans, because the most basic mechanisms are conserved in evolution.

The versatility in function found in the P450 superfamily results primarily from a diversity of sequences encoded by a multiplicity of genes. Although the function of many P450 proteins from vertebrates, fungi, plants, and bacteria is known, only a single P450 from *Drosophila melanogaster*, CYP6A2, has been functionally characterized (Tijet et al., 2001; Wilkinson, 1983; Saner et al., 1996; Dunkov et al., 1997). More than half of the cytochrome P450 genes belong to only two families, CYP4 and CYP6. The CYP6 family is insect specific whereas the CYP4 family genes are also found in vertebrates. The genetic map of the distribution of *D. melanogaster* P450 genes shows (a) the absence of P450 genes on chromosomes 4 and Y, (b) more than half of the P450 genes are found on chromosome 2, and (c) the largest cluster contains nine genes.

Objective of the present investigation

Because cytochrome P450 genes are comprised of such a large and functionally diverse family, the capability of various CYP genes to confer insecticide resistance is difficult to predict. Earlier studies mapped the resistance locus of *Drosophila* close to ~64-67 cM on the right arm of chromosome 2 (Tsukamoto and Ogaki, 1953; Tsukamoto, 1958), however, other studies showed that insecticide resistance is not associated with just one locus, but rather multiple loci located on chromosomes 2 and 3 (Crow, 1957;

King and Somme, 1958). The loci responsible for resistance may vary from strain to strain. While in many strains of *Drosophila*, the resistance locus maps close to 64 m.u. of chromosome 2, in the 91-R strain DDT resistance locus is located at ~ 56 cM on chromosome 2 (Dapkus, 1992). These results suggest that insecticide resistance may be a multifactorial trait. Indeed, Dapkus and Merrel (1977) showed by chromosome substitution experiments that all three major chromosomes are involved in DDT resistance in 91-R strain.

In *Drosophila*, multiple *Cyp* genes which map close to the resistance loci at 64 and 56 cM show significantly higher level of expression at RNA level compared to the susceptible strains (Maitra *et al*., 1996; Dombrowski *et al*., 1998; Pedra et al., 2004). These are *Cyp6a2* and *Cyp6w1* close to locus 56, and *Cyp6g1, Cyp12d1* and *Cyp6a8* close to locus 64. However, it is not known whether only one or multiple *Cyp* genes are involved in DDT metabolism, although CYP6G1 has been shown to confer low levels of DDT resistance (Daborn et al., 2002). Recently, Daborn et al (2007) used the Gal4:UAS system and showed that CYP6G1 and CYP12D1, but not CYP6A2 or CYP6A8, could confer low levels of DDT resistance. However, Amichot et al (2004) showed that CYP6A2 could metabolize DDT when expressed in E. coli.

Cyp6w1 is among one of the genes that is also located on chromosome 2R. It is on band 42A12, which is close the resistance locus at 56 m.u mapped by Dapkus (1992*).* *Cyp6w1* is preferentially expressed in the third antennal segment, but also in the legs and wings, where the taste sensilla are located in this species (Wang *et al*., 1999). Microarray analysis has shown that the DDT resistant strains have much higher level of CYP6W1 RNA than the susceptible strain (Pedra et al., 2004). However, the roles of CYP6W1 in

DDT resistance and inducibility to xenobiotic compounds have not been examined. Therefore, the objectives of this present investigation focus on examining these two aspects of the *Cyp6w1* gene:

1. The role of *Cyp6w1* in DDT resistance by using Gal4:UAS binary expression system, performing DDT bioassays, and measuring the lethal dose at which 50% die at various DDT concentrations.
2. The inducibility of *Cyp6w1* to different xenobiotic compounds by creating *Cyp6w1-luciferase* chimeric reporter plasmids, subjecting to caffeine, phenobarbital, and DDT, and measuring F-luc/R-luc activity.

II. Materials and Methods

Drosophila strains and culture conditions

All *Drosophila* stocks were raised on standard cornmeal-agar-molasses medium at 24º C under a 12-hour dark and 12-hour light cycle. The DDT resistant 91-R strain used in this investigation was obtained from Larry Waters, Oak Ridge National Laboratory. Originally, a population of *Drosophila* was collected from the wild in 1952 in St. Paul, Minnesota. The flies collected were initially separated into two groups. The first group, named 91-R, was subjected to DDT selection while the other group, called 91-C, was maintained on normal food medium (Merrell and Underhill, 1956). For about another 20 years, Dapkus and Merrell (1977) continuously selected 91-R flies that survived increasing doses of DDT exposure. Since 1988 the 91-R strain used in this investigation has not been through anymore DDT selection. However, periodic assays showed that 91-R is still super-resistant strain and can survive high doses of DDT which would normally kill all other strains.

The *Drosophila melanogaster* strain, w^{1118}, was used as a host strain for germ-line transformation studies. The *w; Bl/CyO; +/+* and *yw; +/+; TM6C/+* are the second and third chromosome balancer stocks, respectively, that were used for chromosome linkage analysis. All balancer stocks were obtained from Bloomington Stock Center in Bloomington, IN. Two stocks used for overexpression studies were obtained from Dr. Jae H. Park and Bloomington *Drosophila* Stock Center respectively: Fat body enhancer gal4 (*w; +/+; FB-GAL4/FB-GAL4)* and Tubulin gal4/Sb *(w/w; +/+; Tubulin-GAL4/Sb)*. The Canton S (NY) wild type strain was received from Dr. Indrani Ganguly at Neuroscience Institute, La Jolla.

Isolation of total RNA

Total RNA samples were isolated from 40-50 adult female flies using TRI Reagent (Sigma, St. Louis, MO). The flies were thoroughly homogenized in a 1.5ml eppendorf tube with 500µl of TRI Reagent. Another 500µl of TRI Reagent was added after complete homogenization and vortexed. Centrifugation at 12,000 X g at 4ºC for 10 minutes ensued. The clear supernatant was transferred to a fresh tube and allowed to stand at room temperature for 5 minutes. Afterwards, 200µl of chloroform was added, vortexed, and allowed to sit for 5 minutes at room temperature. Centrifugation followed at 12,000 X g at 4ºC for 15 minutes. The upper aqueous phase was again transferred to a fresh tube with 500µl of isopropanol. Incubation at room temperature for 10 minutes occurred before another centrifugation at 4ºC for 10 minutes that pelleted the RNA. The RNA pellets obtained from this isolation were rinsed with chilled 70% ethanol, dried, and resuspended in an appropriate volume of DEPC treated water that completely dissolved the pellet, usually 100µl.

Synthesis of CYP6W1 cDNA

To study the role of CYP6W1 on DDT resistance, a transgenic approach was used. The overall procedure is outlined in (Figure 2). Total RNA isolated from 91-R strain was utilized for synthesizing first strand cDNA using Invitrogen's Superscript 1st strand synthesis kit. First, about 1µl of total RNA with a concentration of about 3µg was mixed with 1µl of 10mM dNTP mix, 1µl of oligo (dT) $_{12\text{-}18}$, and 7µl of water. The RNA:primer mixture was incubated for 5 minutes at 65ºC, cooled on ice for 1 minute and added to the reaction mixture made with 2µl of 10X RT Buffer, 4µl of 25mM $MgCl_2$, 2µl

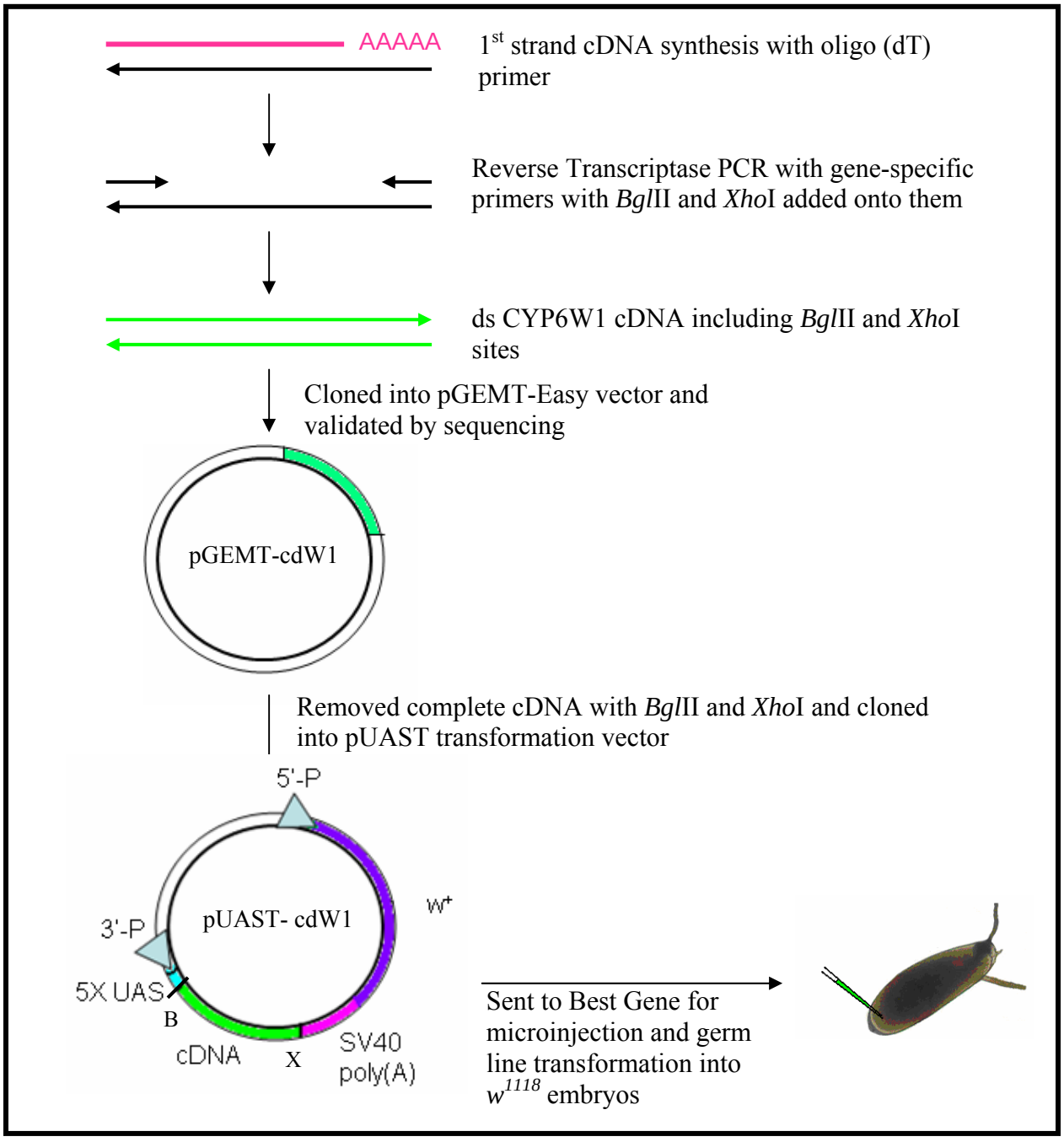


Figure 2. Cloning of CYP6W1 cDNA into pUAST vector for germ line transformation

of 0.1M DDT, and 1µl of RNAse OUT (RNAse inhibitor). The combined mixture was incubated at 42ºC for 2 minutes and then the reverse transcription reaction was started by adding 1µl of Superscript II RT. The final reaction mixture (50µl) was incubated at 42ºC for 50 minutes after which the reaction was terminated by incubating at 70ºC for 15 minutes. The reaction mix was then cooled on ice, mixed with 1µl of RNAse H enzyme and incubated at 37ºC for 20 minutes to digest the RNA present in the RNA:DNA hybrid. The resulting first strand cDNA made against whole cellular RNA was stored at -20ºC until the next procedure.

To synthesize double-stranded CYP6W1 cDNA, 10% (or 2µl) of the first strand reaction was PCR amplified using primers specific for the *Cyp6w1* gene. The sequences of the primers are shown in Table 1. For final cloning into pUAST vector in proper orientation, BglII and XhoI sites were added to the 5'-end of the forward and reverse primers, respectively. The forward and the reverse primers are complimentary to +12/+33 and +1707/+1725 regions located within the 5'- and 3'-UTRs of the *Cyp6w1* gene, respectively. The PCR reaction was done by using reagents obtained from Sigma. To avoid errors during the amplification, high fidelity Platinum PFX DNA polymerase (Invitrogen) was used. After an initial incubation for 2 min at 94^0C, the following cycle parameter was used and repeated 34 times: 94ºC for 1 min, 60ºC for 45 sec, and 72ºC for 2 min. After the completion of 34^{th} cycle, a 3 min incubation at 72ºC was done. The PCR product i.e., CYP6W1 ds-cDNA was purified using QIAquick PCR purification kit (Qiagen, CA) and analyzed on 1% agarose gel for its size.

Primer Name	Restriction enzyme site added	Primer binding regions	Sequence of the primer
1. W1-GENE-F	Bgl II	+12/+33	5'-ggcagatctGCACCCAACATGTTGTTACTGC-3'
2. W1-GENE-R	Xho I	+1707/+1725	5'-ccgctcgagTATTTGGGCGGCTGCTGTG-3'

Table 1. Primers used for the amplification of *Cyp6w1* cDNA. Extra bases added to the 5' end of each primer are shown in lower case and the added restriction enzyme sites are underlined. W1-GENE-F and W1-GENE-R primers bind with 5'- and 3'-UTR of the *Cyp6w1* gene, respectively.

Cloning of CYP6W1 ds-cDNA in pUAST transformation vector

The double-stranded CYP6W1 cDNA synthesized by PCR was cloned into pUAST vector for germ line transformation via a two-step process. First, the PCR product was cloned into pGEMT-easy (Promega) TA cloning vector following the manufacturer's protocol. The colonies collected from the transformation were processed using Wizard Plus SV Miniprep DNA Purification System (Promega). The purified DNA was digested with EcoRI restriction enzyme because the cloning site in pGEMT-easy vector is flanked on either side by an EcoRI site. Clones that gave ~ 1.7-kb DNA fragment following EcoRI digestion were positive. To further verify, both strands of the cDNA insert was sequenced at the Molecular Biology Resource Facility at University of Tennessee, Knoxville. The resulting DNA sequences were analyzed by BLAST program and compared with the CYP6W1 sequences available in the database.

In the second step, CYP6W1 cDNA insert was isolated from the pGEMT vector by cutting with BglII and XhoI, purified by using QIAquick purification kit from Qiagen, and ligated with pUAST vector digested with the same enzyme pair. The ligation mix was used to transform *E. coli* DH5α bacteria and plated on ampicillin-containing agar plate. Plasmid DNA was purified from 10 randomly chosen colonies and examined for the presence of CYP6W1 cDNA insert by digesting with BglII and XhoI. The colony that turned out to be positive was used to isolate large quantities of the plasmid DNA. The plasmid was named pUAST/cd w1.

Germ line transformation and synthesis of transgenic lines

The purified pUAST-CYP6W1 recombinant plasmid was sent to Best Gene Inc (Chino Hills, CA) for their germ line transformation service using *white*^{1118} (*w*^{1118}) as the host strain. In a few weeks, they had about 115 G_0 larvae that survived. The 80 G_0 adult flies that emerged from these larvae were individually crossed to the opposite sex of the *w*^{1118} host strain. If a G_0 embryo carried the transgene in the germ line, some of its progeny (G_1 flies) may have red/orange eye color because the pUAST vector has mini-white$^+$ gene as dominant selectable marker. G_1 transformants with w^+ (red/orange) eye color were collected from the G_0 cross and individually mated with *w*^{1118} strain to establish independent transformant lines.

