MOLECULAR MECHANISMS OF CYTOCHROME P450 MONOOXYGENASE-MEDIATED PYRETHROID RESISTANCE IN THE HOUSE FLY, *MUSCA DOMESTICA* (L.)

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MOLECULAR MECHANISMS OF CYTOCHROME P450 MONOOXYGENASE-MEDIATED PYRETHROID RESISTANCE IN THE HOUSE FLY, *MUSCA DOMESTICA* (L.)

Fang Zhu

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VITA

Fang Zhu, daughter of Tao Zhang and Xingzhi Zhu, was born on April 28, 1976, in Anqiu City, Shandong Province, People's Republic of China. She graduated from Anqiu No. 1 High School in 1995. She attended Shandong Agricultural University in Tai'an, Shandong Province and graduated with a Bachelor of Science degree in Plant Protection in 1999. Then she entered the Graduate School of China Agricultural University in Beijing with major in Entomology. After got her Master degree in 2002, she entered Department of Entomology and Plant Pathology, Auburn University, Alabama, USA, to study Insect Physiology, Toxicology, and Molecular Biology.

DISSERTATION ABSTRACT

MOLECULAR MECHANISMS OF CYTOCHROME P450 MONOOXYGENASE-MEDIATED PYRETHROID RESISTANCE IN THE HOUSE FLY, *MUSCA DOMESTICA* (L.)

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A wild-type pyrethroid resistant house fly strain, ALHF, was collected from a poultry farm in Alabama after control failure with permethrin (a pyrethroid insecticide). It was further selected with permethrin for 6 generations in the laboratory and obtained a very high level of resistance to permethrin. When ALHF was treated with piperonyl butoxide (PBO), an inhibitor of cytochrome P450s, the permethrin resistance in ALHF was reduced dramatically from 1,800- to 100-fold, indicating that P450-mediated detoxification is one of the important mechanisms involved in permethrin resistance in ALHF. In order to characterize P450 genes that are involved in the permethrin resistance of ALHF, I isolated 19 P450 cDNA fragments from ALHF using PCR strategies. This

preliminary study provided a framework for conducting the whole project to investigate the importance of P450 genes in permethrin resistance in ALHF. To characterize the functional importance of these P450 genes in resistance, I examined the expression profiles of these 19 P450 genes between resistant and susceptible house flies. Six of them were constitutively overexpressed in ALHF compared with a susceptible strain, CS, suggesting that multiple genes are involved in the increased detoxification of permethrin in ALHF. To test my hypothesis that insecticide resistant insects may be uniquely resistant to insecticides due to an ability of up-regulation of P450s when challenged with insecticides, I examined P450 gene expression in response to insecticide stimulation. Among these 6 overexpressed P450 genes, 3 of them were further induced by the permethrin stimulation in ALHF, indicating that these 3 genes may play an important role in resistance. Tissue specific analysis indicated that these constitutively expressed and permethrin induced P450 genes were overexpressed in the abdomen tissue, in which the primary detoxification organs of insects are located. This finding strongly suggests the importance of these genes in increased insecticide detoxification in ALHF. Genetic linkage analyses were further conducted to determine the causal link between these constitutively expressed and permethrin induced P450 genes and insecticide resistance. Five of these genes were mapped on autosome 5, which is correlated with the linkage of resistance in ALHF. Taken together, my research provides the first evidence that multiple P450 genes are up-regulated in insecticide resistant house flies through both constitutively overexpression and induction mechanisms, which increase overall expression levels of the P450 genes and the level of detoxification of insecticides in resistant house flies.

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Style manual or journal used	Insect Biochemistry and Molecular Biology
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LIST OF ABBREVIATIONS

3’-RACE	rapid amplification of 3’ cDNA ends
5’-RACE	rapid amplification of 5’ cDNA ends
AChE	acetylcholinesterase
ANOVA	a one-way analysis of variance
ASPCR	allele specific PCR
BC_1	back-cross generation 1
bp	base pair
Bt	*Bacillus thuringiensis*
CA	corpora allata
cDNA	complementary DNA
DDT	dichloro-diphenyl-trichloroethane
DNA	deoxyribose nucleic acid
dsRNA	double strand RNA
E	ecdysone
E20MO	E-20-monoxygenase
GABA	gamma aminobutyric acid
GST	glutathione S-transferase
JH	juvenile hormone
kdr	knockdown resistance

MFO	mixed function oxidases
mRNA	messenger RNA
OP	organophosphate
OR	olfactory receptor
ORF	open reading frame
P450	Cytochrome P450 monooxygenase
PBO	piperonyl butoxide
PCR	polymerase chain reaction
PSMO	polysubstrate monooxygenases
PTX	picrotoxinin
qRT-PCR	quantitative real-time PCR
RISC	RNA induced silencing complex
RNA	ribose nucleid acid
RNAi	RNA interference
RT-PCR	reverse transcription-mediated polymerase chain reaction
siRNA	small interfering RNA

CHAPTER 1: LITERATURE REVIEW

1.1 Insecticide resistance

The definition of resistance by the World Health Organization (WHO) is "the development of an ability in a strain of some organisms to tolerate doses of a toxicant, which would prove lethal to the majority of individuals in a normal population of the same species" (WHO, 1957). Toxicants cannot change any individual but modify the population by selecting individuals with the factor(s) that survive toxicants (Brown and Pal, 1971). Therefore, resistance is a pre-adaptive phenomenon. Insecticide resistance was firstly reported in 1914 about San Jose scale becoming resistant to lime sulfur (Melander, 1914). Up to now, insecticide resistance has occurred to all five major classes of pesticides (cyclodienes, carbamates, dichloro-diphenyl-trichloroethane (DDT) and its analogues, organophosphates (OPs), and pyrethroids) and some relatively new insecticides, such as abamectin (Scott, 1989), fipronil (Liu and Yue, 2000), imidacloprid (Liu and Yue, 2000; Wei et al., 2001; Pan et al., 2003), indoxacarb (Shono et al., 2004), spinosad (Moulton et al., 2000). Resistance has increased the application rate and frequency of insecticides, increased cost and environment contamination, disrupted wildlife and biological control agents (Scott, 1991; Liu et al., 2006). Moreover, resistance will cause outbreaks of human diseases when insect vectors cannot be controlled (Hemingway and Ranson, 2002). Therefore, studying the insecticide resistance and

understanding mechanisms of insecticide resistance are essential to design effective resistance management strategies for the long-term control of agriculturally and medically important pests. They are also important to protect the health of humans and the environment (Scott, 1991; 1999).

1.2 Mechanisms of insecticide resistance

1.2.1 Increased metabolic detoxification

Increased metabolic detoxification is one of the most universal and important mechanisms of insecticide resistance (Hemingway et al., 2004; Enayati et al., 2005; Feyereisen, 2005). There are 3 well-studied enzymes involved in this mechanism, monooxygenases, hydrolases or esterases, and glutathione S-transferases (GSTs) (Scott, 1991; Feyereisen, 1995).

1.2.1.1 Cytochrome P450 monooxygenase-mediated detoxification

There are 2 major types of oxidases, cytochrome P450 and flavin monooxygenases. The role of cytochrome P450 in insecticide resistance was firstly reported in the early 1960's that the carbaryl resistance of house flies was reduced by the P450 inhibitor sesamex (Eldefrawi et al., 1960). Cytochrome P450 monooxygenase-mediated detoxification is a very important resistance mechanism because it can cause high levels of resistance (Brattsten et al., 1977; Scott and Georghiou, 1986) and cross-resistance to unrelated compounds due to the wide variety of substrates that cytochrome P450s can metabolize (Scott, 1991; 1993).

Cytochrome P450s constitute the largest supergene family. To date there are around 1000 P450 genes identified and named from various insects (http://drnelson.utmem.edu/P450.stats.all.htm, updated on January, 2007). Some of these P450s have been isolated from insecticide resistant strains and their functions in the insecticide resistance were investigated. The first insect P450 gene, *CYP6A1*, was cloned from a diazinon resistant house fly strain, Rugters (Feyereisen et al., 1989b). *CYP6A1* was expressed at about 3-fold higher levels in Rugters compared with a susceptible strain, and at even higher levels in the Learn Pyrethroid Resistant (LPR) strain (Carino et al., 1992). In a malathion-resistant strain (91-R) of the fruit fly, *Drosophila melanogaster* (Meigen), *CYP6A2* was expressed 20-30 times more than a susceptible strain (Waters et al., 1992). *CYP6D1* was expressed 9-fold higher in LPR strain of house flies than in a susceptible strain (Liu and Scott, 1998). *CYP4G8*, a P450 gene of Australian cotton bollworm, *Helicoverpa armigera* (Hubner), was 2-fold overexpressed in pyrethroid resistant strain compared with a susceptible strain (Pittendrigh et al., 1997a). *CYP6F1* from the southern house mosquito, *Culex quinquefasciatus* (Say), was expressed 3-fold more in permethrin-resistant strain than in the susceptible strain (Kasai et al., 2000). In malaria mosquitoes, *Anopheles gambiae*, an adult-specific P450 gene, *CYP6Z1*, was expressed approximately 11- and 4.5-fold higher in males and females, respectively, from a pyrethroid-resistant strain compared with a susceptible strain (Nikou et al., 2003). The expression of a German cockroach P450, *CYP4G19*, was about 1.7-fold higher in the nymphs and approximately 5-fold higher in the adults in the pyrethroid-resistant strain compared with a susceptible strain (Pridgeon et al., 2003). Recently, *Cyp6g1* and *Cyp12d1* were reported to be overexpressed in DDT resistant *Drosophila* strains (Festucci-Buselli et al., 2005).

Although overexpression of P450s in resistant strains is so common a phenomenon, it may not absolutely indicate their importance in resistance. P450 involvement in resistance must meet following two criteria: 1) P450s must be shown to metabolize or sequester the compound to which the strain has monooxygenase-mediated resistance, and 2) P450 enzymes in resistant strain must either have higher expression or have a greater catalytic activity compared with a susceptible strain (Scott et al., 1998). According to these two aspects, only *CYP6D1* has been considered to be directly involved in insecticide resistance up to now (Scott et al., 1998; Scott, 1999).

1.2.1.2 Hydrolase-mediated detoxification

Hydrolases or esterases including carboxylesterases and phosphorotriester hydrolases represent a large variety of enzymes present in most organisms (Scott, 1991). Hydrolases are involved in metabolism of OPs, carbamates, pyrethroids, and juvenile hormones (Oppenoorth, 1985; Scott, 1999). There are at least two mechanisms in hydrolase-mediated detoxification, non-elevated and elevated esterase-based mechanisms. Non-elevated esterase-based mechanism is based on the mutation in carboxylesterase causing the loss of its carboxylesterase activity but gaining hydrolase activity. Elevated esterase-based mechanism is to elevate carboxylesterase through gene amplification, protecting insects by binding and sequestering insecticides (Claudianos et al., 1999; Hemingway, 2000; Small and Hemingway, 2000; Liu et al., 2006).

The correlation between non-elevated esterase-based mechanism and OP resistance was initially described in OP-resistant house flies (van Asperen and Oppenoorth, 1959). Subsequently, this correlation was elucidated as “mutant ali-esterase hypothesis”,

describing a mutation in the major ali-esterase (carboxylesterase) that enhances its hydrolase activity while reducing its carboxylesterase activity (Oppenoorth and van Asperen, 1960). This hypothesis was proved to be true in OP-resistant strain of the sheep blowfly, *Lucilia cuprina* Wiedemann, where a single amino acid substitution (glycine 137 to aspartic acid) of the ali-esterase E3 encoded by *LcαE7* lies within the active site of the enzyme and is responsible for both the loss of carboxylesterase activity and the gaining of a novel OP hydrolase activity (Newcomb et al., 1997). The same amino acid substitution encoded by *MdαE7* gene orthologous to *LcαE7* was also found in the OP-resistant strain of house flies (Claudianos et al., 1999). Another point mutation (tryptophan 251 to leucine) in the ali-esterase E3 has been reported to be associated with malathion resistance in the sheep blowfly (Campbell et al., 1998). Lower esterase activity was also demonstrated in the OP-resistant blowfly, *Chrysomya putoria* (Wiedemann) (Townsend and Busvine, 1969) and the parasitoid *Habrobracon hebetor* (Say) (Perez-Mendoza et al., 2000).

In the elevated esterase-based mechanism, the overproduced esterase quickly sequesters OP insecticide with low hydrolysis activity (Claudianos et al., 1999). For instance, carboxylesterase E4 was shown to be overproduced in very large quantity (about 3% of the total protein) in the high OP-resistant peach-potato aphid, *Myzus persicae* (Sulzer) while the catalytic activity of E4 is low. It suggests that the resistance caused by E4 is not conferred by hydrolysis, but by sequestration (Devonshire and Moores, 1982). Most esterases that function by sequestration are raised by gene amplification (Hemingway, 2000). Esterase gene amplification has been well documented in resistant strains of insects (Blackman et al., 1999; Gao and Zhu, 2000; Vontas et al.,

2000; Buss and Callaghan, 2004).

1.2.1.3 Glutathione S-transferase-mediated detoxification

The glutathione S-transferases (GSTs) are a superfamily of enzymes found in most organisms (Hayes and Pulford, 1995; Rauch and Nauen, 2004). In insects, GSTs function as detoxification enzymes by conjugating the reduced glutathione (GSH) to the insecticide (Hemingway, 2000; Hemingway et al., 2004; Enayati et al., 2005), resulting in compounds that are more water-soluble and more easily excreted (Habig et al., 1974; Fournier et al., 1992b). Because GSTs have a broad substrate range of extremely wide variety of potential xenobiotics, they play an important role in insects to detoxify insecticides (Zhou and Syvanen, 1997). In insects, three classes of GSTs, class I (delta class), class II (sigma class) (Chelvanayagam et al., 2001), and class III (epsilon class) (Ranson et al., 2001; Rauch and Nauen, 2004) were identified.

To date, there are numerous cDNA clones of insect GSTs reported, such as from *Drosophila melanogaster* (Meigen) (Toung et al., 1990), yellow fever mosquitoes, *Aedes aegypti* (L.) (Grant et al., 1991), tobacco hornworms, *Manduca sexta* (L.) (Snyder et al., 1995b), yellow mealworm beetles, *Tenebrio molitor* (Kostaropoulos et al., 1996), mosquitoes, *Anopheles dirus* (species B) (Prapanthadara et al., 1996; Udomsinprasert and Ketterman, 2002), German cockroaches, *Blattella germanica* (Yu and Huang, 2000), fall armyworms, *Spodoptera frugiperda* (Smith) (Yu, 2002), red imported fire ants, *Solenopsis invicta* (Valles et al., 2003), two syrphid flies, *Myathropa florae* and *Syrphus ribesii* (Vanhaelen et al., 2004), and tarnished plant bug, *Lygus lineolaris* (Zhu et al., 2007). Although so many GSTs were sequenced or further studied, few cases show a

direct link between the cloned insect GSTs and insecticide metabolism (Rauch and Nauen, 2004).

The molecular basis of GST-based resistance is best-known in house flies and malaria mosquitoes (Hemingway, 2000; Rauch and Nauen, 2004). Four different GST isozymes, MdGST-1, -2, -3, and –4, have been isolated and sequenced from a house fly Cornell-R strain, which is resistant to OP and carbamate insecticides, and their activity has been expressed in *Escherichia coli* (Wang et al., 1991; Syvanen et al., 1994). Among these four GST isozymes, only MdGST-3 and MdGST-4 were proved likely responsible for OP resistance (Syvanen et al., 1994; 1996) and the RNA transcripts for both MdGST-3 and MdGST-4 are considerably overproduced in insecticide resistant strains compared with susceptible strains (Syvanen et al., 1994; Zhou and Syvanen, 1997). Another GST isozyme, MdGST-6A, was purified and identified from a house fly strain Cornell-HR, which is highly resistant against OP insecticides. When expressed in *Escherichia coli*, the cloned MdGST-6A produces an enzyme that conjugates glutathione to the insecticides methyl parathion and lindane. It shows that MdGST-6A probably plays a significant role in OP resistance for Cornell-HR (Wei et al., 2001).

In the malaria mosquito, seven GST genes were identified subsequently from a DDT resistant strain (ZAN/U) (Ranson et al., 1997; 2001). Five of them, agGST1-5, agGST1-6, agGST1-8, agGST1-9, and agGST1-10, belong to the class I family and are located on chromosome 2R (Ranson et al., 1997; 2001). The remaining two genes, agGST3-1 and agGST3-2, belong to the class III and are located on chromosome 3R that contains a major DDT resistance gene. It has been suggested that class III GSTs are involved in resistance. The further study showed that mRNA levels of agGST3-2 are about 5-fold

higher in the DDT strain than a susceptible strain and the recombinant agGST3-2 was demonstrated having very high DDT dehydrochlorinase activity (Ranson et al., 2001). These findings further suggest that there is a strong link between class III GSTs and resistance to DDT in malaria mosquitoes (Ranson et al., 2001). Direct relationship between GST overexpression and insecticide resistance is also reported in the diamondback moth, *Plutella xylostella* L. (Huang et al., 1998).

1.2.2 Target site insensitivity

Target site insensitivity in the nervous system is one of the major mechanisms of insecticide resistance (Feyereisen, 1995). Three important target sites, sodium channel, acetylcholinesterase, and gamma-aminobutyric acid receptor, have been identified to be involved in insecticide resistance associated with point mutations (ffrench-Constant, 1999; ffrench-Constant et al., 1998; 2004).

1.2.2.1 Insensitivity of voltage-gated sodium channel

Voltage-gated sodium channel plays a vital role in the generation and propagation of action potentials in the neurons of both vertebrates and invertebrates (Williamson et al., 1996; Goldin, 2003). The insect voltage-gated sodium channel is the primary target of pyrethroids and DDT (Elliot et al., 1978; ffrench-Constant et al., 2004). Target-site resistance to DDT was first characterized as knockdown resistance (*kdr*) in house flies (Busvine, 1951). Subsequent studies showed that *kdr* trait also conferred resistance to all known natural and synthetic pyrethroids (Soderlund and Knipple, 1999; 2003). Another resistance trait named *super-kdr* was found to confer much greater levels of resistance to

DDT and pyrethriods than *kdr* (Sawichi, 1978; Soderlund and Knipple, 1999; 2003). Both *kdr* and *super-kdr* were confirmed to be involved in the reduction of neuronal sensitivity to insecticides (Scott and Georghiou, 1986a; Soderlund and Knipple, 1999; 2003). Some genetic linkage analysis implicated that the sodium channel gene (designated *Vssc1* or *Msc*) of house fly orthologous to *Drosophila melanogaster* sodium channel gene *para* tightly linkaged with *kdr* and *super-kdr* traits (Williamson et al., 1993; Knipple et al., 1994). Subsequently, the genetic evidence demonstrated that *kdr* and *super-kdr* were caused by mutations at a sodium channel structure gene (Ingles et al., 1996; Williamson et al., 1996).

Two mutations in house fly strains were firstly identified to associate with resistant phenotypes: a mutation of leucine to phenylalanine at amino acid residue 1014 (L1014F, *kdr* mutation) in *kdr* strains, and both L1014F and an additional mutation of methionine to threonine at residue 918 (M918T) in *super-kdr* strains (Ingles et al., 1996; Williamson et al., 1996; Soderlund and Knipple, 1999; 2003). The mutation of L1014F has also been found in seven other pest species, such as all knockdown-resistant house fly strains (Soderlund and Knipple, 1999; 2003), German cockroaches (Dong, 1997), horn flies (Guerrero et al., 1997), malaria mosquitoes (Martinez-Torres et al., 1998), diamondback moths (Schuler et al., 1998), Colorado potato beetles, *Leptinotarsa decemlineata* (Say) (Lee et al., 1999), peach-potato aphids (Martinez-Torres et al., 1999b), and mosquitoes, *Culex pipiens* (Martinez-Torres et al., 1999a) and C*ulex quinquefasciatus* (Xu et al., 2005; Xu et al., 2006a). This mutation has been clearly demonstrated to be associated with resistance to pyrethroid and DDT insecticides. Recently, the molecular basis of the genotype and *kdr*-mediated resistance phenotype relationship was investigated in

pyrethroid resistant mosquitoes, house flies, and German cockroaches (Xu et al., 2006a; 2006b). An obvious correlation between the *kdr* allelic expression and levels of insecticide resistance and susceptibility through RNA allelic variation and RNA editing was revealed, suggesting posttranscritptional regulation connecting the sodium channel genotype and its resistant phenotype (Xu et al., 2006a; 2006b).

Several other sodium channel mutations have been identified associated with knockdown resistance. In pyrethriod-resistant tobacco budworms, *Heliothis virescens* (F.), mutations leucine 1029 to histidine (L1029H, Park and Taylor, 1997), valine 421 to methionine (V421M, Park et al., 1997), aspartic acid 1549 to valine (D1549V, Head et al., 1998), and glutamic acid 1553 to glycine (E1553G, Head et al., 1998), were detected. The last two were also found in Australian cotton bollworm (Head et al., 1998). A mutation leucine 1014 to serine (L1014S) had been identified in populations of common house mosquitoes and malaria mosquitoes (Martinez-Torres et al., 1999a; Ranson et al., 2000). Two mutations methionine 818 to valine (M818V) and leucine 925 to isoleucine (L925I) were identified in some pyrethroid-resistant sweetpotato whiteflies, *Bemisia tabaci* (Gennadius) (Morin et al., 2002; Roditakis et al., 2006). Analysis of sodium channel sequences from pyrethroid-resistant diamondback moths indicated a mutation threonine 929 to isoleucine (T929I) associated with the L1014F mutation in strains with high pyrethroid resistance (Schuler et al., 1998). Four mutations, aspartic acid 58 to glycine (D58G), glutamic acid 434 to lysine (E434K), cysteine 764 to arginine (C764R), and proline 1880 to leucine (P1880L), were found corresponding with the mutation of leucine 993 to phenylalanine (L993F) in pyrethroid resistant strains of the German cockroach (Liu et al., 2000). Another four mutations, isoleucine 253 to asparagine (I253N), alanine

1410 to valine (A1410V), alanine 1494 to valine (A1494V), and methionine 1524 to isoleucine (M1524I), were pyrethroid resistant associated mutations found in strains of *Drosophila melanogaster* (Pittendrigh et al., 1997b). A mutation, methionine 815 to isoleucine (M815I), was identified from pyrethroid resistant populations of the human head louse, *Pediculus capitis* (De Geer) (Lee et al., 2003). Recently, a novel kdr resistant mutation for *Bemisia tabaci*, a threonine to valine substituation at position 929 (T929V, Roditakis et al., 2006) was identified.

1.2.2.2 Insensitivity of acetylcholinesterase

Acetylcholinesterase (AChE) functions by hydrolyzing a neuro-transmitter acetylcholine (ACh) into acetate and choline and terminating signal transduction at the cholinergic synapse of insects (Kozaki et al., 2002; Shi et al., 2002). AChE is the primary target site for carbamate and OP insecticides. These insecticides inhibit AChE activity to cause excitement in nerve system, a blockage of neurotransmission, and then death of insects (Nabeshima et al., 2003). The insensitivity of AChE to insecticides was firstly uncovered in the two-spotted spider mite, *Tetranychus urticae* Koch (Smissaert, 1964), and the sheep blowfly (Schuntner and Roulston, 1968). Since then the insensitivity of AChE had been reported as one of the most important insecticide resistance mechanisms in many insect species (Villatte et al., 2000; Nabeshima et al., 2004).

One mutation (phenylalanine 368 to tyrosine) of AChE gene (*Ace*) was identified from susceptible and malathion-resistant *Drosophila* strains through comparative studies of AChE cDNA sequences (Fournier et al., 1992a). Subsequently, Mutero et al. (1994) reported the sequence analysis of the *Ace* gene in several resistant field strains of

Drosophila melanogaster and identified five mutations associated with insensitivity of AChE. In the Colorado potato beetle, a serine to glycine point mutation of AChE was demonstrated as a major contributing factor in azinphosmethyl resistance (Zhu et al., 1996). This mutation has been found in all individuals of the azinphosmethyl resistant strain but not in the susceptible strain, indicating that it is responsible for the reduced AChE sensitivity (Zhu and Clark, 1997; Zhang et al., 1999). In *Drosophila*, 28 mutations in the *Ace* gene were generated and most of them led to a reduced sensitivity to several carbamate and OP insecticdes (Villatte et al., 2000). cDNAs encoding two AChEs, MpAChE1 and MpAChE2, were isolated from the peach potato aphid, where one single amino acid substitution of serine 431 to phenylalanine on MpAChE2 was found in pirimicarb resistant strains but not in susceptible strains (Nabeshima et al., 2003). Sequence and linkage analyses on mosquitoes, *Culex tritaeniorhynchus*, indicated that the CtAChE2 was likely the primary insecticide target and the phenylalanine 455 to tryptophan (F455W) replacement was possibly responsible for insecticide insensitivity of AChE (Nabeshima et al., 2004). A single mutation (G119S of the ace-1 gene) was reported to confer high resistance in *Culex pipiens*, *Culex quinquefasciatus*, *Anopheles gambiae*, and *Anopheles albimanus* (Weill et al., 2003; 2004; Liu et al., 2005). One point mutation in the S291G substitution was found in the AChE of the azinphosmethyl-resistant strain of Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Kim et al., 2006). Three mutations in the AChE gene of *Bactrocera dorsalis* were identified to be involved in the resistance to organophosphorus insecticides (Hsu et al., 2006). Recently, three mutations of AChE1 (D229G, A298S, and G324A) were identified in the prothiofos-resistant strain of diamondback moth, *Plutella xylostella* (L.) and two of them

were expected to be associated with the resistance (Lee et al., 2007). In *Culex pipiens*, the complete amino acid sequence of the AChE1 was reported and a single Phe-to Val mutation of residue 290 was proved to be involved in the resistance (Alout et al., 2007).

1.2.2.3 Insensitivity of gamma-aminobutyric acid receptor

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in both invertebrates and vertebrates (Usherwood and Grunddest, 1965; Otsuka et al., 1966). GABA receptors function as heteromultimers to form an integral chloride ion channel (ffrench-Constant et al., 1993a). Picrotoxinin (PTX) and cyclodiene are insecticides working as GABA antagonists (Matsumura and Ghasuddin, 1983). A single amino acid substitution (alanine 302 to serine or glycine) in a GABA receptor gene, *Rdl* (resistance to dieldrin), was isolated from the field-collected strains of *Drosophila melanogaster* with high levels of resistance to PTX and cyclodiene (ffrench-Constant et al., 1993b). This amino acid replacement was demonstrated to confer resistance by site directed mutagenesis of the *Rdl* cDNA and functional expression in both *Xenopus oocytes* (ffrench-Constant et al., 1993a) and baculovirus infected cells (Lee et al., 1993). The substitution of alanine 302 to serine as a most common resistance-associated replacement in *Rdl* was also found in other insect species, such as house flies (Thompson et al., 1993b), yellow fever mosquitoes, *Aedes aegypti* (Thompson et al., 1993a), sweet potato whiteflies, *Bemisia tabaci* (Anthony et al., 1995), coffee berry borers, *Hypothenemus hampei* (ffrench-Constant et al., 1994), and red flour beetles, *Tribolium castaneum* (Andreev et al., 1999). The same residue alanine 302 replaced with a glycine has also been found in another fruit fly, *Drosophila simulans* (ffrench-Constant et al., 1993b),

green peach aphids, *Myzus persicae* (Anthony et al., 1998), and cat fleas, *Ctenocephalides felis* (Daborn et al., 2004).

1.2.3 Other mechanisms of insecticide resistance

1.2.3.1 Decreased penetration

Decreased rate of insecticide penetration as a resistance mechanism was firstly described in the early 1960s (Scott, 1991). By itself, this mechanism only can cause negligible levels of resistance. But it is important because it provides protection against insecticides and enhances the level of resistance through other mechanisms (Scott and Georghiou, 1986a; 1986b; Scott, 1991; Wu et al., 1998; Wen and Scott, 1999; Valles et al., 2000). [^{14}C] fipronil had been found to penetrate into resistant house flies more slowly than into susceptible flies (Wen and Scott, 1999) suggesting that decreased penetration might be involved in fipronil resistance of flies. In the German cockroach, decreased penetration was also reported as one of the mechanisms for resistance to fenvalerate and cypermethrin insecticides (Wu et al., 1998; Valles et al., 2000). Scott (1991) hypothesized that almost every membrane had the potential to prevent penetration, although the cuticle was the major barrier responsible for decreased penetration in insects. For example, in the larvae of lepidopterous species, the peritrophic membrane appeared to be a barrier to impede the movement of the *Bacillus thuringiensis* (Bt) toxin protein to the midgut brush border (Granados et al., 2001).

1.2.3.2 Accelerated excretion

Accelerated excretion is another mechanism involved in insecticide resistance. A wide variety of insect species developed resistance to insecticides through this mechanism. For instance, in diazinon resistant thrips, *Frankliniella occidentalis* (Pergande), the diazinon excretion was faster than in the susceptible ones (Zhao et al., 1994). It has been also found that the resistance mechanism of Colorado potato beetles to azinphosmethyl may be potentiated by the generally rapid excretion of aziophosmethyl (Argentine et al., 1994). Compared with those from a susceptible strain, levels of excretion of cypermethrin were higher in larvae of field collecting tobacco budworms (Ottea et al., 1995). The increased excretion rate was also observed in the carbaryl-resistant populations of Western corn rootworm (Scharf et al., 1999).

1.2.3.3 Behavioral resistance

Behavioral resistance describes the development of the ability to avoid a lethal dose to the majority of individuals in a normal population of the same species (Scott, 1991). This mechanism can be divided into stimulus-dependent mechanisms, such as increased repellency and irritancy, which require contact with insecticides and stimulus-independent mechanisms such as exophily that do not require any contact with insecticides (Sparks et al., 1989). Although there are fewer reports of behavioral resistance than other mechanisms, its importance in resistance cannot be ignored. Behavioral resistance plays a role in the multi-resistant horn flies. Initial studies suggested that repellency or irritancy of pyrethroids was greater in resistant horn flies than in susceptible ones (Quisenberry et al., 1984). In the following dose-response studies,

it was demonstrated that the resistant strain of horn flies exhibited a unique behavioral response pattern to four pyrethroids and DDT. It may indicate that there is an underlying behavioral resistance mechanism independent from the processes involved in other mechanisms (Lockwood et al., 1985).

1.3 Interactions between resistance mechanisms

Interactions between different resistance mechanisms are very important because in combination they may provide high levels of resistance (Scott, 1991). For example, combining a resistance mechanism that provided 3.2-fold resistance (increased detoxification) with one that gave < 2-fold resistance (penetration) resulted in nearly 50-fold resistance (Georghiou, 1971). Similarly, separate mechanisms conferring 7.3-, 2.6-, 54-, and 65- fold resistances could combine to produce a 5,000- fold resistance (Scott and Georghiou, 1986a). There are numerous evidences showing that multiple resistance mechanisms are usually responsible for insecticide resistance together (Scott, 1991). However, the molecular basis underlying interactions between resistant mechanisms is still poorly understood (Feyereisen, 1999). Recently an interesting study revealed the molecular mechanism of the relationship between P450 overproduction and point mutation of carboxylesterase in the multi-resistant Rutgers strain of house flies. This study showed that the glycine (Gly) 137 to aspartic acid mutation in *αE7* esterase gene conferred resistance and overproduction of the *CYP6A1* protein. Therefore, the authors hypothesized that it was the absence of the wild-type Gly 137 that releases the transcriptional repression of genes coding for *CYP6A1*, by this means leading to metabolic resistance (Sabourault et al., 2001).

1.4 House fly as an insect pest

1.4.1 Introduction of the house fly

House flies, the most common species in the genus *Musca* (Diptera: Muscidae), occurs throughout the world, but in lower number in Africa (Howard, 1911; Service, 1996). House flies have four different stages during their life, adult, egg, larva, and pupa. House fly is an important pest of humans and domesticated animals and is also a vector of both human and animal diseases (Marcon et al., 2003). It can transmit a large number of diseases to humans and domesticated animals due to the habits of indiscriminately visiting faeces, other unhygienic matter, and human and domesticated animals' food (Service, 1996). Over 100 different pathogen species are harbored by house flies, at least 65 of which are known to be transmitted by flies (Service, 1996; Marcon et al., 2003). House flies can transmit viruses of polio, trachoma, and infectious hepatitis, as well as rickettsiae such as Q fever (*Cuxiella burneti*). House flies also can transmit numerous bacteriae diseases, such as bacillary dysentery (*Shigella*), cholera, enterotoxic *Eschericia coli*, *Campylobacter*, the typhoids and paratyphoids (*Salmonella*), as well as a variety of streptococci and staphylococci (Service, 1996). They may also be vectors of protozoan parasites such as those causing amoebic dysenteries (Entamoeba, Giardia). In addition, they can carry eggs and cysts of a variety of helminthes (Service, 1996). Recently, enterohemorrhagic *Esherichia coli* 0157:H7 (EHEC) was found to proliferate in the mouthparts of the house fly and to be excreted for at least 3 days after ingestion. Further study showed that frequent excretion potentially enhanced the dissemination of EHEC to foods, particularly during the first 24 hours after ingestion of the bacteria (Sasaki et al., 2000). *Yersinia pseudotuberculosis* (Pfeiffer), a pathogen of domestic animals and

humans, was reported to be potentially carried by adult house flies. *Yersinia pseudotuberculosis* did not establish a permanent population in 36 hours after the initial exposure, and flies contaminated their environment for up to 30 hours after the exposure. These results showed that house flies could carry *Yersinia pseudotuberculosis* for a considerable period and must be considered as a potential mechanical vector of pseudotuberculosis infection (Zurek et al., 2001).

1.4.2 Insecticide resistance in house flies

Historically, house fly management relied heavily on chemical control. However, resistance problems increased the cost and diminished the efficacy of insecticides (Geden et al., 1992). Selection pressure from frequent treatments led to widespread resistance in house fly populations (Learmount, 2002). House flies were found to be resistant to DDT, cyclodiene insectices, and gamma-HCH in 1950's (Learmount, 2002). By 1978, strains of house flies were reported to be resistant to all of the major insecticides classes, including DDT and its analogues, cyclodienes, carbamates, organophosphates, and pyrethroids (Georghiou, 1986). Substantial evidence indicates multiple resistance mechanisms or genes are involved in insecticide resistance of individual insect species (Scott, 1999). Resistance conferred by enhanced metabolic detoxification via cytochrome P450 monooxygenases, hydrolase, and GST, target-site (primarily sodium channel) insensitivity, and decreased penetration has been reported in house flies (Scott and Georghiou, 1986a; 1986b; Liu and Pridgeon, 2002; Scott and Zhang, 2003).

