University of Tennessee, Knoxville

Trace: Tennessee Research and Creative Exchange

Doctoral Dissertations

Graduate School

12-2006

Studies on Cytochrome P450 genes in *Drosophila melanogaster*: Relationship between over expression and DDT resistance, and xenobiotic induction

Srilalitha Kuruganti

University of Tennessee - Knoxville

Recommended Citation

Kuruganti, Srilalitha, "Studies on Cytochrome P450 genes in *Drosophila melanogaster*: Relationship between over expression and DDT resistance, and xenobiotic induction. " PhD diss., University of Tennessee, 2006.
https://trace.tennessee.edu/utk_graddiss/1976

This Dissertation is brought to you for free and open access by the Graduate School at Trace: Tennessee Research and Creative Exchange. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of Trace: Tennessee Research and Creative Exchange. For more information, please contact trace@utk.edu.

To the Graduate Council:

I am submitting herewith a dissertation written by Srilalitha Kuruganti entitled "Studies on Cytochrome P450 genes in *Drosophila melanogaster*: Relationship between over expression and DDT resistance, and xenobiotic induction." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.

Ranjan Ganguly, Major Professor

We have read this dissertation and recommend its acceptance:

Bruce McKee, John Koontz, Albrecht von Arnim, Sundar Venkatachalam

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a dissertation written by Srilalitha Kuruganti entitled "Studies on Cytochrome P450 genes in *Drosophila melanogaster*: Relationship between over expression and DDT resistance, and xenobiotic induction." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry, Cellular and Molecular Biology.

Ranjan Ganguly

Major Professor

We have read this thesis
and recommend its acceptance:

Bruce McKee

John Koontz

Albrecht von Arnim

Sundar Venkatachalam

Accepted for the Council:

Linda Painter

Interim
Dean of Graduate Studies

(Original signatures are on file with official student records)

Studies on Cytochrome P450 genes in *Drosophila melanogaster:* Relationship between over expression and DDT resistance, and xenobiotic induction

A Dissertation

Presented for the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Srilalitha Kuruganti

December 2006

To my father,

Kuruganti Prabhakar Sastry,

without whose constant love, encouragement and motivation, this would

have been impossible

ACKNOWLEDGEMENTS

It was a great pleasure working in Dr. Ranjan Ganguly's lab. Dr.Ganguly's constant attention and support have helped me a lot towards my scientific development. He is a great advisor helping students in academics as well as non-academics. He is always available and provided excellent guidance, trouble shooting in all endeavors from painful microinjections to cloning to setting up numerous genetic crosses. I would like to sincerely thank him for all his advice and help. I would also like to thank Dr. Nivedita Ganguly for being so warm and friendly during all my visits to their house.

I would like to thank my committee members, Dr. Bruce McKee, Dr. John Koontz, Dr. Von Arnim and Dr. Sundar Venkatachalam for their encouragement and critical evaluation of my research. My sincere thanks to Dr. Jae Park and Dr. Gyunghee Lee for friendly advice regarding microinjection and also for providing balancer stocks for various host strains that made my research easier. I would like to thank Dr. Liz Howell, Dr. Barry Bruce, Dr. Cynthia Peterson, Dr. Ranjan Ganguly and Dr. Bruce McKee for their excellent teaching that improved my scientific knowledge. I would like to thank Dr. Sharon Thomas for kindly advice during stressful times and for reading my prelim grants critically. I want to thank Dr. Arnold Saxton from the Animal Sciences department for helping me with probit and microarray data analysis in SAS.

I would like to thank Sally Chopra, my friend for being there during the happy and difficult times. I can never forget the chats with her in the department. I would like to thank Srividya Bhaskara, my friend for constant support and for maintaining a friendly atmosphere in the lab. I would like to thank Vita Lam for helping with endless DDT

bioassays and Chandrashis Bhowmick for his help in the lab. I want to thank Charity Olson for helping with the PCR amplification of recombinant lines. My sincere thanks to all the undergraduates, Kim Crowder, Lauren, Maggie, Neha for making fly food that made life much easier. I want to thank Julia Gouffon and Pradeep Chimakurthy for patiently guiding me through the microarray software and data analysis. I would like to thank BCMB department for providing this excellent opportunity and for all the resources.

I would like to specially thank my mother, Swarajya lakshmi for sending me for studies to US. It was a difficult decision and I would not have been here without her courage and support. I would like to extend my sincere thanks and gratitude to my husband, PD Kumar for guiding me and helping me through all the aspects. Being a Ph.D student himself, he always understood what I was going through and encouraged me towards completing my goal. I would like to thank my brother, Teja for being there for me all the time. Without him, this would have been impossible. I would like to thank my sister and my best friend, Srivalli for sharing my happiness and sorrows all the time. I would like to thank all my family members for their love and support. I would like to thank all my friends in Knoxville especially Sampath and Sankar for making my Knoxville stay more fun. I would like to thank my friends, Malathi, Harini, Satya and Priya for making me laugh all the time, even during my bad times and also for helping with my thesis formatting and corrections. I will take these memories with me wherever I go in the future.

ABSTRACT

Cytochrome P450 monooxygenases (CYPs), are involved in the metabolism of a diverse group of endogenous and xenobiotic compounds. In insects, CYPs are involved in conferring resistance against insecticides. In *Drosophila*, the expression of *Cyp6a2, Cyp6a8, Cyp6g1, Cyp6w1* and *Cyp12d1* is higher in the resistant compared to susceptible strains. Recent study by Daborn et al (2002, *Science*, 297, 2253-2256) showed that *Cyp6g1* alone can confer resistance phenotype. The aim of my first objective was to re-examine this claim and second to examine the effect of common xenobiotic compounds on the transcriptome of *Drosophila.*

In first objective, six strains of *Drosophila* were examined for DDT resistance and *Cyp6g1* expression. Results showed that some of the highly susceptible strains showed high level of *Cyp6g1* expression and *Accord* element in the *Cyp6g1* upstream DNA. When *Cyp6g1* allele of the resistant 91-R strain was substituted with that of the susceptible 91-C strain via recombination, the resulting three recombinant lines retained high level of resistance like the 91-R strain, but showed very low *Cyp6g1* expression. This suggests that there is a correlation between overexpression of *Cyp6g1* and the presence of *Accord* transposable element but not DDT resistance.

In the second part of the first objective, I directly examined the role of the *Cyp6a2* and *Cyp6g1* in DDT resistance. Germ line transformation in susceptible strain showed that there was a two-fold increase in DDT resistance (LD_{50}) in transformed flies showing

two-fold higher expression of GAL4/UAS driven CYP6A2 or CYP6G1 cDNA. A cumulative increase (4-fold) in DDT resistance was observed when both cDNAs were overexpressed in the same fly. Results suggest that the expression of multiple *Cyp* genes may be needed to confer a high level of DDT resistance.

In the second objective, microarray was used to examine the transcripts induced by caffeine and phenobarbital. Results showed that genes involved in detoxification, carbohydrate metabolism, signal transduction and *Cyp* genes are induced by caffeine and phenobarbital. These are the same group of genes overexpressed in the resistant 91-R and recombinant strains. These studies shed light on the molecular basis of induction of *Cyp* genes and insecticide resistance.

// COMPREHENSIVE ABSTRACT

Cytochrome P450 monooxygenases or CYPs, a superfamily of enzymes present in all organisms, are involved in the metabolism of a diverse group of endogenous and xenobiotic (foreign) compounds. In insects, CYPs are involved in conferring metabolic resistance against various insecticides. In many insects, over expression of one or more *CYP* gene is found in the resistant strain. In *Drosophila*, the level of *Cyp6a2, Cyp6a8, Cyp6g1, Cyp6w1* and *Cyp12d1* expression is much higher in the resistant strains than in the susceptible ones. However, it is not known how many of these genes are actually involved in resistance, although a recent study Daborn et al (2002, *Science*, 297, 2253-2256) claims that *Cyp6g1* alone can confer resistance phenotype. The aim of the first objective of this investigation has been to re-examine this claim and the aim of second objective has been to examine the effect of two common xenobiotic compounds on the transcription profile of all genes in *Drosophila* including the ones involved in detoxification so that these xenochemicals could be used in future to understand the regulation of *Cyp* and other genes with similar functions.

For the first objective, six strains of *Drosophila* were examined for DDT resistance and *Cyp6g1* expression. The results showed that some strains, which are highly susceptible to DDT showed high level of *Cyp6g1* expression like the super-resistant 91-R strain. These strains also have *Accord* element in the upstream DNA of *Cyp6g1*, which Daborn et al (2002) claimed is needed for high *Cyp6g1* expression and DDT resistance. Cloning and sequencing results showed that *Cyp6g1* alleles of all strains are almost

identical. When *Cyp6g1* allele of the resistant 91-R strain was substituted with that of the susceptible 91-C strain via recombination, the resulting three recombinant lines retained high level of resistance like the 91-R strain. However, they lacked the *Accord* element, and showed very low *Cyp6g1* expression. Taken together, it can be concluded that there is a correlation between overexpression of *Cyp6g1* and the presence of *Accord* transposable element but neither overexpression nor the *Accord* element is necessary for DDT resistance, as claimed by Daborn et al (2002, *Science*, 297, 2253-2256).

In the second part of the first objective, I directly examined the role of the *Cyp6a2* and *Cyp6g1* in DDT resistance because these two genes show over expression in resistant strains and they map close to known resistance loci. Germ line transformation of susceptible strain and GAL4/UAS system were used for this purpose. The results showed that there was a two-fold increase in DDT resistance (LD_{50}) in transformed flies showing two-fold higher expression of GAL4/UAS driven CYP6A2 or CYP6G1 cDNA. A cumulative increase (4-fold) in DDT resistance was observed when both cDNAs were over expressed in the same fly. These results suggest that the expression of multiple *Cyp* genes may be needed to confer a high level of DDT resistance. Since, the level of resistance is several orders of magnitude lower than that found in the 91-R or *Wisconsin* strain, it is concluded that *Cyp6a2* and *Cyp6g1* alone or together cannot give high level of resistance, which appears to be a multifactorial trait.

In the second objective, microarray technique was used to examine how many transcriptomes, especially the CYPs, are induced by two common xenobiotic compounds

such as caffeine and phenobarbital. Although many *Cyp* genes are induced by various xenobiotic compounds, the mechanism of *Cyp* gene regulation in insect is not known. Results showed that the genes involved in different functions such as detoxification, carbohydrate metabolism and signal transduction are induced by both caffeine and phenobarbital. Many *Cyp* genes are also induced by both the chemicals. These data suggest that for all these genes, caffeine and phenobarbital probably use a common regulatory pathway. Interestingly, these are the same group of genes that are overexpressed in the resistant 91-R and recombinant strains. These studies pave the way to understand the molecular basis of xenobiotic induction of *Cyp* genes and insecticide resistance better.

Table of Contents

Chapters **Page**

1. General Introduction

Cytochrome P450 enzymes – discovery and classification 1

Molecular structure of P450s 2

Reactions catalyzed by CYPs 7

Endogenous functions of P450s 8

Role of CYPs in xenobiotic metabolism 14

Induction of CYPs by different xenobiotic compounds 17

CYP gene overexpression and genetics of insecticide resistance in insects 20

Objectives of proposed research 24

2. Investigation of the role of *Cyp6g1* in conferring DDT resistance in *Drosophila melanogaster* 27

Introduction 27

Materials and Methods 29

Drosophila strains 29

Total RNA isolation and northern blot hybridization 30

DDT resistance bioassay 32

Analysis of the upstream DNA of different *Cyp6g1* alleles 32

Protein isolation and western blot analysis 33

Cloning of *Cyp6g1* alleles from different strains 35

Synthesis of stocks with recombinant second chromosome 38

Microarrays 40
Microarray data analysis 42

Results 43
Lack of correlation between CYP6G1 RNA and DDT resistance 43
Lack of correlation between CYP6G1 protein and DDT resistance 49
Association between the presence of *Accord* element, overexpression of *Cyp6g1* and DDT resistance 49
Sequence comparison of *Cyp6g1* alleles from resistant and susceptible strains of *Drosophila* 54
Substitution of the *Cyp6g1* allele of resistant 91-R strain with the allele from the susceptible 91-C strain 57
Genome wide expression analysis of genes differentially expressed genes between 91-R, RC-21 and 91-C strains 60

Discussion 76

3. Overexpression of *Cyp6a2* and *Cyp6g1* genes in DDT susceptible strain of *Drosophila melanogaster* 83

Introduction 83

Materials and Methods 86

Drosophila stocks 86
Total RNA and poly(A)$^+$ RNA isolation 87
Synthesis of *Cyp6a2* and *Cyp6g1* cDNAs and cloning into transformation vector 88
Cloning of *Cyp6g1* and *Cyp6a2* cDNAs into pUAST vector for germ line transformation 91

P-element mediated transformation 92
Chromosomal localization of the transgene 93
Real time Quantitative PCR 98
DDT resistance bioassay 100

Results 101
Characterization of the *Cyp6* cDNAs, and linkage analysis and the chromosomal locations of the transgenes 101
Analysis of *Cyp6a2* and *Cyp6g1* RNA levels in the transgenic lines 109
Investigation of the DDT resistance phenotype in the transgenic lines 113

Discussion 118

4. Effect of caffeine and phenobarbital on the transcriptome of *Drosophila melanogaster.* 123

Introduction 123
Materials and Methods 125
Drosophila strains and treatments 125
Total RNA isolation and northern blot hybridization 126
Microarrays 127
Microarray data analysis 129

Results and Discussion 130
Genes upregulated by both caffeine and phenobarbital 144
Transcripts encoding developmental and proteolysis genes are downregulated by caffeine and phenobarbital. 151
Induction of signal transduction genes by caffeine and repression by 154

phenobarbital

Down regulation of genes involved in transcription by phenobarbital 155

Conclusions 157

5. Conclusions 158

References 166

Vita 191

List of Tables

Table		Page
1-1	Number of families and sequences found in selected taxonomical groups	3
1-2	Different metabolic reactions catalyzed by each family of P450 in humans	11
1-3	Some diseases associated with mutations in Cytochrome P450 enzymes.	12
2-1	Primers used for the amplification of *Cyp6g1* gene in three fragments	37
2-2	Expression of *Cyp6g1* in different resistant and susceptible strains of *Drosophila melanogaster*	46
2-3	DDT resistance bioassay	47
2-4	Nucleotide and amino acid substitutions among *Cyp6g1* alleles (+6/+2803 region) between different resistant and susceptible strains	55
2-5	Comparison of the *Cyp6g1* alleles of three recombinant lines, 91-C and 91-R strains	59
2-6	Comparison of DDT resistance in 91-R, 91-C and recombinant lines	67
2-7	Genes that are commonly upregulated in 91-R and RC-21 strains compared to 91-C	72
2-8	Genes that are commonly repressed in 91-R and RC-21 strains compared to 91-C	74

3-1 Primers used for the amplification of *Cyp6g1* and *Cyp6a2* cDNA. 90

3-2 Primers used to determine the cytological position of the transgene insertion by inverse PCR 97

3-3 Primers used to determine the expression of *Cyp6a2* and *Cyp6g1* in transgenic lines 99

3-4 Statistics of microinjections performed with *Cyp6a2* and *Cyp6g1* cDNA clones 107

3-5 Characterization of *Cyp6g1* and *Cyp6a2* transgenes in *Drosophila melanogaster* 108

3-6 Analysis of transgene expression by Real-time PCR 112

3-7 DDT resistance bioassay of the transgenic lines 115

4-1 List of genes differentially expressed in caffeine treated flies 133

4-2 List of genes differentially expressed in phenobarbital treated flies 138

4-3 Representation of the different categories of genes that are differentially expressed in caffeine and phenobarbital treated flies. 145

4-4 List of genes upregulated by both caffeine and phenobarbital 147

4-5 List of genes downregulated by both caffeine and phenobarbital 152

List of figures

Figures **Page**

1-1 Topology diagram showing the secondary structural alignment of a typical P450 protein. 5

1-2 Ribbon representation of the folded CYP2C5 showing its putative association with the ER membrane (in purple). 6

1-3 Mechanism of catalysis of Cytochrome P450 enzymes. 9

1-4 Positions of major insecticide resistance loci (R1 – R5) in *Drosophila melanogaster* genome 23

2-1 Strategy for cloning and sequencing *Cy6g1* alleles from 91-R, 91-C, *Wisconsin*, Canton SH and Hikone RH strains 36

2-2 Genetic crossing scheme to substitute *Cyp6g1* allele of 91-R strain with the allele from 91-C strain 39

2-3 Northern blot analysis of expression of *Cyp6g1* in various resistant and susceptible strains of *Drosophila melanogaster* 45

2-4 Dose response curves derived from the data in Table 2-3 for DDT resistance in different strains of *Drosophila melanogaster* 48

2-5 Western blot analysis of CYP6G1 protein levels in DDT resistant and susceptible strains of *D. melanogaster* 50

2-6 Agarose gel picture of the PCR products obtained from amplification of the upstream DNA of different *Cyp6gl* alleles 52

2-7 Comparison of the sequence of the insertional DNA (upperline) and *Ninja* transposable element (AF520587) (bottom line) 53

2-8 Northern blot analysis of the expression of *Cyp6a2* in the adult flies of recombinant RC-21, RC-35, RC-48 stocks, 91-C and 91-R strains of *Drosophila melanogaster* 61

2-9 Quantitative analysis of the data shown in Figure 2-8 showing the expression of *Cyp6a2* in recombinant stocks, 91-R and 91-C strains of *Drosophila melanogaster* 62

2-10 Expression of *Cyp6g1* in the adult female flies of recombinant RC-21, RC-35, RC-48 stocks, 91-C and 91-R strains of *Drosophila melanogaster* 63

2-11 Quantitative analysis of the expression of *Cyp6g1* in the recombinant stocks, 91-R and 91-C strains of *Drosophila melanogaster* 64

2-12 Northern blot analysis of the expression of *Cyp6a8* in the adult flies of recombinant RC-21, RC-35, RC-48 stocks, 91-C and 91-R strains of *Drosophila melanogaster* 65

2-13 Quantitative analysis of the expression of the expression of *Cyp6a8* in recombinant stocks, 91-R and 91-C strains of *Drosophila menalogaster* 66

2-14 Dose response curves derived from the data in Table 2-6 for DDT resistance in recombinant stocks compared with 91-R and 91-C of *Drosophila melanogaster* 68

2-15 Number of genes in different categories overexpressed in 91-R strain compared to 91-C strain 70

2-16 Graphical representation of different categories of genes overexpressed in RC-21 strain compared to the 91-C strain 71

3-1 Schematic representation of the steps involved in the synthesis of cDNA and its cloning into transformation vector 89

3-2 Genetic cross employed to determine the chromosome linkage of transgenes 94

3-3 Schematic representation of the strategy of Inverse PCR 96

3-4 Nucleotide sequence comparison of the isolated *Cyp6a2* cDNA with the *Cyp6a2* sequence present in the database 102

3-5 Nucleotide sequence comparison of the isolated *Cyp6g1* cDNA with the *Cyp6g1* sequence present in the database 104

3-6 Genetic crosses of the transgenic lines with tissue specific GAL4 stocks to overexpress *Cyp6a2* or *Cyp6g1* transgene in fat bodies 110

3-7 Genetic cross to generate a stock with *Cyp6a2* and *Cyp6g1* transgenes in the same fly 114

3-8 Dose response curves of the transgenic lines expressing *Cyp6a2* and *Cyp6g1* in fat bodies 116

4-1 Effect of Caffeine and barbiturate compounds (barbital and phenobarbital) on the *Cyp6g1* gene expression in the 91-C strain of *Drosophila melanogaster* 131

4-2 Venn diagram showing the genes differentially regulated upon treatment with caffeine and phenobarbital 146

Chapter I

General Introduction

Cytochrome P450 enzymes – discovery and classification

Cytochrome P450 enzymes (CYPs or P450s) comprise a superfamily of heme proteins involved in the oxidative and reductive metabolism of a diverse group of endogenous and exogenous compounds (Fleming et al., 2006; Guengerich, 2006; Bernhardt, 2006, for review). CYPs were first discovered as a microsomal carbonmonoxide-binding pigment by Klingenberg et al. (1958). When rat liver microsomes were treated with the reducing agent, sodium dithionite, and then gassed with carbonmonoxide, a novel pigment absorbing light at 450nm was observed (Klingenberg, 1958). This pigment was named P450. Later, in 1962, the microsomal carbon monoxide binding pigment P450 was found to contain a heme moiety and it was formally named cytochrome P450 (Omura and Sato, 1962, 1964). In initial experiments, it was found that the CYPs present in the microsomes of adrenal glands catalyzed the hydroxylation of 17-hydroxy progesterone at the C21 position (Estabrook et al., 1963). Later, it was discovered that the liver P450 also functions as a terminal oxidase in the metabolism of codeine, monomethyl-4-aminopyrine and acetanilide (Cooper, 1965).

Cytochrome P450s have been discovered in all taxonomical groups. In almost all living organisms, these enzymes are present in more than one form, thus forming one of the largest families of enzymes. However, the number of families and enzymes varies among different organisms. As of Jul 18, 2006, total number of P450 sequences

discovered in various organisms is 6051 (http://drnelson.utmem.edu). The numbers of different CYP families and genes discovered so far in few representative groups are shown in Table 1-1. The number of different CYP sequences in different species is also variable. It ranges from the highest (323) sequences in rice to none in *Salmonella typhimurium* and *Plasmodium falciparum* (Guengerich, 2003). Humans have 57, mouse has 102 and *Caenorhabditis elegans* has 74 CYP genes (http://drnelson.utmem.edu). Genome sequencing project has identified 90 CYP sequences in *Drosophila melanogaster* of which seven are pseudogenes (Tijet et al., 2001).

Cytochrome P450s are classified based on the amino acid sequence identity. First, to be classified as a CYP, the polypeptide must have CYP-like molecular structure as discussed below. CYPs that show greater than 40% amino acid identity are grouped under same family and those showing greater than 55% identity belong to the same subfamily (Scott and Wen, 2001; Bernhardt 2006, for review). The name of the families are denoted by numerals 1,2, 3 etc, and the subfamilies are denoted by alphabets (Nelson et al., 1996). As of Jul 18, 2006, 711 families and 814 subfamilies of CYPs have been identified (http://drnelson.utmem.edu).

Molecular structure of P450s

The eukaryotic P450s are bound to the membranes of endoplasmic reticulum or mitochondria and the prokaryotic P450s are cytoplasmic (Omura, 1999). All P450s are made of approximately 500 amino acid long single polypeptide chain with a highly

Table 1-1

Number of CYP families and sequences found in selected taxonomical groups

Taxonomical groups	# of families	# of P450 sequences
Animals	99	2279
Plants	97	1932
Fungi	282	1001
Bacteria	177	621
Protists	51	210
Archaea	5	8

conserved C-terminal and less conserved N-terminal regions. All microsomal P450s have a highly hydrophobic "signal anchor sequence" made of 20-25 amino acid residues at the N-terminal end. This sequence targets and anchors the P450 molecules to the microsomal membrane (Sakaguchi et al., 1987). CYPs found in mitochondria are actually encoded by the nuclear genes. These CYPs also have the signal anchor sequence. However, the anchor sequence is proteolytically cleaved after the enzyme is imported to the mitochondria (Omura, 1999).

The first P450 purified and crystallized is a water soluble bacterial P450 from *Pseudomonas putida* (P450cam) (Poulos et al., 1987). Comparison of the three dimensional structures of the different P450s such as P450cam, P450BM-P and P450terp revealed that the shape of the protein is an asymmetrical triangular prism composed of two domains: one that is predominantly α-helical accounting for 70% of the protein and one that is predominantly β-sheet accounting for 22% of the protein (Figure 1-1). The α-helical domain contains helices B' through K, helix L and sheets $\beta_{3,}$ β_4 and $\beta_{5.}$ The β-sheet domain contains sheets $\beta_{1,}$ β_2, and helices A, B and K'. All these elements are connected together by random coils and loops (Graham-Lorence and Petersen, 1996).

The ribbon structure of Cytochrome P450 is shown in Fig. 1-2. Although the CYP proteins have less than 20% sequence identity, all P450s appear to have similar structural fold. CYPs contain a conserved heme-binding domain at the C-terminus containing the axial *Cys* ligand within a conserved sequence (PFXXGXXXCXG). There are only three residues absolutely conserved among the P450 proteins: the cysteine residue present in the heme binding region that serves as a fifth co-ordinating ligand to the heme iron, Glu

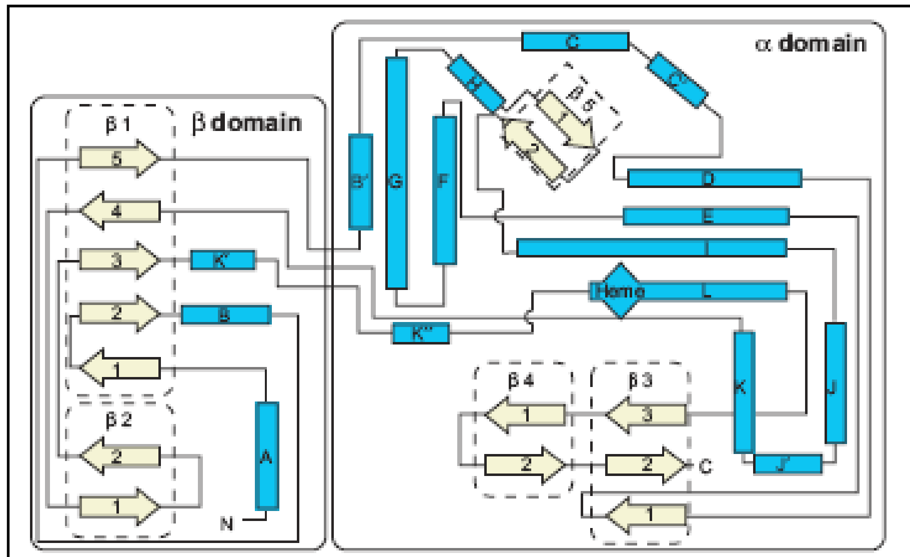


Figure 1-1: Topology diagram showing the secondary structural alignment of a typical P450 protein. The helices are shown in blue boxes, β sheets are shown as yellow arrows with dotted lines. The β-domain is associated with substrate recognition and the access channel and the α-domain is the catalytic center. (adapted from Graham Lorence and Petersen, 1999)

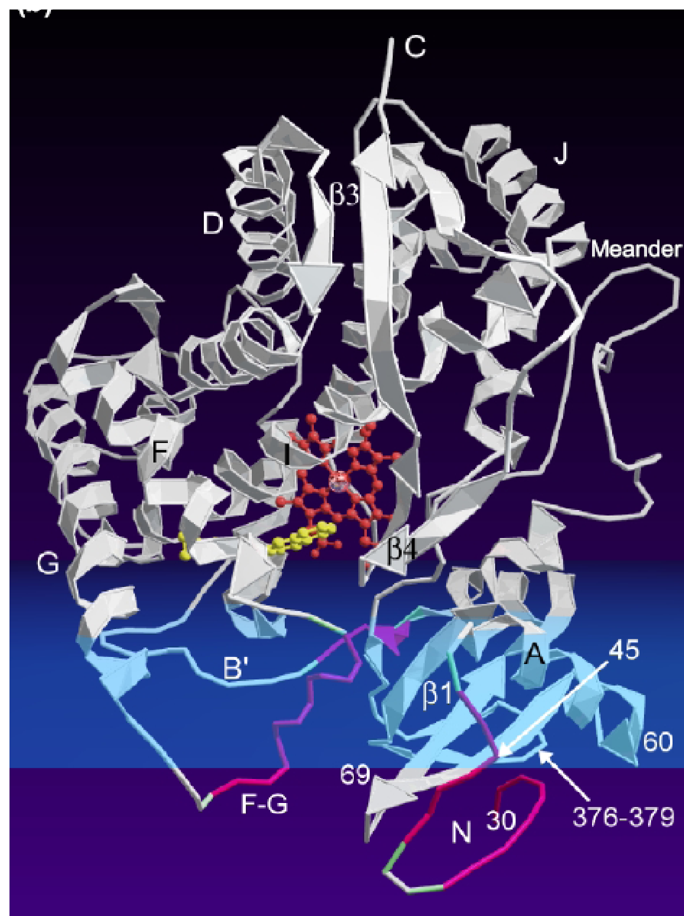


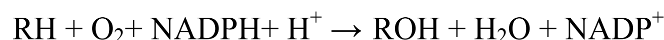
Figure 1-2. Ribbon representation of the folded CYP2C5 showing its putative association with the ER membrane (in purple). The heme moiety is shown in orange and the substrate is shown in yellow. The α-domain is on top left, the β domain more closely associated with the membrane at bottom right. The numbers indicate the positions of the amino acid in the primary sequence. The proximal (back) face of the protein is involved in redox partner recognition and electron transfer to the active site; protons flow into the active site from the distal face (front) (adapted from Williams, 2000).

and Arg in the K helix that forms a salt bridge facing the region named as “meander”. Another highly conserved (A/G)-G-X- (E/D) – T sequence is present in the middle of the I helix, directly over pyrrole ring B of the heme and appears to form a slight bend in the I helix. Most microsomal P450s have a conserved tryptophan residue that may have important function in the transfer of electrons from the reductase to the heme (Lewis, 1996)

Apart from the conserved structure, there are regions with large structural differences that are involved in substrate recognition and binding as well as redox partner binding. In these variable regions such as helices F and G and the F-G loop, the length and positions of the α-helices, β-sheets and loops will differ among different P450s to accommodate for diverse substrates (Graham-Lorence, 1999).

Reactions catalyzed by CYPs

CYPs are known to catalyze more than 60 different types of chemical reactions such as hydroxylations, alkylations, epoxidations, dealkylations and N- and S-oxidation (Estabrook, 1996). The overall reaction catalyzed by P450 enzymes is shown below.



$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

In this reaction, RH is the substrate which is converted to ROH via monooxygenase reaction (Guengerich, 2003, for review). Both microsomal and mitochondrial P450s in eukaryotes utilize NADPH as the electron donor for the monooxygenation reactions, whereas the bacterial P450s utilize NADH. In the case of mitochondrial P450s, the NADPH-linked flavoprotein and a ferredoxin type iron-sulfur

protein, named adrenodoxin catalyze the electron transfer from NADPH. On the other hand, microsomal P450s consists of two membrane bound components, the heme bound to CYP as an electron acceptor and NADPH-cytochrome P450 reductase that acts as an electron donor (Werck-Reichhart, 2000).

Depending on the carrier used for the transfer of electrons from NADPH to the catalytic site, P450s are classified into four classes. Class I proteins require both an FAD-containing reductase and an iron-sulfur redoxin. Class II proteins require only an FAD/FMN containing P450 reductase for the transfer of electrons. Class III enzymes require no electron donor and the Class IV receive electrons directly from NAD(P)H. The detailed mechanism of catalysis is shown in Fig. 1-3 (Werck-Reichhart, 2000).

Endogenous functions of P450s

CYPs are involved in various types of metabolic functions. In mammals, they are involved in the biosynthesis of steroid hormones, vitamins (A and D), prostaglandins, cholesterol, fatty acids, bile acids and other eicosanoids (Guengerich, 2003; Bernhardt, 2006, for review). Microsomal family 4 CYPs present in the liver of humans are involved in the synthesis and metabolism of eicosanoids, for example, ω-hydroxylation of prostaglandins and leukotrienes (Omura, 1999). Mammalian P450, CYP5A1 is involved in the conversion of prostaglandin H2 (PGH2) to thromboxane B2 without the requirement of molecular oxygen or supply of reducing equivalents from NADPH. CYPs catalyze the oxidative removal of 14α-methyl group from the intermediate compounds (lanosterol in the synthesis of cholesterol and ergosterol and obtusiferiol in the synthesis

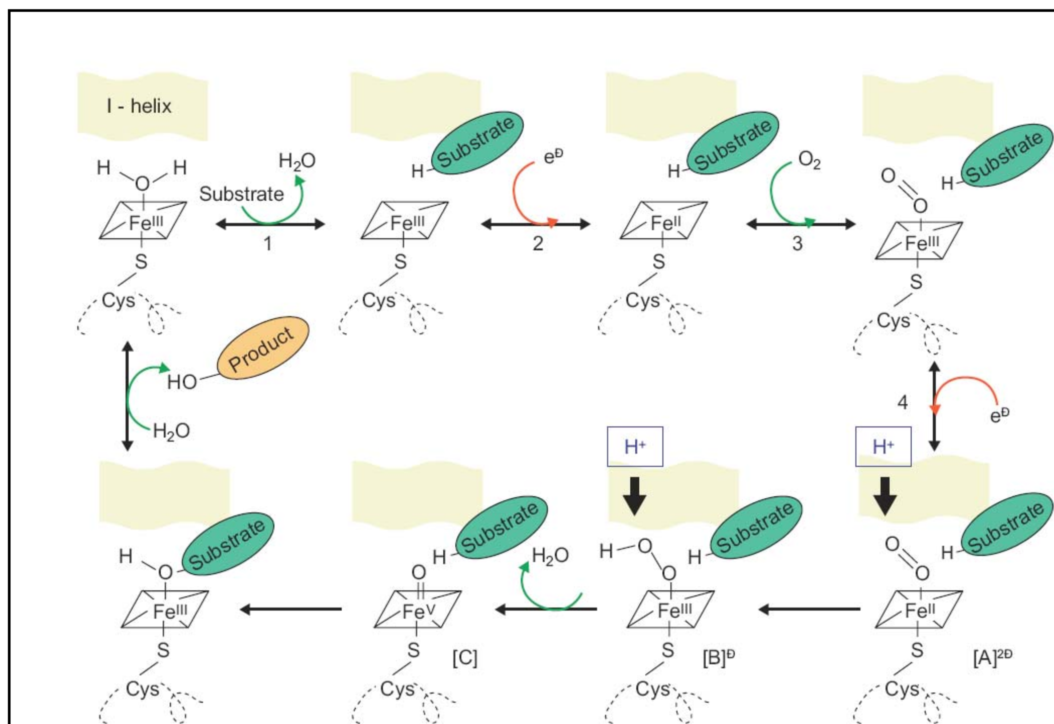


Figure 1-3. Mechanism of catalysis of Cytochrome P450 enzymes. One of the atoms of molecular oxygen is inserted into the substrate and the second atom is reduced to water. The catalyzed reaction is hydroxylation using an electrophilic and highly reactive iron-oxo intermediate (species [C], bottom row). The hydroperoxo form of the enzyme (species $[B]^{D}$) is also an electrophilic oxidant catalyzing H^+ insertion. The dehydration or isomerization and reduction are catalyzed by the oxygen free forms of the enzyme and nucleophilic attack is catalyzed by the species $[A]^{2D}$ and $[B]^{D}$. (Adapted from Werck-Reichhart, 2000)

of phytosterols), which is the common essential step in the biosynthesis of all sterols (cholesterol of animals, ergosterol of fungi and phytosterols in plants). Metabolism of steroid hormones is the major physiological function of the microsomal P450s in the hepatocytes (Omura, 1999). Another major catabolic pathway is the synthesis of bile acids from cholesterol, which is dependent on three P450 catalyzed reactions (Bernhardt, 2006). The list of different metabolic reactions catalyzed by each family of human P450s is given in Table 1-2.

Cytochrome P450s also participate in the biosynthesis and metabolism of various lipid biofactors. The microsomal ω-hydroxylation of fatty acids such as arachidonic acid released from the membrane phospholipids is catalyzed by cytochrome P450 (Estabrook, 1996). The metabolism of vitamin D3 into its physiologically active form, 1, 25-dihydroxy vitamin D3 is catalyzed by two P450s, CYP27A1 in the liver and CYP27B1 in the kidney. P450s are also involved in flower coloring in plants, environmental bioremediation by microorganisms (Graham and Peterson, 1996; Estabrook, 1996, for review). Null mutations in the CYPs with physiological functions often lead to serious diseases, whereas similar mutations in the xenobiotic-metabolizing CYPs will affect drug metabolism and susceptibility to some diseases, without directly causing the disease (Nelson, 1999). Some of the diseases associated with mutations in P450s are given in Table 1-3 (Nebert, 2002).

In plants, P450s are involved in the biosynthesis of UV protectants (flavonoids), pigments (anthocyanins), defense compounds (isoflavonoids, phytoalexins, hydroxamic acids, terpenes), fatty acids, hormones (gibberellins, brassinosteroids),signaling

Table 1-2

Different metabolic reactions catalyzed by each family of P450 in humans.

CYP family	Main functions
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic metabolism Arachidonic acid metabolism
CYP3	Xenobiotic and steroid metabolism
CYP4	Fatty acid hydroxylation
CYP5	Thromboxane synthesis
CYP7	Cholesterol 7α-hydroxylation
CYP8	Prostacyclin synthesis
CYP11	Cholesterol side-chain cleavage Steroid 11β-hydroxylation Aldosterone synthesis
CYP17	Steroid 17α-hydroxylation
CYP19	Androgen aromatization
CYP21	Steroid 21-hydroxylation
CYP24	Steroid 24 hydroxylation
CYP26	Retinoic acid hydroxylation
CYP27	Steroid 27-hydroxylation
CYP39	Unknown
CYP46	Cholesterol 24-hydroxylation
CYP51	Sterol biosynthesis

Data adapted from Gonzalez, 1992; Nelson et al., 1996; Nelson, 1999; Lund et al., 1999; Guengerich, 2003.

Table 1-3

Some diseases associated with mutations in Cytochrome P450 enzymes

Gene involved	Organism	Defect
CYP1B1	Human	Congenital Glaucoma
CYP11A1, CYP21A2	Human	Adrenal hyperplasia
CYP17A1	Human	Mineralocorticoid excess
CYP27B1	Human	Rickets
CYP24A1	Human	Hypervitaminosis
CYP7A1	Human	Hypercholesterolaemia
CYP5A1, 8A1	Human	Clotting and inflammatory disorders, pulmonary hypertension, coronary artery disease
CYP7B1	Human	Severe hyperoxysterolaemia
CYP27A1	Human	Cerebrotendinous xanthomatosis
CYP11A1	Human	Lipoid adrenal hyperplasia
CYP19	Human	Failure of normal female development
CYP84A1	*Arabidopsis thaliana*	Does not accumulate sinapoyl malate; altered lignin composition
CYP90A1	*Arabidopsis thaliana*	De-etiolated in dark and dwarfism; male sterility in the light.
CYP72B1 overactive	*Arabidopsis thaliana*	Suppression of long hypocotyl phenotype of photoreceptor phyB-4 phenotype.
CYP302A1	*Drosophila melanogaster*	Embryonic morphogenesis and cuticle deposition impaired
CYP75A1	*Petunia*	Altered flower color (blue to pink)
CYP504	*Aspergillus nidulans*	Pencillin overproduction

(Adapted from Reichhart, 2000 and Nebert and Russell, 2002).

molecules (salicylic acid, jasmonic acid), accessory pigments (carotenoids) and structural polymers (lignins) (Schuler, 1996). CYP75A is responsible for flower coloring in plants, which led to the generation of blue roses (Bernhardt, 2006). CYP4C1 is proposed to be involved in fatty acid metabolism, CYP12B1 may have a role in calcium homeostasis and CYP18 may play a role in postembryonic development (Scott, 2001).

In insects, P450s are involved in biosynthetic pathways of juvenile hormone and ecdysteroid synthesis. These are two most important hormones which are required for insect growth, development and reproduction. A group of genes known as Halloween genes, have been identified in *Drosophila*; mutation of which results in embryonic lethality. The genes that belong to the Halloween family are: *disembodied (dib) (CYP302A1), shadow (sad) (CYP315a1)*, *shade (shd) (CYP314a1)*, *spook (spk) (CYP307A1)*, *and phantom (phm) (CYP306A1)* (Chavez et al., 2000; Gilbert, 2004). Two of these genes, *dib* and *sad* involved in the synthesis of ecdysone, a polyhydroxylated sterol, which is the precursor of the major molting hormone, 20-hydroxyecdysone (Gilbert, 2004). Via transient expression in S2 cells with the plasmid consisting of *dib* or *sad* cDNA revealed that CYP302A1 (*dib*) is an ecdysteroid C22 hydroxylase involved in the conversion of 2, 22-dideoxyecdysone (Ketotriol) to 2-deoxyecdysone whereas CYP315A1 (*sad*) is a C2 hydroxylase involved in the conversion of the latter into ecdysone. Another enzyme, CYP314A1 encoded by *shade (shd)* is a 20-hydroxylase, which is needed for the conversion of ecdysone to 20-hydroxyecdysone (Warren et al., 2002). Another gene, *phantom (phm*) (CYP306A1), encoding microsomal 25

hydroxylase, is involved in the conversion of 2,22,25-trideoxyecdysone to 2,22-dideoxyecdysone (Warren et al., 2004).

Role of CYPs in xenobiotic metabolism

Apart from their involvement in the endogenous biosynthesis and metabolism, CYPs also play a major role in the metabolism of foreign or xenobiotic compounds. Human CYP1A1 and CYP1B1 are involved in the metabolism of polycyclic aromatic hydrocarbons present in the products of industrial incinerations, cigarette smoke and charcoal. Human CYP3A4 is involved in the detoxification of aflatoxin B1, a hepatocarcinogenic mycotoxin, into non-toxic compounds (Guengerich et al., 1998). Oxidative metabolism of drugs and foreign chemicals by P450s is generally regarded as a detoxification function. However, some of the reactions may release highly reactive metabolites that trigger cytotoxicity and genotoxicity (Omura, 1999). For example, oxidative metabolism of benzo[*a*]pyrene present in cigarette smoke by CYP1A1 releases highly reactive epoxides that serve as carcinogens (Bernhardt, 2006). Among the 57 isoforms of CYPs found in humans, genes belonging to family 1, 2 and 3 are involved in drug metabolism (Nebert and Russell, 2002; Guengerich, 2006). Among the three families, CYP2 is the largest family in humans with 16 genes, 16 pseudogenes in 13 subfamilies comprising of approximately one third of human P450 enzymes (Porter and Coon, 1991; Nelson, 1999). CYP2D6 is the most studied P450 with drug metabolism polymorphism. This P450 can metabolize 70 different drug oxidations. Some of the substrates of CYP2D6 include Flecainide (Antiarrhythmic), Prozac (antidepressant), antipsychotics, beta-blockers and analgesics. CYP2E1 is another human P450 enzyme

induced by alcohol (Porter and Coon, 1991). CYP3A is another important drug metabolizing subfamily in humans. Human CYP3A4 can metabolize more than 120 different drugs. Some of the substrates of CYP3A4 are Acetominophen (Tylenol), Codeine (narcotic), Cyclosporin A (an immunosuppresant), Diazepam (Valium), Erythromycin (antibiotic), Lidocaine (anesthetic), Lovastatin (HMGCoA reductase inhibitor, a cholesterol lowering drug), Taxol (cancer drug), Warfarin (anticoagulant) etc (Guengerich, 2006).

Insect P450s are also involved in the metabolism of various xenobiotic compounds including insecticides (Feyereisen, 1999). This particular property of CYPs makes the insects resistant to insecticides and causes agricultural, health and economic problems (McKenzie and Batterham, 1998). It has been demonstrated that resistant insects are rendered susceptible if they are treated with piperonyl butoxide (PBO), a specific inhibitor of CYPs (Hodgson et al., 1993). This suggests that CYPs play a major role in insecticide resistance. Several studies have emerged to identify the specific P450s involved in the detoxification of insecticides, plant allelochemicals and promutagens using heterologous expression, reconstitution experiments and isoform specific antibodies. In *Musca domestica*, treatment of microsomes from resistant LPR strain with anti-CYP6D1 serum inhibited the metabolism of pyrethroid insecticide, deltamethrin suggesting a strong role of CYPs in pyrethroid resistance (Scott, 1999). When CYP6A2 cDNA from *Drosophil*a was expressed in lepidopteran cells using baculovirus expression system, it metabolized aldrin, dieldrin and diazinon but not DDT (Dunkov, 1997). Metabolism of DDT (dichlorodiphenyltrichloroethane) by an allelic variant of CYP6A2 (CYP6A2SVL) was demonstrated by overexpressing the gene in *E.coli* (Amichot, 2004).