Chromosomal linkage and synthesis of homozygous transgenic lines

The next step involved checking each line for the transgene structure via PCR and sequencing. Out of the ten lines, five were used for the project. In order to determine chromosome linkage, all five transgenic lines' virgins were independently crossed with males from the 2nd or 3rd chromosome balancer stocks (w; Bl/CyO; +/+ or yw; +/+; TM6C, Sb, Tb/+ that has curly wings (Cy) or stubble bristles and tubby (Sb,Tb), respectively, as dominant markers). The segregation of the mini-white gene giving red/orange eye color from the dominant *Cy* or *Sb, Tb* visible marker was observed in the F2 progeny. The segregation pattern determined the linkage of the transgene to one of the four linkage groups. In the cross with the 2nd chromosome balancer w; Bl/CyO; +/+, if F2 Cy flies were always white eyed, then the transgene is located on the 2nd chromosome. However, if the Cy flies were white or orange, then the transgene was not

located on the 2nd chromosome. Yet, if all females, regardless of being Cy or not, are orange eyed (and all males are white eyed), then the transgene is located on the 1st chromosome. In the cross with the 3rd chromosome balancer yw; +/+; TM6C, Sb, Tb/+, if F2 Sb, Tb flies were always white-eyed, then the transgene is located on the 3rd chromosome. On the other hand, if Sb, Tb flies are white or orange, then the transgene was not located on the 3rd chromosome. Nonetheless, if all females, regardless of being Sb, Tb or not, are orange eyed (and all males are white eyed), then the transgene is located on the 1st chromosome (Figure 3). Once the chromosome linkage of the transgene for each line was determined, crosses were done to make each line homozygous for the transgene. The strategy used for this purpose is shown in Figure 4.

Chromosomal localization of CYP6W1 transgene by inverse PCR

To find the cytological position on the 3rd chromosome, I performed inverse PCR (strategy outlined in Figure 5). Berkely Drosophila Genome Project's protocol was used (http://www.fruitfly.org/about/methods/inverse.pcr.html). In brief, thirty unsexed flies were collected and frozen at -80°C. Once frozen, they were homogenized in 200µl Buffer A which consisted of 100mM Tris-HCl, pH 7.5, 100mM EDTA, 100mM NaCl, and 0.5% SDS. Another additional 200µl of Buffer A was added and homogenized until only the cuticles remain. The homogenate was incubated at 65°C for 30 minutes. Then, 800µl LiCl/KAc Solution (1 part 5M KAc stock : 2.5 parts 6M LiCl stock) was added and incubated on ice for at least 10 minutes. Afterwards, the solution was centrifuged for 15 minutes at RT at 12,000 x g. One ml of the supernatant was transferred into a new

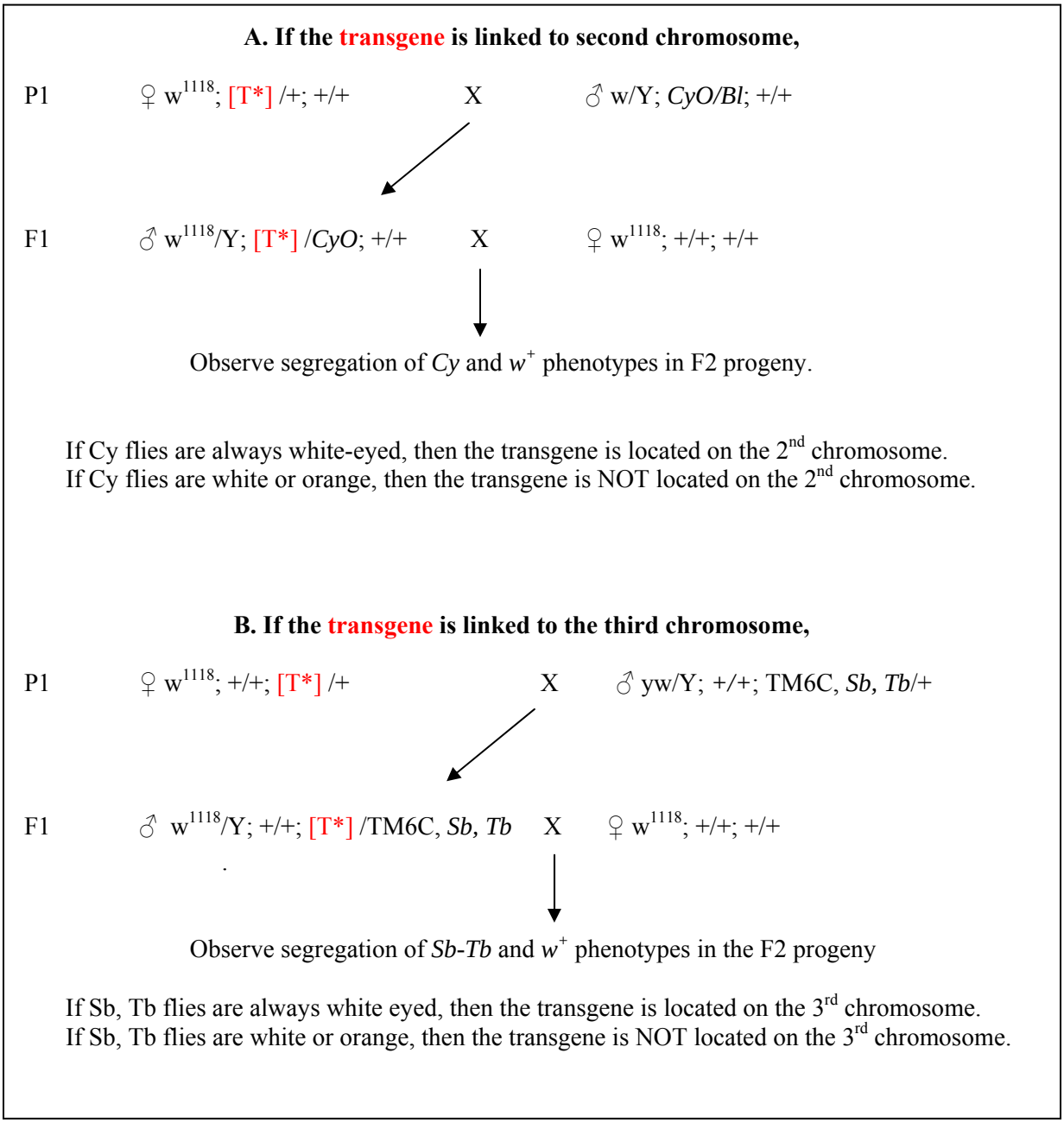


Figure 3. Genetic crosses employed to determine the chromosome linkage of the UAS-CYP6W1-w^+ transgene shown as [T*].

How to make a homozygous stock if the transgene is linked to 2nd chromosome,
(Alternative cross after P1)

P1 ♀ w^{1118}; [T*]/+; +/+ X ♂ w/Y; *CyO/Bl*; +/+

↓

F1 ♂ w/Y; [T*]/*CyO*; +/+ X ♀ w/w; [T*]/*CyO*; +/+

↓

F2 Cross w^+ eyed, Cy^+ males (w/Y; [T*]/[T*]; +//+) with virgin females w^+-eyed and Cy^+ females (w/w; [T*]/[T*]; +/+) to raise the homozygous stock

How to make a homozygous stock if the transgene is linked to 3rd chromosome,
(Alternative cross after P1)

P1 ♀ w^{1118}; +/+; [T*]/+ X ♂ yw/Y; +/+; TM6C, *Sb, Tb*/+

↓

F1 ♂ w/Y; +/+; [T*]/TM6C, *Sb, Tb* X ♀ w/yw; +/+; [T*]/TM6C, *Sb, Tb*

↓

F2 Cross w^+ eyed and Sb^+-Tb^+ males (w /Y; +/+; [T*]/[T*]) with w^+ eyed and Sb^+-Tb^+ females (yw/Y; +/+; [T*]/[T*]) to raise the homozygous stock

Figure 4. Synthesis of transgenic lines homozygous for UAS-CYP6W1-w^+ transgene [T^*].

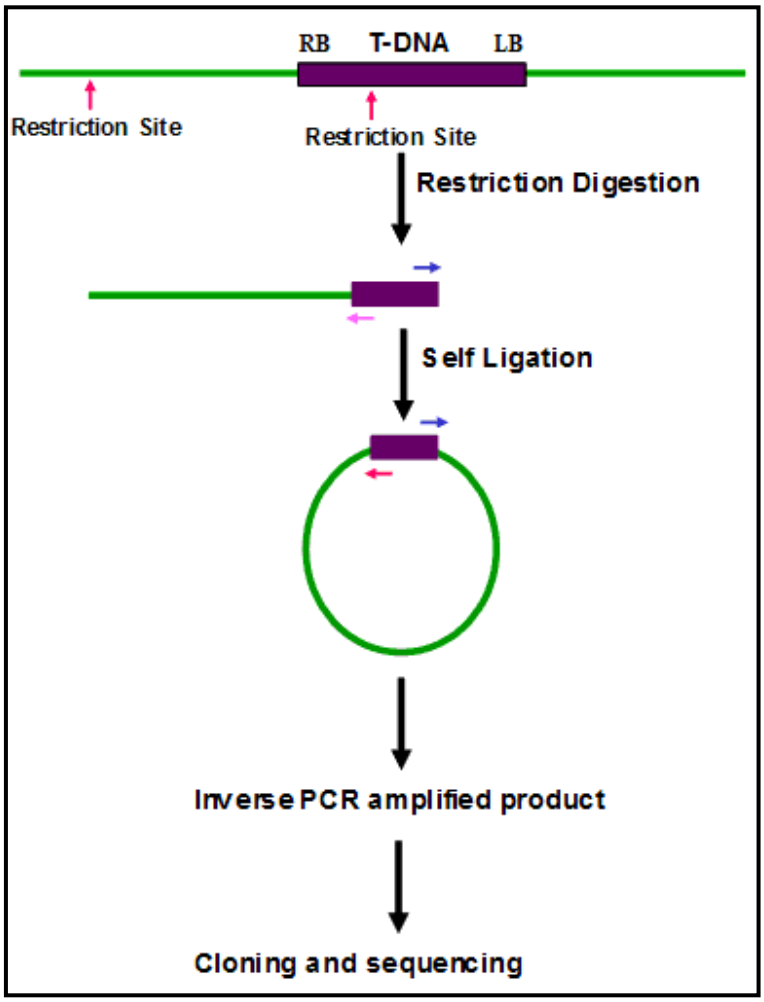


Figure 5. Depiction of Inverse PCR strategy. The restriction enzyme of genomic DNA with an appropriate enzyme that cuts (preferably once within the T-DNA) followed by self-ligation. The circular ligated products are PCR amplified using appropriate primers from the T-DNA region. The flanking DNA is represented by a green line. The appropriate primers (forward and reverse) are indicated by blue and red arrows.

tube, avoiding the floating crud, and 600µl of isopropanol was added to precipitate the DNA, mixed, and spun for 15 minutes at RT to pellet the DNA. The DNA pellet was washed with 70% ethanol, dried, and resuspended in 150µl water.

The isolated genomic DNA from each transformant line was individually digested with Hha I and Msp I for three hours and ligated overnight at 4°C. The ligation mixture was set up in 400µl volume so that intra molecular ligation could occur. The ligated product was ethanol precipitated and resuspended in 150µl of water. PCR was run using Plac4/Plac1 and Pry4/Pry1 primer sets (Table 2) that are specific for the 5' and 3'end, respectively, of the PZ-P elements that flank the transgene. The PCR conditions for Plac4/Plac1 primers were: 30 seconds at 94°C, 1 min at 60°C, and 3 min at 68°C, all repeated 35 times. For Pry4/Pry1 primers, the conditions were exactly the same except for the annealing temperature which was 60°C instead of 55°C. The PCR were sequenced and blasted against the *Drosophila* genome.

Chromosomal localization of CYP6W1 transgene by in situ hybridization

In order to determine the chromosomal location of other transgenes, in situ hybridization technique was tried. For this purpose polytene chromosomes of each line were prepared by squashing the salivary glands in 45% acetic acid. The slides were kept at 4^0C overnight in an acetic acid vapor chamber. On the next day, the slides were placed in liquid nitrogen and the cover slips were removed with a razor blade. The slides were then placed in a fixative, dehydrated, and air-dried. To prepare for hybridization, the slides were incubated for 3 min in 0.07N NaOH at room temperature, rinsed in 2XSSC, dehydrated and air dried. CYP6W1 cDNA was labeled with Biotin using Biotin-High

Primer name	Primer sequence	Melting temperature
5' P element		
Plac4	5'-actgtgcgttaggtcctgttcattgtt-3'	58ºC
Plac1	5'-cacccaaggctctgctcccacaat-3'	61ºC
3' P element		
Pry1	5'-ccttagcatgtccgtggggtttgaat-3'	60ºC
Pry4	5'-caatcatatcgctgtctcactca-3'	53ºC

Table 2. Sequences of primers for inverse PCR that are specific for the 5' and 3'end, respectively, of the PZ-P elements that flank the transgene.

Prime (Roche) and hybridized with the chromosomes. Slides were then washed, and the chromosomes were incubated in solution containing alkaline conjugated Streptavidin. The hybridization signal was detected with BCIP/NBT substrate (Vector Lab). The chromosomes were stained with Giemsa (Sigma).

Real Time Quantitative PCR

For Real time quantitative PCR, the total RNA was used to synthesize first strand cDNA using iScript cDNA Synthesis Kit (Bio-Rad). Total volume of the reaction mixture was 20µl containing 4µl of 5x iScript reaction Mix, 1µl of iScript reverse transcriptase, nuclease-free water, and 1µg total RNA. The reaction mixture was incubated for 5 min at 25°C, 30 min at 42^0C, and 5 min at 85°C. After the reaction, the cDNA was stored at -20^0C until needed.

For the real-time PCR reaction the 1st strand cDNA was diluted 5-fold with DEPC-treated water. Each PCR mix contained 12.5µl 2X Sybergreen Q-PCR mix, 1.25µl each of the forward and reverse primers (10µM), 9µl water, and 1µl of the 5-fold diluted 1st strand cDNA. The reaction was initiated by incubating at 95ºC for 3 min and then the following cycling parameter was repeated 40 times: 95ºC for 30 sec, 59ºC for 30 sec, and 72ºC for 30 seconds. The reaction was ended by raising the temperature from 52ºC-95ºC by 0.5^oC increment in 22 seconds to determine the melting curve. To analyze the expression a gene of interest, triplicate PCR reactions were done with gene-specific primers, and triplicate reactions were done with RP-49 gene-specific primers. RP-49 was used as internal control to normalize the data. Primer details used to determine the expression of *Cyp6w1*, *Cyp6a2*, and *Cyp6g1* in transgenic lines are shown in Table 3.

Primer Name	Primer Sequence	Melting Temperature	Amplicon length
Cyp6w1-F	5'-gccggaccccgagaagta-3' (+1388/+1405)	64.5ºC	60bp
Cyp6w1-R	5'-gttaagattgtcgcggttgct-3' (+1428/+1448)	60.6ºC	
Cyp6a2-F	5'-cgacagagatcccactgaagtatagt-3' (+1458/+1484)	64.6ºC	83bp
Cyp6a2-R	5'-tgcgttccactcgcaagtag-3' (+1520/+1541)	62.4ºC	
Cyp6g1-F	5'-cctgaagccgttctacgactaca-3' (+1381/+1400)	64.6ºC	99bp
Cyp6g1-R	5'-gctgggattggtccagtacttt-3' (+1458/+1480)	62.7ºC	
RP49-F	5'-gcgcaccaagcacttcatc-3' (+405/+423)	62.3ºC	155bp
RP49-R	5'-gacgcactctgttgtcgatacc-3' (+540/+560)	64.5ºC	

Table 3. Primers used to determine the expression of *Cyp6w1*, *Cyp6a2*, and *Cyp6g1* in transgenic lines by real time PCR. The regions of the genes spanned by the primers are shown in parentheses.

DDT resistance bioassay

The DDT resistance bioassay required three consecutive days for the experiment to be done. On the first day, flies (3-5 day old) were etherized and groups of 20 females were sorted into vials containing normal Drosophila medium. To recover from the effects of ether, the flies were left overnight at room temperature. Varying concentrations of stock DDT solutions were made in acetone. To coat the inside of the scintillation vials with a specific quantity of DDT, 200µl of the appropriate DDT solution was added to the vials and swirled slowly until the acetone evaporated completely. The vials were left overnight under a hood for complete drying. On the next morning, the flies (in groups of 20) were transferred live to each DDT-coated scintillation vial. The vials were sealed with cotton plugs moistened with 5% sucrose. The mortality rate was recorded after 24 hour exposure to the DDT. The data was analyzed using Probit analysis in SAS (SAS Institute, 2000) and plotted using Sigma Plot (Systat Software, Inc 2002).