Cytochrome P450 monooxygenases-mediated detoxification is an important mechanism of insecticide resistance in house flies (Scott, 1999) and several P450 genes

have been isolated from resistant house fly strains. The Rutgers strain of house flies originated from the lab of Forgash, A. J. in the early 1960s is one of the most extensively studied strains of house flies. It contained 98- to 125- fold resistance to diazinon and cross-resistance to numerous other insecticides (Forgash et al., 1962). *CYP6A1* is the first insect P450 cloned and sequenced from Rutgers strain (Feyereisen et al., 1989b). *CYP6A1* was expressed at about 3-fold higher levels in resistant strain compared with susceptible strains, and was expressed at even higher levels in the LPR strain of house flies (Carino et al., 1992). However, it is unclear whether the overexpression of *CYP6A1* is involved in insecticide resistance (Scott, 1999). Using a reconstitution system system, including *CYP6A1* and NADPH-cytochrome P450 reductase expressed in *Escherichia coli*, *CYP6A1* was shown to metabolize aldrin and heptachlor, but not seven other compounds including methoxyresorufin and ecdysone (Andersen et al., 1994).

The LPR strain of house flies was collected from a dairy in New York in 1982 following the introduction of permethrin for the fly control. After several generations of selection with permethrin, LPR became more homozygous for the major mechanisms of resistance and attained extremely high levels (>6000-fold) of resistance to pyrethroid insecticides (Scott and Georghiou, 1986a). Permethrin resistance can be suppressed from 5900-fold to 32-fold by the P450 inhibitor piperonyl butoxide (PBO) (Scott and Georghiou, 1986a). *CYP6D1* from the LPR strain was cloned and sequenced (Tomita and Scott, 1995; Tomita et al., 1995; Liu and Scott, 1996; 1997a; 1997b; Smith and Scott, 1997). *CYP6D1* mRNA was expressed at about a 10-fold higher level in LPR flies compared with susceptible flies (Scott, 1999). In vitro studies showed that cytochrome monooxygenase-mediated detoxification of permethrin (Scott and Georghiou, 1986a;

1986b), deltamethrin (Wheelock and Scott, 1992) or cypermethrin (Zhang and Scott, 1996) occurrs at a rate nearly 10-fold greater in LPR compared with a susceptible strain. The enhanced in vivo metabolism of deltamethrin in LPR compared with the susceptible strain has also been demonstrated (Wheelock and Scott, 1992). Substantial evidence indicates that the *CYP6D1* is directly involved in insecticide resistance (Wheelock and Scott, 1992; Zhang and Scott, 1996; Scott, 1999).

The Alabama house fly strain (ALHF) was collected from a poultry farm in Alabama in 1998 after a control failure with permethrin, and further selected in the laboratory with permethrin for five generations. The level of resistance to permethrin in ALHF was increased rapidly to 1,800-fold (Liu and Yue, 2000). The ALHF strain showed a great ability to develop resistance and cross-resistance to different insecticides within and outside the pyrethroid group including some new insecticides. P450 monooxygenases and hydrolases were proved to be involved in resistance in ALHF (Liu and Yue, 2000). *CYP28B1* and *CYP4G13v2* are two P450 genes isolated from the ALHF strain. However, the function of these two genes in house fly resistance remains unclear (Liu and Zhang, 2002).

Scott and Kasai (2004) investigated the genetic basis of resistance in a NG98 house fly strain with 3700-fold resistance to permethrin and other permethrin resistant strains from US and Japan. They found that the resistance of NG98 is due to *kdr* on autosome 3 and monooxygenase-mediated resistance on autosome 1, 2, and 5. They suggested that P450 genes involved in resistance and regulatory factors controlling P450 expression are different among different populations (Scott and Kasai, 2004).

The hydrolase mediated detoxification due to the mutation in carboxylesterase, causing the loss of its carboxylesterase activity and gaining OP hydrolase activity, has been identified as a mechanism of resistance to OP (Oppenoorth, 1985; Claudianos et al., 1999; Hemingway, 2000). The lower esterase activity was firstly detected in the OP-resistant house flies (van Asperen and Oppenoorth, 1959). Subsequent study assumed that there was a mutation in the major ali-esterase to enhance its hydrolase activity, while reducing its carboxylesterase. This was named the "mutant ali-esterase hypothesis" (Oppenoorth and van Asperen, 1960). The house fly *MdαE7* gene encoding the ali-esterase E3 was cloned and characterized. Comparisons between OP susceptible and resistant Rutgers strains indicated that resistance in the house fly was associated with a single amino acid mutation (glycine 137 to aspartic acid) in the ali-esterase E3. The same mutation has been reported in an OP-resistant strain of the sheep blowfly (Claudianos et al., 1999).

The GST mediated detoxification in house flies is well studied at the molecular level (Hemingway, 2000; Rauch and Nauen, 2004). First two classes of GSTs, GST1 and GST2, were identified from the OP and carbamate resistant house fly strain, Cornell-R (Fournier et al., 1992b). Subsequently, MdGST-6A has been proved to play a significant role in OP-insecticide resistance of the Cornell-HR house fly strain (Wei et al., 2001).

The house fly has been used as a model system for the study of sodium channel insensitivity in resistance (Soderlund and Knipple, 1999; 2003). The phenomenon of *kdr* was first observed in a DDT-resistant house fly strain as an ability to resist the rapid knockdown paralysis action of DDT usually observed after DDT exposure (Busvine, 1951). Subsequent studies mapped the *kdr* trait to chromosome 3 (Soderlund and Knipple,

1999). Another resistant trait, *super-kdr*, that confers much greater resistance than *kdr* was also isolated genetically and mapped to chromosome 3 from pyrethroid-resistant house fly strains (Soderlund and Knipple, 1999).

Sodium channel (*para*-type) cDNA of house flies was isolated and two point mutations associated with resistant phenotypes were identified (Ingles et al., 1996; Williamson et al., 1996). The mutation of leucine at position 1014 to phenylalanine (L1014F) is present in both *kdr* and *super-kdr* strains, and an additional mutation of methionine to threonine at residue 918 (M918T) is only present in *super-kdr* strains (Williamson et al., 1996). A 392 bp *para*-type sodium channel gene fragment where *kdr* (L1014F) and *super-kdr* (M918T) mutations reside was generated by RT-PCR from ALHF (Liu and Pridgeon, 2002). Only L1014F was present in ALHF, suggesting that *kdr* mutation is a significant factor for pyrethroid resistance of ALHF. In the YPER strain of house flies, *super-kdr* is demonstrated to confer a >18,400-fold level of permethrin resistance (Shono et al., 2002).

Decreased penetration (*pen*, Farnham, 1973) has been recorded in a number of resistant strains in the house fly (Plapp and Hoyer, 1968; DeVries and Georghiou, 1981; Scott and Georghiou, 1986a; 1986b; Wen and Scott, 1999). By itself, *pen* confers only 2- to 3- fold resistance (Plapp and Hoyer, 1968; Sawicki, 1970). However, it acts as an enhancer of resistance conferred by other mechanisms (Sawicki, 1970; Georghiou, 1971; 1986; Oppenoorth, 1985; Scott, 1991). The molecular basis for this mechanism is not clear. *In vivo* studies of ^{14}C-permethrin have demonstrated that decreased cuticular penetration is one of the resistance mechanisms responsible for pyrethroid resistance in the LPR house fly strain (Scott and Georghiou, 1986a; 1986b).

Using quantitative real-time polymerase chain reaction (qRT-PCR), the genome size of house flies was recently estimated. The size of the house-fly genome was 184 Mb that was only about 1.6-fold larger than that of *Drosophila melanogaster*, indicating house-fly genome size made it an excellent candidate for whole genome sequencing (Gao and Scott, 2006b). Obviously, this effort will strongly enhance our understanding of mechanisms of house fly insecticide resistance and accelerate the progress in controlling this devastating human and animal diseases vector—house-fly.

1.5 Cytochrome P450 monooxygenases in insects

1.5.1 Introduction and terminology

Cytochrome P450 monooxygenases (P450s) are a microsomal pigment (P) and have an absorption peak at 450 nm when reduced and saturated with carbon monoxide (Omura and Sato, 1964). The P450s are known in the literature with various names, such as cytochrome P450 monooxygenases, mixed function oxidases (MFO), polysubstrate monooxygenases (PSMO), microsomal oxidases, and heme thiolate proteins (Feyereisen, 1999; 2005). The P450 system is an extremely important metabolic enzyme system. It is involved in the detoxification of xenobiotics, such as drugs, pesticides, and plant toxins; and in the regulation of endogenous compounds such as hormones, fatty acids, and steroids (Scott, 1999). P450s are found in virtually all aerobic organisms, as diverse as insects, plants, mammals, birds, fishes, and bacteria (Stegeman and Livingstone, 1998). P450 is a hemoprotein and acts as the terminal oxidase in the monooxygenase system (Scott, 1999). In eukaryotes, P450s are typically found in the endoplasmic reticulum of cells. The three most important components of the P450 monooxygenase system are

cytochrome P450, which acts as the substrate binding protein, NADPH-cytochrome P450 reductase (P450 reductase), which transfers electrons from NADPH to cytochrome P450, and cytochrome b5, which transfers electrons from NADH to cytochrome P450 in some P450 monooxygenase systems as an additional potential electron donor (Scott, 1993; Feyereisen, 1999; 2005).

Both mammalian and insect P450 systems have similar mechanism. Outlines of the catalytic cycle are as follows (Ortiz de Monetellano, 1995) (Fig. 1.1): The heme protein in the oxidized form, P450-Fe (III), binds the substrate. The P450-substrate (XH) complex receives a single electron from a redox partner, and P450-Fe (II) then binds oxygen. A second electron reduction step precedes the really fancy chemistry where molecular oxygen is split and a reactive oxygen complex inserts an atom of oxygen into the substrate in a radical reaction. The classical stoichiometry of a P450 monooxygenase, $XH + NADPH + H^+ + O_2 \rightarrow XOH + NADP^+ + H_2O$, is only achieved in tightly coupled reactions, whereas the reactions are uncoupled to varying degrees and superoxide and hydrogen peroxide are generated by the collapse of one- or two- electron-reduced P450-dioxygen complexes (Feyereisen, 1999).

Cytochrome P450 is a hemoprotein that acts as the terminal oxidase in monooxygenase systems (Scott, 1999). The nomenclature of P450 gene universally accepted is introduced by Nebert et al. (1987). All gene members in the P450 superfamily, for example, *CYP6D1*, have a *CYP* prefix, followed by a numeral for the family, a letter for the subfamily, and a number for the individual gene. All members of a family share more than 40% identity at the amino acid sequence level and members of a subfamily share more than 55% identity (Feyereisen, 1999; 2005; Fig. 1.2). A region located

towards the C-terminus of all P450 proteins surrounds a cysteine residue, the fifth ligand of the heme iron. This region shows an invariant sequence F**G*R*C*G, which is not found in other proteins and thus constitutes the P450 "signature" (Feyereisen, 1999; 2005).

The first sequence of the P450 gene, *CYP2B1*, from rat liver was published in 1982 (Fujii-Kuriyama et al., 1982). Subsequently, a large number of mammalian P450s have been characterized and sequenced. Insects as the single largest class of organisms have evolved a larger variety of P450 genes than vertebrates because of their shorter generation time, wider ecological dispersal, and greater diversity of relationships with other organisms, such as plants, fungi, bacteria and their environment (Feyereisen et al., 1989a; Feyereisen, 1991; Scott et al., 1998). In insects, about 1000 P450s have been identified and named (http://drnelson.utmem.edu/P450.stats.2006.htm, updated on January, 2007). Ninety P450 genes scattered in 25 families in the genome of *Drosophila melanogaster* (Meigen) were reported (Tijet et al., 2001). There are also 105 P450s in malaria mosquito (http://P450.antibes.inra.fr, updated on February 26th, 2003).

1.5.2 Functions of insect P450s

Insect P450s are found in the biosynthetic and degradation pathways of endogenous compounds such as juvenile hormones, ecdysteroids, fatty acids, and pheromones, which are at the center stage of insect growth, development, reproduction, and communication (Feyereisen, 1999). P450 enzymes are also involved in the metabolism of insecticides, resulting in either bioactivation or detoxification, which causes resistance to insecticides (Agosin, 1985). Furthermore, P450 metabolism of certain plant secondary metabolites is

often the key to the adaptation of insect herbivores to their host plants (Cohen et al., 1992).

1.5.2.1 Metabolism of endogenous compounds

Juvenile hormones (JHs) play a critical role in insect development, metamorphosis, reproduction, and many other physiological processes (Sutherland et al., 1998; Helvig et al., 2004). JHs are synthesized from endocrine glands, the corpora allata (CA) and degraded by esterases and epoxide hydrolases. Cytochrome P450 enzymes had been proved to play important role in the biosynthesis and degradation pathways of JH (Helvig et al., 2004; Feyereisen, 2005). Methyl farnesoate epoxidation of juvenile hormone (JH) III catalyzed by a P450-like enzyme in the corpora allata (CA) of the giant cave roach, *Blaberus giganteus*, was first identified in 1975 (Hammock, 1975). A later study on the migratory locust, *Locusta migratoria*, corpora allata proved that the epoxidation of methyl farnesoate was indeed catalyzed by a microsomal P450 (Feyereisen et al., 1981). When a biorational insecticide was used to inhibit the P450 epoxidase in order to block the synthesis of JH, it caused precocious metamorphosis in immature stages and sterility in adults, also suggesting the role of P450 in the biosynthesis of JH (Hammock and Mumby, 1978). Recently a P450, *CYP15A1*, was purified and reconstituted from the corpora allata of the Pacific beetle cockroach, *Diploptera punctata*. It had been found that *CYP15A1* catalyzed the last step of JH biosynthesis, metabolizing methyl farnesoate to JH III (Helvig et al., 2004). P450s are not only involved in JH biosynthesis but also involved in the JH degradation (Hodgson, 1985). A house fly P450, *CYP6A1*, was shown to epoxidize a wide variety of terpenoids including JH I and JH III. *CYP6A1* can

metabolize both JH I and III to their diepoxides (Andersen et al., 1997). *CYP4C7* was isolated from the corpora allata of reproductively active Pacific beetle cockroaches. *CYP4C7* protein expressed in a heterologous system metabolized JH III and JH precursors to their 12-*trans*-hydroxy metabolites (Sutherland et al., 1998), playing a significant role in regulating levels of JH synthesis during special developmental stages (Sutherland et al., 1998).

Because insects are characterized by exoskeleton, molting becomes an important issue in the development process of insects. This molting process and many other developmental events in insects are triggered by the insect molting hormone, 20-hydrooxyecdysone (20E) (Gilbert, 2004). Ecdysone (E) is a precursor of 20E. And E is synthesized from cholesterol or plant sterols in the prothoracic glands. The conversion of cholesterol to 7-dehydrocholesterol is the first step of E biosynthesis both in the prothoracic glands and the ovaries (follicle cells) (Rees, 1995). The conversion of E to the major active form, 20E, is catalyzed by a P450 enzyme, E-20-monoxygenase (E20MO) (Feyereisen and Durst, 1978; Williams et al., 1997). *CYP6H1* cloned from malpighian tubules of the migratory locust was the first P450 proved to be involved in 20E biosynthesis. *CYP6H1* is predominantly expressed at larval instars and in malpighian tubules. The protein encoded by *CYP6H1* had been identified as an E-binding cytochrome P450 protein (Winter et al., 1999). Using a combination of molecular and biochemical approaches, a series of cytochrome P450 enzymes named Halloween genes were identified to be involved in the E biosynthesis in *Drosophila melanogaster* (Chavez et al., 2000; Warren et al., 2002; Petryk et al., 2003; Niwa et al., 2004; Warren et al., 2004). When these Halloween genes were mutated, insects failed to form a normal cuticle,

resulting in embryonic lethality due to low E level (Wieschaus et al., 1984). These genes are *phantom* (*phm*) coding for CYP306A1, *disembodied* (*dib*) coding for CYP302A1, *shadow* (*sad*) coding for CYP315A1, and *shade* (*shd*) coding for CYP314A1. Among these 4 P450s, *phm*, *dib*, and *sad* are expressed in the lateral prothoracic gland, which are responsible for E biosynthesis and they catalyze the last three steps of ecdysteroidogenesis, the 25-, 22-, and 2- hydroxylation steps, respectively. *shd* had been characterized as the E20MO, which is expressed mainly in the larval gut and fat body but not in the prothoracic gland (Petryk et al., 2003). Recently, *CYP306A1*, *CYP302A1*, *CYP315A1*, and *CYP314A1* were also identified and characterized mediating E biosynthesis in the tobacco hornworm, *Manduca sexta* (Rewitz et al., 2006a; 2006b). The expression of these Halloween genes in the prothoracic glands of *Manduca* undergoes dramatic changes that correspond temporally to changes in the hemolymph ecdysteroid level.

Olfaction is very important for insects to feed, mate, defend, and communicate, as well as to learn and memory. Insect olfactory system is extremely sensitive and specific, enabling insects to identify minute concentrations of relevant compounds. Several P450s were investigated predominantly expressed in the antennae, such like *CYP9A13*, *CYP4G20* in male moth *Mamestra brassicae* (Maibeche-Coisne et al., 2005), and *CYP341A2* in the swallowtail butterfly, *Papilio xuthus* L. (Ono et al., 2005), suggesting their functions in olfactory systems. Recently, one new cytochrome P450, *CYP6AM1*, was characterized in the damp-wood termite, *Hodotermopsis sjostedti*. It was specifically expressed in the fat body of pseudergates and soldiers, indicating the putative function of this P450 in the caste differentiation system (Cornette et al., 2006).

1.5.2.2 Metabolism of foreign compounds

The metabolism of foreign compounds (xenobiotics) to less toxic metabolites is the best-known function of P450 enzymes (Feyereisen, 1999). The function of insect P450 enzymes associated with detoxification of insecticides has been introduced in detail in the Section 1.2.1.1 of this review. Besides detoxification, P450 enzymes can activate insecticides to more active toxicants, such as acting on phosphorothioate and cyclodiene insecticides (Neuman et al., 1985). For OP insecticides, the activation of oxygen substituting sulphur on the double bond with phosphate (P=S to P=O) by P450 enzymes results in a notably increased activity, for example the activation of malathion to malaoxon. In addition *CYP6A1*, *6A2*, *6A5*, and *12A1* also possess some degree of cyclodiene epoxidase activity (Feyereisen, 1999; 2005).

P450 enzymes are involved in the detoxification of certain plant secondary metabolites as well. Induction of P450 enzymes plays important roles in the insect-plant interactions (Brattsten et al., 1977). Induction of P450 enzymes is a phenomenon that the levels of certain P450 gene expression and monooxygenase activities can be increased considerably upon exposure to particular chemicals, such as plant toxicants, xenobiotics, fungal metabolites and hormones (Feyereisen, 1999; 2005). Phenobarbital (PB) is a well studied inducer in both mammals and insects. Numerous insect P450s had been reported to be induced by PB, such as *CYP4D10*, *CYP6A1*, *CYP6A2*, *CYP28A1*, *CYP28A2*, and *CYP28A3* in *Drosophila* (Danielson *et al.,* 1997; 1998; Dombrowski *et al.,* 1998), *CYP6B2* in *Helicoverpa armigera* (Wang and Hobbs, 1995), *CYP6D1* and *CYP6D3* in *Musca domestica* (Liu and Scott, 1997b; Kasai and Scott, 2001). Recently, microarray experiment showed that another 11 *Drosophila* P450 genes can be significantly induced

by PB (Goff *et al*., 2006). It has been demonstrated that the PB induction of *CYP6D1* is due to a *trans* acting factor on autosome 2 of the house fly (Liu and Scott, 1997b). A large number of studies have established that P450s in certain insects also can be induced by their host plant allochemicals that is suggested a mechanism for insect herbivores surviving their host plants (Cohen *et al*., 1992; Hung *et al*., 1995; Li *et al*., 2000, 2002a). As the same principle, some P450s responsible for insecticide detoxification can be induced by insecticides that they act upon because many organic insecticides resemble plant allochemicals, such as pyrethroids (Schuler, 1996). For example, 3 CYP4 genes are induced by pentamethyl benzene in western corn rootworms (Scharf *et al*., 2001). *CYP6B7* can be induced by α–pinene and pyrethriods, fenvaleate, cypermethrin, and permethrin observed in fat body culture in *Helicoverpa armigera* (Ranasinghe and Hobbs, 1999). And significant induction of *CYP6BG1* and *CYP6BF1v4* by permethrin in susceptible strains of diamondback moth has been identified (Bautista *et al.,* 2007). A detail of introduction of P450 enzymes in plant-insect interactions is in the Section 1.8 of this review.

1.6 Cytochrome P450-mediated resistance in other insects

The 91-R strain of *Drosophila melanogaster* was selected with DDT in laboratory (Merrell and Underhill, 1956). The level of DDT resistance in the 91-R strain was less than 10-fold (Dapkus and Merrell, 1977; Merrell and Underhill, 1956). 91-R also has 100-fold cross-resistance to malathion (OP) (Sundseth et al., 1989). Two P450s, P450s-A and P450s-B were revealed in 91-R (Sundseth et al., 1989). The P450s-B immunoreactive protein was shown to be expressed at higher levels in 91-R compared

with a susceptible strain, while the P450s-A immunoreactive protein was expressed at similar levels in both strains (Sundseth et al., 1989; Waters et al., 1992). *CYP6A2* was cloned by screening a library with the P450s-B specific monoclonal antibody (Waters et al., 1992). Northern blot analysis indicated that *CYP6A2* was expressed at much higher levels in 91-R compared with a susceptible strain (Waters et al., 1992). *CYP6A2* was subsequently shown to be inducible by Phenobarbital (PB) in resistant and susceptible strains although the level of induction was greater in the susceptible strain (Brun et al., 1996).

The 2500-fold resistance to permethrin in the Jpal-per strain of southern house mosquitoes (Kasai et al., 1998a) could be reduced to 43-fold by PBO, indicating that P450 mediated detoxification was one of the important mechanisms in permethrin resistance of this strain. *CYP6F1* from the southern house mosquito larvae was overexpressed in a permethrin-resistant strain compared with a susceptible strain; however, its function in resistance is unclear (Kasai et al., 2000).

Synergism studies indicated that P450-mediated detoxification was involved in insecticide resistance of the German cockroach (Wei, et al., 2001). Several P450s, *CYP6L1*, *CYP6J1*, *CYP6K1*, *CYP9E2*, *CYP4C21*, and *CYP4G19* have been isolated from the German cockroach (Wen and Scott, 2001a; 2001b; Wen et al., 2001; Pridgeon et al., 2003). *CYP4G19* was found to be overexpressed in a pyrethriod-resistant Apyr-R strain compared with a susceptible strain, and the overexpression was developmentally and tissue-specifically regulated (Pridgeon et al., 2003). *CYP9A1* was isolated from the Herbert strain of tobacco budworms which had >150-fold and 90-fold resistance to thiodicarb and cypermethrin, respectively (Rose et al., 1997). *CYP6B2*, *CYP6B6*, and

CYP6B7 were cloned from Australian cotton bollworm (Ranasinghe et al., 1997; Ranasinghe and Hobbs, 1998). However, whether these P450s are involved in insecticide resistance remains unknown (Scott, 1999).

1.7 Regulation of P450 gene expression

The expression of P450 genes is under complex and distinct regulation during development, either following exposure of organisms to various xenobiotics or in response to vital endogenous signals (Nebert, 1987). Developmental regulation of P450 genes was first reported by the pioneering studies by Conney et al. (1969). It was showed that the level of hydroxylated testosterone metabolites generated from rat liver microsomes varys with age and sex. To date, the diversity in the developmental regulation of insect P450s has been well documented. *CYP4D1* is expressed in a very similar prototype at all life stages (Gandhi et al., 1992). The expression of *CYP4G19* is developmentally regulated with very low level in eggs, rising in nymphs, and reaching a maximum in both male and female adults (Pridgeon et al., 2003). *CYP6A1* is expressed at a very low level in eggs, increasing in larvae with a maximum in day 4 larvae, steep decline in pupae, and a rise in adults with no obvious difference between males and females (Carino et al., 1992). *CYP6B* genes are expressed significantly in larval stages with highest levels in early larval instars, but no expression in other developmental stages (Harrison et al., 2001). *CYP6D1* is expressed in both male and female adults, hardly detectable in pupae, and could not be detected in larvae (Wheelock et al., 1991). *CYP6D3* is expressed in larvae as well as male and female adults, with no expression in eggs (Kasai and Scott, 2001). *CYP6L1* is not expressed in eggs nor nymphs and adult females

but only expressed in male adults (Wen and Scott, 2001a). *CYP6Z1* is adult-specific in its expression with higher level in males than in females (Nikou et al., 2003). Recently a sex (male) specific expression of *CYP312A1* was observed in three *Drosophila* strains (Kasai and Tomita, 2003).

The expression of P450 genes also shows great variation as to the tissues. Levels of *CYP4G19* mRNA were detectable in head+thorax tissues and about 5-fold increased in the abdomens of both susceptible and pyrethroid resistant German cockroach strains (Pridgeon et al., 2003). In the Canton strain (wild type) of *Drosophila melanogaster*, when insects were treated with phenobarbital, *CYP6A2* was expressed more in the midgut, the pericuticular fat bodies, and the Malpighian tubules (Brun et al., 1996). In the $RDDT^R$ strain of *Drosophila melanogaster*, which is resistant to DDT, *CYP6A2* is constitutively overexpressed in the same tissues (Brun et al., 1996). *CYP6B1* transcripts in black swallowtail butterfly, *Papilio polyxenes* (Fabr.), are induced by xanthotoxin in all tissues examined, including the midgut, fat body, and integument while *CYP6B3* transcripts are only induced in the fat body (Petersen et al., 2001). The house fly specific gene *CYP6D1* is found in abdomen, all tagmata, and thoracic ganglia (Korytko and Scott, 1998; Scott et al., 1998). *CYP6L1* is specifically expressed in the reproductive tissues (testes and accessory glands) of adult male German cockroaches (Wen and Scott, 2001a). High *CYP18* expression was observed in body wall and gut while insignificant expression was detectable in salivary glands and fat body of *Drosophila melanogaster* (Bassett et al., 1997). The expression of some certain P450 has been proved under the regulation in response to important endogenous signals. In adult males of the tropical cockroach, *Blaberus discoidalis*, the expression of *CYP4C1* is up-regulated by the hypertrehalosemic

hormone (HTH) (Bradfield et al., 1991; Lu et al., 1995; 1996; 1999).

The study of regulation of P450 expression at the molecular level has been focused on house flies and fruit flies. In house flies, it has been demonstrated that a *trans*-acting factor from chromosome 2 regulates the expression of *CYP6A1*, which is located on chromosome 5 (Cohen et al., 1994). The *CYP6D1* gene, located on chromosome 1 of LPR strain (Thomas et al., 1995), is regulated by a factor located on chromosome 2, suggesting that it is also under the regulation of a *trans*-acting factor (Liu and Scott, 1995; 1997a; 1997b; 1998). In *Drosphila melanogaster*, overexpression of *CYP6A2*, *CYP4E2*, and *CYP6A9* in resistant strains has been observed and the down-regulation in *trans* in the susceptible strain has been suggested (Maitra et al., 1996). Point mutations in the coding sequence of the *CYP6A2* gene have been reported and may contribute to the resistance of the Raleigh-DDT strain of *Drosphila melanogaster* (Berge et al., 1998).

1.8 Cytochrome P450 in plant-insect interactions

Cytochrome P450 monooxygenases are one of the most important enzymes utilized by insect herbivores to detoxify plant secondary metabolites (Cohen et al., 1992). Herbivorous insects encounter a wide diversity of plant secondary metabolites. They must, therefore, have developed mechanisms to survive in their hosts. Xanthotoxin, a linear furanocoumarins, is toxic to a variety of organisms because of its ability to cross-link DNA strands in the presence of ultraviolet light, effectively preventing transcription and replication (Berenbaum, 1978; Berenbaum et al., 1990). However, it is not toxic to the larvae of black swallowtail butterflies, *Papillo polyxenes* (Berenbaum and Feeny, 1981) due to the fact that P450 enzymes are involved in xanthotoxin metabolism in the

midgut of the larvae (Ivie et al., 1983; Bull et al., 1984, 1986). In addition, those cytochrome P450s involved in the metabolism of xanthotoxin were induced by xanthotoxin dose-dependently (Cohen et al., 1989). The induction of xanthotoxin metabolism in the black swallowtail caterpillar is chemical specific and many synthetic inducers, such as indole-3-carbinol and phenobarbital, are not the inducers of the xanthotoxin-metabolizing cytochrome P450. In contrast, substances with similar structures to xanthotoxin, such as its 5-methoxy isomer bergapten, can induce xanthotoxin-metabolizing cytochrome P450 activities to varying degrees (Berenbaum et al., 1990). In 1992, *CYP6B1* was isolated and sequenced from the black swallowtail caterpillar. *CYP6B1* is highly induced by xanthotoxin, a secondary metabolite abundant in the host plants of this specialized insect herbivore (Cohen et al., 1992). Besides the linear furanocoumarins, P450 enzymes also metabolize and are induced by angular furanocoumarins (Hung et al., 1995). In the *CYP6B1* gene, there is a complex array of transcripts, derived from a minimum of two loci to be differently induced by linear and angular furanocoumarins (Hung et al., 1995). It has been found that phenylalanine (Phe) 116 and histidine 117 contained within substrate recognition site 1 (SRS1), Phe 371 contained within SRS5, and Phe 484 contained within SRS6 of *CYP6B1* contribute to the formation of a resonant aromatic network that stabilizes the catalytic site, which is critical for furanocoumarin metabolism (Chen at al., 2002; Pan et al., 2004).

Induction of P450 genes by plant secondary substance in many insects has been taken as an indication of their roles in the metabolism of host plant secondary metabolites (Stevens et al., 2000). For example, members of the *CYP4* family in the tobacco hornworm, *Manduca sexta* (Snyder et al., 1995a), the *CYP6* family in black swallowtail

butterflies (Petersen et al., 2001; 2003), Canadian tiger swallowtails, *Papilio Canadensis* and Eastern tiger swallowtails, *Papilio glaucus* (Hung et al., 1996; 1997; Li et al., 2001; 2002), corn earworms, *Helicoverpa zea* (Li et al., 2000; 2002a) and Australian cotton bollworms (Li et al., 2002a), parsnip webworms, *Depressaria pastinacella* (Li et al., 2004), the *CYP9* family in tobacco hornworms (Stevens et al., 2000), and the *CYP28* family in *Drosophila* species (Danielson et al., 1997) have been reported to be inducible by host plant secondary metabolites. The broadly polyphagous corn earworm has been revealed to have a more complicated mechanism to protect if from plant defense. Jasmonate and salicylate were stolen by corn earworms as signals to trigger its P450 genes responsible for plant chemical metabolism either before or at the same time with the biosynthesis of plant chemicals, which are also activated by jasmonate and salicylate (Li et al., 2002b).

FIGURE LEGENDS

Figure 1.1. A simplified diagram of the P450 catalytic cycle shows the steps involved in the metabolism of substrate (XH).

Figure 1.2. Scheme of the P450 nomenclature (Feyereisen, 2005).

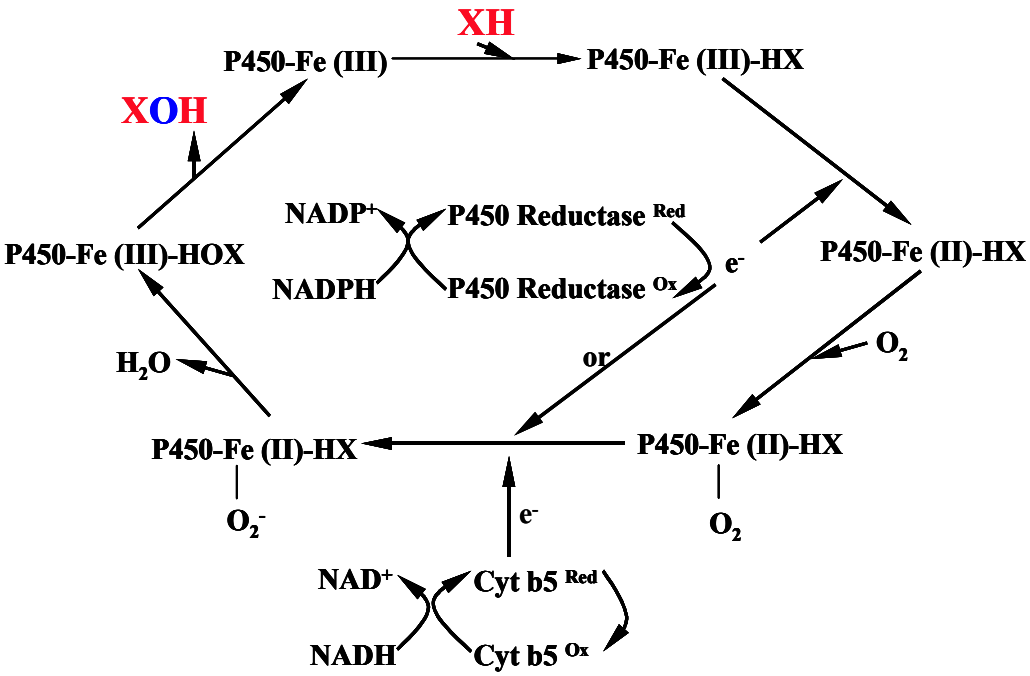
XH
P450-Fe (III)
P450-Fe (III)-HX
XOH
NADP+
P450 Reductase Red
P450-Fe (III)-HOX
NADPH
P450 Reductase Ox
e-
P450-Fe (II)-HX
H2O
or
O2
P450-Fe (II)-HX
P450-Fe (II)-HX
O2-
e-
O2
NAD+
Cyt b5 Red
NADH
Cyt b5 Ox

Figure 1.1

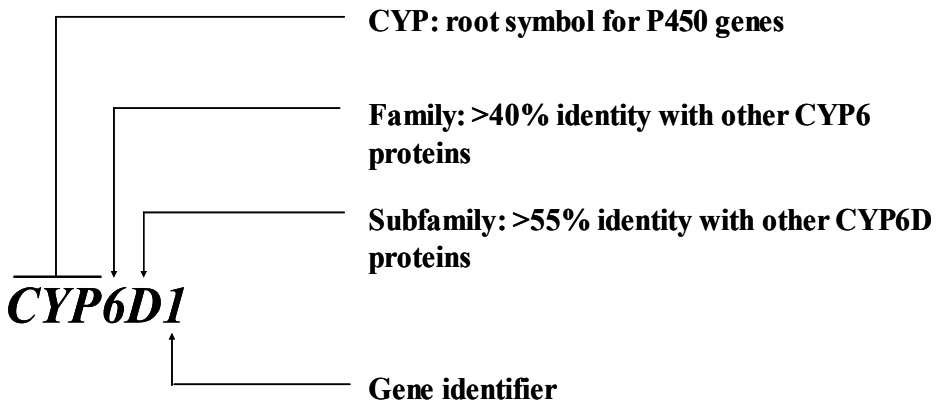
CYP: root symbol for P450 genes
Family: >40% identity with other CYP6 proteins
Subfamily: >55% identity with other CYP6D proteins
CYP6D1
Gene identifier

Figure 1.2

CHAPTER 2: RESEARCH GOAL AND SPECIFIC OBJECTIVES

2.1 Introduction

Insecticide resistance is an immensely practical problem associated with the chemical control of agriculturally and medically important insect pests. The cytochrome P450-mediated detoxification is one of the most significant mechanisms that have been studied in insecticide resistance (Scott, 1993). The house fly is not only a serious pest at livestock and poultry facilities but also a significant public health pest (Marcon et al., 2003). It has been found that house flies carry over 100 pathogen species and transmit more than 60 of them (Service, 1996; Marcon et al., 2003). One house fly strain ALHF was collected in Alabama in 1998, selected with permethrin for 6 generations after collection to reach a 6,600-fold resistance (Liu and Yue, 2000; 2001). When ALHF house flies were treated with PBO, an inhibitor of cytochrome P450, the resistance was reduced dramatically (Liu and Yue, 2000). Therefore, I hypothesize that P450-mediated detoxification is the most important mechanism involved in permethrin resistance in ALHF. Two questions are raised: 1) which P450s are involved in ALHF resistance? and 2) how are these P450s expressed and regulated in ALHF?