When CYP6A2 was co-expressed along with human P450 reductase in *Saccharomyces cerevisiae*, it metabolized aflatoxin B1, 7,12- dimethylbenzanthracene and 3-amino-1-methyl-5H pyrido (4,3-b) indole (Saner, 1996). Overexpression of CYP6D1 from *Musca domestica* in yeast showed metabolism of chlorpyrifos, benzo[*a*]pyrene, deltamethrin and cypermethrin insecticides (Scott and Wen, 2001). When CYP12A1 expressed in *E.coli* was reconstituted with bovine mitochondrial adrenodoxin reductase and adrenodoxin, it metabolized diazinon, heptachlor, aldrin, progesterone and testosterone but failed to metabolize DDT (Guzov et al., 1998). Expression of CYP6A8 from susceptible OregonR-C strain of *Drosophila* in yeast system has shown that it metabolizes lauric acid but not DDT or heptachlor (Helvig et al., 2004). These studies indicate that CYPs play a prominent role in the metabolism of insecticides *in vitro.*

CYPs also play a major role in insect-plant interactions (Li et al., 2004). Many plants use their CYPs to synthesize various toxic allelochemicals and alkaloids as a defense against herbivorous insects (Morant et al., 2003). Interestingly, these herbivorous insects use their CYPs to detoxify the plant chemicals which are present in their diet as xenobiotic compounds. Insect P450s can metabolize wide range of plant allelochemicals including furanocoumarins, terpenoids, indoles, glucosinolates, flavonoids, alkaloids and lignans (Li et al., 2006, for review). For example, *Papilio polyxenes* (black swallow butterfly) and *Papilio glaucus* (tiger swallowtail butterfly) feed on plants that are rich in linear (xanthotoxin and bergapten) and angular (angelicin and sphondin) furanocoumarins (Ma et al., 1994**.** These chemicals are highly toxic to wide variety of organisms including plants, insects, birds and mammals since they react directly with pyrimidine bases in DNA after photoactivation (Berenbaum, 1981). However, the

swallowtail butterflies produce high levels of CYP6B1 and CYP6B4 respectively to be able to digest the toxin producing plants (Li et al., 2001). Heterologous expression of CYP6B1 and CYP6B4 in the baculovirus-infected Sf9 cells showed metabolism of furanocoumarins (Wen et al., 2003; Hung et al., 1997).

Induction of CYPs by different xenobiotic compounds

CYP genes of different species are known to be induced by various xenobiotic compounds such as barbiturates (Kim and Fulco, 1983), polyaromatic hydrocarbons (Gautier et al., 1996), plant allelochemicals (Hung et al., 1995; 1997), DDT (Brandt et al., 2002), caffeine (Goasduff et al., 1996; Bhaskara et al., 2006) and etc. Although the molecular basis of most of these CYP gene induction is not known, considerable progress has been made on the induction of bacterial and mammalian CYP genes. Induction studies have helped scientists to better understand the mechanism of regulation (Porter and Coon, 1991).

Phenobarbital (PB) is used as a prototype for large subset of structurally related chemicals that induce the expression of CYP genes such as CYP3A, CYP2A, 2B, 2C, 2H and CYP102/CYP106. Bacterial PB induction mechanisms have been elucidated at the molecular level and several central factors have been identified (Sueyoshi and Negishi, 2001). In *Bacillus megaterium*, a repressor Bm3R1 binds to the operator sites of BM-1 and BM-3 genes, which also have a 17 bp *cis*-regulatory element called as Barbie box. When barbiturates are added to the medium, the repressor fails to bind to the operator site or barbie box and therefore results in the induction of BM1 and BM3 (Shaw and Fulco,

1992). In addition, barbiturates induce the synthesis of positive transcription factors, BM1P1, BM1P2, and BM1P3, which competes with the repressor to bind to the BM-1 operator site and to the Barbie box thereby increasing the expression of BM-1 gene (He et al., 1995).

In mammals, these barbie box sequences are present in the proximal promoter regions of many CYP genes. However, the mutation or deletion of these sequences did not affect the PB response in mammalian CYP2B family. Using mouse PB-inducible *Cyp2b10* gene, the minimum sequence required for PB induction is found to be a 51-bp sequence named as Phenobarbital responsive enhancer module (PBREM) (Zelco, 2000). These PBREs are composed of a central binding site for nuclear factor NF1 flanked by two nuclear receptor binding sites, known as NR1 and NR2 (Sueyoshi and Negishi, 2001). These sequences are conserved in mice, rat and human CYP2B genes. Recent evidence suggests that the PB induction may involve orphan nuclear receptors such as constitutive active receptor (CAR), Pregnane X receptor (PXR) and Peroxisome proliferator activator receptor (PPAR) (Waxman, 1999). These receptors bind to the dimerization partner, retinoid X receptor (RXR) and the resultant heterodimer binds to the NR sites in the 5' regulatory region of CYP genes, and activate transcription (Kakizaki et al., 2002; Swales and Negishi, 2004). The orphan nuclear receptor CAR is implicated in the PB-mediated induction of CYP2B and CYP3A genes (Honkakoshi et al., 1998).

The most extensively characterized P450 with regard to regulation is the CYP1A1. This is the only P450 for which the receptor-mediated mechanism of induction has been clearly demonstrated, via the Ah or TCDD receptor. CYP1A1 is induced to high

levels by polyaromatic hydrocarbons such as benzo[*a*]pyrene and TCDD (2,3,7,8,-tetrachlorodibenzo-*p*-dioxin) (Guengerich, 2006, for review). Individuals with highly inducible forms of CYP1A1 enzyme seem to be more susceptible to lung cancers. The receptor for TCDD is the aryl hydrocarbon (Ah) receptor. Normally, the Ah receptor is bound to heat shock protein 90 (hsp90) in the cytoplasm (Whitlock, 1999). Upon ligand binding, the AhR sheds the chaperone proteins and binds to AhR nuclear translocator (ARNT). The complex of AhR-ARNT-TCDD then enters the nucleus and binds to the xenobiotic response elements (XREs) in the upstream regulatory region of CYP1A1 gene and induces its transcription (Hankinson, 1994).

Caffeine is another widely used compound induced by cytochrome P450 enzymes. Almost all of the caffeine comes from dietary sources such as chocolate, coffee, tea and beverages (Lorist and Tops, 2003). Several mechanisms of action were proposed for the induction by caffeine; a) ability to block adenosine receptors, b) inhibition of phosphodiesterase and c) mobilization of intracellular calcium. Caffeine interacts with the neurotransmission in different regions of the brain and promotes motor functions such as attention, mood, arousal and alertness (Fisone, 2004). The exact mechanism of CYP gene induction by caffeine is not known. Induction studies of *CYP1A1* and *CYP1A2* with caffeine showed increased expression in rat liver and kidney (Goasduff et al.,1996). When primary murine neuronal and astroglial cells were treated with caffeine, the upregulation of sonic hedgehog RNA was observed. (Sahir et al., 2004). In *Drosophila melanogaster*, *Cyp4e2* gene is upregulated in response to caffeine (Shaw et al., 2000). *Cyp6a2, Cyp6a8* genes were also found to be upregulated in response to caffeine suggesting their role in the metabolism of this psychostimulant (Bhaskara et al*.,* 2006). In

order to understand the mechanism of induction of CYP genes by caffeine and discover the genes overtranscribed in *Drosophila melanogaster*, we performed whole genome microarrays using Affymetrix *Drosophila* genome 2.0 chips.

CYP gene overexpression and genetics of insecticide resistance in insects

Overexpression of one or more CYP genes was observed in resistant strains compared to the susceptible strains in insects. However, studies to identify a single P450 gene responsible for DDT resistance have not been successful. In *Musca domestica*, P450 mediated resistance maps to autosomes 1 and 2, while *kdr* and *pen* map to autosome 3 (Scott et al., 1984; Scott and Georghiou, 1986; Liu and Scott, 1995). In *Musca domestica*, CYP6A1 is overproduced in resistant Rutgers strain and CYP6D1 in LearnPyrR compared to the susceptible strain (Berge, 1998). In *Drosophila*, *Cyp6a2, Cyp6a8*, *Cyp12d1, Cyp6g1* and *Cyp6w1* are some of the genes overexpressed in resistant strains compared to the susceptible ones (Waters, 1992; Maitra, 1996, 2000; Dombrowski, 1998; Daborn, 2002; Pedra, 2004; Festussi-Buselli, 2005).

Although these examples suggest that there is a correlation between the overexpression of CYP genes and resistance, the molecular and genetic basis of CYP gene regulation is not understood. Chromosome substitution studies in *Musca* and *Drosophila* have suggested that there are trans-regulatory loci that influence the expression of CYP genes. In *Musca domestica*, *CYP6A1* gene present on chromosome 5 was regulated by an incompletely dominant locus on chromosome 2 (Carino et al., 1994; Feyereisen et al., 1995). Liu and Scott (1998) found that *CYP6D1* present on chromosome 1 is regulated both in *cis* and in *tran*s by the master regulatory gene on

chromosome 2. Several genes coding for detoxifying enzymes (P450s, glutathione S-transferases such as DDT-dehydrochlorinase) are under the regulation of a master gene on chromosome 2 in housefly (Plapp, 1984). Since the chromosome 2 of housefly is related to chromosome 3 of *Drosophila*, it is possible that the *trans*-regulatory loci for the second chromosome linked *Cyp6a2* and *Cyp6a8* genes are located on third chromosome. Recent work by Dombrowski et al. (1998) and Maitra et al. (2000) suggested that a repressor present on the third chromosome regulates the constitutive and induced expression of *Cyp6a2* and *Cyp6a8* genes present on the second chromosome. The wild type function of these loci is to repress the expression of these two CYP genes and the overexpression of *Cyp6* genes in resistant 91-R strain is due to the mutation in these regulatory loci (Maitra et al., 2000). *To date, no trans-regulatory genes have been identified.*

Using *Drosophila* as a model organism, several studies attempted to map the loci conferring resistance to DDT, organophosphates and various other insecticides. In the field collected *Drosophila*, a resistance locus was mapped close to ~64-67 cM on the right arm of chromosome 2 and named as R1 (Tsukamoto and Ogaki, 1953; Tsukamoto, 1958). Earlier investigations have concluded that DDT resistance in *Drosophila* is inherited not as a single gene but as a polygenic complex (Crow, 1957; Oshima, 1954; King and Somme, 1958). Using laboratory-selected resistant strain such as 91-R, Dapkus and Merrell (1977) showed that DDT resistance in *Drosophila* is multi-factorial and each of the three major chromosomes (X, 2 and 3) are involved. However, later studies using the same 91-R strain showed that the DDT resistance is monofactorial and the resistance

locus maps to 56 ±1 m.u. on the right arm of second chromosome (R2) (Dapkus, 1992). Since P450 enzymes were known to metabolize insecticides, mapping of the loci was done using the increased P450 content and activity as the phenotypes of insecticide resistance. These studies revealed that multiple loci (R1-R5) located on the 2nd and 3rd chromosomes influence P450 content and activity (Fig. 1-4) (Hallstrom, 1985; Houpt *et al*., 1988; Waters and Nix, 1988). Interestingly, one of the loci maps near the 65cM of the second chromosome. The third chromosome resistance loci R4 and R5 might be responsible for malathion resistance (Dapkus, 1992). Many studies were initiated to study the overexpression of the CYP genes present at or close to this locus in the resistant strains with a vision to link the resistance phenomena to these overexpressing genes. However, these studies did not provide any clue whether the gene (s) located at these loci are directly involved in the metabolism of insecticides or regulating the expression of CYP genes located elsewhere in the genome. *In summary, the genetic basis of insecticide resistance remains unresolved and it is not known whether DDT resistance in Drosophila is conferred by a single gene or multiple genes.*

Recently, Daborn et al., (2001) identified a new DDT resistance allele called *Rst(2)DDT*^{EMS1} using chemical mutagenesis. This allele maps close to the ~64.5 cM on the right arm of chromosome 2 at 48D5-6 to 48F3-6 on the polytene chromosome. Gene expression studies at this locus identified that *Cyp6g1* is overexpressed in the resistant strain compared to the susceptible strain. *However, it is not known whether resistance was associated with the Cyp6g1 gene.* In a more recent study, Daborn et al. (2002) collected several DDT resistant strains of *Drosophila* from different regions of the globe

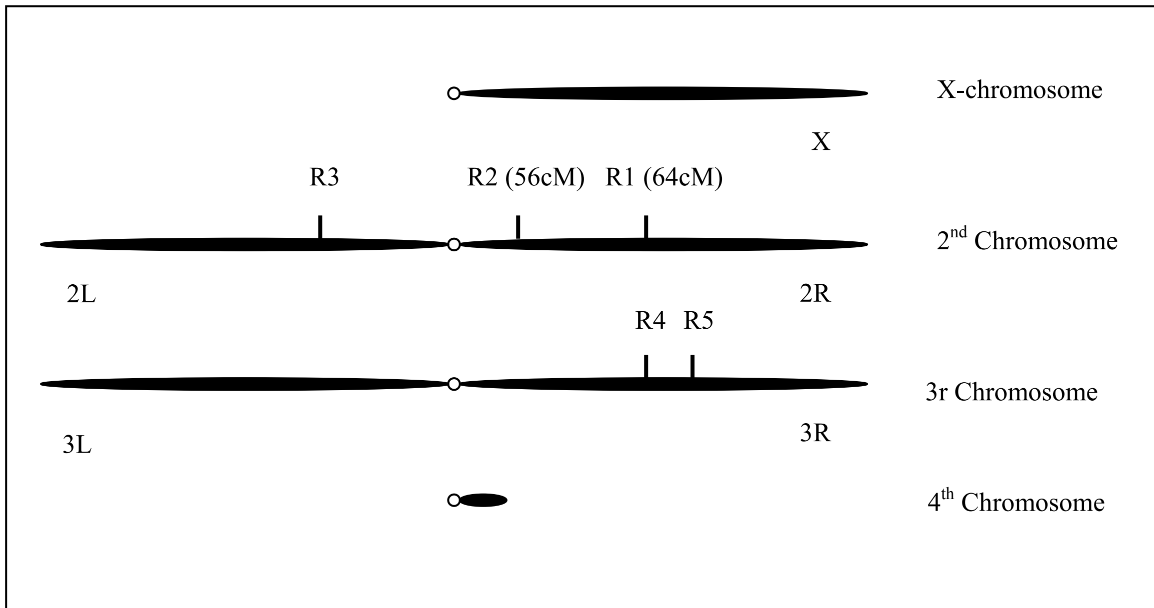


Figure 1-4: Positions of major insecticide resistance loci (R1 – R5) in *Drosophila melanogaster* genome. The map position of the loci is given in parentheses.

and showed that DDT resistance is associated with high-level of CYP6G1 RNA. Using GAL4/UAS system, they overexpressed the *Cyp6g1* transgene and showed that it confers resistance to 10µg DDT. Based on these results, they concluded that expression of *Cyp6g1* alone is necessary and sufficient to confer DDT resistance phenotype in *Drosophila melanogaster.* My preliminary observations showed that *Cyp6g1* is highly expressed in the susceptible Canton S strain. This observation raised a concern to reexamine the DDT resistance phenomenon.

Objectives of proposed research

The major focus of the proposed research is to investigate the molecular basis of cytochrome P450-mediated DDT resistance in *Drosophila melanogaster.* In *Drosophila*, overexpression of atleast five CYP genes, *Cyp6a2, Cyp6g1, Cyp6w1*, *Cyp6a8* and *Cyp12d1* are observed in resistant strains compared to the susceptible ones (Waters et al., 1992; Maitra et al., 1996, 2000; Dombrowski et al., 1998; Brandt et al., 2002; Daborn et al., 2002; Le Goff et al., 2003; Pedra et al., 2004). Although studies by Daborn et al. (2002) suggest that DDT resistance is monofactorial and it is mediated by *Cyp6g1* alone, there are a few unresolved issues. They collected fourteen resistant and six susceptible strains from different regions of the globe and measured the expression of *Cyp6g1* by real-time quantitative PCR. Microarray analysis was performed on two out of the fourteen strains and found that *Cyp6g1* is the only gene overtranscribed in the resistant strains compared to susceptible ones. Since microarray was done only on two of the

fourteen resistant strains, it is not known whether any gene other than *Cyp6g1* is overexpressed in the twelve resistant strains that were examined by real-time PCR. In order to support that *Cyp6g1* confers DDT resistance, transgenic flies showing 100-fold higher expression of *Cyp6g1* were treated with 10 μg DDT. Since higher doses were not used on the transgenic lines, it is not known whether *Cyp6g1* can confer the high level of resistance that is found in the lab-selected 91-R and *Wisconsin* strains. Typically, field collected or laboratory strains show resistance to atleast 50-fold higher amounts of DDT. By sequencing the alleles from the resistant strains, they identified the presence of an insertion in the 5' end of *Cyp6g1* that shows homology to the terminal direct repeat of an *Accord* transposable element. By PCR analysis, they showed that there is a perfect correlation between the presence of this insertion and DDT resistance. In view of these observations, it is important to investigate whether resistance to high level of DDT is conferred by *Cyp6g1* alone or by multiple genes. Hence, our first objective is to investigate the role of *Cyp6g1* in conferring DDT resistance.

In the 91-R strain, four of the above-mentioned five genes, except *Cyp12d1* show overexpression compared to the susceptible 91-C strain. In addition to the known genes, there may be other unidentified genes that may play a role in resistance. Hence, we compared the transcriptome of the resistant 91-R and the susceptible 91-C strains using microarrays. Several CYP genes showing overexpression has been implicated in insecticide resistance but in most cases it was not proved by a direct experimental approach (e.g., gene disruption or transgenics) whether a specific CYP confers resistance or metabolize insecticide *in vivo*. The second part of the first objective of the present

investigation is to overexpress the candidate CYP genes using transgenic technology and examine the level of DDT resistance conferred by these genes.

CYPs are involved in the metabolism of xenobiotics (see introduction). Induction of expression of CYPs by xenobiotics and understanding the molecular basis of it is of great interest. However, not much is known about the detailed mechanism of CYP gene regulation in insects. Hence, our second objective is to compare the transcriptome profile of caffeine and phenobarbital treated adult Canton S strain. Results obtained in this analysis can be used in future studies to understand the regulatory mechanism of xenobiotic metabolism by CYP gene induction.

Chapter II

Investigation of the role of *Cyp6g1* in conferring DDT resistance in *Drosophila melanogaster*

Introduction

Cytochrome P450s or CYPs are family of enzymes (CYPs) found in all living organisms from bacteria to man. CYPs are heme proteins and they are involved in the biosynthesis of different hormones, prostaglandins, pigments and other endogenous substances. Apart from the endogenous compounds, CYPs are involved in the detoxification of various foreign chemicals including drugs and toxic chemicals (Agosin et al., 1985; Guengerich, 2006, Lewis, 1996). Considering their biochemical properties, CYPs are also expected to metabolize various insecticides. Using heterologous expression systems, it has been demonstrated that housefly and *Drosophila* CYP*s* can metabolize various insecticides, e.g., aldrin, heptachlor, etc (Dunkov 1997). In addition, a positive correlation between the quantity of CYP mRNA or protein, and the resistance phenotype has been also observed (Carino et al., 1992; Waters et al., 1992; Maitra et al., 1996; Maitra et al 2000), implying that the resistant phenotype may be conferred by CYPs. However, it is not known whether the resistant phenotype in a given species is conferred by one or more than one CYP. In *Drosophila melanogaster,* resistant phenotype may be a multifactorial trait because loci present both on the 2nd and 3rd chromosomes has been implicated in DDT and organophosphate resistance (Dapkus and Merrell, 1977; Waters and Nix, 1988**;** Houpt et al., 1988; Dapkus 1992).

In a recent study, Daborn et al., (2002) compared several DDT resistant and susceptible strains of *Drosophila* collected from different regions around the globe and found that the DDT resistant strains have much higher levels of CYP6G1 than the susceptible strain and overexpression of a CYP6G1 cDNA in a susceptible strain confers resistance only to 10µg DDT (Daborn et al., 2002), which is several hundred fold lower amount of DDT that a resistant strain can tolerate. Although the studies made by Daborn et al. (2002) may suggest that DDT resistance phenotype in *Drosophila* is monofactorial and mediated by *Cyp6g1* alone, later studies demonstrated a lack of correlation between *Cyp6g1* expression and DDT resistance (Schlenke and Begun, 2004; Festucci-Buselli et al., 2005). In their studies, Festucci-Buselli et al. (2005) used RNA and western blot analysis and showed that the expression of *Cyp6g1* was high in DDT susceptible Canton-S and Hikone-R strains. However, in this study CYP6G1 RNA and protein were not quantified to compare different strains (Festucci-Buselli et al, 2005). In addition, it is not known whether there are any differences in the sequence of the *Cyp6g1* alleles between the resistant and susceptible strains. It is possible that the high expressing *Cyp6g1* alleles in Canton-S and Hikone-R strains are hypomorphic.

In order to resolve the role of *Cyp6g1* in DDT resistance, the strains used by Festucci-Buselli et al (2005) were used in the present investigation. However, in the present investigation a more rigorous quantitative assessment of DDT resistance and *Cyp6g1* expression in different resistant and susceptible strains was examined. Analysis of CYP6G1 RNA and protein were done to compare different strains. In addition, organization and nucleotide sequence of *Cyp6g1* alleles of different strains were compared in the present investigation in order to investigate the allelic differences.

Finally, the role of *Cyp6g1* in DDT resistance was examined by using recombinant stocks that are homozygous for the *Cyp6g1* allele of susceptible 91-C strain, and the X and 3rd chromosomes from the resistant 91-R strain. Additionally, microarray analysis of the recombinant stocks was performed and compared with that of resistant 91-R and susceptible 91-C strain. Our results demonstrate that there is no correlation between *Cyp6g1* expression and DDT resistance, and the *Cyp6g1* alleles from the resistant and susceptible strains are identical in amino acid sequence. As observed by Daborn et al (2002), we also found that there is an association between high *Cyp6g1* expression and the presence of an *Accord/Ninja*-like transposable element in the upstream DNA of the *Cyp6g1* gene. However, DDT resistance is not associated with the presence of this transposable element in the upstream DNA of *Cyp6g1* as claimed by other investigators (Daborn et al., 2002; Catania et al., 2004). Microarray analysis revealed several other candidate *Cyp* and defense response genes that may have potential role in insecticide resistance.

Materials and Methods

Drosophila strains

Drosophila stocks were raised on standard cornmeal-agar-molasses medium at 24^{0}C under 12-hour light and 12-hour dark cycle. In the present investigation, Canton-SH, Hikone-RH, 91-C, 91-R, *Wisconsin* (also previously referred to as *RstIIDDT* ^{Wisconsin} or *Rst(2)DDT*^{Wisconsin}) and *ry*^{506} were used. The first four strains were obtained from Larry Waters, Oak Ridge National Laboratory in 1992. The Canton-SH and Hikone-RH

strains are the renamed wild type Canton-S (BG) and Hikone-R (BG), which were originally obtained from the Mid-America *Drosophila* Stock Center at Bowling Green, Ohio. They have been renamed because in the present investigation they have been found to show high *Cyp6g1* expression. The laboratory selected DDT-resistant 91-R and susceptible 91-C strains are genetically similar and have been described previously (Dapkus, 1992; Maitra et al., 2002). A population of flies was collected in St. Paul, Minnesota in 1952 and split into two groups. One group was subjected to DDT selection (91-R) and the other group was maintained in normal medium (91-C) (Merrell and Underhill, 1956). DDT selection of the 91-R strain continued for another 20 years (Dapkus and Merrell, 1977). Although the 91-R strain has not been under DDT selection since 1985, it still shows resistance to malathion and DDT (Sundseth et al., 1989; Ganguly, unpublished observations). The *Wisconsin* and ry^{506} were obtained from Barry Pittendrigh (Purdue University) and John Lucchesi (Emory University), respectively. The details of the field-collected DDT resistant *Wisconsin* strain have been described previously (Pittendrigh, 1999) and the description of ry^{506} allele can be found in Lindsley and Zimm (1992).

Total RNA isolation and northern blot hybridization

From each strain, three total RNA samples were isolated. Briefly, each RNA sample was isolated from a pool of 40-50 adult female flies (5-10 day old) using TRI® Reagent (Sigma, St. Louis, MO) and following the manufacturer's protocol. The resulting RNA pellets were rinsed with chilled 70% ethanol, dried and dissolved in appropriate volume of RNase-free water. Three RNA samples were fractionated on three separate

northern gels and blotted. For each northern blot, a set of RNA samples, comprising one RNA sample from each strain, were fractionated on the 1.2% agarose–2.2M formaldehyde denaturing gel as described (Maitra et al., 2000). Each RNA sample was loaded in triplicate in each gel. Before loading, each RNA sample (20µg per lane) was dried, dissolved in 20µl 1X Formaldehyde loading dye (Ambion Inc., Austin, TX) and incubated at 65^0C for 15 minutes before loading. After electrophoresis, RNA was blotted onto Hybond (Amersham) nylon, cross-linked with UV and the rRNA band, which co-migrates with CYP mRNAs, was visualized with a long wave UV lamp. The blots were divided into upper and lower halves by cutting about 1.0 cm below the ribosomal RNA bands. The upper and lower blots were prehybridized in separate hybridization bottles for 1 hour at 37^0C in Northern Max™ prehybridization/hybridization buffer (Ambion Inc., Austin, TX). After prehybridization, the upper blots were hybridized with ^{32}P-labeled 0.6-kb N' terminal DNA of the desired *Cyp* gene and the lower blots were hybridized with ^{32}P-labeled RP49 (ribosomal protein) cDNA. The *Cyp* and *RP-49* gene probes were labeled by using the Strip-EZ® random prime labeling kit from Ambion (Austin, TX) and a nick-translation labeling kit from Invitrogen (Carlsbad, CA), respectively. Similar quantity and activities (cpm) of radioactive probes were used in all three sets of blots, and all probes used were in excess over the RNA on the blots. Hybridization was done for 30h at 37^0C. After hybridization, the blots were washed under stringent conditions. The low stringency first wash was at room temperature with Buffer I (2X SSC, 0.1% SDS) consisting of four washes with 10 min per wash. The high stringency second wash was at 65^0C with Buffer II (0.2X SSC, 0.1% SDS) consisting of four washes with 15 min per wash. The hybridization signals on the blots were quantified with a radioanalytical

imager as described earlier (Dombrowski et al., 1998; Maitra et al., 2000). RP49 signal was used as the internal control to normalize for the RNA loading errors. The CYP/RP49 values of three sets of northern blots made with three sets of RNA samples were used to determine the mean value and compare *Cyp* gene expression in different strains. Data were analyzed by ANOVA test.

DDT resistance bioassay

For the DDT resistance assay, stock solutions of different concentrations of DDT were prepared in acetone. From each solution, 100µl was added into individual glass scintillation vials. In order to obtain uniform coating of the pesticide inside the vial, the vials were swirled continuously until acetone evaporated. The vials were left in a fume-hood overnight for complete drying. Mature flies (5-10 days old) were etherized and female flies in groups of 20 were sorted in vials containing fresh *Drosophila* medium. These vials were left overnight at room temperature to allow the flies to recover from the ether shock and feed on fresh medium. Next day, live flies were directly transferred to the DDT-coated scintillation vials, which were sealed with cotton plugs soaked in 5% sucrose. Mortality (dead flies and flies that could not move or stand up) was recorded after 24 hr exposure. Vials coated with acetone only were used as the controls. The data were analyzed using probit analysis in SAS (SAS Institute, 2000).

Analysis of the upstream DNA of different *Cyp6g1* alleles

Genomic DNA was isolated from adult unsexed flies of each strain using DNAzol® reagent (Invitrogen, Carlsbad, CA) and following manufacturer's protocol.

Briefly, the flies were homogenized in DNAzol® reagent and the homogenate was centrifuged at 13,000 x g for 10 min. The supernatant was transferred into a fresh tube and precipitated using 100% ethanol. The DNA pellet obtained was washed with chilled 75% ethanol, dried and dissolved in appropriate volume of sterile water. The genomic DNA obtained from each strain was used as a template for PCR amplification. To analyze the upstream DNA, PCR amplification was performed with forward primer 5'F-CAGCAAACGCAACAATAATG-3' starting at -373 bp and reverse primer 5'R-CCACAGCAAATCCAGAGGG-3' starting at -123 bp region of the *Cyp6g1* gene. The template DNA and primers were added to Ready-to-Use PCR tubes (MBP) and incubated at 94^0C for 3 min. After adding Taq DNA polymerase, thirty PCR cycles were run using the following cycling parameter: 94^0C for 30 s, 50^0C for 45 s, 72^0C for 1 min. The amplified DNA was purified by using a commercially available PCR clean-up kit (Qiagen, Valencia, CA), quantified by fluorometry and electrophoresed on 1% agarose gel for size determination.

Protein isolation and western blot analysis

Approximately 40 adult females from each strain were homogenized in a pH 7.4 protein extraction buffer (PEB) containing 100mM K_2PO_4-KH_2PO_4; 10% (v/v) glycerol; 1mM PMSF; 0.1mM DTT; 10mM EDTA; 0.1% (v/v) Triton X-100 and 10mM β-mercaptoethanol. The resulting homogenate was centrifuged at 4^0C for 10 min at 15,000 x g. The supernatant was then transferred into a fresh tube and centrifuged at 4^0C for 60 min at 38,000-x g to separate the microsomal fraction from the soluble fraction. The microsomal pellet was washed twice with PEB and subsequently resuspended in PEB.

The protein concentration was determined by Bradford method using Bovine serum albumin (BSA) as standard.

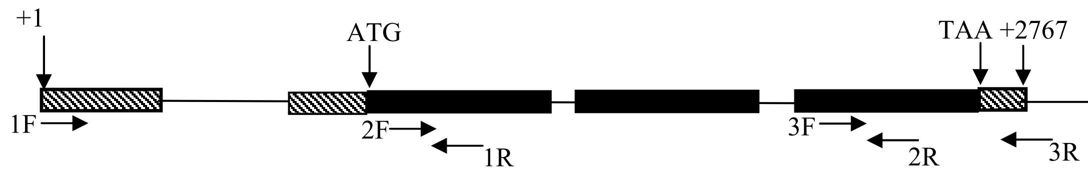
Western blot analyses were carried out following the separation of 35 μg denatured *Drosophila* microsomal proteins on 8 % SDS-PAGE gels (Laemmli, 1970). Proteins were transferred from the gel to a nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany) at constant 80 mA, and 4°C overnight by a wet-cell transfer apparatus (Bio-Rad, Hercules, CA). Transfer occurred in Tris-glycine buffer containing 20% (v/v) methanol, and transfer was confirmed by reversible staining with Ponceau-S (Sigma, St. Louis, MO). The membrane was briefly washed in phosphate-buffered saline (PBS) and blocked for 1h at ambient temperature in 50 ml of PBST (PBS + 0.05% v/v Tween-20) containing 15mg/ml non-fat dried milk (Bio-Rad, Hercules, CA). Membrane was then excised into two pieces by a sterile blade at the position around 55 kDa based on the low range prestained SDS-PAGE Standards as well as Kaleidoscope Prestained Standards (Bio-Rad, Hercules, CA). The upper and lower halves of the membrane were incubated for 1 hr respectively with primary antisera for CYP6G1 (~60 kDa, target protein) and JLA20 (~43 kDa, actin) prepared at 1:500 dilution. Both halves were washed three times (5 min each) in PBST, and incubated for an hour in their respective secondary antisera of goat anti-rabbit IgG (for the CYP6G1 antiserum detection) or goat anti-mouse IgM (for the JLA 20 antiserum detection) alkaline phosphatase conjugate (Boehringer-Mannheim; Indianapolis, IN) at 1:2000 dilution. The membrane was washed five more times (5 min each) in PBST before developing in the BCIP-NBT substrate (Sigma, St. Louis, MO) to visualize antibody-conjugate. In this study, actin was used as the loading control to normalize the data. All western blot

analyses were performed in Dr. Barry Pittendrigh's lab, Purdue University, LaFayette, IN.

Cloning of *Cyp6g1* alleles from different strains

The strategy for cloning *Cyp6g1* alleles from different strains is shown in Fig 2-1. Genomic DNA from unsexed flies of different strains of *Drosophila* was isolated by using DNAzol® reagent (Invitrogen, Carlsbad, CA) and used as templates for PCR amplification of the *Cyp6g1* gene. Sequence of the *Drosophila* genomic DNA scaffold available at database (accession number AE003823) was used to design the PCR primers. In this genomic DNA scaffold, base numbers 189952 and 192718 correspond to the bases +1 and +2767 of the *Cyp6g1* gene. Three pairs of gene-specific PCR primers were designed to amplify the *Cyp6g1* gene into three overlapping fragments: 5'-third, middle third and 3'-third. The sequences of primers used to amplify the *Cyp6g1* gene are described in Table 2-1. To prevent PCR based mutations in the alleles; PCR kit containing high fidelity Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) was used to amplify the genomic DNA. The DNA was denatured at 94^0C for 2 minutes and then subjected to 35 cycles of PCR amplification. Parameter for each cycle was as follows: 94^0C for 45 s, 48.4^0C for 45 s and 68^0C for 2 min.

All PCR products were purified and cloned into pGEMT-Easy vector (Promega, Madison, WI). Two clones for each of the three regions of the *Cyp6g1* gene were randomly picked, DNA was purified and both strands of each cloned DNA were sequenced with ABI Prism 3100 Analyzer (Fig. 2-1). Thus, for each PCR fragment,



PCR amplification of *Cyp6g1* gene in three overlapping fragments

T-A cloning

Double strand sequencing of two independent clones

Comparison of sequences using ClustalW program

Figure 2-1. Strategy for cloning and sequencing of *Cyp6g1* alleles from 91-R, 91-C, *Wisconsin*, Canton SH and Hikone RH strains.

Table 2-1

Primers used for the amplification of *Cyp6g1* gene in three fragments

Primer name	Region amplified	Sequence of the primer
1. 6g1-1F	+6/+1148	5’-AAGTGCGGGTGCGTAGAGC-3’ (+6/+23)
2. 6g1-1R		5’-GAAGAACAGGTTATTATAGCC-3’ (+1127/+1148)
3. 6g1-2F	+1064/+2085	5’-ATCAAGGACTTCAATCGGTTC-3’(+1064/+1084)
4. 6g1-2R		5’-ATAGAT GGGTATGAACACAGGG-3’ (+2064/+2085)
5. 6g1-3F	+2012/+2803	5’-AGCCAGACTTGAGCCTGAAG-3’(+2012/+2031)
6. 6g1-3R		5’-TGTTACATTTGGGAGATGCC-3’(+2784/+2803)

The region the primer spans is given in brackets.

four sequences were obtained for each strain, which were then analyzed and compiled into a single sequence. The final sequences of the overlapping 5'-third, middle third and 3'-third fragments of each *Cyp6g1* allele were then analyzed and linked by using the Sequencher program (Applied Biosystem, Foster City, CA). The final sequence of each *Cyp6g1* allele was analyzed by BLAST program (NCBI). Nucleotide and amino acid sequences of the *Cyp6g1* alleles from four strains were compared by CLUSTALW program (http://www.ebi.ac.uk/clustalw).

Synthesis of stocks with recombinant second chromosome

The crossing scheme shown in Fig 2-2 was used to generate *R; rx; R* recombinant stocks. In these stocks, the *R* chromosomes represent the X and 3rd chromosomes from the 91-R strain and the *rx* chromosome is a product of recombination between the 2nd chromosomes of DDT-resistant 91-R and susceptible 91-C strains. To synthesize these recombinant stocks, *RCR* females were crossed to *R; R/Cy; R* males and the F1 *R; R/C; R* females with straight (Cy^+) wings were collected. These females were then crossed to *R; R/Sco; R* males and the F2 *R; u2/Sco; R* males with *Sco* phenotype were collected. The symbol *u2* stands for the second chromosome with unknown genotype, which could be a non-recombinant chromosome (*R* or *C*) or could be a product of recombination between the *R* and *C* second chromosomes in the F1 *R; R/C; R* females. To recover the *u2* chromosome in pure form, the F2 *R; u2/Sco; R* males were selected because crossing over does not take place in male *Drosophila*. Therefore, fifty F2 males (*R; u2/Sco; R*) were selected randomly and singly crossed to several *R;R/Cy;R* virgins to make fifty

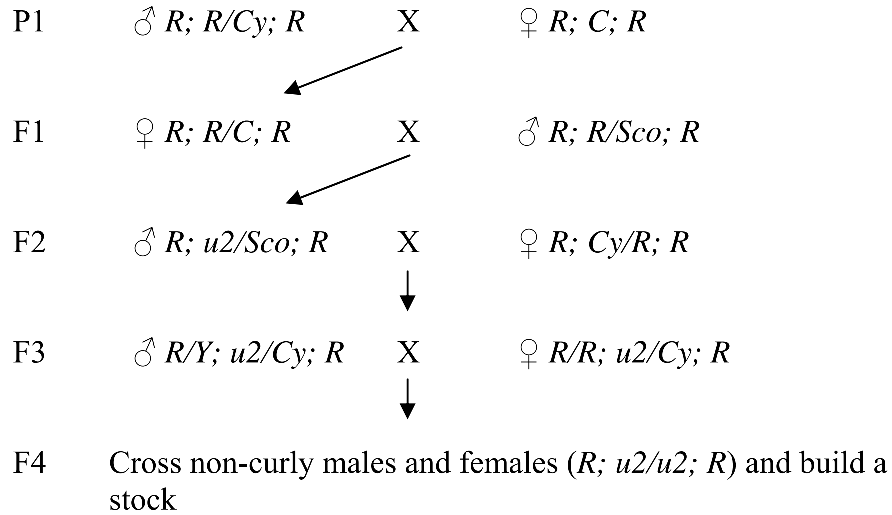


Figure 2-2: Genetic crossing scheme to substitute *Cyp6g1* allele of 91-R strain with the allele from 91-C strain. Fifty F2 *R; u2/Sco; R* males were individually mated with several *R; Cy/R; R* virgin females to establish fifty individual *R; u2/u2; R* lines in the F4 generation. Each line was examined for insertion in the *Cyp6a2* and *Cyp6g1* genes via PCR (see Results). Three lines (RC-21, RC-35 and RC-48) were products of recombination and homozygous for the *Cyp6a2-91R* and *Cyp6g1-91C* alleles of 91-R and 91-C strains, respectively.

independent lines. In the F3 generation, curly-winged *R; u2/Cy; R* males and females of each line were collected and mated to recover F4 non-curly male and female progeny. These flies were used to build a stock. The genotype of each line was *R; u2/u2; R*. As mentioned above, *u2* chromosome could be a product of recombination between the 2nd chromosomes of 91-R in 91-C origin in the F1 female, and this may create a stock that is homozygous for the *Cyp6a2* allele of the 91-R and *Cyp6g1* allele of the 91-C. Therefore, to determine the genotype of the *Cyp6a2* and *Cyp6g1* alleles in the putative recombinant lines, gene-specific molecular markers were examined. Waters et al (1992) have shown that the 91-C but not the 91-R strain carries insertion of LTR of *17.6* mobile element in the 3'-UTR of the *Cyp6a2* gene. On the other hand, in this study we found that the 91-R but not the 91-C strain has *Accord/Ninja* insertion in the upstream DNA of the *Cyp6g1* gene. Therefore, genomic DNA was isolated from each line and their *Cyp6a2* and *Cyp6g1* genes were examined for the insertional element via PCR (see Results section).

Microarrays

The quality of all total RNA samples was assessed using an Agilent 2100 RNA BioAnalyzer (Agilent Technologies, CA). Total RNA (3.5 µg) was used to setup first strand cDNA synthesis reaction with T7-oligo (dT) primer (Affymetrix) and Superscript II reverse transcriptase (First strand cDNA synthesis kit, Invitrogen). The final reaction mixture consists of 2µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$ (5mM final concentration), 2 µl of 0.1 M DTT (0.01 M final concentration) 1 µl of RNAseOUT RNAse inhibitor, 1 µl of Superscript II RT enzyme were added to a final volume of 20

μl. The second strand synthesis reaction was setup by the addition of dNTPs, Second Strand Reaction Buffer, *E. coli* DNA Ligase, *E. coli* DNA polymerase I and *E. coli* RNase H (Invitrogen) to the first strand reaction according to standard Affymetrix protocols. The double-stranded cDNA synthesized was purified using Affymetrix GeneChip sample cleanup modules. Biotin-Labeled cRNA was prepared using an ENZO BioArray High Yield RNA Transcript Labeling Kit (ENZO Life Sciences, Inc., Farmingdale, NY). The *in vitro* transcription reaction (IVT) was used to synthesize the cRNA from the double stranded cDNA. After cleanup of the *in vitro* transcription products, the purified cRNA was fragmented to a size ranging from 35 to 200 bases using fragmentation buffer at 94°C for 35 minutes. The extent of fragmentation was assessed by loading the fragmented cRNA on a BioAnalyzer. Fifteen micrograms of the fragmented cRNA was mixed into a hybridization cocktail containing hybridization buffer, B2 oligo control RNA, herring sperm DNA, and BSA (Invitrogen, Carlsbad, CA). The solution was hybridized to a GeneChip *Drosophila* Genome 2.0 Array (Affymetrix, Santa Clara, CA) at 45°C for 16 hours at a setting of 60 rpm in a hybridization chamber. *Drosophila* Genome 2.0 Array consists of 18,500 transcripts based on the recent annotation (release 3.1) of the *Drosophila melanogaster* genome by Berkeley *Drosophila* genome project (BDGP) and Flybase. Fourteen pairs of perfect matched and mismatched oligonucleotide probes present on the arrays were used to measure the transcription level of each representative sequence. The mismatched probe consists of a single nucleotide mismatch at position 13 of the oligonucleotide. After hybridization, the GeneChips were washed using the Affymetrix Fluidics 450 wash station (Affymetrix Fluidics Protocol Midi_EUK2V3_450) and stained with streptavidin-phycoerythrin (Molecular Probes,

Carlsbad, CA), followed by a wash with biotinylated antibody goat IgG to remove unbound streptavidin. Phycoerythrin is a compound that emits fluorescence that is scanned by a GeneChip 3000 High-Resolution Scanner. The scanned images were quantified using GeneChip Operating software/ Microarray analysis suite (GCOS or MAS 5.0). The individual GeneChip scans were quality checked for the intensity of the control genes and background signal values. The signal intensity values for the 5' probe sets of Actin and GAPDH genes were compared with their corresponding 3' probe sets. The ratio of the 3' probe set to the 5' probe set was identified to be less than 3, which validates the RNA sample and assay quality. The GeneChips were processed at the Affymetrix core facility at University of Tennessee, Knoxville.