Construction of *Cyp6w1-luciferase* chimeric reporter plasmids

To measure the promoter activity of different regions of the upstream DNA of the *Cyp6w1* gene, pGL2-basic vector (Promega, WI) carrying firefly *luciferase* (*luc*) gene was used. For this purpose genomic DNA isolated from Canton S (NY) strain was used as the template to PCR amplify the upstream DNA of *Cyp6w1* gene with three different forward and one common reverse primers (Table 4). To the 5'-ends of each forward primer, a MluI restriction site was added, whereas a XhoI site was added to the 5'-end of the reverse primer which starts at -1 considering ATG as +1. These restriction sites were used to clone the upstream DNA fragments in front of the luciferase gene in the proper

Primer Name	Primer Sequence	Melting Temperature	Amplicon length
6w1 (-1/-22)R	5’-ccgctcgagGTTGGGTGCTTAGAAGTTAGGG-3’	72.6ºC	
6w1(-74/-95)F	5’-ggcacgcgtCTACATATGCGGCCTGGAAAGC-3’	73.9ºC	95bp
6w1(-488/-510)F	5’-ggcacgcgtGCTCGGTTCGCAATTGATTCAGC-3’	73.6ºC	510bp
6w1(-916/-937)F	5’-ggcacgcgtGCAAGATTAATATCGCCACGCC-3’	72.6ºC	937bp

Table 4. Primers used to amplify *Cyp6w1* upstream DNA. Extra bases added at the 5’ end of each primer are shown in lower case. The underlined bases represent added restriction enzyme sites which are *Xho*I and *Mlu*I in reverse (R) and forward (F) primers, respectively. The gene regions spanned by the primers are shown in parentheses.

5'-3' polarity. Three PCR primers amplified the -1/-95, -1/-510 and -1/-937 upstream DNAs of the *Cyp6w1* gene and have been referred hereafter as -0.1, -0.5 and -0.9 kb, respectively. For cloning, each PCR amplified upstream DNA product and the pGL2-basic vector were digested with MluI and XhoI and ligated by using T4 DNA ligase. The ligated chimeric reporter plasmid was transformed into competent DH5α strain of *E. coli* (Invitrogen). The transformed cells were plated on an LB-plate supplemented with ampicillin. Ten colonies were selected randomly and plasmid DNA was isolated by rapid boiling method, and the presence of the upstream DNA insert in the plasmid was checked by digesting the DNA with MluI and XhoI and electrophoresing the digests on 1% agarose gel. DNA from the positive colony for each upstream DNA was reisolated, purified using Wizard Plus SV Miniprep DNA Purification System (Promega), and both strands were sequenced using Applied Biosystems' 3100 capillary electrophoresis DNA analyzer at the Molecular Biology Resource Facility at University of Tennessee, Knoxville. The obtained sequences were analyzed by BLAST program and compared with the *Cyp6w1* upstream sequences available in the database.

Transient transfection of S2 cells with reporter plasmids and treatments with different chemicals

Drosophila Schneider 2 (S2) cells (Invitrogen) were used in this present investigation and raised in HyQ SFX insect cell culture medium (HyClone). Cells of 3-4 day old culture were dispersed, washed with 1 x PBS and resuspended into fresh medium. Approximately 5 to 6 million cells were dispensed into each well of a 6-well cell culture plate and fresh medium was added into each well to bring the total volume to 2.5ml per

well. The plate was gently swirled to mix and incubated at 25ºC. In the mean time, transient transfection was performed using Effectene Transfection Reagent (Qiagen). For this purpose 1µg of the DNA to be transfected was mixed with 0.1µg of pRL null vector (Promega, WI), dried down, and redissolved in 10µl of water. The pRL null vector, which has the *Renilla luciferase* gene, was used as an internal control to normalize the data. To the 10µl plasmid DNA mixture, 140µl of buffer EC and 8µl of buffer Enhancer were added, vortexed, and quickly spun down to pull down any stray droplets. The mixture was incubated at room temperature for 5 min after which 25µl Effectene was added, mixed and incubated for 10 min. The total volume of the reaction mixture was brought up to 500µl by adding 317µl fresh media. The transfection mix was gently pipetted up and down, and added dropwise to the appropriate wells that were incubating at 25ºC in the 6-well plate. The plate was gently swirled to mix and incubated 18-24 hours at 25ºC. On the following day, the transfected cells were dispersed, and 150µl was dispensed into each well of a 48-well plate to which an aliquot of 32mM caffeine, 8mM phenobarbital (PB) or 2mM DDT was added alone or in combination for a desired final concentration of the chemical. The volume in each well was brought up to 0.5ml with fresh media. The chemicals were mixed by gently swirling the plate which was then incubated for 24 hours at 25ºC and assayed for the firefly (F-luc) and *Renilla* luciferase (R-luc) activities.

Preparation of cell extracts and dual luciferase assay

After overnight exposure to the xenobiotic compounds, S2 cell extracts were made. Each well was gently dispersed by pipetting up and down and transferred to

labeled 1.5ml eppendorf tubes. The cells were centrifuged at 400 x g for 5min at room temperature to pellet down the cells. The supernatant was then removed and 500µl of 1X PBS was added to each tube, but it was not vortexed. Another centrifugation was done at 400 x g at room temperature for 5min. The supernatant was again removed and 80µl of 1X PLB (Passive Lysis Buffer, Promega, WI) was added to each tube and vortexed until the pellet dissolved. The tubes were then centrifuged at 13k RPM for 1min. Approximately 70µl of the supernatant was transferred to fresh 0.5ml eppendorf tubes. The PLB lysate is now ready for the dual luciferase assay.

Since the S2 cells were transfected with firefly as well as *Renilla* luciferase (internal control), the cell lysates were assayed at room temperature using the Dual-Luciferase Reporter Assay kit from Promega (WI). To assay, 25µl of luciferase assay reagent (LARII, Promega, WI) and 5µl of cell lysate was gently mixed in a 1.5 ml eppendorf tube by finger flicking. The firefly luciferase (F-luc) activity was measured first by placing the eppendorf tube in the single-tube luminometer (Zylux Corp). After 15 sec reading of the F-luc activity, 25µl of Stop & Glo Reagent (Promega, WI) was dispensed into the same tube, mixed gently by finger flicking and the R-luc activity was measured. A ratio of the F-luc/R-luc activity was calculated with the R-luc activity being used to normalize the data.

Statistical analysis

For each experiment at least three biological replicates were done. The means were gathered for a data set and analyzed by ANOVA or t-test.

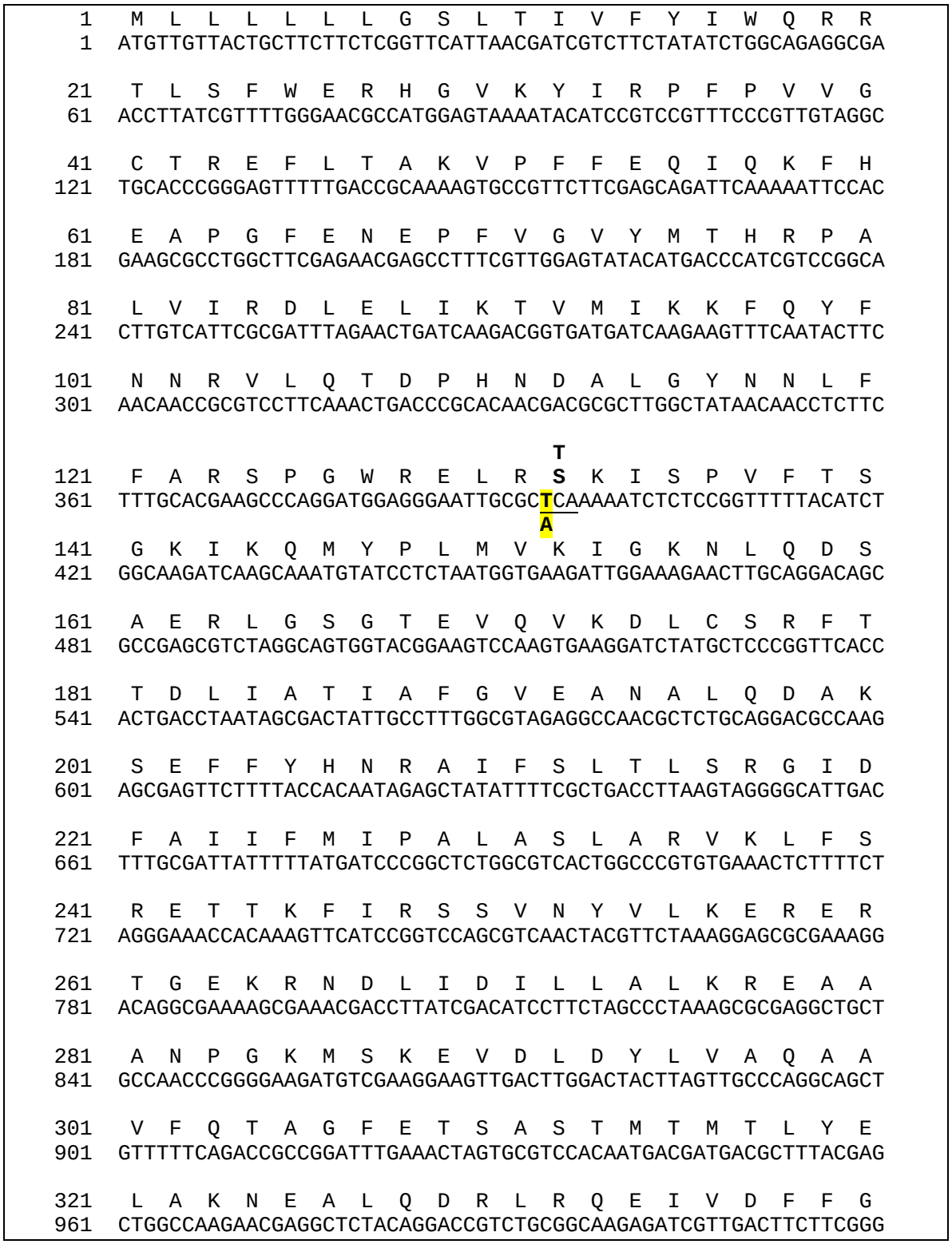
III. Results

A. Cyp6 genes and DDT resistance

Cloning and characterization of CYP6W1 cDNA for germ line transformation

To determine whether CYP6W1 has any role in DDT resistance in *Drosophila*, Gal4:UAS binary expression strategy was followed. First, total RNA was isolated from the 91-R strain which is highly resistant to DDT (Kuruganti et al., 2007). The RNA was used as a template to synthesize the double-stranded CYP6W1 cDNA using gene-specific primers complimentary to the 5'- and 3'-UTRs. The 5'-ends of these primers had engineered *Bgl*II and *Xho*I sites, respectively. The double-stranded cDNA was first cloned into pGEMT vector by TA cloning procedure and it was excised from this vector with *Bgl*II and *Xho*I and cloned into the pUAST transformation vector.

To validate, the putative cDNA was sequenced and checked against *Drosophila* genome sequence database using the BLAST program. The results showed that the nucleotide sequence of the cDNA is almost identical to the *Cyp6w1* gene sequence present in the database (Figure 6). Conceptual translation showed that CYP6W1 polypeptide is made up of 514 amino acids and like other CYP enzymes, CYP6W1 polypeptide encoded by the cDNA also has the invariant amino acid sequence, FXXGXXXCIG, spanning positions 443 to 452 (Figure 10). This conserved sequence constitutes the heme binding site of the CYP enzymes (Maitra et al., 1996). Comparison of the amino acid sequence of the cloned cDNA and the CYP6W1 sequence present in the database revealed that the cloned CYP6W1 cDNA has base substitutions at positions 394, 1109 and 1377. While the fist two base substitutions changed serine to threonine, and alanine to valine, respectively, substitution of the base at position 1377 did not


1 M L L L L L L G S L T I V F Y I W Q R R
1 ATGTTGTTACTGCTTCTTCTCGGTTCATTAACGATCGTCTTCTATATCTGGCAGAGGCGA
21 T L S F W E R H G V K Y I R P F P V V G
61 ACCTTATCGTTTTGGGAACGCCATGGAGTAAAATACATCCGTCCGTTTCCCGTTGTAGGC
41 C T R E F L T A K V P F F E Q I Q K F H
121 TGCACCCGGGAGTTTTTGACCGCAAAAGTGCCGTTCTTCGAGCAGATTCAAAAATTCCAC
61 E A P G F E N E P F V G V Y M T H R P A
181 GAAGCGCCTGGCTTCGAGAACGAGCCTTTCGTTGGAGTATACATGACCCATCGTCCGGCA
81 L V I R D L E L I K T V M I K K F Q Y F
241 CTTGTCATTCGCGATTTAGAACTGATCAAGACGGTGATGATCAAGAAGTTTCAATACTTC
101 N N R V L Q T D P H N D A L G Y N N L F
301 AACAACCGCGTCCTTCAAACTGACCCGCACAACGACGCGCTTGGCTATAACAACCTCTTC
T
121 F A R S P G W R E L R S K I S P V F T S
361 TTTGCACGAAGCCCAGGATGGAGGGAATTGCGCTCAAAAATCTCTCCGGTTTTTACATCT
A
141 G K I K Q M Y P L M V K I G K N L Q D S
421 GGCAAGATCAAGCAAATGTATCCTCTAATGGTGAAGATTGGAAAGAACTTGCAGGACAGC
161 A E R L G S G T E V Q V K D L C S R F T
481 GCCGAGCGTCTAGGCAGTGGTACGGAAGTCCAAGTGAAGGATCTATGCTCCCGGTTCACC
181 T D L I A T I A F G V E A N A L Q D A K
541 ACTGACCTAATAGCGACTATTGCCTTTGGCGTAGAGGCCAACGCTCTGCAGGACGCCAAG
201 S E F F Y H N R A I F S L T L S R G I D
601 AGCGAGTTCTTTTACCACAATAGAGCTATATTTTCGCTGACCTTAAGTAGGGGCATTGAC
221 F A I I F M I P A L A S L A R V K L F S
661 TTTGCGATTATTTTTATGATCCCGGCTCTGGCGTCACTGGCCCGTGTGAAACTCTTTTCT
241 R E T T K F I R S S V N Y V L K E R E R
721 AGGGAAACCACAAAGTTCATCCGGTCCAGCGTCAACTACGTTCTAAAGGAGCGCGAAAGG
261 T G E K R N D L I D I L L A L K R E A A
781 ACAGGCGAAAAGCGAAACGACCTTATCGACATCCTTCTAGCCCTAAAGCGCGAGGCTGCT
281 A N P G K M S K E V D L D Y L V A Q A A
841 GCCAACCCGGGGAAGATGTCGAAGGAAGTTGACTTGGACTACTTAGTTGCCCAGGCAGCT
301 V F Q T A G F E T S A S T M T M T L Y E
901 GTTTTTCAGACCGCCGGATTTGAAACTAGTGCGTCCACAATGACGATGACGCTTTACGAG
321 L A K N E A L Q D R L R Q E I V D F F G
961 CTGGCCAAGAACGAGGCTCTACAGGACCGTCTGCGGCAAGAGATCGTTGACTTCTTCGGG

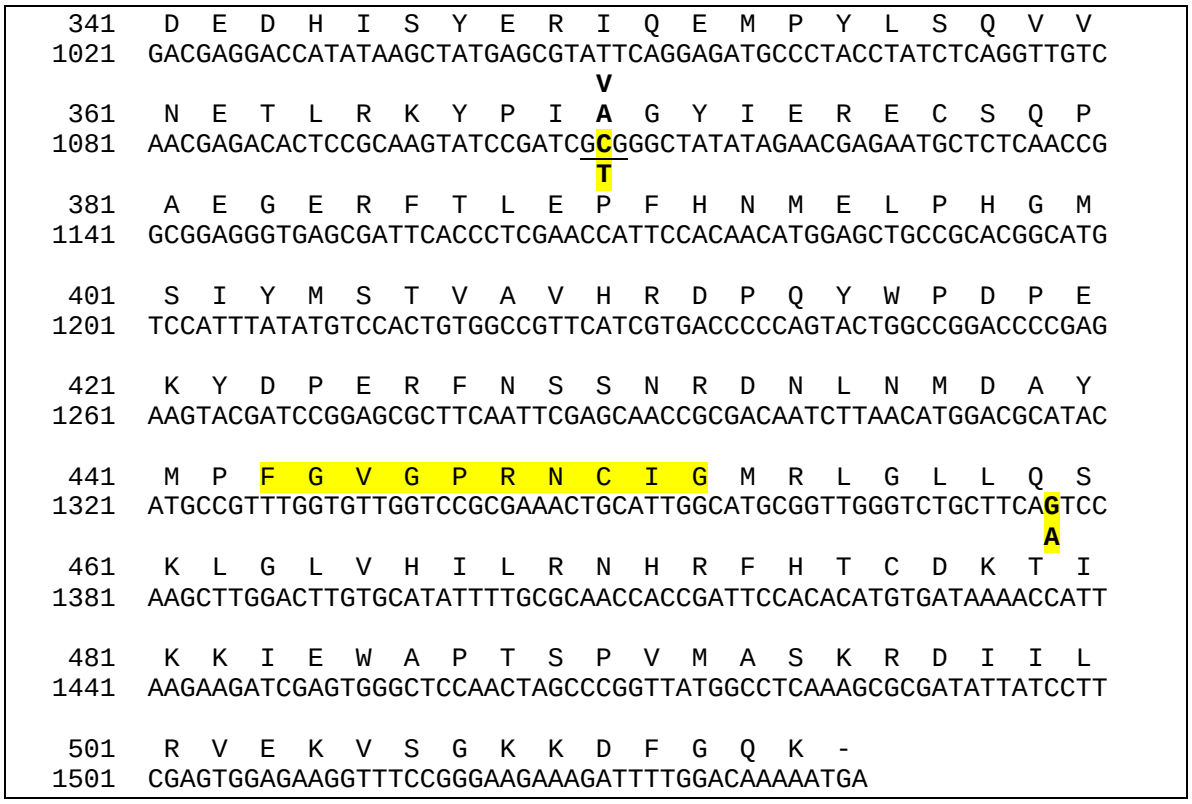


Figure 6. Comparison of the CYP6W1 cDNA sequence with the sequence present in the Drosophila genome database. Changed bases and amino acids are shown in bold. The conserved heme binding site is highlighted.

change the amino acid.