2.2 The goal of research and specific objectives

In order to answer these two questions in 2.1 and understand the importance of P450 genes in insecticide resistance, the long-term goal of my project is to determine P450 gene expression in the house fly in response to insecticides and investigate the regulation of P450 gene expression. To achieve the long-term goal, the following objectives will be performed: 1) isolating the putative P450 fragments from house flies; 2) investigating family 6 P450 genes that are overexpressed in resistant house flies; 3) characterizing P450 genes in response to insecticide stimulation in resistant house flies; 4) identifying functions of P450 alleles in resistant house flies; 5) analyzing the sequence and structure of P450 genes.

2.2.1 Isolating the putative P450 fragments from house flies

To date, there are about 1000 P450s that have been identified and named in insects. For example, ninety P450 genes (including seven pseudogenes) are present in the *Drosophila melanogaster* genome (Tijet et al., 2001), one hundred and eleven P450 genes (including five pseudogenes) are present in *Anopheles gambiae* (Ranson et al., 2002), and 86 P450 genes are present in *Bombyx mori* (Li et al., 2005). However, there are only 18 P450 genes cloned and named in house flies. In the current study, a pyrethroid resistant house fly strain, ALHF, will be used. ALHF is a wild-type strain collected from a poultry farm after failure control with permethrin in Alabama in 1998. ALHF obtained a 6,600-fold resistance after selection with permethrin for 6 generations, and then were maintained under biannual selection with permethrin (Liu and Yue, 2000; 2001). To clone the P450 genes, total RNA will be extracted from whole bodies of ALHF

flies using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott, 1997a). mRNA will be isolated with oligotex-dT suspension as described by manufacturer (QIAGEN). The reverse transcription-mediated polymerase chain reaction (RT-PCR) will be performed. The first strand cDNA will be synthesized with SuperScript II reverse transcriptase and an antisense 5'-anchored oligo (dT) primer (5' TAATACGA CTCACTATAGGGAGATTTTTTTTTTTTTT 3') (Tomita and Scott, 1995), using ALHF mRNAs as templates. The first strand cDNA will be amplified with either the C2 primer (5' TAATACGACTCACTATAGGGAGA 3') and internal degenerated sense primer, Flyh1 (5'-GGICCIAGIAACTG CATIGG-3') (or Flyc1 primer (5' GGAAGTNG ACACNTTYATGTT 3')) or two internal degenerated primers, antisense Heme R1 (5' CCIATGCAGTTICTIGGICC 3') and a sense degenerated primer designed based on a conserved amino acid region in house fly and *Drosophila* P450s. Flyh1 and Heme R1 will be designed according to the P450 heme binding consensus sequence. Flyc1 will be designed based on a conserved 13 amino acid region found in rat, human, and insect P450 sequences (Falackh et al., 1997). The PCR products will be cloned into PCR™ 2.1 Original TA cloning vector (Invitrogen) and sequenced by automated sequencing (Genomic and Sequencing Lab, Auburn University).

2.2.2 Investigating family 6 P450 genes that are overexpressed in resistant house flies

Increased levels of total P450s and P450 activities that are responsible for insecticide resistance are clearly attributable to an increased level of P450 gene expression (i.e., constitutive overexpression of P450 genes). Up to now, five of the 18 house fly P450 genes, *CYP6A1*, *CYP6A24*, *CYP6D1*, *CYP6D3*, and *CYP12A1* (Carino et

al., 1992; Guzov et al., 1998; Liu and Scott, 1998; Kamiya et al., 2001; Kasai and Scott, 2001), have been found to be constitutively overexpressed in insecticide resistant house flies, indicating that P450s in family 6 play important roles in insecticide resistance. Therefore, I hypothesize that there should be several P450 genes in family 6 that are overexpressed in the ALHF permethrin resistant strain compared to a susceptible one, and that these P450 genes may play critical roles in the ALHF resistance.

Three house fly strains will be used in this study. CS is a wild-type susceptible strain. aabys is a mutant susceptible strain with 5 recessive morphological markers, ali-curve (ac), aristapedia (ar), brown body (bwb), yellow eyes (ye), and snipped wings (sw), on autosomes 1, 2, 3, 4, and 5, respectively. Both susceptible strains were obtained from Dr. J. G. Scott (Cornell University). ALHF is a wild-type resistant strain (Liu and Yue, 2000; 2001). In order to test my hypothesis, total RNAs will be extracted from adults of CS and ALHF house flies using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott, 1997a). The developmental and tissue specific expression pattern of constitutively overexpressed P450 genes in ALHF will be further characterized by Northern blotting analysis according to Sambrook (1989). Total RNAs will be extracted from $1^{st}/2^{nd}$ instar larvae, 3^{rd} instar larvae, pupae, and adults of CS and ALHF house flies as well as the head+thorax and abdomen (tissues) from CS and ALHF using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott, 1997a) and then Northern blotting analysis will be carried out.

The 3' and/or 5' end of putative family 6 P450s that are overexpressed in ALHF will be cloned by 3'-RACE and/or 5'-RACE. The specific primer will be generated based on sequences of the putative P450 cDNAs. The full length of the putative P450 cDNA in CS

and ALHF will be generated by RT-PCR using one specific primer pair synthesized based on the 5' and 3' end sequences of the putative P450 gene.

To examine a causal link between the overexpressed P450 genes and insecticide resistance, I will examine the genetic linkage of P450 genes with 5 house fly lines derived from crosses of ALHF and a susceptible morphological marker strain, aabys, using allele specific PCR determination. A genetic cross experiment will be conducted as described previously (Liu and Scott, 1995; Liu and Yue, 2001). Allele specific PCR (ASPCR) will be carried out according to the method of Liu and Scott (1995). Two rounds of PCR will be performed. In the first PCR reaction, allele independent primers will be used as those used for cloning the full length of these P450s. In the second PCR reaction, allele specific primers will be designed according to the sequence where nucleotide polymorphism resides.

2.2.3 Characterizing P450 genes in response to insecticide stimulation in resistant house flies

The levels of certain P450 gene expression and monooxygenase activities can be significantly increased when these genes are exposed to particular xenobiotics, a phenomenon named induction (Feyereisen, 2005). Induction of P450 is probably another mechanism involved in the P450-mediated insecticide resistance because it economically provides energy by enhancing the activity of the detoxification system only when a chemical stimulus occurs (Brattsten, 1979). Therefore, I hypothesize that there should be certain P450 genes that can be significantly induced by permethrin stimulation in ALHF stain.

To test my hypothesis, three house fly strains, CS, ALHF, and aabys, will be used. Total RNAs will be extracted from permethrin-treated and non-treated adults of CS and ALHF house flies by using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott, 1997a). The developmental and tissue specific expression pattern of permethrin induced P450 genes will be further characterized by Northern blotting analysis according to Sambrook et al. (1989). The full length of these genes will be isolated by 3'-RACE and 5'-RACE as described in section 2.2.2.

To confirm a causal link between permethrin induced P450 genes and insecticide resistance, I will examine the genetic linkage of P450s with 5 house fly lines derived from crosses of ALHF and a susceptible morphological marker strain, aabys, using the single nucleotide polymorphism (SNP) determination with an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer (A&B Applied Biosystems).

2.2.4 Identifying functions of P450 alleles in resistant house flies

Cytochrome P450s constitute the largest gene superfamily found in all living organisms examined (Feyerence, 2005). Genome sequencing indicates that multiple P450 genes exist in an individual insect species. For example, 90 P450 genes (including 7 pseudogenes) are present in the *Drosophila melanogaster* genome (Tijet et al., 2001), 111 P450 genes (including 5 pseudogenes) are present in *Anopheles gambiae* (Ranson et al., 2002), and 86 P450 genes are present in *Bombyx mori* (Li et al., 2005). It has been hypothesized that all these P450 genes evolved from a common ancestor by gene duplication (Feyerence, 1999). Since gene duplication commonly occurs in the P450

kingdom and many P450 genes share high sequence similarity, the question arises: how can the expression of each of these P450 genes with high sequence similarity be precisely examined?

To answer this question, the expression pattern of P450 alleles isolated from Section 2.2.1 will be examined in non-permethrin treated and permethrin treated CS and ALHF house flies by Northern blotting analysis and/or quantitative real-time PCR (qRT-PCR). The alleles that are overexpressed and/or induced in the resistant strain will be further characterized for their tissue specific expression. The full length of these alleles will be isolated by 3'-RACE and 5'-RACE as described in section 2.2.2. Moreover, the genetic linkage of these alleles will be examined using ASPCR and/or SNP determination method(s).

2.2.5 Analyzing the sequence and structure of P450 genes

A pseudogene is a DNA sequence that resembles one or more paralogous functional genes, but does not produce a functional, full-length protein (Mighell et al., 2000). Pseudogenes are the consequence of gene duplication occurring in two main ways, retrotransposition and duplication of genomic DNA. Duplication of genomic DNA segments is an essential step in the development of complex genomes and the generation of gene families descended from a common ancestral gene (Mighell et al., 2000), such as the cytochrome P450 gene superfamily. Pseudogenes commonly exist in the P450 gene superfamilies. For example, there are 7 P450 pseudogenes in *Drosophila melanogaster* genome (Tijet et al., 2001) and 5 P450 pseudogenes in *Anopheles gambiae* (Ranson et al.,

2002). Therefore, I hypothesize that there should be certain P450 pseudogenes in house flies.

To test my hypothesis, three house fly strains, CS, ALHF, and aabys, will be used. I will perform 3'-RACE and/or 5'-RACE to get the full lengths of the genes isolated in section 2.2.1 from CS, ALHF, and aabys house flies as described in section 2.2.2. The sequences will be analyzed by bioinformatics tools. The expression patterns and genetic linkage of interesting genes will be investigated as described in sections 2.2.2 and 2.2.3.

2.3 Significance

Insect cytochrome P450s are critical for detoxification and/or activation of xenobiotics and play the fundamental role in biosynthesis and degradation of endogenous compounds. This proposed research is a comprehensive effort to decipher P450 genes and understand their functions in house flies. Cytochrome P450s involved in insecticide resistance are not only overexpressed in the resistant strain but also can be induced by the insecticides to which the insects are resistant. This study will provide a novel and dynamic view of the P450-mediated mechanisms in insecticide resistance.

CHAPTER 3: ISOLATION OF THE PUTATIVE P450 FRAGMENTS FROM HOUSE FLIES, *MUSCA DOMESTICA*

3.1 Introduction

Cytochrome P450s constitute one of the largest gene superfamilies found in all living organisms examined, including mammals, fish, arthropods, fungi, plants, and bacteria, and possess a great diversity of physiological and biochemical functions (Feyereisen, 2005). In insects, more than 1000 P450s have been identified and are distributed throughout over 150 subfamilies of 40 known P450 gene families (http://drnelson.utmem.edu/P450.stats.2006.htm). It has been found that an insect genome carries multiple P450 genes. For example, ninety P450 genes scattered in 25 families in the genome of *Drosophila melanogaster* (Meigen) were reported (Tijet et al., 2001) and 111 P450 genes exist in the genome of *Anopheles gambiae* (Ranson et al., 2002). However, there are only 18 P450 geness scattered in 4 families named and sequenced from house flies. To better understand the roles of P450 genes in physiological and toxicological processes, more individual house fly P450 genes need to be discovered.

At first, one method used to clone insect P450 genes was screening of cDNA expression libraries. For example, *CYP6A1* was isolated from *Musca domestica* cDNA libraries with polyclonal antibodies to purify P450 proteins of insecticide resistant flies (Feyereisen et al., 1989b), and *CYP6A2* was cloned and sequenced from *Drosophila*

cDNA libraries with monoclonal antibodies (Waters et al., 1992). In this classical cloning method, the initial P450 sequences were used as probes to screen the cDNA libraries for isolating related sequences in the same or phylogenetically close species (Cohen and Feyereisen, 1995; Hung et al., 1995; 1996; Li et al., 2001). Another method to clone new P450 genes by PCR with degenerated primers arose by 1994 and since then more and more vertebrate and insect P450 sequence information has become available (Feyereisen, 2005). In this method, the usual degenerated primers were designed according to the sequences of P450 conserved motifs PFxxGxRxCxG/A and GxE/DTT/S (Feyereisen, 2005). Plenty of new P450s were successfully cloned with this PCR method, such as 17 *CYP4* genes in *Anopheles albimanus* (Scott et al., 1994), 8 *CYP* genes from *Helicoverpa armigera* (Pittendrigh et al., 1997a), 14 P450 fragments from *Ceratitis capitata* (Danielson et al., 1999) and 95 P450 sequences from 16 *Drosophila* species (Fogleman et al., 1998). In this study, I chose the PCR method with a modification to clone the P450 genes from house flies.

3.2 Materials and methods

3.2.1 The house fly strain

ALHF was a wild-type house fly strain collected from a poultry farm in Alabama in 1998. The ALHF strain was selected with permethrin for 6 generations after collection, obtained a 6,600-fold resistance, and then was maintained under biannual selection with permethrin (Liu and Yue, 2000; 2001).

3.2.2 PCR method strategies

Three strategies were used to clone the P450 fragments from house flies by PCR according to the method of Snyder et al. (1996). In strategy I, a primer, Flyh1, designed based on the P450 heme binding region was used as a sense primer (Table 3.1). Oligo(dT) primer (for cDNA), C2, was used as antisense primer. In strategy II, Flyc1 was designed as the sense primer based on a conserved 13 amino acid region found in rat, human, and insect P450 sequences (Falackh et al., 1997). The antisense primer was C2 as well. In strategy III, one degenerated primer designed according to the conserved region of insect P450 family 6 was used as a sense primer. An antisense primer, Heme R1, was designed based on the complementary sequences of P450 heme binding region (Fig. 3.1; Table 3.1).

3.2.3 Cloning and sequencing of P450 genes from house flies

Putative house fly P450 cDNA fragments were amplified from ALHF by RT-PCR with 3 PCR strategies (section 3.2.2). The PCR products were cloned into PCR™ 2.1 Original TA cloning vector (Invitrogen) and sequenced by automated sequencing (Genomic and Sequencing Lab, Auburn University). Then the sequence information was submitted to the NCBI gene bank database to perform BLAST search.

3.3 Results and discussion

Nineteen cDNA fragments were successfully cloned and sequenced from ALHF house flies (Table 3.2). Blast analysis of the amino acid sequences predicted from these partial putative P450 cDNAs showed that these cDNA fragments exhibit significant

protein homologies to the members in cytochrome P450 families 4, 6, and 9 with similarities ranging from 50% to 98%, indicating that these P450s are new P450s. The P450 protein signature motif, FXXGXRXCXG, is present in all P450 fragments. In strategy I, Flyh1 and C2 served as primers, and PCR products of about 230-400 bp (including a variable 3' UTR sequence) that code for around 60 amino acids were isolated. In strategy II, Flyc1 and C2 serving as primers, fragments of about 650-950bp (including a variable 3' UTR sequence) were produced. The fragment of 12' only has 382bp possibly because of exon skipping in the 3' end. In strategy III, one degenerated primer was designed according to the conserved region of insect P450 family 6 and the reverse primer, Heme R1, was designed based on the P450 heme binding region (Fig. 3.1; Table 3.1). The nucleotide sequence of PCR products was around 450-970 bp that coded for about 150-320 amino acids in the middle region. The 3' and/or 5' end of these putative P450s will be cloned by 3'-RACE and/or 5'-RACE.

Nineteen novel P450 fragments scattered in families 4, 6, and 9 were cloned from house flies using the PCR method indicating that this method is a successful approach for P450 cloning. Ten of 19 P450s were cloned by strategy I. However, the fragments cloned by this strategy are very short and therefore the full length of sequence is not easy to be obtained. Strategy II is a better one to clone new CYP4 genes. The fragments cloned by strategy II are long and about half of the full-length of P450 genes. Most fragments cloned by strategy III are CYP6 genes because the forward primer in this strategy was designed according to the conserved region of insect P450 family 6. The lengths of P450 fragments in this strategy are about 440-970 bps. Because it is not far from both 3' and 5' ends of the sequence, the full length of these fragments can easily be obtained. If the

degenerated primer designed according to the conserved region of other families besides family 6 of P450 is used as forward primers in strategy III, many new P450s scattered in other families will be isolated.

In conclusion, nineteen new P450 fragments were isolated from house flies with 3 PCR strategies. Based on these partial P450 cDNA sequences, I will characterize the P450 gene expressional patterns in different development stages, tissues, and in response to permethrin treatment for resistant and susceptible house fly strains. This study provided a framework for conducting the whole project to understand functions of P450 genes in permethrin resistance of house flies.

Table 3.1. Degenerated primers used for amplifying P450 gene fragments in house flies

Strategies	Name of Primers	Sequence of Primers
I	Flyh1	5'-GGICCIAGIAACTGCATIGG-3'
II	Flyc1	5'-GGAAGTNGACACNTTYATGTT-3'
III	Heme R1	5'-CCIATGCAGTTICTIGGICC-3'
	CYP6A1	5'-CYTTTGGCATTGARTGCARKAG-3'
	CYP6AD1	5'-CVTCNGSHAARATKAARHNNATG-3'
	CYP6D1	5'-GATCGYGGSVTBTAYGTKGAYG-3'

Table 3.2. Nineteen P450 cDNA fragments isolated from house flies

Number	Sense Primer	Anti-sense Primer	Homologous Protein	Similarity (%)	Length (bp)
1	Flyh1	C2	CYP6N3v3	57	308
2	Flyh1	C2	CYP6G1	50	238
3	Flyh1	C2	CYP9	51	273
4	Flyh1	C2	CYP4D1	60	285
5	Flyh1	C2	CYP4D14	75	289
7	Flyh1	C2	CYP9	56	301
8	Flyh1	C2	CYP4D14	69	405
9	Flyh1	C2	CYP6A25	92	277
10	Flyh1	C2	CYP6A5	98	274
11	Flyc1	C2	CYP4D1	66	658
12	Flyc1	C2	CYP4D8	57	874
12'	Flyc1	C2	CYP4D8	87	382
13	Flyc1	C2	CYP4AC1	63	796
14	Flyc1	C2	CYP4P3	53	975
15	CYP6AD1	Heme R1	CYP6A9	62	448
15'	Flyh1	C2	CYP6A9	66	231
16	CYP6A1	Heme R1	CYP6A24	96	976
17	CYP6AD1	Heme R1	CYP6A24	87	672
20	CYP6D1	Heme R1	CYP4G1	80	583

FIGURE LEGEND

Fig 3.1. Three strategies used to amplify P450 gene fragments. The blank box represents P450 sequence. The small boxes with different patterns indicate conserved regions in which the degenerated primers were designed. Degenerated or C2 primer for PCR was represented by arrows.

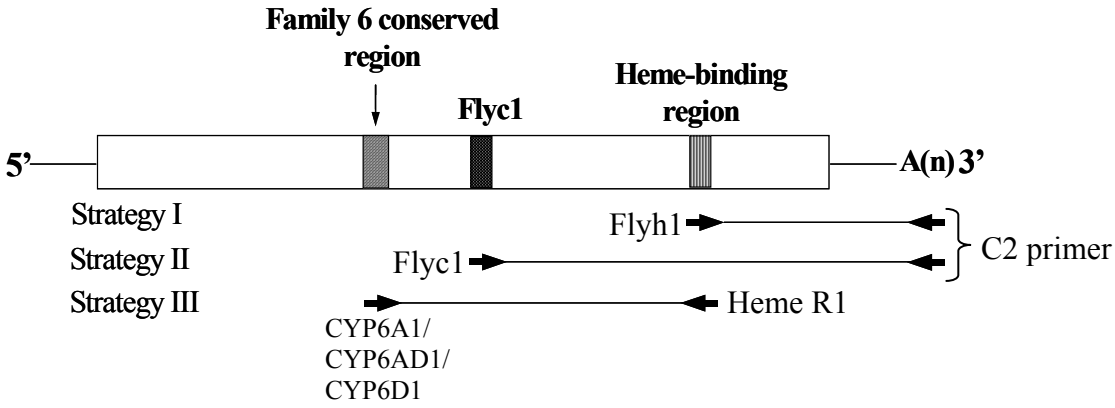
Family 6 conserved region
Heme-binding region
Flyc1
5'
A(n) 3'
Strategy I
Strategy II
Strategy III
Flyh1
Flyc1
C2 primer
Heme R1
CYP6A1/
CYP6AD1/
CYP6D1

Figure 3.1

CHAPTER 4: CHARACTERIZATION OF TWO NOVEL CYTOCHROME P450 GENES IN INSECTICIDE RESISTANT HOUSE FLIES

4.1 Introduction

Insect cytochrome P450s are known to play an important role in detoxifying exogenous compounds such as insecticides (Scott, 1999; Feyereisen, 2005) and plant toxins (Berenbaum, 1991; Schuler, 1996). Increased P450 proteins and P450 activities resulting from transcriptional up-regulation of P450 genes are involved in enhanced metabolic detoxification of insecticides and plant toxins in insects, leading to the development of resistance to insecticides (Carino et al., 1994; Liu and Scott, 1997; Liu and Scott, 1998; Kasai et al., 2000; Feyereisen, 2005) and tolerance to plant toxins (Li et al., 2002b; Wen et al., 2003). Insect P450s are also an important part of the biosynthesis and degradation pathways of endogenous compounds such as pheromones, 20-hydroxyecdysone, and juvenile hormone (JH) (Reed et al., 1994; Sutherland et al., 1998; Winter et al., 1999; Gilbert, 2004; Niwa et al., 2004) and thus play important roles in insect growth, development, and reproduction. Although the importance of P450s in insect physiology and toxicology is widely recognized, there are enormous gaps in our knowledge of insect P450s, their roles, and regulation processes in the evolution of insecticide resistance, which typically requires the interaction of multiple genes. As

more P450 sequences become available, the better our understanding of the roles of P450s in physiological and toxicological processes. Consequently, the characterization of P450 gene regulation and interactions in resistance is fundamental for achieving an understanding of the complex processes responsible for the evolution of resistance.

The house fly *Musca domestica* is not only a serious pest affecting livestock and poultry facilities, but also poses a major threat to human health by transmitting the pathogens that cause many human diseases (Kettle, 1984; Burgess, 1990). The house fly has shown a remarkable ability to develop resistance to insecticides (Liu and Yue, 2000; Shono et al., 2004). To date, five P450 genes, *CYP6A1* (in diazinon resistant Rugters strain), *CYP6A24* (in pyrethoroid resistant YPER, YS, and Hachinohe strains), *CYP6D1* (in Learn Pyrethroid Resistant (LPR) strain), *CYP6D3* (in diazinon resistant Rugters strain and LPR strain), and *CYP12A1* (in diazinon resistant Rugters strain), have been found to be overexpressed in insecticide resistant house flies (Carino et al., 1992; Guzov et al., 1998; Liu and Scott, 1998; Kamiya et al., 2001; Kasai and Scott, 2001). Two of these genes, *CYP6A1* and *CYP6D1*, have been documented to metabolize xenobiotics in house flies (Feyereisen, 1999; Scott, 1999). Isolation and characterization of additional P450 genes involved in insecticide resistance in house flies will be an essential step towards understanding their role in the life of house flies and the true importance of P450s in the integration of insecticide resistance.

The house fly strain ALHF exhibits high levels of resistance to pyrethroids (Liu and Yue, 2000). Previous research using piperonyl butoxide (PBO), an inhibitor of cytochrome P450s, suggested that P450-mediated detoxification may be one of the major mechanisms involved in pyrethroid resistance in ALHF (Liu and Yue, 2000). Genetic

linkage analysis points to the localization of PBO-suppressible-P450-mediated resistance on autosomes 1, 2, and 5 of ALHF (Liu and Yue, 2001), and factors on autosome 5 are known to play a major role in P450-mediated resistance. The current study, therefore, focused on the isolation and characterization of individual P450 genes from ALHF that are responsible for P450-mediated resistance. Two novel P450 genes, *CYP6A36* and *CYP6A37* were isolated from ALHF; changes in the expression profile of these 2 genes during a house fly's life cycle, among different tissues, and between resistant and susceptible house flies were characterized; and genetic linkage studies conducted as part of the search for a causal link between the P450 genes and insecticide resistance in ALHF house flies.

4.2 Materials and methods

4.2.1 House fly strains

Three house fly strains were used in this study. ALHF, a wild-type strain collected from a poultry farm in Alabama in 1998, was selected with permethrin for 6 generations after collection to reach a 6,600-fold resistance, and maintained under biannual selection with permethrin (Liu and Yue, 2000; 2001). CS is a wild type insecticide-susceptible strain. aabys is an insecticide-susceptible strain with recessive morphological markers ali-curve (*ac*), aristapedia (*ar*), brown body (*bwb*), yellow eyes (*ye*), and snipped wings (*sw*) on autosomes 1, 2, 3, 4, and 5, respectively. Both CS and aabys were obtained from Dr. J. G. Scott (Cornell University).

4.2.2 RNA extraction, cDNA preparation, and the 3' half of the putative P450 gene fragment isolation

Total RNAs were extracted from house flies using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott, 1997b). mRNA was isolated with oligotex-dT suspension (QIAGEN). Rapid amplification of 3' cDNA ends (3'-RACE) was performed by reverse transcription-mediated polymerase chain reaction (RT-PCR). The first strand cDNA was synthesized with SuperScript II reverse transcriptase and an antisense 5'-anchored oligo(dT) primer (Table 4.1) (Tomita and Scott, 1995), using ALHF mRNAs as templates. The first strand cDNA products were amplified by PCR with the C2 primer and 2 internal degenerated sense primers, CYP6AD1 and Flyh1, (Table 4.1). The CYP6AD1 was designed based on a conserved amino acid region found in house fly, *Drosophila,* and mosquito P450 sequences after alignment of these insect P450 genes and Flyh1 was designed based on the heme binding consensus sequence (Liu and Zhang, 2002). The PCR products were cloned into PCR™ 2.1 Original TA cloning vector (Invitrogen) and sequenced by automated sequencing (Genomic & Sequencing Lab, Auburn University). Cloning and sequence analyses of P450 gene fragments were repeated at least three times with different preparations of mRNAs. Three TA clones from each replication were sequenced.

4.2.3 Cloning the 5' half of the putative P450 gene fragments and the full length of the putative P450 cDNAs

To clone the 5' half of the putative P450 gene fragments, 5'-RACE was carried out using the Marathon™ cDNA Amplification Kit (Clontech) (Liu and Zhang, 2002). The

first strand cDNAs were synthesized with AMV reverse transcriptase using ALHF mRNAs as templates. The primers, P450HF15R and P450HF15R2 (Table 4.1), were generated based on sequences of the 3' putative P450 cDNA fragments. The double strand cDNA was synthesized following the protocol described by the manufacturer (Clontech). Adaptors were ligated to both ends of the double strand cDNA as described by the manufacturer. The double strand cDNAs were amplified by PCR with the primer pairs, P450HF15R/AP1 (based on the sequence of the adaptor) and P450HF15R2/AP1. The PCR products were cloned into PCR™ 2.1 Original TA cloning vector (Invitrogen) and sequenced. The full length of the 2 putative P450 cDNAs was generated by RT-PCR using two specific primer pairs, AP450HF15F/ AP450HF15R and AP450HF15'F/ AP450HF15'R (Table 4.1), synthesized based on the 5'and 3'end sequences of the 2 putative P450 genes. The PCR products were cloned and sequenced. Cloning and sequence analyses of the P450 cDNA fragments were repeated at least three times with different preparations of mRNAs, and three TA clones from each replication were verified by sequencing.

4.2.4 Cloning and sequencing the 5' flanking region of the P450 gene, CYP6A36, from ALHF and aabys

In order to clone the 5' flanking regions of *CYP6A36*, 5 adaptor-ligated ALHF genomic DNA libraries were constructed using the Universal GenomeWalker™ Kit (Clontech). House fly genomic DNAs were isolated as described by Liu and Scott (Liu and Scott, 1995) and then digested with 5 different restriction enzymes (provided in the kit) to generate 5 pools of blunt-end fragments of ALHF genomic DNAs. For each pool,

the genomic DNA fragments were ligated to GenomeWalker adaptors to form a GenomeWalker library. The adaptor ligated DNA fragments in the GenomeWalker libraries were amplified by PCR with Advantage *Tth* polymerase (Clontech), the antisense primer, P450HF15P (Table 4.1) based on the 5' coding region of the *CYP6A36*, and a sense primer, AP1, based on the sequence of the adaptor. The PCR products were cloned into the TA cloning vector (Invitrogen) and sequenced. Cloning and sequence analyses of PCR products were repeated at least three times each with three TA clones from each replication. The 5' flanking region of *CYP6A36* in aabys was subsequently generated by PCR from the genomic DNA using a primer pair, P450HF15P-F/ P450HF15P (Table 4.1) designed according to the 5' flanking region of *CYP6A36* in ALHF.

4.2.5 Northern blotting analysis

Northern blotting analyses were performed according to Sambrook et al. (Sambrook et al., 1989). Twenty micrograms of total RNA from each sample were fractionated on 1% formaldehyde denaturing agarose gel and transferred to Nytran membranes (Schleicher and Schuell) (Sambrook et al., 1989). The P450 cDNA fragments were labeled with [α-^{32}P] dCTP using a Primer-It II Random Primer Labeling kit (Stratagene) and hybridized with RNA blots using QuickHyb solution (Stratagene). The amount of RNA loaded in each lane was standardized by comparing the density of the 18S ribosomal RNA band on agarose gel under UV light before transfer (Spiess and Ivell, 1998). All Northern blot analyses were repeated three times with different preparations of RNA samples. The radiographic signal intensity was quantitatively analyzed by

QuantiScan v3.0 (Biosoft) as previously (Liu and Zhang, 2004). Statistical significance of the gene expressions was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant.

4.2.6 Genetic linkage analysis of cytochrome P450 genes

To determine genetic linkage of P450 genes, a genetic cross experiment was conducted (Liu and Scott, 1995; Liu and Yue, 2001; Fig. 4.1). Briefly, virgin females were collected within 8 h after emergence. Reciprocal crosses between ALHF and aabys were conducted. F_1 males were back-crossed to aabys females that were isolated every 8 h. Five phenotypes were saved from the back-cross generation (BC_1) with the genotypes of: *ac/ac*, +/*ar*, +/*bwb*, +/*ye*, +/*sw* (A2345); +/*ac*, *ar/ar*, +/*bwb*, +/*ye*, +/*sw* (A1345); +/*ac*, +/*ar*, *bwb/bwb*, +/*ye*, +/*sw* (A1245); +/*ac*, +/*ar*, +/*bwb*, *ye/ye*, +/*sw* (A1235); and +/*ac*, +/*ar*, +/*bwb*, +/*ye*, *sw/sw* (A1234). Since crossing over does not or very rarely occurs in male flies (Gao and Scott, 2006), the presence of a mutant phenotype indicated that the respective autosome with a mutant-type marker was derived from aabys. The genotype of each line was homozygous for the recessive mutant allele from aabys and heterozygous for the dominant wild-type alleles from ALHF. These lines were named according to the autosomes bearing wild-type markers from ALHF. For example, A1234 strain had wild-type markers on autosomes 1, 2, 3, and 4 from ALHF and the recessive mutant marker on autosome 5 from aabys.

Allele specific PCR was conducted for genetic mapping of P450 genes (Liu and Scott, 1995). Two rounds of PCR were conducted. For the first PCR reaction, the allele-

independent primer pairs, P450HF15P-F/P450HF15P and AP450HF15'F/AP450HF15'R (Table 4.1), were used to generate *CYP6A36 and CYP6A37* cDNA fragments, respectively. The second PCR were employed with 0.5 μl of the first round PCR reaction solution and the allele specific primer pairs, AP450HF15PNF-1/P450HF15P and AP450HF15'NLF-1/ AP450HF15'NLR (Table 4.1). One of each allele specific primer pair was designed based on the specific sequence of the P450 gene from ALHF by placing a specific nucleotide polymorphism at the 3' end of the primer to permit preferential amplification of P450 allele from ALHF (Figs. 4.7A and 4.8A). Each experiment was repeated three times with different preparations of genomic DNAs (for *CYP6A36*) or mRNAs (for *CYP6A36*). To confirm that the PCR products were in fact the P450 gene fragments, the PCR products were sequenced at least once each.

4.3 Results

4.3.1 Cloning and sequencing of P450 genes from house flies

Two distinct partial putative house fly P450 cDNA fragments were amplified from ALHF by RT-PCR with 3' RACE using a 5'-anchored oligo(dT) primer and 2 internal degenerate primers, CYP6AD1 and Flyh1. BLAST analysis of the amino acid sequences predicted from these partial putative P450 cDNAs showed that these 2 sequences encoded the C-terminal ends of putative P450s with 50-70% identity to the CYP6 family of insects. To isolate and amplify the 5' ends of these two putative P450 genes, we conducted a 5' RACE reaction for each putative P450 gene using a specific antisense primer synthesized based on the sequence of the 3' putative P450 cDNA fragment and a sense primer, AP1, synthesized based on the sequences of the adaptor (Clontech). The

sequence of each 5' RACE reaction amplified cDNA overlapped with its corresponding 3' putative P450 cDNA fragment, identifying them as the 5' ends of the putative P450 genes. An entire cDNA fragment for each of the putative P450 genes was subsequently amplified from both ALHF and aabys house flies by PCR using the primers synthesized based on the respective 5' and 3' end sequences of the putative P450 genes. The sequences of the entire cDNA fragments perfectly overlapped with the 3' and 5' sequences of the putative P450 genes generated by 3' and 5' RACE (Figs. 4.2 and 4.3). The sequences were named *CYP6A36* and *CYP6A37* (accession numbers: DQ642009 and DQ642010, respectively) by the P450 nomenclature committee (Dr. D. Nelson, personal communication).