Microarray data analysis

Genes were represented as probe sets with more than one transcript for each gene on the *Drosophila* Genome 2.0 chip. Each probe set consists of fourteen pairs of perfect match (PM) and mismatch (MM) oligonucleotides. The MAS 5.0 software was used for background subtraction of all the chips (nine GeneChips with three chips for each of the samples; 91-R, 91-C and recombinant RC-21 strain) followed by GC-robust multiarray analysis (GC-RMA) for linear multi-chip normalization. The intensity value is the ratio of the difference between the perfect match and mismatch nucleotides to the total hybridization intensity. The data was also checked for the presence of outliers using a residual cut off of 2500 i.e., if the residual is greater than 2500 or less than –2500, it will be indicated as an outlier. The outliers were examined and eliminated from further analysis. Univariate method was used to investigate normal distribution of the residuals

with a 0.9 cut-off for Shapiro-Wilkes test. For each observation in the dataset, a linearized model of ANOVA i.e., $y_{ij} = \mu + T_i + R(T)_{ij}$ (where y represents the observation on the ith replicate for the jth treatment, μ is the overall mean, T is the ith treatment effect and R(T) is the residual error) was fit. The F values obtained from the above equation represent the ratio of the mean expression of the treatments to the mean expression of the residuals. The F-value obtained was used to identify the genes that showed significant differences between the control and treated samples. Simultaneously, t-test was performed to individually compare the means of 91R and RC-21 with the 91C as control and obtained a raw p-value. Further, a p-value correction was performed by Bonferroni, False discovery rate (FDR) and Benjamin- Hochberg methods. The Bonferroni method is overly conservative and leads to false negatives when large numbers of genes are involved. Hence, the corrected p-values obtained from false discovery rate with a cut off of 0.01 (99% confidence level) and an F-test with $p<0.05$ were used for further analysis. All the data analyses were performed in SAS (SAS institute, Cary, NC).

Results

Lack of correlation between CYP6G1 RNA and DDT resistance

To examine whether there is any correlation between *Cyp6g1* expression and DDT resistance, 91-R, 91-C, *Wisconsin*, ry^{506}, Canton-SH and Hikone-RH strains were examined. While the 91-R and *Wisconsin* are highly resistant and the 91-C strain shows low levels of resistance to DDT, no published reports are available on the DDT resistance phenotype of the ry^{506}, Canton-SH and Hikone-RH strains that have been maintained in

our lab for the past twelve years. However, periodic assays showed that these three strains are susceptible to DDT (Ganguly, unpublished observations). Therefore, *Cyp6g1* expression was examined in all these strains by three independent northern blot analyses. The results (Figure 2-3 and Table 2-2) showed that as expected, CYP6G1 mRNA level were significantly higher in the DDT resistant 91-R and *Wisconsin* strains than in the susceptible ry^{506} and 91-C strains. Surprisingly, the level of CYP6G1 mRNA in susceptible Canton-SH and Hikone-RH strains was also very high. In fact, the level of CYP6G1 mRNA in Hikone-RH was as high as that found in the resistant 91-R strain and the level of Canton SH strain is same as that of *Wisconsin* strain (Table 2-2). Since current data have apparent discrepancies with the published report (Daborn et al. 2002) that a positive correlation exists between *Cyp6g1* expression and DDT resistance and no detailed resistance assay data are available for Canton-S and Hikone-R strains, a rigorous DDT bioassay was done on all six strains. The results are shown in Table 2-3 and Fig 2-4. It is clear from the data that there is a lack of correlation between CYP6G1 RNA level and DDT resistance. For example, the *Wisconsin* and Canton-SH strains have more or less similar level of CYP6G1 RNA, but Canton-SH is highly susceptible (LC_{50} = 12.9) and *Wisconsin* is highly resistant (LC_{50} = 447). Similarly, Hikone-RH and 91-R have similar levels of CYP6G1 RNA, but they are highly susceptible and resistant, respectively (Table 2-3). The LC_{50} value of the 91-R is almost 1000-fold greater than the LC_{50} value of the Hikone-RH strain. Based on these data we conclude that there is a lack of correlation between the high level of CYP6G1 mRNA and DDT resistance ($r=0.49$, $b=0.0001 \pm 0.00005$, $P>0.32$).

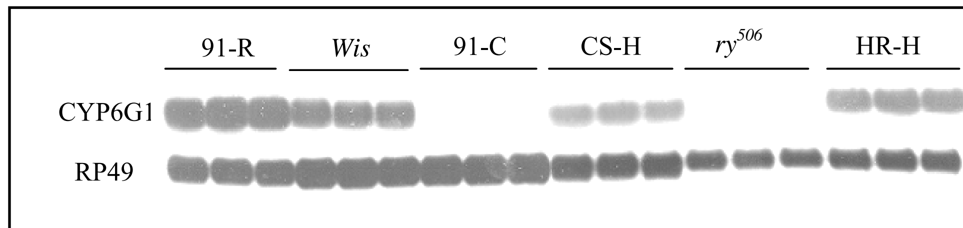


Figure 2-3: Northern blot analysis of expression of *Cyp6g1* in various resistant and susceptible strains of *Drosophila melanogaster*. Total RNA was isolated from the adult female flies (5-10 days old) using TRI-Reagent (Sigma) and electrophoresed on a 2.2M-1.2% formaldehyde agarose gel and hybridized with N-terminal 700 bp of *Cyp6g1* and RP49 as described in Materials and Methods. *Wis* = *Wisconsin*, CS-H= Canton SH, HR-H=Hikone RH strains.

Table 2-2

Expression of *Cyp6g1* in different resistant and susceptible strains of *Drosophila melanogaster*

Strain	N[a]	*Cyp6g1* counts	RP49 counts	*Cyp6g1*/RP49	Mean Cyp6g1/RP49	S.D.	D[b]
ry[506]	1	2,764	105,358	0.03	0.03	0.003	1
	2	2,833	92,257	0.03			
	3	2,488	81,682	0.03			
91-C	1	22,247	446,745	0.05	0.046	0.004	1.5
	2	18,547	418,587	0.04			
	3	18,700	435,762	0.043			
Canton SH	1	93,610	187,150	0.50	0.56	0.049	18.7
	2	113,409	192,166	0.59			
	3	103,517	178,329	0.58			
Hikone RH	1	100,928	108,434	0.93	0.91	0.023	30.3
	2	105,039	118,690	0.88			
	3	101,821	111,571	0.91			
Wisconsin	1	196,365	463,901	0.42	0.43	0.006	14.3
	2	188,676	433,886	0.43			
	3	190,209	440,954	0.43			
91-R	1	208,889	256,413	0.81	0.87	0.052	29
	2	252,569	275,071	0.92			
	3	243,647	276,460	0.88			

Background counts for one experiment- 1753 counts

[a] Number of samples loaded for each strain.). [b] Fold greater than *ry*[506]. The radioactivity on the blots was counted using Packard Radioanalytical imager. The counts obtained from the imager after background subtractions are given. Three independent northern blots with different isolates of RNA were performed and the mean *Cyp6g1*/RP49 values of three sets of northern blots were used to compare the *Cyp6g1* gene expression in different strains ($p<0.0001$ by ANOVA test).

Table 2-3

DDT resistance bioassay

Strain	N	Slope (± S.E)	LC_{25} (95% CI)[a]	LC_{50} (95% CI)[b]	RR50[c]
ry^{506}	600	2.5 (0.3)	0.47 (0.36-0.57)	0.74 (0.65-0.85)	1
Hikone RH	900	1.9 (0.03)	5.87 (4.44-6.95)	8.94 (7.9-10.1)	12
Canton SH	1320	2.6 (0.01)	4.4 (0.12-7.5)	12.9 (9.9-16.5)	17
91-C	1320	1.7 (0.01)	10.5 (6.2-14.4)	20.9 (16.8-26.5)	28
Wisconsin	1000	0.1 (0.003)	138 (14.2-239.7)	447 (343-543.4)	604
91-R	1260	0.12 (0.02)	2860 (1458-4401)	8348 (6369 - 12130)	11281

LC_{25} and LC_{50} are the doses of DDT conferring 25% and 50% mortality respectively. N, number of adult females tested. [a] μg DDT giving 25% mortality. [b] μg DDT giving 50% mortality [c] Resistance factor relative to *ry^{506}*.

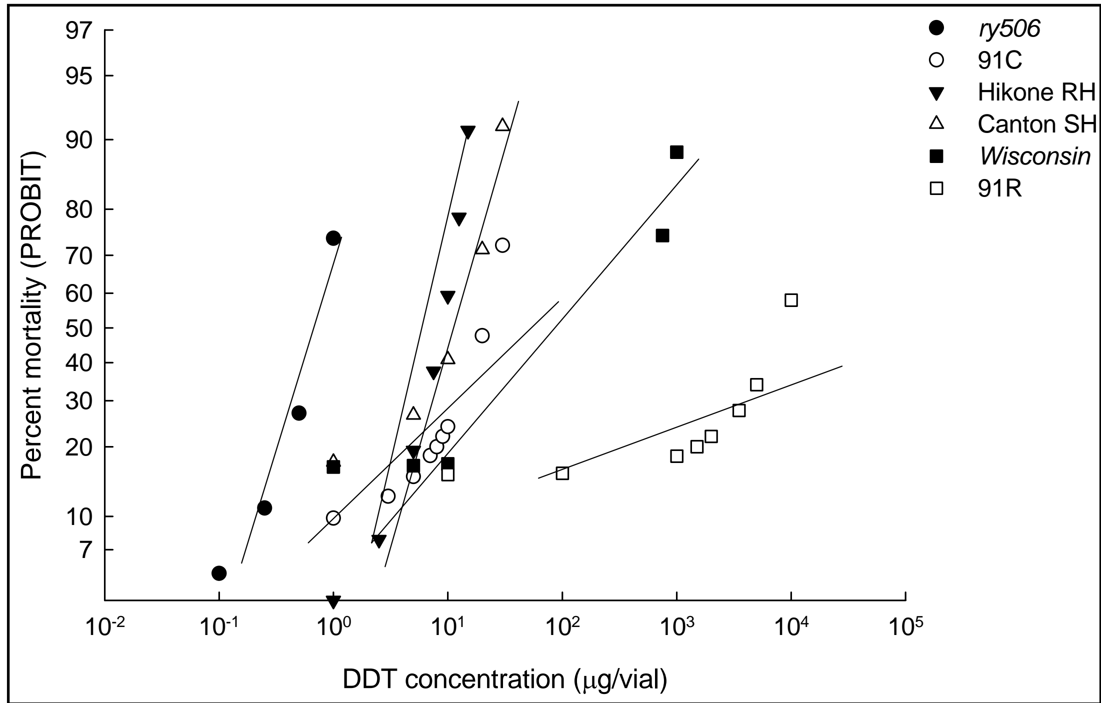


Figure 2-4: Dose response curves derived from the data in Table 2-3 for DDT resistance in different strains of *Drosophila melanogaster*. The predicted mortality values from the probit analysis were plotted against DDT concentration (μg/vial) using SigmaPlot 9.0.

Lack of correlation between CYP6G1 protein and DDT resistance

High levels of mRNA expression may not necessarily result in high levels of polypeptide. Therefore, the level of CYP6G1 peptide expression in 91-R, 91-C, Hikone-RH, Canton- SH and *Wisconsin* strains was investigated by western blot analysis (Figure 2-5). The results showed that the DDT-susceptible 91-C strain, which produces very low level of CYP6G1 mRNA, had a barely detectable level of CYP6G1 protein (Figure 2-5). On the other hand, the 91-R, Hikone-RH, Canton-SH and *Wisconsin* strains, which are high producers of CYP6G1 mRNA, also produced high and similar level of CYP6G1 protein. However, such high levels of CYP6G1 protein or mRNA expressions do not correlate with the DDT resistance because Canton-SH and Hikone-RH are susceptible to DDT (Table 2-3 and Figure 2-4) but high producers of CYP6G1 mRNA and protein (lack of correlation between resistance and protein level: $r=0.24$, $b=21.51 \pm 49.69$ S.E., $P>0.25$).

Association between the presence of *Accord* element, overexpression of *Cyp6g1* and DDT resistance

Daborn et al. (2002) concluded that there is a strong correlation between high level of *Cyp6g1* expression, DDT resistance and the presence of the insertional element *Accord* in the upstream DNA of *Cyp6g1*. In another study, Catania et al (2004) found a 100% correlation between DDT resistance and the presence of the *Accord* element in the *Cyp6g1* gene. To examine this phenomenon, a pair of primers flanking between -123 and -373 bp regions of the *Cyp6g1* gene were designed to amplify the upstream DNA of six strains, which show high and low expression of *Cyp6g1*. Results showed that the

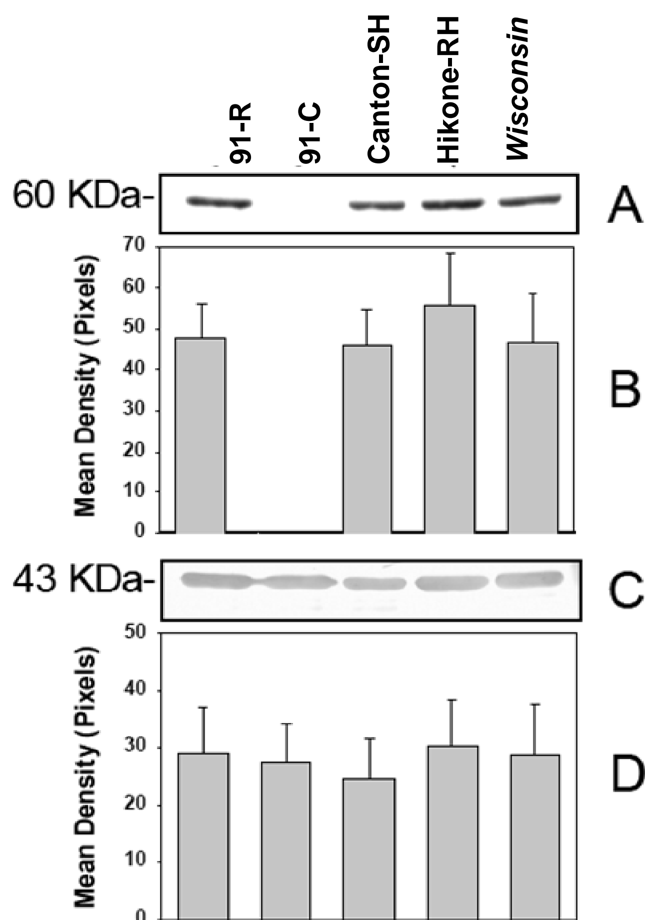


Figure 2-5: Western blot analysis of CYP6G1 protein levels in DDT resistant and susceptible strains of *D. melanogaster*. Microsomal protein preparations isolated from forty female flies of each strain were fractionated on SDS-PAGE and blotted on to nitrocellulose membrane. The upper half of the blot was probed with anti-CYP6G1 antibody (A), whereas the lower half was probed with anti-actin antibody (C), which was used as a loading control. Secondary antibodies conjugated with alkaline phosphatase were used to quantify the CYP6G1 (B) and actin (D) in each fly extract as described in Materials and Methods. The bars represent means of three independent experiments done with three independent fly extracts (+ standard error bars).

upstream DNA of strains exhibiting low *Cyp6g1* expression (91-C and ry^{506}) produced an ~250 bp amplified product (Figure 2-6). Since the PCR primers are 250 bp apart, we conclude that these two strains do not have any insertional DNA between -123 and -373 bp regions of the *Cyp6g1* gene. However, the upstream DNA from the other four strains (91-R, *Wisconsin*, Canton-SH and Hikone-RH) showing high expression of *Cyp6g1* produced a ~700 bp PCR product (Figure 2-6), suggesting the presence of a ~450 bp insertional DNA between -123 and -373 bp region of the *Cyp6g1* gene. To analyze further, the PCR products from all four strains (91-R, *Wisconsin*, Canton-SH and Hikone-RH) were cloned and sequenced. Results showed that the length of the insertional DNA in all strains is 492 bp and their sequences are almost identical except for a few single nucleotide mismatches. In addition, insertional elements from all strains showed about 90% sequence identity with the insertional DNA identified by Daborn et al. (2002) in the upstream DNA of the *Cyp6g1* allele (accession number AY131284). BLAST analysis showed that the 492 bp insertional elements from all four strains had about 89% sequence identity with the reverse complement of the terminal 480 bases of *Accord* element reported in the *Drosophila* genome sequence database (AE003820, nt 46563 - 51781). Based on the sequence data, we found that the insertional DNA is present 283 bp upstream of the 5'UTR of *Cyp6g1* gene. Additionally, insertional elements of all four strains also had sequence similarity with a portion of the *Ninja* retrotransposon (Figure 2-7) found in the *D. melanogaster* genome (accession number AF520587). Our results are consistent with the observations made by Daborn et al. (2002) that there is a positive correlation between the presence of an *Accord* element in the upstream DNA of *Cyp6g1* gene and high expression of *Cyp6g1* (Figure 2-6 and Table 2-2). However, our data show

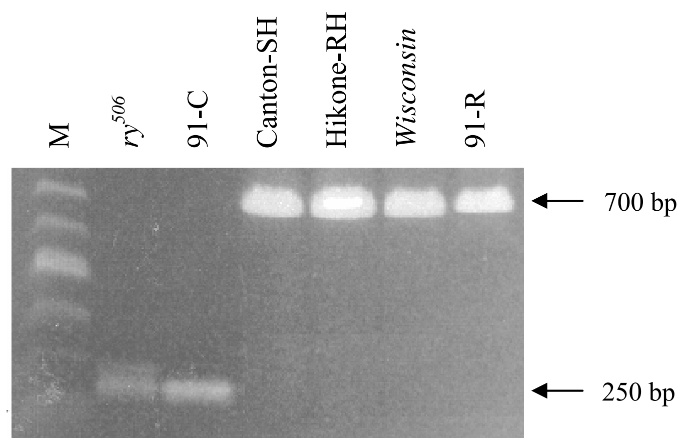


Figure 2-6: Agarose gel picture of the PCR products obtained from amplification of the upstream DNA of different *Cyp6g1* alleles. Genomic DNA isolated from adult flies of each strain was used as templates to amplify –123 to –373 region of the *Cyp6g1* gene with the primer pairs and the PCR parameters described in Methods. Strains without any insertion are expected to produce 250-bp PCR product.

```
              .         .         .         .         .
   4 TACGGGTGCCTCCGTTGGATCGGGGGTCC.TCCTTTCTCTACTCTTAGTT 52
     ||  ||||||||||||| |||||| |||| ||||||||||||||||||||
1311 taacggtgcctccgttgaatcgggcgtccttcctttctctactcttagtt 1262
              .         .         .         .         .
  53 AGTTGCTGCCCAGG.................................... 66
     ||||||||||||||
1261 agttgctgcccaggctgatctgatgggagtcactaatgactttattgtgt 1212
              .         .         .         .         .
  67 ...................................TTTAAATAAATCAAC 81
                                        |||||||||||||||
1211 acttatgatatatgtaggttatatatatttgccactttaaataaatcaac 1162
              .         .         .         .         .
  82 TGCTCGGTTTGGTGTCAAGATCAAGAATGATGAAAGCTTTATTCTTAATG 131
     ||||||||||||||||||||||||||||||||||||||||||||| ||||
1161 tgctcggtttggtgtcaagatcaagaatgatgaaagctttattctcaatg 1112
              .         .         .         .         .
 132 TATACGCACGGGCAAGCAGCAGTGCTTTTGATCCCGTTAGTTCTAGATAG 181
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1111 tatacgcacgggcaagcagcagtgcttttgatcccgttagttctagatag 1062
              .         .         .         .         .
 182 CGAGTCTAGACGAGGACGTCGCGATTACGCAGTGTGTATGTGTGTGTGTA 231
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1061 cgagtctagacgaggacgtcgcgattacgcagtgtgtatgtgtgtgtgta 1012
              .         .         .         .         .
 232 GGTTCATGTTAATTTCAACGTCGAGAGCGTCGCCACCTCGCAGTATGTGT 281
     ||||||||||||||||||||||||||||||||||||| ||| ||||||||
1011 ggttcatgttaatttcaacgtcgagagcgtcgccaccacgcggtatgtgt 962
              .         .         .         .         .
 282 GAATATGTGTGTGTGTGTGTGTTCATGCCGATTTCATCATCGGGTGCAAC 331
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 961 gaatatgtgtgtgtgtgtgtgttcatgccgatttcatcatcgggtgcaac 912
              .         .         .         .         .
 332 AGAGTTTCAGGTAGCGGGGGACGAGGCTATGCTGGCATGGTAGCTCGTGA 381
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 911 agagtttcaggtagcgggggacgaggctatgctggcatggtagctcgtga 862
              .         .         .         .         .
 382 TCAATATATATATATACAGCGTTGGCACTTTGCTGATGTCGCCTACCGGG 431
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 861 tcaatatatatatatacagcgttggcactttgctgatgtcgcctaccggg 812
              .         .         .         .         .
 432 CGGGCTTCATAGGCGGAGGTGGTTGTTGAGGGGGGTTAATGCTGGGCATG 481
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 811 cggcttcataggcggaggtggttgttgaggggggttaatgctgggcatg 762
              .
 482 GTAACTCGTGC 492
     ||||||| |.|
 761 gtaactcctcc 751
```

Figure 2-7: Comparison of the sequence of the insertional DNA (upperline) and *Ninja* transposable element (AF520587) (bottom line).

that presence of an *Accord* element in the upstream DNA of *Cyp6g1* does not necessarily confer DDT resistance (Figure 2-6 and Table 2-3).

Sequence comparison of *Cyp6g1* alleles from resistant and susceptible strains of *Drosophila*

The level of CYP6G1 mRNA in the strains examined so far is correlated with the levels of polypeptide. However, the amino acid sequence of CYP6G1 proteins of the susceptible Hikone-RH and Canton-SH strains may differ significantly with the amino acid sequence of the CYP6G1 proteins from the resistant 91-R and *Wisconsin* strains. To examine the allelic differences, we completely sequenced the *Cyp6g1* alleles from 91-R, 91-C, Hikone-RH, Canton-SH and *Wisconsin* strains (Table 2-4) For each strain, the *Cyp6g1* allele was PCR amplified into three overlapping fragments by using three pairs of gene-specific primers as described in Methods and explained in Fig 2-1. These three overlapping PCR fragments represent the +6/+1148, +1064/+2085 and +2021/+2803 regions of the *Cyp6g1* gene, which were individually cloned into pGEMT-Easy vector (Promega). Two clones for each PCR fragment of each strain were randomly picked, and both DNA strands of each clone were sequenced. Thus, for each fragment four nucleotide sequences were obtained and compared to have accurate sequence data. The sequences from all five strains were compiled and compared using the Sequencher program. The results (Table 2-4) show that the coding regions of the *Cyp6g1* alleles of five strains are almost identical except for nine codons located at base numbers 949, 1088, 1142, 1344, 1392, 1479, 1786, 1827 and 1911. These codons are identical in

Table 2-4

Nucleotide and amino acid substitutions among *Cyp6g1* alleles (+6/+2803 region) between different resistant and susceptible strains

Gene regions	Base number	Strains					Amino acid	Changed codon
		91-C	91-R	*Wisconsin*	C-SH[#]	H-RH[#]		
5'-UTR	60	A	T	T	T	T		
	70	C	A	A	A	A		
Intron 1	241	a	c	c	c	c		
	262	g	c	c	c	c		
	336	t	c	c	c	c		
	389	g	a	a	a	a		
	397	42nt[*]	-	-	-	-		
	480	g	t	t	t	t		
	553	c	t	t	t	t		
	596	a	g	g	g	g		
Exon 1	762	T	A	A	A	A		
	949	C	C	C	C/T	C/T	C or T = His	
	1088	A	A	A/C	A	A	A = Asn, C = His	N105H
	1142	T	T	T/G	T	T	T = Phe, G = Val	F123V
Intron 2	1253	g	g	g	g/a	g		
	1277	g	g	a	a	a/g		
	1279	-	-	-	g	-		
	1283	t	t	a	a	a/t		
Exon 2	1344	A	A	C	C	A/C	A or C = Gly	
	1392	T	T	C	C	C/T	C or T = Ala	
	1479	T	C	T	T	C	T or C = Tyr	
	1786	A	A	T	A	A	A = Thr, T = Ser	T316S
	1827	C	C/T	C	C	T	C or T = Ala	
	1911	A	A/G	A	A	G	A or G = Lys	

Table 2-4 continued

Gene regions	Base number	Strains					Amino acid	Changed codon
		91-C	91-R	*Wisconsin*	C-SH#	H-RH#		
Intron 3	2117	a	c	c	c	c		
	2121	t	g	g	g	g		
	2156	g	a	a	a	a		
3'-UTR	2558	G	-	-	-	-		

C-SH and H-RH refer to Canton-SH and Hikone-RH strains, respectively.
*Indicates insertion of a 42 bp sequence in 91-C strain. All sequences have been submitted to GenBank. The accession numbers are AY842137 – AY842141 Bases in introns are shown in lower cases and those in the 5'-UTR are shown in upper case and italics. Dashes indicate missing bases. The amino acid substitutions occurred due to the polymorphism is indicated in the last column. The sequences have been submitted to GenBank with accession numbers AY842137-AY84214.

most alleles except in one or two alleles the third base is substituted with a different base. Due to the third letter degeneracy, these substitutions usually do not change the amino acids. However, in the allele of the resistant *Wisconsin* strain, codons located at base numbers 1088, 1142 and 1786 show first letter substitution. As a result, the amino acids encoded by codons 105, 123 and 316 are substituted compared to the alleles from the other strains (Table 2-4). However, it should be noted that two clones isolated for the allele of *Wisconsin* showed polymorphism for amino acids 105 and 123, whereas in both clones amino acid 316 is a serine (Table 2-4). Based on the sequence data, we conclude that the five alleles of *Cyp6g1* cannot be grouped into resistant and susceptible classes, and their peptide sequences are almost identical except the allele of the resistant *Wisconsin* strain.

Substitution of the *Cyp6g1* allele of resistant 91-R strain with the allele from the susceptible 91-C strain

It is clear from the results discussed above that a strain showing high levels of CYP6G1 protein such as Canton-SH or Hikone-RH may also be highly susceptible to DDT. However, it is not known whether a strain with low *Cyp6g1* expression levels can be highly resistant to DDT. More accurately, we surmised what would be the resistance phenotype of a modified 91-R strain if it's highly active *Cyp6g1* allele (*Cyp6g1-91R*) was substituted with the low transcribing allele from the 91-C strain (*Cyp6g1-91C*). To synthesize such a strain a crossing scheme as shown in Figure 2-2 (see Methods) was followed. In this cross, F1 *R; R/C; R* females were used so that crossing over between the 2nd chromosomes of 91-C and 91-R origin could produce a recombinant chromosome

carrying high transcribing *Cyp6a2-91R* and low transcribing *Cyp6g1-91C* alleles. The crossing scheme shows the strategy to recover the putative recombinant 2nd chromosome (*u2*) in homozygous condition in individual F4 *R; u2/u2; R* lines. It is known that the 3'-UTR of *Cyp6a2-91C* allele of 91-C strain has an insertion of 0.5-kb LTR of *17.6* mobile element (Waters et al., 1992), and the upstream DNA of the *Cyp6g1-91R* allele of the 91-R strain has an insertion of a 450 bp *Accord* element (Fig. 2-6). Therefore, *Cyp6a2* and *Cyp6g1* alleles of fifty F4 *R; u2/u2; R* lines were genotyped via PCR using primers flanking the insertional sites. In twenty-two lines *Cyp6a2* had the inserted *17.6* mobile element, but *Cyp6g1* did not have any. Thus, these lines were homozygous for the alleles of both *Cyp6a2* and *Cyp6g1* from the 91-C strain. On the other hand, twenty-five lines were homozygous for the *Cyp6a2* and *Cyp6g1* alleles of 91-R strain because their *Cyp6a2* allele did not have the *17.6* mobile element but *Accord* insertion was present in their *Cyp6g1* allele. In the remaining three lines, no insertional DNA was found in their *Cyp6a2* and *Cyp6g1* alleles. Thus, these lines were homozygous for a recombinant 2nd chromosome carrying the *Cyp6a2* allele of the 91-R and *Cyp6g1* allele of the 91-C strain. These three recombinant lines, RC-21, RC-35 and RC-48, were also homozygous for the X and 3rd chromosomes from the 91-R strain and their chromosomal composition is *R; rx; R* where *rx* refer to the recombinant 2nd chromosome. To verify further, -1200/+4 region, 5'-UTR, intron 1 and intron 3 of the *Cyp6g1* allele of each recombinant line were sequenced and compared with the sequence of the *Cyp6g1* alleles of the 91-C and 91-R strains. Results showed that the *Cyp6g1* alleles of the three recombinant lines lack the *Accord* element but have all the sequence features of the *Cyp6g1* allele of the 91-C strain (Table 2-5). To examine the effects of *Cyp6g1* allele substitution, the recombinant lines

Table 2-5

Comparison of the *Cyp6g1* alleles of three recombinant lines, 91-C and 91-R strains.

Gene region	Position	91-R	91-C	RC-21	RC-35	RC-48
Upstream DNA	-1044	t	c	c	c	c
	-1042	t	g	g	g	g
	-1041	t	g	g	g	g
	-1037	a	g	g	g	g
	-352	c	a	a	a	a
	-331	t	c	c	c	c
	-265	c	t	t	t	t
	-251	a	t	t	t	t
	-124	t	g	g	g	g
	-123	c	g	g	g	g
5'-UTR	+60	T	A	A	A	A
	+70	A	C	C	C	C
Intron 1	+241	c	a	a	a	a
	+262	c	g	g	g	g
	+336	c	t	t	t	t
	+389	a	g	g	g	g
	+397	-	+	+	+	+
Intron 3	+2117	c	a	a	a	a
	+2121	g	t	t	t	t
	+2156	a	g	g	g	g

+ , 42 bp insertion (see Table 2-4)

were compared with the 91-R and 91-C strains for *Cyp6* gene expression and DDT resistance. In all three recombinant lines, expression *Cyp6a2* was as high as in the 91-R strain (Fig 2-8 and 2-9). However, the expression of *Cyp6g1* in the recombinant lines was more or less similar to that observed in the 91-C strain and 6.5- to 8-fold lower than that found in the 91-R strain (Fig 2-10 and 2-11). These results are expected because the recombinant lines have high-transcribing *Cyp6a2-91R* and low-transcribing *Cyp6g1-91C* alleles. I also compared these strains for *Cyp6a8* expression. Like the 91-C strain, all three recombinant lines showed lower *Cyp6a8* expression compared to the 91-R strain (Fig 2-12 and 2-13). Thus, the recombinant lines are similar to the 91-C strain with respect to *Cyp6g1* and *Cyp6a8* expression when compared to the 91-R strain. Surprisingly, however, two of the three recombinant lines (RC-21 and RC-35) showed even higher levels of DDT resistance than the 91-R strain (Table 2-6 and Figure 2-14). The 3rd recombinant line, RC-48, also showed a high level of DDT resistance and its LC_{25} and LC_{50} values were similar to those observed for the 91-R strain. Compared to the 91-C strain, all three recombinant lines showed several hundred-fold higher levels of DDT resistance (Table 2-6 and Figure 2-14).

Genome wide expression analysis of genes differentially expressed genes between 91-R, RC-21 and 91-C strains

Expression of genome wide profile for the resistant 91-R strain, susceptible 91-C strain and recombinant RC-21 strain was compared using Affymetrix microarrays. The *Drosophila* genome 2.0 chip consists of 18,500 transcripts based on the recent annotation

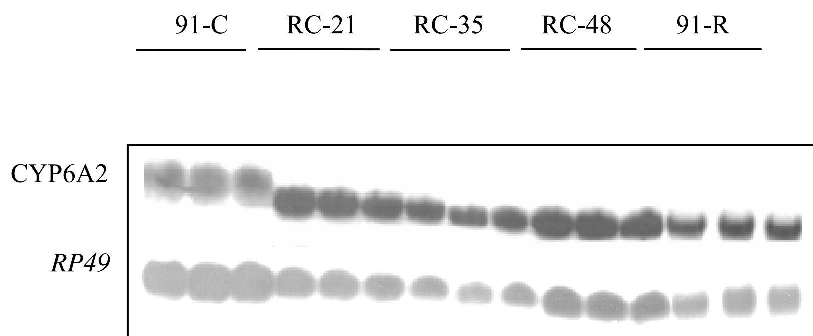


Figure 2-8: Northern blot analysis of the expression of *Cyp6a2* in the adult flies of recombinant RC-21, RC-35, RC-48 stocks, 91-C and 91-R strains of *Drosophila melanogaster*. Total RNA was isolated and electrophoresed on 1.2% agarose-2.2M formaldehyde, blotted onto nylon membrane and hybridized with *Cyp6a2* and RP49 probes as described in Methods. RP49 is used as an internal control to correct for loading errors.

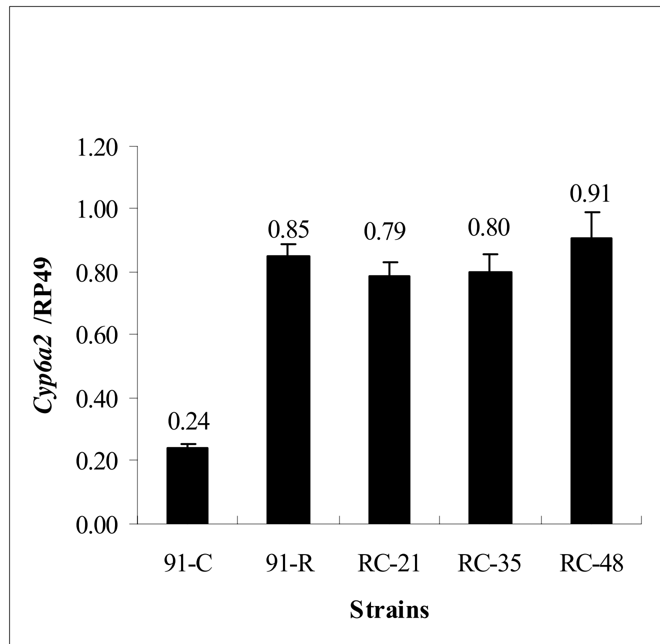


Figure 2-9: Quantitative analyses of the data shown in Figure 2-8 showing the expression of *Cyp6a2* in recombinant stocks, 91-R and 91-C strains of *Drosophila melanogaster*. The blots were scanned using Packard Radioanalytical imager. Each bar represents the mean ± S.D. of *Cyp6a2*/RP49 counts from three independent RNA samples examined by northern blot analyses.

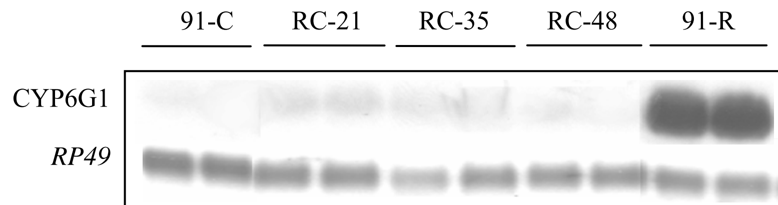


Figure 2-10: Expression of *Cyp6g1* in the adult female flies of recombinant RC-21, RC-35, RC-48 stocks, 91-C and 91-R strains of *Drosophila melanogaster*. Total RNA was isolated from the strains mentioned above and electrophoresed on 1.2% agarose-2.2M formaldehyde, blotted onto nylon membrane and hybridized with *Cyp6g1* probe. RP49 is used an internal control to correct for loading errors. Quantitative analyses of the results are shown in Figure 2-11.

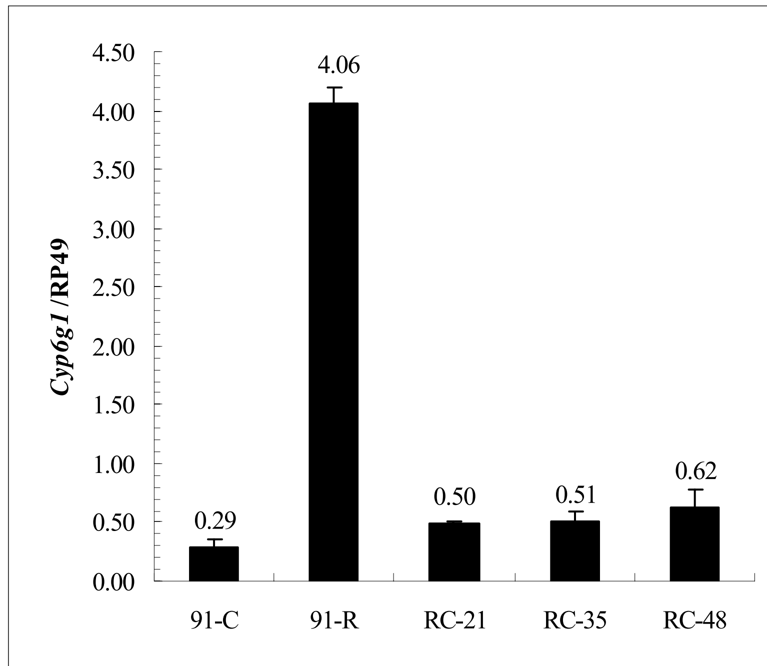


Figure 2-11: Quantitative analyses of the expression of *Cyp6g1* in recombinant stocks, 91-R and 91-C strains of *Drosophila melanogaster*. The blots shown in Figure 2-10 were scanned using Packard Radioanalytical imager. Each bar represents the mean ± S.D. of *Cyp6g1*/RP49 counts from three independent RNA samples examined by northern blot analyses.

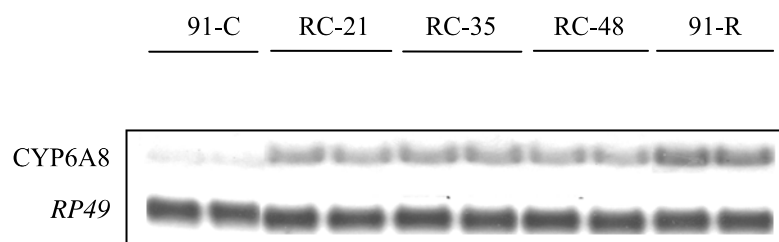


Figure 2-12: Northern blot analysis of the expression of *Cyp6a8* in the adult flies of recombinant RC-21, RC-35, RC-48 stocks, 91-C and 91-R strains of *Drosophila melanogaster*. Total RNA was isolated and electrophoresed on 1.2% agarose-2.2M formaldehyde, blotted onto nylon membrane and hybridized with *Cyp6a8* and RP49 probes as described in Methods. RP49 is used as an internal control to correct for loading errors.

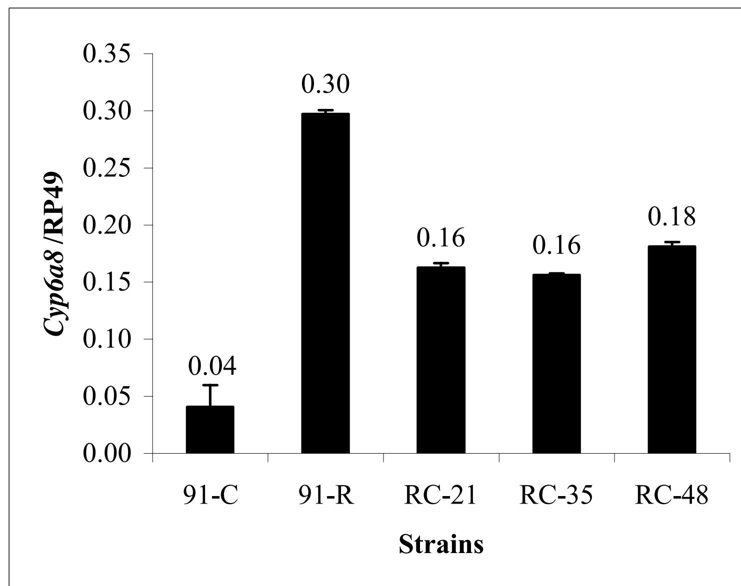


Figure 2-13: Quantitative analyses of the expression of *Cyp6a8* in recombinant stocks, 91-R and 91-C strains of *Drosophila melanogaster*. The blots shown in Figure 2-12 were scanned using Packard Radioanalytical imager. Each bar represents the mean ± S.D. of *Cyp6a8*/RP49 counts from three independent RNA samples examined by northern blot analyses.

Table 2-6

Comparison of DDT resistance in 91-R, 91-C and recombinant lines

Strain	N	Slope (± S.E)	LC_{25} (95% CI) in mg	LC_{50} (95% CI) in mg
RC-21	620	0.043 (0.01)	9.2 (5.7-15.8)	25 (17.5-47.7)
RC-35	660	0.1 (0.01)	8.2 (4.9-13.6)	20.3 (14.5-36.9)
RC-48	620	0.08 (0.01)	5.3 (2.8-7.9)	14.2 (11-20)
91R	660	0.123 (0.02)	5.1 (3.4-9.9)	11.2 (7.6 - 25)
91-C	1320	1.7 (0.01)	0.011 (0.006-0.014)	0.021 (0.017-0.027)

N= number of adult female flies tested, LC_{25} and LC_{50}= Dose of DDT (mg) that caused 25% and 50% mortality respectively, 95% CI = 95% confidence interval. RC-21, RC-35 and RC-48 are the recombination stocks generated by the cross described in Fig.2-1.

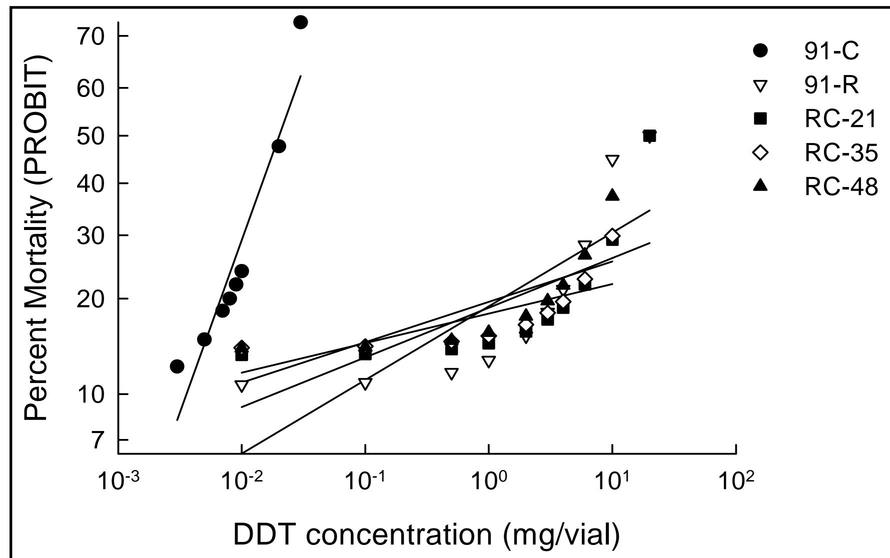


Figure 2-14: Dose response curves derived from the data in Table 2-6 for DDT resistance in recombinant stocks compared with 91-R and 91-C of *Drosophila melanogaster*. The predicted mortality values from the probit analysis were plotted against DDT dose (mg) using SigmaPlot 9.0.

of BDGP (Berkeley Drosophila Genome project). Analysis of the microarray data in SAS showed that 104 genes were upregulated and 110 genes were downregulated in the 91-R strain compared to the 91-C strain. The differentially expressed genes were grouped into different categories based on the biological process. The overexpressed genes in 91-R strain belonged to defense and stress response, carbohydrate metabolism and development. The number of genes overexpressed in each category in the 91-R strain compared to 91-C strain is shown in Fig. 2-15. The repressed genes in 91-R strain belonged to the signal transduction, development, protein folding, transport genes, lipid and steroid metabolism and proteolysis. Comparison of expression of genes between RC-21 and 91-C strain showed that there were 743 genes upregulated and 315 genes downregulated in RC-21 strain compared to the 91-C strain. The graphical representation of different categories of genes upregulated in the RC-21 strain compared to the 91-C strain is shown in Figure 2-16. Among the genes upregulated in 91-R and RC-21 strains compared to the 91-C strain, the genes commonly overexpressed between 91-R and RC-21 strains were analyzed. Results showed that there are 51 genes commonly overexpressed in the 91-R and RC-21 strains compared to the 91-C strain. Among these genes, 20 do not have the functional annotation and will not be discussed further. Among the remaining 31 genes, majority of genes are involved in carbohydrate metabolism, cytochrome P450 genes, defense and stress response and developmental genes. The list of genes that are commonly upregulated in 91-R and RC-21 strain are shown in Table 2-7. There are 34 genes that are commonly downregulated in both 91-R and RC-21 compared to 91-C strain. The repressed genes belong to different categories such as proteolysis, development and signal transduction as shown in Table 2-8. Since the major resistance

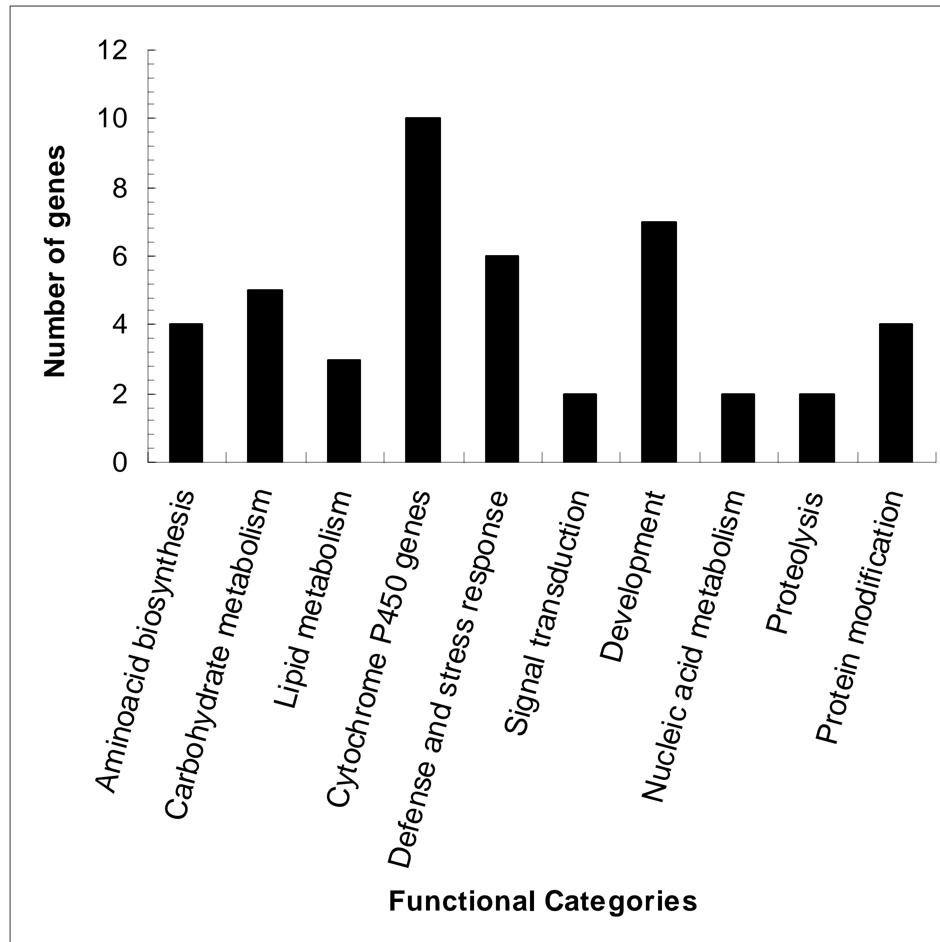


Figure 2-15: Number of genes in different categories overexpressed in 91-R strain compared to 91-C strain.