Sequence of the cloned CYP6W1 cDNA was also compared with *Drosophila* genome using BLAST program. Results showed that CYP6W1 has 44% and 34% amino acid sequence identity to the CYP6G1 and CYP6A2 polypeptides, respectively (Figure 7). Higher sequence identity with CYP6G1 may make one speculate that CYP6W1 has some role in DDT resistance because transgenic experiments have shown that CYP6G1 could confer low level of DDT resistance in adult flies (Daborn et al., 2002). However, it should be noted that the first 14 amino acids of CYP6W1 and CYP6G1 are completely different.

Synthesis of transgenic lines homozygous for the CYP6W1 cDNA of the 91-R strain

To examine the role of CYP6W1 in DDT resistance, a Gal4:UAS binary expression system was used. For this purpose CYP6W1 cDNA cloned into pUAST transformation vector was used to transform w^{1118} stock. Best Gene Inc. (Chino Hills, CA) microinjected 200 embryos of w^{1118} stock and supplied 115 larvae from which 80 adult flies emerged (Table 5). In the end, ten transgenic lines were obtained from this experiment. Five of the ten transformants were chosen and characterized. Using 2nd or 3rd chromosomal balancer stocks, CYP6W1 transgene in 4 of the 5 transgenic lines was mapped to the 3rd chromosome with one remaining unresolved (Table 6). Inverse PCR method was tried to map the transgenes to specific site on the chromosome, but it did not yield any conclusive results except for line T(w^{+})6w1-CS. Based on the sequence data of inverse PCR this transgene was mapped to the right arm of chromosome 3, section 34 of 118 of the complete sequence. After determining the chromosome linkage, genetic

Figure 7. Alignment of the amino acid sequences of the CYP6W1, CYP6G1, and CYP6A2 polypeptides. The conserved sequences found in most CYP enzymes are shown in red and blue (the heme binding site).

cDNA	# of injections	# larvae survived	# adults emerged and crossed to w^{1118}	# of transformants
Cyp6w1	200	115	80	10

Table 5. The statistics of microinjections performed with pUAST/CYP6W1 plasmid.

Transgenic lines	Location
1. T(w+)6w1-AZ	3rd chromosome
2. T(w+)6w1-BQ	Not studied
3. T(w+)6w1-CS	3rd chromosome
4. T(w+)6w1-DK	Not studied
5. T(w+)6w1-ER	Not studied
6. T(w+)6w1-FP	Not studied
7. T(w+)6w1-GV	ambiguous
8. T(w+)6w1-HX	3rd chromosome
9. T(w+)6w1-IY	3rd chromosome
10. T(w+)6w1-JW	Not studied

Table 6. Chromosomal localization of the UAS-CYP6W1-w^+ transgene.

crosses were done to make the transgenic lines homozygous for the CYP6W1 transgene. The details of the crosses are shown in the Materials and Methods section (Figures 3, 4).

Analysis of CYP6W1, CYP6A2, and CYP6G1 RNA levels in the transgenic lines by Gal4:UAS system

Gal4:UAS binary system was used to overexpress the CYP6W1 transgene in adult flies. For this purpose CYP6W1 cDNA was cloned into the pUAST vector under the control of five copies of yeast upstream activating sequences or 5X UAS (Figure 8). UAS is the binding site for GAL4 transcription factor of yeast. Since GAL4 is not present in *Drosophila*, it has to be supplied. Therefore, two GAL4 transgenic lines of *Drosophila*, FB-GAL4 and Tu-GAL4, were used. While in FB-GAL4 line, GAL4 cDNA is under the control of fat body-specific enhancer, in Tu-GAL4 line it is under the control of tubulin promoter which is active in all tissues.

In order to activate and overexpress the CYP6W1 transgenes, crosses were done to bring the FB-GAL4 or Tu-Gal4 driver and UAS-CYP6W1 transgenes into the same genome. Details of these crosses are shown in Figure 9. Since in all four lines the UAS-CYP6W1 transgene is linked to the third chromosome, virgin females of each homozygous transgenic line were crossed with the males from the 3rd chromosome balancer stock *yw; +/+; Ly/TM6C, Tb, Sb*. The w^+ (red eye) phenotype was the visible marker used to track the UAS-CYP6W1 transgene. In the F1 generation, *w/Y; +/+; w1/TM6C, Tb, Sb* males with stubble (Sb), tubby (Tb), and red eyes (w^+) phenotypes were collected and crossed to virgin females of FB-GAL4 (*w/w; +/+; FB-Gal4/FB-Gal4*) or TU-GAL4 (*w/w; +/+; TU-GAL4/Sb*) driver stock. In the F2 generation of FB-

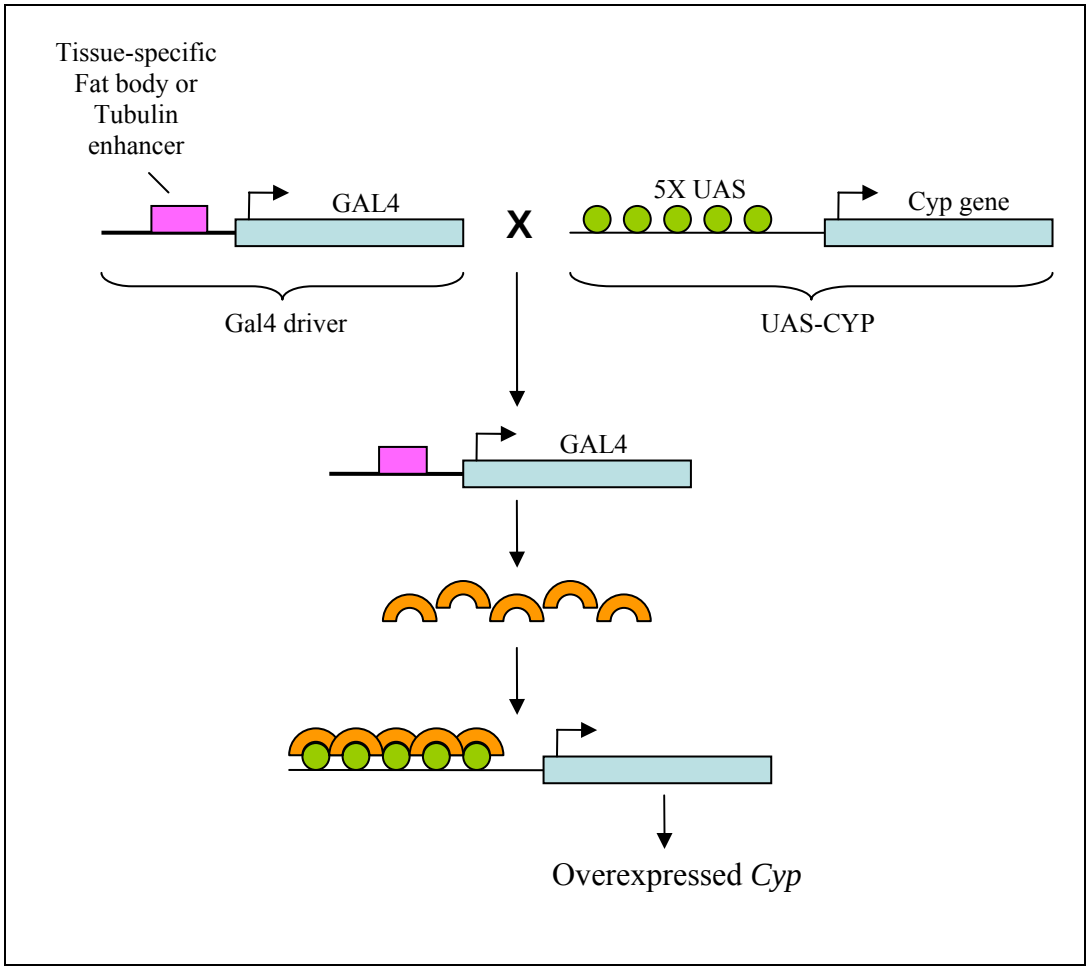


Figure 8. Gal4:UAS binary expression system. The Cyp gene of interest can be expressed in a target tissue by crossing the UAS-CYP transgenic flies with a Gal4 driver line in which GAL4 cDNA is under the control of a tissue-specific or ubiquitous enhancer.

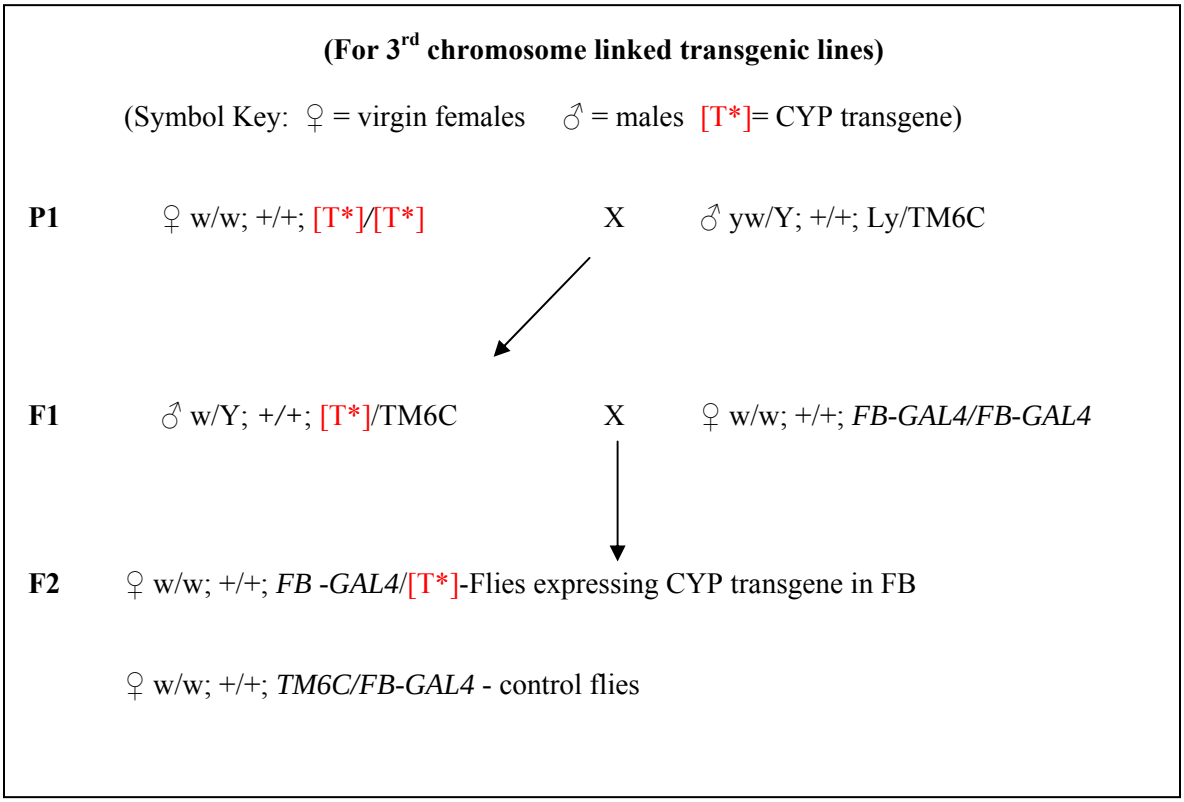


Figure 9. Genetic crosses to place UAS-CYP6W1-w^+ transgene [T*] and FB-Gal4 in the same genome. In the F2 generation, female flies expressing *Cyp6w1, Cyp6a2,* or *Cyp6g1* and the control female flies (GAL4 alone) were collected for RT-PCR and DDT Bioassays. The same exact strategy was used for the tubulin driver but TU-GAL4/Sb replaced the FB-GAL4 virgins in the F1 generation.

GAL4 cross, *w/w; +/+; FB-Gal4/UAS-6w1* females with w^+- Sb^+-Tb^+ phenotypes were collected for analysis. As a control, *w/w; +/+; FB-Gal4/TM6C, Sb, Tb* F2 females with *w-Sb-Tb* phenotype were used. In the case of TU-GAL4 driver cross, *w/w; +/+; TU-GAL4/UAS-6w1* F2 females with w^+- Sb^+-Tb^+ phenotypes were collected. The *w/w; +/+; TU-GAL4/TM6C, Sb, Tb* F2 females with *w-Sb-Tb* phenotypes were used as control. In each cross, the control females have the GAL4 driver but not the UAS-CYP6W1 transgene.

I also did genetic crosses to synthesize two different doubly-transgenic stocks. Both of these stocks have UAS-CYP6W1 plus UAS-CYP6A2 or UAS-CYP6G1 transgene in the same genome. The UAS-CYP6A2 and UAS-CYP6G1 lines were originally synthesized by Srilalitha Kuruganti, a former graduate student in the lab. In these lines the transgenes are linked to the 2nd chromosome. The details of the crosses to place two different CYP transgenes and GAL4 driver into the same genome are shown in Figure 10.

Before examining DDT resistance, transgenic and control females were analyzed for the expression of the transgenes at the RNA level. Total RNA was isolated from these females and CYP6W1, CYP6A2, and CYP6G1 RNA levels were quantified by using quantitative real-time PCR or qRT-PCR. Table 7 shows the expression data obtained with FB-GAL4 driver. It is clear that the T(w^+) 6w1-AZ, T(w^+) 6w1-CS and T(w^+)6w1-HX lines had significantly higher levels (1.9 to 3.0-fold) of CYP6W1 RNA compared to their respective controls. However, the level of CYP6W1 RNA in T(w^+)6w1-IY line was only 1.3-fold higher than the control flies, which was not statistically significant (p = 0.393).