4.3.2 Structural analysis of CYP6A36 and CYP6A37 from house flies

The cDNA sequences of *CYP6A36* and *CYP6A37* have open reading frames (ORFs) of 1521 and 1518 nucleotides encoding proteins of 507 and 506 residues, respectively (Fig. 4.4). The putative protein sequences of CYP6A36 and CYP6A37 deduced from the cDNA sequences shared 59 and 66% identities with *Drosophila melanogaster* CYP6A21 and CYP6A9, respectively. CYP6A36 and CYP6A37 protein sequences have a highly hydrophobic N terminus that is characteristic of membrane-bound P450 proteins and functions as a membrane-anchor signal (Sakaguchi et al., 1987). The P450 protein signature (heme binding) motif, FXXGXRXCXG (Feyereisen, 1999), is presented at amino acid residues 445-454 of CYP6A36 and 444-453 of CYP6A37. The cysteine residue in this motif is known to be an important ligand for heme binding. The other important motif, YXXAXXXEXXR, the conserved region of P450 sequences coinciding

with Helix K in P450cam (Gotoh and Fujii-Kuriyama, 1989), is present at amino acid residues 362-372 of CYP6A36 and 361-371 of CYP6A37, with the same alanine residue change to glutamine (Fig. 4.4). A typical aromatic sequence of P450s with 3 aromatic (A) residues and 2 highly conserved proline residues, $A_1XXPXXA_2XPXBA_3$ (Gotoh and Fujii-Kuriyama, 1989), is present at amino acid residues 418-429 of CYP6A36 and 417-428 of CYP6A37. Finally, another conserved 17- residue sequence, corresponding to the central part of the I-helix, was found at amino acid residues 306-322 of CYP6A36 and 305-321 of CYP6A37 (Fig. 4.4). This motif is particularly conserved among family 6 (Cornette et al., 2006) and is thought to be involved in proton delivery (Gorokhov et al., 2003). Comparison of the deduced protein sequences of both CYP6A36 and CYP6A37 from resistant ALHF and susceptible aabys house flies revealed identical protein sequences between these two strains, although two nucleotide polymorphisms of A to C and T to G were found 222 and 1020 nucleotides downstream of the ATG translation start codon of *CYP6A37*, respectively, between the two strains.

4.3.3 Developmental expression of CYP6A36 and *CYP6A37 in resistant and susceptible house flies*

Diversity in the developmental expression and regulation of insect P450s is well established, so expression patterns of *CYP6A36* and *CYP6A37* were examined for different stages in the house fly's life cycle. Northern blot analysis was performed to compare expression levels of the 2 P450 genes for 4 development stages of both the CS and ALHF strains. There were significant differences in the expression of *CYP6A36* among developmental stages and between susceptible CS and resistant ALHF strains.

The expression of *CYP6A36* was detectable in the 3rd instar of ALHF but not in CS, not detected in the pupa of either strain, and then rose to a maximum in both CS and ALHF adults (Fig. 4.5A, C) with a significant overexpression in ALHF flies (2.7-fold) compared to the CS susceptible flies. In contrast, the expression of *CYP6A37* was detected in all the tested life stages of both CS and ALHF strains (Fig. 4.5A, B) and no significant difference in the expression of *CYP6A37* was detected between CS and ALHF.

4.3.4 Tissue specific overexpression of CYP6A36 between ALHF and CS house flies

Insect P450s may also vary as to the tissues where they are expressed in response to physiological and environmental stimulators. In insects, the midgut and fat body tissue have generally been considered to be the primary detoxification organs (Hodgson, 1985) where most insect detoxification P450s are expressed (Scott et al., 1998). To investigate whether the overexpression of *CYP6A36* is tissue specific, RNAs from the abdomen and head+thorax of adults of both the CS and ALHF strains were subjected to Northern blot analyses and revealed no significant difference in the expression of *CYP6A36* between the head+thorax and abdomen tissues of the CS strain (Fig. 4.6A, B). Whereas, the expression of *CYP6A36* was lower in the head+thorax tissue and higher in the abdomen tissue of ALHF (Fig. 4.6A, B). However, significant overexpression was more evident in the ALHF strain in both tissues compared with the CS strain. The expression of *CYP6A36* was about 2-fold higher in the head+thorax and ~5.6-fold higher in the abdomen tissue of the ALHF strain compared to the CS strain (Fig. 4.6B).

4.3.5 Chromosomal linkage of CYP6A36 and CYP6A37

To confirm a causal link between P450 genes and insecticide resistance, we examined the genetic linkage of both *CYP6A36* and *CYP6A37* with 5 house fly lines derived from crosses of ALHF and a susceptible morphological marker strain, aabys, using allele specific PCR determination. Sequence comparison of *CYP6A36* and *CYP6A37* between ALHF and aabys reveled two nucleotide polymorphisms of A to C and T to G, respectively, that were presented at 222 and 1020 nucleotides downstream of the ATG translation start codon of *CYP6A37* and no nucleotide polymorphisms were identified in the coding region of *CYP6A36*. Thus, we cloned a ~700 bp 5' flanking region of *CYP6A36* using a Genome Walking method (Liu and Zhang, 2002) in order to genetically map the *CYP6A36* gene. Comparison of the nucleotide sequence of the 5' flanking region of *CYP6A36* uncovered several deletions (or insertions) and single nucleotide polymorphisms between ALHF and aabys house flies. Based on the sequence differences of both *CYP6A36* and *CYP6A37* between ALHF and aabys, specific primer pairs were designed for allele specific PCR reactions by placing single mismatched bases at the 3' ends of primers that permitted preferential amplification of ALHF alleles over aabys' (Fig. 4.7A, B). To increase primer specificity and avoid false-negative results, two rounds of PCR amplification were performed. In the first PCR reaction, universal primer sets (P450HF15P-F/P450HF15P (Table 4.1) and AP450HF15'F/AP450HF15'R (Table 4.1) for *CYP6A36* and *CYP6A37*, respectively, were used and the expected products were obtained in all strains tested (Figs. 4.7 and 4.8). The second PCR reactions were conducted using the first PCR products as the templates and ALHF allele-specific primer sets, AP450HF15PNF-1(ALHF allele specific)/P450HF15P and AP450HF15'NLF-1

(ALHF allele specific)/AP450HF15'NLR (Table 4.1, Figs. 4.7A and 4.8A), for *CYP6A36* and *CYP6A37*, respectively. The ALHF allele-specific primer sets for both *CYP6A36* and *CYP6A37* amplified specific DNA fragments only in flies having the autosome 5 wild type marker (i.e., *sw*) (Figs. 4.7 and 4.8), demonstrating that both *CYP6A36* and *CYP6A37* are located on autosome 5.

4.4 Discussion

Previous research indicated that resistance in the ALHF strain could be largely suppressed by PBO, an inhibitor of cytochrome P450s. Further, P450-mediated resistance has been mainly linked to autosome 5, with minor factors linked to autosomes 1 and 2 (Liu and Yue, 2001). Nevertheless, no individual P450s have been identified in the ALHF strain as being responsible for resistance. In the current study, we have cloned, sequenced, and characterized two novel P450 genes, *CYP6A36* and *CYP6A37*, from ALHF and susceptible house flies. In many cases, increased levels of P450 gene expression (i.e., overexpression of P450 genes) have resulted in increased levels of total P450s and P450 activities that are responsible for insecticide resistance (Carino et al., 1992; Liu and Scott, 1997b; Feyereisen, 2005). For example, in house flies, a link between *CYP6A1* and *CYP6D1* and insecticide resistance is suggested by evidence that resistant strains have greater metabolic detoxification, higher P450 content, and more P450 transcripts than susceptible strains (Carino et al., 1992; Carino et al., 1994; Liu and Scott, 1997b). A similar linkage has also been reported in other insect species (Festucci-Buselli et al., 2005). Accordingly, our finding of the lack of a significant difference in the expression of *CYP6A37* between resistant ALHF and susceptible CS house flies may

suggest less importance of this gene in the resistance of ALHF. In contrast, the overexpression of *CYP6A36* in both larvae and adult stages of ALHF suggests its important role in ALHF resistance. If *CYP6A36* is involved in xenobiotic metabolism, the overexpression of *CYP6A36* in larval instars and adult ALHF house flies may indicate the adaptive ability of ALHF to metabolize insecticides after exposure from the larval stage onward.

In insects, the midgut has generally been considered to be the primary detoxification organ (Hodgson, 1985) where most insect detoxification P450 genes are expressed (Scott et al., 1998). Fat body tissue has been identified as another major organ for insect P450s (Scott et al., 1998). So to ascertain the importance of the overexpression of *CYP6A36* in metabolizing insecticides and the development of resistance, the tissue specific expression of *CYP6A36* was explored. We found that *CYP6A36* was much more significantly overexpressed in abdomen tissue than in the head+thorax in ALHF house flies and the overexpression of *CYP6A36* was more evident in both head+thorax and abdomen tissues of ALHF compared with CS. As midgut and most fat body components are located in the abdomen of insects, relatively high levels of *CYP6A36* in the abdomen of ALHF may suggest that the midgut and fat body are of primary importance in detoxification-related functions in this strain. Detoxification-related tissue specific overexpression of *CYP6A36* further suggests the importance of the gene in increasing metabolic detoxification of insecticide in ALHF house flies compared with the susceptible CS strain.

Genetic linkage between an overexpressed P450 gene or protein and insecticide resistance is an important step in establishing a causal link between a P450 gene and its

role in resistance (Carino et al., 1994; Liu and Scott, 1996; Rose et al., 1997; Guzov et al., 1998; Maitra et al., 2000; Feyereisen, 2005). We therefore examined the linkage of *CYP6A36* with 5 house fly lines derived from crosses of ALHF and a susceptible morphological marker strain, aabys, using allele specific PCR determination and found it to be located on autosome 5. In a previous study, we demonstrated that P450-mediated resistance (or PBO suppressible resistance) in the ALHF house flies was linked to autosomes 1, 2, and 5 (Liu and Yue, 2001) and that the factor(s) on autosome 5 played a major role in PBO-suppression (Liu and Yue, 2001). Given that *CYP6A36* is overexpressed in ALHF, specifically in abdomen tissue, and that *CYP6A36* is located on autosome 5, which is correlated with the linkage of resistance in ALHF, it seems likely that the overexpression of *CYP6A36* plays an important role in insecticide resistance in ALHF house flies. Although *CYP6A37* was also linked to autosome 5, the lack of a significant difference in the expression of *CYP6A37* between ALHF and CS house flies suggests the reduced importance of *CYP6A37* in resistance in ALHF.

In addition to the gene(s) on autosome 5, our previous studies have demonstrated that P450-mediated resistance (or PBO suppressible resistance) in ALHF house flies is linked to autosomes 1 and 2, strongly suggesting that other genes or factors are involved in PBO suppressible resistance in ALHF. Many studies have revealed that the up-regulation of P450 genes in resistant insects is regulated by *trans* or *cis* regulatory genes. The up-regulation of 2 P450 genes, *CYP6A1* and *CYP6D1*, in insecticide resistant house flies is known to be *trans*-regulated by one or more factors on autosome 2 (Carino et al., 1994). The up-regulation of *CYP6A2* and *CYP6A8* in the insecticide resistant fruit fly *Drosophila melanogaster* is also known to be regulated by *trans*-regulatory factors

(Maitra et al., 1996; Maitra et al., 2000). Thus, we cannot exclude the possibility that the factors or genes on autosomes 1 and 2 may be involved in the regulation of *CYP6A36*. Although we detected no differences in the deduced protein sequence of CYP6A36 between susceptible aabys and resistant ALHF house flies, we identified significant differences – deletions (or insertions) and single nucleotide polymorphisms - in the 5' flanking region of *CYP6A36* between the two strains. Further characterization of the overexpression regulation, especially the importance of factors on autosomes 1 and 2 (Liu and Yue, 2001), as well as the function of different elements on the 5' flanking region of *CYP6A36* between ALHF and aabys house flies is therefore necessary to improve our understanding of P450 gene regulation in resistance.

Since the level of resistance in ALHF is dramatically decreased by PBO from 1800-fold to 100-fold (Liu and Yue, 2000) and the level of *CYP6A36* expression is only about 3- to 4-fold higher compared with the susceptible CS strain, we further suggest that *CYP6A36* may only play a partial role in resistance in the ALHF strain. Multiple P450s have been identified in individual insect species (Feyereisen, 2005); for example, overexpression of *CYP6A1*, *CYP6D1*, and *CYP6D3* has been reported in a resistant LPR house fly strain (Carino et al., 1992; Liu and Scott, 1996; Kasai and Scott, 2001). These studies implicate multiple P450 genes in the detoxification of insecticides in a single organism and, consequently, in the development of resistance in individual organisms. Thus further characterization of the other P450 genes, in addition to *CYP6A36*, involved in PBO-suppressible resistance in ALHF will be extremely important for understanding the overall P450-mediated resistance in house flies.

Insect P450s have been implicated in the biosynthesis and degradation pathways of endogenous compounds such as pheromones, 20-hydroxyecdysone, and juvenile hormone (JH) (Reed et al., 1994; Sutherland et al., 1998; Feyereisen, 1999; Sutherland et al., 2000; Gilbert, 2004; Feyereisen, 2005). Recently, *CYP18*, *CYP6H1*, and several Halloween P450 genes from insects have been implicated in the biosynthesis of ecdysone (Bassett et al., 1997; Helvig et al., 2004; Niwa et al., 2004; Niwa et al., 2005). A common feature of these P450 genes is that there is an almost perfect spatio-temporal correlation between the dynamics of ecdysone production and the expression of the Halloween P450 genes (Parvy et al., 2005). Expression of *CYP18* in *Drosophila melanogaster* is tightly associated with ecdysteroid pulses in the 1^{st}, 2^{nd}, and 3^{rd} larval instars, at the time of pupariation, and in pupae (Bassett et al., 1997). Like the genes involved in ecdysone biosynthesis, P450 genes, such as *CYP4C1* (Lu et al., 1999), *CYP4C7* (Sutherland et al., 2000), and *CYP15A1* (Helvig et al., 2004), that have been implicated in JH biosynthesis also display a temporal correlation between expression of the gene and the dynamics of juvenile hormone production. Although *CYP6A36* and *CYP6A37* may be involved in hormone biosynthesis, the lack of expression of *CYP6A36* in the 1^{st} and 2^{nd} larval instars and pupae may not suggest this function. However, the significant expression of *CYP6A37* in all life stages of house flies may merit more consideration of its role in hormone biosynthesis. A detailed quantitative analysis of *CYP6A37* expression throughout the life stages, as well as tissue specific expression corresponding to hormone production in house flies, therefore warrants further investigation.

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Table 4.1. The information of primers used for the cloning and genetic linkage analysis of *CYP6A36* and *CYP6A37*

Gene	Primer Names	Primer Sequences	Positions (nt)
CYP6A36	P450HF15P-F	5' CTTATAGAGCTCGGAACTACCTTTG 3'	-611 to -586
	AP450HF15PNF-1	5' GGAGTGAATTTCGGTTTCCATTG 3'	-482 to -459
	AP450HF15F	5' ATGATCGCTCTGGCAATACTACTCAG 3'	1 to 26
	P450HF15P	5' CACTCAATAAACGGCCAATGTAAGC 3'	70 to 46
	CYP6AD1	5' GTNATHGGHHNBTGYGCHTTYGG 3'	559 to 581
	P450HF15R	5' CTGCTCATTTAGGGCTTTGCGTC 3'	654 to 632
	AP450HF15R	5' TTCAAATCCTCTCCACACGCAAATAAATAC 3'	1525 to 1496
CYP6A37	AP450HF15'F	5' ATGTCTTTGTCGTTCGTGTTATTTACGATCG 3'	1 to 31
	AP450HF15'NLF-1	5' GCATTGAGTGCAGTAGCCTTAAG 3'	578 to 600
	AP450HF15'NLR	5' GATACTTCTCCAATTTCTCCAAA 3'	1042 to 1020
	Flyh1	5' GGICCIAGIAACTGCATIGG 3'	1339 to 1358
	P450HF15R2	5' ACGAGGGCTTGCATTTTGCCAAAACGC 3'	1388 to 1362
	AP450HF15'R	5' TTTAATTTTTTTCAACCCTCAAATAAATACCCTTATC 3'	1522 to 1486
Common	Oligo (dt)	5' TAATACGACTCACTATAGGGAGATTTTTTTTTTTTTTT 3'	
	C2	5' TAATACGACTCACTATAGGGAGA 3'	
	AP1 (RACE)	5' CCATCCTAATACGACTCACTATAGGGC 3'	
	AP1 (GenomeWalking)	5' GTAATACGACTCACTATAGGGC 3'	

FIGURE LEGENDS

Figure 4.1. Diagrammatic representation of genetic isolation of five house fly lines by crossing the resistant ALHF and susceptible aabys strains. These lines are named according to the autosomes bearing wild-type markers from ALHF. Note that crossover does not occur in male house flies.

Figure 4.2. The cDNA/deduced protein sequences/5' flanking region of *CYP6A36* in ALHF and aabys. Invariant and highly conserved motifs in all P450 proteins are doubly underlined and nucleotide polymorphisms are highlighted. The primers used for generating the *CYP6A36* cDNA fragments are indicated by arrowheads.

Figure 4.3. The cDNA/deduced protein sequences of *CYP6A37* in ALHF and aabys. Invariant and highly conserved motifs in all P450 proteins are doubly underlined and nucleotide polymorphisms are highlighted. The primers used for generating the *CYP6A37* cDNA fragments are indicated by arrowheads.

Figure 4.4. Alignment of the deduced amino acid sequences of CYP6A36, CYP6A37, CYP6A9 and CYP6A27. Conserved amino acid residues among all four and more than two of the P450 proteins are indicated by boxes (gray) and asterisks, respectively. Invariant and highly conserved motifs in the P450 proteins are underlined.

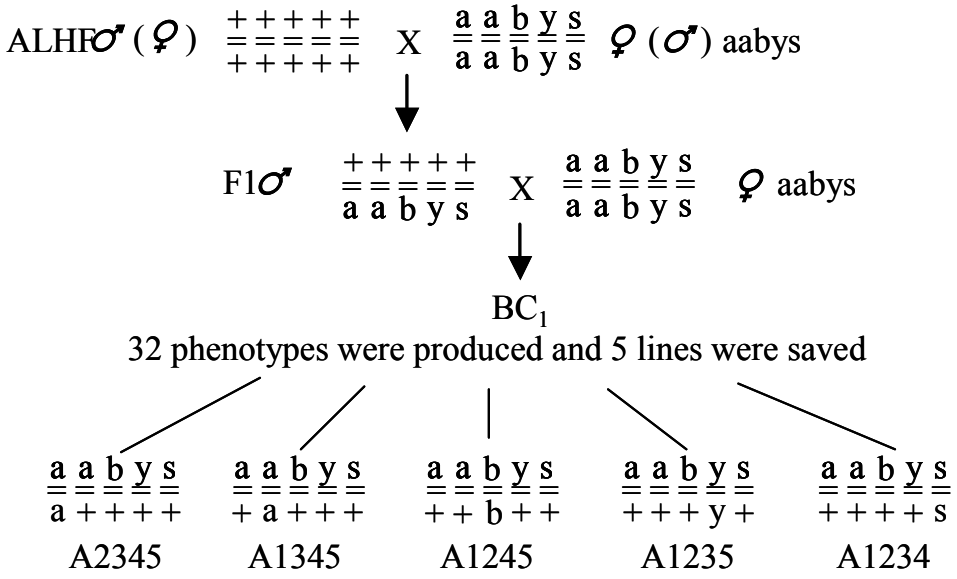
Figure 4.5. Expression analysis of *CYP6A36* and *CYP6A37* in different life stages of CS and ALHF house flies. A: Northern blot analysis of *CYP6A36* and *CYP6A37* in 1st and 2nd larval instars, 3rd instars, pupae, and adults of CS and ALHF. Blots were hybridized with the cDNA probes of *CYP6A36* and *CYP6A37* fragments. The ethidium bromide stain of 18S ribosomal RNA in agarose gel is shown at the bottom. B: Relative *CYP6A37* RNA levels for different life stages of house flies. C. Relative *CYP6A36* RNA levels for different life stages of house flies. The blots from three independent experiments were scanned. The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of P450 gene expression among the samples with the same alphabetic letter.

Figure 4.6. Expression of *CYP6A36* in head+thorax and abdomen tissue of adult house flies. A: Northern blot analysis of *CYP6A36* in head+thorax and abdomen tissue of CS and ALHF. Blots were hybridized with the cDNA probes of *CYP6A36* fragment. The ethidium bromide stain of 18S ribosomal RNA in agarose gel is shown at the bottom. B: Relative *CYP6A36* RNA levels in head+thorax and abdomen tissue. The blots from three independent experiments were scanned. The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of P450 gene expression among the samples with the same alphabetic letter.

Figure 4.7. Genetic linkage analysis of *CYP6A36*. A: Graphic representation of *CYP6A36*, showing locations and sequences of ALHF allele-specific primers. B: PCR fragments generated using the *CYP6A36* allele-independent primer set. C: PCR fragments

generated using the *CYP6A36* allele-specific primer set. The DNA templates used in the PCR reaction were from the following house flies: ALHF, aabys, and the 5 house fly lines - A2345, A1345, A1245, A1235 and A1234 - generated from reciprocal crosses between ALHF and aabys.

Figure 4.8. Genetic linkage analysis of *CYP6A37*. A: Graphic representation of *CYP6A37*, showing locations and sequences of ALHF allele-specific primers. B: PCR fragments generated using the *CYP6A37* allele-independent primer set. C: PCR fragments generated using the *CYP6A37* allele-specific primer set. The DNA templates used in the PCR reaction were from the following house flies: ALHF, aabys, and the 5 house fly lines - A2345, A1345, A1245, A1235 and A1234 - generated from reciprocal crosses between ALHF and aabys.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BC1
32 phenotypes were produced and 5 lines were saved
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A2345
a a b y s / + a + + +
A1345
a a b y s / + + b + +
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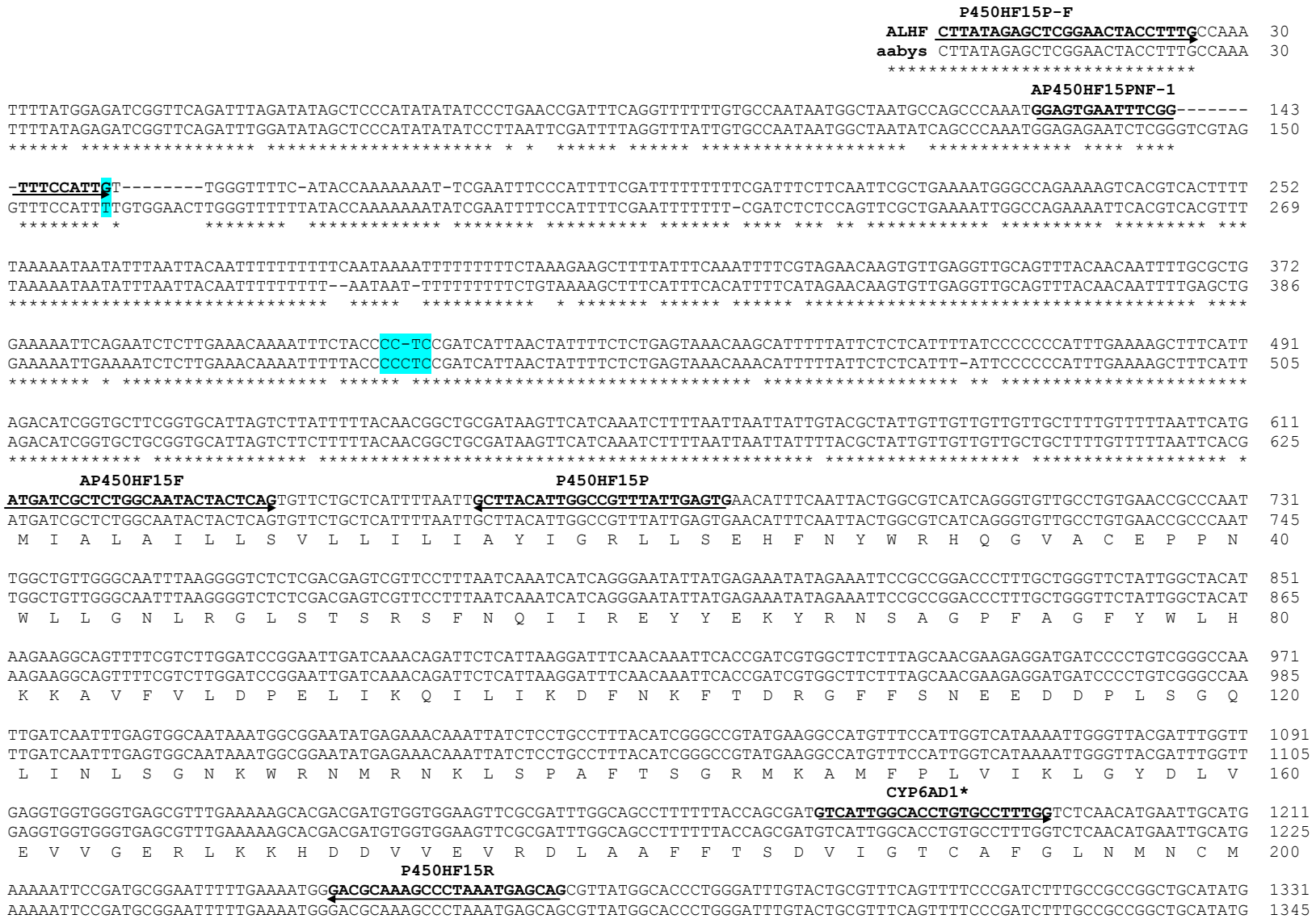
a a b y s / + + + y +
A1235
a a b y s / + + + + s
A1234

Figure 4.1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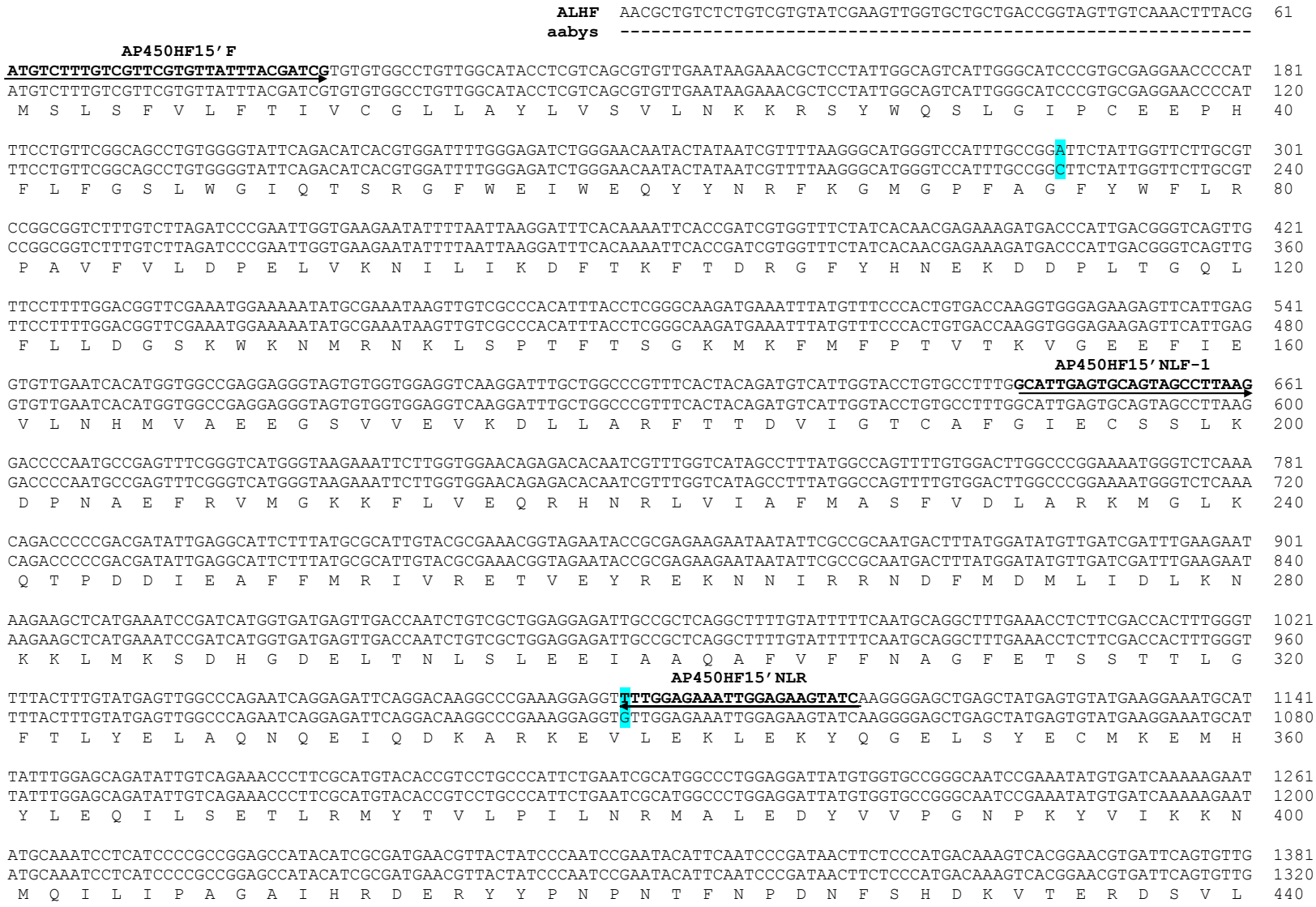
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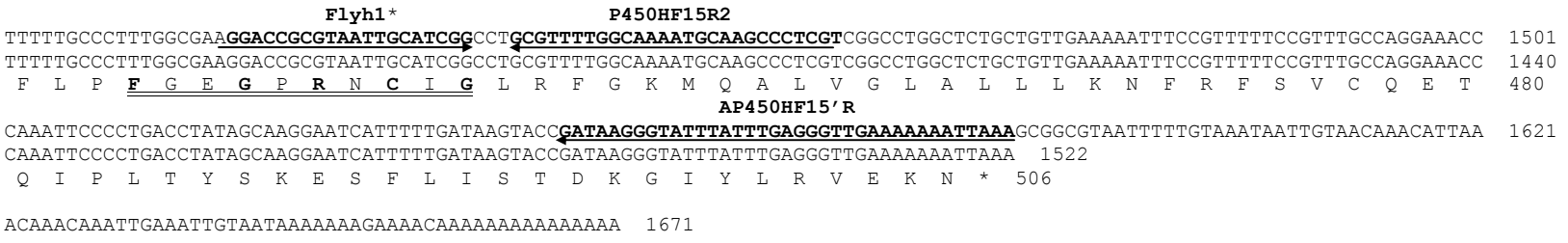
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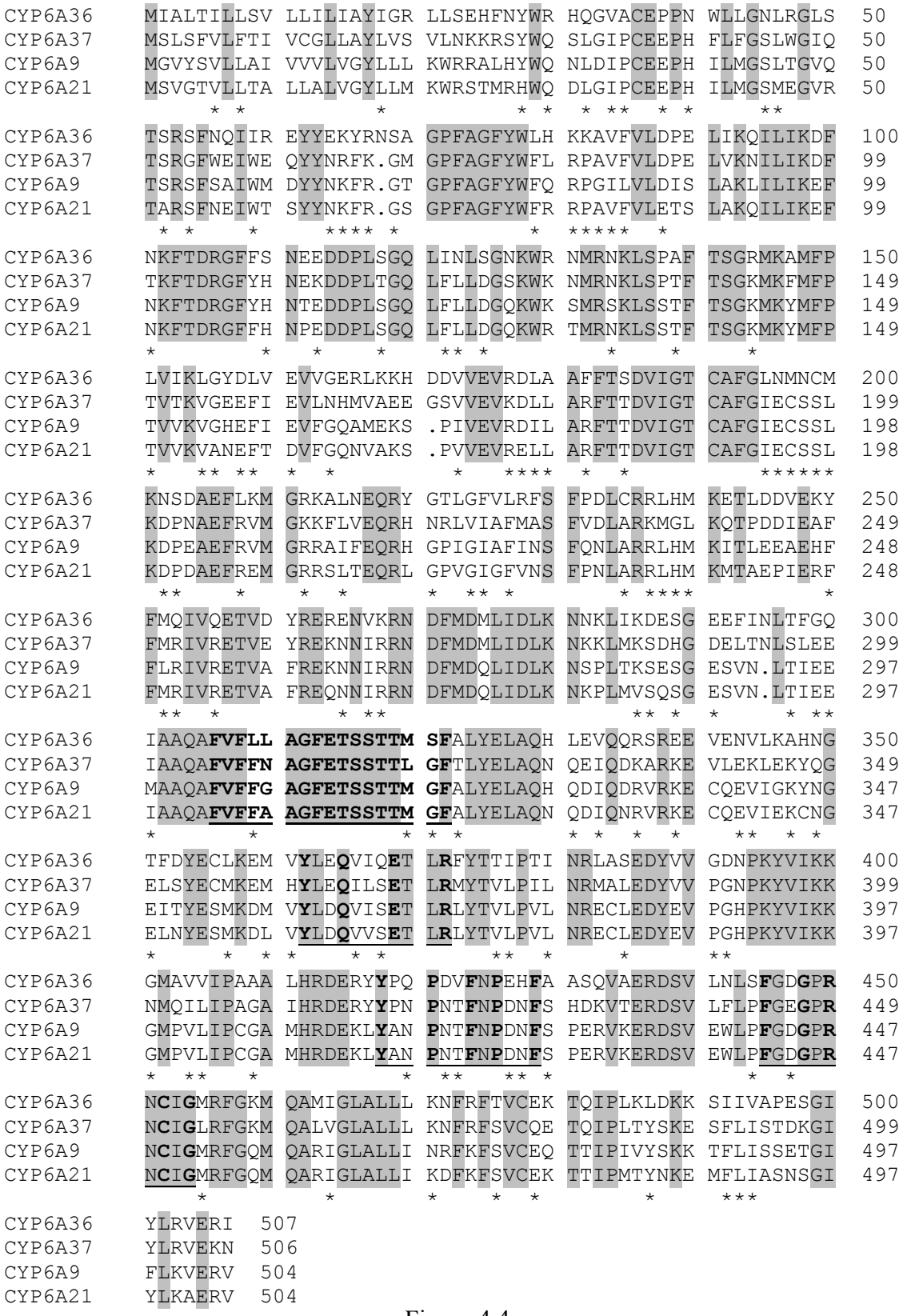
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Figure 4.4