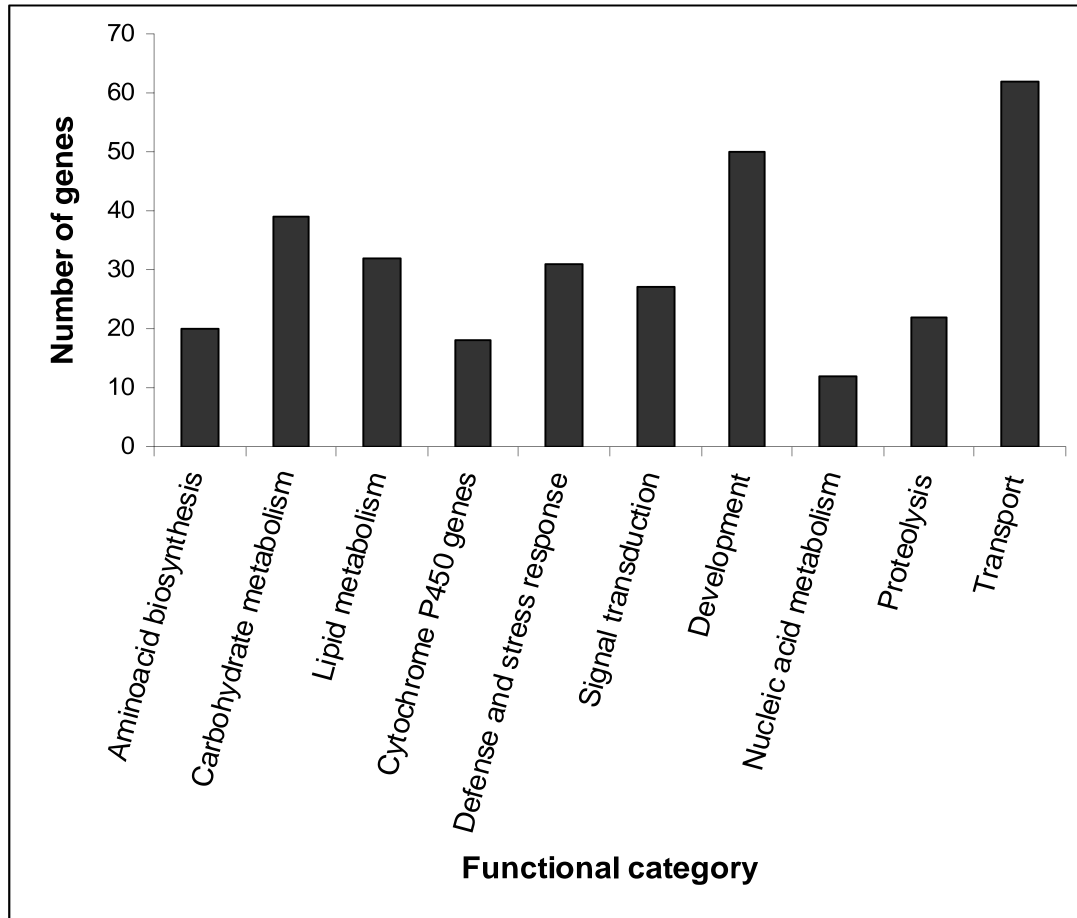


Figure 2-16: Graphical representation of different categories of genes overexpressed in RC-21 strain compared to the 91-C strain.

Table 2-7

Genes that are commonly upregulated in 91-R and RC-21 strains compared to 91-C strain

Gene Title	Gene Symbol	p-value	A*	B†	Location	GO: Molecular Function
Unknown function						
---	CG16898	1.37E-03	2.91	12.4	chr2R:	
---	CG9616	2.40E-04	10.59	5.04	chr3R:	
---	CG13658	6.93E-04	9.80	4.76	chr3R:	
---	CG15281	6.30E-04	6.41	4.71	chr2L:	
---	CG33091	3.54E-03	3.15	4.44	chr3R:	
---	CG14191	2.21E-02	2.98	3.92	chrX:1	
---	CG4210	2.05E-06	2.94	3.76	chr3R:	
---	CG3348	2.42E-02	3.56	3.61	chr3R:	
---	CG15293	2.52E-04	5.12	3.53	chr2L:	
---	---	1.75E-04	6.08	3.08	chr2L:	
---	CG31205	8.19E-05	2.04	3.01	chr3R:	
---	CG6834	1.36E-04	5.13	2.91	chr3R:	
---	CG31077	1.72E-02	3.54	2.74	chr3R:	
---	CG7214	1.69E-03	80.48	2.57	chr2L:	
---	---	1.85E-02	3.80	2.54	chrX:1	
---	CG11458	4.57E-07	24.98	2.5	chr3L:	
---	CG11315	4.96E-05	5.47	2.16	chr3R:	
---	CG18628	2.13E-02	2.90	2.14	chr3L:	
---	CG32594	5.24E-03	3.66	2.11	chrX:1	
---	CG13656	5.88E-03	2.55	2.06	chr3R:	
Amino acid biosynthesis and catabolism						
---	CG8745	5.78E-05	16.31	5.19	chr3L:	ornithine-oxo-acid transaminase
---	CG8129	2.03E-03	2.55	2.77	chr3R:	nitrilase
Proteolysis						
Jonah 65Aii	Jon65Aii	4.47E-04	5.61	2.64	chr3L:	serine-type endopeptidase
Lipid and steroid metabolism						
--- /// ---	CG31809 CG31810	4.60E-05	4.79	4.98	chr2L:	oxidoreductase
Defense and stress response						
--- /// ---	PGRP-SC1a	1.70E-05	16.63	7.6	chr2R:	receptor activity
Galactose-specific C-type lectin	Lectin-galC1	6.90E-07	5.02	2.25	chr2L:	galactose binding
drosomycin-4	dro4	1.07E-04	3.21	2.17	chr3L:	
Development						
---	CG30174	6.82E-04	7.79	2.36	chr2R:	
prickle	pk	2.21E-04	3.10	2.31	chr2R:	structural constituent of cytoskeleton
Egghead	egh	1.18E-02	2.74	1.91	chrX:2	beta-1,4-mannosyltransferase

Table 2-7 continued

Gene Title	Gene Symbol	p-value	A*	B†	Location	GO: Molecular Function
Protein biosynthesis and translation						
---	CG15261	6.76E-03	5.85	2.5	chr2L:	
Signal transduction						
---	CG7650	5.63E-04	2.69	8.14	chr3L:	small regulatory GTPase
Carbohydrate metabolism						
---	CG11909	1.58E-06	10.89	6.94	chr3R:	alpha-glucosidase
---	CG11669	1.32E-06	36.10	6.69	chr2R:	alpha-glucosidase
---	CG12780	2.11E-03	4.24	3.19	chr2R:	glucosidase activity
---	CG30438	4.92E-03	4.26	2.43	chr2R:	transferase activity
---	CG30360	4.36E-04	5.87	2.01	chr2R:	transporter activity
Cytochrome P450 genes						
Cytochrome P450-4p1	Cyp4p1	9.10E-05	25.75	23	chr2R:	
Saccharomyces cerevisiae UAS construct of Daborn	Cyp6g1	5.12E-05	2.64	8.38	chr2R:	
Cytochrome P450-6a8	Cyp6a8	2.07E-02	2.23	6.58	chr2R:	
---	Cyp6w1	5.22E-04	3.80	4.82	chr2R:	
---	Cyp309a1	2.57E-03	3.76	4.75	chr2L:	
Cytochrome P450-4d1	Cyp4d1	1.65E-05	2.05	1.91	chrX:1	
---	Cyp4d20	3.33E-03	3.22	1.84	chr3L:	
Cytochrome P450-6a2	Cyp6a2	2.73E-05	1.75	1.8	Chr2R	
DNA replication and repair						
Companion of reaper	Corp	3.92E-02	2.05		chrX:8	
Homeostasis, cell adhesion, motility and contraction						
Larval serum protein	Lsp2	1.92E-03	24.40	19.6	chr3L:	nutrient reservoir
---	TpnC4	7.98E-05	17.31	4.1	chr2R:	calmodulin binding
Odorant-binding protein 57a	Obp57a	2.38E-03	2.20	2.45	chr2R:	odorant binding
---	CG32372	5.30E-03	3.67	2.05	chr3L:	
Myosin light chain 2	Mlc2	4.96E-06	11.28	2.04	chr3R:	calmodulin binding

Table 2-8

Genes that are commonly repressed in 91-R and RC-21 strains compared to 91-C strain.

Gene Title	Gene Symbol	GO: Molecular Function	p-value	A*	B†	Location
Jonah 99Fi	Jon99Fi	serine-type endopeptidase	1.54E-02	4.58	1.99	chr3R:
Cytochrome P450 genes						
--- /// ---	Cyp12d1	oxidoreductase	7.73E-03	3.90	2.51	chr2R:
---	Cyp4d14	oxidoreductase	1.04E-02	2.04	2.52	chrX:1
Carbohydrate metabolism						
---	CG3168	transporter	9.98E-04	1.92	3	chrX:6
---	CG3797	Glucuronosyl transferase	9.63E-04	4.65	2.02	chr3L:
Transport proteins						
---	CG11163	zinc ion transporter	8.32E-06	2.02	2.53	chr2R:
DNA replication and repair						
Pontin	pont	nucleic acid binding	3.18E-05	4.28	2.76	chr3R:
---	CG17227		6.73E-04	2.84	2.15	chr3R:
Signal transduction						
Drosulfakinin	Dsk	neuropeptide hormone activity	2.69E-03	2.83	2.06	chr3R:
---	CG15534	sphingomyelin phosphodiesterase	2.98E-02	3.99	3.49	chr3R:
Transcription-associated						
Ribosomal protein L28	RpL28	nucleic acid binding	7.37E-04	4.04	3.02	chr3L:
Protein folding, assembly and modification						
Calcineurin A1	CanA1	calcium-dependent protein serine/threonine phosphatase	1.19E-05	4.43	6.32	chr3R:
Development						
Imaginal disc growth factor	Idgf1	chitinase activity	1.67E-05	6.35	2.74	chr2L:
---	CG10592	nucleotide phosphatase activity	1.87E-05	3.14	2.79	chr3L:

Table 2-8 continued

Gene Title	Gene Symbol	GO: Molecular Function	p-value	A*	B†	Location
---	CG5150	nucleotide phosphatase	1.02E-06	3.90	3.31	chr3L:
Defense and stress response						
---	CG8193	monophenol monooxygenase	4.05E-04	4.02	2.28	chr2R:
Defensin	Def		9.38E-07	4.41	3.16	chr2R:
---	CG2064	oxidoreductase	6.85E-04	6.28	6.49	chr2R:
Lipid and steroid metabolism						
---	CG17192	triacylglycerol lipase	1.82E-03	5.72	2.22	chr3R:
---	CG33116	CDP-alcohol phospho transferase	6.86E-04	16.63	16.02	chr2L:
Proteolysis						
---	CG8773	glutamyl aminopeptidase	5.33E-04	2.31	2.01	chr3R:
Trypsin	Try	trypsin activity	2.13E-04	4.57	2.3	chr2R:
---	CG17571	serine-type endopeptidase	2.02E-03	2.17	2.11	chrU:2
---	CG7631	Metallo endopeptidase	1.24E-02	2.47	2.22	chr2L:
---	CG4563	ligase activity	9.10E-06	2.90	3.44	chr2R:
Amino acid biosynthesis and metabolism						
---	vanin-like	pantetheinase	1.95E-03	2.26	2.09	chrX:5
---	CG2191	cation transporter	9.26E-06	6.24	4.26	chr3R:
Unknown function						
---	CG33109		3.33E-03	5.24	2.07	chr3R:
---	---		1.63E-02	2.02	2.09	chr2h:
---	CG11399		8.75E-03	3.81	2.31	chr3L:
---	CG31259		3.51E-02	2.66	2.34	chr3R:
---	CG6839		9.75E-03	2.67	2.44	chr3L:
---	CG32984		1.57E-02	2.83	3.11	chr2L:
---	CG11825		2.78E-03	9.70	8.35	chr2R:

*Ratio of REC/91-C i.e., overexpression of recombinant strain compared to 91-C strain

† Ratio of 91-R/91-C i.e., overexpression of 91-R strain compared to 91-C strain

locus was discovered on the second chromosome (See Introduction), the *Cyp* genes overexpressed in resistant strains and present on the second chromosome were analyzed. The cytochrome P450 genes that are commonly overexpressed between 91-R and RC-21 strains and present on the second chromosome are *Cyp4p1, Cyp6w1, Cyp6a2* and *Cyp309a1* (Table 2-7). Although *Cyp6g1* and *Cyp6a8* are upregulated in the RC-21 strain, it is not as high as in the 91-R strain (Table 2-7). The partial overexpression of the 91-C allele of *Cyp6g1* and *Cyp6a8* in the background of 91-R strain is due to the effect of the third chromosome that influences the expression of *Cyp* genes on the second chromosome (Maitra *et al*., 1996; Dombrowski *et al.,* 1998).

Discussion

Daborn et al (2001, 2002) suggested that DDT resistance phenotype in *Drosophila* is monofactorial and the overexpression of *Cyp6g1* alone is 'necessary and sufficient' to confer resistance. First, Daborn *et al* (2002) examined only two out of fourteen resistant strains by microarray analysis and found that *Cyp6g1* is the only P450 gene that is overtranscribed in the resistant strains relative to the susceptible ones. However, in the other twelve resistant and six susceptible strains, expression of *Cyp6g1* alone was measured via RT-PCR. These resistant strains do show overtranscription of *Cyp6g1* relative to the susceptible strains, but it is not known whether any other *Cyp* gene is also upregulated in these twelve DDT resistant strains. Recent work by Pedra et al. (2004) has revealed that more than just *Cyp6g1* is over-transcribed in DDT resistant strains. Third, to support the hypothesis that *Cyp6g1* confers DDT resistance, transgenic flies showing 100-fold higher expression of *Cyp6g1* were challenged with low dose

(10μg) of DDT to demonstrate that they are resistant (Daborn et al. 2002). Since higher doses of DDT were not tested against the transgenic flies, nor were LC_{50}s presented, it is not known whether overexpression of *Cyp6g1* alone can confer a high level of DDT resistance that is found in the lab-selected 91-R or the field collected *Wisconsin* strains (Festucci-Buselli et al., 2005). The bioassay results of the present investigation also showed that the 91-R and *Wisconsin* strains are highly resistant with their LC_{50} values of 8348 μg and 447 μg respectively (Table 2-3).

Recently, it has been shown that there is a lack of correlation between high level of *Cyp6g1* expression and DDT resistance in *Drosophila* (Festucci-Buselli et al., 2005). Since in this study the authors did not present any quantitative data on *Cyp6g1* expression, we reexamined this issue and quantified *Cyp6g1* expression at RNA and protein levels in multiple strains. Consistent with the observations made by Festucci-Buselli et al (2005) we find that Canton-SH and Hikone-RH strains have high level of CYP6G1 protein yet they are as susceptible as the 91-C strain, which shows barely detectable level of *Cyp6g1*expression. We compared the *Cyp6g1* alleles of all susceptible and resistant strains rigorously by DNA sequencing and did not find any significant difference. Surprisingly, the amino acid sequences of CYP6G1 polypeptides of the susceptible and the resistant strains are found to be almost identical and *Cyp6g1* alleles cannot be grouped into susceptible and resistant classes based on their amino acid sequence (Table 2-4). Thus, susceptible phenotype of Canton-SH and Hikone-RH strains showing high *Cyp6g1* expression is not due to mutation that may inactivate the CYP6G1 protein. These data led us to conclude that high expression of a *Cyp6g1* allele that is

almost identical to the allele of a resistant strain does not guarantee DDT resistance phenotype.

Lack of correlation between DDT resistance and *Cyp6g1* expression has been observed in other situations as well. Microarray analysis of a laboratory selected DDT resistant strain (*Wis1 lab*) showed that *Cyp6a8* was selected as the overexpressing *Cyp* gene instead of *Cyp6g1* (Le Goff et al., 2003). It may be argued that selection of DDT resistance with laboratory strain may co-select a *Cyp* gene other than *Cyp6g1* whereas selection of DDT resistance in the field strains tends to co-select the *Cyp6g1* gene. However, it should be noted that *Cyp6a8* failed to metabolize DDT when it was expressed in yeast (Helvig et al., 2004). Thus, the biological significance of co-selection of overexpressing *Cyp6a8* allele in the laboratory-selected DDT resistant strain obtained by Le Goff et al (2003) is not understood. Several strains of *D. simulans* and *D. melanogaster* collected from California and Africa do not always show a tight correlation between DDT resistance and *Cyp6g1* overexpression (Schlenke and Begun, 2004). For example, CS1 strain of *D. simulans*, with high *Cyp6g1* expression shows 84% mortality when exposed to 20μg DDT for 18 hours. Similarly, CM2 and AM3 lines of *D. melanogaster* showing high *Cyp6g1* expression are found to be highly susceptible to 20 μg DDT (Schlenke and Begun, 2004).

Since long terminal repeats (LTR) of many transposable elements are known to upregulate transcription of nearby genes (Sverdlov, 1998), the *Accord* element found in the upstream DNA of *Cyp6g1* alleles of the resistance strains (Daborn et al, 2002) may also upregulate *Cyp6g1* expression. If overexpression of *Cyp6g1* is necessary for DDT resistance and *Accord* insertion is necessary for *Cyp6g1* overexpression, DDT resistant

strains are expected to have *Accord* insertion in their *Cyp6g1* locus. Indeed, a 100% correlation between *Accord* insertion and DDT-resistance (24 hour – 5 μg DDT assay) was found when 673 strains of *Drosophila* collected from different regions of the globe were examined (Catania et al., 2004). Since CYP6G1 RNA levels in these strains were not determined, it is not known whether there is also a 100% correlation between *Accord* insertion and *Cyp6g1* overexpression. In another study, however, *Accord* insertion was not always associated with DDT resistance in field-collected strains of *D. melanogaster* (Schlenke and Begun, 2004). In this study, 40% strains with *Accord* insertion were susceptible and showed 80% - 100% mortality when exposed to 20 μg DDT for 18 hours (Schlenke and Begun, 2004). We examined *Accord* insertion in the *Cyp6g1* alleles of all the strains we used. Our results show that there is a positive correlation between *Accord* insertion and high expression of *Cyp6g1*. However, the presence of *Accord* element does not necessarily confer DDT resistance. For example, Canton-SH and Hikone-RH strains have *Accord* insertion in their *Cyp6g1* alleles yet both strains are highly susceptible to DDT.

Northern blot and microarray analysis have shown previously that expression of atleast four *Cyp* genes, *Cyp6a2, Cyp6a8*, *Cyp6g1* and *Cyp6w1*, is much higher in the laboratory-selected 91-R strain compared to the susceptible ones (Waters et al., 1992; Maitra et al., 1996; Dombrowski et al., 1998; Pedra et al., 2004; Festucci-Buselli et al., 2005). However, it is not known how many of these four *Cyp* genes are necessary for DDT resistance. In the present investigation, we examined the role of *Cyp6g1*. To do this, we synthesized three recombinant lines, which are homozygous for the low expressing *Cyp6g1* allele of the susceptible 91-C strain, and the X and 3rd chromosomes

of the resistant 91-R strain. All three lines are found to be as resistant as the 91-R strain although they have very low level of CYP6G1 RNA. Thus a laboratory strain could be highly resistant to DDT even if it shows very low *Cyp6g1* expression. This observation does not agree with the hypothesis proposed by Daborn et al (2002) that *Cyp6g1* is involved in DDT resistance in field-collected strain. The observed difference between two studies may be a result of genetic difference between the field-selected and laboratory-selected strains. A long-term DDT selection in laboratory, as it has been done to obtain the 91-R strain (Dapkus and Merrell, 1977; Dapkus, 1992), is expected to select genetic factors that are directly or indirectly involved in DDT resistance. If it is assumed that DDT resistance is a multifactorial trait, some of these selected factors may be involved in DDT metabolism and the others may act as modifiers. Since the selection pressure put on a laboratory strain for DDT resistance is more intense than the populations in the wild, laboratory-selected and field-selected resistant strains may select different number and/or types of genetic factors. This difference may also make the laboratory-selected strain such as 91-R more resistant than the field-selected strain. Since P450s are known to be involved in xenobiotic metabolism, selection of overexpressing alleles of *Cyp* genes in natural and laboratory population of *Drosophila* is quite possible.

In order to investigate the categories of genes overexpressed in resistant 91-R and RC-21 strains, microarray was performed on the 91-R, RC-21 and 91-C strains. Comparison of the transcriptome showed that several groups of genes such as *Cyp* genes, carbohydrate metabolism, defense and stress response genes etc are overexpressed in both the resistant strains compared to the 91-C strain (Table 2-7). A proteolytic gene, Jonah 65Aii was overexpressed in both resistant strains. This finding is consistent with

Ahmed et al. (1998) and Saleem et al. (1994) where they reported that increased proteolytic activities in DDT- resistant houseflies compared to susceptible ones. This increase in proteolytic activities may be necessary to meet the energy demands of the cell to cope with the xenobiotic stress, thereby balancing the protein synthesis and degradation. Intracellular proteases play a role in the protein biosynthesis as well as to change the conformation of the enzymes in response to stress (Jensen et al., 2006). Future studies involving the increased protease expression in relation to DDT resistance will help resolve the possible role of proteases in insecticide resistance.

There are several *Cyp* genes that are commonly overexpressed in the 91-R and RC-21 strain. One of the *Cyp* genes, *Cyp4p1* (25 fold in 91-R and 23-fold in RC-21) showed the highest *Cyp* expression. However, it is not known whether *Cyp4p1* has any implication in conferring resistance in *Drosophila*. Overexpression of *Cyp4* family of genes was previously implicated in resistance to methyl parathion and carbaryl in the Nebraska western corn rootworm (Scharf et al., 2001). Additionally, *Cyp6a2* and *Cyp6w1* also showed high and similar expression in the 91-R and RC-21 strains. As described earlier, heterologous expression of *Cyp6a2* in *E.coli* showed metabolism of DDT (Amichot et al., 2004). Pedra et al (2004) reported that the expression of *Cyp6g1, Cyp12d1* and *Cyp6a2* is high in their microarray experiments suggesting their role in DDT resistance. However, in the 91-R strain, the expression of *Cyp12d1* is very low and in the RC-21 strain, the level of *Cyp6g1* is low. These two strains are highly resistant to DDT and it is noteworthy that *Cyp6a2* is overexpressed in both the resistant strains (Table 2-7). Hence, further investigation of *Cyp6a2* by germline transformation will be required to test its role in resistance. Taken together, microarray results suggest that

multiple P450s are overexpressed in resistant strains that may contribute to the DDT resistance phenotype.

However, it is not understood why high *Cyp6g1* expression is not associated with DDT resistance in laboratory and some field-collected strains discussed above. If CYP6G1 is the only CYP that metabolizes DDT, it should be a required factor in any resistant strain no matter whether they are field- or laboratory-selected population. Only explanation one may come up with is that in *Drosophila* DDT can be metabolized by more than one factor, and the lab- and field-selected strains may select these factors differentially. In that case, DDT resistance in lab- and field-selected strains may be conferred by different combinations of genetic factors and the level of resistance may be a function of number and type of factors a strain accumulates during the selection process. We believe that DDT resistance in *Drosophila* is a complex phenomenon and may be a polygenic trait, in keeping with almost 50 years of literature on the topic (Crow, 1957; King and Somme, 1958; Dapkus and Merrell, 1977, Dapkus, 1992). It is known that one P450 enzyme can metabolize more than one xenobiotic compound and multiple P450s can metabolize the same xenobiotic compound (Vickers et al., 1999; Nebert and Russel, 2002). If the latter phenomenon is operating in *Drosophila*, resistance to DDT may be a quantitative trait and the level of resistance to DDT (i.e., LC_{50}) in a given strain is expected to be directly proportional to the combined activities of all factors involved in DDT metabolism. We conclude that *Cyp6g1* may be one of these factors involved in DDT resistance phenotype in some fly strains and its overexpression is not sufficient to confer resistance. The molecular basis of DDT resistance remains to be determined.

Chapter III

Overexpression of *Cyp6a2* and *Cyp6g1* genes in DDT susceptible strain of *Drosophila melanogaster*

Introduction

Insecticide resistance phenotype allows insects to survive high doses of insecticides. Every year a large number of different types of insecticides are manufactured and applied in agricultural fields to kill insects. Although many insecticides are highly effective as insecticides, routine application of these chemicals has adverse effects also. Firstly, many insecticides are hazardous for other living organisms including humans; some of them are neurotoxins and some are potential carcinogens. Secondly, repeated use of insecticides actually helps select the resistance genes present in the population of an insect species, which eventually leads to the evolution of a resistant strain (Scott, 2001; Wilson, 2001). There are three mechanisms of insecticide resistance: target site insensitivity, increased metabolism and reduced penetration through the cuticle. Target site insensitivity is the modification of the affinity of the insecticide to its receptor site or a mutation in the target molecule that decreases binding of the toxin. For example, the *kdr* (knock down resistance) mutation in *Musca domestica* attributes resistance to pyrethroids by mutation of the gene encoding voltage-sensitive sodium channel (Amichot et al., 1992). Another example is the resistance to cyclodiene insecticides due to the mutation in the 'resistance to dieldrin' (*Rdl*) gene in *Drosophila melanogaster*. Reduced penetration through the cuticle is another mechanism of

resistance where the insect's exoskeleton is modified to inhibit insecticide penetration. In *Musca domestica*, resistance to DDT and dieldrin is conferred through this mechanism. Later, it was shown that this mechanism is controlled by gene named *pen* on chromosome III in *Musca* (Farnham, 1973). Of these three mechanisms, increased metabolism is the major mechanism conferring insecticide resistance, which involves glutathione S transferase and cytochrome P450 monooxygenases or CYPs. In many insect species, the resistant strains are rendered susceptible if they are treated with P450-specific inhibitor, piperonyl butoxide (Berge *et al.*, 1998)**,** suggesting that CYPs play a major role in resistance phenotype.

One of the problems to understand CYP-mediated insecticide resistance is the fact that CYPs belong to multigene family and multiple *CYP* genes are found in a given insect. Attempts have been made in *Drosophila* and *Musca domestica* to genetically map the insecticide resistance locus. In *Drosophila*, a resistance locus was mapped close to ~64-67 cM on the right arm of chromosome 2 (Tsukamoto and Ogaki, 1953; Tsukamoto, 1958). In 91-R strain, Dapkus and Merrell (1992) showed that DDT resistance is monofactorial and mapped at 56.1 ± 1 m.u. on the right arm of chromosome 2. Increased P450 content and activity were used as markers by several investigators to map the resistance loci. These studies showed that the insecticide resistance is associated with multiple loci on 2nd and 3rd chromosome suggesting that resistance is a polygenic trait (Hallstrom, 1985; Houpt et al., 1988; Waters and Nix, 1988; Crow, 1957). Based on these studies, it can be concluded that the mechanism of DDT resistance in *Drosophila* is unresolved.

Recent study by Daborn et al (2002) showed that overexpression of *Cyp6g1* alone is sufficient to confer DDT resistance in *Drosophila melanogaster*. Using transgenic technology, they overexpressed *Cyp6g1* in a susceptible strain and showed that it confers resistance to only 10 μg DDT. However, the levels of resistance observed with the 91-R and *Wisconsin* strains are much higher than what Daborn et al (2002) observed with the transgenic flies. It is possible that there are factors other than CYP6G1, which are needed for a full-blown resistance. These factors could be other *CYP* genes or unknown factors. To date, no *Drosophila* CYP has been identified that can metabolize DDT or insecticides *in vivo*. However, CYP6A2 expressed in lepidopteran cells (Sf21) could metabolize aldrin, dieldrin and diazinon. Recently, Amichot et al (2004) isolated an allele of *Cyp6a2* of *Drosophila* named *Cyp6a2SVL*, which metabolized DDT when expressed in bacteria. These studies suggest that CYP6A2 may play an important role in conferring insecticide resistance in *Drosophila melanogaster*.

Microarray analysis described in Chapter 2 showed that the expression of four 2R chromosome-linked genes, *Cyp6a2*, *Cyp6w1*, *Cyp6g1* and *Cyp6a8* is much higher in the resistant 91-R than in the susceptible 91-C strain. The *Cyp6a2* and *Cyp6w1* also show much higher expression in the resistant second chromosome stocks than in the susceptible 91-C strain (Chapter II). If there is a correlation between *Cyp* gene overexpression and DDT resistance, one of these genes showing overexpression may be involved in DDT resistance. Although data presented in Chapter 2 showed a lack of correlation between *Cyp6g1* expression and DDT resistance in the laboratory strains, *Cyp6g1* may still be one of the players in conferring resistance phenotype because it gave a low level of resistance in transgenic flies (Daborn et al., 2002). In addition, *Cyp6g1* maps close to the major

resistance locus at 65 cM. *Cyp6a2* may be another gene involved in DDT resistance. Firstly, it shows high expression in the 91-R and 2nd chromosome recombinant strain described in Chapter 2, and secondly, it is located close to the resistance locus in the 91-R strain mapped by Dapkus (1992). In view of these, in the present investigation, we used transgenic technology to introduce and overexpress both *Cyp6a2* and *Cyp6g1* genes singly or together in a susceptible strain by using the GAL4/UAS system. The results obtained were surprising; only 2.7-fold increase in LD_{50} was conferred by each *Cyp6* gene. However, overexpression of both *Cyp6* genes showed cumulative effect with further increase in LD_{50} value.

Materials and methods

Drosophila stocks

Two strains of *Drosophila melanogaster*- *yw* and w^{1118} were used as host strains for transformation studies. The second and third chromosome balancer stocks used for chromosome linkage analysis respectively are *w; Bl/CyO; +/+* and *yw; +/+; Ly/TM6C, Sb, Tb*. For further genetic manipulation of the transgenic lines, a stock carrying both balanced second and third chromosomes, *w/w; SM6, Cy/Sco; MKRS, Sb/TM6, Tb*, was used. All these balancer stocks were obtained from Bloomington Stock Center, Bloomington, IN. A stock homozygous for GAL4 driver under the control of fat body (fb) enhancer (*y w; +/+; fb-GAL4/fb-GAL4*) was obtained from Dr. Jae H. Park, University of Tennessee. This stock was used to overexpress the CYP6A2 and CYP6G1 cDNA in the transgenic lines. All stocks and transformant lines were maintained on standard corn meal-agar-molasses medium at 25^{0}C under 12 h dark and 12 h light cycle.

Total RNA and poly(A)$^{+}$ RNA isolation

Total RNA was isolated from a group of 40-50 adult female flies of the DDT resistant 91-R strain using TRI® Reagent (Sigma, St. Louis, MO) and following manufacturer's protocol. Briefly, flies were homogenized in 1 ml of TRI reagent and centrifuged at 12,000 x g for 10 min at 4^{0}C to remove the insoluble debris. To the clear supernatant, 200 µl of chloroform was added and vortexed for 15 sec. This mixture was centrifuged at 12,000 x g for 15 min and the aqueous phase on the top was transferred into a fresh eppendorf tube. In order to precipitate RNA, isopropanol at RT was added to the aqueous phase and incubated at room temperature for 15 min. The mixture was centrifuged at 12,000 x g at 4^{0}C for 10 min. The resulting RNA pellet was washed with 70% ethanol, dried and dissolved in appropriate volume of RNAse free DEPC- treated water. The quantity and concentration of the total RNA was measured using UV-spectrophotometry.

The isolated total RNA was then fractionated through oligo(dT) cellulose chromatography to obtain poly(A)$^{+}$ RNA. Briefly, the total RNA was reconstituted in buffer A (0.5 M NaCl, 0.01M Tris, pH 7.5, 1mM EDTA, 0.1% SDS), denatured at 65°C for 10 min, cooled to room temperature and fractionated on oligo (dT) column which was equilibrated in buffer A. The column was washed with 5 volumes each buffer A and buffer B (same as buffer A without the SDS) to remove non-poly (A) RNAs. The bound poly(A)$^{+}$ RNA was eluted with 10mM Tris, pH 7.5, mixed with 0.1 vol of 2M NaOAc, pH 7.5 and two volumes of chilled 95% ethanol. After keeping at -20°C overnight RNA

was pelleted by centrifuging at 12,000 x g for 10 min. The pelleted RNA was washed with 70% ethanol, dried and dissolved in DEPC treated water.

Synthesis of CYP6A2 and CYP6G1 cDNAs and cloning into transformation vector

The strategy for the synthesis of CYP6A2 and CYP6G1 cDNAs and cloning of these cDNAs into transformation vector pUAST is shown in Fig 3-1. The poly$(A)^+$ RNA isolated from the 91-R strain was used for the synthesis of first strand cDNA. Briefly, 300 ng of poly $(A)^+$ RNA, 1 µl of oligo $(dT)_{12-18}$ (0.5 µg/µl) and 1µl of 10mM dNTP mix were added in an eppendorf tube and incubated at 65^0C for 5 min to denature secondary structures. To this mixture, 2 µl of 10X RT buffer, 4 µl of 25mM $MgCl_2$ (5mM final concentration), 2 µl of 0.1M DTT (0.01M final concentration), 1 µl of RNAseOUT RNAse inhibitor, 1 µl of Super Script II RT enzyme were added to a final volume of 20 µl. The reaction mixture was incubated at 42^0C for 50 min and terminated by incubating the reaction mixture at 70^0C for 15 min. The RNA present in the RNA: DNA hybrids were digested by incubating the reaction mixture with RNAse H enzyme at 37^0C for 20 min. The resulting first strand cDNA (0.1 vol) was PCR amplified using gene specific primers for *Cyp6g1* and *Cyp6a2* to obtain the specific cDNAs. High fidelity Platinum Taq polymerase was used to avoid errors during amplification. The sequences of these primers, restriction enzymes added to each of the primers are shown in Table 3-1. The conditions used for each PCR cycle to amplify CYP6G1 and CYP6A2 cDNAs were as follows: 94^0C for 2 min, 60^0C for 45 sec and 72^0C for 2 min. After 34 cycles, the PCR products were purified by using Qiaquick PCR purification kit (Qiagen) and analyzed on

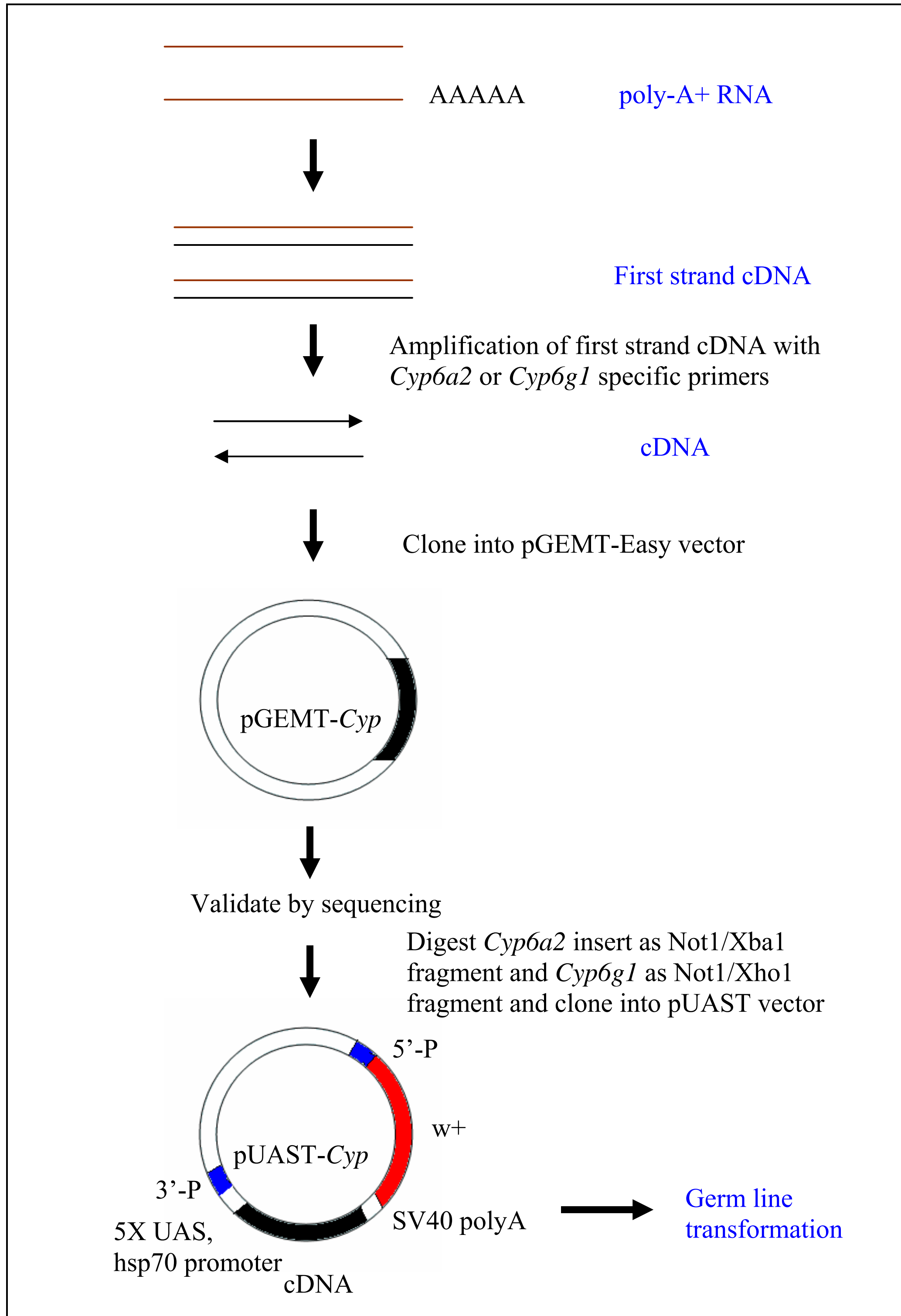


Figure 3-1: Schematic representation of the steps involved in the synthesis of cDNA and its cloning into transformation vector.

Table 3-1

Primers used for the amplification of *Cyp6g1* and *Cyp6a2* cDNA.

Primer name	Restriction enzyme site added	Region amplified	Sequence of the primer
1. G1-DNAR	Sal1	+6/+1944	5'-ccggtcgacCTAGGCGCCGCTTCTAACAC-3' (+1926/+1944)
2. G1-UTR	Not1		5'-ggcgcggccgcAAGTGCGGGTGCGTAGAGC-3' (+6/+23)
3. A2-1F	Not1	+1/+1602	5'-cgggcggccgcCGAAAAGGGAGCCAGCTACGC-3' (+1/+20)
4. A2-1578R	Xba1		5'-cggtctagaGTACATCACTTTAGCTTTGGATCC-3' (+1578/+1602)

Extra bases added at the 5' end are shown in lower case and the restriction sites added to the primers are underlined. The gene regions spanned by the primers are shown in parentheses. Primers 1 and 2 were used to amplify CYP6G1 cDNA, whereas primers 3 and 4 were used for CYP6A2 cDNA amplification.

1% agarose gel and characterized by digesting with restriction enzymes. The PCR products were ligated into pGEMT-Easy vector and incubated overnight at 4^0C. (Promega Inc). The ligated DNA was used to transform competent DH5α strain of *E. coli* bacteria (Invitrogen). The plasmids were screened for the presence of the cDNA insert by using appropriate restriction enzymes. Clones turned out to be positive were sequenced on both strands using ABI Prism 3100 analyzer at the Molecular biology resource facility, University of Tennessee, Knoxville. The sequences obtained were compiled by using Sequencher program and analyzed by BLAST program. The cDNA sequences were conceptually translated using Translate program at EBI and compared with the conceptual amino acid sequences of the CYP6G1 and CYP6A2 polypeptides available in the database.

Cloning of CYP6G1 and CYP6A2 cDNAs into pUAST vector for germ line transformation

For germ line transformation CYP6G1 and CYP6A2 cDNAs were cloned into pUAST plasmid vector (Fig. 3-1). In this vector, cDNA is cloned downstream from 5X sequence of yeast upstream activator sequence (UAS), which is the binding site for the GAL4 transcription factor of yeast. In addition, the vector also has Hsp70 minimal promoter to drive the cDNA and SV40 poly (A) signal (Fig. 3-1). The plasmid also has a mini-*white*$^+$ gene as a dominant selectable marker for germ line transformation. All these elements are flanked by P elements for transposition into the genome. To clone, CYP6G1 cDNA was excised from the pGEMT vector by cutting with *Not*I and *Sal*I, whereas

CYP6A2 cDNA was isolated by cutting with *Not*I and *Xba*I. The excised CYP6G1 and CYP6A2 cDNA fragments were then ligated with *Not*I/*Sal*I and *Not*I/*Xba*I cut pUAST vector, respectively (Fig. 3-1). The resulting recombinant plasmids were purified using plasmid midi kit (Qiagen) and quantified by flourimetry.