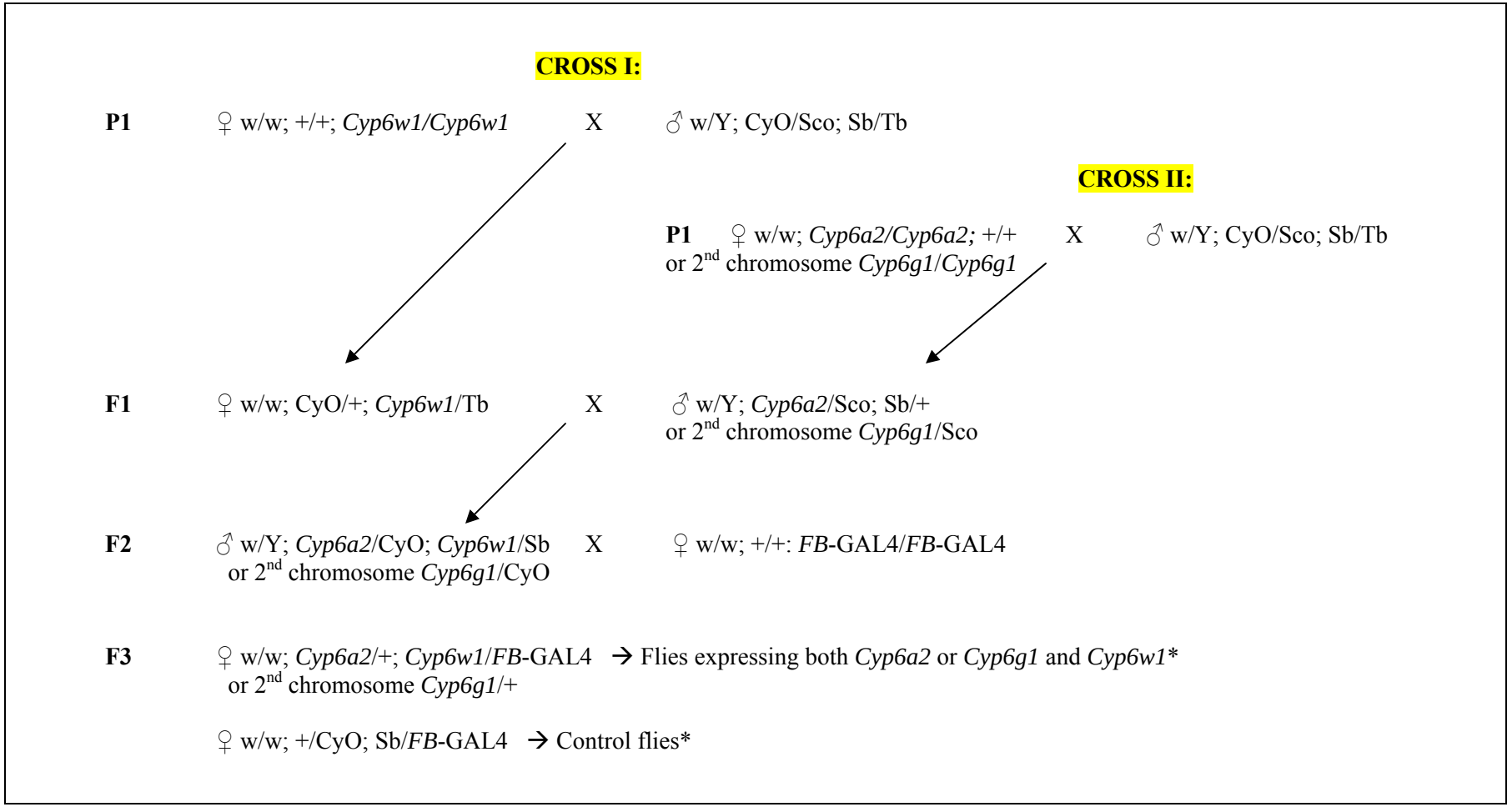


Figure 10. Genetic crosses to synthesize doubly transgenic flies carrying both UAS-CYP6W1-w^+ and UAS-CYP6A2-w^+ or UAS-CYP6G1-w^+ transgene in the same genome. Two different F3 females were tested for DDT resistance and RNA expression by real-time PCR.

Table 7. Analysis of *Cyp* gene expression in Fat Bodies by real-time PCR

Samples[1]	*Cyp* gene analyzed	Avg. ΔCt	St. Dev.	ΔΔCt	$2^{-\Delta\Delta Ct}$ Fold difference	p-value
1. T(w^+)6w1-AZ	*Cyp6w1*	1.910	0.381	-0.953	1.936	0.032*
Control	*Cyp6w1*	2.863	0.342			
2. T(w^+)6w1-CS	*Cyp6w1*	2.517	0.240	-0.903	1.870	0.013*
Control	*Cyp6w1*	3.420	0.272			
3. T(w^+)6w1-HX	*Cyp6w1*	2.053	0.071	-1.590	3.010	0.001*
Control	*Cyp6w1*	3.643	0.329			
4. T(w^+)6w1-IY	*Cyp6w1*	3.100	0.524	-0.350	1.275	0.393
Control	*Cyp6w1*	3.450	0.355			
5. UAS-6a2-1C[s]	*Cyp6a2*	2.710	0.446	-0.960	2.000	0.010*
Control[s]	*Cyp6a2*	3.670	0.543			
6. UAS-6g1-1C[s]	*Cyp6g1*	4.154	0.379	-0.740	1.700	0.010*
Control[s]	*Cyp6g1*	4.893	0.283			
7. UAS-6a2/6g1[s]	*Cyp6a2*	3.150	0.440	-1.000	2.700	0.0001*
	Cyp6g1	5.990	0.580	-0.100	1.100	0.300
Control[s]	*Cyp6a2*	4.140	0.280			
	Cyp6g1	6.100	0.270			
8. T(w^+)6w1/6a2	*Cyp6w1*	2.687	0.270	-4.643	24.991	0.000*
	Cyp6a2	6.693	0.172	1.303	0.405	0.003*
Control	*Cyp6w1*	7.330	0.541			
	Cyp6a2	5.390	0.314			
9. T(w^+)6w1/6g1	*Cyp6w1*	3.700	0.989	-1.510	2.848	0.064
	Cyp6g1	4.617	0.875	-1.230	2.346	0.085
Control	*Cyp6w1*	5.210	0.287			
	Cyp6g1	5.847	0.335			

[1] Flies other than control flies have the FB-Gal4 driver and the *Cyp* transgene with T or UAS as prefix. The control flies have the FB-Gal4 driver but no *Cyp* transgene.
*indicates that the fold difference is significant ($p<0.05$)
s indicates the data collected by Srilalitha Kuruganti
RP49 is used as an internal control and amplified along with the *Cyp* genes for each of the samples.
Ct is the cycle number below the threshold value.
ΔCt= Cyp Ct- RP49 Ct
ΔΔCt= ΔCt of the transgene expressing line- ΔCt of its control.
Since the Ct value is in $\log_2$ scale, the fold difference was obtained by calculating $2^{-\Delta\Delta Ct}$.

Because T(w+)6w1-HX showed the highest level of overexpression for CYP6W1 RNA, it was used in the crosses that placed both CYP6W1 and CYP6A2 (or CYP6G1) cDNA transgenes together with the FB-Gal4 driver in the same genome (Figure 10). The levels of CYP6W1 and CYP6A2 (or CYP6G1) mRNA were also measured by qRT-PCR method. The doubly-transgenic lines carrying both UAS-CYP6W1 and UAS-CYP6A2 transgenes showed a 25-fold higher expression for CYP6W1 relative to the control, while CYP6A2 mRNA decreased by 0.4-fold (Table 7). Similar results were obtained when qRT-PCR experiment was repeated (data not shown). The reason for the dramatic change in the expression level of CYP6W1 and CYP6A2 is not understood. No such dramatic shift in the expression level was observed in the other doubly-transgenic line. When combined with UAS-CYP6G1 transgene, the level of CYP6W1 and CYP6G1 mRNA was found to be 2.8- and 2.3-fold higher than in the control flies, respectively (Table 7). For comparison purpose, qRT-PCR data collected previously in the lab on CYP6G1 and CYP6A2 expression (Kuruganti and Ganguly, unpublished results) are also shown in Table 4. The data showed that in UAS-6a2-1C line, CYP6A2 expression was 2-fold higher and in UAS-6g1-1C line, CYP6G1 expression was 1.7-fold higher than the controls. When these two transgenes were placed in the same genome, CYP6A2 expression increased from 2.0- to 2.7-fold, while CYP6G1 expression decreased from 1.7- to 1.1-fold.

Although FB-GAL4 driver gave a significant increase in CYP6W1 expression, it was not very high in singly-transgenic lines. Therefore, for higher level of expression, UAS-CYP6W1 transgene was placed in the same genome (Figure 9) with TU-GAL4 driver because tubulin enhancer is ubiquitously expressed. Table 8 shows the data for the

Table 8. Analysis of *Cyp6w1* expression driven by TU-Gal4 driver

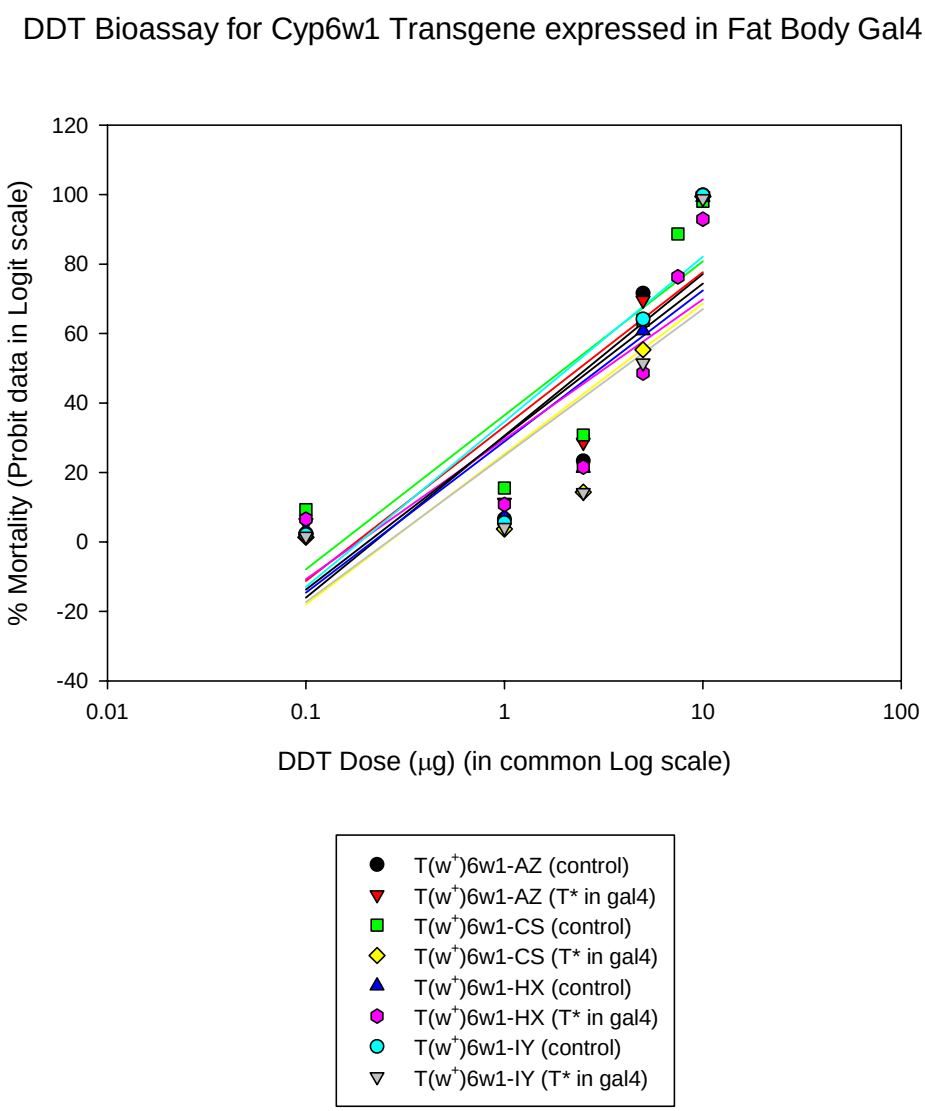
Samples[1]	Gene analyzed	Avg. ΔCt	St. Dev.	ΔΔCt	$2^{-\Delta\Delta Ct}$ Fold difference	p-value
1. T(w^+)6w1-AZ	*Cyp6w1*	0.917	0.660	-3.347	10.173	0.004*
Control	*Cyp6w1*	4.263	0.732			
2. T(w^+)6w1-CS	*Cyp6w1*	0.973	0.538	-2.977	7.872	0.005*
Control	*Cyp6w1*	3.950	0.733			
3. T(w^+)6w1-HX	*Cyp6w1*	0.380	0.896	-4.540	23.264	0.001*
Control	*Cyp6w1*	4.920	0.249			
4. T(w^+)6w1-IY	*Cyp6w1*	1.363	0.192	-5.410	42.518	0.000*
Control	*Cyp6w1*	6.773	0.298			

[1] Fly names starting with T have both UAS-CYP6W1 transgene and TU-Gal4 driver. The control flies have the TU-Gal4 driver but not transgene.
*indicates that the fold difference is significant ($p<0.05$)
RP49 is used as an internal control and amplified along with the *Cyp* genes for each of the samples.
Ct is the cycle number below the threshold value.
ΔCt= Cyp Ct- RP49 Ct
ΔΔCt= ΔCt of the transgene expressing line- ΔCt of its control.
Since the Ct value is in $\log_2$ scale, the fold difference was obtained by calculating $2^{-\Delta\Delta Ct}$.

CYP6W1 mRNA levels in the transgenic flies in the presence of TU-GAL4 driver. It is clear from the data that TU-GAL4 driver gave a significantly higher level of CYP6W1 expression, which ranged from about 8- to 42-fold compared to the TU-GAL4 control flies lacking the UAS-CYP6W1 transgene. In summary, results presented in Tables 7 and 8 show that TU-GAL4 driver gives higher level of expression than the FB-GAL4 driver.

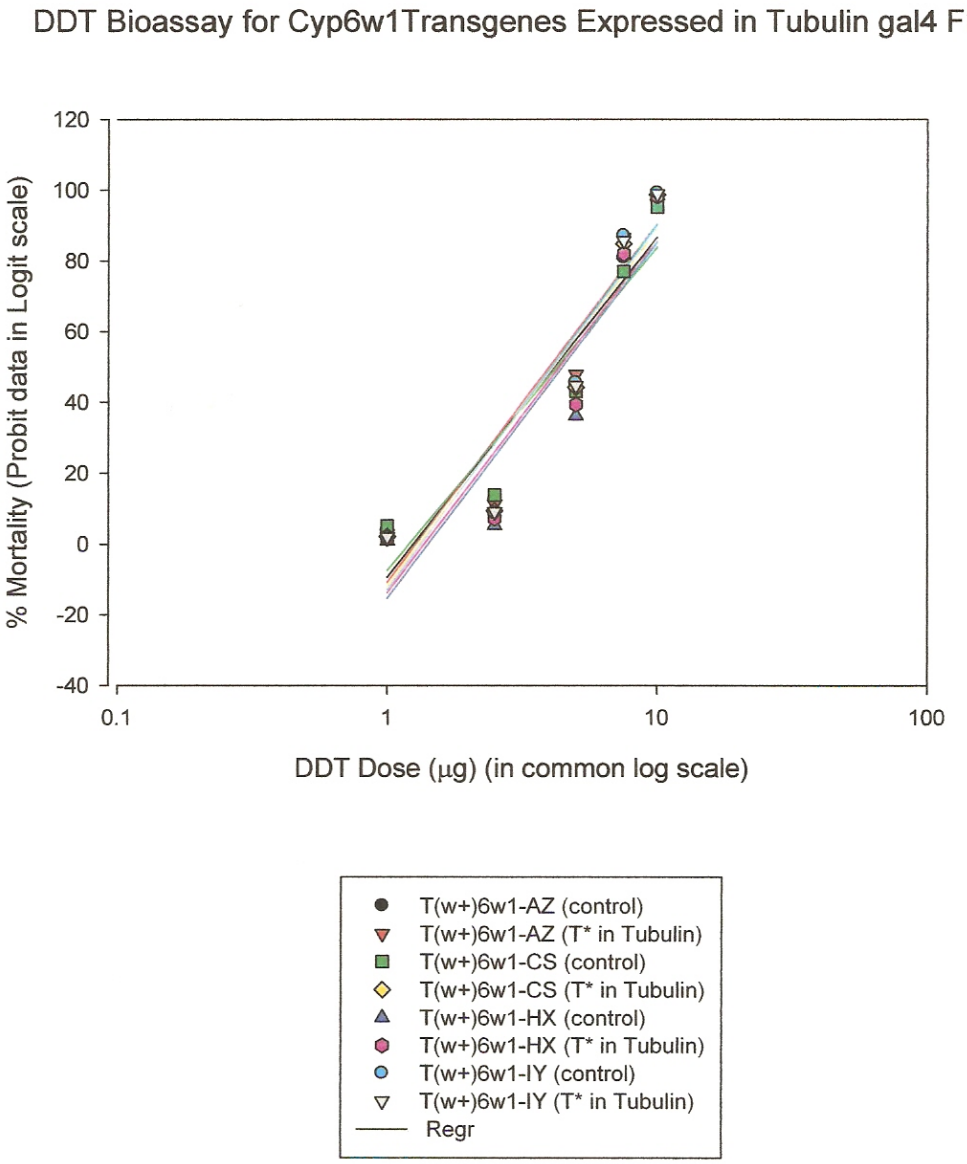
DDT resistance bioassays in flies overexpressing *Cyp6w1*, *Cyp6a2*, and *Cyp6g1* transgenes