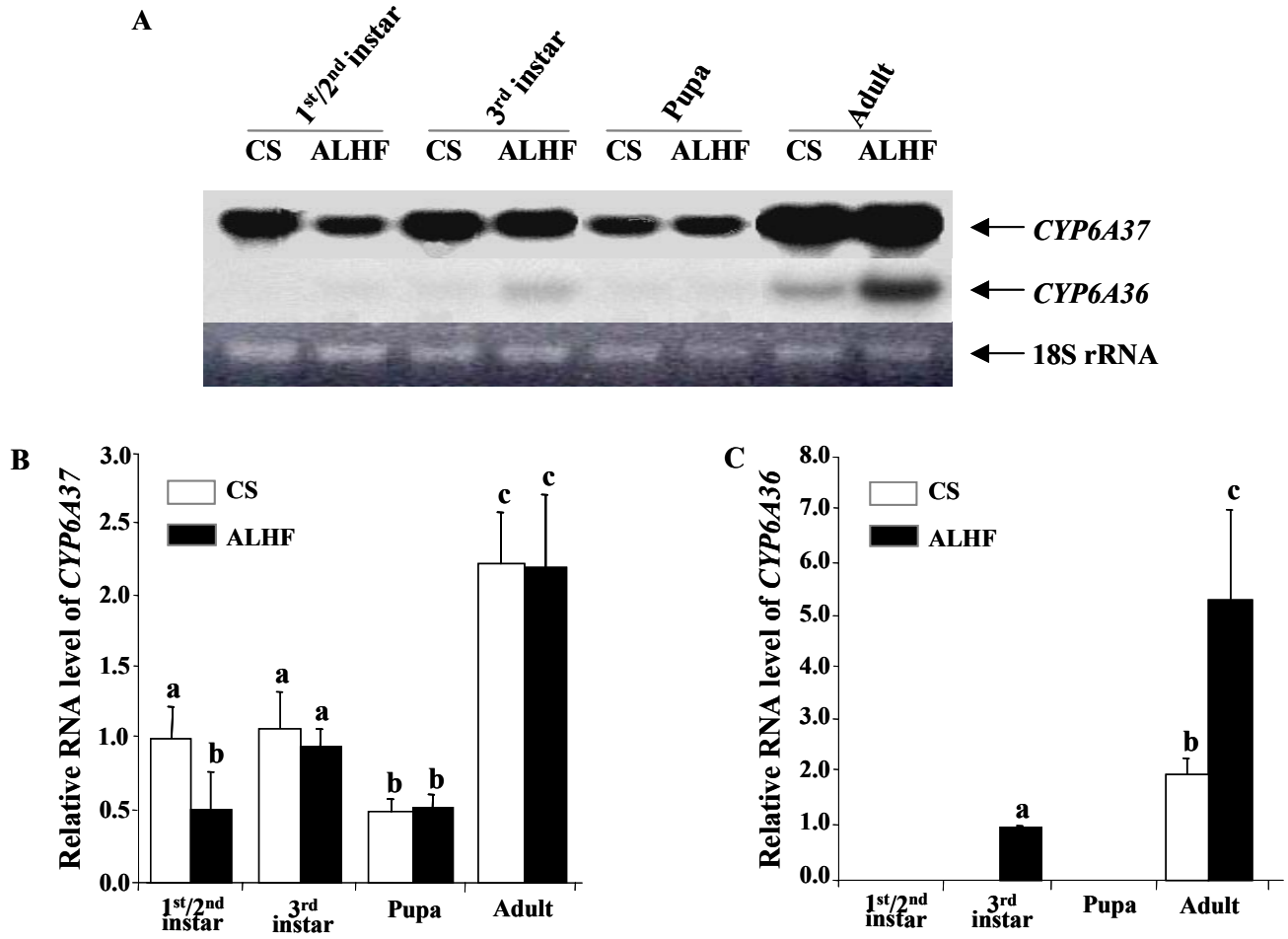
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Figure 4.5

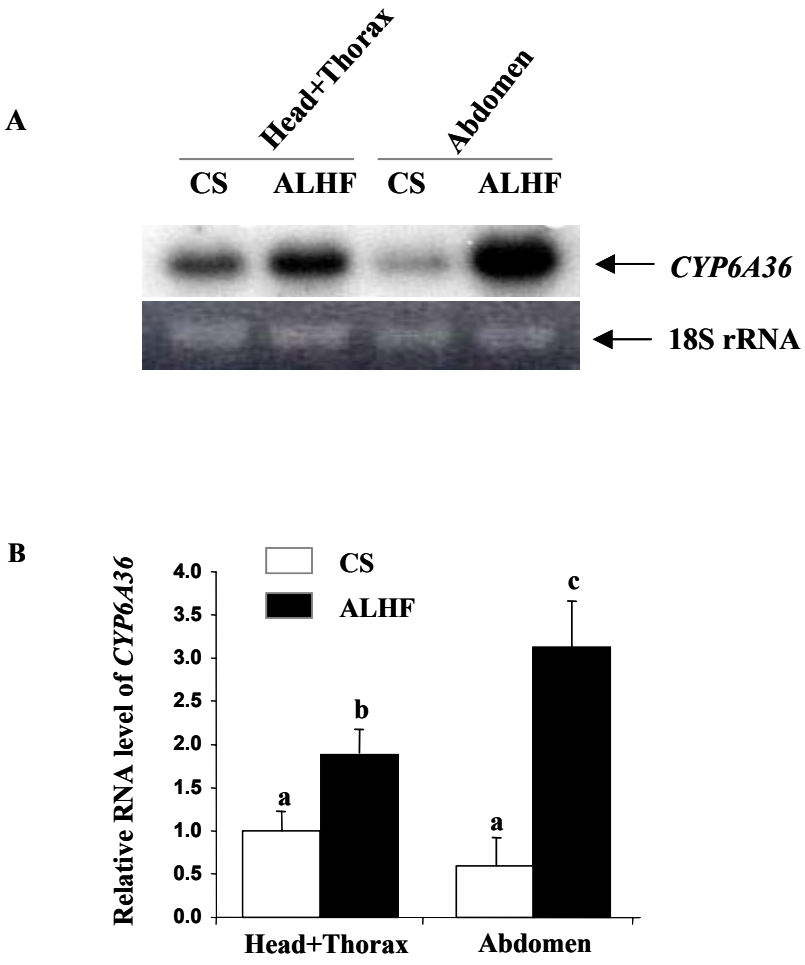
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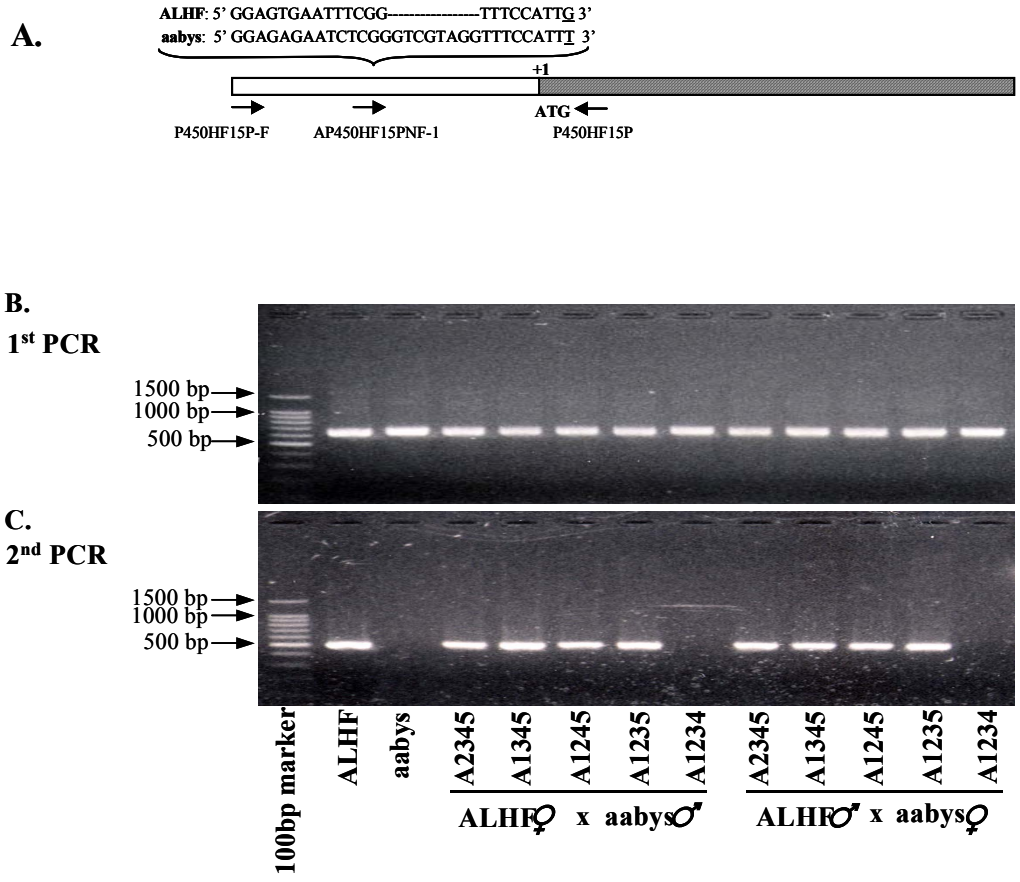
Figure 4.6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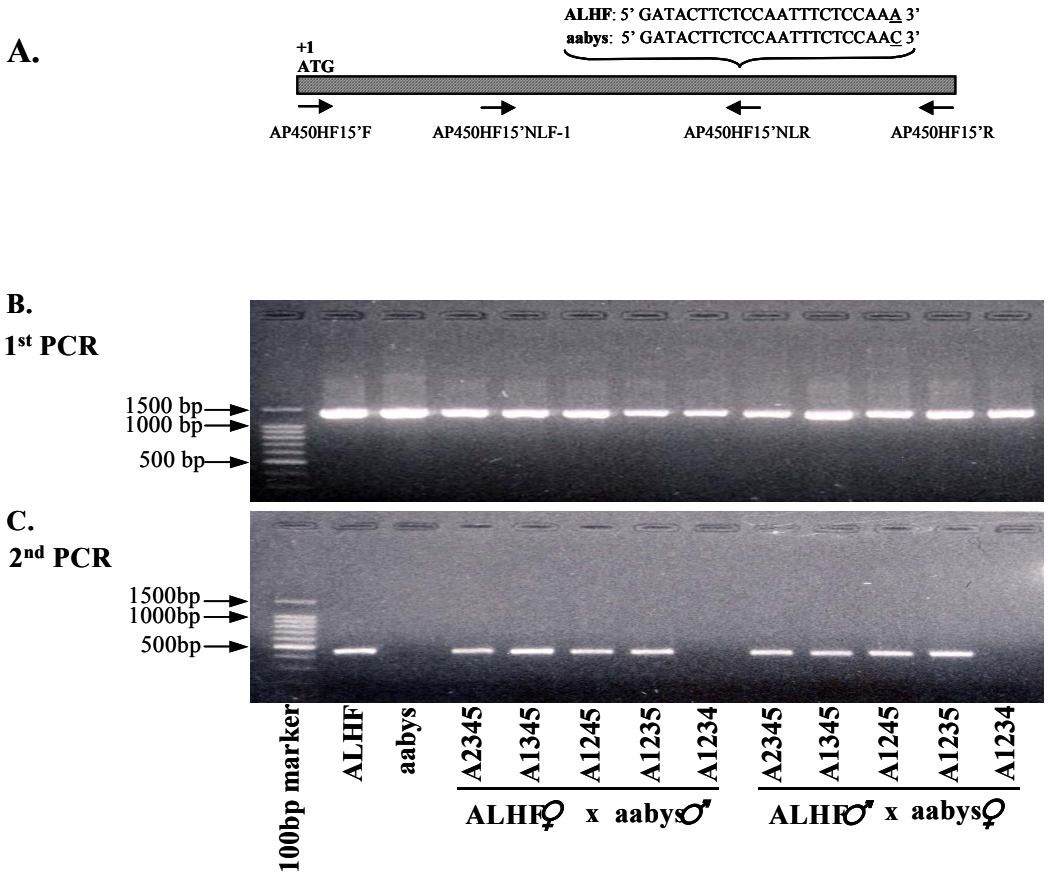
ALHF♂ x aabys♀

Figure 4.7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A1235
A1234
ALHF♀ x aabys♂
ALHF♂ x aabys♀

Figure 4.8

CHAPTER 5: PERMETHRIN INDUCTION AND CONSTITUTIVE OVEREXPRESSION OF THREE NOVEL CYTOCHROME P450 GENES IN PERMETHRIN RESISTANT HOUSE FLIES

5.1 Introduction

Cytochrome P450s constitute the largest gene superfamily and are found in a variety of organs and tissues of many organisms, including mammals, fish, plants, arthropods, fungi, and bacteria (Agosin, 1985). Cytochrome P450s have long been of particular interest because they are critical for the detoxification and/or activation of xenobiotics such as drugs, pesticides, plant toxins, chemical carcinogens and mutagens; and for metabolizing endogenous compounds such as hormones, fatty acids, and steroids. Insect cytochrome P450s are known to play an important role in detoxifying exogenous compounds such as insecticides (Scott, 1999; Feyereisen, 2005) and plant toxins (Berenbaum, 1991; Schuler, 1996), resulting in the development of resistance to insecticides (Carino et al., 1994; Liu and Scott, 1997; 1998; Kasai et al., 2000; Feyereisen, 2005) and facilitating the adaptation of insects to their plant hosts (Li et al., 2002; Wen et al., 2003). A significant characteristic of insect P450s that is associated with enhanced metabolic detoxification of insecticides in insects is the constitutively increased P450 proteins and P450 activities that result from constitutively transcriptional overexpression of P450 genes in insecticide resistant insects (Carino et al., 1994; Liu and Scott, 1997; 1998; Kasai et al., 2002; Feyereisen, 2005). Another feature of insect P450 genes is that the expression of some P450 genes can be induced by exogenous and endogenous compounds (Feyereisen, 2002), a phenomenon known as induction. It has

been suggested that the induction of P450s and their activities in insects is involved in the adaptation of insects to their environment (Terriere, 1983; 1984).

While all insects probably possess some capacity to detoxify insecticides and xenobiotics, the degree to which insects can metabolize and detoxify these toxic chemicals is of considerable importance to their survival in a chemically unfriendly environment (Terriere, 1984) and to the development of resistance. Both the constitutively increased expression and induction of P450s are thought to be responsible for increased levels of detoxication of insecticides. However, unlike the constitutively overexpressed P450 genes, whose expression association with insecticide resistance has been extensively studied, the induction of P450s is, although documented in insects (Scott et al., 1996; Ranasinghe and Hobbs, 1999; Scharf et al., 2001; Zhu and Snodgrass, 2003; Goff et al., 2006; Bautista et al., 2007), not well characterized in insecticide resistance. In contrast to the fairly well defined response of insect P450 gene induction to host plant secondary substances (Feyereisen, 2005), the connection of P450 gene induction in response to insecticide challenge and insecticide resistant is not fully understood.

It has been proposed that many chemical inducers act as substrates for P450s that they induce and that the induction of the P450s by the substrates will, in turn, reduce the effects of the substrates by enhancing substrate metabolism (Okey, 1990). It has also been suggested that the induction of P450s is probably one of the mechanisms involved in the P450-mediated resistance in insects because induction of P450s reflects a good compromise between energy saving (i.e., enhancing the activity of the detoxification system only when a chemical stimulus occurs) and adjustment to a rapidly changing

environment (i.e., insecticide application) (Brattsten, 1979; Depardieu et al., 2007). Thus, we hypothesized that insecticide resistant insects may be uniquely resistant to insecticides due to their ability to mount an adequate cellular response, for example the ability to up-regulate their production of P450s, when challenged with insecticides. In order to test our hypothesis, the current study is focused on the characterization of individual P450 genes from house flies that are induced in response to permethrin treatment in a pyrethroid resistant house fly strain, ALHF (Liu and Yue, 2000; 2001). We isolated 3 novel P450 genes, *CYP4D4v2*, *CYP4G2*, and *CYP6A38*, whose expression was constitutively overexpressed and further induced by permethrin treatment in resistant ALHF house flies. In addition, their expression profiles in different tissues and in both resistant and susceptible house flies were characterized. Genetic linkage studies were conducted to identify a further possible causal link between the P450 genes in response to insecticide treatment and the development of insecticide resistance in ALHF.

5.2 Materials and methods

5.2.1 House fly strains

Three house fly strains were used in this study. ALHF, a wild-type strain collected from a poultry farm in Alabama in 1998, selected with permethrin for 6 generations after collection to reach a 6,600-fold resistance, and maintained under biannual selection with permethrin (Liu and Yue, 2000; 2001). CS is a wild type insecticide-susceptible strain. aabys is an insecticide-susceptible strain with recessive morphological markers ali-curve (*ac*), aristapedia (*ar*), brown body (*bwb*), yellow eyes (*ye*), and snipped wings (*sw*) on

autosomes 1, 2, 3, 4, and 5, respectively. Both CS and aabys were obtained from Dr. J. G. Scott (Cornell University).

5.2.2 Permethrin challenge experiments

Two-day old resistant ALHF and susceptible CS house flies were treated with permethrin by topical application (Liu and Yue, 2000; 2001) with 0.5 *ul* permethrin solution (in acetone) dropped on the thoracic notum. Preliminary dose range, time course, and P450 gene induction assays were performed with a dose range of sublethal dose, LD_{10}, LD_{50}, and LD_{90}. Based on results of the pilot experiment, in which the induction of P450s was noticed 12, 24, and 48 h after flies were treated with permethrin at the doses of $\geq LD_{50}$, two doses, 20 *ug*/fly and 10 *ng*/fly that resulted in 50-60% mortality for ALHF and CS house fly strains, respectively, were chosen for challenge. The surviving flies were collected for RNA extraction after 24 h permethrin challenge according to a preliminary time course study. The control flies without permethrin treatment or treated with acetone alone were collected at the same day as the permethrin treated flies. The experiments were repeated three times.

5.2.3 RNA extraction, cDNA preparation, and the putative P450 gene isolation

Total RNAs were extracted from the house flies using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott, 1997). mRNA was isolated with oligotex-dT suspension as described by the manufacturer (QIAGEN). The first strand cDNA was synthesized with SuperScript II reverse transcriptase (Stratagene) and an antisense 5'-anchored oligo(dT) primer (Table 5.1) (Tomita and Scott, 1995), using

ALHF mRNAs as templates. The PCR products were amplified using three primer pairs of antisense C2/ Flyh1, antisense C2/Flyc1, and antisense HemeR1/CYP6AD1 (Table 5.1; Figs. 5.1, 5.2, and 5.3). CYP6AD1 was designed based on a conserved amino acid region found in the house fly, *Drosophila*, and mosquito P450 sequences after alignment of these insect P450 genes; Flyh1 was designed based on the heme binding consensus sequence (Liu and Zhang, 2002); HemeR1 was generated based on the complementary sequence of Flyh1; and Flyc1 was synthesized based on a conserved 13 amino acid region found in rat, human, and insect P450 sequences (Liu and Zhang, 2002). The PCR products were cloned into PCR^{TM} 2.1 Original TA cloning vector (Invitrogen) and sequenced. Cloning and sequence analyses of P450 gene fragments were repeated at least three times with different preparations of mRNAs. Three TA clones from each replication were sequenced.

5.2.4 Rapid amplification of cDNA ends (RACE) of the putative P450 gene fragments

RACE was carried out using the Marathon^{Tm} cDNA Amplification Kit (Clontech) as described by the manufacturer and Liu and Zhang (Liu and Zhang, 2002). The first strand cDNAs were synthesized with AMV reverse transcriptase using ALHF mRNAs as templates. The double strand cDNA was synthesized following the protocol described by the manufacturer (Clontech). Adaptors were ligated to both ends of each double strand cDNA using T4 DNA ligase as described by the manufacturer. The 5' and/or 3' ends of the P450 cDNA fragments were amplified by PCR using primers (Table 5.1; Figs. 5.1, 5.2, and 5.3) generated based on the 5' and/or 3' end sequences of the putative P450 cDNA fragments as described by the manufacture (Clontech). The full length of each

putative P450 cDNA was subsequently generated by RT-PCR using specific primer pairs (Table 5.1; Figs. 5.1, 5.2, and 5.3) synthesized based on the 5'and 3'end sequences of the putative P450 genes. Cloning and sequence analyses of the P450 cDNAs were repeated at least three times with different preparations of mRNAs, and three TA clones from each replication were verified by sequencing.

5.2.5 Cloning and sequencing the 5' flanking region of CYP6A38 from ALHF and aabys

House fly genomic DNAs were digested with 5 different restriction enzymes using the Universal GenomeWalker^{TM} Kit (Clontech) and generated 5 adaptor-ligated ALHF genomic DNA libraries as described by the manufacture. The adaptor ligated DNA fragments in the GenomeWalker libraries were amplified by PCR with Advantage *Tth* polymerase (Clontech), the antisense primer, P450HF17P (Table 5.1; Fig. 5.3) based on the 5' coding region of the *CYP6A38*, and a sense primer, AP1, based on the sequence of the adaptor. The PCR products were cloned into the TA cloning vector (Invitrogen) and sequenced. Cloning and sequence analyses of PCR products were repeated at least three times each with three TA clones from each replication. The 5' flanking region of *CYP6A38* in aabys was subsequently generated by PCR from the genomic DNA using a primer pair, P450HF17P-F/P450HF17P (Table 5.1; Fig. 5.3) designed according to the 5' flanking region of *CYP6A38* in ALHF.

5.2.6 Northern blotting analysis

Northern blotting analyses were performed according to Sambrook et al. (1989). Twenty micrograms of total RNA from each sample were fractionated on 1%

formaldehyde denaturing agarose gel and transferred to Nytran membranes (Schleicher and Schuell) (Sambrook et al., 1989). The P450 cDNAs were labeled with [α-^{32}P] dCTP using a Primer-It II Random Primer Labeling kit (Stratagene) and hybridized with RNA blots using QuickHyb solution (Stratagene). The amount of RNA loaded in each lane was standardized by comparing the density of the 18S ribosomal RNA band on agarose gel under UV light before transfer (Spiess and Ivell, 1998). All Northern blot analyses were repeated three times with different preparations of RNA samples. The radiographic signal intensity was quantitatively analyzed by QuantiScan v3.0 (Biosoft) as described previously (Liu and Zhang, 2004). The statistical significance of the gene expression was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant.

5.2.7 Quantitative Real-time PCR (qRT-PCR)

Total RNA samples (0.5 μg/sample) were reverse-transcribed using SuperScript II reverse transcriptase (Stratagene) in a total volume of 20 μl. The cDNAs were measured using a spectrophotometer prior to qRT-PCR. qRT-PCR was performed with the SYBR Green master mix Kit and ABI 7500 Real Time PCR system (Applied Biosystems). Each qRT-PCR reaction (25 μl final volume) contained 1x SYBR Green master mix, 1 μl of cDNA, and gene specific primers (Table 5.1; Figs. 5.1, 5.2, and 5.3) at a final concentration of 3-5 μM. A 'no-template' negative control and all samples were performed in triplicate. The reaction cycle consisted of a melting step of 50ºC for 2 min then 95 °C for 10 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min.

Specificity of the PCR reactions was assessed by a melting curve analysis for each PCR reaction using Dissociation Curves software (Wittwer et al., 1997). Relative expression levels for specific genes were calculated by the $2^{-\Delta\Delta C(t)}$ method using SDS RQ software (Livak and Schmittgen, 2001). The *β*-actin gene, an endogenous control, was used to normalize expression of target genes (Aerts et al., 2004; Dorak, 2006). The preliminary assay had shown that the *β*-actin gene remained constant in different tissues and in both permethrin treated and untreated house flies and could therefore be used for internal normalization in qRT-PCR assays. Each experiment was repeated three times with different preparations of RNA samples. The statistical significance of the gene expressions was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant.

5.2.8 Genetic linkage analysis of cytochrome P450 genes

To determine the genetic linkage of P450 genes, a genetic cross experiment was conducted (Liu and Scott, 1995; Liu and Yue, 2001). Briefly, reciprocal crosses between ALHF and aabys were conducted and F_1 males were back-crossed to aabys females. Five lines were saved from the back-cross generation (BC_1) with the genotypes of: *ac/ac*, +/*ar*, +/*bwb*, +/*ye*, +/*sw* (A2345); +/*ac*, *ar/ar*, +/*bwb*, +/*ye*, +/*sw* (A1345); +/*ac*, +/*ar*, *bwb/bwb*, +/*ye*, +/*sw* (A1245); +/*ac*, +/*ar*, +/*bwb*, *ye/ye*, +/*sw* (A1235); and +/*ac*, +/*ar*, +/*bwb*, +/*ye*, *sw/sw* (A1234). Since crossing over does not or very rarely occurs in male flies (Gao and Scott, 2006), the presence of a mutant phenotype indicated that the respective autosome with a mutant-type marker was derived from the aabys females. The genotype of each

line was homozygous for the recessive mutant allele from aabys and heterozygous for the dominant wild-type alleles from ALHF. These lines were named according to the autosomes bearing wild-type markers from ALHF. For example, the A1234 strain had wild-type markers on autosomes 1, 2, 3, and 4 from ALHF and the recessive mutant marker on autosome 5 from aabys.

The P450 specific alleles in ALHF and aabys and five house fly BC_1 lines were genetically mapped by single nucleotide polymorphism (SNP) determination using an ABI Prism SNaPshot Multiplex Kit and analyzed on the ABI Prism® 3100 Genetic Analyzer using Genemapper software according to the manufacture's instructions (A&B Applied Biosystems). Briefly, the cDNA fragments, which covered the SNP sites of P450 cDNAs, were generated by PCR. The primer for SNP determination of each P450 gene was designed according to the sequence immediately upstream of the single nucleotide polymorphism of each P450 gene. Three replications of the SNP determination were carried out with different preparations of the PCR templates. To confirm that the PCR products used for the SNP determination were in fact the P450 gene fragments, the PCR products were sequenced at least once each.

5.3 Results

5.3.1 Cloning and sequencing three novel P450 genes in response to permethrin challenge

To characterize the P450 genes in response to the insecticide treatment, we used the PCR technique with combination of different degenerated PCR primer pairs (Snyder et al., 1996) to amplify P450 cDNAs from house flies. We initially isolated a total of 19

P450 cDNA fragments from ALHF house flies using three primer pairs, C2/Flyh1, C2/Flyc1, and HemeR1/CYP6AD1 (Tables 3.1 and 5.1). To identify the P450 genes that were induced in response to insecticide treatment, we conducted Northern blot analysis using 19 P450 cDNAs as probes to compare expression levels of these genes in both permethrin treated and untreated ALHF house flies. Three of the 19 P450 genes were found to be significantly induced in the permethrin treated ALHF house flies after 24 hours treatment (Fig. 5.4) at the doses that cause ≥50% mortality of each house fly strain. The preliminary data on dose range, time course, and induction experiments, indicated no induction was detected at the sublethal dose or LD_{10} of permethrin (data not show). No response of P450 genes to permethrin treatment at lower dose in house flies is probably because of the rapid metabolism of permethrin at these doses (Yu and Terriere, 1973; Kasai and Scott, 2001), which can never reach a threshold dose for induction (Islam et al., 2006). BLAST analysis of the putative amino acid sequences predicted from partial cDNA sequences of these three permethrin inducible P450 genes showed that one sequence encoded the C-terminal end of a putative P450 with 50-70% identity to the CYP4 family of insects and the other 2 sequences encoded middle sections of putative P450s with 50-60% and 90% identity to the CYP4 and CYP6 families, respectively.

To isolate the full length of the three putative P450 genes, we conducted 5'and/or 3' RACE. The primer P450HF5R (Table 5.1; Fig. 5.2) was generated based on the 5' end sequence of the putative P450 cDNA fragment with the identity to the CYP4 family. The primers P450HF20F and P450HF20R (Table 5.1; Fig. 5.1) were used for 3' and 5' RACE for the putative P450 cDNA fragment that had the similarity to the CYP4 family, and the primers P450HF17R and P450HF17F (Table 5.1; Fig. 5.3) were used for 3' and 5' RACE

for the putative P450 cDNA fragment that had the similarity to the CYP6 family. The sequences of the 5' and/or 3' RACE amplified cDNAs overlapped with their corresponding putative P450 cDNA fragments. An entire cDNA fragment for each of the putative P450 genes was subsequently amplified from both ALHF and aabys house flies by PCR using the primer pair synthesized based on the respective 5' and 3' end sequences of the putative P450 genes. The sequences were named *CYP4D4v2, CYP4G2*, and *CYP6A38* (accession numbers: EF615001, EF615002, and EF615003, respectively) by the P450 nomenclature committee (Dr. D. Nelson, personal communication). The cDNA sequences of *CYP4D4v2*, *CYP4G2*, and *CYP6A38* have open reading frames of 1515, 1647, and 1500 nucleotides encoding proteins of 505, 549, and 500 residues, respectively. The putative protein sequences of CYP4D4v2 shared 57% identity to *Drosophila melanogaster* CYP4D14 (Adams, 2000), CYP4G2 shared 65% and 71% identities with *Musca domestica* CYP4G13v2 (Liu and Zhang, 2002) and *Drosophila melanogaster* CYP4G1 (Dunkov et al., 1996), respectively, and CYP6A38 shared 98% similarity to *Musca domestica* CYP6A24 (Kamiya et al., 2001). The P450 heme binding motif, FXXGXRXCXG (Gotoh and Fujii-Kuriyama, 1989), was presented at amino acid residues 446-455, 482-491, and 438-447 of CYP4D4v2, CYP4G2, and CYP6A38, respectively. Comparison of the deduced protein sequences of CYP4D4v2, CYP4G2, and CYP6A38 between ALHF and aabys revealed identical protein sequences, although several nucleotide polymorphisms were found in the coding regions of *CYP4D4v2* and *CYP4G2* between the two strains.

5.3.2 Response of P450 genes to permethrin challenge in resistant and susceptible house flies

We had previously observed that *CYP4D4v2, CYP4G2*, and *CYP6A38* expression could be induced by permethrin treatment in ALHF house flies (Fig. 5.4). Therefore, we considered it necessary to test whether or not the three genes could also be induced in CS susceptible strains. We hypothesized that these genes would not be induced by or respond to a permethrin challenge in susceptible strains if the genes had a role in insecticide resistance. We thus compared the inducibility of expression of the three genes between resistant ALHF and susceptible CS house flies. Our results detected no significant induction in the expression of the three P450 genes in CS susceptible house flies that had either been treated with acetone alone or with permethrin solution in acetone compared with untreated house flies (Fig. 5.5). Similarly, no significant induction was obtained in acetone treated ALHF house flies compared with untreated flies (Fig. 5.5). However, the expression of these three genes was induced at a variety of levels in permethrin treated ALHF house flies compared with untreated or acetone treated ALHF flies. A ~4-fold significant induction in the expression of both *CYP4D4v2* and *CYP6A38* were detected in permethrin treated ALHF house flies compared with untreated or acetone treated ALHF flies (Fig. 5.5B, C), whereas, a low level (~1.5-fold; $*p \leq 0.05$) of induction for *CYP4G2* was detected in the permethrin treated ALHF house flies (Fig. 5.5A). The significant induction of the three P450 genes only in ALHF house flies suggests the importance of these genes in response to permethrin treatment in the resistant ALHF house flies. In addition, our results also showed that expression of *CYP4D4v2, CYP4G2*, and *CYP6A38*, respectively, was ~1.5-, ~15-, and ~3-fold higher in ALHF compared with CS even

before the permethrin treatment (Fig. 5.5). This result implies that multiple P450 genes are constitutively overexpressed in resistant ALHF house flies, which is consistent with previous studies (Carino et al., 1992; Liu and Scott, 1996; Kasai and Scott, 2001).

5.3.3 Tissue specific expression of the P450 genes in resistant and susceptible house flies

In insects, the midgut and fat body tissue have generally been considered to be the primary detoxification organs (Hodgson, 1985) where most insect detoxification P450s are expressed (Scott et al., 1998). Thus, we considered it necessary to evaluate whether the overexpression of the P450 genes in ALHF specifically occurs in this detoxification tissue. We examined the expression of the three P450 genes in the abdomen and head+thorax tissues of both the CS and ALHF strains by qRT-PCR. Our results revealed 2 to 3-fold higher expression of both *CYP4D4v2* and *CYP6A38* in the abdomen tissue of both CS and ALHF flies compared with head+thorax tissues (Fig. 5.6B, C). However, significant overexpression was more evident in ALHF in both tissues compared with CS. The expression of *CYP4D4v2* was ~15-fold higher in both head+thorax and abdomen tissues of ALHF compared to the corresponding tissues in CS (Fig. 5.6B) and the expression of *CYP6A38* was ~4- and 6-fold higher in the head+thorax and abdomen tissues, respectively, of ALHF (Fig. 5.6C). In contrast, a significant difference in the *CYP4G2* expression was obtained in between head+thorax and abdomen tissues of both CS and ALHF flies, with the expression of *CYP4G2* being ~15- and 20-fold higher in the abdomen tissue of CS and ALHF, respectively, compared with the expression in head+thorax tissues of the same strains (Fig. 5.6A). However, a ~1.5-fold overexpression was obtained only in the abdomen tissue of ALHF compared with that of CS (Fig. 5.6A).

As the midgut and fat body tissue have generally been considered to be the primary detoxification organs and as midgut and most fat body components are located in the abdomen of insects, relatively high levels of *CYP4D4v2, CYP4G2*, and *CYP6A38* in the abdomen of ALHF may suggest the importance of the genes in increasing metabolic detoxification of insecticide in ALHF house flies compared with the susceptible CS strain.

5.3.4 Chromosomal linkage and allele determination of three P450 genes

We previously demonstrated that permethrin resistance in ALHF house flies was linked on autosomes 1, 2, 3, and 5, with major factors on autosomes 3 and 5 (Liu and Yue, 2001). We also demonstrated that the P450-mediated resistance in ALHF was predominantly linked on autosome 5 (Liu and Yue, 2001). To determine whether there is a causal link between the P450 genes and insecticide resistance, we examined the genetic linkage of *CYP4D4v2*, *CYP4G2*, and *CYP6A38* with 5 BC_1 house fly lines derived from crosses of ALHF and a susceptible morphological marker strain, aabys, by allele specific SNP determination. Sequence comparisons of the three genes between ALHF and aabys revealed several nucleotide polymorphisms in the coding regions of *CYP4D4v2* and *CYP4G2*, while no nucleotide polymorphisms were identified in the coding region of *CYP6A38*. Thus, we cloned a ~700 bp 5' flanking region of *CYP6A38* in order to genetically map the *CYP6A38* gene. Comparison of the nucleotide sequence of the 5' flanking region of *CYP6A38* uncovered several deletions (or insertions) and single nucleotide polymorphisms between ALHF and aabys (data not shown). Therefore, the nucleotide polymorphisms, C to T, C to T, and G to T, in *CYP4D4v2*, *CYP4G2*, and

CYP6A38, respectively, in ALHF relative to aabys (Fig. 5.7), were used to determine the linkage of P450 genes relative to the recessive morphological markers in the aabys strain. The SNP determination reactions were conducted for each of the genes using a specific primer (Fig. 5.7) designed according to the sequences immediately upstream of the nucleotide polymorphism to distinguish the single nucleotide polymorphism for the P450 allele in each house fly strain or line. Our results showed that the BC_1 lines with the genotypes of *ac/ac*, +/*ar*, +/*bwb*, +/*ye*, +/*sw* (A2345), +/*ac*, *ar/ar*, +/*bwb*, +/*ye*, +/*sw* (A1345), +/*ac*, +/*ar*, *bwb/bwb*, +/*ye*, +/*sw* (A1245), and +/*ac*, +/*ar*, +/*bwb*, *ye/ye*, +/*sw* (A1235) were heterozygous for *CYP4D4v2* and *CYP6A38*, whereas the BC_1 line with the genotype of +/*ac*, +/*ar*, +/*bwb*, +/*ye*, *sw/sw* (A1234) was homozygous for both the *CYP4D4v2* and *CYP6A38* alleles from aabys (Table 5.2). These results strongly indicate that both *CYP4D4v2* and *CYP6A38* are located on autosome 5 in house flies. The BC_1 lines of A2345, A1345, A1234, and A1235 were heterozygous for *CYP4G2*, whereas the A1245 line was homozygous for the *CYP4G2* allele from aabys (Table 5.2), indicating that *CYP4G2* is located on autosome 3 in house flies.