P-element mediated transformation

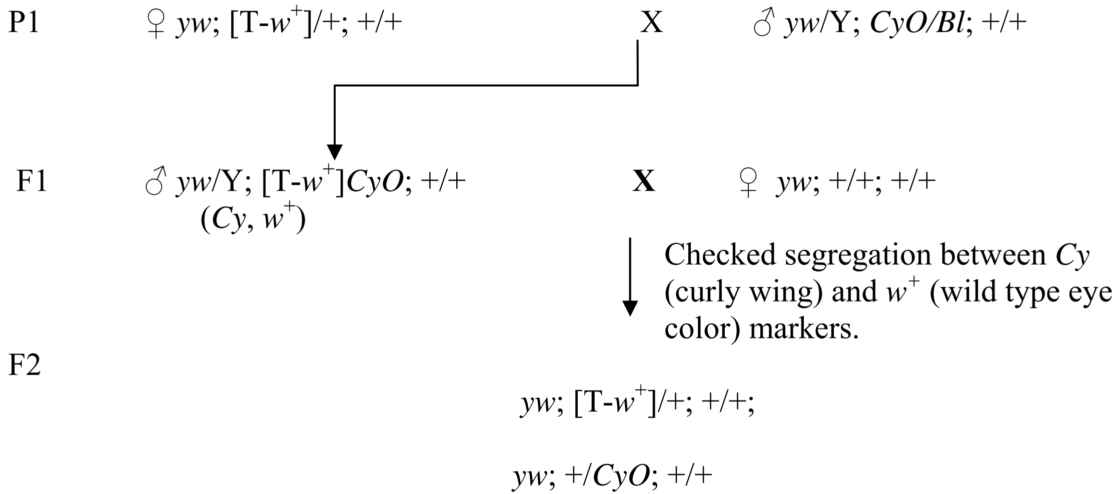
Germ line transformation of w^{1118} strain with the pUAST-*Cyp6a2* plasmid carrying CYP6A2 cDNA was done at the microinjection facility at Duke University. The microinjected G_0 larvae were received from this facility, which I raised, characterized and made homozygous for the transgene in our laboratory as described below. Transformation with the pUAST-*Cyp6g1* plasmid was done in our laboratory. The plasmid was mixed with pπ25.7wc helper plasmid in proportion 3:1 in 10X injection buffer (1mM NaH_2PO_4, pH 6.8 and 50 mM KCl) (Karess and Rubin, 1984). The DNA mixture was then injected into preblastoderm embryos of the *yw* strain. The helper plasmid has a defective P-element and cannot transpose into the genome. However, it has an active transposase gene that synthesizes transposase required for the transposition of the CYP6G1 cDNA from the pUAST plasmid to any chromosome (Sprandling and Rubin, 1982; Karess and Rubin, 1984; Karess, 1985). In the pUAST vector CYP6G1 cDNA is cloned between two P elements that allow transposition. In both transformation experiments, G_0 flies were mated with the opposite sex of the white-eyed host strain (w^{1118} or *yw*) and G1 progeny were screened for the presence of wild type (w^+) eye color because the pUAST vector carries a w^+ minigene as a dominant selectable marker. Since

the expression of the w^+ minigene is affected by the chromosomal context, the eye color of the transgenic flies ranges between orange and typical red. The G1 progeny with these eye colors, considered be wild type, were backcrossed singly to the host strain *yw* flies to synthesize individual transformant lines. At every generation afterwards, males and females with wild type colors were chosen to propagate the line until each one was made homozygous for the transgene.

Chromosomal localization of the transgene

Chromosomal linkage of the transgene in each transgenic line was determined by genetic crosses and the cytological localization was determined by inverse PCR. To determine the linkage to the 2nd or 3rd chromosome genetically, each line was crossed with the white-eyed second (*w*; *CyO/Bl*; +/+) or third (yw; +/+; Ly/TM6C) chromosome balancer stocks carrying *Cy* (curly wing) or *Sb* (stubble bristle) as dominant visible markers (Fig 3-2). The F1 red-eyed (w^+) virgins were crossed with the males of the white-eyed host strain (w^{1118} or *y w*) and segregation of the *Cy* or *Sb* with respect to the w^+ allele was examined in the F2 progeny. Segregation of w^+ away from the dominant marker means that the transgene and the dominant marker belong to the same linkage group (Fig 3-2). If all w^+ flies in F2 are straight-winged (Cy^+) or non-*Cy*, it would mean that the transgene is linked to the 2nd chromosome and segregates away from the *Cy* marker on the 2nd chromosome. Similarly, if the transgene is linked to 3rd chromosome, it will segregate away from the 3rd chromosome-linked *Sb* marker and all F2 w^+ flies will be non-*Sb* or Sb^+ (Fig 3-2). In order to identify the X-linkage of the transgene, the red-

If the transgene is linked to the second chromosome,



If the transgene is linked to the third chromosome,

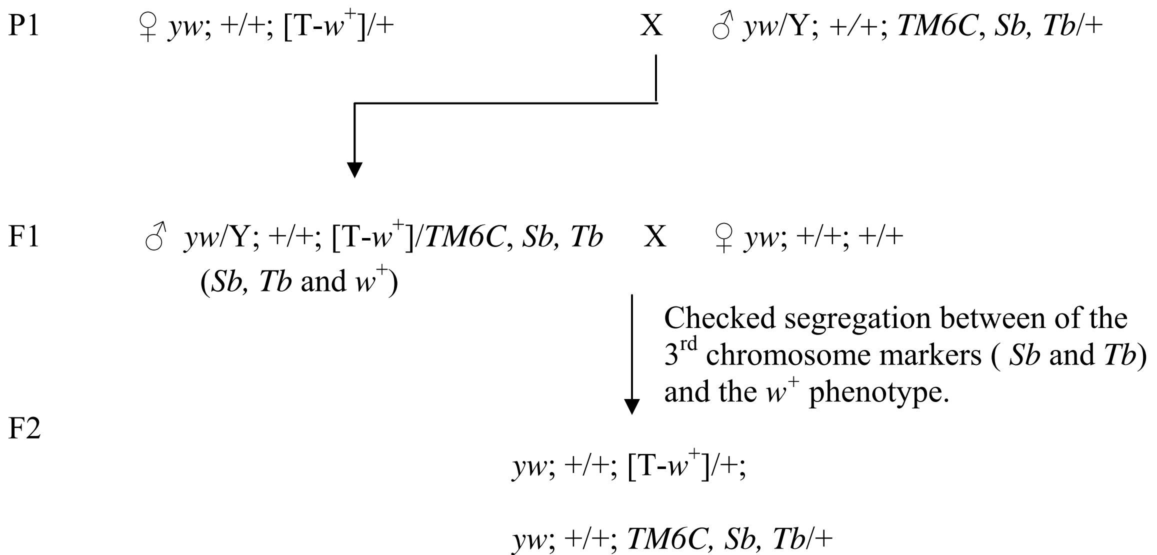


Figure 3-2: Genetic cross employed to determine the chromosome linkage of the transgenes. The transgenic lines were crossed independently with the 2nd or 3rd chromosome balancer stocks and segregation of the visible markers was observed in the F2 progeny. The chromosomes are written in the order of X; 2; 3. T-w^+= *Cyp6a2* or *Cyp6g1* transgene with w^+

eyed transgenic males will be crossed with *yw* virgin females. In the next generation, the transgene will segregate away from the males and only the females will have the w^+ eye color. No X-linked transgene was observed in the present study. The cytological position of the transgene insertion was determined by Inverse PCR method (Fig. 3-3). Genomic DNA was isolated from 30 unsexed flies using the method available at http://www.fruitfly.org/about/methods/inverse.pcr.html, a web page of Berkeley Drosophila Genome Project (BDGP). Briefly, flies were homogenized in buffer A containing 100 mM Tris-HCl, pH 7.5, 100 mM EDTA, 100 mM NaCl and 0.5% SDS. The homogenate was incubated at 65^0C for 30 minutes and then, 800µl of LiCl/KAc (1 part 5M KAc stock: 2.5 parts 6M LiCl stock) solution was added. The mixture was incubated on ice for 10 minutes and then it was centrifuged for 15 min at room temperature at 12,000 x g. The supernatant was transferred to a fresh tube and the DNA present in the supernatant was precipitated using 600µl of isopropanol at room temperature. The mixture was centrifuged for 15 min at room temperature to pellet the DNA. The resultant DNA pellet was washed with 70% ethanol, dried and dissolved in 150µl TE. The isolated genomic DNA from each transformant line was digested individually with *Sau3*A1, *Hin*P1 or *Msp*1 for 3 hr at 37^0C and ligated overnight at 4^0C. The ligation reaction was set up in large volumes (400 µl) in order to facilitate intra molecular ligation. The ligated products were precipitated and PCR was run on the samples using Pry2/Pry1 or Pry4/Pry1 primers sets (Table 3-2) that are specific for the P element bordering the transgene. The conditions used for each PCR cycle with Pry2/Pry1 primers were as follows: 3 min at 94^0C, 45 sec at 55^0 C and 2 min at 68^0 C. On the other hand, for Pry4/Pry1 primer each cycle had the following incubation regimens: 3 min at

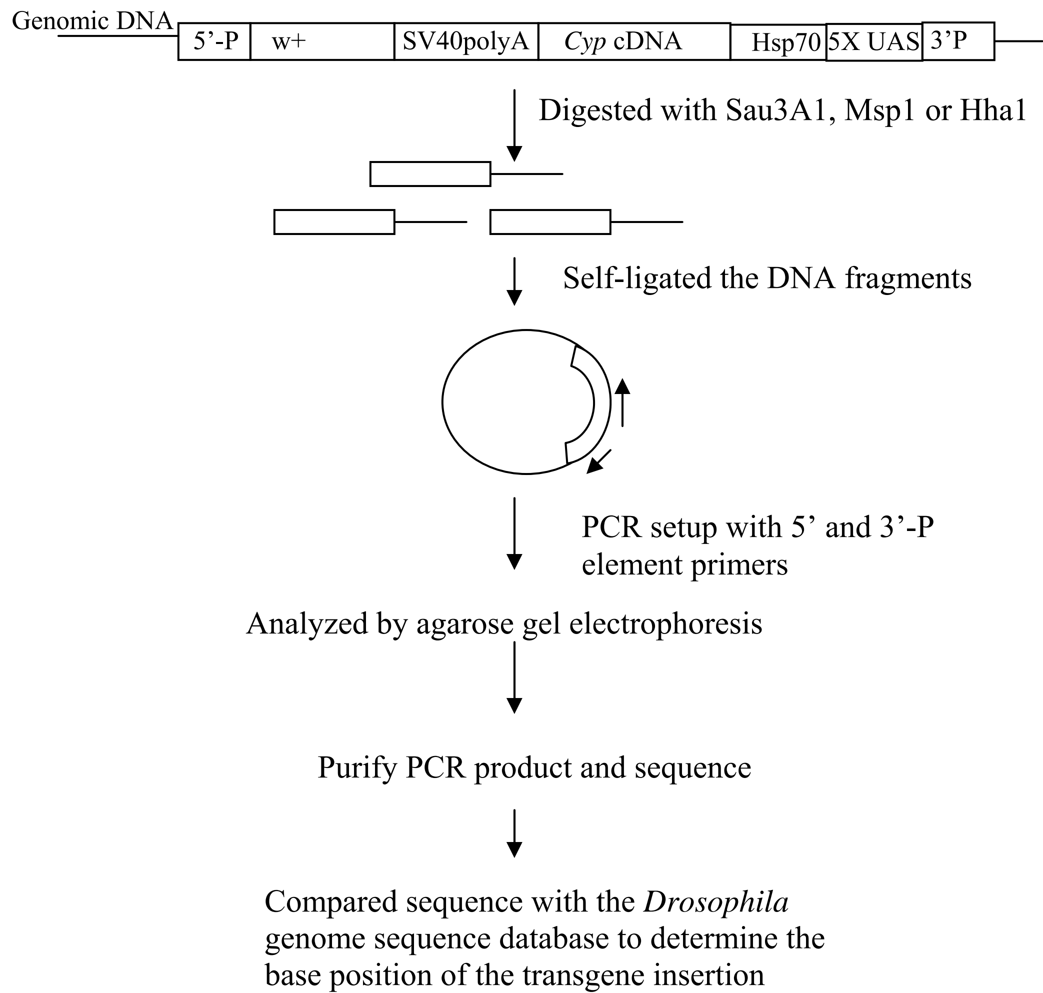


Figure 3-3: Schematic representation of the strategy of Inverse PCR.

Table 3-2

Primers used to determine the cytological position of the transgene insertion by Inverse PCR

Primer name	Primer sequence	Melting temperature T_m, ^0C
5'-P element		
For1	5'-gcacgtttgcttgttgagag-3'	57.4
Rev1	5'-ctcccaaatttgtgataccc-3'	55.1
3'-P element		
Pry1	5'-ccttagcatgtccgtggggtttga-3'	73.2
Pry2	5'-gatgtctcttgccgacgggaccac-3'	75.4
Pry4	5'-caatcatatcgctgtctcactcag-3'	64.7

The genomic DNA flanking the 5' side of the transgene was amplified by using For1 and Rev1 primer pairs. The Pry1 and Pry2 primers are used to amplify the 3'-end of the transgene.

94^0C, 45 sec at 60^0C and 2 min at 72^0C for 2 min. PCR with both sets of primers was run for 34 cycles. The length and number of PCR products produced by each ligated DNA sample was examined by running an aliquot of the PCR reaction on an agarose gel. The PCR products were then sequenced and the sequences were compared with the *Drosophila* genome sequence database using BLAST program to identify the cytological position of the transgene insertion.

Real time Quantitative PCR

For real time PCR, two total RNA samples, each from a group of 40-50 females, were isolated using TRI reagent (Sigma). Each total RNA sample was used for the synthesis of first strand cDNA as described above. The synthesized first strand cDNA was serially diluted five-fold to obtain three different dilutions (25, 5 and 1) where 25 is the arbitrary number given to undiluted sample followed by the two serial dilutions. PCR reactions were setup for each dilution with a total of six reactions for each sample. The diluted first strand cDNA samples were amplified with *Cyp6a2*, *Cyp6g1* and *RP49* gene-specific primers, which were synthesized using Primer Express software (Applied Biosystems). RP49 RNA was used as an internal control to normalize the data. The primer sequences and reactions conditions are given in Table 3-3. The product accumulated during the PCR cycles was detected using SYBR Green I dye and quantified using ABI 7000 sequence detection system from Applied Biosystems. Since SYBR Green dye will detect all double-stranded DNA, including non-specific reaction products, a well-optimized reaction is essential for accurate results. The design of gene specific

Table 3-3

Primers used to determine the expression of *Cyp6a2* and *Cyp6g1* in transgenic lines

Primer name	Primer Sequence	Melting temperature	Amplicon length
Cyp6g1-F	5'-CCTGAAGCCGTTCTACGACTACA-3' (+1381/+1400)	64.6	100 bp
Cyp6g1-R	5'-GCTGGGATTGGTCCAGTACTTT-3' (+1458/+1480)	62.7	
Cyp6a2-F	5'-CGACAGAGATCCCACTGAAGTATAGT-3' (+1458/+1484)	64.6	85 bp
Cyp6a2-R	5'-TGCGTTCCACTCGCAAGTAG-3' (+1520/+1541)	62.4	
RP49-F	5’-GCGCACCAAGCACTTCATC- 3’ (+405/+423)	60.0	155 bp
RP49-R	5’-GACGCACTCTGTTGTCGATACC-3’ (+540/+560)	61.0	

The regions of the genes spanned by the primers are shown in parentheses. The primers were designed using Primer Express software (Applied Biosystems)

primers and optimization of the reaction conditions was performed at the UT Genomics Hub, University of Tennessee at Knoxville. After real-time PCR, Ct values for the target and the reference gene were extracted with auto baseline and manual threshold. The data was validated by statistical analyses using SAS software (SAS institute, Cary, NC).

DDT resistance bioassay

For the DDT resistance assay, different concentrations of DDT solutions were made in acetone. Different volumes of DDT solutions were added to individual glass scintillation vials to coat the vials with specific amounts of DDT. The vials were rolled continuously until the acetone evaporated. Mature female flies (5-10 days old), collected in groups of 20, were sorted into vials containing regular *Drosophila* medium and left overnight at room temperature for the flies to recover from ether shock. Next day, these flies were directly transferred to the DDT-coated vials without etherization. The vials were sealed with cotton plugs soaked in 5% sucrose. Mortality was recorded after 24h exposure. Vials coated with acetone only were used as the controls. The data were analyzed using probit analysis in SAS (SAS Institute, 2000).

Results

Characterization of the CYP6 cDNAs, linkage analysis and the chromosomal locations of the transgenes

Before cloning into pUAST vector for germ line transformation, first the CYP6A2 and CYP6G1 cDNAs were sequenced and compared with the *Cyp6a2* and *Cyp6g1* sequences available at the database. The results showed that the nucleotide sequence of both cDNAs matched 99% with their respective gene sequences available at the database (Fig 3-4, 3-5). However, conceptual translation of the cDNA sequences with Translate program showed that both cDNAs are 100% identical to the amino acid sequence of their respective polypeptide sequence available at the database. Therefore, CYP6A2 and CYP6G1 cDNAs were excised from the pGEMT-Easy vector as a *Not*1/*Xba*I and *Not*1/*Sal*1 fragments, respectively. These fragments were then cloned respectively into *Not*1/*Xba*I and *Not*1/*Sal*1 cut pUAST transformation vector. The recombinant plasmids were purified and used for germ line transformation. Table 3-4 shows the results of the transformation experiments. While five transformed lines were obtained for *Cyp6a2*, *Cyp6g1* produced only two lines. However, three out of five *Cyp6a2* transgenic lines did not survive and could not be analyzed further. Chromosomal linkage and cytological positions of the transgenes in two transformed lines of each *Cyp* gene were determined by genetic crosses and inverse PCR as described in the Methods section. For each *Cyp* gene, the transgene was linked to the 2nd chromosome in one line and to the 3rd chromosome in the other line (Table 3-5).

```
Query  1     ATGTTTGTTCTAATATACCTGTTGATCGCGATCTCCTCGCTTTTGGCCTACTTGTACCAC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24    ATGTTTGTTCTAATATACCTGTTGATCGCGATCTCCTCGCTTTTGGCCTACTTGTACCAC  83

Query  61    CGCAACTTCAACTACTGGAATCGCCGCGGCGTACCACACGATGCTCCTCACCCACTGTAT  120
             |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Sbjct  84    CGCAACTTCAACTACTGGAATCGCCGCGGCGTGCCACACGATGCTCCTCACCCACTGTAT  143

Query  121   GGCAACATGGTCGGGTTCCGGAAGAACCGGGTGATGCACGACTTCTTCTACGACTACTAC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  144   GGCAACATGGTCGGGTTCCGGAAGAACCGGGTGATGCACGACTTCTTCTACGACTACTAC  203

Query  181   AACAAGTACCGGAAGAGCGGCTTTCCCTTCGTAGGCTTTTACTTTCTGCACAAGCCGGCC  240
             |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||
Sbjct  204   AACAAGTACCGGAAGAGCGGCTTTCCCTTCGTGGGCTTTTACTTTCTGCACAAGCCGGCC  263

Query  241   GCCTTCATCGTGGACACCCAGCTGGCCAAGAACATCCTGATCAAGGATTTCTCGAACTTT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264   GCCTTCATCGTGGACACCCAGCTGGCCAAGAACATCCTGATCAAGGATTTCTCGAACTTT  323

Query  301   GCCGATCGTGGCCAGTTTCACAACGGGCGCGACGACCCGCTCACGCAGCACCTGTTCAAC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  324   GCCGATCGTGGCCAGTTTCACAACGGGCGCGACGACCCGCTCACGCAGCACCTGTTCAAC  383

Query  361   CTGGACGGAAAGAAGTGGAAGGACATGCGCCAGAGGCTGACGCCGACTTTCACCTCGGGC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  384   CTGGACGGAAAGAAGTGGAAGGACATGCGCCAGAGGCTGACGCCGACTTTCACCTCGGGC  443

Query  421   AAGATGAAGTTCATGTTCCCGACGGTGATCAAGGTGTCCGAGGAGTTCGTCAAGGTGATC  480
             |||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||
Sbjct  444   AAGATGAAGTTCATGTTCCCGACGGTGATCAAGGTGTCTGAGGAGTTCGTCAAGGTGATC  503

Query  481   ACGGAGCAGGTGCCCGCCGCCCAGAACGGCGCTGTGCTCGAGATCAAGGAGCTGATGGCC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  504   ACGGAGCAGGTGCCCGCCGCCCAGAACGGCGCTGTGCTCGAGATCAAGGAGCTGATGGCC  563

Query  541   AGGTTCACCACCGATGTGATTGGCACCTGTGCCTTCGGCATTGAGTGTAACACGCTGCGC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  564   AGGTTCACCACCGATGTGATTGGCACCTGTGCCTTCGGCATTGAGTGTAACACGCTGCGC  623

Query  601   ACCCCTGTCAGTGATTTCCGCACCATGGGACAGAAGGTGTTCACCGATATGCGCCACGGG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  624   ACCCCTGTCAGTGATTTCCGCACCATGGGACAGAAGGTGTTCACCGATATGCGCCACGGG  683

Query  661   AAACTGCTGACCATGTTCGTGTTCAGCTTTCCCAAGCTGGCCAGCAGGTTGAGAATGCGC  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  684   AAACTGCTGACCATGTTCGTGTTCAGCTTTCCCAAGCTGGCCAGCAGGTTGAGAATGCGC  743

Query  721   ATGATGCCCGAGGACGTCCACCAGTTCTTCATGCGCCTGGTCAACGACACGATTGCCCTC  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  744   ATGATGCCCGAGGACGTCCACCAGTTCTTCATGCGCCTGGTCAACGACACGATTGCCCTC  803

Query  781   AGGGAGCGGGAGAACTTCAAGAGGAACGACTTCATGAACCTGCTGATTGAACTGAAGCAG  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  804   AGGGAGCGGGAGAACTTCAAGAGGAACGACTTCATGAACCTGCTGATTGAACTGAAGCAG  863

Query  841   AAGGGGCGCGTCACCCTGGACAACGGAGAGGTGATCGAGGGCATGGACATCGGCGAACTG  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  864   AAGGGGCGCGTCACCCTGGACAACGGAGAGGTGATCGAGGGCATGGACATCGGCGAACTG  923
```

Figure 3-4: Nucleotide sequence comparison of the isolated *Cyp6a2* cDNA with the *Cyp6a2* sequence present in the database (Accession number: NM_078904). Query – Sequenced DNA; Subject- *Cyp6a2* sequence from database.

```
Query  901   GCCGCCCAGGTGTTCGTCTTTTATGTGGCCGGATTTGAGACCTCCTCCTCGACAATGAGT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  924   GCCGCCCAGGTGTTCGTCTTTTATGTGGCCGGATTTGAGACCTCCTCCTCGACAATGAGT  983

Query  961   TACTGCCTGTATGAGTTGGCTCAGAATCAGGACATTCAGGACAGGCTGCGCAACGAGATC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  984   TACTGCCTGTATGAGTTGGCTCAGAATCAGGACATTCAGGACAGGCTGCGCAACGAGATC  1043

Query  1021  CAAACGGTGCTGGAGGAACAGGAGGGGCAGCTAACGTACGAATCCATCAAGGCCATGACC  1080
             |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  1044  CAAACGGTGCTGGAGGAACAGGAGGGGCAGCTAACGTACGAATCCATCAAAGCCATGACC  1103

Query  1081  TACTTGAACCAGGTCATCTCAGAAACCCTGAGGCTCTACACACTGGTGCCCCACCTCGAA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1104  TACTTGAACCAGGTCATCTCAGAAACCCTGAGGCTCTACACACTGGTGCCCCACCTCGAA  1163

Query  1141  CGGAAGGCCCTCAACGACTACGTGGTGCCGGGCCATGAAAAGCTTGTGATTGAGAAGGGC  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1164  CGGAAGGCCCTCAACGACTACGTGGTGCCGGGCCATGAAAAGCTTGTGATTGAGAAGGGC  1223

Query  1201  ACACAGGTCATAATCCCCGCTTGCGCCTACCACCGCGACGAGGATCTTTATCCGAATCCG  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1224  ACACAGGTCATAATCCCCGCTTGCGCCTACCACCGCGACGAGGATCTTTATCCGAATCCG  1283

Query  1261  GAGACCTTTGATCCGGAGCGCTTCTCGCCGGAGAAAGTGGCCGCCCGGGAGTCCGTGGAG  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1284  GAGACCTTTGATCCGGAGCGCTTCTCGCCGGAGAAAGTGGCCGCCCGGGAGTCCGTGGAG  1343

Query  1321  TGGCTGCCCTTCGGCGACGGGCCGCGGAACTGCATCGGGATGCGGTTTGGACAAATGCAG  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1344  TGGCTGCCCTTCGGCGACGGGCCGCGGAACTGCATCGGGATGCGGTTTGGACAAATGCAG  1403

Query  1381  GCTCGCATCGGTTTGGCTCAGATCATCAGCCGGTTCAGGGTATCCGTCTGCGATACGACA  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1404  GCTCGCATCGGTTTGGCTCAGATCATCAGCCGGTTCAGGGTATCCGTCTGCGATACGACA  1463

Query  1441  GAGATCCCACTGAAGTATAGTCCCATGTCCATAGTTTTGGGCACCGTTGGGGGCATCTAC  1500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1464  GAGATCCCACTGAAGTATAGTCCCATGTCCATAGTTTTGGGCACCGTTGGGGGCATCTAC  1523

Query  1501  TTGCGAGTGGAACGCATCTA  1520
             ||||||||||||||||||||
Sbjct  1524  TTGCGAGTGGAACGCATCTA  1543
```

Figure 3-4- continued.

```
Query  9     GGTGCGTAGAGCTTTAATTGTCGGTTGTGTACGCGGGTGCTCAGAATTTATAGATCCAAT  68
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12    GGTGCGTAGAGCTTTAATTGTCGGTTGTGTACGCGGGTGCTCAGAATTTATAGATCCAAT  71

Query  69    AAAAGTTTCCTTGAAATTGCTGGACAAACTTGTTGCGAATTAGGCCAGTTGCAAATAAAT  128
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  72    AAAAGTTTCCTTGAAATTGCTGGACAAACTTGTTGCGAATTAGGCCAGTTGCAAATAAAT  131

Query  129   TGTGTGACTAAAAAAACCTGTATATTTTCAAAAGTGGCGATACCCATTACAACGACATCC  188
             ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  132   TGTGTGACTAAAAAAACCTGTATATTTTCAAAAGTGGCGATACCCATTACATCGACATCC  191

Query  189   CCAAAATGGTGTTGACCGAGGTCCTCTTTGTGGTGGTCGCCGCACTGGTGGCGCTCTACA  248
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  192   CCAAAATGGTGTTGACCGAGGTCCTCTTTGTGGTGGTCGCCGCACTGGTGGCGCTCTACA  251

Query  249   CTTGGTTCCAGCGCAACCATAGCTACTGGCAACGCAAGGGCATACCCTATATTCCGCCCA  308
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  252   CTTGGTTCCAGCGCAACCATAGCTACTGGCAACGCAAGGGCATACCCTATATTCCGCCCA  311

Query  309   CGCCGATCATTGGCAACACCAAGGTGGTCTTCAAGATGGAGAACTCCTTTGGGATGCATC  368
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  312   CGCCGATCATTGGCAACACCAAGGTGGTCTTCAAGATGGAGAACTCCTTTGGGATGCACC  371

Query  369   TATCGGAGATATACAATGATCCGCGGCTGAAGGACGAGGCTGTGGTGGGCATCTACTCCA  428
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  372   TATCGGAGATATACAATGATCCGCGGCTGAAGGACGAGGCTGTGGTGGGCATCTACTCCA  431

Query  429   TGAACAAGCCCGGCTTGATAATACGCGACATAGAGCTGATCAAATCCATTCTGATCAAGG  488
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  432   TGAACAAGCCCGGCTTGATAATACGCGACATAGAGCTGATCAAATCCATTCTGATCAAGG  491

Query  489   ACTTCAATCGGTTCCACAACCGATACGCCCGCTGCGATCCCCATGGCGATCCATTGGGCT  548
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  492   ACTTCAATCGGTTCCACAACCGATACGCCCGCTGCGATCCCCATGGCGATCCATTGGGCT  551

Query  549   ATAATAACCTGTTCTTCGTCAGGGATGCCCATTGGAAGGGAATTCGCACCAAGCTGACTC  608
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  552   ATAATAACCTGTTCTTCGTCAGGGATGCCCATTGGAAGGGAATTCGCACCAAGCTGACTC  611

Query  609   CCGTTTTCACCAGCGGCAAGGTCAAGCAGATGTACACCCTTATGCAGGAGATTGGAAAGG  668
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  612   CCGTTTTCACCAGCGGCAAGGTCAAGCAGATGTACACCCTTATGCAGGAGATTGGAAAGG  671

Query  669   ATCTGGAGCTGGCACTGCAGAGGCGTGGAGAGAAGAACTCTGGGAGTTTCATTACGGAGA  728
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  672   ATCTGGAGCTGGCACTGCAGAGGCGTGGAGAGAAGAACTCTGGGAGTTTCATTACGGAGA  731

Query  729   TTAAGGAGATCTGCGCTCAGTTCTCCACGGACAGCATAGCCACGATTGCATTTGGCATTC  788
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  732   TTAAGGAGATCTGCGCTCAGTTCTCCACGGACAGCATAGCCACGATTGCATTTGGCATTC  791

Query  789   GTGCTAACAGCCTAGAGAATCCCAACGCAGAGTTCCGTAACTACGGACGCAAGATGTTCA  848
             ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  792   GTGCTAACAGCCTAGAGAATCCCAACGCAGAGTTCCGTAACTATGGACGCAAGATGTTCA  851

Query  849   CCTTCACCGTAGCGCGTGCCAAGGACTTCTTTGTGGCCTTCTTCCTGCCCAAGCTGGTGT  908
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  852   CCTTCACCGTAGCGCGTGCCAAGGACTTCTTTGTGGCCTTCTTCCTGCCCAAGCTGGTGT  911
```

Figure 3-5: Nucleotide sequence comparison of the isolated *Cyp6g1* cDNA with the *Cyp6g1* sequence present in the database (Accession number: NM_136899.2). Query – Sequenced DNA; Subject- *Cyp6g1* sequence from database.

```
Query  909   CGCTGATGCGCATCCAGTTCTTCACGGCGGACTTTTCCCACTTTATGCGCAGCACCATTG  968
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  912   CGCTGATGCGCATCCAGTTCTTCACGGCGGACTTTTCCCACTTTATGCGCAGCACCATTG  971

Query  969   GTCACGTTATGGAGGAGCGAGAGCGATCGGGCCTGCTCCGCAATGATCTCATAGATGTCT  1028
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  972   GTCACGTTATGGAGGAGCGAGAGCGATCGGGCCTGCTCCGCAATGATCTCATAGATGTCT  1031

Query  1029  TGGTGAGTCTGCGCAAAGAGGCGGCTGCCGAGCCTTCGAAGCCTCACTATGCCAAGAACC  1088
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1032  TGGTGAGTCTGCGCAAAGAGGCGGCTGCCGAGCCTTCGAAGCCTCACTATGCCAAGAACC  1091

Query  1089  AGGACTTCCTGGTGGCTCAGGCGGGCGTGTTTTTTACGGCGGGTTTCGAGACCTCCTCCT  1148
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1092  AGGACTTCCTGGTGGCTCAGGCGGGCGTGTTTTTTACGGCGGGTTTCGAGACCTCCTCCT  1151

Query  1149  CGACCATGTCTTTTGCCCTGTACGAGATGGCTAAGCATCCAGAGATGCAGAAACGCCTGC  1208
             ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  1152  CGACCATGTCTTTTGCCCTGTACGAGATGGCCAAGCATCCAGAGATGCAGAAACGCCTGC  1211

Query  1209  GCGACGAGATCAACGAAGCTTTGGTGGAGGGCGGTGGGTCATTGAGCTACGAGAAGATCC  1268
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  1212  GCGACGAGATCAACGAAGCTTTGGTGGAGGGCGGTGGGTCATTGAGCTACGAGAAAATCC  1271

Query  1269  AGTCCCTGGAGTATCTGGCCATGGTGGTGGACGAGGTGCTGCGCATGTATCCGGTGCTGC  1328
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1272  AGTCCCTGGAGTATCTGGCCATGGTGGTGGACGAGGTGCTGCGCATGTATCCGGTGCTGC  1331

Query  1329  CGTTCCTGGACCGCGAGTACGAGAGCGTGGAGGGACAGCCAGACTTGAGCCTGAAGCCGT  1388
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1332  CGTTCCTGGACCGCGAGTACGAGAGCGTGGAGGGACAGCCAGACTTGAGCCTGAAGCCGT  1391

Query  1389  TCTACGACTACACTCTCGAGAACGGAACCCCTGTGTTCATACCCATCTATGCACTGCATC  1448
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1392  TCTACGACTACACTCTCGAGAACGGAACCCCTGTGTTCATACCCATCTATGCACTGCATC  1451

Query  1449  ATGATCCAAAGTACTGGACCAATCCCAGCCAATTCGATCCGGAGCGTTTCTCACCCGCGA  1508
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1452  ATGATCCAAAGTACTGGACCAATCCCAGCCAATTCGATCCGGAGCGTTTCTCACCCGCGA  1511

Query  1509  ACCGCAAGAACATAGTGGCCATGGCATATCAACCCTTCGGATCTGGGCCGCACAACTGCA  1568
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1512  ACCGCAAGAACATAGTGGCCATGGCATATCAACCCTTCGGATCTGGGCCGCACAACTGCA  1571

Query  1569  TTGGCAGCCGGATTGGCCTGCTACAGAGCAAACTGGGCCTGGTCAGCCTGCTGAAGAATC  1628
             ||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  1572  TTGGCAGCCGGATTGGCCTGCTACAGAGCAAACTGGGACTGGTCAGCCTGCTGAAGAATC  1631

Query  1629  ACTCAGTGCGCAACTGCGAGGCCACCATGAAGGACATGAAATTCGATCCCAAGGGTTTCG  1688
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1632  ACTCAGTGCGCAACTGCGAGGCCACCATGAAGGACATGAAATTCGATCCCAAGGGTTTCG  1691

Query  1689  TGCTCCAGGCAGATGGTGGCATACATTTGGAGATAGTCAACGATCGCCTCTACGATCAGA  1748
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1692  TGCTCCAGGCAGATGGTGGCATACATTTGGAGATAGTCAACGATCGCCTCTACGATCAGA  1751

Query  1749  GCGCTCCATCGCTCCAATGAATTTGAATCGCATGAACTGTGTGATCTGTATGGATACACA  1808
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1752  GCGCTCCATCGCTCCAATGAATTTGAATCGCATGAACTGTGTGATCTGTATGGATACACA  1811
```

Figure 3-5-continued.

```
Query  1809  TATGGATACATATATGTACATATATGGAATAAGTGATTAGTTGATGGGGTAATCGCCTTC  1868
             ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1812  TATGGATACATATATGTACATATATGGAATAAGTGATTAGTTGATGGGGCAATCGCCTTC  1871

Query  1869  TGACAGCTGGCATTTGCCTGACTTATGATGCAGATTATATAATGATAATCCGTGTGTTAG  1928
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1872  TGACAGCTGGCATTTGCCTGACTTATGATGCAGATTATATAATGATAATCCGTGTGTTAG  1931

Query  1929  AAGCGGCGCCTAG  1941
             |||||||||||||
Sbjct  1932  AAGCGGCGCCTAG  1944
```

Figure 3-5-continued.

Table 3-4

Statistics of microinjections performed with *Cyp6a2* and *Cyp6g1* cDNA clones.

Name of cDNA	# of injections	# of adults emerged	# sterile	# of transformants
Cyp6a2	110	50	7	5
Cyp6g1	900	75	20	2

Table 3-5

Characterization of *Cyp6g1* and *Cyp6a2* transgenes in *Drosophila melanogaster*

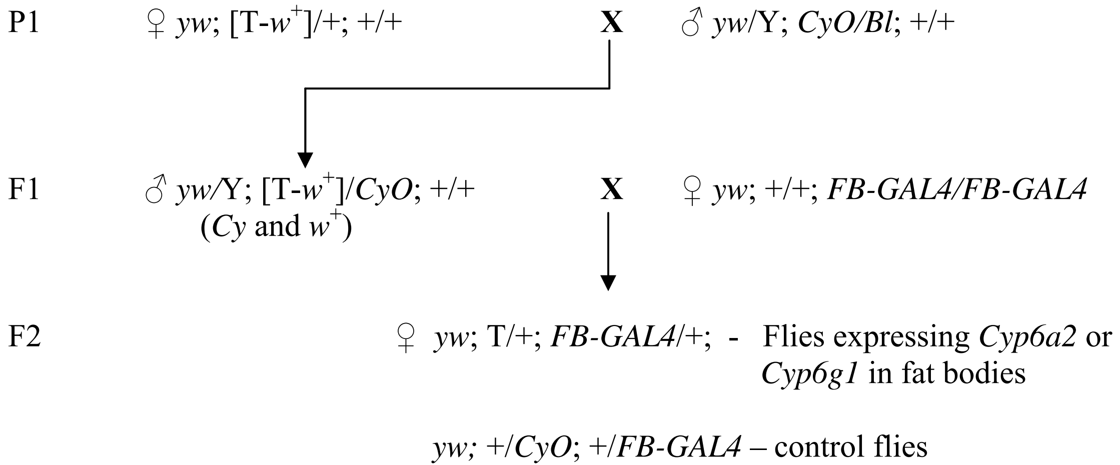
Transgenic lines	Linkage group	Cytological position	Base position in genome
UAS-6g1-1C	2R	52B3-B5	258047 position of AE003810.3 genome scaffold, in the intronic region of fus gene
UAS-6g1-2D	3	-	-
UAS-6a2-1C	2R	48B2	170666 position of AE003825.3 genome scaffold
UAS-6a2-4B	3R	93E5	138409 position of AE003735.2 genome scaffold

The chromosome linkage was determined by genetic crosses and the cytological position of insertion was mapped using Inverse PCR.

Analysis of CYP6A2 and CYP6G1 RNA levels in the transgenic lines

The CYP6A2 and CYP6G1 cDNAs in the UAS-CYP6A2 and UAS-CYP6G1 transgenic lines are under the control of 5X upstream activating sequences (UAS) of yeast that can be activated by GAL4 protein. Because GAL4 activator protein is not present in *Drosophila* these cDNAs are not expected to be transcribed in the transgenic flies. Therefore, to express the cDNAs, the transgene of each line and GAL4 cDNA under the control of fat body enhancer were brought in the same genome via genetic crosses. In these crosses, second and third chromosome balancer stocks carrying dominant visible markers were used to follow these chromosomes, and w^+ (red eye) phenotype was used to follow the CYP cDNA transgene. Since the transgenic lines were not made homozygous for the transgene, crossing strategy shown in Fig 3-2 was followed. Virgin females of UAS-6g1-1C and UAS-6a2-1C transgenic lines carrying transgene on the second chromosome (Table 3-5) were crossed to the males of *y w; CyO/Bl; +/+* second chromosomal balancer line (Fig 3-6). The F1 curly-winged and red or orange-eyed males (*y w; T/CyO; +/+*) were collected. The orange or red eyes indicate that these flies carry the cDNA transgene (*T*), which has the w^+ gene as a selectable marker. These F1 males were crossed with the virgin females of GAL4 driver stock (*y w; +/+; FB-GAL4/FB-GAL4*) in which GAL4 cDNA is linked to the 3rd chromosome and under the control of fat body enhancer. About 50% of these F2 flies had straight wings (*y w; T/+; FB-GAL4/+*) indicating that they had a copy of the CYP6 cDNA transgene. On the other hand the other 50% had curly wings meaning that they had no CYP6 cDNA transgene (*y w; +/CyO; FB-GAL4/+*). However, all F2 progeny were heterozygous for the third chromosome-linked *FB-GAL4* transgene. The curly-winged flies with no CYP

For 2nd chromosome linked transgenic lines



For 3rd chromosome linked transgenic lines

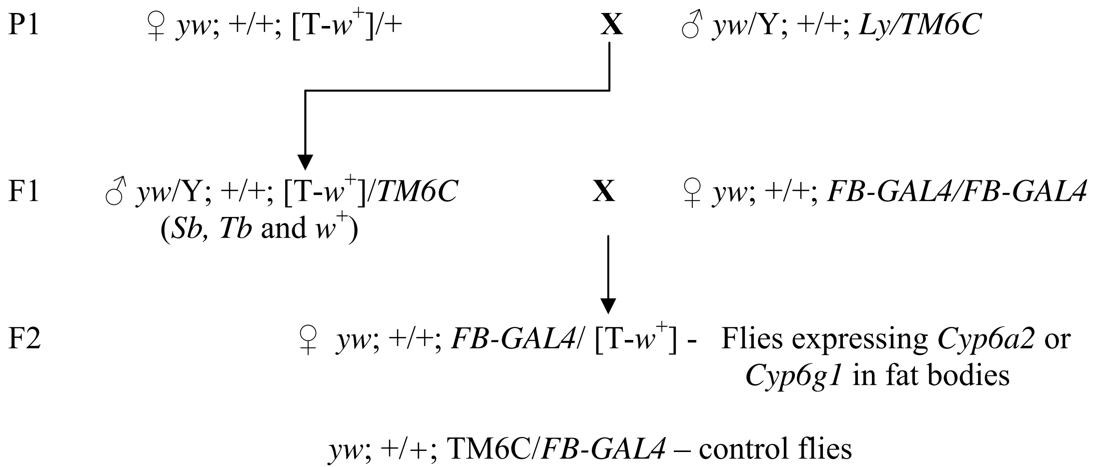


Figure 3-6: Genetic crosses of the transgenic lines with tissue specific GAL4 stocks to overexpress *Cyp6a2* or *Cyp6g1* transgene in fat bodies. Crosses were setup with the transgenic lines *Cyp6g1* or *Cyp6a2* transgene. In the F2 generation, female flies expressing *Cyp6a2* or *Cyp6g1* and the control female flies (GAL4 alone) were collected. (Symbol Key: [T-w^+] = transgene (*Cyp6a2* or *Cyp6g1*) with w^+ marker.

cDNA transgene were used as control for the flies with the transgene. For the transgenic lines with third chromosome-linked CYP cDNA (UAS-6g1-2D and UAS-6a2-4B), similar types of crosses were done except that the virgin females of the transgenic lines were crossed to the males of the *y w; +/+; Ly/TM6C, Sb Tb* third chromosome balancer stock (Fig 3-6). The third chromosome-linked dominant *Sb* (stubble bristles) and *Tb* (tubby body) markers were used to select F2 females carrying both the transgene and GAL4 driver (*y w; +/+; FB-GAL4/T*), or the GAL4 driver alone (*y w; +/+; TM6C, Sb Tb/FB-GAL4*). Flies carrying GAL4 driver alone were used as control. To examine the expression of CYP cDNA transgene, total RNA was isolated from the transgenic and control F2 females of the crosses shown in Fig 3-6 and CYP6G1 or CYP6A2 RNA was quantified by using quantitative real time PCR or qRT-PCR as detailed in Materials and Methods section. The C_t values of the control and transgenic RNA samples were obtained for comparison. The plot of cycle number versus the $\log_2$ based transformed fluorescent signal with each of the samples gives the linear range at which the log-fluorescent signal is linearly correlated with the original template amount. The Ct number is defined as the cycle number at the threshold level of the log-based fluorescence. The higher the Ct value, the lower is the expression of the gene and vice-versa. The difference of the cycle number between the target gene and the control gene is named as ΔCt. The difference between the ΔCt values of the test strain and the control strain is known as ΔΔCt. Since the cycle number was measured on a log2 scale, the fold difference was calculated using $2^{-\Delta\Delta Ct}$. The data for the mRNA expression of the transgenic lines is shown in Table 3-6. Between the two lines expressing *Cyp6a2,* we observed that the UAS-*6a2-1C* showed a two-fold overexpression whereas the UAS-*Cyp6a2-4B* showed a 1.4-fold overexpression.