To examine the role of *Cyp6w1*, *Cyp6a2*, and *Cyp6g1* in DDT resistance, the F2 and F3 females from the genetic crosses in Figure 9 and 10, respectively, were analyzed for DDT resistance. The adult females were 3-5 days old and subjected to varying concentrations of DDT; mortality after 24 hours was recorded as described in the Methods section. The sigma plot in Figure 11 shows that when CYP6W1 alone was expressed in the fat bodies by using FB-GAL4 driver, no significant increase in the LC_{50} value, compared to the control flies, was observed. All four transgenic lines gave the same results. It was assumed that 1.3- to 3.0-fold overexpression of CYP6W1 mediated by FB-GAL4 driver (Table 7) was not sufficient enough to confer DDT resistance. However, this may not be the case because no significant increase in DDT resistance was observed in TU-GAL4:UAS-CYP6W1 transgenic flies either (Fig. 12) which showed 8- to 42-fold overexpression of CYP6W1 RNA (Table 8). Interestingly, however, when UAS-CYP6W1 transgene of T(w^+)6w1-HX line was combined with UAS-CYP6A2 or UAS-CYP6G1, almost a 2-fold increase in DDT resistance was observed in the doubly-



Strain	N	Slope (± S.E.)	LC50 (95% CI)	RR50
T(w$^+$)6w1-AZ (control)	520	0.520 (0.065)	3.90 (3.42-4.54)	1.00
T(w$^+$)6w1-AZ (T* in gal4)	680	0.430 (0.090)	3.81 (2.91-4.97)	0.97
T(w$^+$)6w1-CS (control)	600	0.342 (0.067)	3.97 (3.00-5.30)	1.00
T(w$^+$)6w1-CS (T* in gal4)	600	0.480 (0.068)	4.72 (4.10-5.52)	1.19
T(w$^+$)6w1-HX (control)	380	0.429 (0.085)	4.36 (3.34-6.07)	1.00
T(w$^+$)6w1-HX (T* in gal4)	660	0.301 (0.050)	5.12 (4.26-6.32)	1.17
T(w$^+$)6w1-IY (control)	300	0.489 (0.065)	4.26 (3.56-5.14)	1.00
T(w$^+$)6w1-IY (T* in gal4)	380	0.444 (0.054)	4.91 (4.33-5.65)	1.15

Figure 11. DDT resistance assay in UAS-CYP6W1/FB-GAL4 transgenic flies



Strain	N	Slope (± S.E.)	LC50 (95% CI)	RR50
T(w^+)6w1-AZ (control)	440	0.407 (0.030)	5.35 (4.95-5.76)	1.00
T(w^+)6w1-AZ (T* in gal4)	900	0.464 (0.043)	5.12 (4.71-5.51)	0.96
T(w^+)6w1-CS (control)	620	0.366 (0.033)	5.48 (4.98-6.00)	1.00
T(w^+)6w1-CS (T* in gal4)	880	0.470 (0.042)	5.31 (4.96-5.67)	0.97
T(w^+)6w1-HX (control)	620	0.504 (0.046)	5.71 (5.36-6.07)	1.00
T(w^+)6w1-HX (T* in gal4)	940	0.475 (0.043)	5.57 (5.20-5.94)	0.98
T(w^+)6w1-IY (control)	500	0.498 (0.057)	5.23 (4.67-5.76)	1.00
T(w^+)6w1-IY (T* in gal4)	760	0.481 (0.043)	5.28 (4.90-5.67)	1.01

Figure 12. DDT resistance assay in UAS-CYP6W1/TU-GAL4 transgenic flies

transgenic flies (Fig. 13, Table 9). These results are puzzling because the T(w^+)6w1 line did not show much resistance when the transgene was driven either by FB-GAL4 or TU-GAL4 driver (Figures 11, 12). It is possible that UAS-CYP6G1 or UAS-CYP6A2 present in the doubly-transgenic line (Table 9) is responsible for DDT resistance because it has been observed that UAS-CYP6A2 and UAS-CYP6G1 can confer 2.7-fold increase in DDT resistance when driven by FB-GAL4 driver (Kuruganti and Ganguly, unpublished results). The summary of the results of all experiments described above and the unpublished results obtained by Kuruganti and Ganguly are shown in Table 10.

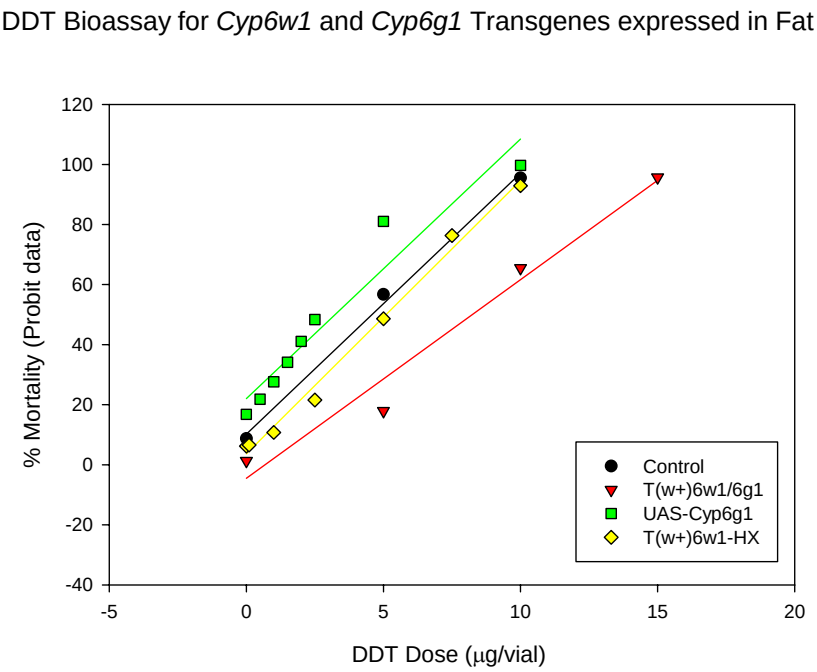
B. Analysis of *Cyp6w1* upstream DNA for xenobiotic induction

Constitutive promoter activities of different lengths of *Cyp6w1* upstream DNAs

In order to map the cis-regulatory sequences involved in constitutive expression and xenobiotic induction process, 0.1-, 0.5- and 0.9-kb of the upstream DNA of *Cyp6w1* gene was PCR amplified from the genomic DNA of the wild type Canton S (NY) strain. The nucleotide sequence of 0.9-kb upstream DNA of *Cyp6w1* that was amplified is detailed in Figure 14. These DNA fragments were then cloned in front of the firefly *luciferase* (F-luc) reporter gene of the pGL2-basic vector. Sequence comparison showed that the amplified products and the upstream DNA of the *Cyp6w1* gene present in *Drosophila* genome sequence database are 99% identical.

Before the promoter activities of 0.1, 0.5, and 0.9 kb upstream DNA of the *Cyp6w1* gene are compared, promoter activity of the 0.9luc/w1 plasmids was determined. For this purpose, *Drosophila* S2 cells were co-transfected with the 0.9luc/w1 and pRL-null plasmid carrying *Renilla luciferase* gene (R-luc) gene. Extracts prepared from the

A.



B.

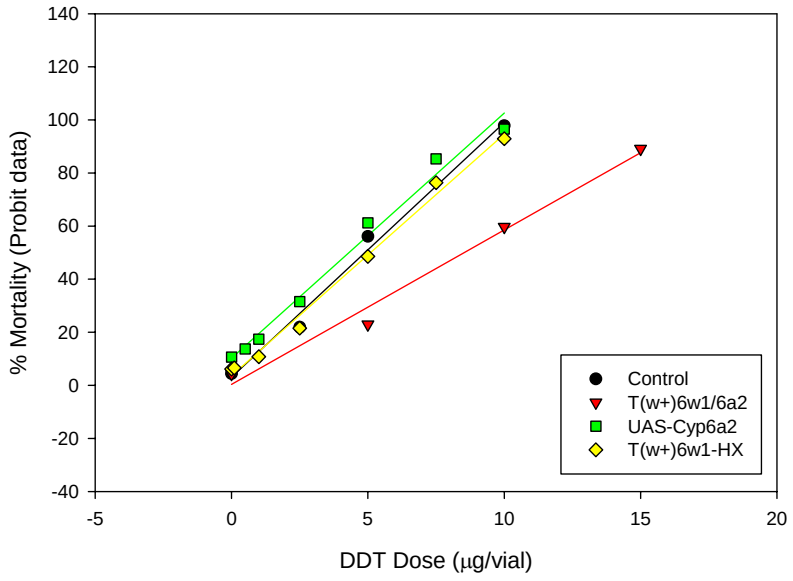


Figure 13. Dose response curves of the transgenic lines expressing *Cyp6w1*, *Cyp6g1*, and *Cyp6a2* in fat bodies. A) Transgenic lines expressing *Cyp6w1* and *Cyp6g1* B) Transgenic lines expressing *Cyp6w1* and *Cyp6a2*. UAS-Cyp6g1 and UAS-Cyp6a2 data was collected from Srilalitha Kuruganti.

DDT resistance assay in different UAS-CYP/FB-GAL4 transgenic flies					
Strain	**N**[a]	**P-value**	**Slope (± SE)**	**LD50 (95% CI)**[b]	**Fold Resistance**[c]
UAS-6a2-1C[d]	447	<0.0001	.31 (0.046)	4.07 (3.3-5.1)	2.7
Control-6a2-1C[d]	221	0.005	.72 (0.26)	1.49 (0.57-3.4)	
UAS-6g1-1C[d]	462	0.0004	0.37 (0.097)	2.6 (1.83-3.85)	2.7
Control-6g1-1C[d]	360	<0.0001	1.64 (0.34)	0.97 (0.74-1.22)	
T(w^+)6w1-HX (T* in gal4)	660	<0.0001	0.301 (0.050)	5.12 (4.26-6.32)	1.2
T(w^+)6w1-HX (Control)	380	<0.0001	0.429 (0.085)	4.36 (3.34-6.07)	
UAS- 6a2/6g1[d]	446	0.0001	0.25 (0.06)	1.7 (0.1-3.2)	4.3
Control[d]	170	<0.0001	7.9 (1.4)	0.4 (0.3-0.44)	
T(w+)6w1/6g1 (T* in gal4)	280	<0.0001	0.26 (0.045)	8.49 (6.88-10.21)	1.9
Control	240	<0.0001	0.31 (0.07)	4.45 (2.35-6.29)	
T(w+)6w1/6a2 (T* in gal4)	300	<0.0001	0.197 (0.045)	8.75 (6.64-11.69)	1.9
Control	260	<0.0001	0.372 (0.077)	4.59 (3.26-5.93)	

a- Number of female flies tested

b- Dose of DDT in µg that gives 50% mortality

c- The resistance compared to its control (test LD50/control LD50)

d-Data collected by Srilalitha Kuruganti

Statistical analysis were performed using probit analysis in SAS (SAS Insitute, Cary, NC)

Table 9. DDT resistance assay in transgenic flies carrying UAS-CYP6W1 transgene and in combination with UAS-CYP6A2 or UAS-CYP6G1 transgene. FB- GAL4 driver was used to express the transgenes.

Sample name	Gene expression analyzed	RNA (fold difference) [1]	RNA (fold difference)[2]	DDT resistance[1] (fold difference)	DDT resistance[2] (fold difference)
UAS-6g1-1C[s]	*Cyp6g1*	1.7		2.7	
Control[s]	*Cyp6g1*				
UAS-6a2-1C[s]	*Cyp6a2*	2.0		2.7	
Control[s]	*Cyp6a2*				
UAS-6g1/Cyp6a2[s]	*Cyp6g1*	1.1		4.3	
	Cyp6a2	2.7			
Control[s]	*Cyp6g1*				
	Cyp6a2				
T(w+)6w1-AZ	*Cyp6w1*	1.9	10.2	1.0	0.96
Control	*Cyp6w1*				
T(w+)6w1-CS	*Cyp6w1*	1.9	7.9	1.2	0.97
Control	*Cyp6w1*				
T(w+)6w1-HX	*Cyp6w1*	3.0	23.3	1.2	0.98
Control	*Cyp6w1*				
T(w+)6w1-IY	*Cyp6w1*	1.3	42.5	1.2	1.01
Control	*Cyp6w1*				
UAS-6g1/6w1	*Cyp6g1*	2.3		1.9	
	Cyp6w1	2.8			
Control	*Cyp6g1*				
	Cyp6w1				
UAS-6a2/6w1	*Cyp6a2*	0.4		1.9	
	Cyp6w1	25.0			
Control	*Cyp6a2*				
	Cyp6w1				

Table 10. Comparison of all transgenic lines for CYP RNA expression and DDT resistance. Gal4 driver used: 1, FB-Gal4; 2, TU-Gal4.

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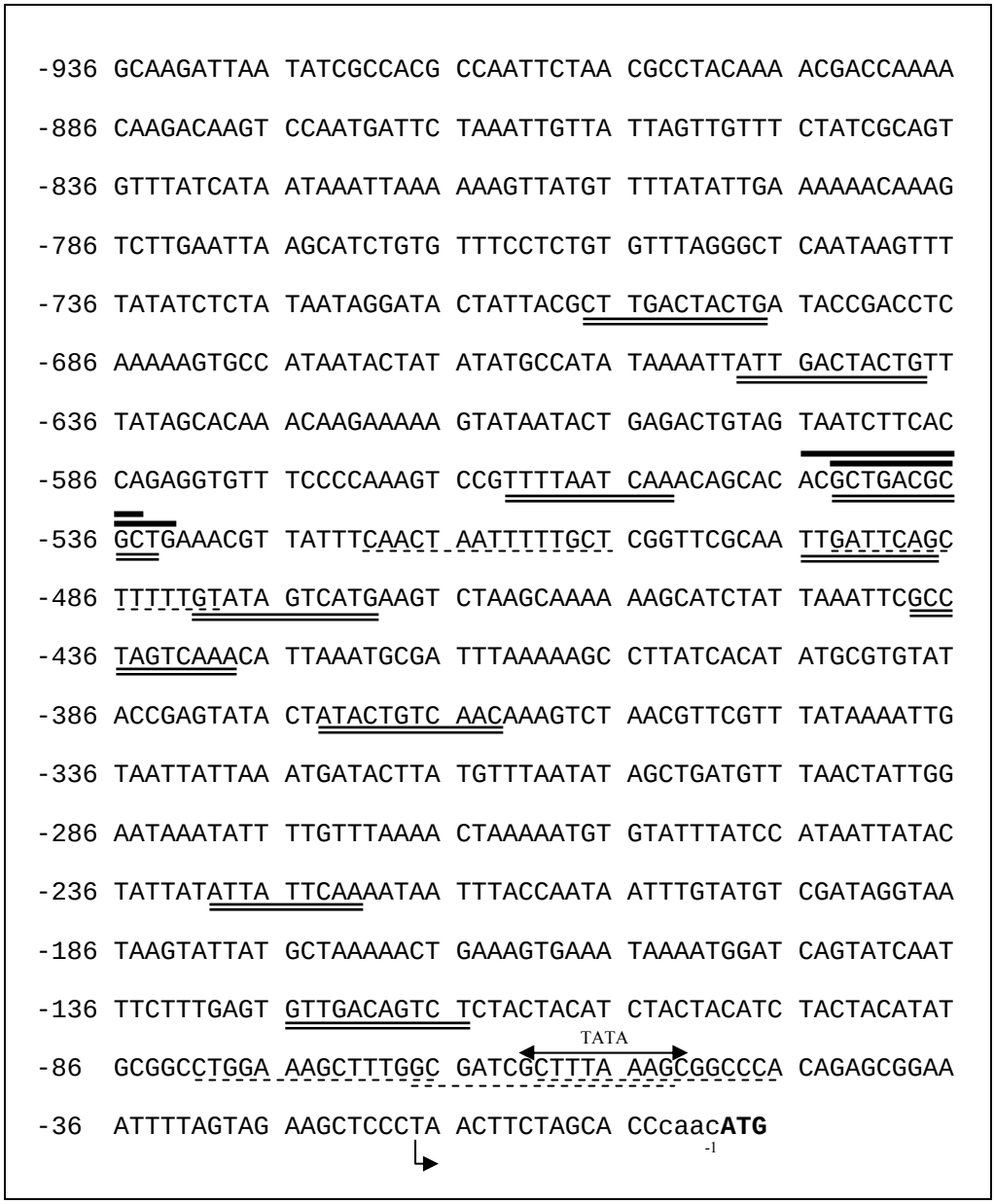

Figure 14. Nucleotide sequence of 0.9-kb upstream DNA of *Cyp6w1* from Canton S (NY). The ATG start codon at +1 is shown in bold. The putative translational start point is shown in lower case letters. The bent arrow indicates putative transcriptional start point. The putative TATA box is shown with a double-arrowhead line. The dashed lines indicate the putative Barbie boxes. Putative AP-1 sites are double-and the CREB sites are labeled overhead with a bold solid line.

transfected cells were then assayed for F-luc and R-luc activities as described in the Methods. The R-luc activity was used as an internal control to normalize the data which were expressed as F-luc/R-luc ratios. Figure 15 shows that the activity of the 0.9luc/w1 plasmid was almost 50-times greater than the activity of the pGL2 (N)-basic empty vector that was used to clone the 0.9-kb upstream DNA of the *Cyp6w1* gene. These results suggest that the 0.9-kb upstream DNA has the sequences that give high level of constitutive promoter activity.