5.4 Discussion

The primary goal of this study was to investigate the hypothesis that insecticide resistant insects may be uniquely resistant to insecticides due to their ability to mount an adequate cellular response by up-regulating the production of P450s when challenged with insecticides. It appears that the induction of gene expression probably reflects a good compromise between energy saving (i.e., enhancing the activity of the detoxification system only when a chemical stimulus occurs) and adjustment to a rapidly

changing environment (Brattsten, 1979). Multiple P450 genes that are induced in insects in response to host plant allelochemicals or secondary products have been extensively studied (Cohen et al., 1992; Ma et al., 1994; Snyder et al., 1995a; Li et al., 2000; 2001; Stevens et al., 2000; Feyereisen 2005) and are fairly well documented in terms of their function in the adaptation of insects in "animal-plant warfare" (Gonzalez and Nebert, 1990) and in the co-evolution of insects and plants (Li et al., 2004). In contrast, P450 gene induction in response to insecticide resistance is less well understood. The current study, therefore, focused on characterization the P450 genes induced in response to the challenge of insecticides in insecticide resistant house flies. We restricted this response to permethrin treatment because it is the insecticide that the house flies are resistant to. We found that only resistant house flies exposed to permethrin responded by up-regulating a set of P450 genes compared with flies that had not been challenged with permethrin, whereas susceptible house flies showed no significant response. This finding indicates that permethrin induces multiple P450 genes' response at different levels in a resistance-specific manner, strongly suggesting that permethrin may serve as an inducer of resistance to itself by modulating P450 gene expression and detoxification. In addition, our results also indicated that these three genes were overexpressed in the resistant strain compared with the susceptible strains before permethrin treatment. The overexpression of all three genes was far more significantly expressed in abdomen tissue where the primary detoxification organs of midgut and most fat body components are located (Hodgson, 1985) and most insect detoxification P450 genes are expressed (Scott et al., 1998).

It has been proposed that induction or constitutive overexpression of P450s is the response for the adaptation of insects to their environment (Terriere, 1983; 1984). Further

more, in many cases, increased levels of P450 gene expression have resulted in increased levels of both total P450s and the P450 activities and have been strongly suggested as a major cause of insecticide resistance (Brattsten et al., 1986; Carino et al., 1992; Liu and Scott, 1997; Festucci-Buselli et al., 2005; Feyereisen, 2005). Terriere (1983) proposed that there could be similar regulatory mechanisms existing between P450 constitutive overexpression and induction, and that both led to insecticide resistance. Accordingly, our findings of permethrin induction and constitutive overexpression of three P450 genes in resistant house flies suggest the important role of these three genes in increasing metabolic detoxification of permethrin in resistant ALHF house flies and imply the role of these genes in the development of insecticide resistance. Our study also strongly supports the suggestion that a significant resistance level obtained by an insect organism is unlikely to be conferred by only one or two overexpressed genes and multiple P450s are usually involved in the resistance simultaneously (Terriere, 1983; 1984).

Early studies in our laboratory on permethrin resistance in ALHF house flies had led to the identification of permethrin resistance that could be largely suppressed by piperonyl butoxide (PBO), an inhibitor of cytochrome P450s (Liu and Yue, 2000). Furthermore, genetic linkage studies had linked permethrin resistance in ALHF to the autosomes 3 and 5, and PBO-suppressible resistance (or P450-mediated resistance) has been mainly linked to autosome 5, with minor factors linked to autosomes 1 and 2 (Liu and Yue, 2001). The genetic linkage between an overexpressed P450 gene or protein and insecticide resistance has been an important step in establishing a causal link between a P450 gene and its role in resistance (Carino et al., 1994; Liu and Scott, 1996; Rose et al., 1997; Guzov et al., 1998; Maitra et al., 2000; Feyereisen, 2005). We, therefore, further

examined the linkage of the three P450 genes with 5 house fly BC_1 lines derived from crosses of ALHF and a susceptible morphological marker strain, aabys, using allele specific PCR determination. We identified that both *CYP4D4v2* and *CYP6A38* were located on autosome 5, whereas *CYP4G2* was located on autosome 3 of house flies. Given that *CYP4D4v2* and *CYP6A38* are significantly overexpressed in ALHF, specifically in abdomen tissue, highly induced, and located on autosome 5, which is correlated with the linkage of P450-mediated resistance in ALHF, it seems likely that the overexpression of *CYP4D4v2* and *CYP6A38* plays an important role in insecticide resistance in ALHF house flies. Compared to the significant overexpression and induction of the other two P450 genes, relatively lower levels (~1.5-fold; $*p \leq 0.05$) of overexpression and induction of *CYP4G2* obtained in the ALHF house flies may suggest a relatively minor role of this gene in resistance. *CYP4G2* has been linked to autosome 3. To our knowledge, this is the first report of a P450 gene that is located on autosome 3 of house flies. An earlier study indicated that although the factors on autosome 3 were very important in overall permethrin resistance in ALHF house flies (Liu and Yue, 2001), the resistance in ALHF governed by them was not suppressed by PBO (Liu and Yue, 2001). These conflicting results may suggest that a ~1.5-fold level of *CYP4G2* overexpression or induction may only play a minor role in resistance, which is too small to be detected by a synergism study. Alternatively, it may also suggests that PBO, as been proposed (Brown et al., 1996; Zhang et al., 1997; Feyereisen, 2005), may not be a perfect inhibitor for some P450s responsible for resistance, so that the product of *CYP4G2v2* may not be sensitive to the inhibition of PBO.

In conclusion, our study provides the first evidence that multiple P450 genes are up-regulated in insecticide resistant house flies through both constitutively overexpression and induction mechanisms, which increase overall expression levels of the P450 genes and the level of detoxification of insecticides in resistant house flies. Taken together with the significant constitutive overexpression and induction of *CYP4D4v2*, *CYP4G2*, and *CYP6A38* only in resistant house flies, the expression of the three genes in detoxification-related specific tissue, and the correlation of the linkage of the genes with that of resistance and/or P450-mediated resistance in ALHF, this study strongly suggests the functional importance of these 3 P450 genes in the increased detoxification of insecticides in ALHF. Our study strongly suggests the existence of the variability of P450 gene expression in natural populations of insects, which thus provide a rich substrate for evolutionary insecticide selection, supporting the hypothesis that both induction and overexpression of P450s are the responses for the adaptation of insects to the changing environments (Terriere, 1983; 1984).

ACKNOWLEDGEMENTS

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Table 5.1. The primers used for cloning P450 genes, qRT-PCR reactions, and SNP determinations

Gene	Primer Names	Functions	Primer Sequences	Positions (nt)
CYP4G2	AP450HF20F	Full length	5' ATGGATAGTGCAAACAATTCAACTGCG 3'	1 to 27
	SNP450HF20F	SNaP determination	5' AAATGGCCATTTGGTGGCCCAT 3'	174 to 195
	RTP450HF20F	qRT-PCR	5' CGAGGAGGATGATGAAATAAGCAAGC 3'	837 to 862
	RTP450HF20R	qRT-PCR	5' TTGGACATGGCCATCATGGCATCT 3'	957 to 980
	P450HF20R	5'-RACE	5' GCAGGAAGTTGTCACCAAAGATG 3'	1140 to 1162
	P450HF20F	3'-RACE	5' CGTGCATCGCAATCCCCAATAC 3'	1341 to 1362
	AP450HF20R	Full length	5' TTACATAGCTTTCATGGCTTCGGGTC 3'	1625 to 1650
CYP4D4v2	AP450HF5F	Full length	5' ATGTTATTTGAATTCCTGGTGGGTC 3'	1 to 25
	SNP450HF5F	SNaP determination	5' ACATGACACCACGACAAGTGG 3'	948 to 968
	RTP450HF5F	qRT-PCR	5' AGGATAAGGAGAAACCGGTGACC 3'	1052 to 1074
	RTP450HF5R	qRT-PCR	5' CAATTGTCGGCACCGATGGATAC 3'	1156 to 1134
	P450HF5R	5'-RACE	5' AGCAACTCAAAATGGCGTACC 3'	1430 to 1410
	AP450HF5R	Full length	5' TAACTACTTGCGAACTCTCAAACCC 3'	1521 to 1497
CYP6A38	P450HF17P-F	5' flanking region	5' TGGTCTTCTAGGGGAGAAGACTACCTGC 3'	-676 to -649
	SNP450HF17PF	SNaP determination	5' TTCAGGATTGCTGGGTAGCT 3'	-69 to -50
	P450HF16F-3	Full length	5' CATTATGGAGACTTCGGGAGTTTTG 3'	-4 to 21
	P450HF17P	5' flanking region	5' CGTAGGTTCCTCATGGGGTATACCCAGC 3'	120 to 93
	RTP450HF17F	qRT-PCR	5' CCCTGATGGGCAACATGAATGGAT 3'	122 to 145
	RTP450HF17R	qRT-PCR	5' TAGTTGTTTGTCCAGCAGCACCAC 3'	270 to 247
	P450HF17R	5' RACE	5' CGCTGTACTTCAATAGATTTCCTGC 3'	650 to 626
	P450HF17F	3' RACE	5' TGAGGGTGATACAAAACCAAGC 3'	1017 to 1038
	AP450HF17R	Full length	5' GAGATAATCTCCCACCCCTAAATCG 3'	1520 to 1496
Common	Flyh1		5' GGICCIAGIAACTGCATIGG 3'	
	Flyc1		5' GGAAGTNGACACNTTYATGTT 3'	
	CYP6AD1		5' GTNATHGGHHNBTGYGCHTTYGG 3'	
	HemeR1		5' CCIATGCAGTTICTIGGICC 3'	
	Oligo (dt)		5' TAATACGACTCACTATAGGGAGATTTTTTTTTTTTTTT 3'	
	C2		5' TAATACGACTCACTATAGGGAGA 3'	
	AP1 (RACE)		5' CCATCCTAATACGACTCACTATAGGGC 3'	
	AP1 (GenomeWalking)		5' GTAATACGACTCACTATAGGGC 3'	
	ActinS1/AS1		5' AGGCGAATCGCGAGAAGATG 3'/ 5' TCAGATCACGACCAGCCAGATC 3'	

Table 5.2. The expression of the nucleotide(s) at the polymorphism site of each P450 gene in different house fly strains and lines generated by the crosses of resistant ALHF and susceptible aabys strains.

House Fly Strain		P450 Gene		
		CYP4G2	*CYP4D4v2*	*CYP6A38*
ALHF		C	C	G
aabys		T	T	T
BC_1 Lines (ALHF ♀x aabys ♂)	A2345	C/T	T/C	G/T
	A1345	C/T	T/C	G/T
	A1245	T	T/C	G/T
	A1235	C/T	T/C	G/T
	A1234	C/T	T	T
BC_1 Lines (ALHF ♂x aabys ♀)	A2345	C/T	T/C	G/T
	A1345	C/T	T/C	G/T
	A1245	T	T/C	G/T
	A1235	C/T	T/C	G/T
	A1234	C/T	T	T

FIGURE LEGENDS

Figure 5.1. The cDNA/deduced protein sequences of *CYP4G2* in ALHF and aabys. Invariant and highly conserved motifs in all P450 proteins are doubly underlined and nucleotide polymorphisms are highlighted. The primers used for generating the *CYP4G2* cDNA fragments are indicated by arrowheads.

Figure 5.2. The cDNA/deduced protein sequences of *CYP4D4v2* in ALHF and aabys. Invariant and highly conserved motifs in all P450 proteins are doubly underlined and nucleotide polymorphisms are highlighted. The primers used for generating the *CYP4D4v2* cDNA fragments are indicated by arrowheads.

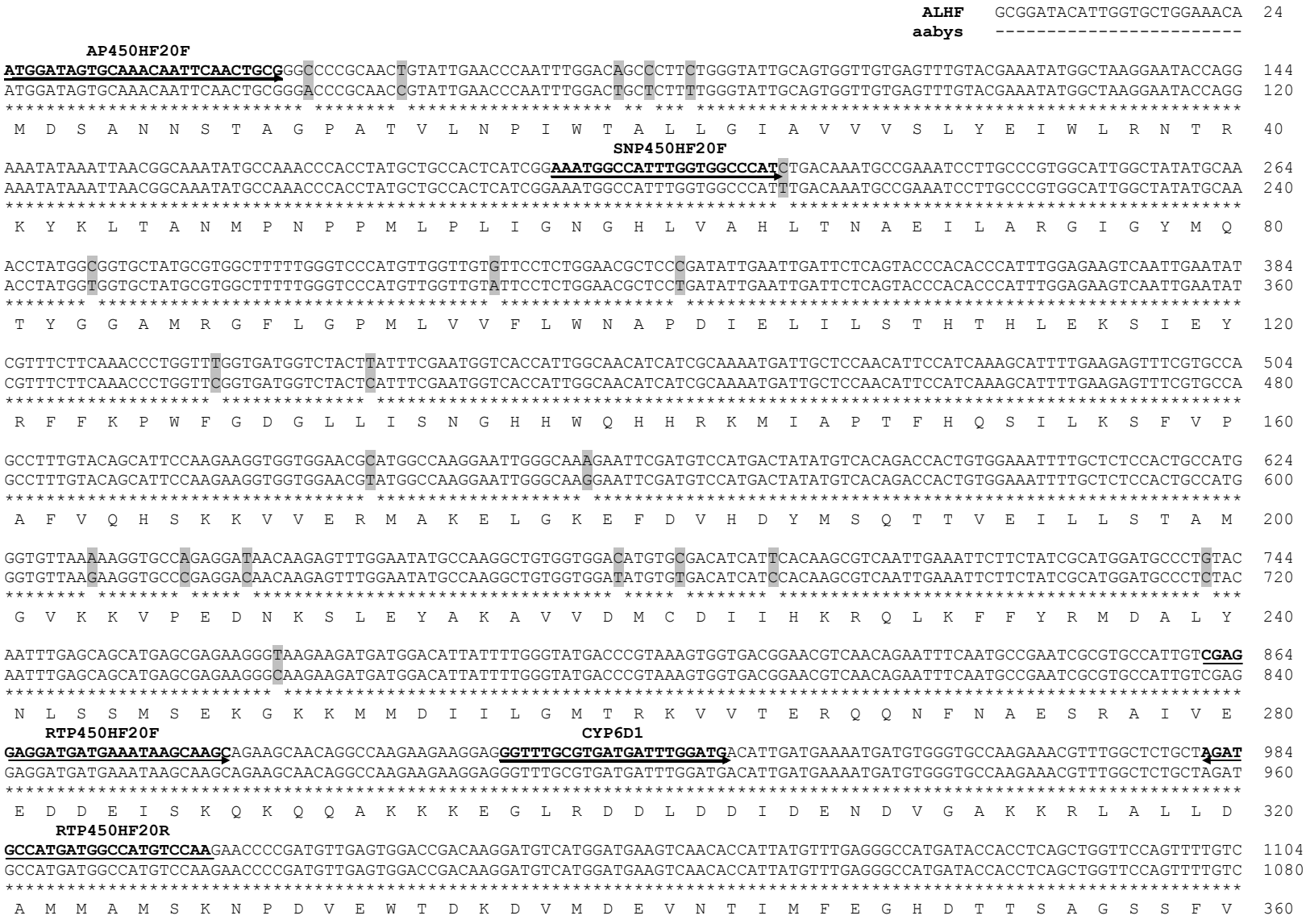
Figure 5.3. The cDNA/deduced protein sequences/5' flanking region of *CYP6A38* in ALHF and aabys. Invariant and highly conserved motifs in all P450 proteins are doubly underlined and nucleotide polymorphisms are highlighted. The primers used for generating the *CYP6A38* cDNA fragments are indicated by arrowheads.

Figure 5.4. Northern blot analysis of differentially expressed patterns of *CYP4G2*, *CYP4D4v2*, and *CYP6A38* between permethrin treated and untreated ALHF house flies. mRNAs were isolated from the whole bodies of 20 surviving house flies 24 h after permethrin treatment with 2μg/fly. Blots were hybridized with the cDNA probes derived from three P450 gene fragments. The ethidium bromide stain of 18s ribosomal RNA in agarose gel is shown at the bottom.

Figure 5.5. Induction of *CYP4G2*, *CYP4D4v2*, and *CYP6A38* in CS and ALHF house flies following treatment with permethrin. The expression of three genes was analyzed by qRT-PCR as described in the materials and methods. A: Relative expression of *CYP4G2* in permethrin treated and untreated susceptible CS and resistant ALHF house flies. B. Relative expression of *CYP4D4v2* in permethrin treated and untreated CS and ALHF house flies. C. Relative expression of *CYP6A38* in permethrin treated and untreated CS and ALHF house flies. The relative levels of gene expression are shown as a ratio in comparison with that in untreated CS flies. The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of P450 expression among the samples with the same alphabetic letter (i.e., a, b, or c).

Figure 5.6. Expression of *CYP4G2*, *CYP4D4v2*, and *CYP6A38* in head+thorax and abdomen tissue of adult house flies. The expression of these three genes was analyzed by qRT-PCR as described in the materials and methods. A: Relative expression of *CYP4G2* in head+thorax and abdomen tissue of CS and ALHF. susceptible CS and resistant ALHF house flies. B. Relative expression of *CYP4D4v2* in head+thorax and abdomen tissue of CS and ALHF. C. Relative expression of *CYP6A38* RNA in head+thorax and abdomen tissue of CS and ALHF. The relative levels of gene expression are shown as a ratio in comparison with that in untreated CS flies. The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of P450 expression among the samples with the same alphabetic letter (i.e., a, b, or c).

Figure 5.7. Graphic representation of *CYP4G2*, *CYP4D4v2*, and *CYP6A38*, showing locations and sequences of SNP-specific primers for SNP determinations and genetic linkage analyses of three P450 genes. A: *CYP4G2*. B: *CYP4D4v2*. C: *CYP6A38*.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CYP6D1
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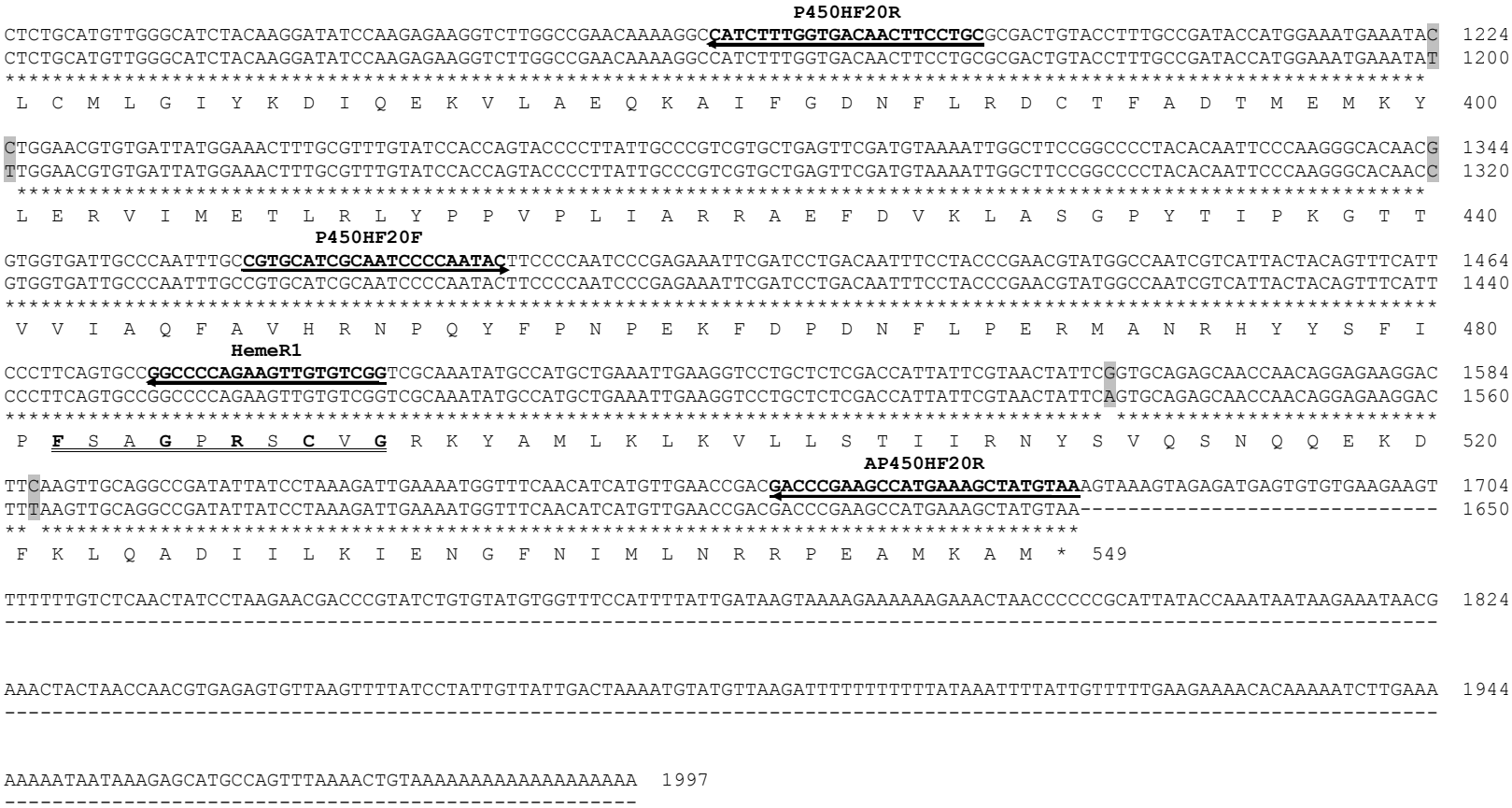
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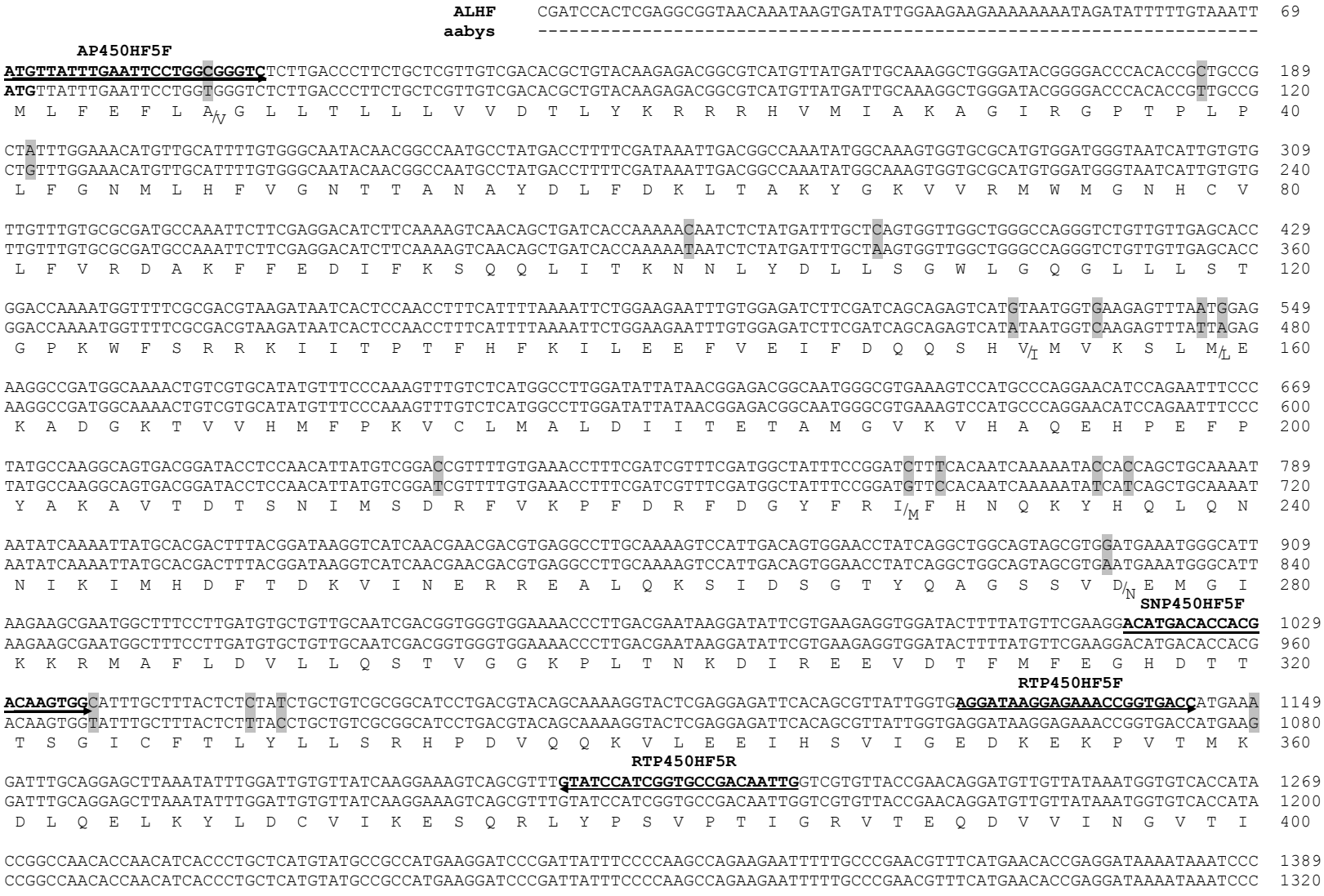
Figure 5.1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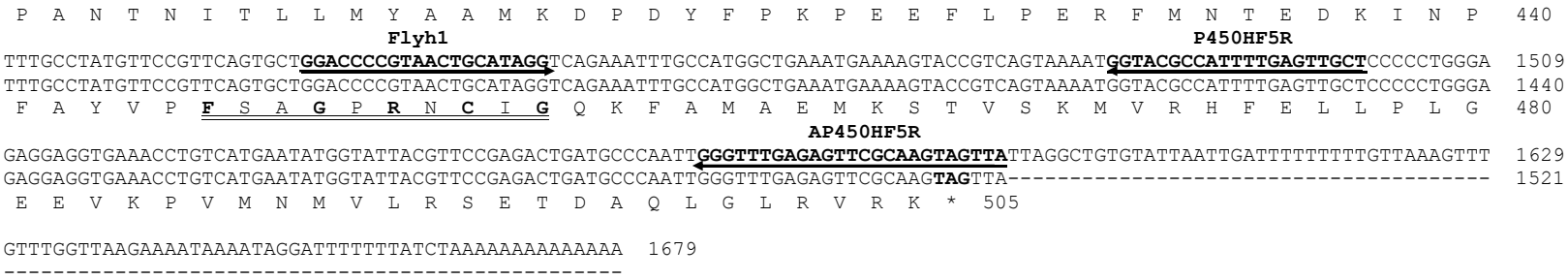
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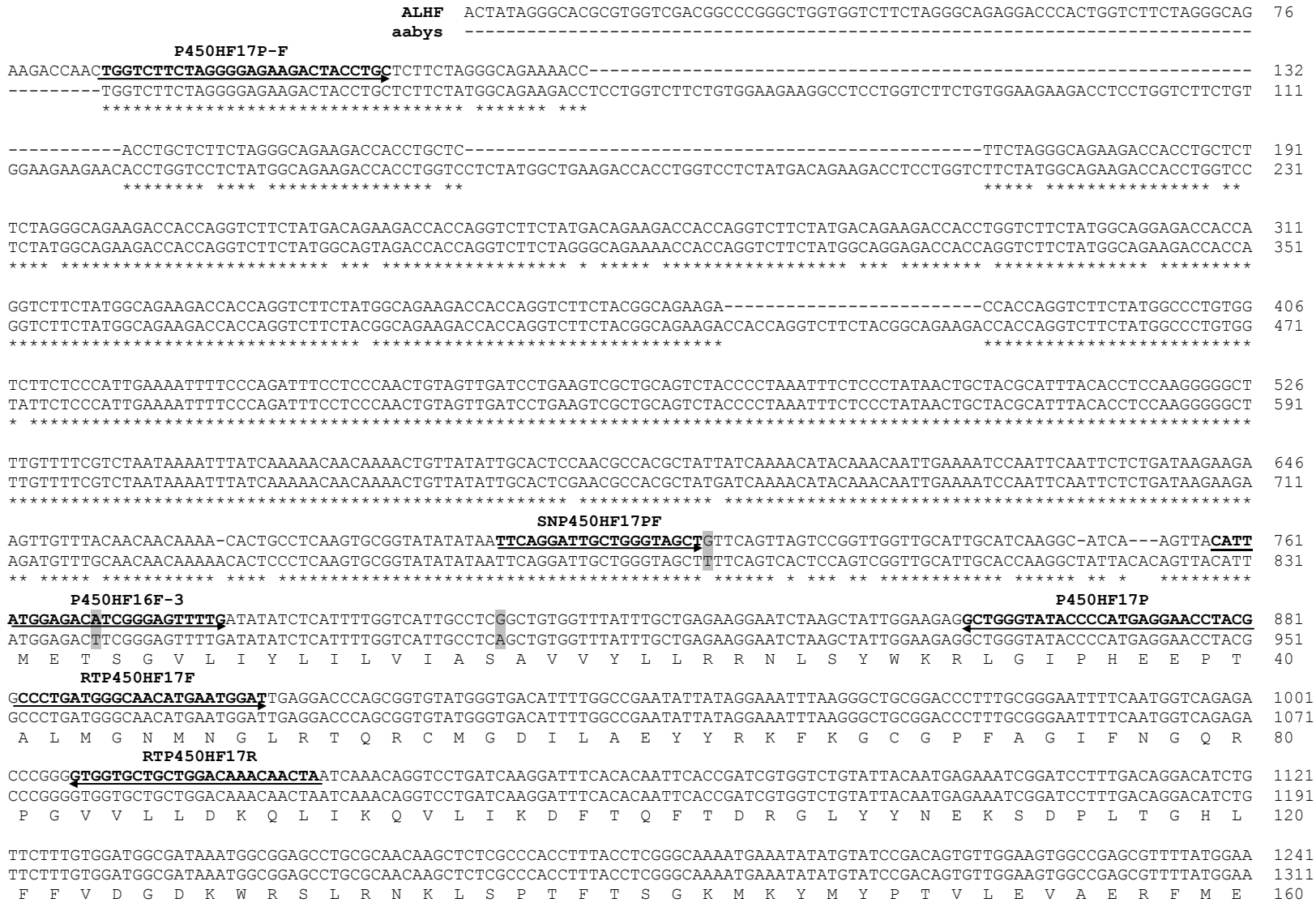
Figure 5.2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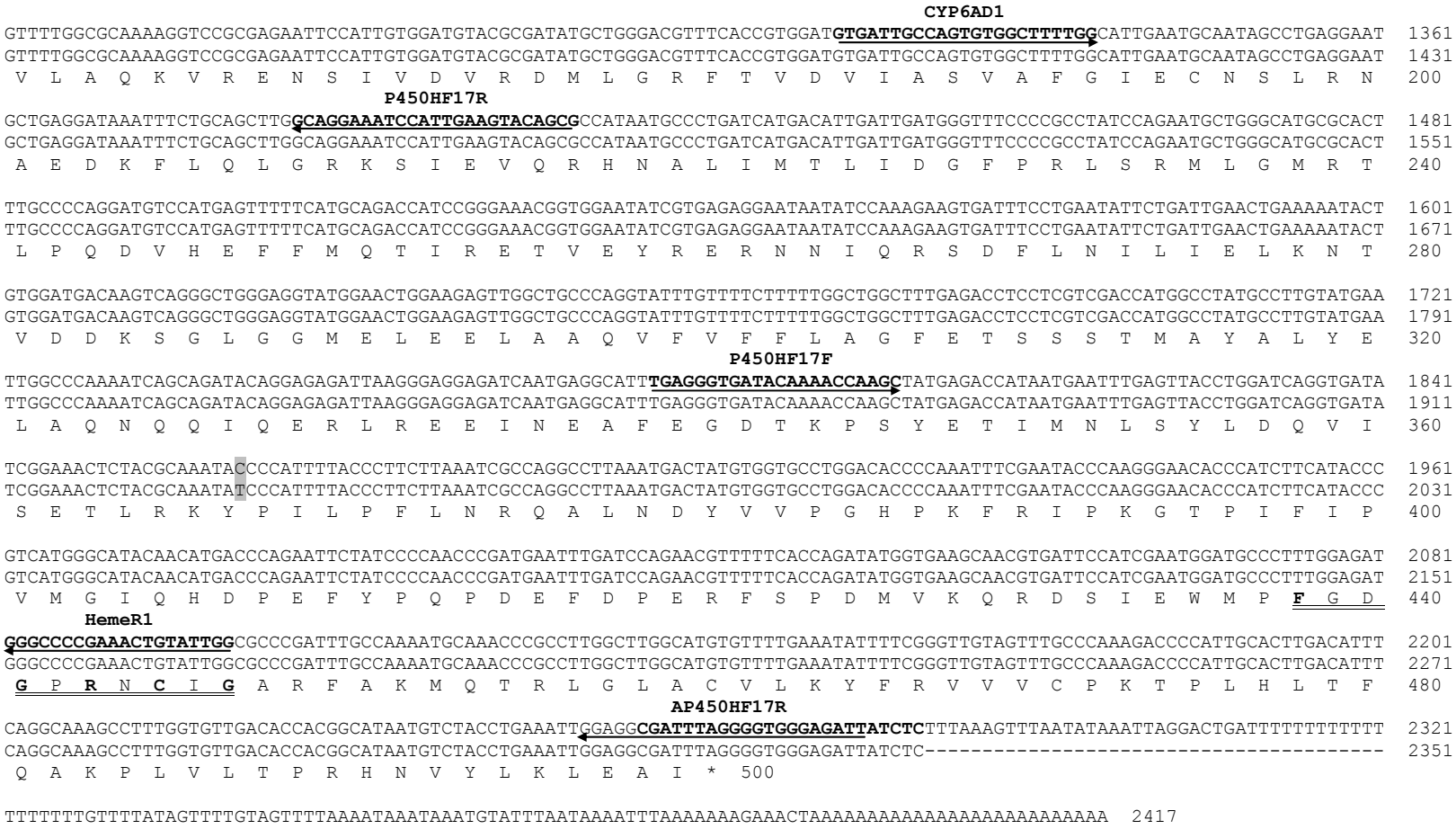
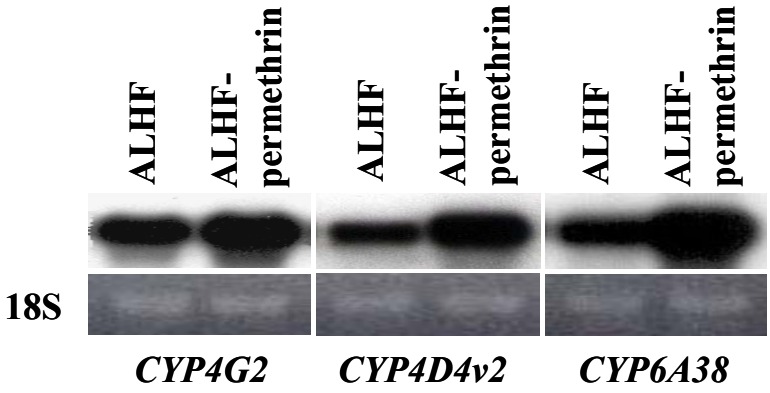