Table 3-6

Analysis of transgene expression by Real-time PCR

Sample name	Gene amplified	Avg. ΔCt	Stdev	ΔΔCt	$2^{-\Delta\Delta Ct}$ Fold difference	p-value
1. UAS-6g1-1C	*Cyp6g1*	4.154	0.379	-0.74	1.7	0.01*
Control	*Cyp6g1*	4.893	0.283			
2. UAS-6g1-2D	*Cyp6g1*	4.997	0.368	-1.21	2.3	0.0005*
Control	*Cyp6g1*	6.204	0.368			
3. UAS-6a2-1C	*Cyp6a2*	2.71	0.446	-0.96	2	0.01*
Control	*Cyp6a2*	3.67	0.543			
4. UAS-6a2-4B	*Cyp6a2*	2.35	0.304	-0.36	1.4	0.2
Control	*Cyp6a2*	2.71	0.286			
5.UAS-6g1/Cyp6a2	*Cyp6g1,* *Cyp6a2*	5.99, 3.15	0.58, 0.44	-0.1, -1	1.1 2.7	0.3 0.0001*
Control	*Cyp6g1,* *Cyp6a2*	6.10, 4.14	0.27, 0.28	-4.4, -1.6		
6. 91-R	*Cyp6g1,* *Cyp6a2*	1.73, 2.54	0.42, 0.9		79 5	<0.0001* <0.0001*

* indicates that the fold difference is significant ($p<0.05$)

RP49 is used as an internal control and amplified along with the *Cyp* genes. Ct is the cycle number below the threshold value. ΔCt = Cyp Ct - RP49 Ct; ΔΔCt = ΔCt of the transgene expressing line – ΔCt of the control. Since the Ct value is in $\log_2$ scale, the fold difference was obtained by the calculation of $2^{-\Delta\Delta Ct}$. Statistical analyses to validate the results were performed in SAS (SAS institute, Cary, NC).

Statistical analysis revealed that the overexpression in UAS-*Cyp6a2-4B* is not significant (Table 3-6). In case of *Cyp6g1* transgenic lines, the UAS-6g1-1C showed a 1.7 fold overexpression compared to its control whereas UAS-6g1-2D showed a 2.3-fold overexpression. These fold differences are found to be statistically significant. Genetic crosses were also set up to place both CYP6G1 and CYP6A2 cDNA transgenes and the GAL4 driver in the same genome (Fig 3-7). When the levels of CYP6G1 and CYP6A2 mRNA in the doubly-transgenic female flies were measured, a 2.7-fold overexpression of the CYP6A2 RNA was found relative to the control flies. However, the level of CYP6G1 RNA was only 1.1- fold higher than the control. Although the expression of both CYP6 cDNA was not very high despite the fact they were under the control of 5X UAS, the F2 flies used above were examined for DDT resistance.

Investigation of the DDT resistance phenotype in the transgenic lines

To examine the role of *Cyp6a2* and *Cyp6g1* in DDT resistance, the F2 female flies (Fig 3-6 and 3-7) used for RNA analysis were also analyzed for the DDT resistance. The adult F2 females (2-5 days old) were treated with different concentrations of DDT and mortality was measured after 24 h as described in Methods. It is clear from the results (Table 3-7, Fig 3-8) that the *UAS-CYP6A2* and *UAS-CYP6G1* transgenes give a statistically significant but not tremendously higher level of DDT resistance relative to their respective controls. The $L.D_{50}$ values of UAS-CYP6G1-1C and UAS-CYP6G1-2D lines were 2.7 and 1.6-fold higher than the $L.D_{50}$ values of their respective controls. These lines also had 1.7 and 2.3-fold higher level of CYP6G1 RNA, respectively. For

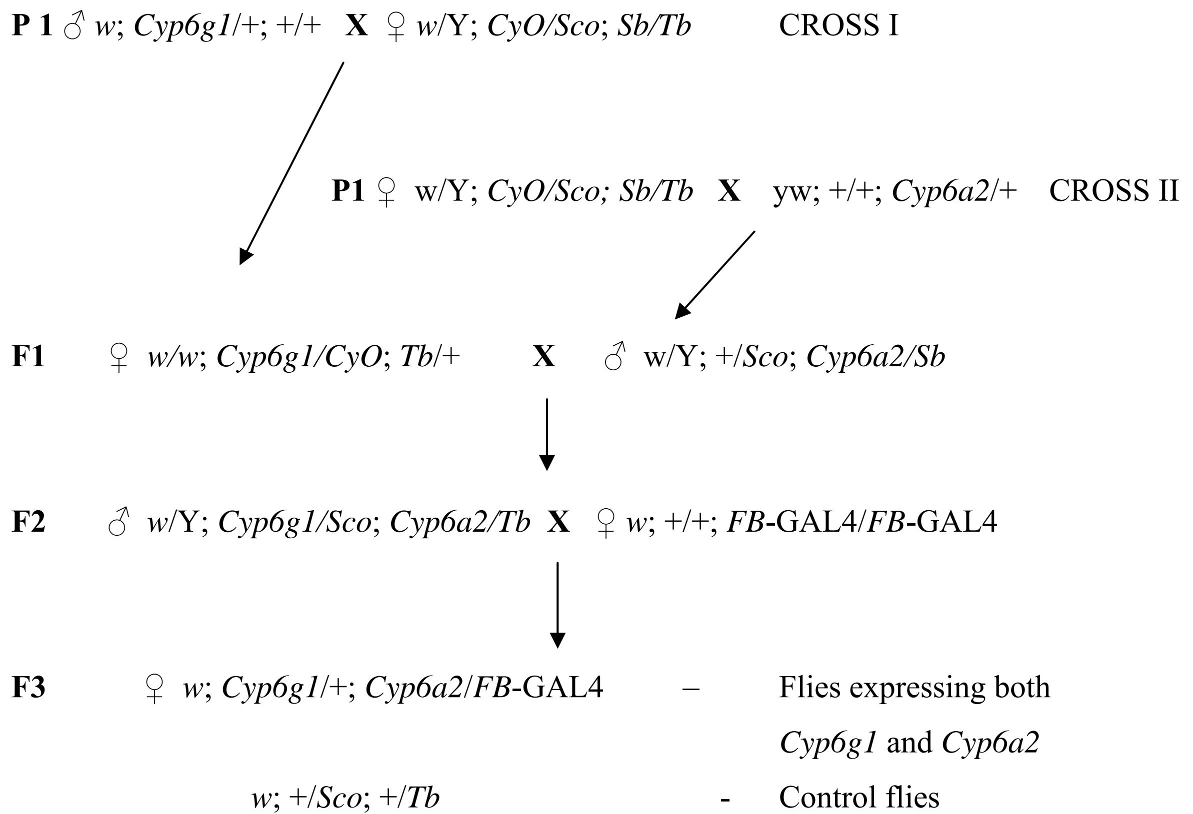


Figure 3-7: Genetic crosses to generate a stock with *Cyp6a2* and *Cyp6g1* transgenes in the same fly. Different progeny tested in the F3 generation were shown. The F3 progeny were tested for DDT resistance and RNA expression by real time PCR.

Table 3-7

DDT resistance bioassay of the transgenic lines

Strain	N[a]	p-value	Slope (± SE)	LD50 (95% CI)[b]	Fold resistance[c]
UAS- 6a2-4B	642	<0.0001	0.673 (0.103)	2.51 (2.18-2.94)	0.97
Control 6a2-4B	176	0.0001	0.39 (0.12)	2.59 (1.4-4.96)	
UAS- 6a2-1C	447	<0.0001	0.31 (0.046)	4.07 (3.3 – 5.1)	2.7
Control 6a2-1C	221	0.005	0.72 (0.26)	1.49 (0.57– 3.4)	
UAS-6g1-2D	520	0.0004	0.4 (0.1)	2.45 (1.46 – 3.84)	1.6
Control 6g1-2D	267	<0.0001	1.1 (0.2)	1.56 (1.2 – 1.99)	
UAS-6g1-1C	462	0.0004	0.37 (0.097)	2.6 (1.83 – 3.85)	2.7
Control 6g1-1C	360	<0.0001	1.64 (0.34)	0.97 (0.74-1.22)	
UAS- 6a2/6g1	446	0.0001	0.25 (0.06)	1.7 (0.1-3.2)	4.3
Control	170	<0.0001	7.9 (1.4)	0.4 (0.3-0.44)	

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). Statistical analyses were performed using probit analysis in SAS (SAS institute, Cary, NC).

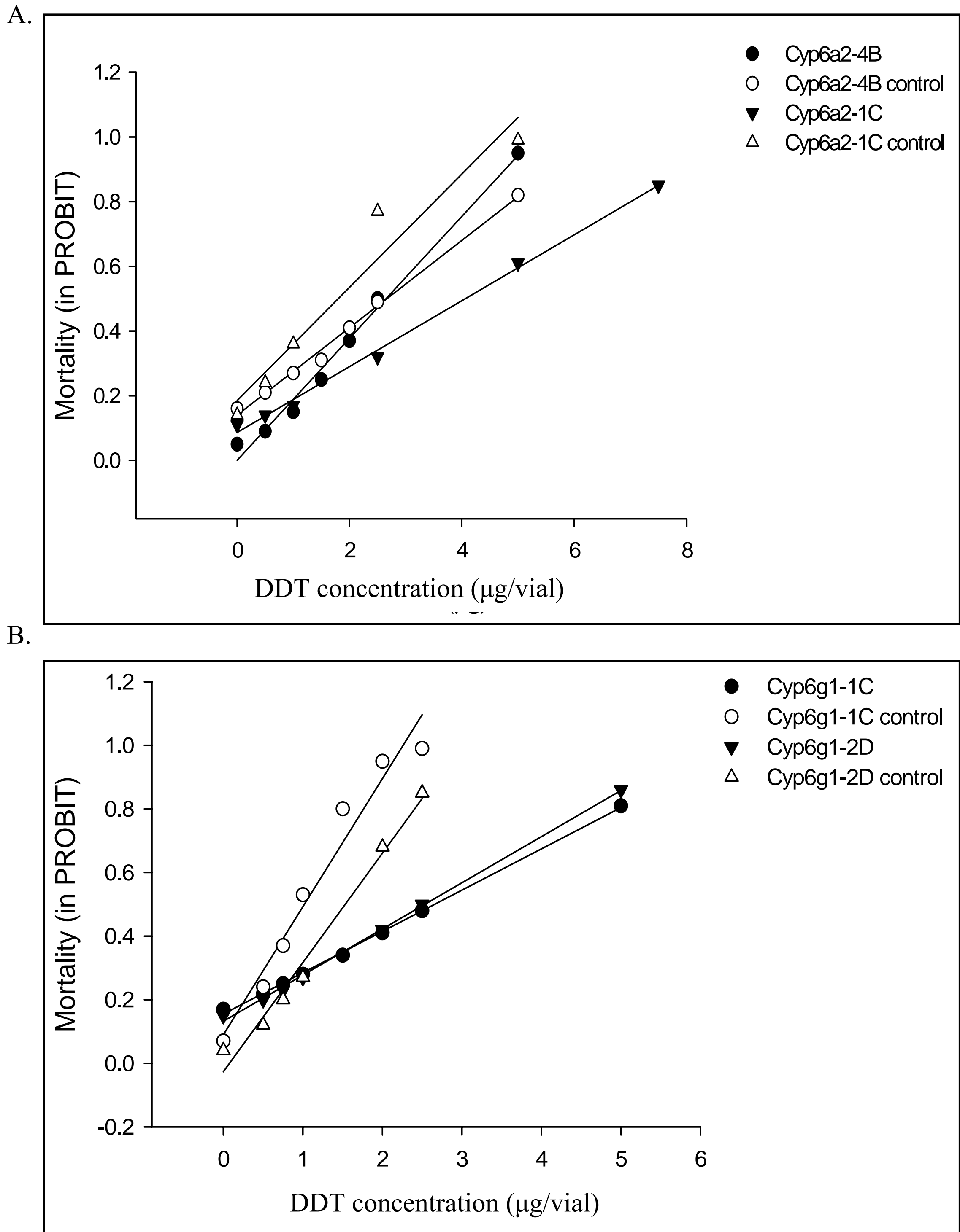


Figure 3-8: Dose response curves of the transgenic lines expressing *Cyp6a2* and *Cyp6g1* in fat bodies. A) Transgenic lines expressing *Cyp6a2* B) Transgenic lines expressing *Cyp6g1*

C.

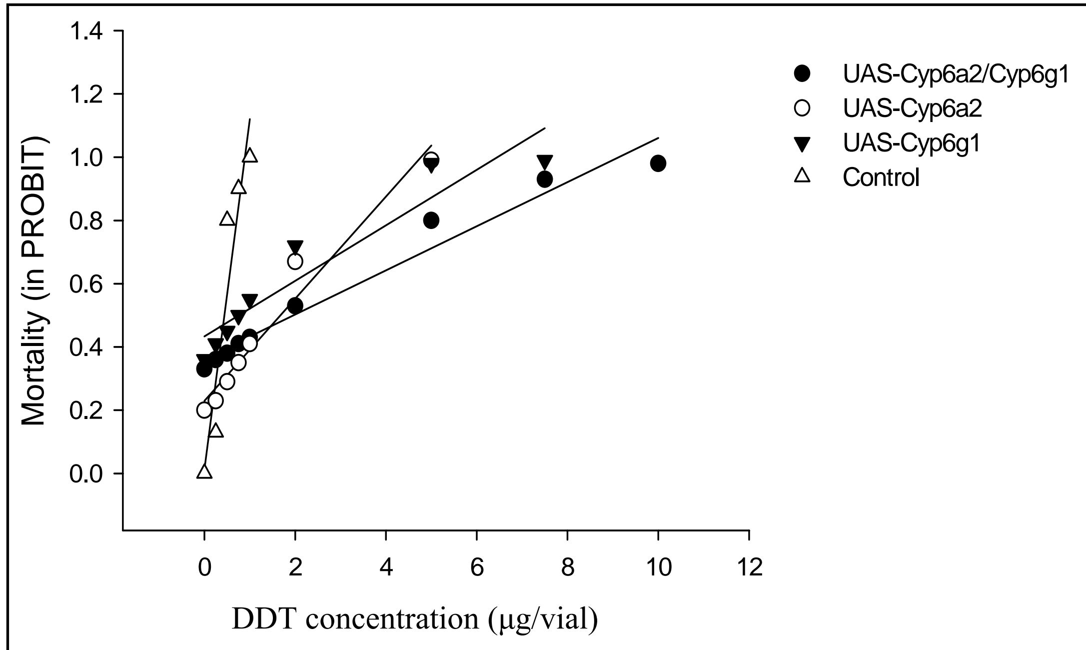


Figure 3-8-continued: C) Transgenic lines expressing both *Cyp6a2* and *Cyp6g1*

the transgenic flies carrying CYP6A2 cDNA, only UAS-CYP6A2-1C line, which had 2-fold higher level of CYP6A2 RNA than the control, also had 2.7-fold higher LD_{50} value (Table 3-7). On the other hand UAS-CYP6A2-4B line with similar level of CYP6A2 RNA like the control also had similar LD_{50} (Table 3-7). When both UAS-CYP6G1 and UAS-CYP6A2 transgenes were genetically placed into the same genome with GAL4 driver, an additive effect on DDT resistance was observed. The LD_{50} of these doubly transgenic flies was 4.3-fold greater than the LD_{50} of the control flies. Since there is not very high level of overexpression, the resistance phenomenon cannot be associated with either of the genes. However, when the *Cyp6a2* and *Cyp6g1* are manipulated in the same fly, there is additive effect showing four-fold resistance compared to control.

Discussion

Evidence that cytochrome P450 enzymes are involved in conferring insecticide resistance is based on the observation that resistant insects are rendered susceptible when they are treated with P450-specific inhibitor such as piperonyl butoxide or PBO (Hodgeson et al., 1993). The other connection between P450s and insecticide resistance is that one or more *CYP* gene is overexpressed in resistant strain compared to the susceptible ones. In *Drosophila*, at least five 2nd chromosome-linked *Cyp* genes, *Cyp6w1*, *Cyp6a2*, *Cyp12d1*, *Cyp6g1* and *Cyp6a8* show overexpression in resistant strains compared to the susceptible ones (Waters et al., 1992; Maitra et al, 1996; Dombrowski et al., 1998; Daborn et al., 2002; Pedra at al., 2004). While *Cyp6w1* and *Cyp6a2* are located close to a DDT resistance locus at 56 m.u that Dapkus (1992) has mapped in the 91-R

strain, *Cyp12d1* and *Cyp6g1* are located close to a major resistance locus at 64 m.u that many investigators have mapped in different strains of *Drosophila* but 91-R (Tsukamoto and Ogaki, 1953; Tsukamoto, 1958). Although these observations suggest that these P450s mapping close to the resistance loci may play a positive role, the actual molecular basis of DDT resistance in *Drosophila* is still not well understood. Multiplicity of P450s has made it difficult to identify the one that is actually responsible for resistance to an insecticide such as DDT.

Various investigators tried to resolve this issue by examining the metabolic property of a given P450 expressed in a heterologous system such as lepidopteran cells and *E. coli*. Homologous expression system has also been tried; *Drosophila Cyp* gene has been expressed in *Drosophila* S2 cells in culture to examine the metabolic properties (Saner, 1996; Dunkov et al., 1997). Dunkov et al (1997) showed that lepidopteran cells expressing CYP6A2 cDNA of *Drosophila* could metabolize organophosphorus insecticides such as dieldrin, heptachlor and diazinon, but not DDT. Recently, Amichot et al (2004) isolated a variant of CYP6A2 with three amino acid substitutions (R335S, L336V, V476L) from DDT resistant $RDDT^R$ strain. By expressing in *E. coli* they demonstrated that this CYP6A2vSVL allele could metabolize DDT (Amichot 2004).

Although the above observations suggest that CYP6A2 can metabolize certain insecticides in heterologous cell culture system, these data do not give any clue about the role of CYP6A2 in insecticide resistance in a fly strain. In addition, the observations made with the *Cyp6a2svl* allele do not agree with the other observations. First, Dunkov et al (1997) did not find metabolism of DDT in cells expressing CYP6A2. It may be

argued that the *Cyp6a2* allele used by Dunkov et al (1997) could not metabolize DDT because it did not have the amino acid substitutions found in the *Cypp6a2svl* allele. However, it should be noted that the *Cyp6a2-91R* allele found in the super DDT-resistant 91-R strain also does not have these amino acids substitutions, and the sequence of the *Cyp6a2* allele of a wild type strain published by Dunkov et al (1997) is same as the sequence of the allele found in the resistant 91-R strain. In view of this, it may be argued that *Cyp6a2* is not responsible for DDT resistance in *Drosophila* and DDT metabolism activity observed with the *Cyp6a2svl* allele in *E. coli* (Amichot et al., 2004) may be a result of some peculiarity associated with the heterologous system.

A straightforward approach to determine the role of a P450 in insecticide resistance is to examine the resistance phenotype of a susceptible strain transformed with that P450. Thus, in the present investigation, we used transgenic technology to introduce the CYP6A2 cDNA of the DDT resistant 91-R strain into the susceptible w^{1118} strain. The cDNA transgene under the control of yeast UAS was overexpressed by bringing it into the same genome with a yeast GAL4 cDNA driven by *Drosophila* fat body enhancer. Analysis of the expression by real-time quantitative PCR showed that there was a two-fold overexpression of *Cyp6a2* in the transgenic lines compared to their controls. DDT resistance bioassay showed that the LD_{50} of the transgenic flies was about two-fold than that of the control flies. Although the expression levels may be correlated with the resistance phenotype, the level of resistance was much lower than that observed in the resistant 91R or the *Wisconsin* strains. Almost similar results were obtained when CYP6G1 cDNA transgene was expressed in the transgenic flies. Although the cDNA

was driven by GAL4 cDNA under the control of a fat body enhancer, only two-fold increase both in cDNA expression and LD_{50} was observed. Interestingly, when the CYP6A2 and CYP6G1 cDNAs were expressed together in the same fly and tested for DDT resistance, an additive effect was observed; the doubly transgenic flies showed a four-fold increase in LD_{50} compared to the control. Although the GAL4 driver did not increase the CYP6A2 and CYP6G1 RNA level as high as found in the 91R strain, the additive effect on resistance suggests that both *Cyp6a2* and *Cyp6g1* are involved in DDT resistance in *Drosophila*.

However, in the doubly transformed flies, the level of resistance is low and several hundred-fold lower than the resistance found in laboratory-selected (91R) or field-collected (*Wisconsin*) resistant strains. Daborn et al (2002) overexpressed *Cyp6g1* in *yw* strain using a GAL4 driver under the control of a heat shock promoter. Although tthe heat-shocked transgenic flies showed about a 100-fold overexpression of CYP6G1 RNA compared to the control, they were resistant only to a low dose (10 μg) of DDT. The results of the present investigation also showed that *Cyp6a2* or *Cyp6g1* confer a low level of resistance in the transformed flies. Low level of resistance in the transformed flies observed in the present investigation may be a result of low expression of CYP6A2 or CYP6G1 transgene. However, similar low level of resistance was also observed by Daborn et al (2002) in their transgenic lines showing 100-fold higher level of expression than the control flies. Taken together, it may be suggested that for high level of DDT resistance factors other than *Cyp6a2* and *Cyp6g1* are needed. Such factors could be modifiers and/or specific alleles of other *Cyp* genes which may be missing in the

transgenic flies carrying overexpressing CYP6A2 and CYP6G1 cDNAs. The other *Cyp* gene that may play role in DDT resistance is *Cyp6w1*, which shows higher level of expression in the resistant 91R strain compared to the susceptible 91C strain (Awwad and Ganguly, unpublished observations, and Chapter III). Interestingly, *Cyp6w1* is located close to a DDT resistance locus mapped by Dapkus (1992) at 56 ± 1 m.u of the second chromosome of 91R strain. This close proximity of *Cyp6w1* to the DDT resistance locus makes *Cyp6w1* another candidate gene which may be involved in DDT resistance. It is possible that for DDT resistance, overexpression of *Cyp6w1* is also necessary besides other genetic factors. Since susceptible 91-C strain is genetically similar to the resistant 91-R strain, it may have these unknown genetic factors. Therefore, 91-C may be a better host for germ line transformation than the *white* strain used in the present investigation to determine the role of a specific CYP in DDT resistance.

Chapter IV

Effect of caffeine and phenobarbital on the transcriptome of *Drosophila melanogaster*

Introduction

Like many other genes, cytochrome P450 or *CYP* genes are also environment sensitive. They are known to be induced by various xenobiotic compounds. Initially, in 1960's, only one P450 enzyme was identified when P450 research just started. At that time only two chemicals were identified as the inducers for CYPs, one is phenobarbital and the other is 3-methylcholanthrene. Over the years, the number of CYPs discovered in different organisms increased dramatically and so did the number of inducers. Interestingly, large number of drugs, polyaromatic hydrocarbon found in many environmental pollutants, and many phytochemicals present in vegetables and fruits are turned out to be inducers for different P450s. A list of inducers and substrates for humans P450 can be found at http://medicine.iupui.edu/flockhart/table.htm. Many endogenous compounds also act as inducers for some P450s (Schuetz, 2001).

CYPs are known to be involved in the metabolism of various xenobiotic compounds including drugs used for medical reasons (Danielson 2002, for review). In mammals, they are also involved in other routine endogenous functions such as cholesterol biosynthesis, vitamin D metabolism, steroid biosynthesis etc (Nebert and Russell, 2002). The reason research on P450 induction grew very fast is the fact that some P450s metabolize xenobiotic compounds that also act as inducers, and induction of

a P450 may compromise the efficacy of drugs used to treat clinical conditions (Guengerich, 2003; Murray, 2006). Many of these inducers turned out to be valuable tools and have been used extensively to dissect out the molecular mechanism of *CYP* gene regulation. Using dioxin, phenobarbital-like compounds such as TCPOBOP and other lipophilic polyaromatic hydrocarbons as inducers, a large number of nuclear receptors have been identified in mammals which regulate multiple P50 genes (Schuetz, 2001; Sueyoshi and Negishi, 2001; Fujii-Kuriyama and Mimura, 2005, for review). These studies showed that many *CYP* genes in mammals appear to be regulated by a common mechanism. This knowledge has been very helpful for proper drug design.

Compared to the mammals, very little is known about the mechanism of *CYP* gene regulation in insects. So far, only phenobarbital and DDT has been used as inducers to examine whether they can induce P450 genes in insects such as house fly and *Drosophila* (Carino et al., 1992; Maitra et al., 1996; Liu and Scott, 1995; Dunkov et al., 1997; Dombrowski et al., 1998; Pedra et al., 2004). In the present study, we used two xenobiotic compounds, caffeine and phenobarbital. Caffeine is one of the most widely consumed psychostimulant xenobiotic compounds (Lorist and Tops, 2003). It is found in coffee, tea, soft drinks and chocolate. The psychostimulant properties of caffeine is due to its ability to interact with the neurotransmission in different regions of the brain promoting change in different behavioral functions such as attention, mood, arousal and alertness (Fisone, 2004). Caffeine fights fatigue, and prolongs the time to fall asleep. The effect of phenobarbital is opposite; it is an anesthetic compound and it makes the subject sleepy. It is also used to control epileptic seizure. In the present study, using microarray technology we compared transcriptome profiles in caffeine- and

phenobarbital-treated adult *Drosophila*. Results showed that several stress response genes, *Cyp* genes, genes involved in carbohydrate and lipid metabolism are upregulated by both caffeine and phenobarbital. There are also genes which are downregulated by these chemicals. Thus, in future caffeine and phenobarbital may be used as tools to understand the regulatory mechanisms of various groups of genes, especially the *Cyp* genes that have been implicated in DDT resistance.

Materials and methods

Drosophila strains and treatments

The *Canton- S* strain used in the present study was obtained from *Drosophila* stock center (Bloomington, Indiana) and 91-C strain was obtained in 1992 from Larry Waters, Oak Ridge National Laboratory. These stocks were maintained on cornmeal-agar-molasses medium at 24°C under 12-hour light and 12-hour dark cycle. For xenobiotic induction studies, 5-10 day old female flies were treated with 16 mM caffeine, 4 mM phenobarbital, 4 mM barbital or water. Briefly, 5-10 days old flies from three different cultures were combined, etherized and four groups of randomly picked 200-300 females were sorted and transferred into four individual bottles containing fresh *Drosophila* medium. These bottles were left overnight at room temperature to allow the flies to recover from ether shock. Next day, three different types of media, each containing 16 mM caffeine or 4 mM barbital or 4 mM phenobarbital, were prepared using instant fly food (Carolina Biologicals). Fly food made with water was used as control. After recovery from the ether shock, sorted females were directly transferred to each of the four types of fly food without etherization and allowed to feed on the media

for 24h in a dark cabinet at room temperature. After the treatment, flies from each media bottle were sorted into three groups of approximately fifty flies in each group and transferred into eppendorf tubes for RNA isolation.

Total RNA isolation and northern blot hybridization.

Briefly, total RNA was isolated from female flies (5-10 day old) sorted above using TRI® Reagent (Sigma, St. Louis, MO) and following the manufacturer's protocol. The final RNA pellets were rinsed with chilled 70% ethanol, dried and dissolved in appropriate volume of RNase-free water. For each northern blot, a set of RNA samples, comprising one RNA sample from each treatment, were fractionated on the 1.2% agarose–2.2M formaldehyde denaturing gel as described. For each lane, 20 μg of total RNA was dried, dissolved in 20 μl of 1X formaldehyde loading dye (Ambion Inc., Austin, TX) and incubated at 65^0C for 15 minutes. Each RNA sample was loaded in triplicate in the gel and electrophoresed. After electrophoresis, RNA was blotted onto Hybond (Amersham) nylon, cross-linked with UV and the rRNA band, which co-migrates with CYP mRNAs, was visualized with a long wave UV lamp. The blots were divided into upper and lower halves by cutting about 1.0 cm below the ribosomal RNA bands. The upper and lower blots were prehybridized in separate hybridization bottles for 1 hour at 37^0C in Northern-Max prehybridization/hybridization buffer (Ambion Inc., Austin, TX). After prehybridization, the upper blots were hybridized with ^{32}P-labeled 0.6-kb N' terminal DNA of the desired *Cyp* gene and the lower blots were hybridized with ^{32}P-labeled RP49 (ribosomal protein) cDNA. The *Cyp* and *RP-49* gene probes were labeled using the Strip-EZ® random prime labeling kit (Ambion, Austin, TX) and a nick-

translation labeling kit from Invitrogen (Carlsbad, CA) respectively. Similar quantities (CPM) of radioactive probes with similar specific activities were used in all three sets of blots, and all the probes used were in excess over the RNA on the blots. Hybridization was done for 30h at 37^0 C. After hybridization, the blots were washed under stringent condition and hybridization signals on the blots were quantified with a radioanalytical imager as described. RP49 signal was used as the internal control to normalize for the RNA loading errors. The CYP/RP49 values of three sets of northern blots made with three sets of RNA samples were used to determine the mean value and compare *Cyp* gene expression between control and treated flies.

Microarrays

The quality of all total RNA samples was assessed using an Agilent 2100 RNA BioAnalyzer (Agilent Technologies, CA). Total RNA (3.5 μg) was used to set up first strand cDNA synthesis reaction with T7-oligo (dT) primer (Affymetrix) and Superscript II reverse transcriptase (First strand cDNA synthesis kit, Invitrogen). The method was described in detail in Chapter 2. Briefly, second strand cDNA was synthesized from the first strand described above according to standard Affymetrix protocols. The double-stranded cDNA synthesized was purified using Affymetrix GeneChip sample cleanup modules. Biotin-Labeled cRNA was prepared using an ENZO BioArray High Yield RNA Transcript Labeling Kit (ENZO Life Sciences, Inc., Farmingdale, NY). After cleanup of the *in vitro* transcription products, the purified cRNA was fragmented to a size ranging from 35 to 200 bases using fragmentation buffer at 94°C for 35 minutes. The extent of fragmentation was assessed by loading the fragmented cRNA on a BioAnalyzer. Fifteen

micrograms of the fragmented cRNA was mixed into a hybridization cocktail containing hybridization buffer, B2 oligo control RNA, herring sperm DNA, and BSA (Invitrogen, Carlsbad, CA). The solution was hybridized to a GeneChip Drosophila Genome 2.0 Array (Affymetrix, Santa Clara, CA) at 45°C for 16 hours at a setting of 60 rpm in a hybridization chamber. Drosophila Genome 2.0 Array consists of 18,500 transcripts based on the recent annotation (release 3.1) of the *Drosophila melanogaster* genome by Berkeley Drosophila genome project (BDGP) and Flybase. Fourteen perfect matched probes and fourteen mismatched probes with a single nucleotide mismatch at position 13 were used to measure the transcription levels of each representative sequence (See data analysis below). After hybridization, the GeneChips were washed using the Affymetrix Fluidics 450 wash station (Affymetrix Fluidics Protocol Midi_EUK2V3_450) and stained with streptavidin-phycoerythrin (Molecular Probes, Carlsbad, CA). The GeneChips were scanned with a GeneChip 3000 High-Resolution Scanner. The scanned images were quantified using GeneChip Operating software/ Microarray analysis suite (GCOS or MAS 5.0). The individual GeneChip scans were quality checked for the intensity of the control genes and background signal values. The signal intensity values for the 5' probe sets of actin and GAPDH genes were compared with their corresponding 3' probe sets. The ratio of the 3' probe set to the 5' probe set was identified to be less than 3, which validates the RNA sample and assay quality. The entire procedure was carried out at the Affymetrix core facility at University of Tennessee, Knoxville.

Microarray data analysis

Genes were represented as probe sets with more than one transcript for each gene on the Drosophila Genome 2.0 chip. Each probe set consists of fourteen pairs of perfect match (PM) and mismatch (MM) oligonucleotides. The MAS 5.0 software was used for background subtraction of all the chips (nine GeneChips with three chips for each of the treatments; water, caffeine and phenobarbital) followed by GC-robust multiarray analysis (GC-RMA) for linear multi-chip normalization. The intensity value is the ratio of the difference between the perfect match and mismatch nucleotides to the total hybridization intensity. The data was also checked for the presence of outliers using a residual cut off of 2500 i.e., if the residual is greater than 2500 or less than –2500, it will be indicated as an outlier and eliminated from further analysis. Univariate method was used to investigate normal distribution of the residuals with a 0.9 cut-off for Shapiro-Wilkes test. For each observation in the dataset, a linearized model of ANOVA i.e., yij = (μ + Ti + R (T)ij (where y represents the observation on the ith replicate for the jth treatment, (μ is the overall mean, T is the ith treatment effect and R(T) is the residual error) was fit. The F values obtained from the above equation represent the ratio of the mean expression of the treatments to the mean expression of the residuals. The F-value obtained was used to identify the genes that showed significant differences between the control and treated samples. Simultaneously, T-test was performed to individually compare each of the treatment means with the control and obtained a raw p-value (Canton S caffeine treated, Phenobarbital treated and water control). Further, a p-value correction was performed by Bonferroni, False discovery rate (FDR) and Benjamini- Hochberg methods. The Bonferroni method is overly conservative and leads to false negatives when large

numbers of genes are involved. Hence, the corrected p-values obtained from false discovery rate with a cut off of 0.01 (99% confidence level) and an F-test with $p<0.05$ were used for further analysis. All the data analyses were performed in SAS (SAS institute, Cary, NC).

Results and Discussion

Previous studies in our laboratory demonstrated that *Cyp6a2* and *Cypa8* promoters are induced by phenobarbital, barbital and caffeine (Maitra et al., 1996; Dombrowski et al., 1998; Bhaskara et al., 2006). These data suggested that promoters of most *Cyp* genes in *Drosophila* may be activated by these compounds. Precisely, the objective of this investigation has been to examine this possibility. However, before examining the change in genome-wide transcriptome profile following treatment with these chemicals, induction of *Cyp6g1* was examined initially because compared to *Cyp6a2* and *Cyp6a8* genes, this gene shows much higher level of expression in the DDT resistant 91-R strain. Therefore, effects of phenobarbital and caffeine on the expression of *Cyp6g1* in the DDT susceptible *91-C* strain were examined. Results (Fig 4-1) showed that there was a two-fold increase in *Cyp6g1* RNA in response to 16mM caffeine. Barbital and caffeine treatment also induced *Cyp6g1* expression and showed 3.4- and 2-fold induction, respectively (Fig. 4-1). Thus, like *Cyp6a2* and *Cyp6a8*, *Cyp6g1* is also induced by all three chemicals.

In order to examine genome-wide effect of caffeine and phenobarbital on the transcriptome profile, we used Affymetrix oligonucleotide arrays containing probes for

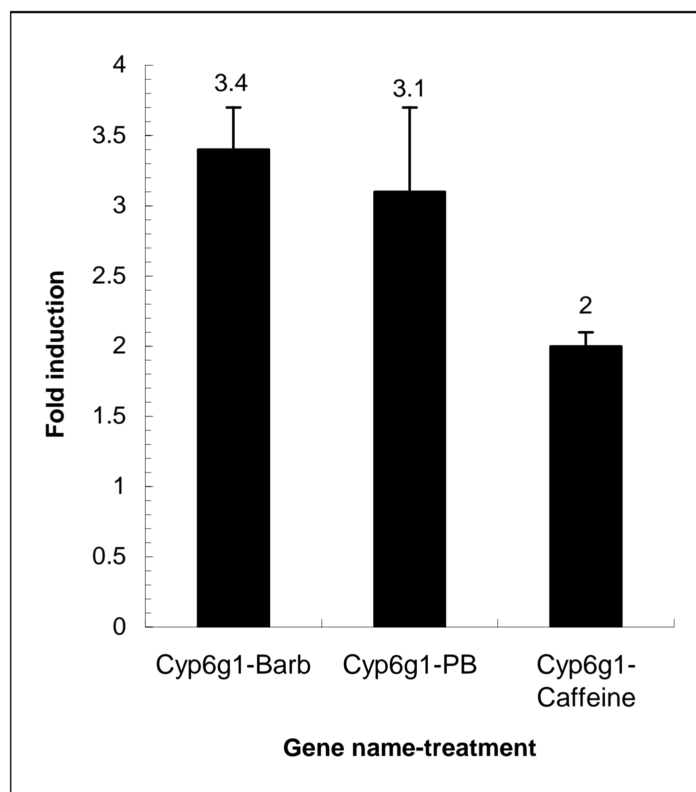


Figure 4-1: Effect of caffeine and barbiturate compounds (barbital and phenobarbital) on the *Cyp6g1* gene expression in the 91-C strain of *Drosophila melanogaster.* Total RNA was isolated from the adult females of the treated and untreated flies using TRI® Reagent (Sigma) as described in Methods. RNA was electrophoresed on formaldehyde-agarose gel and hybridized with *Cyp6g1* gene probe. RP49 was used as an internal control to normalize for the RNA loading errors. The data shown represent the mean ± S.D of three Northern blots with three independent isolates of RNA from each strain. Barb- barbital, PB- phenobarbital.