In order to compare the three upstream DNAs of *Cyp6w1* for constitutive promoter activity, 0.1luc/w1, 0.5luc/w1 and 0.9luc/w1 plasmids were individually used to co-transfect the S2 cells with pRL-null plasmid. To minimize the variations, the same batch of cells was used for all three plasmids and three different batches of cells were used. The relative constitutive promoter activities of the three upstream DNAs are shown in Figure 16. The data show that the activities of the 0.5luc/w1 and 0.9luc/w1 plasmids were similar and significantly higher (1.84 to 1.89-fold) than the activity of the 0.1luc/w1 plasmid. These results lead us to presume that beyond -100bp of the upstream DNA, there are important sequences for transcription factors that allow for greater constitutive expression of the *Cyp6w1* gene. However, the DNA between -500bp and -900bp regions of the *Cyp6w1* gene probably does not have additional sequences for more transcription factors because no significant difference in promoter activities was observed between 0.5luc/w1 and 0.9luc/w1 plasmids.

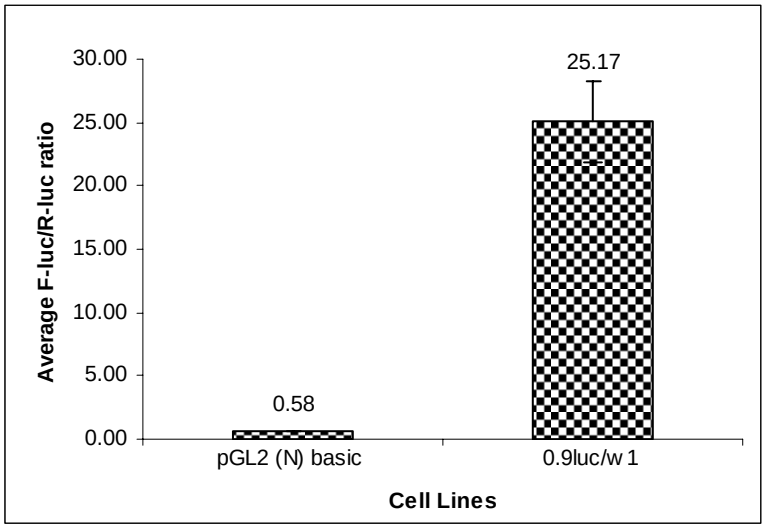

Figure 15. Comparison of luciferase activity in the cells transfected with pGL2(N)basic empty vector and 0.9luc/w1 plasmid. Each bar represents the mean of triplicate samples ± S.D.

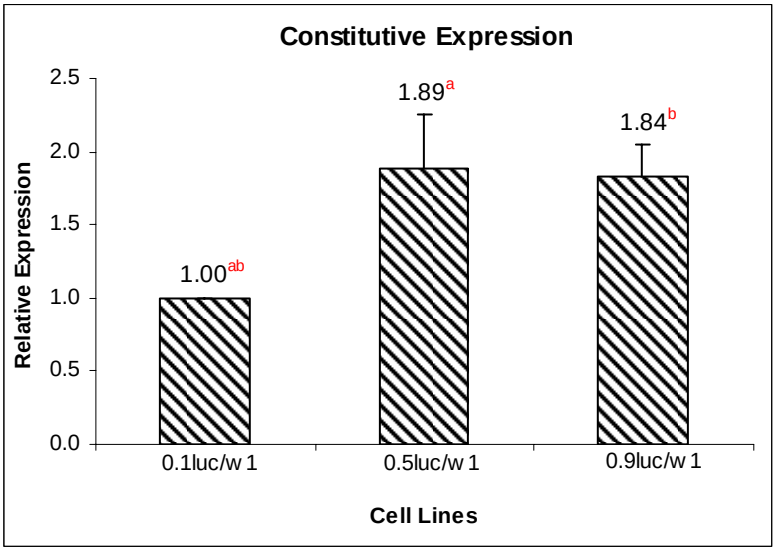

Figure 16. Constitutive expression of luciferase reporter gene in cells individually transfected with 0.1luc/w1, 0.5luc/w1 or 0.9luc/w1 plasmid. Values represent expression relative to 0.1luc/w1 plasmid. The bars represent mean ± S.D. of three biological samples. *P* values (Student's t-test): a < 0.01; and b < 0.002.

Dose response assays with caffeine, phenobarbital and DDT

In order to determine the specific concentration of each xenobiotic chemical that would give maximal induction, an initial dose response assay for each chemical was performed. In this assay, only 0.9/w1 plasmid was used. Results (Fig. 17) show that caffeine gave a steady rise in induction as its concentration was increased. However, induction tends to plateau between 4mM and 6mM concentration. Therefore, 4mM dose, giving about 4-fold induction was chosen for future experiments. In contrast, induction with PB increased linearly as the dose was increased to 1mM. This dose which gave about 5-fold induction was used in subsequent experiments. Induction with DDT was not as robust as it was seen with caffeine or PB. Maximum induction of the promoter was barely 2-fold at 0.02mM concentration and no further increase in induction was observed when the concentration was raised to 0.04mM. Therefore, 0.02mM DDT was used in future experiments.

Induction of different upstream DNAs of *Cyp6w1* by different xenobiotic chemicals

To measure the induction of 0.1, 0.5, and 0.9 kb upstream DNA of the *Cyp6w1* gene by different xenobiotic chemicals, S2 cells were individually transfected with 0.1luc/w1, 0.5luc/w1 and 0.9luc/w1 plasmids. The pRL-null plasmid was also used for co-transfection. Following 24 hours of transfection time, the cells were treated with 4mM caffeine, 1mM PB or 0.02mM of DDT. After 24h treatment the cells were harvested, the extracts were prepared, and dual luciferase assay was done to measure firefly and *renilla* luciferase activity. All experiments were done three times using three different batches of S2 cells, and each time three technical replicates were done for each

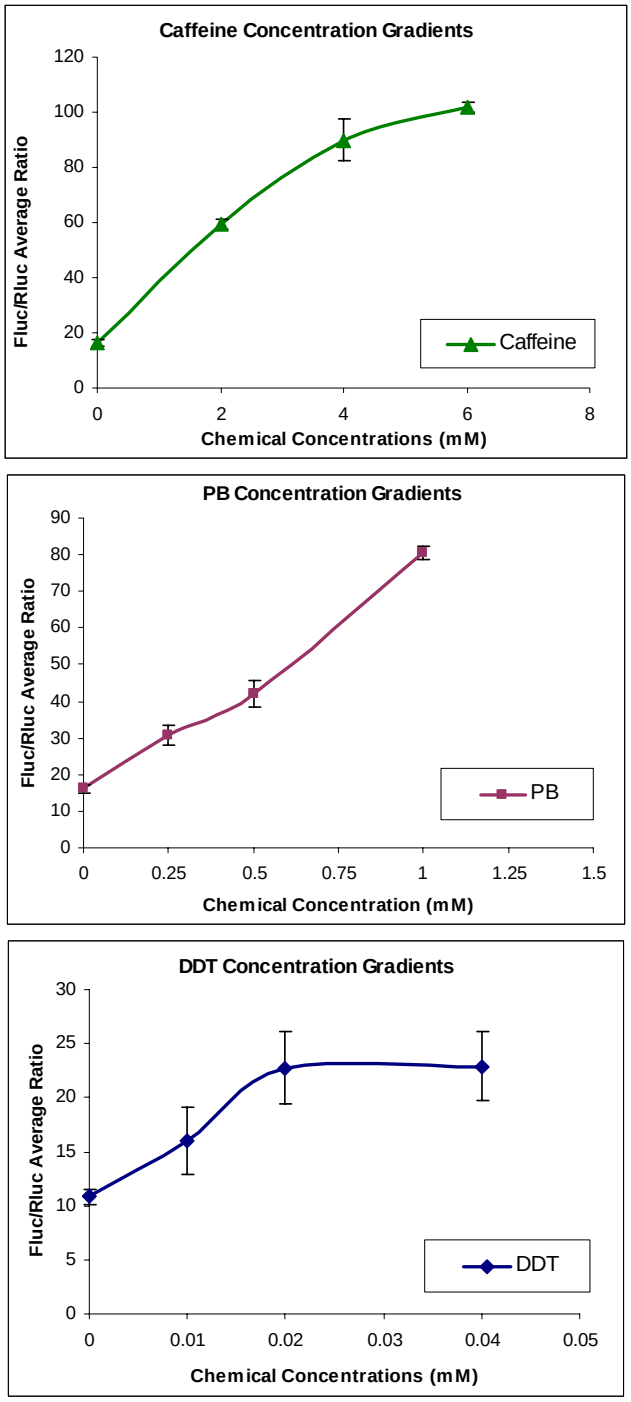

Figure 17. Induction of luc activity in S2 cells by different doses of caffeine, PB, and DDT. Cells transfected with 0.9luc/A8 plasmid were used. Data points represent mean ± S.D of triplicate samples.

treatment. Figure 18 shows the results of caffeine induction. Compared to its constitutive expression, 0.1luc/w1 showed an average of 1.57-fold induction when exposed to 4mM caffeine, 0.5luc/w1 exhibited a 1.8-fold induction, and 0.9luc/w1 had a 2.91-fold induction. Student's t-test showed that there was no significant difference between 0.1luc/w1 and 0.5luc/w1 plasmids for caffeine inducibility. However, the difference in inducibility between 0.1luc/w1 and 0.9luc/w1, as well as between 0.5luc/w1 and 0.9luc/w1, was significant. ANOVA statistical test also demonstrated that among all the constructs, the entire data set is significant.

When treated with 1mM PB, 0.1luc/w1 was induced 1.26-fold, 0.5luc/w1 by 1.44-fold, and 0.9luc/w1 had the highest induction of 2.81-fold (Figure 19). Again, student's t-test showed that there was no significant difference in PB inducibility between 0.1luc/w1 and 0.5luc/w1 plasmids. However, difference in PB inducibility between 0.1luc/w1 and 0.9luc/w1, as well as between 0.5luc/w1 and 0.9luc/w1, was found to be statistically significant. ANOVA statistical test also demonstrated that among all the plasmid constructs, this entire data set is also significant.

The final chemical tested for induction was DDT. After being subjected to 0.02mM DDT for 24 hours, luciferase activity was measured and an average fold induction for each plasmid construct was determined (Figure 20). A 1.13, 1.22, and 1.46-fold induction were seen for 0.1, 0.5, and 0.9luc/w1 constructs respectively. However, student's t-tests and ANOVA analysis showed that the difference in DDT inducibility between constructs was not statistically significant. It is to be noted that the level of induction observed with the 0.9luc/w1 plasmid was highest and a similar level of induction was observed in the dose response experiment (Fig.17).

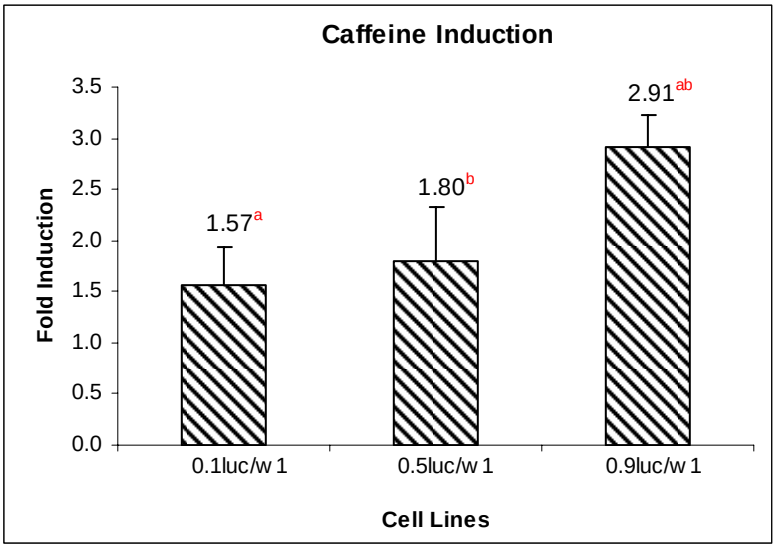

Figure 18. Comparison of 4mM caffeine-induced luc activities in S2 cells transfected with 0.1luc/w1, 0.5luc/w1, or 0.9luc/w1 reporter plasmids. Each bar represents the fold-induction observed with each plasmid relative to its untreated control. Values represent mean fold induction ± S.D of three experiments done with different batches of cells. Student's t-test was done to compare the mean fold inductions. The *P* values are: a < 0.008; and b < 0.03.

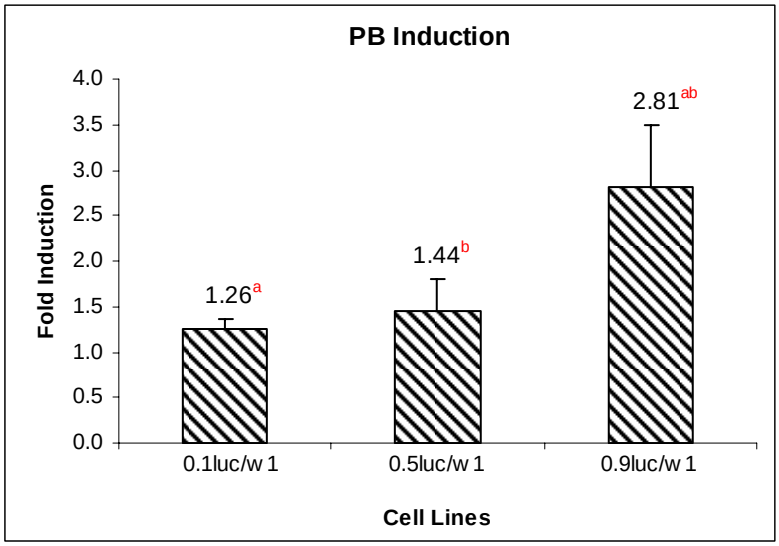

Figure 19. Comparison of 1mM phenobarbital-induced luc activities in S2 cells individually transfected with 0.1luc/w1, 0.5luc/w1, and 0.9luc/w1 reporter plasmids. Each bar represents fold induction relative to untreated control. Values represent mean fold induction ± S.D of three experiments done with different batches of cells. Student's t-test was done to compare the fold inductions. *P* values are: a < 0.02; and b < 0.04.

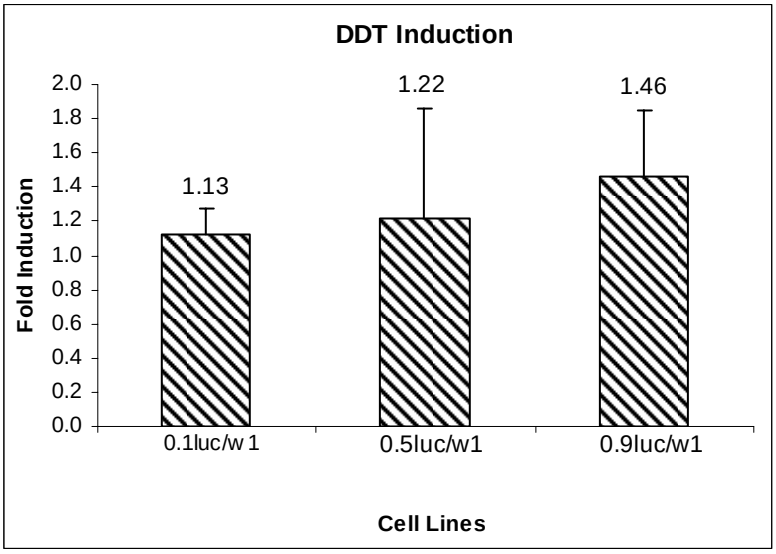

Figure 20. Comparison of 0.02mM DDT-induced luc activities in S2 cells individually transfected with 0.1luc/w1, 0.5luc/w1, and 0.9luc/w1 reporter plasmids. Each bar represents fold induction relative to untreated control. Values represent mean fold-induction ± S.D. of three experiments done with different batches of cells. Student's t-test was done to compare the fold inductions observed with three different plasmids. The *P* values were > 0.05.