Figure 5.3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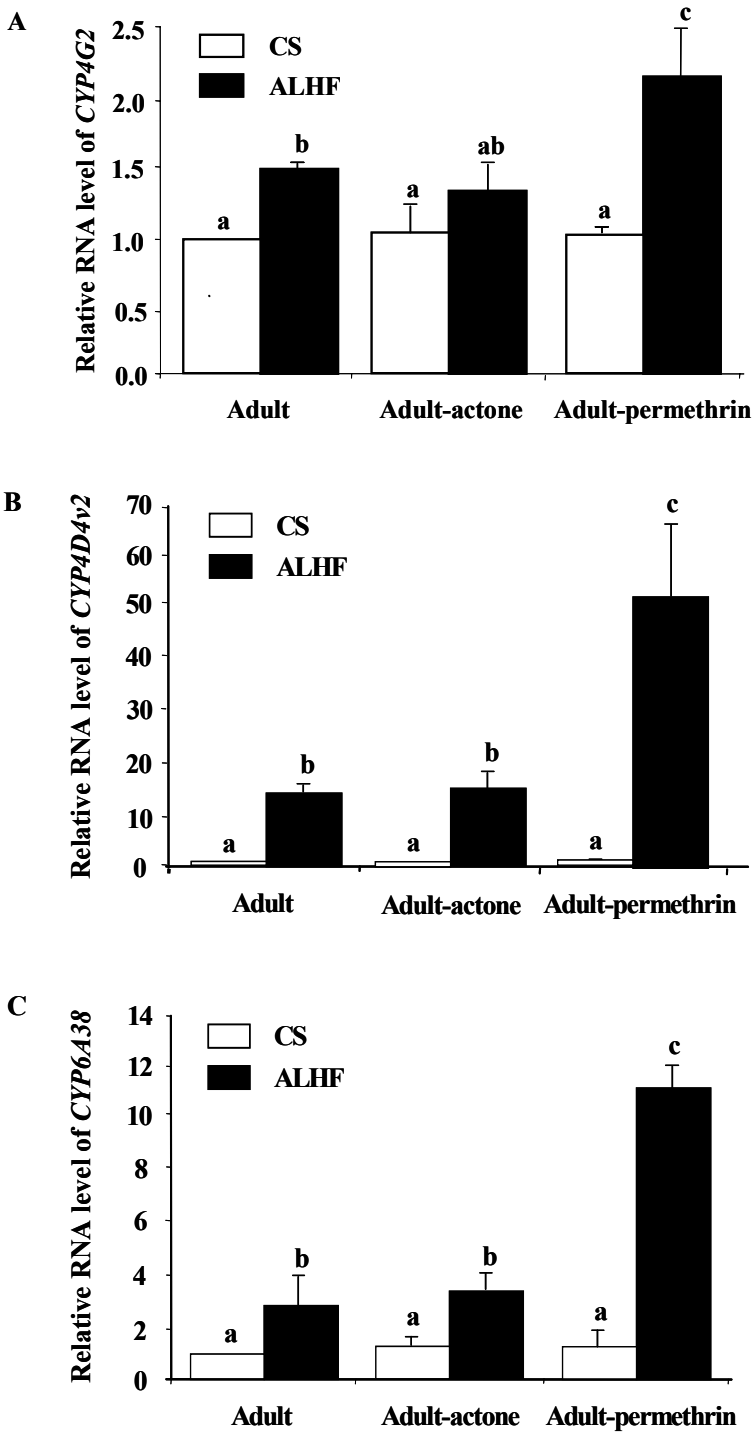
ALHF-permethrin
ALHF
ALHF-permethrin
ALHF
ALHF-permethrin
18S
CYP4G2
CYP4D4v2
CYP6A38

Figure 5.4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1.5
1.0
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0.0
CS
ALHF
a
b
a
ab
a
c
Adult
Adult-actone
Adult-permethrin
B
Relative RNA level of CYP4D4v2
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60
50
40
30
20
10
0
CS
ALHF
a
b
a
b
a
c
Adult
Adult-actone
Adult-permethrin
C
Relative RNA level of CYP6A38
14
12
10
8
6
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a
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Adult
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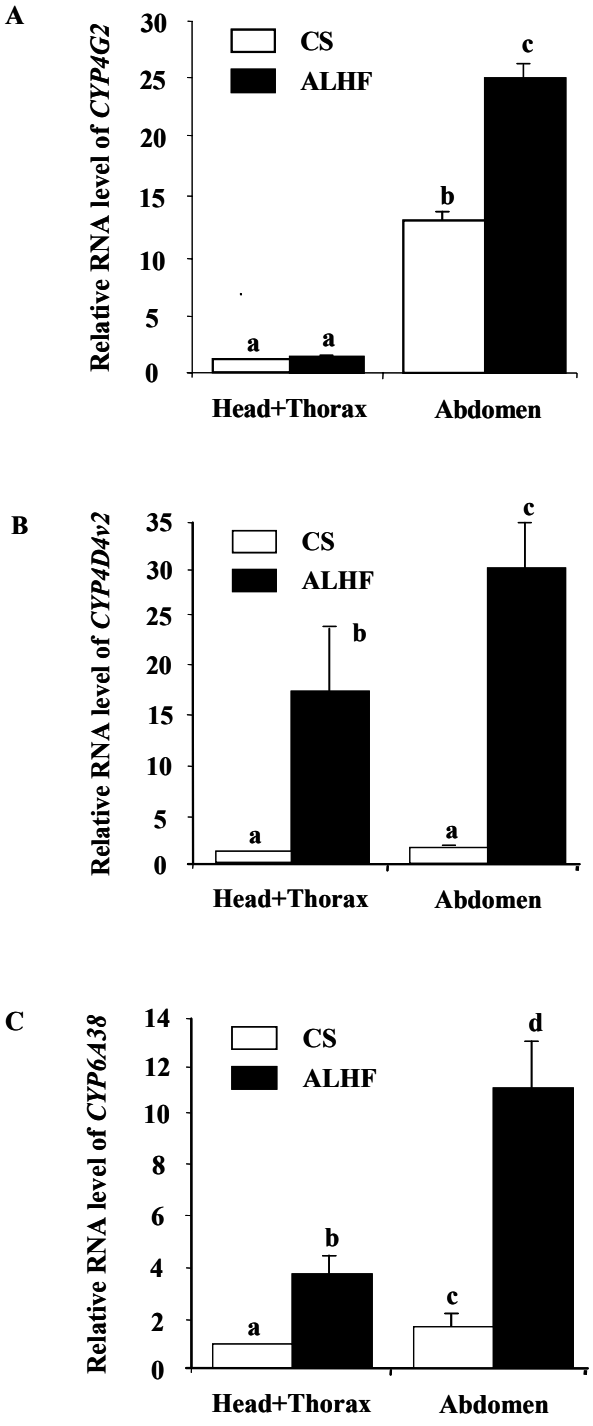
Adult-permethrin

Figure 5.5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CS
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a
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30
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a
b
a
c
Head+Thorax
Abdomen
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Relative RNA level of CYP6A38
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ALHF
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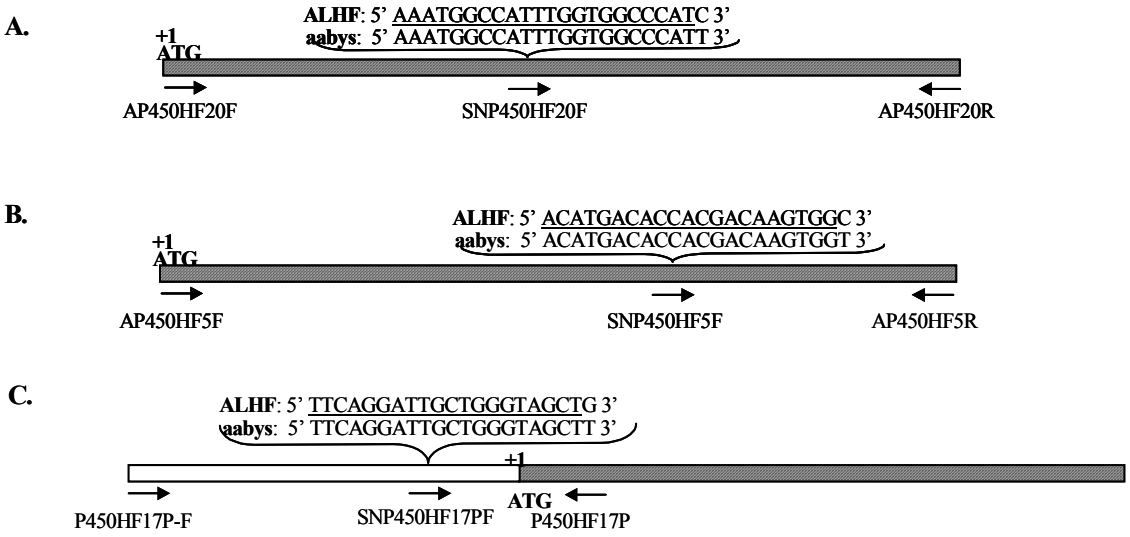
c
d
Head+Thorax
Abdomen

Figure 5.6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AP450HF20R
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ATG
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SNP450HF5F
AP450HF5R
C.
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+1
ATG
P450HF17P-F
SNP450HF17PF
P450HF17P

Figure 5.7

CHAPTER 6: DIFFERENTIAL EXPRESSION OF TWO *CYP6A5* ALLELES IN PYRETHROID RESISTANT HOUSE FLIES

6.1 Introduction

Insect cytochrome P450s are known to play an important role in detoxifying insecticides (Scott, 1999; Feyereisen, 2005) and plant toxins (Berenbaum, 1991; Schuler, 1996), resulting in the development of resistance to insecticides (Brattsten et al., 1986; Carino et al., 1994; Liu and Scott, 1997; 1998; Kasai et al., 2000; Feyereisen, 2005) and facilitating the adaptation of insects to their plant hosts (Li et al., 2002; Wen et al., 2003). A significant characteristic of insect P450s that is associated with enhanced metabolic detoxification of insecticides in insects is the constitutively increased P450 proteins and P450 activities that result from constitutively transcriptional overexpression of P450 genes in insecticide resistant insects (Carino et al., 1994; Liu and Scott, 1997; 1998; Kasai et al., 2000; Feyereisen, 2005). Cytochrome P450s constitute the largest gene superfamily found in all living organisms examined (Feyereisen, 2005). There are more than 1000 P450s that have been identified in insects, distributed throughout more than 150 subfamilies of 40 known P450 gene families (http://dmelson.utmem.edu/nelsonhomepage.html). For example, ninety P450 genes (including 7 pseudogenes) are present in the *Drosophila melanogaster* genome (Tijet et al., 2001), one hundred and eleven P450

genes (including 5 pseudogenes) are present in *Anopheles gambiae* (Ranson et al., 2002), and 86 P450 genes are present in *Bombyx mori* (Li et al., 2005). Because these P450 genes perhaps evolve from a common ancestor by gene duplication (Gonzalez and Nebert, 1990; Feyereisen, 1999), some of them share very high similarity at both nucleotide and amino acid sequence levels. For example, two *Drosophila melanogaster* P450 genes, *CYPq3l1* and *CYPq3l2*, share 99.8% nucleotide similarity resulting in only one amino acid difference (Ranson et al., 2002). A question emerging from such a high sequence similarity shared by P450 genes is: how can the expression of each of these P450 genes with high sequence similarity be precisely examined?

A house fly strain, ALHF, exhibits very high levels of resistance to pyrethroids (Liu and Yue, 2000). Early studies in our laboratory on permethrin resistance in ALHF house flies had led to the identification of permethrin resistance that could be largely suppressed by piperonyl butoxide (PBO), an inhibitor of cytochrome P450s, (Liu and Yue, 2000; 2001). These studies suggest that cytochrome P450-mediated detoxification might be one of the major mechanisms involved in pyrethroid resistance in ALHF. Furthermore, genetic linkage studies had linked PBO-suppressible resistance (or P450-mediated resistance) to autosome 5, with minor factors linked to autosomes 1 and 2 (Liu and Yue, 2001). In the current study, we isolated 2 P450 alleles, *CYP6A5v2* and *CYP6A5* (Cohen and Feyereisen, 1995) from ALHF house flies. *CYP6A5v2* and *CYP6A5* share 98% similarity in amino acid sequence. To accurately evaluate the expression profiles of *CYP6A5* and *CYP6A5v2* between resistant and susceptible house flies, quantitative real-time PCR (qRT-PCR) was chosen to characterize the expression patterns of these two P450 genes. The expression profiles of these two genes in different tissues and in both

resistant and susceptible house flies were characterized. Genetic linkage studies were conducted to providing the further information on a possible genetic link of *CYP6A5v2* and the pyrethroid resistance in ALHF.

6.2 Materials and methods

6.2.1 House fly strains

Three house fly strains were used in this study. ALHF, a wild-type strain collected from a poultry farm in Alabama in 1998, was selected with permethrin for 6 generations after collection to reach a 6,600-fold resistance, and was maintained under biannual selection with permethrin (Liu and Yue, 2000; 2001). CS is a wild type insecticide-susceptible strain. aabys is an insecticide-susceptible strain with recessive morphological markers ali-curve (*ac*), aristapedia (*ar*), brown body (*bwb*), yellow eyes (*ye*), and snipped wings (*sw*) on autosomes 1, 2, 3, 4, and 5, respectively. Both CS and aabys were obtained from Dr. J. G. Scott (Cornell University).

6.2.2 RNA extraction, cDNA preparation, and P450 gene fragment isolation

Total RNAs were extracted from house flies using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott, 1997). mRNA was isolated with oligotex-dT suspension (QIAGEN). The first strand cDNA was synthesized with SuperScript II reverse transcriptase and an antisense 5'- anchored oligo(dT) primer (5' TAATACGACT CACTATAG GGAGATTTTTT TTTTTTTTTT 3') (Tomita and Scott, 1995), using ALHF mRNAs as templates. The first strand cDNA products were amplified by PCR with the C2 primer (5' TAATACGACTCACTATAGGGAGA 3') and internal

degenerated sense primer, Flyh1 (5' GGICCI AGIAACTGCATIGG 3', Fig. 6.1). The Flyh1 was designed based on the heme binding consensus sequence (Liu and Zhang, 2002). The PCR products were cloned into PCR™ 2.1 Original TA cloning vector (Invitrogen) and sequenced (Genomic & Sequencing Lab, Auburn University). Cloning and sequence analyses of P450 gene fragments were repeated at least three times with different preparations of mRNAs. Three TA clones from each replication were sequenced.

6.2.3 Rapid amplification of 5' cDNA ends (5'-RACE) of the putative P450 gene fragments

To clone the 5' half of the putative P450 gene fragments, 5'-RACE was carried out using the Marathon™ cDNA Amplification Kit (Clontech) as described by the manufacturer and Liu and Zhang (2002). The first strand cDNAs were synthesized with AMV reverse transcriptase using ALHF mRNAs as templates. The double strand cDNA was synthesized following the protocol described by the manufacturer (Clontech). Adaptors were ligated to both ends of each double strand cDNA using T4 DNA ligase as described by the manufacturer. The 5' end of the P450 cDNA fragment was amplified by PCR using the primer pair, P450HF10R/AP1 (based on the sequence of the adaptor). The primer P450HF10R (5' TCGCAGCAGGGATACCAAAC 3', Fig. 6.1) was generated based on the 5' end sequences of the putative P450 cDNA fragment as described by the manufacturer (Clontech). The full length of the P450 cDNA was subsequently generated in the ALHF and susceptible aabys strains by RT-PCR using a specific primer pair, AP450HF10-3F/AP450HF10-3R (Fig. 6.1), synthesized based on the 5'and 3'end

sequences of the putative P450 gene from ALHF. Cloning and sequence analyses of the P450 cDNAs were repeated at least three times with different preparations of mRNAs, and three TA clones from each replication were verified by sequencing.

6.2.4 Northern blotting analysis

Northern blotting analyses were performed according to Sambrook et al. (Sambrook et al., 1989). Twenty micrograms of total RNA from adults of CS and ALHF were fractionated on 1% formaldehyde denaturing agarose gel and transferred to Nytran membranes (Schleicher and Schuell) (Sambrook et al., 1989). The P450 cDNA fragments were labeled with [α-^{32}P] dCTP using a Primer-It II Random Primer Labeling kit (Stratagene) and hybridized with RNA blots using QuickHyb solution (Stratagene). The amount of RNA loaded in each lane was standardized by comparing the density of the 18S ribosomal RNA band on agarose gel under UV light before transfer (Spiess and Ivell, 1998). All Northern blot analyses were repeated three times with different preparations of RNA samples. The radiographic signal intensity was quantitatively analyzed by QuantiScan v3.0 (Biosoft) as previously described by Liu and Zhang (2004). Statistical significance of the gene expressions was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.0\,5$ was considered statistically significant.

6.2.5 Gene expression analysis by quantitative real-time PCR (qRT-PCR)

Total RNA samples (0.5 μg/sample) were reverse-transcribed using SuperScript II reverse transcriptase (Stratagene) in a total volume of 20 μl. The quantity of cDNAs was measured using a spectrophotometer prior to qRT-PCR. qRT-PCR was performed with the SYBR Green master mix Kit and ABI 7500 Real Time PCR system (Applied Biosystems). Each qRT-PCR reaction (25 μl final volume) contained 1x SYBR Green master mix, 1 μl of cDNA, and a gene specific primer pair, RTP450HF10-3F/RTP450HF 10-3R (for *CYP6A5v2*, Fig. 6.2A) or RTP450HF10-10F/RTP450HF10-10R (for *CYP6A5*, Fig. 6.2B) at a final concentration of 3-5 μM. Each P450 gene specific primer pair was designed based on the specific sequence of each gene by placing a specific nucleotide at the 3' end of each primer to permit preferential amplification of that specific P450 gene (Fig. 6.2). A 'no-template' negative control and all samples were performed in triplicate. The reaction cycle consisted of a melting step of 50ºC for 2 min then 95°C for 10 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. Specificity of the PCR reactions was assessed by a melting curve analysis for each PCR reaction (Wittwer et al., 1997). Relative expression levels for specific genes were calculated by the $2^{-\Delta\Delta C(t)}$ method using SDS RQ software (Livak and Schmittgen, 2001). The β-actin gene, an endogenous control, was used to normalize expression of target genes (Aerts et al., 2004; Dorak, 2006). The preliminary assay had shown that the β-actin gene remained constant in different tissues and in both resistant and susceptible house flies. Thus, it could be used for internal normalization in qRT-PCR assays. Each experiment was repeated three times with different preparations of RNA samples. The statistical significance of the gene expressions was calculated using a Student's *t*-test for all 2-sample comparisons and a

one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant.

6.2.6 Genetic linkage analysis of CYP6A5v2

To determine the genetic linkage of *CYP6A5v2*, a genetic cross experiment was conducted (Liu and Scott, 1995; Liu and Yue, 2001). Briefly, reciprocal crosses between ALHF and aabys were conducted and F_1 males were back-crossed to aabys females. Five lines were saved from the back-cross generation (BC_1) with the genotypes of *ac/ac*, +/*ar*, +/*bwb*, +/*ye*, +/*sw* (A2345); +/*ac*, *ar/ar*, +/*bwb*, +/*ye*, +/*sw* (A1345); +/*ac*, +/*ar*, *bwb/bwb*, +/*ye*, +/*sw* (A1245); +/*ac*, +/*ar*, +/*bwb*, *ye/ye*, +/*sw* (A1235); and +/*ac*, +/*ar*, +/*bwb*, +/*ye*, *sw/sw* (A1234). Since crossing over does not or very rarely occurs in male flies (Gao and Scott, 2006), the presence of a mutant phenotype indicated that the respective autosome with a mutant-type marker was derived from the aabys females. The genotype of each line was homozygous for the recessive mutant allele from aabys and heterozygous for the dominant wild-type alleles from ALHF. These lines were named according to the autosomes bearing wild-type markers from ALHF. For example, the A1234 strain had wild-type markers on autosomes 1, 2, 3, and 4 from ALHF and the recessive mutant marker on autosome 5 from aabys.

Allele specific PCR was conducted for genetic mapping of P450 genes (Liu and Scott, 1995). Two rounds of PCR were conducted. For the first PCR reaction, the allele-independent primer pair, AP450HF10-3F/AP450HF10-3R (Fig. 6.1), was used to generate *CYP6A5v2* cDNA fragments. The second PCR was employed with 0.5 μl of the first round PCR reaction solution with 1000-fold dilution and an allele specific primer

pair, AP450HF10-3NLF/AP450HF10-3NLR (Figs. 6.1 and 6.6A). The allele specific primer pair was designed based on the specific sequence of *CYP6A5v2* in ALHF by placing a specific nucleotide polymorphism at the 3' end of each primer to permit preferential amplification of the P450 allele from ALHF. Each experiment was repeated three times with different preparations of mRNAs. To confirm that the PCR products were in fact the P450 gene fragments, the PCR products were sequenced at least once each.

6.3 Results and discussion

6.3.1 Cloning and sequencing of P450 genes from house flies

One partial putative house fly P450 cDNA fragment was amplified from ALHF by 3' RACE using a 5'-anchored oligo(dT) primer and an internal degenerate primer, Flyh1. BLAST analysis of the amino acid sequence predicted from the partial putative P450 cDNA fragment showed that the sequence encoded the C-terminal end of a novel putative P450 with 98% identity to *CYP6A5* (accession number: U09343) from a susceptible house fly strain, *sbo* (Cohen and Feyereisen, 1995). To isolate and amplify the 5' end of this P450 gene, 5'-RACE was conducted using a specific antisense primer synthesized based on the sequence of the novel putative P450 cDNA and a sense primer, AP1, synthesized based on the sequence of the adaptor (Clontech). Two cDNA products were amplified by 5'-RACE. One was 100% overlapped with its corresponding 3' putative P450 cDNA fragment, identifying it as the 5' end of the novel P450 gene. The other, however, was 98% overlapped with its corresponding 3' putative P450 cDNA but was 100% identical to the sequence of *CYP6A5*, indicating that it was *CYP6A5*. Because of

the perfectly matched sequences at 3' and 5' ends of both the putative P450 gene and *CYP6A5*, entire cDNA fragments for both genes were subsequently amplified from ALHF and aabys house flies by PCR using a primer pair of AP450HF10-3F/AP450HF10 -3R (Fig. 6.1) synthesized according to the respective 5' and 3' end sequences shared by both genes. Ten TA clones generated from the full length of PCR products were sequenced from each house fly strain. The sequences of 6 clones of the entire cDNA fragment from ALHF were perfectly overlapped with the 3' and 5' sequences of the novel putative P450 gene generated by RACE. The sequence was named *CYP6A5v2* (accession number: EF615004) by the P450 nomenclature committee (Dr. D. Nelson, personal communication). The sequences of other 4 clones were identical with *CYP6A5*. Comparison of the nucleotide and deduced protein sequences of *CYP6A5v2* between ALHF and aabys revealed several nucleotide polymorphisms, 6 of which resulted in 6 animal acid substitutions (Fig. 6.1).

Comparison of the cDNA sequences of both *CYP6A5* and *CYP6A5v2* revealed an identical open reading frame (ORF) of 1521 nucleotides encoding proteins of 507 residues (Fig. 6.3). The putative protein sequences of *CYP6A5* and *CYP6A5v2* deduced from the cDNA sequences shared 98% identity. Five consensus motifs that were found in insect P450 proteins (Feyereisen, 2005) were identified in both CYP6A5 and CYP6A5v2 (Fig. 6.3). WxxR motif located in C-helix is presented at amino acid residues 27-30 and the arginine (R) in this motif can form a charge pair with the propionate of the heme (Feyereisen, 2005). GxE/DTT/S motif that is presented at amino acid residues 313-317 is located in I-helix and this motif is thought to be involved in proton delivery and catalysis (Gorokhov et al., 2003). ExLR and PxxFxPE/DRF motifs, which are located at amino

acid residues 336-339 and 421-429, respectively, are thought to form a set of salt bridge interactions (E-R-R) for stabilizing the overall structure of protein (Hasemann et al., 1995). The P450 heme binding motif, FXXGXRXCXG (Gotoh and Fujii-Kuriyama 1989), is presented at amino acid residues 444-454 of both CYP6A5 and CYP6A5v2. The cysteine residue in this motif is known to be an important ligand for heme binding. Finally, another conserved 17- residue sequence, corresponding to the central part of the I-helix, is found at amino acid residues 307-323 of CYP6A5 and CYP6A5v2 (Fig. 6.3). This motif is particularly conserved among family 6 (Cornette et al., 2006) and is thought to be involved in proton delivery (Gorokhov et al., 2003).

6.3.2 Expressional profiles of CYP6A5 and CYP6A5v2 between ALHF and CS house flies

To investigate the possible role of CYP6A5 and CYP6A5v2 in the resistance of ALHF, Northern blot analysis and qRT-PCR were performed to examine the expression patterns of these two alleles in CS and ALHF house flies. Northern blot analysis revealed a similar expression pattern for both alleles (data not show). In contrast, qRT-PCR with an allele specific primer pair for each allele (Figs. 6.1 and 6.2) revealed different expression patterns for two alleles. We found no significant difference in the expression of *CYP6A5* between susceptible CS and resistant ALHF strains (Fig. 6.4A), which was consistent with a previous study on other house fly strains by Cohen and Feyereisen (1995). However, we identified significant overexpression (~1000-fold) of *CYP6A5v2* in ALHF compared to CS (Fig. 6.4B). It has been proposed that constitutive overexpression of P450s is one of the responses for the adaptation of insects to their environment (Terriere, 1983; 1984). Further more, in many cases, increased levels of P450 gene

expression have resulted in increased levels of both total P450s and the P450 activities and have been strongly suggested as a major cause that are responsible for insecticide resistance (Brattsten, 1986; Carino et al., 1992; Liu and Scott, 1997; Scott, 1999; Festucci-Buselli et al., 2005; Feyereisen, 2005). Accordingly, overexpression of *CYP6A5v2* in ALHF may suggest the importance of the gene in increasing metabolic detoxification of insecticides in ALHF house flies compared with the susceptible ones.

Our controversial results generated by Northen blot analyses and qRT-PCR is likely because that the probes used in Northen blot analyses for detecting both genes shared the high sequence similarity, resulting in lacking the sensitivity of probes to specifically detect their own RNA transcripts. Thus, the similar expression patterns for both *CYP6A5* and *CYP6A5v2* detected by Northen blot analyses were actually resulted from the hybridization of both trascripts by each probe. The same shortcoming of detecting expression of genes that sharing high sequence similarity by Northen blot analysis has also been pointed out by Wen et al. (2001). In contrast, because of primer specificity for each gene, qRT-PCR can highly guarantee the sucessful amplification and quantification of a specific product for a given DNA molecule. Besides, the specificity of the qRT-PCR products can be identified by sequencing. These two characters of qRT-PCR allow it serves as a more sensitive method to discriminate the expression profiles of genes sharing high degree of sequence similarity.

6.3.3 Tissue specific expression of CYP6A5v2 between ALHF and CS house flies

In insects, the midgut and fat body tissue have generally been considered to be the primary detoxification organs (Hodgson, 1985) where most insect detoxification P450s

are expressed (Scott et al., 1998). To investigate whether the overexpression of *CYP6A5v2* is detoxification-tissue specifically related, we examined the expression of the gene in the abdomen and head+thorax tissues of both CS and ALHF strains by qRT-PCR. Our results revealed ~16- and ~5-fold higher expression of *CYP6A5v2* in the abdomen tissue of CS and ALHF flies, respectively, compared with their head+thorax tissues (Fig. 6.5). However, significant overexpression was more evident in both tissues of ALHF compared with CS. The expression of *CYP6A5v2* was ~500 and ~150-fold higher in both head+thorax and abdomen tissues, respectively, of ALHF compared to the corresponding tissues in CS (Fig. 6.5). As the midgut and fat body tissue have generally been considered to be the primary detoxification organs and as both components are located in the abdomen of insects, relatively high levels of *CYP6A5v2* in the abdomen of ALHF may suggest the importance of the genes in increasing metabolic detoxification of insecticides in ALHF house flies compared with the susceptible CS strain.

6.3.4 Chromosomal linkage of CYP6A5v2

Genetic linkage between an overexpressed P450 gene and insecticide resistance is an important step in establishing a possible link between a P450 gene and its role in resistance (Carino et al., 1994; Liu and Scott, 1996; Rose et al., 1997; Guzov et al., 1998; Maitra et al., 2000; Feyereisen, 2005). Our previous research indicated that resistance in the ALHF strain could be largely suppressed by PBO, an inhibitor of cytochrome P450s. Further, genetic linkage studies had linked PBO-suppressible resistance (or P450-mediated resistance) to autosome 5, with minor factors linked to autosomes 1 and 2 (Liu and Yue, 2001). To confirm a likely link between *CYP6A5v2* and insecticide resistance in

ALHF, an allele specific PCR (ASPCR) determination was performed to examine the genetic linkage of *CYP6A5v2* with 5 house fly lines derived from crosses of ALHF and a susceptible morphological marker strain, aabys. To increase primer specificity and avoid false-negative results, two rounds of PCR amplification were performed. In the first PCR reaction, a universal primer set, AP450HF10-3F/AP450HF10-3R (Fig. 6.1), was used and the expected products were obtained in all strains tested (Fig. 6.6B). The second PCR reactions were conducted using the first PCR products as the templates and an ALHF allele-specific primer pair of AP450HF10-3NLF/AP450HF10-3NLR (Fig. 6.6A). The ALHF allele specific primer pair was designed based on the specific sequence of the P450 gene from ALHF by placing a specific nucleotide polymorphism at the 3' end of each primer to permit preferential amplification of P450 allele from ALHF. Our results showed that the ALHF allele-specific primer set for *CYP6A5v2* amplified specific DNA fragments only in flies having the autosome 5 wild-type marker from ALHF (Fig. 6.6C). The PCR products were further sequenced and revealed that they were *CYP6A5v2* fragments. This study demonstrates that *CYP6A5v2* is located on autosome 5. Given that *CYP6A5v2* is overexpressed in ALHF, specifically in abdomen tissue, and that *CYP6A5v2* is located on autosome 5, which is correlated with the linkage of P450-mediated resistance in ALHF (Liu and Yue 2001), it suggests that the overexpression of *CYP6A5v2* plays an important role in insecticide resistance in ALHF house flies. *CYP6A5,* on the other hand, has also been linked to autosome 5 (Cohen and Feyereisen, 1995). However, the lack of a significant difference in the expression of *CYP6A5* between ALHF and CS house flies indicates the reduced importance of *CYP6A5* in resistance in ALHF compared with that of *CYP6A5v2*.

6.4 Conclusion

In the current study, we have characterized the expression of *CYP6A5* and *CYP6A5v2* from a pyrethroid resistant house fly strain, ALHF. Our study provides the first direct evidence that two sequence-closely-related *CYP6A5* genes are differentially expressed in insecticide resistant house flies, suggesting the dissimilar functions of these two genes in a resistant organism. Taken together with the significant constitutive overexpression of *CYP6A5v2* only in resistant house flies, the expression of the gene in detoxification-related specific tissue, and the correlation of the linkage of the gene with that of P450-mediated resistance in ALHF, this study strongly suggests the functional importance of *CYP6A5v2* in the increased detoxification of insecticides in ALHF. It has been proposed that the cytochrome P450 superfamily is likely evolved through extensive duplication and differentiation (Gonzalez and Nebert 1990; Hung et al., 1996; Ranson et al., 2002) and P450 genes, which share high similarity and are originally attributed to allelic variation, may represent recently diverged P450 genes (Ranson et al., 2002). It has been also proposed that the simplest way for an enzyme to gain new functions is to duplicate the entire genes and differentiate them into other genes through nucleotide substitution (Ohta, 1991). Accordingly, the significant difference in the expression of *CYP6A5* and *CYP6A5v2* in the resistant house flies identified in the current study suggests that these two genes may be of recently diverged P450 genes through gene duplication with different functions.

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FIGURE LEGENDS

Figure 6.1. The cDNA/deduced protein sequences of *CYP6Av2* in ALHF and aabys. Heme binding motif that is highly conserved in all P450 proteins is doubly underlined. Nucleotide polymorphisms between ALHF and aabys are highlighted. The primers used for generating the *CYP6Av2* cDNA fragments, qRT-PCR reactions, and ASPCR are indicated by arrowheads.