18,500 transcripts of *Drosophila melanogaster*. Total RNA samples isolated from female flies treated with caffeine, phenobarbital or water were used to generate cRNAs which were then hybridized with probes on the microarrays as described in Materials and Methods. Triplicate arrays were used for each treatment. Genes that are differentially expressed by caffeine and phenobarbital alone are shown in Tables 4-1 and 4-2. The results showed that caffeine treatment upregulated 162 and downregulated 54 genes (Tables 4-1). In case of phenobarbital treatment, we found that 94 genes were upregulated and 173 were downregulated (Table 4-2). Gene Ontology (GO) database search revealed that about one-third of the genes upregulated by caffeine (57/162) or phenobarbital (32/94) do not have any known or predicted molecular function. Therefore, these genes will not be discussed hereafter. The remaining 105 and 62 genes upregulated by caffeine and phenobarbital, respectively belong to different functional categories such as protein, lipid, carbohydrate and nucleic acid metabolism, amino acid biosynthesis, transport, signal transduction and defense response (Tables 4-1 and 4-2). Both treatments also induced 13-15 cytochrome P450 genes, including *Cyp6a2*, *Cypa8* and *Cyp6g1*. Previous studies (Maitra et al., 1996; Dunkov et al., 1997; Dombrowski et al., 1998) and present investigation (Fig 4-1) also showed that these genes are induced by caffeine and barbiturates, as determined by northern blot analysis. It is to be noted that a large proportion (21%) of upregulated genes in both treatments are involved in carbohydrate metabolism (Tables 4-1 and 4-2). A large proportion of downregulated genes, also do not have any known or predicted function. In caffeine-treated flies, 29 out of 54 (54%) and in phenobarbital-treated flies, 70 out of 173 (41%) downregulated genes belong to this class. With caffeine, the remaining 25 genes out of the 54 downregulated

Table 4-1

List of genes differentially expressed in caffeine treated flies

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Upregulated genes				**Induction**
Amino acid biosynthesis and metabolism				
methyltransferase activity	pug	8.09E-06	8E-04	2.50
cysteine dioxygenase activity	CG5493	3.15E-05	0.001	2.11
amino acid transporter activity	CG1139	4.26E-06	5E-04	4.08
cation transporter activity	CG15088	7.19E-05	0.003	2.24
aminomethyltransferase activity	CG6415	1.62E-04	0.003	2.01
lyase activity	CG5793	2.81E-05	0.001	2.65
Carbohydrate metabolism				
alpha-amylase activity	Amyrel	1.31E-04	0.003	7.28
alpha-glucosidase activity	CG14934	2.19E-06	4E-04	4.76
	LvpH	2.47E-06	4E-04	3.02
	CG8690	2.92E-09	1E-05	7.96
	CG11909	5.26E-08	9E-05	6.97
fructose transporter activity	CG15406	1.93E-07	2E-04	2.56
glucose transporter activity	CG8249	1.87E-04	0.004	2.27
	CG1208	1.07E-07	1E-04	5.49
	CG6484	3.54E-06	5E-04	4.34
transporter activity	CG31106	8.37E-05	0.002	2.26
	CG3285	1.43E-06	3E-04	3.24
isomerase activity	CG9008	3.67E-06	5E-04	2.28
kinase activity	CG9886	1.09E-04	0.002	2.49
L-iditol 2-dehydrogenase activity	Sodh-1	2.76E-05	0.002	6.79
oxidoreductase activity	CG6910	6.09E-05	0.002	4.40
oxidoreductase activity	CG9331	1.16E-05	8E-04	2.39
serine-pyruvate transaminase activity	Spat	1.08E-04	0.002	2.13
aldose 1-epimerase activity	CG32444	2.58E-05	0.001	4.56
alpha alpha-trehalase activity	CG16965	2.33E-05	0.001	2.99
amidophosphoribosyltransferase activity	Prat2	1.80E-06	4E-04	2.00
galactokinase activity	CG5288	1.62E-04	0.003	2.18
2-hydroxyacylsphingosine 1-beta-galactosyltransferase	Ugt86Dc	2.92E-04	0.004	3.51
chitinase activity	CG9307	5.51E-04	0.007	2.26
glucuronosyltransferase activity	Ugt86Dh	8.98E-05	0.002	2.13
Gram-negative bacterial binding	CG12780	1.05E-04	0.002	2.15
Coenzyme and prosthetic group metabolism				
carrier activity	CG15018	1.75E-05	9E-04	2.25
cation transporter activity	CG2196	2.52E-08	4E-05	2.41

Table 4-1 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Upregulated genes				**Induction**
Defense response				
glutathione transferase activity	GstD1	1.37E-05	9E-04	2.04
	GstD5	1.77E-04	0.003	6.12
receptor activity	CG10824	3.84E-07	2E-04	2.35
Transport				
ATP-binding cassette (ABC) transporter activity	CG9270	5.96E-04	0.006	2.79
	CG10226	5.28E-05	0.002	3.18
xenobiotic-transporting ATPase activity	Mdr49	2.48E-05	0.001	2.14
long-chain fatty acid transporter activity	CG6300	8.43E-06	7E-04	3.61
monosaccharide transporter activity	CG15407	1.05E-04	0.002	3.13
Lipid metabolism				
long-chain-fatty-acid-CoA ligase	CG4500	3.64E-04	0.006	6.21
acyltransferase activity	CG18609	2.01E-06	4E-04	2.49
oxidoreductase activity acting on CH-OH group of donors	CG7322	8.06E-05	0.002	2.49
structural molecule activity	CG9914	1.76E-05	9E-04	2.48
triacylglycerol lipase activity	CG10357	5.09E-06	9E-04	2.28
	CG6283	1.06E-05	8E-04	2.29
	CG8093	1.28E-06	3E-04	3.43
	Lip3	7.62E-04	0.007	3.24
Proteolysis and peptidolysis				
enteropeptidase activity	CG9649	3.41E-04	0.004	2.03
metalloendopeptidase activity	CG14528	2.08E-06	4E-04	3.35
trypsin activity	CG6041	1.37E-04	0.003	2.36
Nucleic acid metabolism				
phosphoribosylformylglycinamidine synthase activity	ade2	2.38E-04	0.005	2.01
xanthine dehydrogenase activity	ry	2.13E-04	0.003	2.03
oxidoreductase activity	CG18522	2.80E-06	5E-04	2.29
Cytochrome P450 genes				
electron transporter activity	Cyp309a2	1.52E-06	3E-04	3.54
	Cyp6a20	1.99E-05	9E-04	2.10
	Cyp309a1	4.33E-07	2E-04	16.83
oxidoreductase activity	Cyp28d1	3.58E-05	0.001	2.13
	Cyp12a5	4.97E-07	2E-04	2.67
electron transporter activity	Cyp9b1	2.00E-04	0.003	2.84
Oxidoreductase activity				
oxidoreductase activity	CG13091	5.17E-06	9E-04	6.50
	CG3609	1.05E-07	1E-04	4.91
	CG18547	5.32E-06	5E-04	3.52
	CG2064	3.64E-05	0.001	2.08
	CG3699	9.85E-06	8E-04	5.90

Table 4-1 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Upregulated genes				**Induction**
Signal transduction				
4-nitrophenylphosphatase	CG5577	4.04E-05	0.002	2.16
adenosine deaminase activity	Adgf-D	3.33E-06	4E-04	2.49
inositol-trisphosphate 3-kinase	IP3K2	3.64E-05	0.002	2.04
calcium ion binding	smp-30	1.27E-05	8E-04	7.14
neprilysin activity	CG3775	1.94E-05	0.001	2.59
peptidyl-dipeptidase A activity	Ance-4	7.42E-06	7E-04	2.71
phospholipase A2 activity	CG11124	2.46E-04	0.004	2.11
protein dimerization activity	CG17836	1.52E-04	0.003	2.10
Unknown function				
	CG2650	5.91E-05	0.003	2.33
	CG15263	1.52E-07	2E-04	16.79
	CG32647	5.40E-04	0.009	2.44
	CG1979	5.87E-04	0.009	2.72
	---	3.60E-05	0.002	2.54
	CG11594	1.18E-04	0.003	2.77
	CG4213	3.04E-04	0.006	2.07
	---	5.44E-07	2E-04	2.38
	---	2.46E-06	5E-04	73.61
	---	1.08E-05	9E-04	2.92
	CG30019	3.24E-05	0.002	3.50
	CG9119	1.45E-05	0.001	3.77
	---	7.28E-05	0.002	2.06
	CG33085	1.41E-04	0.003	2.89
	CG10912	2.24E-04	0.003	2.06
	---	3.92E-04	0.005	2.06
	CG1468	2.84E-04	0.004	2.08
	CG16836	5.34E-04	0.006	2.18
	CG18279	5.49E-04	0.006	2.62
	CG33091	2.13E-07	1E-04	3.00
	CG4377	2.04E-07	2E-04	5.74
	JhI-26	3.63E-04	0.005	2.06
	comm2	1.57E-04	0.003	2.60
	CG11878	6.00E-06	6E-04	4.65
	CG6830	4.39E-08	7E-05	3.16
	---	1.03E-04	0.002	2.49
	---	2.99E-07	2E-04	88.50
	CG9691	1.20E-04	0.003	2.12
	---	1.71E-05	0.001	2.10
	CG10562	1.19E-05	8E-04	2.29
	CG9396	6.46E-04	0.007	2.51
	---	2.46E-06	4E-04	2.84
	CG13658	8.94E-04	0.008	3.61
	CG15407	4.09E-05	0.002	2.32
	CG4363	2.94E-06	4E-04	5.90
	---	2.32E-06	5E-04	2.84

Table 4-1 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Upregulated genes				**Induction**
	---	2.86E-04	0.004	8.22
	Jheh2	6.64E-07	3E-04	7.67
Downregulated genes				**Repression**
Development				
alkaline phosphatase activity	CG10592	1.7E-05	0.006	3.23
	CG5150	3.3E-05	0.009	3.23
DNA binding	Alhambra	0.00027	0.004	8.3
galactose binding	lectin-24A	6.4E-05	0.003	14.3
Proteolysis and peptidolysis				
metallocarboxypeptidase activity	CG14820	2.5E-05	0.001	2.5
metalloendopeptidase activity	CG7631	4.4E-05	0.001	4.5
serine-type endopeptidase activity	CG11911	1.8E-06	4E-04	2.2
	CG7118	1.6E-05	0.001	4.2
	Ser4	0.00055	0.006	5.3
	ndl	0.00062	0.008	3.3
	CG10475	0.00028	0.004	2.7
	CG8869	1.8E-07	1E-04	20
	CG5246	2.4E-07	1E-04	5
	CG6580	0.00024	0.005	2.2
Miscellaneous				
sulfotransferase activity	CG6704	0.00016	0.003	4
high affinity inorganic phosphate:sodium symporter activity	CG9825	1.1E-05	0.003	4.5
receptor binding	CG5550	8.6E-07	2E-04	2.6
ligase activity	CG4830	4.9E-05	0.003	3.3
oxidoreductase activity	CG8303	8.4E-06	8E-04	2.2
transcription regulator activity	sug	1.3E-05	0.002	3.6
triacylglycerol lipase activity	CG17192	0.00019	0.004	3.3
Unknown function				
	CG13912	2.8E-06	0.001	2.4
	CG31041	0.00027	0.005	3.3
	CG11892	8.3E-05	0.002	2.1
	---	1.9E-06	4E-04	4.5
	yellow-k	0.00062	0.008	3.1
	Obp19c	0.00093	0.009	4.8
	CG13992	0.00109	0.009	2.3
	fit	0.00025	0.004	4.5
	Ag5r2	6.5E-06	5E-04	2.1
	CG9850	0.00041	0.005	2.1
		6.5E-05	0.002	2.5
	---	5.5E-05	0.002	2.6
	---	1.4E-06	3E-04	2.5
	CG14205	1.7E-06	3E-04	12.5
		8.2E-05	0.007	2.6
	Cad74A	0.00024	0.004	2.9
	CG14834	0.00038	0.005	4.3
		5.4E-05	0.002	2.6

Table 4-1 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Downregulated genes				**Repression**
---	CG13784	8.92E-06	8.38E-04	2.6
---	CG12505	5.64E-08	2.09E-04	4.2
---	---	3.80E-04	4.71E-03	4.2
---	CG1347	6.93E-07	2.09E-04	3.8
---	CG32469	6.26E-05	2.83E-03	3.6
---	CG14023	6.23E-04	6.38E-03	2.5
---	CG8516	4.00E-06	4.28E-04	3.0
---	CG1863	2.88E-06	4.23E-04	4.5
---	CG8949	4.34E-04	5.16E-03	2.3

Table 4-2

List of genes differentially expressed in phenobarbital treated flies

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Upregulated genes				**Induction**
Carbohydrate metabolism				
beta-galactosidase activity	Gal	2.15E-04	5.89E-03	2.03
hydrolase activity, hydrolyzing N-glycosyl compounds	CG9463	6.15E-04	6.59E-03	6.35
Lipid metabolism				
palmitoyl-CoA oxidase activity	CG5009	4.87E-04	6.00E-03	2.19
FAD binding	CG9509	2.73E-04	3.82E-03	2.18
oxidoreductase activity	CG31810	2.77E-06	3.88E-04	5.61
Defense and stress response				
Glucuronosyltransferase activity	Ugt36Bb	1.91E-06	3.91E-04	5.08
	Ugt36Bc	2.68E-06	3.91E-04	2.11
	GstE5	1.07E-06	5.43E-04	2.02
glutathione transferase activity	GstE7	2.41E-05	1.90E-03	2.04
	GstD7	5.92E-06	5.40E-04	8.48
	CG6776	3.73E-05	1.29E-03	2.14
	DyakGstE3	2.66E-05	1.22E-03	2.64
	DyakGstE2	7.62E-06	1.89E-03	2.15
	GstD2	1.25E-05	8.28E-04	33.56
glutathione peroxidase activity	Obp99b	4.84E-04	7.83E-03	3.67
odorant binding	Jheh1	6.64E-07	2.87E-04	7.87
epoxide hydrolase activity	Hsp70Bbb	1.35E-03	9.91E-03	4.48
ATP binding	Mdr50	4.77E-05	1.50E-03	2.23
Cytochrome P450 genes				
monooxygenase activity	Cyp6a21	1.68E-04	2.95E-03	4.83
	Cyp4e3	4.62E-04	5.33E-03	2.79
	Cyp4p3	7.28E-04	1.14E-02	2.06
Transport proteins				
transporter activity	CG31272	7.81E-06	9.50E-04	2.37
monosaccharide transporter activity	CG33281	8.66E-05	2.84E-03	2.69
organic cation porter activity	CG8654	2.17E-04	7.29E-03	2.15
Miscellaneous groups				
transcriptional repressor activity	Her	4.10E-05	1.39E-03	1.98
carbohydrate kinase activity	Sk1	1.95E-04	3.30E-03	2.43
trypsin activity	CG9377	1.90E-09	1.29E-05	10.22
pantetheinase activity	vanin-like	3.70E-05	3.54E-03	2.63
nucleotide phosphatase activity	CG3290	1.86E-05	1.49E-03	4.09
zinc ion binding	CG18473	1.01E-05	1.06E-03	2.08
structural constituent of pupal cuticle	Pcp	9.62E-05	2.46E-03	1.99
Unknown function				
---	---	1.12E-06	3.57E-04	15.25
---	---	1.54E-06	4.57E-04	2.30
---	CG7272	7.03E-06	5.80E-04	2.04
---	CG10182	4.29E-06	1.11E-03	2.22
---	CG13325	2.08E-05	1.33E-03	2.73
---	---	1.84E-05	1.51E-03	2.87

Table 4-2 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Upregulated genes				**Induction**
---	CG15203	9.27E-05	2.52E-03	3.61
---	CG13324	1.14E-04	2.64E-03	2.10
---	CG9498	2.03E-04	3.50E-03	3.18
---	CG31975	5.04E-04	5.71E-03	3.11
---	CG13845	4.66E-04	7.92E-03	2.49
---	CG13656	6.78E-04	9.37E-03	1.97
---	CG18410	1.11E-03	9.52E-03	2.33
Downregulated genes				**Repression**
Protein biosynthesis and modification				
---	raptor	1.03E-04	2.29E-03	3.7
protein serine	CG1906	1.01E-07	1.30E-04	2.6
protein tyrosine phosphatase activity	Ptp4E	6.32E-05	2.27E-03	2.3
hematopoietin	Ptp10D	1.56E-04	3.28E-03	2.9
protein serine	CG17698	4.08E-06	4.75E-04	3.3
protein kinase activity	Gyc76C	3.75E-05	1.32E-03	4.3
mRNA binding	Upf2	9.11E-06	8.48E-04	3.0
asparagine synthase (glutamine-hydrolyzing) activity	asparagine-synthetase	5.39E-05	1.87E-03	2.2
Proteolysis				
ligase activity	CG8188	7.82E-06	6.45E-04	2.3
ubiquitin-protein ligase activity	CG3099	1.66E-05	8.53E-04	2.1
ubiquitin-protein ligase activity	CG4238	6.18E-05	1.86E-03	2.2
nucleic acid binding	CG11360	5.29E-05	1.53E-03	5.0
peptidase activity	RN-tre	1.10E-04	2.34E-03	2.0
zinc ion binding	l(3)IX-14	1.70E-05	1.24E-03	2.3
cysteine-type endopeptidase activity	Dab	2.08E-04	3.62E-03	2.3
carboxypeptidase A activity	svr	3.59E-06	4.31E-04	2.6
Lipid metabolism				
acyltransferase activity	CG5326	3.57E-06	3.94E-04	2.4
stearoyl-CoA 9-desaturase activity	CG15531	3.09E-06	3.91E-04	2.0
catalytic activity	CG33174	1.30E-04	2.52E-03	2.2
ATP binding	CG33298	1.64E-07	1.35E-04	10.0
oxysterol binding	CG1513	6.82E-05	1.97E-03	2.2
lipoprotein binding	LpR1	5.25E-05	1.99E-03	20.0
catalytic activity	CG32394	1.23E-04	2.53E-03	2.8
Transcription				
transcription regulator activity	trr	2.91E-04	3.95E-03	2.3
	rno	5.77E-05	1.60E-03	2.3
transcription regulator activity	CG5319	1.11E-04	2.67E-03	4.0
translation elongation factor activity	CG31054	2.00E-05	9.96E-04	3.8
translation initiation factor activity	Rbp2	1.41E-04	2.73E-03	2.1
	CG10192	2.25E-04	3.80E-03	4.0
tRNA ligase activity	Top3	5.29E-05	1.67E-03	2.0
poly(A) binding	su(f)	3.31E-05	1.51E-03	2.1
mRNA binding	CG32423	6.13E-04	6.52E-03	2.0
	sqd	6.49E-05	1.99E-03	2.2

Table 4-2 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Downregulated genes				**Repression**
	B52	2.87E-05	1.14E-03	3.7
	ps	8.87E-06	1.36E-03	3.0
	how	1.57E-04	3.05E-03	2.9
	Fmr1	7.08E-05	1.83E-03	4.0
protein transporter activity	CG32135	2.77E-04	5.04E-03	3.4
RNA polymerase II transcription factor activity	tj	1.42E-04	2.74E-03	3.1
DNA binding	CG9727	7.61E-07	2.45E-04	5.6
	Mnt	2.06E-05	1.24E-03	2.4
pyrimidine-specific mismatch base pair DNA N-glycosylase activity	Thd1	2.84E-04	6.93E-03	2.4
zinc ion binding	CTCF	3.52E-04	4.60E-03	3.1
	MESR4	2.85E-05	1.14E-03	2.4
	CG10543	1.10E-05	7.26E-04	2.3
	CG2926	7.80E-05	1.92E-03	2.1
dihydropyrimidinase activity	CRMP	3.71E-05	3.09E-03	2.3
ATP binding	bel	6.52E-06	5.64E-04	2.2
AMP deaminase activity	CG32626	2.36E-05	1.33E-03	6.3
Development				
---	Bsg	1.52E-05	1.55E-03	2.9
structural molecule activity	shg	1.30E-05	8.11E-04	3.0
structural constituent of cytoskeleton	Dys	9.73E-05	2.90E-03	2.2
---	mud	1.41E-05	8.11E-04	5.9
ubiquitin thiolesterase activity	faf	8.39E-05	2.32E-03	2.1
transcription factor activity	bcd	9.18E-07	2.31E-04	2.9
transcription factor activity	NfI	2.56E-05	5.53E-03	3.4
actin binding	spir	2.04E-04	3.97E-03	2.9
	Tm1	9.31E-05	2.17E-03	2.1
ATP binding	Src64B	1.32E-05	8.44E-04	2.1
	hep	5.91E-05	1.71E-03	3.2
Transport				
inorganic anion exchanger activity	CG8177	1.80E-04	3.00E-03	2.3
monocarboxylic acid transporter activity	CG3409	6.67E-07	2.09E-04	4.3
protein kinase activity	CG30078	2.50E-04	4.04E-03	2.3
protein binding	pyd	5.30E-05	1.57E-03	2.9
cation transporter activity	CG32000	1.35E-06	3.79E-04	3.4
mRNA binding	xmas-2	4.18E-05	1.36E-03	2.7
guanyl-nucleotide exchange factor activity	garz	2.40E-04	3.50E-03	4.3
hydrogen-transporting ATPase activity	Sin3A	2.44E-06	3.66E-04	4.0
Cell cycle, cell proliferation				
---	larp	3.77E-06	4.43E-04	3.2
nucleic acid binding	CG32767	2.72E-04	4.60E-03	2.3
microtubule binding	nuf	2.01E-04	5.25E-03	2.6
---	oaf	1.54E-06	3.16E-04	2.2

Table 4-2 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Downregulated genes				**Repression**
structural constituent of cytoskeleton	CG38596	8.28E-07	2.25E-04	2.8
receptor binding	Eb1	5.66E-05	1.81E-03	2.1
small GTPase regulator activity	CG11727	1.00E-04	2.17E-03	2.3
---	CG3950	3.09E-05	1.56E-03	3.7
protein-tyrosine kinase activity	CG15072	2.59E-04	3.67E-03	2.6
Cell motility				
zinc ion binding	lola	1.39E-05	8.22E-04	2.4
transcription cofactor activity	CG33182	3.00E-04	4.04E-03	2.9
ATP binding	Pka-C1	9.51E-06	8.42E-04	2.3
transcription factor activity	CrebB-17A	6.25E-05	1.72E-03	2.4
Signal Transduction				
protein binding	CG31304	7.47E-06	5.64E-04	2.1
GTPase activity	Mnn1	9.71E-08	1.25E-04	6.7
signal transducer activity	gce	3.92E-07	3.02E-04	2.9
zinc ion binding	CG5316	7.86E-05	2.28E-03	3.0
protein binding	Gef26	7.39E-04	7.07E-03	2.8
ATP binding	Tao-1	2.79E-05	1.10E-03	2.3
protein phosphatase type 2A regulator activity	wdb	1.43E-05	8.28E-04	7.1
receptor binding	RhoGAP1A	1.29E-06	3.01E-04	9.1
receptor signaling protein activity	CG11940	2.72E-06	3.74E-04	3.4
structural molecule activity	caps	6.92E-05	2.27E-03	2.4
peroxidase activity	kek5	1.02E-03	8.46E-03	2.6
structural constituent of cytoskeleton	hts	3.74E-06	4.43E-04	3.8
sphingomyelin phosphodiesterase activator activity	CG1332	6.34E-04	7.05E-03	2.4
calcium ion binding	CG2165	6.63E-07	2.64E-04	2.1
Miscellaneous				
receptor activity	Lectin-galC1	8.11E-06	1.36E-03	2.4
glutathione transferase activity	gfzf	3.50E-07	1.64E-04	3.1
---	l(3)82Fd	2.26E-06	3.54E-04	5.9
biotin binding	CG1516	2.17E-04	3.69E-03	4.3
Unknown function				
---	CG5521	1.26E-04	2.46E-03	2.4
---	CG12717	5.23E-06	4.77E-04	2.9
---	CG17839	5.73E-06	1.55E-03	2.1
---	CG8034	1.17E-05	9.28E-04	2.3
---	CG18584	4.72E-04	5.59E-03	3.0
---	CG32629	4.39E-08	7.46E-05	7.1
---	CG13680	4.31E-04	5.24E-03	2.4
---	CG14446	4.11E-04	5.36E-03	3.4
---	CG40178	9.21E-06	1.76E-03	2.1
zinc ion binding	CG14306	1.94E-08	3.84E-05	3.8
---	---	2.48E-05	1.84E-03	4.5
---	CG40178	7.09E-06	1.06E-03	2.1
transcription factor binding	CG32133	2.67E-05	1.08E-03	2.5

Table 4-2 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Downregulated genes				**Repression**
---	---	1.86E-04	3.69E-03	2.5
---	CG14435	4.68E-04	7.51E-03	2.3
DNA binding	CG8765	2.76E-06	4.23E-04	4.5
---	CG14559	1.39E-06	4.49E-04	2.5
---	---	1.73E-05	9.31E-04	2.8
---	CG33224	4.01E-04	5.60E-03	7.7
---	Fancd2	1.38E-03	1.00E-02	2.8
zinc ion binding	CG11676	5.22E-06	6.31E-04	3.6
---	CG3805	2.98E-05	1.91E-03	2.3
---	CG1531	8.36E-06	6.30E-04	2.0
---	CG7546	7.27E-06	5.74E-04	2.0
---	CG3304	3.50E-05	1.32E-03	2.3
---	CG32822	1.49E-05	8.43E-04	2.1
transmembrane receptor activity	Ect4	5.36E-05	3.08E-03	2.3
protein binding	CG32611	6.65E-05	1.84E-03	3.0
mRNA binding	CG1316	9.49E-06	8.11E-04	2.6
---	CG33229	1.99E-04	5.38E-03	10.0
---	CG33090	6.72E-06	5.60E-04	4.5
DNA binding	CG8290	7.85E-07	2.15E-04	5.0
---	CG14713	1.27E-05	9.92E-04	3.7
metal ion binding	CG1407	8.20E-05	2.19E-03	2.1
---	CG9028	5.18E-05	1.60E-03	7.7
---	CG15744	1.33E-04	2.82E-03	2.2
---	CG31635	1.40E-06	2.84E-04	2.6
zinc ion binding	CG14200	5.04E-06	5.60E-04	2.7
receptor signaling protein activity	DpseGA21487	2.44E-04	4.19E-03	3.3
zinc ion binding	CG6791	4.34E-06	4.43E-04	3.2
---	CG6630	5.51E-05	2.01E-03	2.4
---	CG1308	4.67E-04	6.27E-03	2.1
---	CG31195	3.43E-06	3.91E-04	2.9
---	CG33521	2.76E-05	7.97E-03	2.3
---	CG12945	6.00E-06	5.60E-04	2.9
---	CG30422	2.95E-04	5.18E-03	3.0
---	CG1531	3.71E-05	1.32E-03	3.1
oxidoreductase activity	CG8303	8.44E-06	1.17E-03	1.9
---	CG6151	3.52E-04	4.70E-03	2.3
---	CG11505	1.12E-04	2.33E-03	3.8
---	CG8538	1.11E-03	8.90E-03	2.1
---	CG12418	3.00E-05	2.82E-03	3.0
---	CG8116	7.69E-05	1.91E-03	2.0
---	CG31035	1.27E-04	3.06E-03	2.0
---	CG7029	2.90E-04	5.79E-03	2.8
catalytic activity	CG33096	4.06E-06	4.28E-04	2.2
---	CG13784	8.92E-06	8.38E-04	2.6
---	CG12505	5.64E-08	2.09E-04	4.2
---	---	3.80E-04	4.71E-03	4.2
---	CG1347	6.93E-07	2.09E-04	3.8
---	CG32469	6.26E-05	2.83E-03	3.6

Table 4-2 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold Difference
Downregulated genes				**Repression**
---	CG14023	6.23E-04	6.38E-03	2.5
---	CG8516	4.00E-06	4.28E-04	3.0
---	CG1863	2.88E-06	4.23E-04	4.5
---	CG8949	4.34E-04	5.16E-03	2.3

genes belonged to either development or proteolysis function. Seven of the fifty-four genes could not be included in any specific groups and were grouped as miscellaneous function.

Out of the different functional categories induced or repressed by caffeine and phenobarbital, genes involved in amino acid biosynthesis, nucleic acid metabolism and oxidoreductase activity are induced only by caffeine (Table 4-3). Genes involved in carbohydrate metabolism, defense response and cytochrome P450 genes are upregulated by both caffeine and phenobarbital. Another interesting observation is that the genes involved in signal transduction are induced by caffeine but repressed by phenobarbital. Genes involved in development are downregulated by both caffeine and phenobarbital whereas the genes involved in cell motility, cell cycle and proliferation and protein biosynthesis are downregulated only by phenobarbital (Table 4-3).

Genes upregulated by both caffeine and phenobarbital

Data were also examined for genes that are upregulated by both caffeine and phenobarbital using Venn diagram (Fig. 4-2). The results showed that there are fifty genes that are induced by both caffeine and phenobarbital. Of the fifty genes upregulated both by caffeine and phenobarbital, nineteen genes have no known molecular function. Nine genes are thought to be involved in carbohydrate metabolism, which includes alpha-glucosidase activity, transporter activity and glucuronosyl transferase activity (Table 4-4). Previous studies have demonstrated the role of caffeine and phenobarbital in the upregulation of carbohydrate metabolic enzymes. One such study (Kalamidas *et al*.,

Table 4-3

Representation of the different categories of genes that are differentially expressed in caffeine and phenobarbital treated flies.

Functional categories	Caffeine		Phenobarbital	
	Induction[a] (% Total)[c]	Repression[b] (% Total)[c]	Induction[a] (% Total)[c]	Repression[b] (% Total)[c]
Amino acid biosynthesis and metabolism	6 (3.7)	-	-	-
Carbohydrate metabolism	34 (21.1)	-	11 (11.7)	-
Lipid metabolism	8 (5)	-	3 (3.2)	7 (4.1)
Co-enzyme and prosthetic group metabolism	2 (1.2)	-	-	-
Defense response	5 (3.1)	-	15 (16)	-
CytochromeP450 genes	16 (9.9)	-	13 (13.8)	-
Nucleic acid metabolism	3 (1.9)	-	-	-
Oxidoreductase activity	7 (4.3)	-	-	-
Signal transduction	11 (6.8)	-	-	14 (8.1)
Transport	6 (3.7)	-	3 (3.2)	8 (4.6)
Proteolysis	3 (1.9)	10 (18.5)	-	8 (4.6)
Development		8 (14.8)	-	15 (8.7)
Protein biosynthesis and modification	-	-	-	8 (4.6)
Transcription	-	-	-	26 (15)
Cell cycle and proliferation	-	-	-	9 (5.2)
Cell motility	-	-	-	4 (2.3)
Others	4 (2.5)	7 (13)	17 (18.1)	4 (2.3)
Unknown	57 (35)	29 (53.7)	32 (34)	70 (40.5)
Total	162 (100)	54 (100)	94 (100)	173 (100)

a, b represent the number of genes that showed induction or repression in a particular category.
c represents the percentage of total number of genes in each functional category.
- (hyphen) represents that there is no change in a particular category for the respective treatment

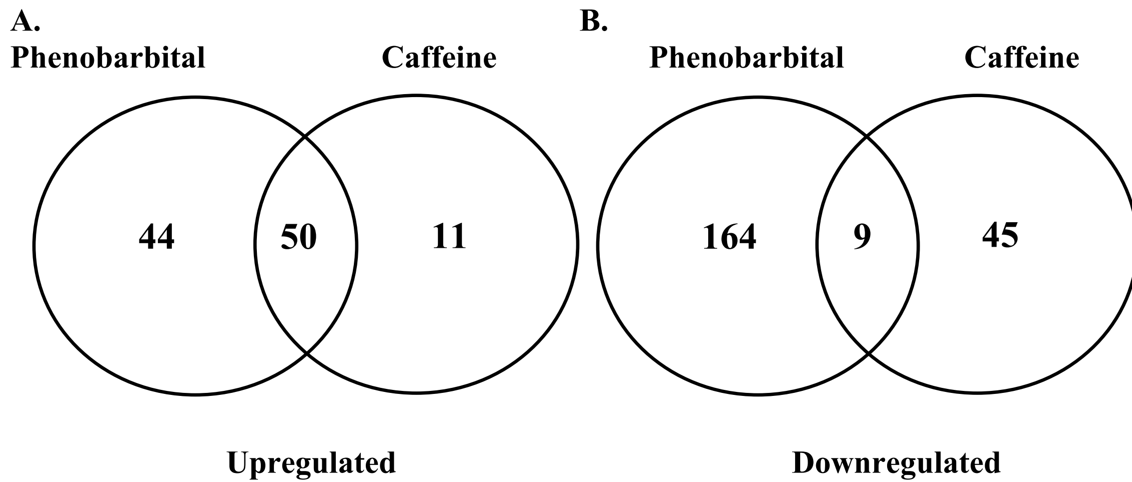


Figure 4-2: Venn diagram showing the genes differentially expressed upon treatment with caffeine and phenobarbital. **A)** Represents the genes upregulated by caffeine and phenobarbital. The values shown in each circle represent the number of genes upregulated by phenobarbital and caffeine alone. The number of common genes upregulated by both caffeine and phenobarbital is shown in the overlapping portion of the two circles. **B)** Number of genes downregulated by caffeine and phenobarbital are shown in the circles. The number of common genes downregulated by both caffeine and phenobarbital is shown in the overlapping region of the two circles.

Table 4-4

List of genes upregulated by both Caffeine and Phenobarbital

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold induction Caffeine	Fold induction Phenobarbital
Carbohydrate metabolism					
alpha-glucosidase activity	CG11669	7.86E-10	1.29E-05	10.65	2.52
	CG8693	3.28E-07	2.08E-04	3.00	2.81
	LvpD	2.65E-06	3.69E-04	3.40	2.22
	LvpL	1.53E-05	8.85E-04	2.96	2.40
transporter activity	CG30360	9.41E-08	1.09E-04	4.27	2.08
glucuronosyltransferase activity	CG15661	3.20E-04	5.36E-03	2.25	2.05
	Ugt37b1	2.63E-05	3.96E-03	2.63	3.43
	Ugt86Dd	1.75E-07	1.39E-04	4.62	3.61
	CG5724	6.53E-06	1.53E-03	5.01	6.54
Detoxification enzymes					
electron transporter activity	Cyp6a23	5.31E-05	1.86E-03	2.71	2.36
	Cyp4e2	2.36E-05	1.59E-03	2.85	2.88
	Cyp4d2	1.57E-05	1.53E-03	2.69	2.89
	Cyp4p1	9.90E-05	5.25E-03	2.68	3.09
	Cyp6a2	1.69E-05	8.53E-04	7.96	4.62
	Cyp6a8	8.61E-07	3.63E-04	11.15	11.89
	Cyp6d5	2.34E-07	1.64E-04	4.24	3.76
	Cyp6g1	3.64E-06	4.88E-04	3.73	3.27
	Cyp4d14	2.04E-06	3.05E-03	2.80	4.91
	Cyp6w1	1.84E-07	1.72E-04	14.69	14.44
epoxide hydrolase activity	Jheh2	3.11E-05	2.23E-03	2.90	3.14
glutathione transferase activity	GstD9	2.84E-06	3.82E-04	2.67	2.01
Miscellaneous enzymes					
glucosidase activity	CG31148	1.45E-04	6.13E-03	2.76	3.13
phosphatidate phosphatase activity	CG11426	1.43E-05	1.32E-03	2.44	2.53
cation transporter activity	CG2191	3.23E-06	4.99E-04	3.85	3.57
glycine N-methyltransferase activity	CG6188	2.99E-06	1.09E-03	2.59	1.38
oxidoreductase activity	CG2065	1.83E-05	9.22E-03	9.17	18.14
oxidoreductase activity	CG9360	5.74E-06	8.95E-04	13.40	14.79
aldehyde reductase activity	CG12766	1.02E-09	1.29E-05	9.49	2.22
beta-galactosidase activity	CG9092	2.15E-04	4.95E-03	2.09	2.03
galactose binding	lectin-28C	9.31E-05	9.42E-03	2.10	2.74
	CG1942	1.94E-06	2.55E-03	3.66	6.50
Unknown function					
	---	1.07E-06	2.84E-04	2.34	2.02
	---	2.41E-05	1.37E-03	2.15	2.04
	---	1.01E-05	9.52E-04	2.12	2.08

Table 4-4 continued

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold induction Caffeine	Fold induction Phenobarbital
Unknown function					
	CG15784	1.19E-06	2.64E-04	2.92	2.10
	CG16898	5.93E-07	2.08E-04	3.31	2.10
	---	7.62E-06	6.29E-04	2.66	2.15
	---	7.81E-06	8.38E-04	2.44	2.37
	CG15281	4.66E-04	8.88E-03	2.43	2.49
	---	2.66E-05	2.88E-03	2.24	2.64
	CG15407	8.66E-05	3.53E-03	2.58	2.69
	CG31104	1.30E-06	2.77E-04	6.69	3.56
	CG10553	1.45E-05	1.11E-03	3.98	3.82
	Obp56e	5.19E-05	9.19E-03	2.75	4.06
	CG13659	1.93E-05	1.01E-03	5.42	4.22
	CG10560	1.11E-06	3.16E-04	5.25	4.69
	CG11893	3.54E-07	1.64E-04	8.14	4.96
	CG31288	1.48E-06	3.91E-04	5.34	5.34
	---	6.64E-07	3.05E-04	7.67	7.87
	CG6908	7.65E-07	3.73E-04	10.42	11.67

1994) which investigated the glycogen breakdown in lysosomes of rat hepatocytes, showed an increase in the acid glucosidase activity following caffeine treatment.

However, further investigation is required to determine the significance of caffeine-induced upregulation of alpha-glucosidase activity in *Drosophila melanogaster*. Studies with phenobarbital also suggested that the alpha-glucosidase activity is increased in the developing rat liver at prenatal stage (Friedrich-Freska, 1976). Apart from the genes products with alpha glucosidase activity, several categories of genes such as aldehyde reductase activity (CG12766), transporter activity (CG31148, CG2191) and glucuronosyl transferase activity were upregulated by both caffeine and phenobarbital. The glucuronosyl transferases are microsomal enzymes that are involved in glucuronidation of exogenous substrates. This increases the polarity and solubility of substrates and helps in the elimination of foreign products from the body.

In addition to these enzymes, several other detoxification enzymes such as cytochrome P450s are upregulated by caffeine and phenobarbital. In mammals also, several *CYP* genes including CYP2C19, CYP2C9 and CYP3A4 are upregulated by barbiturate compounds (Guengerich, 2003). In *Drosophila*, out of the eighty-three P450 enzymes, *Cyp6a2* and *Cyp6a8,* are known to be induced by barbital and phenobarbital (Maitra et al., 1996). Our microarray results showed that ten of the eighty-three P450 enzymes were upregulated by both caffeine and phenobarbital (Table 4-4). However, there are some *Cyp* genes that are induced by either caffeine or phenobarbital. The lists of genes that are upregulated by either caffeine or PB are shown in Table 4-1 and 4-2. It is possible that these genes which are induced either by caffeine or by phenobarbital fall under separate regulatory pathway. Conversely, genes which are induced by both the

chemicals may be regulated by a common pathway. Different or separate pathway may constitute different *cis*- and/or *trans*-regulatory factors, whereas the common pathway may include similar regulatory sequences and/or factors. It has been shown that 0.2- and 0.8-kb upstream DNAs of *Cyp6a8* of *Drosophila* are induced by phenobarbital (Maitra et al., 2002). A recent study (Bhaskara et al., 2006) from our laboratory has shown that these two upstream DNAs of *Cyp6a8* are also induced by caffeine. Although the mechanism is not known in *Drosophila*, in mammals, phenobarbital induction of *CYP* genes is mediated by phenobarbital responsive unit or PBRU (Kim et al., 2001). The critical sequences in PBRU are NF1 and NR sites. Sequence analysis of the 0.2 kb upstream DNA of *Cyp6a8* revealed the presence of a NF1 site and four imperfect steroid binding half sites (Maitra et al; manuscript in progress). It is possible that these putative sequence motifs are responsible for phenobarbital induction. However, the *cis*-elements involved in caffeine induction are not known. It would be interesting to examine in future studies whether the putative NF1 and NR sites are involved in both caffeine and phenobarbital induction.

Apart from the Cytochrome P450 enzymes, other detoxification enzymes such as Glutathione S transferases and enzymes with oxidoreductase activity were upregulated by both caffeine and phenobarbital (Table 4-4). Among the enzymes involved in detoxification, *Cyp6w1, Cyp6a8* and *CG9360* showed the highest level of induction by both caffeine and phenobarbital suggesting an important role in the metabolism of these compounds. The expression of genes involved in oxidoreductase activity is expected to increase in response to physiological stress such as xenobiotic treatment.

Transcripts encoding developmental and proteolysis genes are downregulated by caffeine and phenobarbital.

Microarray data showed that genes involved in development are downregulated by both caffeine and phenobarbital. Out of the fifty-four genes that are downregulated by caffeine, eight of them are involved in development. The Vm34Aa, Vm26Ab, Vm26Aa and Vm32E genes are downregulated by both caffeine and phenobarbital and form the structural constituent of the vitelline membrane (Table 4-5). Among these, the proteins Vm34Ca, Vm26Aa and Vm26Ab are expressed from stage 8 to stage 10 of oogenesis and Vm32E is expressed only at stage 10. These proteins are involved in eggshell morphogenesis. The activity of Vm26Ab protein is required for the proper assembly of Vm32E protein. The VM domain present in these proteins play an important role in holding the vitelline membrane proteins together by disulfide cross-linking of cysteine residues. The expression of all these proteins is required for proper assembly of vitelline membrane (Andrenacci, 2001). Whether the downregulation of these proteins by caffeine and phenobarbital has any affect on the vitelline membrane assembly and eggshell morphogenesis needs further investigation.

Apart from the genes involved in development, proteolysis transcripts were downregulated by both caffeine and phenobarbital. However, there are no specific genes downregulated by both caffeine and phenobarbital (Table 4-1 and 4-2). A serine type protease, *nudel (ndl)* is downregulated three fold by caffeine. It is expressed in follicle cells surrounding the oocyte and is involved in the establishment of dorso-ventral axis of the embryo. Mutation in *nudel* protease results in the failure of crosslinking of vitelline membrane in the laid egg (Andrenacci, 2001). This suggests that this protease might play

Table 4-5

List of genes downregulated by both caffeine and phenobarbital

Molecular function (GO)	Gene Symbol	F value	FDR p value	Fold repression Caffeine	Fold repression Phenobarbital
Development					
structural constituent of vitelline membrane (sensu Insecta)	Vm32E	1.98E-04	3.62E-03	5.56	2.63
	Vm34Ca	1.15E-05	8.85E-04	3.13	2.44
	Vm26Ab	1.69E-05	1.14E-03	3.03	2.56
	Vm26Aa	3.55E-05	1.93E-03	2.94	2.94
Unknown function					
	CG12506	1.22E-05	1.11E-03	2.27	2.27
	CG13997	1.60E-04	3.83E-03	3.57	2.86
	CG13947	1.46E-04	4.86E-03	2.78	3.13
	CG13946	2.92E-07	2.09E-04	5.56	5.56
	CG38687	1.16E-05	9.21E-04	4.00	2.94

an important role in eggshell biogenesis. It is also not known if the *ndl* protease is a regulator of the vitelline membrane proteins such as Vm32E, Vm26Ab that are involved in crosslinking of the vitelline membrane to the laid egg. However, the effect of downregulation of this serine protease on eggshell biogenesis and the dorsoventral patterning is not known. There are several other proteolysis genes upregulated by caffeine that has putative metallocarboxypeptidase activity, metalloendopeptidase activity and serine type endopeptidase activity. However, no studies have been present so far regarding the physiological function of these genes. There is an upregulation of some of the proteolytic genes such as CG9649, CG14528 and CG6041 that possess enteropeptidase, endopeptidase and trypsin activity respectively.

In *Musca domestica*, the increased proteolytic activity in response to stress is to cope with the energy demands and to further balance the protein degradation and synthesis (Ahmed et al., 1998). The role of induction of these enzymes by caffeine is not known. Several proteolytic enzymes such as ubiquitin protein ligases and carboxypeptidases were downregulated by phenobarbital. However, the physiological significance of the downregulation is not known. Carboxypeptidases are required for processing the neuropeptide hormones and other proteins. One of the carboxypeptidase genes named as *Silver (svr)*, is downregulated 2.5 fold by phenobarbital and is enriched in the central nervous system. It was shown that this gene is important for viability in *Drosophila melanogaster* (Stephen, 1995).

Induction of signal transduction genes by caffeine and repression by phenobarbital

Several genes involved in signal transduction such as IP3K2, phosphatases, and calcium ion binding proteins are upregulated greater than two-fold by caffeine (Table 4-1). The intracellular calcium levels are increased in response to caffeine, which in turn triggers the increase of calcium ion binding proteins. In our microarray experiment, we found that *smp-30*; a calcium ion binding protein is increased seven-fold in response to caffeine. This validates the previous observations in mice that the intracellular calcium levels are increased in response to caffeine and several calcium binding proteins such as calmodulin, parvalbumin, troponin C are upregulated in order to transform the increase in intracellular calcium into a molecular cascade. In addition to the calcium binding proteins, IP3K2, which acts to release the intracellular calcium, is upregulated by caffeine. This suggests that caffeine triggers the molecular signaling cascade by the release of intracellular calcium. Apart from the genes involved in calcium signaling cascade, genes with adenosine deaminase activity were also upregulated. The gene, Adgf-D (Adenosine deaminase growth factor-D) is upregulated by caffeine and is involved in the polarization and serum- independent proliferation of imaginal disc and embryonic cells *in vitro* (Zurovec, 2002). Enzyme activity of Adgf-D is required for the mitogenic function of the cells.

The genes involved in signal transduction are downregulated in response to phenobarbital. The list of genes downregulated by phenobarbital is shown in Table 4-2. *Disabled (Dab)* is one of the genes downregulated 2.3 fold by phenobarbital. It is involved in the ommatidial development in the SEV (sevenless tyrosine receptor kinase) signaling pathway. DAB may function downstream of many RTKs, including ones

required for proper development of the *Drosophila* central nervous system. *Mnn1* gene with GTPase activity is downregulated six-fold by phenobarbital. A recent study demonstrated that the function of this gene is to maintain genome integrity and is similar to BRCA and HNPCC genes in humans (Busygina, 2004). The expression of this gene is regulated in response to stress such as heat shock in *Drosophila* and the *Mnn* gene in turn controls the expression of heat shock proteins (Papaconstantinou, 2005). The effect of the repression of this gene in response to phenobarbital is not known. *Gce* (*germ-cell expressed*) is another gene that is downregulated 2.9 fold in response to phenobarbital. This gene has signal transducer activity and is suggested to be involved in insect development or metamorphosis as juvenile hormone receptor components (Godlewski, 2006). Apart from these several other genes such as *gef26, Tao-1, wdb, RhoGAP1A, caps, kek5* and *hts* are downregulated significantly in response to phenobarbital and the effect of this downregulation needs to be investigated.

Downregulation of genes involved in transcription by phenobarbital

Genes coding for transcription factor were downregulated by phenobarbital whereas no effect was observed following caffeine treatment. The genes downregulated by phenobarbital belong to different classes such as transcription regulators, t-RNA ligases, mRNA binding factors, Zinc ion and ATP binding factors. *Rhinoceros (rno)* is one of the transcription factors downregulated by phenobarbital and is involved in regulating the EGFR signaling pathway in the eye. It has a PHD zinc finger domain, a motif commonly found in chromatin remodeling factors (Voas, 2003). Rbp2, a retinoblastoma binding protein is also downregulated by phenobarbital and *Squid* (*sqd*)

gene, repressed by two-fold is known to bind to several cellular RNAs such as *gurken (grn)* RNA and plays a specific developmental role in the determination of dorsoventral axis formation during *Drosophila* oogenesis (Steinhauer, 2005). CTCF binds to the insulators and blocks the enhancer activity thereby repressing transcription of the downstream genes (Yusufzai, 2004). The top3 cDNA is approximately 60% identical to the mammalian topoisomerases. They are involved in the relaxation of hypernegatively supercoiled DNA. These proteins are suggested to play important role in the strand separation processes such as recombination and chromosome segregation (Wilson, 2000). The top3 protein is downregulated two-fold by phenobarbital and the implication of this downregulation is not known. A trithorax related gene in *Drosophila* encodes *Trr,* which is a histone methyltransferase involved in the methylation at lysine 4 of histone H3. It functions as a transcriptional co-activator to EcR (ecdysone receptor) by altering the chromatin structure at the ecdysone response promoters. It acts upstream of hedgehog (hh) signaling in retinal differentiation in the morphogenic furrow progression and post furrow photoreceptor differentiation (Sedkov and Jaynes, 2003). B52 is another protein in *Drosophila* which functions as a regulator of 5' splice site *in vivo* and acts as a general splicing factor. Experiments to overexpress this gene in the developmental stages resulted in adverse affects suggesting that B52 plays a major role in development (Kraus, 1994). In our microarray experiments, we observed that there is a four-fold downregulation of B52 by phenobarbital. Whether this downregulation has any effect on the development or in the splicing phenomenon is not understood.

Conclusions

Microarray technology was used to study the induction of genes by xenobiotics such as caffeine and phenobarbital. In humans, these xenobiotics were widely used to understand the mechanism of *Cyp* gene regulation. The present study showed that the defense response genes such as cytochrome P450, glutathione S transferases, and carbohydrate metabolism genes were overexpressed by both caffeine and phenobarbital. Also, developmental genes, proteolytic genes were downregulated by both caffeine and phenobarbital. Signal transduction genes were upregulated by caffeine but downregulated by phenobarbital. In order to exactly understand the mechanism of induction by caffeine and phenobarbital, these candidate genes need to be investigated. However, there is a large set of genes for which the physiological function or the phenotype is not known. This "phenotype gap" is hampering the implementation of functional genomics approach, hence the studies using "reverse genetics" will aid in closing the gap. This will help in better understanding of the inter-related molecular networks in multicellular model organisms.