Examination of synergistic effect of caffeine and PB on *Cyp6w1* promoter activity

To study the synergistic effect of caffeine and PB together on the reporter plasmids, S2 cells were individually transfected with each of 0.1luc/w1, 0.5luc/w1, and 0.9luc/w1 plasmids, and the pRL-null control plasmid. One day after transfection, the cells were treated with 4mM caffeine or 1mM PB or a mixture of both 4mM caffeine and 1mM PB media in individual wells. The fold-induction for each plasmid with caffeine alone, PB alone, and caffeine plus PB were compared to see whether there is any synergistic effect. It is clear from the results (Figure 21) that there was no synergistic effect of caffeine and PB in cells transfected with 0.1luc/w1 and 0.5luc/w1, although the latter plasmid showed significant (1.8-fold) when treated with caffeine alone. The cells transfected with 0.9luc/w1, however, showed a synergistic effect of the two chemicals in addition to significant induction by caffeine and PB alone.

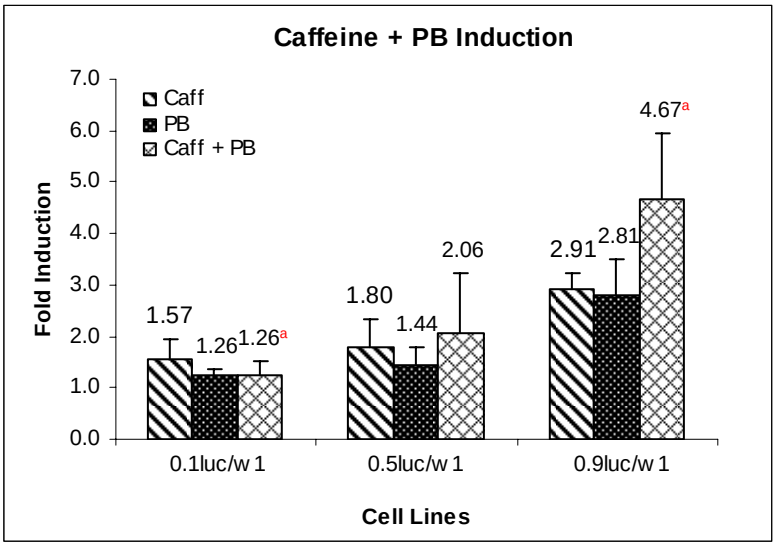

Figure 21. Synergistic effect of caffeine and PB on luc activities in S2 cells individually transfected with 0.1luc/w1, 0.5luc/w1, and 0.9luc/w1 reporter plasmids. Each bar represents fold-induction relative to the untreated controls. Values represent mean fold induction ± S.D. obtained with three different batches of cells. *P*-value of a: < 0.02.

IV. Discussion

A. The role of *Cyp6w1* in DDT resistance

It has been long understood that cytochrome P450 monooxygenases have been involved in the metabolism of virtually all insecticides, either leading to an activation of a molecule or more often a detoxification (Wilkinson and Brattsten, 1972; Hodgson, 1985; Agosin, 1985; Berge et al., 1998). For some insects, this detoxification is so active that the insecticide does not reach its molecular target before being metabolized and degraded, thus causing the insects to become resistant to the insecticides (Taylor and Feyereisen, 1996; Berge et al., 1998).

A study done by Daborn et al (2002) suggested that DDT resistance in field-collected strains of *Drosophila* is a monogenic trait produced by the overexpression of a single P450 gene, C*yp6g1*. A year later, Le Goff et al. (2003) suggested that although resistance in field-collected strains of *Drosophila melanogaster* may be associated with overtranscription of *Cyp6g1*, the DDT-selected strains created in a laboratory gives a different result. It was found that *Cyp6a8* gene was co-selected as an overexpressing P450 gene when the laboratory strain was put through five generations of DDT selection. Different results were also obtained by Daborn et al (2007) in their recent experiments. By using UAS:GAL4 binary system they found that transgenic flies over-expressing *Cyp6g1* or *Cyp12d1* had an increased survival rate when challenged with DDT (Daborn et al., 2007). However, neither *Cyp6a2* nor *Cyp6a8* conferred any resistance to DDT in the same experiment (Daborn et al., 2007). Although it appears that *Cyp6g1* is associated with DDT resistance, other studies tested Daborn et al.'s single gene theory and found that the phenomenon of DDT resistance in *Drosophila* is much more complex than a

single gene theory (Brandt et al., 2002; Pedra et al., 2004; Kuruganti et al., 2007).

To date, eight out of 90 P450 genes in the *Drosophila melanogaster* have been implicated in insecticide resistance and xenobiotic metabolism: CYP4E2, CYP6A2, CYP6A8, CYP6A9, CYP6G1, CYP6W1, CYP12A4, and CYP12D1 (Amichot et al., 1994; Maitra et al., 1996 and 2000; Brandt et al., 2002; Daborn et al., 2002; Pedra et al., 2004; Catania et al., 2004; Schlenke et al., 2004; Bogwitz et al., 2005; Festucci-Buselli et al., 2005; Chen et al., 2007). Although *Cyp6w1* maps close to a DDT resistance locus at 56 m.u. (mapped by Dapkus, 1992) in the resistant 91-R strain and has been shown to be overexpressed in resistant strains compared to susceptible ones, along with *Cyp6a2*, *Cyp12d1*, *Cyp6g1*, and *Cyp6a8* (Waters et al., 1992; Maitra et al., 1996; Dombrowski et al., 1998; Daborn et al., 2002; Pedra et al., 2004), not much is known about *Cyp6w1*'s role in resistance. It would seem that this close proximity to the DDT resistance locus would make it another candidate gene that may be involved in DDT resistance. Thus, in the present investigation, I used a transgenic approach to bring in the CYP6W1 cDNA of the DDT resistant 91-R strain into the susceptible w^{1118} strain. The CYP6W1 cDNA transgene was overexpressed in the fat body or in all cells using the GAL4/UAS system.

Analysis of CYP6W1 expression by qRT-PCR showed that when *Cyp6w1* is overexpressed in fat bodies, there was 1.3 to 3.0 fold expression in the individual transgenic lines compared to their controls. Expression levels among the independently transformed lines were not always consistent and it may be a result of position effect. However, DDT resistance bioassay data showed that the LD50 values of the transgenic lines showing overexpression of CYP6W1 were not different from the values found in the control flies inferring that there was no DDT resistance when CYP6W1 was

overexpressed alone. Nonetheless, when TU-GAL4 driver was used, the fold expression dramatically increased, but again the DDT resistance bioassays showed no significant increase in DDT resistance.

Interestingly, when doubly transformed flies were generated where *Cyp6w1* is overexpressed with *Cyp6g1* in the fat bodies, expression for *Cyp6w1* and *Cyp6g1* was 2.8- and 2.3-fold greater than the control flies, respectively. DDT bioassays show that resistance in these doubly transgenic flies was 1.9-fold greater than in the control flies. However, it is to be noted that the LD50 value of the flies transformed with *Cyp6g1* alone is 2.7-fold higher than the control (Kuruganti and Ganguly, unpublished results), whereas the flies transformed with *Cyp6w1* alone had a 1.2-fold lower LD50 value. Surprisingly, expression of CYP6W1 in flies doubly transformed with *Cyp6w1* and *Cyp6a2* increased 25-fold, while CYP6A2's expression dramatically fell to 0.4-fold. These doubly transformed flies also showed a 1.9-fold increase in DDT resistance. It is not completely understood why CYP6W1 had such a pronounced overexpression when combined with CYP6A2 but not CYP6G1. Nonetheless, since both doubly transgenic flies showed a 1.9-fold higher resistance, it may be concluded that the resistance trait was a result of CYP6W1 overexpression. However, if it was the case, one would expect increased DDT resistance in the flies transformed with CYP6W1 alone. Unfortunately, these singly-transgenic flies showed only 1.2-fold higher CYP6W1 expression, perhaps too low to confer the 1.9-fold increase in DDT resistance. All these data may suggest that perhaps *Cyp6w1* has no significant role in DDT resistance when expressed alone, but may play a role in resistance when it interacts synergistically with other CYP genes. Future work may resolve this issue.

B. The inducibility of *Cyp6w1* upstream DNA by xenobiotic compounds

Three xenobiotic compounds, caffeine, PB, and DDT, were used to study their effects on the transcriptional activity of the *Cyp6w1* upstream DNA. These xenobiotic compounds are some of the most commonly known chemicals that have been used to study the inducibility of various CYP genes (Dapkus, 1992; Goasduff et al., 1996; Pedra et al., 2004; Bhaskara et al., 2006, 2008; Willoughby et al., 2006; Chen and Li, 2007). In this present investigation, 0.1-, 0.5- and 0.9-kb upstream DNAs of the *Cyp6w1* gene of the DDT susceptible Canton S (NY) strain were studied. The results (Figure 16) showed that the 0.5- and 0.9-kb upstream DNAs had almost a 2-fold greater constitutive promoter activity compared to the 0.1-kb of upstream DNA. This suggests that the 0.1-kb DNA has just the basal promoter whereas the other DNAs have sequences that potentiate the basal promoter.

To identify putative sequence motifs that participate in constitutive expression and xenobiotic induction, the upstream DNA was analyzed by using MATCH program set at different stringencies. The results obtained with 0.85 matrix and 0.95 core similarities are shown in Table 11. Although this analysis did not identify any TATA boxes, a putative TATA box sequence (gcTTTAAAgc) is seen at position -62/-53 when the stringency of the search is reduced to 0.75 – 0.80. The location of this sequence is about 33 bp upstream of the putative transcription start site CTA. Sequence analysis also showed that the 0.1-kb DNA has the putative TATA box, but the 0.5-kb and 0.9-kb DNAs also have a putative CAAT box located about 150 bp upstream to the putative TATA box. CAAT boxes are generally present ~ 100 – 150 upstream of TATA in many

Gene	Position strand)	Core match	Matrix match	Sequence[a] (+ strand)	Factor name
Cyp6w1	708 (+)	1.000	0.899	ctTGACTactg	AP-1
	649 (+)	1.000	0.930	atTGACTactg	AP-1
	562 (+)	0.964	0.879	tttAATCAa	AP-1
	546 (+)	1.000	0.957	acgcTGACGcgc	CREB
	544 (+)	1.000	0.882	gcTGACGcgctg	CREB
	544 (+)	0.967	0.931	gcTGACGcgct	AP-1
	521 (-)	0.966	0.900	caactaACTTTtgct	Barbie Box
	496 (+)	0.955	0.965	ttgATTCAg	AP-1
	496 (-)	0.964	0.957	tTGATTcag	AP-1
	494 (-)	1.000	0.895	gattcaGCTTTttgt	Barbie Box
	481 (-)	1.000	0.900	gtatAGTCAtg	AP-1
	439 (-)	1.000	0.892	gcctAGTCAaa	AP-1
	374 (-)	0.967	0.907	atacTGTCAac	AP-1
	230 (+)	0.955	0.876	attATTCAa	AP-1
	126 (+)	0.967	0.907	gtTGACAgtct	AP-1
	81 (+)	1.000	0.867	ctggAAAGCtttggc	Barbie Box
	68 (-)	1.000	0.856	gcgatcGCTTTaaag	Barbie Box
	61 (+)	1.000	0.856	ctttAAAGCggccca	Barbie Box

Table 11. Locations of putative binding sites for transcription factors AP1 and CREB, and the Barbie box sequence motif in the 0.9-Kb upstream DNA of *Cyp6w1*. that participate in cAMP-mediated transcriptional regulation. MATCH program set at 0.85 matrix and 0.95 core similarities was used.

[a] The capital letters indicate the positions in the sequence which match with the core sequence of the matrix.

genes and are known to give increased transcription. It is possible the CAAT box that is present in both 0.5- and 0.8-kb upstream DNA plays a similar role, and for that reason both 0.5- and 0.8-kb DNA shows higher constitutive expression than the 0.1-kb upstream DNA.

Of the three DNA fragments, 0.9-kb only showed some induction with DDT, although it was not statistically significant compared to the control. Results from DDT treated cells generally gave inconsistently high levels of variation. This may be due to the toxicity of the chemical. For this reason it cannot be said conclusively whether the 0.9-kb upstream DNA is induced with DDT. Caffeine and PB, however, give a significant level of induction. With the 0.9-kb upstream DNA, the level of induction was about 3-fold for both chemicals. 0.5-kb DNA also showed about a 2-fold induction with caffeine, but only 1.4-fold induction with PB. The level of induction with 0.1-kb upstream DNA for both chemicals was very low. These results suggest that the regulatory sequences giving caffeine and PB induction are mostly located in the DNA between -500 to -900 bp regions.

Sequence analysis (Table 11) showed that the first 0.1-kb of upstream DNA does not have putative sequence motifs other than Barbie boxes that may give caffeine induction. Bhaskara et al (2006, 2008) have shown that activator protein (AP) Jun and cAMP are involved in the induction of *Drosophila Cyp6a8* gene. It is known that effect of Jun and cAMP is mediated through cis-acting sequence for AP-1 and CREB, respectively. Caffeine has been shown to increase the steady-state levels of two CYP mRNAs and the RNAs for FOS and JUN family proteins (Svenningsson et al., 1995, Goasduff et al., 1996). AP-1 is a hetero- or homodimer composed of JUN and FOS

proteins which must first dimerize in order to bind to the AP-1 recognition site on the DNA. AP-1 controls both basal and inducible transcription of several genes (Sng et al., 2004; Bhaskara et al., 2006). CREB (cAMP response element-binding) proteins are transcription factors which bind to certain DNA sequences called cAMP response elements (CRE) and thereby increase or decrease the transcription of certain genes (Sng et al., 2004). Because significant caffeine induction is only found within the 0.9-kb of upstream DNA, the AP-1 sites found within about -510 bp upstream DNA of *Cyp6w1* may not be involved in caffeine induction. Table 11 shows that the 0.5-kb DNA has 7 and 0.9-kb DNA has 11 putative binding sites for AP-1. No binding site is present in the 0.1-kb DNA. The region between 0.5-kb and 0.9-kb DNA also houses two CREB sites which are not present in the 0.5- or 0.1-kb DNA. If these sequences are functionally meaningful, they would be expected to give more inducibility of 0.9-kb DNA with caffeine compared to the other two DNA fragments.

Although PB has been shown to induce many *Drosophila* and *Musca CYP* genes (Scott and Wen, 2001), no DNA sequence elements have been discovered yet that may mediate the induction process. However, it has been indicated by various authors that a sequence called Barbie box may be responsible for PB induction. The Barbie box element originally discovered in the 5'-regulatory regions of cytochrome P450BM-1 and P450BM-3 genes of *Bacillus megaterium* is recognized by a barbiturate-regulated protein (He and Fulco, 1991). There are five putative Barbie boxes present in the 0.9-kb DNA of which three are present in the 0.1-kb DNA. If these sequences are functionally meaningful, 0.1-kb DNA is expected to show significant level of PB induction. But the

induction was very low. Therefore, the putative Barbie box may not be involved in PB induction.

A previous study showed that PB treatment increases protein binding with the putative AP-1 site found in the promoter of CYP2B2 promoter of rat (Roe et al., 1996). Since six putative AP-1 sites are present in the region between 0.5- and 0.9-kb DNA, it may be speculated that these AP-1 sites are not only involved in caffeine induction, but also PB induction. This, however, may not be the case because when S2 cells are treated with a mixture of caffeine and PB, a synergistic effect is seen, suggesting that these two chemicals may induce through different DNA elements. Similar synergistic effects of caffeine and PB have also been observed in the *Cyp6a8* promoter (Morra and Ganguly, unpublished results). Future site directed mutagenesis studies may uncover the sequences involved in caffeine and PB induction.

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