Chapter V

Conclusions

Insecticide resistance is the ability of the individuals to survive the doses of insecticides which are otherwise toxic to the normal population. Several mechanisms of resistance have been postulated in insects (Feyereisen, 1999). These are target site insensitivity, reduced penetrance and metabolic detoxification (Scott, 2001). However, the metabolic detoxification is the major contributor of insecticide resistance in insects (Chapter 1). Enzymes such as glutathione S transferases, carboxylesterases and cytochrome P450 monooxygenases are induced by xenobiotics, which in turn detoxify them (Feyereisen, 1999). Increased detoxification is brought by the modification of P450 enzymes already present in the wild type strains or by increase in the amount of the enzyme in the resistant strains. The increased resistance will lead to increased use of pesticide which is toxic to human health and environment (McKenzie and Batterham, 1998). Understanding the molecular mechanisms of resistance as well as the regulation of P450 enzymes will aid in the management of insecticide resistance. This helps to prevent or reverse the development of resistance in pests (Elzen and Hardee, 2003). The strategy to select for resistance in the laboratory before the chemical is released into the environment will allow us to anticipate the likely mechanisms of resistance before they evolve in natural populations (McKenzie and Batterham, 1998). To date, the molecular mechanisms that regulate insecticide resistance in insects are largely unknown.

To design strategies to control resistance, a thorough understanding of the molecular mechanisms governing insecticide resistance is required as also the factors that determine the evolution of resistance (Scott, 1999). Since overexpression of CYPs is identified to be one of the mechanisms of resistance, strategies to regulate the levels of CYPs might be an important strategy for the design of novel insect control agents. The molecular analysis of the cytochrome P450-mediated insecticide resistance will pave a way to achieve the long term goal of devising strategies to control resistance. Systematic genetic and molecular studies are necessary to examine the role of CYPs in resistance especially in agriculturally or economically important insects. However, the genetics of many economically important pests is not understood and may not be amenable to molecular and genetic studies. Hence, *Drosophila* is used as a model insect to study the mechanism of CYP-mediated insecticide resistance in *Drosophila.* The genetics of *Drosophila* is well known and its genome is completely sequenced which offers a vast array of molecular tools to manipulate the genome and understand any biological phenomenon.

In this dissertation, the molecular mechanism of cytochrome P450-mediated insecticide resistance was studied in *Drosophila melanogaster*. Studies from many years could not resolve whether DDT resistance is mono or multi-factorial. (Tsukamoto and Ogaki, 1953; Tsukamoto, 1958; Crow, 1957; Oshima, 1954; King and Somme, 1958). The present investigation stems from a recent report by Daborn et al., (2002), which showed that expression of *Cyp6g1* is necessary and sufficient to confer DDT resistance in *Drosophila melanogaster* (Chapter 1 and 2). The present investigation showed that there is no correlation between the expression of *Cyp6g1* and DDT resistance. A direct

correlation between the presence of an *Accord* transposable element in the *Cyp6g1* upstream DNA, expression of *Cyp6g1* and DDT resistance was reported (Daborn et al., 2002). Another study has examined 673 strains for all around the world for the presence of an *Accord* transposable element in the upstream DNA of *Cyp6g1* (Catania et al., 2004). They observed a 100% correlation between the presence of transposable element and the resistance to DDT. In my study, I examined this phenomenon and observed that the Canton SH and Hikone RH strains that have an *Accord* transposable element in the *Cyp6g1* upstream DNA show high expression of *Cyp6g1* but they are highly susceptible to DDT (Chapter 2). This suggests that in the laboratory strains I examined; the presence of *Accord* transposable element correlates with the elevated expression level of *Cyp6g1* but not with DDT resistance.

Lack of correlation between the expression of *Cyp6g1* gene and insecticide resistance is observed in other situations as well. The present study supports the study by Festucci-Buselli et al (2005), where they showed that the strains that show high expression of *Cyp6g1* does not necessarily show high level of DDT resistance. However, they did not provide quantitative data and the fold difference in the expression relative to the resistance was not accurately determined. Hence, we reexamined this issue and quantified the *Cyp6g1* expression at the RNA and protein levels in different strains. Another study also showed that a complete correlation does not occur between the presence of *Accord* transposable element in the *Cyp6g1* upstream DNA and DDT resistance (Schlenke and Begun, 2004). In their study, CS1 strain from *Drosophila simulans* with high *Cyp6g1* expression showed 84% mortality when exposed to 20μg

DDT for 18 hrs. Similarly, CM2 and AM3 lines of *D.melanogaster* showing high expression of *Cyp6g1* are found to be susceptible to 20µg DDT.

Although the present study shows that there is no correlation between the DDT resistance phenotype and expression of *Cyp6g1*, we do not completely rule out the possibility of the role of *Cyp6g1* in DDT resistance. As proposed earlier, DDT resistance in *Drosophila* is complex and may be polygenic trait (Crow, 1957; Oshima, 1954; King and Somme, 1958). One hypothesis is that *Cyp6g1* may be a team player and the expression of other *Cyp* genes or other detoxifying enzymes such as GSTs are required to confer full-blown resistance in *Drosophila melanogaster*. In the highly resistant 91-R strain, the *Cyp6a2, Cyp6w1, Cyp6a8* and *Cyp6g1* show high level of expression compared to the *Wisconsin* strain, where the *Cyp12d1* is highly expressed (Chapter 2, Pedra et al., 2004). The LC_{50}s of 91R and *Wisconsin* are 8348µg and 447µg respectively. This study leads to a hypothesis whether DDT metabolism is a quantitative trait and depends on the expression of multiple *Cyp* genes. It is possible that the level of DDT resistance is directly correlated to the number of *Cyp* genes that show highest expression in an organism. In order to further investigate whether high expression of *Cyp6g1* is necessary for resistance, we recombined the *Cyp6g1* allele of the resistant 91-R strain with that of the susceptible 91-C strain. This recombinant strain (RC-21) showed high level of resistance similar to 91-R strain but low expression of *Cyp6g1* (Chapter 2). This suggests that genes other than *Cyp6g1* may play key role in DDT resistance. To identify the genes overexpressed in the RC-21 strain, we performed whole genome microarray of this strain along with 91-R and 91-C strains. Microarray analysis revealed that 10 *Cyp* genes that are overexpressed in 91-R strain compared to 91-C strain. *Cyp 6* family genes,

Cyp6a2, Cyp6a8, Cyp6w1, Cyp6g1 and *Cyp4* family of genes such as *Cyp4p1, Cyp4p2, Cyp4d1* and *Cyp4d20* were upregulated in 91-R strain compared to 91-C strain. Examination of the common upregulated genes between RC-21 and 91-R strains compared to the 91-C strain showed that *Cyp6a2*, *Cyp6w1* and *Cyp4p1*, *Cyp309a1* and *Cyp4d1* are the *Cyp* genes upregulated in both the strains (Chapter 2). Overexpression of these genes was observed in other cases as well. Microarray analysis by Pedra et al., (2004) showed that *Cyp6w1, Cyp6a2* and *Cyp4p1* are overexpressed in the resistant *Wisconsin* and 91-R strains compared to the susceptible Canton S strain. Microarray analysis also revealed that carbohydrate metabolism genes are upregulated in both RC-21 and 91-R strain compared to the susceptible 91-C strain. It is known that DDT metabolism is associated with glucose utilization and the activity of isocitrate and glucose-6-phospate dehydrogenases was increased in *Pseudomonas aeruginosa* strains capable of metabolizing DDT (Maltseva and Golovleva, 1982). Studies of DDT metabolism in mammals have shown to affect β-oxidation of fatty acids (Oda et al., 1994).

In order to test the *in vivo* role of *Cyp6a2* in DDT resistance, we transformed *Cyp6a2* in the w^{1118} strain using GAL4/UAS system. Results showed that there is a two-fold increase in the *Cyp6a2* expression as well as resistance (Chapter 3). The polygenic nature of DDT resistance was tested by double transforming the *Cyp6a2* and *Cyp6g1* transgenic flies into the same genetic background. Results showed that there is an additive effect on resistance when *Cyp6g1* and *Cyp6a2* were overexpressed in the same fly (Chapter 3). This may suggest the polygenic nature of the DDT resistance phenotype which was hypothesized earlier (Crow 1957; King an Somme, 1958). Since the high

expression of *Cyp* genes was not observed in the transgenic lines, it cannot be concluded whether the expression of a single allele can confer DDT resistance in *Drosophila* (Chapter 3). However, the results of the present investigation suggest that DDT metabolism in *Drosophila* requires the expression of more than one *Cyp* gene or some other unknown factors. Previous studies have shown the presence of a master regulator on the second chromosome of housefly that regulates the expression of CYP6D1 and CYP6A1 in *Musca domestica* (Carino et al., 1994; Liu and Scott, 1995). Studies by Maitra et al., (2000) and Dombrowski et al (1998) in *Drosophila* have shown that the expression of *Cyp6a2* and *Cyp6a8* genes on the second chromosome is influenced by factors on the third chromosome. Deficiency mapping experiments and further sequencing will help understand the regulatory gene (s) present on the third chromosome.

Several P450 genes such as *Cyp6a2, Cyp6w1, Cyp6g1* and *Cyp6a8* that are observed in high levels in the resistant strains are induced by xenobiotics such as barbiturate compounds and caffeine. It was shown that induction with phenobarbital causes increase in P450 activity as well as increased resistance to DDT and pyrethroids (Amichot et al., 1994, 1998). Studies of phenobarbital induction in *Musca* have suggested the presence of a trans-regulatory factor that influences the level of PB-mediated CYP6D1 induction (Liu and Scott, 1995). It is also known that a master regulator on second chromosome influences the constitutive expression of CYP6D1 and CYP6A1 in the resistant strains of *Musca domestica*. Taken together, it may be hypothesized that the mechanism of resistance and PB induction may share some common regulatory pathways. Earlier studies have suggested that same regulatory gene may be involved in both xenobiotic induction and metabolic resistance (Terriere, 1983). It was also suggested

that the receptor involved in xenobiotic induction may be altered in resistant insects (Plapp, 1984). However, the link between xenobiotic induction and the metabolic resistance remains unresolved.

Microarray analysis of Canton S strain induced with PB and caffeine revealed two groups that are induced by both these chemicals. They are carbohydrate metabolism and detoxification enzymes. Among the detoxifying enzymes, the *Cyp* genes that are induced belong to *Cyp6* and *Cyp4* families (Chapter 3). Most of the *Cyp* genes that showed induction with phenobarbital and caffeine are constitutively expressed at high levels in insecticide resistant strains. Comparison of the microarray data of resistant 91-R and RC-21 strains with that of the susceptible 91-C strain showed that detoxification enzymes and carbohydrate metabolism genes are overexpressed in both the resistant strains (Chapter 2). This result is similar to that observed for the induction studies with phenobarbital and caffeine in *Drosophila*. Although several hypotheses exist that there is a link between resistance phenotype and xenobiotic induction, it is not known whether there is an underlying common mechanism between the two phenomena. Studies by Kacew and Singhal (1973) have shown that the DDT treatment of the liver and kidney tissues leads to increase in the cyclic AMP levels that will increase carbohydrate metabolism. Increase in cAMP levels leads to increase in phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (Wicks, 1969). These enzymes catalyze the first two steps of the gluconeogenesis pathway. It is known that induction with caffeine also results in elevated levels of cyclic AMP (Fredholm et al., 1998). Although the downstream effectors of the DDT- mediated increase of cAMP is not known, it is possible that the induction by caffeine and PB may share a common regulatory pathway with insecticide resistance.

The results presented in this dissertation have advanced our knowledge about the relationship between overexpression of *Cyp* genes and DDT resistance in *Drosophila melanogaster*. These studies have shed more light on the xenobiotic induction and advanced our understanding of the *Cyp* mediated insecticide resistance. The present research has opened lot of avenues that can be explored in the area of xenobiotic induction and insecticide resistance in *Drosophila melanogaster*.

REFERENCES

Agosin, M. (1985). "Role of microsomal oxidations in insecticide degradation." Comprehensive insect physiology, biochemistry and pharmacology **12**: 647-712.

Ahmed, S., Wilkins R.M and Mantle D (1998). "Comparison of proteolytic enzyme activities in adults of insecticide resistant and susceptible strains of the housefly *M.domestica* L." Insect biochemistry and molecular biology **28**(9): 629-639.

Amichot, M., Tares, S., Brun-Barale, A., Arthaud, L., Bride, J-M. & Berge, J-B (2004). "Point mutations associated with insecticide resistance in the *Drosophila* cytochrome P450 *Cyp6a2* enable DDT metabolism." European Journal of Biochemistry **271**: 1250-1257.

Andrenacci, D., Cerniloger, M.F, Taddei, C, Rotoli, D, Cavaliere, V, Graziani, F, Gargiulo, G (2001). "Specific domains drive VM32E protein distribution and integration in *Drosophila* eggshell layers." Journal of cell science **114**(15): 2819-2829.

Berenbaum, MC. (1981). " Criteria for analyzing interactions between biologically active agents". Adv Cancer Res.**35**: 269-335

Berenbaum, M. (1995). "Chemical defense: theory and practice." Proc. Natl. Acad. Sci. USA **92**: 2-8.

Berge, J. B., Feyereisen, R., Amichot, M. (1998). "Cytochrome P450 monooxygenases and insecticide resistance in insects." Philosophical transactions research society London B **353**: 1701-1705.

Bernhardt, R. (2006). "Cytochromes P450 as versatile biocatalysts." Journal of Biotechnology **124**: 128-145.

Bhaskara, S., Dean, ED, Lam, V, Ganguly, R. (2006). "Induction of two cytochrome P450 genes, *Cyp6a2* and *Cyp6a8*, of *Drosophila melanogaster* by caffeine in adult flies and in cell culture." Gene **377**: 56-64.

Brand, A. H., and Dormand, E.-L., (1995). "The GAL4 system as a tool for unraveling the mysteries of the *Drosophila* nervous system." Current opinion in neurobiology **5**: 572-578.

Brand, A. (1993). "Targeted gene expression as a means of altering cell fates and dominant phenotypes." Development **118**: 401-415.

Brandt, A., Scharf M, Pedra JH, Holmes G, Dean A, Kreitman M, Pittendrigh BR (2002). "Differential expression and induction of two *Drosophila* cytochrome P450 genes near the Rst(2)DDT locus." Insect molecular biology **11**(4): 337-41.

Busygina, V., Suphapeetiporn, K, Marek, LR, Stowers, RS (2004). "Hypermutability in a *Drosophila* model for multiple endocrine neoplasia type I." Human molecular genetics **13**(20): 2399-2408.

Carino, F., Koener, JF, Plapp, FW Jr, Feyereisen, R. (1994). "Constitutive overexpression of the cytochrome P450 gene CYP6A1 in a house fly strain with metabolic resistance to insecticides." Insect Biochem Mol Biol **24**(4): 411-8.

Carino, F., Koener, J.F., Plapp, F.W., and Feyereisen, R. (1992). "Expression of the cytochrome P450 gene *CYP6A1* in the housefly, *Musca Domestica*." 31-40.

Catania, F., Kauer, M.O., Daborn, P.J., Yen, J.L., Ffrench-constant, R., Schlotterer, C. (2004). "World-wide survey of an *Accord* insertion and its association with DDT resistance in *Drosophila melanogaster*." Molecular ecology **13**: 2491-2504.

Chavez, V. M., Marques, G, Delbecque, JP, Kobayashi, K, Hollingsworth, M, Burr, J, Natzle, JE, O'Connor, MB. (2000). "The *Drosophila* disembodied gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels." Development. **127**(19): 4115-26.

Cooper, D. Y., Levin, S., Narasimhulu, S., Rosenthal, O.,Estabrook, R.W. (1965). "Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems." Science **147**: 400-402.

Crow, J. F. (1954). "Analysis of a DDT-resistant strain in *Drosophila*." J Econ Entomol **47**: 393-398.

Crow, J. F. (1957). "Genetics of insecticide resistance to chemicals." Annual reviews in Entomology **2**: 227-246.

Daborn, P., Boundy, S, Yen, J., Pittendrigh, B., ffrench-Constant, R. (2001). "DDT resistance in *Drosophila* correlates with *Cyp6g1* overexpression and confers cross resistance to the neonicotinoid imidacloprid." Molecular genetics and genomics **266**: 556-563.

Daborn, PJ, Yen, J., Bogwitz MR, Le Goff, G, Feil, E, Jeffers, S, Tijet, N, Perry, T, Heckel D, Batterham, P, Feyereisen, R, Wilson, TG, ffrench-Constant, RH. (2002). "A single p450 allele associated with insecticide resistance in *Drosophila*." Science **297**(5590): 2253-6.

Danielson, P. B. (2002). "The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans." Current drug metabolism **3**(6): 561-597.

Danielson, P. B., MacIntyre, R.J., Fogelman, J.C (1997). "Molecular cloning of a family of xenobiotic-inducible drosophilid cytochrome P450s: Evidence for involvement in host-plant allelochemical resistance." Proceedings of national academy of sciences **94**: 10797-10802.

Dapkus, D. (1992). "Genetic localization of DDT resistance in *Drosophila melanogaster* (Diptera: Drosophilidae)." J. Econ. Entomol **85**: 340-347.

Dapkus, D. and Merrell, D.J., (1977). "Chromosomal analysis of DDT-resistance in a long-term selected population of *Drosophila melanogaster*." Genetics **87**: 685-697.

Denholm, I., Devine, GJ, Williamson, MS. (2002). "Evolutionary genetics. Insecticide resistance on the move." Science **297**(5590): 2222-2223.

Dombrowski, S., Krishnan, R, Witte, M, Maitra, S, Diesing, C, Waters, LC, Ganguly, R. (1998). "Constitutive and barbital-induced expression of the *Cyp6a2* allele of a high producer strain of CYP6A2 in the genetic background of a low producer strain." Gene **221**(1): 69-77.

Dunkov, B. C., Guzov, V.M., Mocelin, G., Shotkoski, F., Brun, A., Amichot, M., Ffrench constant, R.H., and Feyerisen R. (1997). "The *Drosophila* cytochrome P450 gene *Cyp6a2*: structure, localization, heterologous expression and induction by phenobarbital." DNA cell biology **16**: 1345-1356.

Elzen, G., Hardee, DD. (2003). "United states department of agriculture- agricultural research service research on managing insect resistance to insecticides." Pesticide management science **59**(6-7): 770-776.

Estabrook, R., Cooper DY, Rosenthal O. (1963). "The light reversible carbon monoxide inhibition of the steroid C21 hydroxylase system of the adrenal cortex." Biochemical and biophysical research communications **338**: 741-55.

Estabrook, R. W. (1996). "The remarkable P450s: a historical overview of these versatile hemeprotein catalysts." FASEB journal **10**: 202-204.

Festucci-Buselli, R., Carvalho-Dias AS, de Oliveira-Andrade M, Caixeta-Nunes C, Li HM, Stuart JJ, Muir W, Scharf ME, Pittendrigh BR. (2005). "Expression of *Cyp6g1* and *Cyp12d1* in DDT resistant and susceptible strains of *Drosophila melanogaster*." Insect molecular biology **14**(1): 69-77.

Feyereisen, R. (1995). "Molecular biology of insecticide resistance." Toxicol Lett **82-83**: 83-90.

Feyereisen, R. (1999). "Insect P450 enzymes." Annual reviews in Entomology **44**: 507-533.

Fisone, G., Borgkvist, A., Usiello, A. (2004). "Caffeine as a psychomotor stimulant: mechanism of action." Cellular and molecular life sciences **61**: 857-872.

Fleming, B., Johnson DL, Bond AM, Martin LL. (2006). "Recent progress in cytochrome P450 enzyme electrochemistry." Expert Opin Drug Metab Toxicol. **2**(4): 581-9.

Fujii-Kuriyama, Y., and Mimura, J. (2005). "Molecular mechanisms of AhR functions in the regulation of cytochrome P450 genes." Biochemical and Biophysical research communications **338**(1): 311-317.

Fulco, B. (1983). "Induction by barbiturates of a cytochrome p-450-dependent fatty acid monooxygenase in *Bacillus megaterium*: relationship between barbiturate structure and inducer activity." Biochemical and biophysical research communications **116**(3): 843-850.

Gautier, J., Leocoeur, S., Cosme, J., Perret, A., Urban P., Beaume P., Pompon, D. (1996). "Contribution of human cytochrome P450 to benzol[a] pyrene and benzol[a]pyrene7,8-dihydrodiol metabolism as predicted from heterologous expression in yeast." Pharmacogenetics **6**: 489-499.

Gilbert, L. I. (2004). "Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*." Molecular and cellular endocrinology **215**: 1-10.

Goasduff, T., Yvonne Dr, Guillois B, Franfois J and Berthou F (1996). "Induction of liver and kidney CYP1A1/1A2 by Caffeine in Rat." Biochemical Pharmacology **52**: 1915-1919.

Godlewski, J., Wang S, Wilson TG (2006). "Interaction of bHLH-PAS proteins involved in juvenile hormone reception in *Drosophila*." Biochemical and Biophysical research communications **342**(4): 1305-1311.

Gonzalez, F. (1992). "Human cytochromes P450: problems and prospects." Trends Pharmacol Sci. **13**(9): 346-52.

Gore, M. A., Morshedi, M.M., Reidhaar-Olson, J.F. (2000). "Gene expression changes associated with cytotoxicity identified using cDNA arrays." Funct Integr Genomics **1**: 114-126.

Graham-Lorence, S., Petersen, J.A. (1999). "How similar are P450s and what can their differences teach us?" Archives of biochemistry and biophysics **369**: 24-29.

Graham-Lorence, S., Petersen, J.A. (1999). "P450s: structural similarities and functional differences." FASEB journal **10**: 206-214.

Guengerich, F. (2003). "Cytochromes P450, drugs, and diseases." Molecular interventions **3**(4): 194-204.

Guengerich, F. (2006). "Cytochrome P450s and other enzymes in drug metabolism and toxicity." AAPs J **8**(1): E101-11.

Guengerich, F., Johnson WW, Shimada T, Ueng YF, Yamazaki H, Langouet S (1998). "Activation and detoxication of aflatoxin B1." Mutat Res. **402**(1-2): 121-8.

Guzov, V., Unnithan GC, Chernogolov AA, Feyereisen R (1998). "CYP12A1, a mitochondrial cytochrome P450 from the house fly." Arch Biochem Biophys. **359**(2): 231-40.

Hallstrom, I. (1985). "Genetic regulation of the cytochrome P450 system in *Drosophila melanogaster*. II. Localization of some genes regulating cytochrome P450 activity." Chemical and biological interactions **56**: 173-184.

Hankinson (1994). "A genetic analysis of processes regulating cytochrome P4501A1 expression." Adv Enzyme Regul. **34**: 159-71.

He JS, Liang Q., Fulco AJ. (1995). "The molecular cloning and characterization of BM1P1 and BM1P2 proteins, putative positive transcription factors involved in barbiturate-mediated induction of the genes encoding cytochrome P450BM-1 of Bacillus megaterium." J Biol Chem. **270**(31): 18615-25.

Helvig , C., Tijet N, Feyereisen R, Walker FA, Restifo LL. (2004). "*Drosophila melanogaster* CYP6A8, an insect P450 that catalyzes lauric acid (omega-1)-hydroxylation." Biochem Biophys Res Commun. **325**(4): 1495-502.

Hodgson E., Rose. RL., Goh DK, Rock GC, Roe RM. (1993). "Insect cytochrome P-450: metabolism and resistance to insecticides." Biochem Soc Trans. **21**(4): 1060-5.

Honkakoski P, Z. I., Sueyoshi T, Negishi M. (1998). "The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene." Mol Cell Biol. **18**(10): 5652-8.

Houpt, D. R., Pursey, J. C. and Morton, P. A. (1988). "Genes controlling malathion resistance in a laboratory selected population of *Drosophila melanogaster*." Genome **30**: 844-853.

Hung, C.-F., Berenbaum MR, Schuler MA. (1997). "Isolation and characterization of CYP6B4, a furanocoumarin-inducible cytochrome P450 from a polyphagous caterpillar (Lepidoptera: Papilionidae)." Insect Biochem. Mol. Biol. **27**: 377-85.

Hung, C.-F., Harrison TL,Berenbaum MR,Schuler MA (1995). "CYP6B3: a second furanocoumarin-inducible cytochrome P450 expressed in *Papilio polyxenes*." Insect Mol.Biol **4**: 149-60.

Jensen, H. R., Scott, I.M., Sims, S., Trudeau, V.L., Arnason, J.T. (2006). "Gene expression profiles of *Drosophila melanogaster* exposed to an insecticidal extract of *Piper nigrum*." Journal of Agricultural and food chemistry **54**: 1289-1295.

Kakizaki S, Karami S, Negishi M. (2002). " Retinoic acids repress constitutive active receptor-mediated induction by 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene of the CYP2B10 gene in mouse primary hepatocytes". Drug Metab Dispos. 30(2):208-11

Kalamidas, S., Kotoulas OB, Kotoulas AO, Maintas DB. (1994). "The breakdown of glycogen in the lysosomes of newborn rat hepatocytes: the effects of glucose, cyclic 3',5'-AMP and caffeine." Histol Histopathol. **9**(4): 691-8.

Karess, R. (1985). "P element mediated germ line transformation of *Drosophila*." DNA cloning: A practical approach: 121-141.

Karess, R., and Rubin, G.E. (1984). "Analysis of P transposable element functions in *Drosophila*." Cell **38**: 135-146.

Kim, J., Min, G., Kemper, B. (2001). "Chromatin assembly enhances binding to the CYP2B1 phenobarbital responsive unit (PBRU) of nuclear factor-1 which binds simultaneously with constitutive androstane receptor (CAR)/Retinoid X receptor

(RXR) enhances CAR/RXR mediated activation of PBRU." Journal of biological chemistry **276**(10): 7559-7567.

King, J. and Somme, L (1958). "Chromosomal analysis of the genetic factors for resistance to DDT in two resistant lines of *Drosophila melanogaster*." Genetics **43**: 577-593.

Klingenberg, M. (1958). "Pigments of rat liver microsomes." Archives of biochemistry and biophysics **75**: 376-386.

Korytko, P. J., Maclntyre, R.J. and Scott, J.G. (2000). "Expression and activity of a house-fly cytochrome P450, CYP6D1, in *Drosophila melanogaster*." Insect molecular biology **9**(5): 441-449.

Kraus, M., Lis JT (1994). "The Concentration of B52, an essential splicing factor and regulator of splice site choice *in vitro*, is critical for *Drosophila* development." Molecular and cellular biology **14**(8): 5360-5370.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**: 680-685.

Leemans, R., Egger, B., Loop, T., Kammermeier, L., He, H., Hartmann, B., Certa, U., Hirth, F., Reichert, H. (2000). "Quantitative transcript imaging in normal and heat shocked *Drosophila* embryos by using high density oligonucleotide arrays." Proceedings of the national academy of sciences **97**(22): 12138-12143.

LeGoff, G., Boundy, S., Daborn, PJ., Yen JL, Sofer, L et al (2003). "Microarray analysis of cytochrome P450 mediated insecticide resistance in *Drosophila*." Insect Biochemistry and molecular biology **33**: 701-708.

LeMosy, E. K. and Hashimoto, C. (2000). "The Nudel protease of *Drosophila* is required for eggshell biogenesis in addition to embryonic patterning." Developmental biology **217**: 352-361.

Lewis, D. (1996). "Cytochromes P450: Structure, function and mechanism." Taylor and Francis, Bristol, PA.

Li, W., Berenbaum, MR, Schuler, MA. (2001). "Molecular analysis of multiple CYP6B genes from polyphagous *Papilio* species." Insect Biochem Mol Biol **31**(10): 999-1011.

Li, X, Baundry, J., Berenbaum, MR, Schuler, MA. (2004). "Structural and functional divergence of insect CYP6B proteins: From specialist to generalist cytochrome P450." Proc Natl Acad Sci U S A. **101**(9): 2939-44.

Li, X., Schuler, MA, Berenbaum, MR. (2006). "Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics." Annu Rev Entomol. Aug 22 (Epub ahead of print).

Lindsley, D. L., and Zimm, G.G. (1992). "The genome of *Drosophila melanogaster*." Academic press Inc.

Liu, N., Scott, J.G (1995). "Genetics of resistance to pyrethroid insecticides in the house fly, *Musca domestica*." Pestic. Biochem. Physiol **52**: 116-124.

Liu, N., Scott, JG (1998). " Increased transcription of CYP6D1 causes cytochrome P450-mediated insecticide resistance in house fly". Insect Biochem Mol Biol. **28**(8):531-5

Lorist, M., Tops, M. (2003). "Caffeine, fatigue, and cognition." Brain Cogn. **53**(1): 82-94.

Lund, E., Guileyardo, JM, Russell, DW. (1999). "cDNA cloning of cholesterol 24-hydroxylase, a mediator of cholesterol homeostasis in the brain." Proc Natl Acad Sci U S A. **96**(13): 7238-43.

Ma, R., Cohen, M.B., Berenbaum, MR, Schuler, MA. (1994). "Black swallowtail (Papilio polyxenes) alleles encode cytochrome P450s that selectively metabolize linear furanocoumarins." Arch Biochem Biophys. **310**(2): 332-40.

Maitra, S., Dombrowski SM, Basu M, Raustol O, Waters LC, Ganguly R. (2000). "Factors on the third chromosome affect the level of *Cyp6a2* and *Cyp6a8* expression in *Drosophila melanogaster*." Gene **248**(1-2): 147-56.

Maitra, S., Dombrowski SM, Waters LC, Ganguly R. (1996). "Three second chromosome-linked clustered Cyp6 genes show differential constitutive and

barbital-induced expression in DDT-resistant and susceptible strains of *Drosophila melanogaster*." Gene **180**(1-2): 165-71.

Maitra, S., Price C, Ganguly R. (2002). "*Cyp6a8* of *Drosophila melanogaster*: gene structure, and sequence and functional analysis of the upstream DNA." Insect Biochemistry and molecular biology **32**(8): 859-70.

McKenzie, J., Batterham, P. (1998). "Predicting insecticide resistance: mutagenesis, selection and response." Philosophical transactions research society London B **353**(1376): 1729-1734.

Merrell, D. J., and Underhill, J.C. (1956). "Selection for DDT resistance in inbred, laboratory and wild type stocks of *Drosophila melanogaster*." Journal of economic entomology **49**(3): 300-306.

Morant, M., Bak S, Moller BL, Werck-Reichhart D. (2003). "Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation." Curr Opin Biotechnol. **14**(2): 151-62.

Murray, M. (2006). "Role of CYP pharmacogenetics and drug-drug interactions in the efficacy and safety of atypical and other antipsychotic agents." Journal of Pharmacy and pharmacology **58**(7): 871-85.

Mutch, E., Williams, FM. (2006). "Diazinon, Chlorpyrifos and parathion are metabolized by multiple cytochromes P450 in human liver." Toxicology **224**(1-2): 22-32.

Nebert, D. W. (1994). "Drug metabolizing enzymes in ligand-modulated transcription." Biochemical pharmacology **47**: 25-37.

Nebert, D. W., Russell, D.W. (2002). "Clinical importance of cytochrome P450." The Lancet **360**: 1155-62.

Nelson, D. (1999). "Cytochrome P450 and the individuality of species." Arch Biochem Biophys. **369**(1): 1-10.

Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., Nebert, D.W. (1996). "P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature." Pharmacogenetics **6**: 1-42.

Oakeshott, J. G., Horne I, Sutherland, T.D., Russell, R.J. (2003). "The genomics of insecticide resistance." Genome biology **4**(1): 202.

Omura, T. (1999). "Forty years of cytochrome P450." Biochemical and Biophysical research communications **266**(3): 690-698.

Omura, T., Sato, R. (1964). "The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemeprotein nature." Journal of biological chemistry **239**: 2370-2378.

Omura, T. and Sato, R. (1962). “A new cytochrome in liver microsomes." J Biol Chem. **237**: 1375-6.

Oshima, C. (1954). "Genetical studies on DDT-resistance in populations of *Drosophila melanogaster*." Botyu-Kagaku **19**: 93-100.

Papaconstantinou M, Wu, Y., Pretorius HN, Singh N, Gianfelice G, Tanguay RM, Campos AR, Bedard PA. (2005). "Menin is a regulator of the stress response in *Drosophila melanogaster*." Molecular and cellular biology **25**(22): 9960-9972.

Pedra, J. H. F., McIntyre, L.M., Scharf, M.E., Pittendrigh, B.R. (2004). "Genome wide transcription profile of field and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant *Drosophila*." Proceedings of national academy of sciences **101**(18): 7034-7039.

Pittendrigh, B., Aronstein K, Zinkovsky E, Andreev O, Campbell B, Daly J, Trowell S, Ffrench-Constant RH. (1997). "Cytochrome P450 genes from Helicoverpa armigera: expression in a pyrethroid-susceptible and -resistant strain." Insect Biochemistry and molecular biology **27**(6): 507-512.

Plapp, f. (1984). "The genetic basis of insecticide resistance in the housefly: evidence that a single locus plays a major role in metabolic resistance to insecticides." Pesticide biochemistry and physiology **22**: 194-201.

Porter, TD and Coon, MJ (1991). "Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms." Journal of Biological Chemistry **266**(21): 13469-13472.

Poulos, T., Finzel BC, Howard AJ (1987). "High-resolution crystal structure of cytochrome P450cam." J Mol Bio. **195**(3): 687-700.

Prapaipong, H., Berenbaum, M.R., Schuler, M.A (1994). "Transcriptional regulation of the Papilio polyxenes Cyp6B1 gene." Nucleic acids research **22**: 3210-3217.

Rose, R., Goh D, Thompson DM, Verma KD, Heckel DG, Gahan LJ, Roe RM, Hodgson E. (1997). "Cytochrome P450 (CYP)9A1 in Heliothis virescens: the first member of a new CYP family." Insect Biochemistry and molecular biology **27**(6): 605-615.

Sahir N, E. P., Gressens P. (2004). "Caffeine induces sonic hedgehog gene expression in cultured astrocytes and neurons." J Mol Neurosci. **24**(2): 201-5.

Sakaguchi, M., Mihara, K, Sato R (1987). "A short amino-terminal segment of microsomal cytochrome P-450 functions both as an insertion signal and as a stop-transfer sequence." EMBO journal **6**(8): 2425-2531.

Saner, C., Weibel B, Wurgler F, Sengstag C. (1996). "Metabolism of promutagens catalyzed by *Drosophila melanogaster* CYP6A2 enzyme enzyme in Saccharomyces cerevisiae." Environmental and Molecular mutagenesis **27**: 46-58.

SAS, Institute. (2000). Cary, NC.

Scharf, M. E., Parimi, S., Meinke, L.J., Chandler, L.D., Siegfried, B.D. (2001). "Expression and induction of three family 4 cytochrome P450 (CYP4) genes

identified from insecticide resistant and susceptible western corn rootworms." Insect molecular biology **10**: 139-146.

Schaub J, Freksa AF. (1976). "The influence of phenobarbital on carbohydrate metabolism in developing rat liver." European journal of pediatrics **122**: 249-256.

Scheutz, E., Strom, S., Yasuda, K., Lecureur, V., Assem, M., Brimer, C., Lamba, J., Kim, RB, Ramachandran, V., Komoroski, BJ., Venkataramanan, R, Cai, H., Sinal, CJ., Gonzalez, FJ., Scheutz, JD (2001). "Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters and cytochrome P450." Journal of biological chemistry **276**(42): 39411-39418.

Schlenke, T. A., Begun, D.J. (2004). "Strong selective sweep associated with a transposon insertion in *Drosophila simulans*." Proc Natl Acad Sci U S A. **101**(6): 1626-1631.

Schneider, I. (1972). "Cell lines derived from late embyonic stages of *Drosophila melanogaster*." Journal of embryology and experimental morphology **27**: 353-365.

Schuler, M. (1996). "The role of cytochrome P450 monooxygenases in plant-insect interactions." Plant physiology **112**: 1411-1419.

Scott, J. (1999). "Cytochromes P450 and insecticide resistance." Insect Biochemistry and molecular biology **29**: 757-777.

Scott, J., Wen Z (2001). "Cytochromes P450 of insects: the tip of the iceberg." Pest management science **57**: 958-967.

Scott, J. G., Georghiou, G.P (1986). "The biochemical genetics of permethrin resistance in the Learn-PyR strain of house fly." Biochem.Genet. **24**: 25-37.

Scott, J. G., Shono, T., Georghiou, G.P (1984). "Genetic analysis of permethrin resistance in the house fly, *Musca domestica*." L. Experientia **40**: 1416-1418.

Sedkov, Y., Cho E, Petruk S, Cherbas L, Smith ST, Jones RS, Cherbas P, Canaani E, and M. A. Jaynes JB (2003). "Methylation at lysine 4 of histone H3 in ecdysone-dependent development of *Drosophila*." Nature **426**(6962): 78-83.

Shaw , G., Sung, CC, Liu, CH, Lin, CH (1998). "Evidence against the Bm1p1 protein as a positive transcription factor for barbiturate-mediated induction of cytochrome P450BM-1 in *Bacillus megaterium*." J.Biol.Chem **273**: 7996-8002.

Shaw, GC, Fulco, A. (1992). "Barbiturate-mediated regulation of expression of the cytochrome P450BM-3 gene of *Bacillus megaterium* by Bm3R1 protein." J Biol Chem **267**(8): 5515-26.

Shaw, P., Cirelli C, Greenspan RJ, Tononi G (2000). "Correlates of Sleep and Waking in *Drosophila melanogaster*." Science **287**: 1834-1837.

Snyder, M., Stevens JL, Andersen JF, Feyereisen R. (1995). "Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, *Manduca sexta*." Archives of biochemistry and biophysics **321**(1): 13-20.

Spradling, A. C., and Rubin, G.M. (1982). "Transposition of cloned P elements into *Drosophila* germline chromosomes." Science **218**: 341-347.

Steinhauer, J., Kalderon D (2005). "The RNA-binding protein Squid is required for the establishment of anteroposterior polarity in the *Drosophila* oocyte." Development **132**(24): 5515-25.

Stephen, H., Setirle, jr., Green MM, and Burtist KC. (1995). "The *silver* gene of *Drosophila melanogaster* encodes multiple carboxypeptidases similar to mammalian prohormone-processing enzymes." Proceedings of national academy of sciences **92**: 9470-9474.

Sueyoshi, T., Negishi, M (2001). "Phenobarbital response elements of cytochrome P450 genes and nuclear receptors." Annual reviews in pharmacology and toxicology **41**: 123-143.

Sundseth, S. S., Kennel, S.J., and Waters, L.C. (1989). "Monoclonal antibodies to resistance-related forms of cytochrome P450 in *Drosophila melanogaster*." Pesticide Biochemistry and Physiology **33**(2): 176-188.

Sverdlov, E. (1998). "Perpetually mobile footprints of ancient infections in human genome." FEBS letters **428**(1-2): 1-6.

Swales, K and Negishi, M. (2004). "CAR, driving into the future." Mol Endocrinol. **18**(7): 1589-98.

Terriere, L. (1983). Enzyme induction, gene amplification and insect resistance to insecticides. Pest resistance to pesticides. G. P. Georghiou, Saito., T., Plenum press, NY**:** 265-297.

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

Tsukamoto, M. (1958). *Drosophila* information service **32**: 87.

Tsukamoto, M. and Ogaki, M. (1953). "Inheritance of resistance to DDT in *Drosophila melanogaster*." Botyu-Kagaku **18**: 39-44.

Vickers, A., Sinclair, J.R., Zollinger, M., Heitz, F., Glanzel, U., Johanson, L., Fischer, V. (1999). "Multiple Cytochrome P450s involved in the metabolism of Terbinafine suggest a limited potential for drug-drug interactions." Drug metabolism and disposition **27**(9): 1029-1038.

Voas, M., Rebay, I. (2003). "The Novel Plant Homeodomain Protein Rhinoceros Antagonizes Ras Signaling in the *Drosophila* Eye." Genetics **165**(4): 1993-2003.

Wang, X., Hobbs, AA. (1995). "Isolation and sequence analysis of a cDNA clone for a pyrethroid inducible cytochrome P450 from *Helicoverpa armigera.*" Insect Biochemistry and molecular biology **25**(9): 1001-1009.

Warren, J., Petryk A, Marques G, Parvy JP, Shinoda T, Itoyama K, Kobayashi J, Jarcho M, Li Y, O'Connor MB, Dauphin-Villemant C, Gilbert LI. (2004). "Phantom encodes the 25-hydroxylase of *Drosophila melanogaster* and Bombyx mori: a P450 enzyme critical in ecdysone biosynthesis." Insect Biochemistry and molecular biology **34**(9): 991-1010.

Waters, L. C., Zelhof, A.C., Shaw, B.J., Ch'ang, L.Y. (1992). "Possible involvement of the long terminal repeat of transposable element 17.6 in regulating expression of an insecticide resistance-associated P450 gene in *Drosophila*." Proceedings of the national academy of sciences **89**: 4855-4859

Waters, L. C. and Nix, C.E., (1988). "Regulation of insecticide resistance-related cytochrome P450 expression in *Drosophila melanogaster.*" Pesticide Biochemistry and Physiology **30**: 214-227.

Waxman, D. (1999). "P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR." Arch Biochem Biophys. **369**(1): 11-23.

Wen, Z, P. L., Berenbaum MR, Schuler MA. (2003). "Metabolism of linear and angular

furanocoumarins by *Papilio polyxenes* CYP6B1 coexpressed with NADPH cytochrome P450 reductase." Insect Biochem. Mol. Biol. **33**: 937-47.

Werck-Reichhart, D. and Feyereisen R. (2000). "Cytochromes P450: a success story." Genome biology **1**(6): 3003.1-3003.9.

Whitlock, J. J. (1999). "Induction of cytochrome P4501A1." Annu Rev Pharmacol Toxicol **39**: 103-25.

Williams, P., Cosme J, Sridhar V, Johnson E, McRee DE (2000). "Mammalian microsomal cytochrome P450 monooxygenase: structural adaptations for membrane binding and functional diversity." Molecular cell **5**: 121-131.

Wilson, T., Chen AD, Hsieh T. (2000). "Cloning and characterization of *Drosophila* topoisomerase IIIbeta. Relaxation of hypernegatively supercoiled DNA." Journal of biological chemistry **275**(3): 1533-40.

Yusufzai, T., Tagami H, Nakatani Y, Felsenfeld G (2004). "CTCF Tethers an Insulator to Subnuclear Sites, Suggesting Shared Insulator Mechanisms across Species." Molecular cell **13**(2): 291-298.

Zelco, I., Negishi, M. (2000). "Phenobarbital elicited activation of nuclear receptor CAR in induction of Cytochrome P450 genes." Biochemical and Biophysical research communications **277**: 1-6.

Zhou, S., Gao, Y., Jiang, W., Huang, M., Xu, A., Paxton, JW. (2003). "Interactions of herbs with cytochrome P450." Drug metabolism reviews **35**(1): 35-98.

Zurovec, M., Dolezal T. Gazi M, Pavlova E, Bryant PJ (2002). "Adenosine deaminase-related growth factors stimulate cell proliferation in *Drosophila* by depleting extracellular adenosine." Proceedings of national academy of sciences **99**(7): 4403-4408.

VITA

Srilalitha Kuruganti was born in Eluru, AP, India on June 26th, 1977, where she attended high school. She received her Bachelor of Science degree from Osmania University, India. She ranked first in the Bachelor's degree examinations held by Osmania University and was honored with Goldmedal for academic excellence. She received Masters in Biotechnology from Pondicherry Central University; India.She was ranked second in the examination held by Pondicherry Central University. Later she joined as a Research Associate in Shantha biotechnics Pvt. Ltd., India. In August 2001, she started her doctoral studies in The University of Tennessee, Knoxville in Dr.Ranjan Ganguly's lab. She attended several meetings where she presented several posters and also gave presentations on her work. She received the Doctor of Philosophy degree in December 2006. She will further continue her research in Biochemistry as a post-doctoral research trainee.