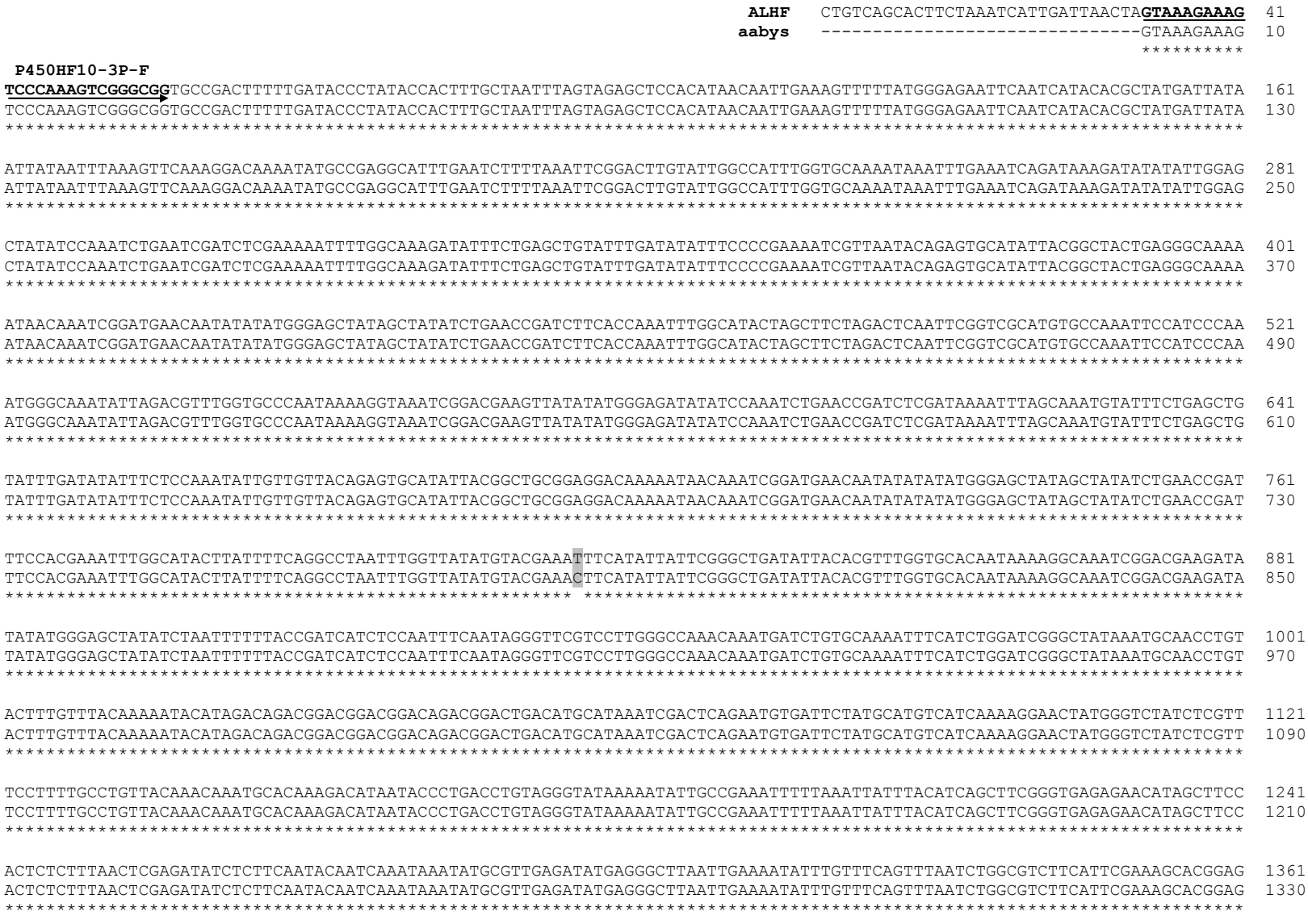
Figure 6.2. Graphic representation of *CYP6A5v2* and *CYP6A5*, showing locations and sequences of each gene specific primer pair for qRT-PCR. A: The locations and sequences of *CYP6A5v2* allele-specific primer pair in *CYP6A5v2*. **The sequences of *CYP6A5* at the corresponding sets of the *CYP6A5v2* allele-specific primer pair. B: The locations and sequences of *CYP6A5* allele-specific primer pair in *CYP6A5*. **The sequences of *CYP6A5v2* at the corresponding sets of the *CYP6A5* allele-specific primer pair.

Figure 6.3. Alignment of the deduced amino acid sequences of CYP6A5 and CYP6A5v2. All identical amino acid residues are shaded. Five important consensus motifs are framed, including motif I (WxxR motif), motif II (GxE/DTT/S motif), motif III (ExLR motif), motif IV (PxxFxPE/DRF motif), motif V (heme binding motif). A conserved 17- residue sequence for family 6 is underlined.

Figure 6.4. Expression analysis of *CYP6A5* and *CYP6A5v2* in CS and ALHF house flies. The expression of the two genes was analyzed by qRT-PCR as described in the materials and methods. A: Relative expression of *CYP6A5* in susceptible CS and resistant ALHF house flies. B. Relative expression of *CYP6A5v2* in CS and ALHF house flies. The relative levels of gene expression are shown as a ratio in comparison with that in CS flies. The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of P450 gene expression among the samples with the same alphabetic letter.

Figure 6.5. Relative expression of *CYP6A5v2* in head+thorax and abdomen tissues of CS and ALHF adult house flies. The expression of these two genes was analyzed by qRT-PCR as described in the materials and methods. The relative levels of gene expression are shown as a ratio in comparison with that in CS flies. The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of P450 gene expression among the samples with the same alphabetic letter.

Figure 6.6. Genetic linkage analysis of *CYP6A5v2*. A: Graphic representation of *CYP6A5v2*, showing locations and sequences of ALHF allele-specific primers. B: PCR fragments generated using the *CYP6A5v2* allele-independent primer set. C: PCR fragments generated using the *CYP6A5v2* allele-specific primer set. The DNA templates used in the PCR reaction were from the following house flies: ALHF, aabys, and the 5 house fly lines - A2345, A1345, A1245, A1235 and A1234 - generated from reciprocal crosses between ALHF and aabys.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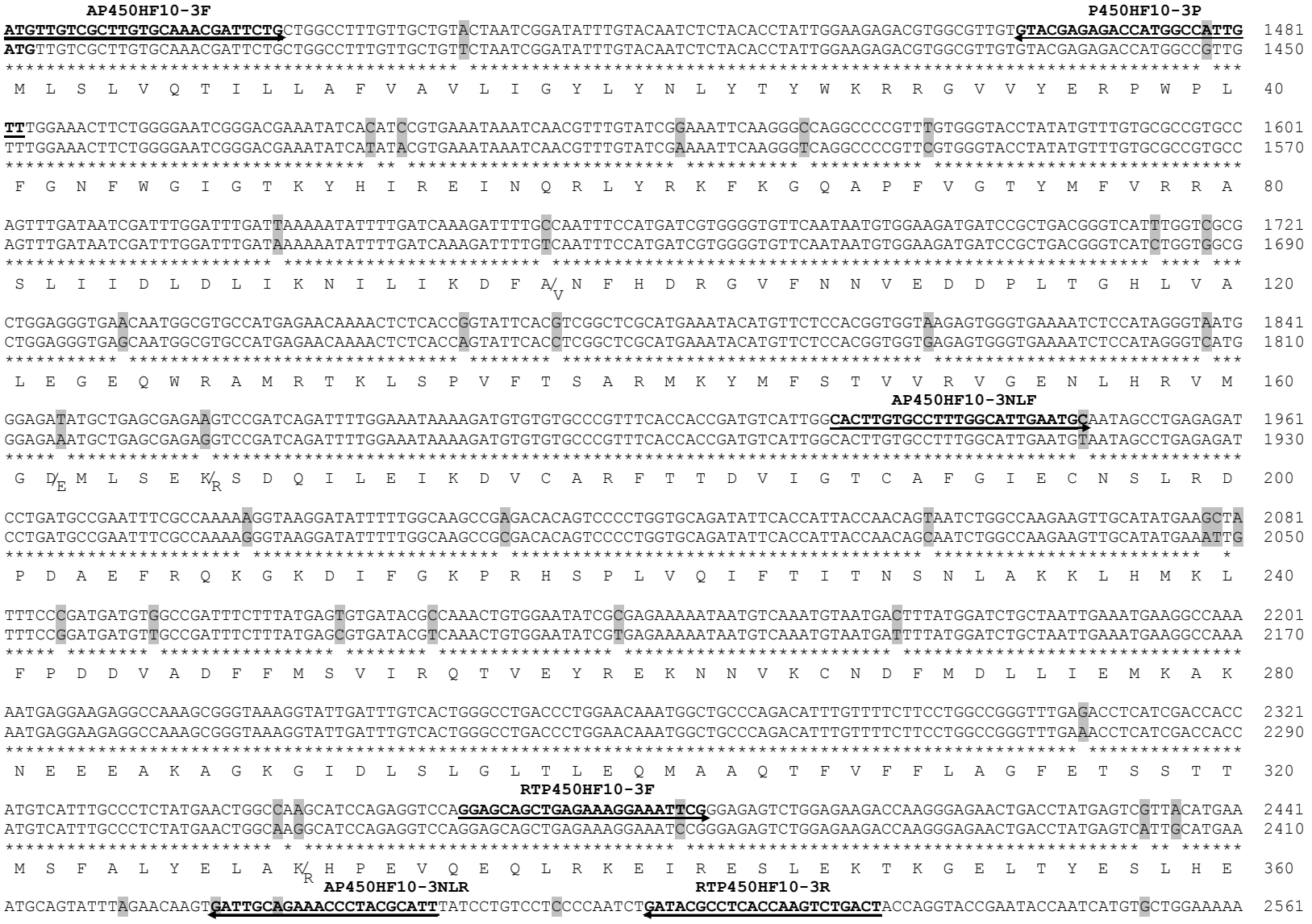
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P450HF10-3P
AP450HF10-3NLF
RTP450HF10-3F
AP450HF10-3NLR
RTP450HF10-3R

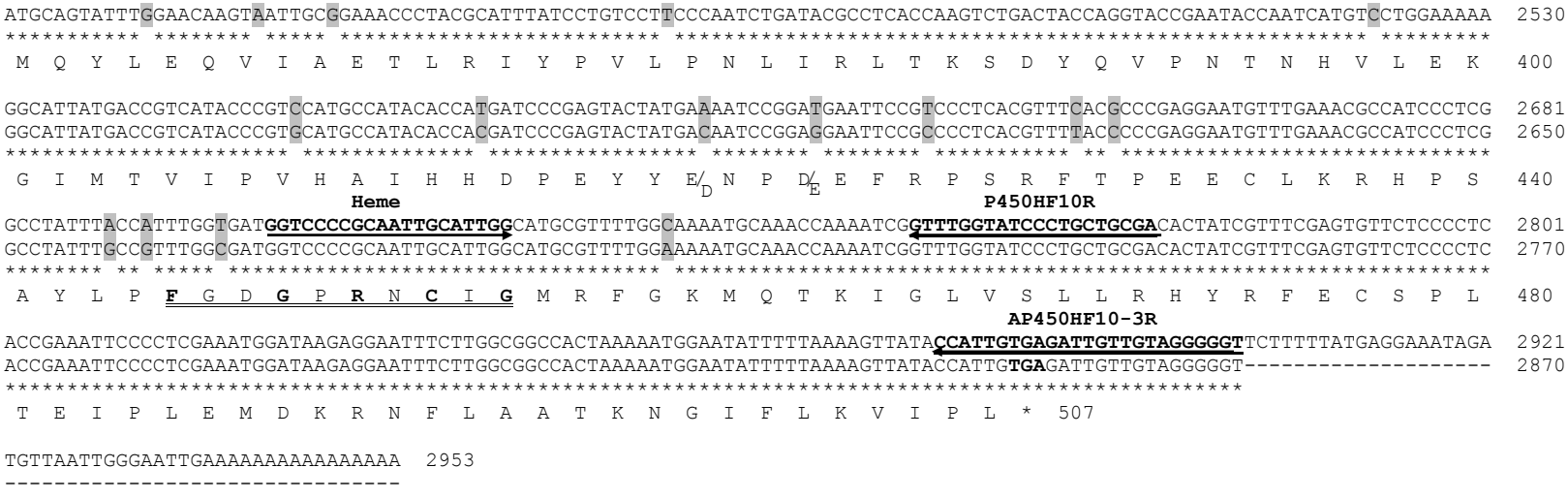

Figure 6.1

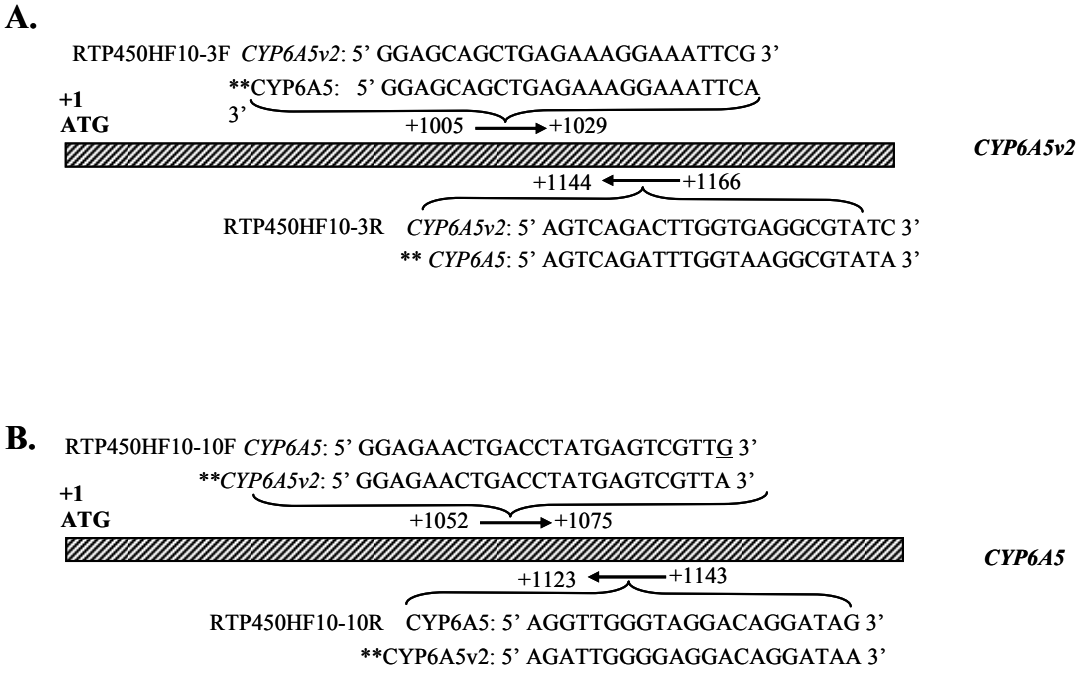
A.
RTP450HF10-3F CYP6A5v2: 5' GGAGCAGCTGAGAAAGGAAATTCG 3'
**CYP6A5: 5' GGAGCAGCTGAGAAAGGAAATTCA
3'
+1
ATG
+1005
+1029
CYP6A5v2
+1144
+1166
RTP450HF10-3R CYP6A5v2: 5' AGTCAGACTTGGTGAGGCGTATC 3'
** CYP6A5: 5' AGTCAGATTTGGTAAGGCGTATA 3'
B.
RTP450HF10-10F CYP6A5: 5' GGAGAACTGACCTATGAGTCGTTG 3'
**CYP6A5v2: 5' GGAGAACTGACCTATGAGTCGTTA 3'
+1
ATG
+1052
+1075
CYP6A5
+1123
+1143
RTP450HF10-10R CYP6A5: 5' AGGTTGGGTAGGACAGGATAG 3'
**CYP6A5v2: 5' AGATTGGGGAGGACAGGATAA 3'

Figure 6.2


Motif I
CYP6A5 MLSLVQTILL AFVAVLIGYL YNIYTYWKRR GVVYERPWPL FGNFWGIGTK 50
CYP6A5v2 MLSLVQTILL AFVAVLIGYL YNLYTYWKRR GVVYERPWPL FGNFWGIGTK 50
CYP6A5 YHIREINQRL YRKFKGQAPF VGTYMFVRRA SLIIDLDLIK NILIKDFANF 100
CYP6A5v2 YHIREINQRL YRKFKGQAPF VGTYMFVRRA SLIIDLDLIK NILIKDFANF 100
CYP6A5 HDRGVFNNVE DDPLTGHLVA LEGEQWRAMR TKLSPVFTSA RMKYMFSTVV 150
CYP6A5v2 HDRGVFNNVE DDPLTGHLVA LEGEQWRAMR TKLSPVFTSA RMKYMFSTVV 150
CYP6A5 RVGENLHRVM GEMLSERSDQ ILEIKDVCAR FTTDVIGTCA FGIECNSLRD 200
CYP6A5v2 RVGENLHRVM GDMLSEKSDQ ILEIKDVCAR FTTDVIGTCA FGIECNSLRD 200
CYP6A5 PDAEFRQKGK DIFGKPRHSP LVQIFTITNS NLAKKLHMKL FPDDVADFFM 250
CYP6A5v2 PDAEFRQKGK DIFGKPRHSP LVQIFTITNS NLAKKLHMKL FPDDVADFFM 250
CYP6A5 SVIRQTVEYR QKNNVKCNDF MDLLIEMKAK NEEEAKAGKG IDLSLGLTLE 300
CYP6A5v2 SVIRQTVEYR EKNNVKCNDF MDLLIEMKAK NEEEAKAGKG IDLSLGLTLE 300
Motif II
Motif III
CYP6A5 QMAAQTFVFF LAGFETSSTT MSFALYELAK HPEVQEQLRK EIRESLEKTK 350
CYP6A5v2 QMAAQTFVFF LAGFETSSTT MSFALYELAK HPEVQEQLRK EIRESLEKTK 350
CYP6A5 GELTYESLHE MQYLEQVIAE TLRIYPVLPN LIRLTKSDYQ VPNTNHVLEK 400
CYP6A5v2 GELTYESLHE MQYLEQVIAE TLRIYPVLPN LIRLTKSDYQ VPNTNHVLEK 400
Motif IV
Motif V
CYP6A5 GIMTVIPVHA IHHDPEYYDN PEEFRPSRFT PEECLKRHPS AYLPFGDGPR 450
CYP6A5v2 GIMTVIPVHA IHHDPEYYEN PDEFRPSRFT PEECLKRHPS AYLPFGDGPR 450
CYP6A5 NCIGMRFGKM QTKIGLVSLL RHYRFECSPL TEIPLEMDKR NFLAATKNGI 500
CYP6A5v2 NCIGMRFGKM QTKIGLVSLL RHYRFECSPL TEIPLEMDKR NFLAATKNGI 500
CYP6A5 FLKVIPL 507
CYP6A5v2 FLKVIPL 507

Figure 6.3

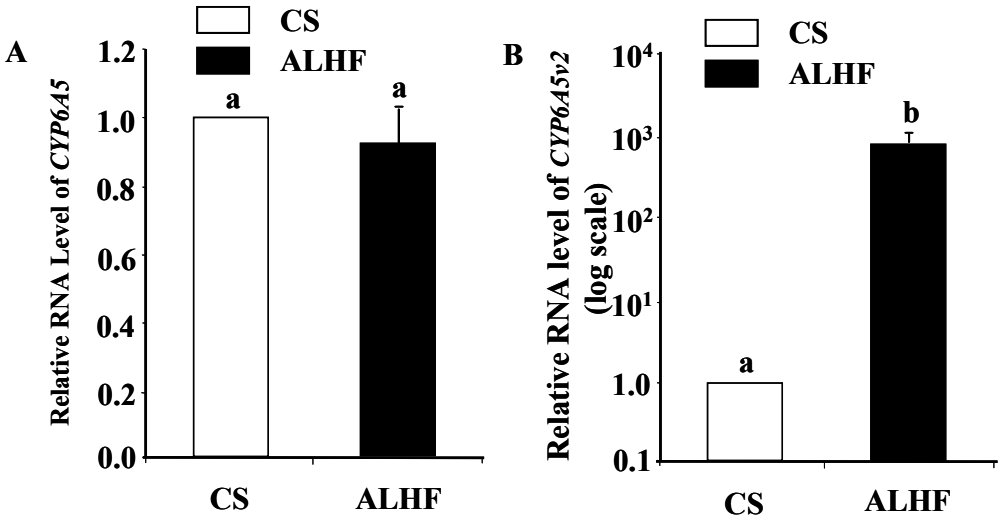

Figure 6.4

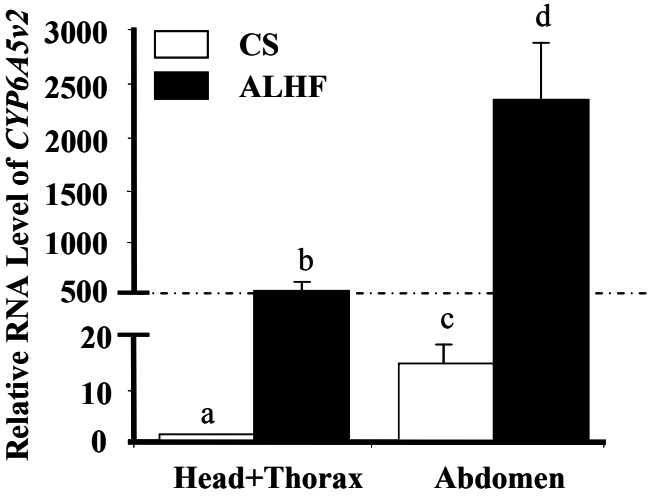

Figure 6.5

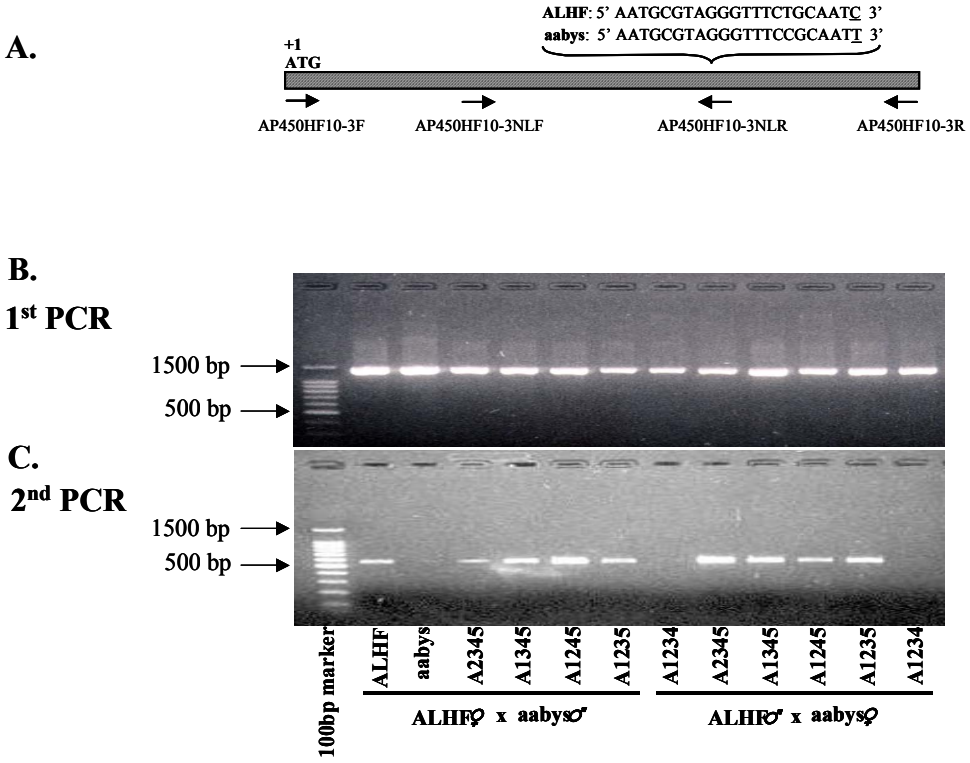
A.
ALHF: 5' AATGCGTAGGGTTTCTGCAATC 3'
aabys: 5' AATGCGTAGGGTTTCCGCAATT 3'
+1
ATG
AP450HF10-3F
AP450HF10-3NLF
AP450HF10-3NLR
AP450HF10-3R
B.
1st PCR
1500 bp
500 bp
C.
2nd PCR
1500 bp
500 bp
100bp marker
ALHF
aabys
A2345
A1345
A1245
A1235
A1234
A2345
A1345
A1245
A1235
A1234
ALHF♀ x aabys♂
ALHF♂ x aabys♀

Figure 6.6

CHAPTER 7: CYTOCHROME P450 PSEUDOGENES AND INSECTICIDE RESISTANCE IN THE HOUSE FLY

7.1 Introduction

Cytochrome P450s compose one of the largest gene superfamilies found in all living organisms examined (Feyereisen, 2005). The diversity of cytochrome P450 genes in various organisms and within a single species mirrors the ancient origin of the P450 superfamily (Cohen and Feyereisen, 1995). It has been proposed that all current P450 genes descended from a common ancestral P450 gene evolved more than 2 billion years ago (Nebert et al., 1989). The formation of the considerable number of P450 genes is due to gene duplication and adaptive diversification (Gotoh, 1993) that are accelerated either by the removal of selective constraints or by positive selection for new functions (Feyereisen, 1999). Cytochrome P450s play important roles in the interaction of insects and their environment (Schuler, 1996; Feyereisen, 2005). The biosynthetic repertoire of plants, fungi, and bacteria has been a strong selective force driving the diversification of insect P450s during hundreds of millions of years' evolution (Cohen and Feyereisen, 1995). Another selective force, synthetic insecticides, became even much stronger especially to the insects important in public health and agriculture in the last 60 years. A multitude of factors related to insecticide resistance was selected and maintained in the

resistant strains during the processes of P450 evolution (Liu et al., 2007), and these factors may harbor all of the mechanisms involved in the insecticide resistance.

A pseudogene is a DNA sequence that resembles one or more paralogous functional genes, but does not produce a functional, full-length protein (Mighell et al., 2000). The lack of function in pseudogenes is due to either failure of transcription or translation or both (Wilde, 1986). Pseudogenes are the consequence of gene duplication occurring in two main ways, retrotransposition and duplication of genomic DNA. The pseudogenes arising by retrotransposition are also described as processed pseudogenes that lack both 5'-promoter sequence and introns (Mighell et al., 2000; Gerstein and Zheng, 2006). Duplication of genomic DNA segments is an essential step in the development of complex genomes and the generation of gene families descended from a common ancestral gene (Mighell et al., 2000), such as the cytochrome P450 gene superfamily. Pseudogenes commonly exist in various organisms. For example, the human genome is estimated to include more than 20,000 pseudogenes (Harrison et al., 2002), and approximately 100 pseudogenes were identified from *Drosophila melanogaster* (Harrison et al., 2003). However, the biological functions of pseudogenes remain largely unknown.

Up to now, several insect P450 pseudogenes were identified from *Drosophila melanogaster* (Adams et al., 2000) and German cockroaches, *Blattella germanica* (Wen et al., 2001). In the current study, two P450 genes, *CYP4D35* and *CYP4D36*, were isolated from the house fly, *Musca domestica*. The cDNA sequence of CYP4D35 in the resistant house fly strain, ALHF, has an open reading frame (ORF). However, the cDNA sequences of *CYP4D35* in both susceptible strains, CS and aabys, have no ORFs to encode an entire protein, suggesting that this gene is perhaps a pseudogene in both

strains. The cDNA sequence of *CYP4D36*, however, was found to lack the heme binding motif (P450 signature) in all three strains (ALHF, CS, and aabys), indicating that *CYP4D36* may not be a functional gene. The functions of these two P450 genes and the possible relationship between pseudogenes and insecticide resistance will be discussed.

7.2 Materials and methods

7.2.1 House fly strains

Three strains of house flies were used in this study. ALHF is a pyrethroid-resistant strain collected from a poultry farm near Grant, in Marshall County, Alabama, in 1998 (Liu and Yue, 2000; 2001), that was selected with permethrin for 6 generations and then maintained under biannual selection with permethrin. CS and aabys are two insecticide-susceptible strains. Both CS and aabys were obtained from Dr. J. G. Scott (Cornell University).

7.2.2 RNA extraction, cDNA preparation, and P450 cDNA fragment isolation

Total RNAs were extracted from house flies using the acidic guanidine thiocyanate-phenol-chloroform method (Liu and Scott, 1997). mRNA was isolated with oligotex-dT suspension as described by the manufacturer (QIAGEN). Rapid amplification of 3' cDNA ends (3'-RACE) was performed using the Marathon^{TM} cDNA Amplification Kit (Clontech) as described by the manufacturer and Liu and Zhang (2002). The first strand cDNA was synthesized with SuperScript II reverse transcriptase and an antisense 5'-anchored oligo(dT) primer (5' TAATACGACTCACTATAGGGAGATTTTTTTTTTTT TTTT 3') (Tomita and Scott, 1995), using ALHF mRNAs as templates. The first strand

cDNA products were amplified by PCR with the C2 primer (5'TAATACGACTCACTAT AGGGAGA 3') and internal degenerated sense primer Flyc1 (5'GGAAGTNGACACNT TYATGTT 3'), synthesized based on a conserved 13 amino acid region found in rat, human, and insect P450 sequences (Liu and Zhang, 2002). The PCR products were cloned into PCR™ 2.1 Original TA cloning vector (Invitrogen). The sequences of cDNA fragments obtained from the TA clones were verified by automated sequencing (Genomic and Sequencing Lab, Auburn University). Sequence analyses of the P450 gene fragments were repeated at least three times with different preparations of mRNAs, and three TA clones from each replication were verified by sequencing.

7.2.3 Cloning the 5' half and the full length of the putative P450 cDNAs

To clone the 5' half of the putative P450 gene fragments, 5'-RACE was carried out using the Marathon™ cDNA Amplification Kit (Clontech) as described by the manufacturer. The first strand cDNAs were synthesized with SuperScript II reverse transcriptase using ALHF mRNAs as templates. The double strand cDNA was synthesized following the protocol described by the manufacturer (Clontech). Adaptors were ligated to both ends of the double strand cDNA using T4 DNA ligase as described by the manufacturer. The double strand cDNAs were amplified by PCR with the primer pair, P450HF12R/AP1 (AP1 is based on the sequence of the adaptor) and P450HF12'R/AP1. P450HF12R (Fig. 7.1) and P450HF12'R (Fig. 7.2) were generated based on sequences of the 3' ends of the putative P450 cDNA fragments. The PCR products were cloned into PCR™ 2.1 Original TA cloning vector (Invitrogen) and sequenced. The full lengths of the putative P450 cDNAs were generated by RT-PCR

using the specific primer pair synthesized based on the 5'and 3'end sequences of the putative P450 genes. The PCR products were cloned and sequenced. Cloning and sequence analyses of the P450 cDNA fragment were repeated at least three times with different preparations of mRNAs, and three TA clones from each replication were verified by sequencing.

7.2.4 Northern blotting analysis

Northern blotting analyses were performed according to Sambrook et al. (1989). Twenty micrograms of total RNA from each sample were fractionated on 1% formaldehyde denaturing agarose gel containing ethidium bromide and transferred to Nytran membranes (Schleicher and Schuell) as described by Sambrook et al. (1989). The P450 cDNA fragments were labeled with [α-^{32}P] dCTP using a Primer-It II Random Primer Labeling kit (Stratagene) and hybridized with RNA blots using QuickHyb solution (Stratagene). The amount of RNA loaded in each lane was standardized by comparing the density of the 18S ribosomal RNA band on agarose gel under UV light before transferring (Spiess and Ivell, 1998). All Northern blot analyses were repeated three times with different preparations of RNA samples. The radiographic signal intensity was quantitatively analyzed by QuantiScan v3.0 (Biosoft) as done previously (Liu and Zhang, 2004). Statistical significance of the gene expressions was calculated using a Student's *t*-test for all 2-sample comparisons and a one-way analysis of variance (ANOVA) for multiple sample comparisons (SAS v9.1 software); a value of $P \leq 0.05$ was considered statistically significant.

7.3 Results

7.3.1 Cloning and sequencing of P450 genes from house flies

Two distinct partial putative ALHF P450 cDNA fragments were amplified by RT-PCR with 3' RACE using a 5'-anchored oligo(dT) primer and one internal degenerate primer, Flyc1 (based on a conserved sequence found in rat, human, and insect P450s) (Liu and Zhang, 2002; Fig. 7.1). BLAST analysis of the amino acid sequences predicted from the putative P450 cDNAs showed that the sequence encoded the C-terminal ends of putative P450s with 50-60% identity to the CYP4 family. To isolate and amplify the 5' ends of 2 putative P450 genes, we conducted a 5' RACE reaction using primer pairs of P450HF12R/AP1 (Fig. 7.1). The 5' RACE reaction amplified two cDNA fragments that overlapped with their corresponding 3' putative P450 cDNA fragments, indicating that they were the 5' ends of the putative P450 genes. An entire cDNA fragment for each of the putative P450 genes was subsequently amplified from ALHF, CS, and aabys house flies by PCR using the primer pairs, AP450HF12F-3/AP450HF12R-3 and AP450HF12'F-1/AP450HF12'R-1 (Figs. 7.1 and 7.2), synthesized based on the respective 5' and 3' end sequences of the putative P450 genes. The sequences of the entire cDNA fragments perfectly overlapped with the 3' and 5' sequences of the putative P450 genes generated by 3' and 5' RACE. The sequences were named *CYP4D35* and *CYP4D36* (accession numbers: DQ642007, DQ642008, respectively) by the P450 nomenclature committee (Dr. D. Nelson, personal communication).

7.3.2 Structural analysis of P450s from house flies

The cDNA sequences of *CYP4D35* and *CYP4D36* in ALHF have ORFs of 1518 and 1212 nucleotides encoding proteins of 506 and 404 residues, respectively (Figs. 7.1 and 7.2). The putative protein sequences of CYP4D35 and CYP4D36 shared 50% of their identities with *Drosophila melanogaster* CYP4D8 (Fig. 7.3). However, CYP4D36 may have skipped the last exon because the putative stop codon appeared at the intron boundary before the last exon (Dr. D. Nelson, personal communication). The P450 protein signature (heme binding) motif, FXXGXRXCXG (Feyereisen, 1999), is present at amino acid residues 445-454 of CYP4D35. The cysteine residue in this motif is known to be an important ligand for heme binding. However, because of the lack of the last exon sequences in CYP4D36, the heme binding motif, which is located in the last exon, is missing in CYP4D36 in all three strains, ALHF, CS, and aabys. These results suggest that CYP4D36 may not be a functional gene. The other important motif, YXXAXXXEXXR, which is known as the conserved region of P450 sequences and coincides with Helix K in P450cam (Gotoh and Fujii-Kuriyama, 1989), is present at amino acid residues 362-372 and 370-380 of CYP4D35 and CYP4D36, respectively, with one amino acid residue change from alanine to cysteine at positions 365 and 373, respectively (Fig. 7.3). A typical aromatic sequence of P450s with 3 aromatic (A) residues and 2 highly conserved proline residues, $A_1XXPXXA_2XPXBA_3$ (Gotoh and Fujii-Kuriyama, 1989), is present at amino acid residues 420-431 of CYP4D35. However, the third aromatic residue (A_3), phenylalanine, which is a characteristic residue within most cytochrome P450s (Gotoh and Fujii-Kuriyama, 1989), is changed to histidine in CYP4D35, as well as in CYP4D8 (Fig. 7.3). A similar modification has also been identified in CYP4G19 (Pridgeon et al.,

2003). An important characteristic of family 4 that is not shared by other P450s is a conserved 13-residue sequence, EVDTFMFEGHDTT (Bradfield et al., 1991; Fig. 7.3). This motif is perfectly present for amino acid residues 310-322 of CYP4D35 with one amino acid change, from glutamic acid to aspartic acid at position 312. It is also present at amino acid residues 311-323 of CYP4D36 with two amino acid changes, from phenylalanine to serine at position 317 and from glutamic acid to alanine at position 318 (Fig. 7.3). Finally, a very interesting and important finding is that cDNA sequences of CYP4D35 in both susceptible CS and aabys house flies do not obtain an ORF to encode an entire protein. In contrast, the cDNA sequence of CYP4D35 in resistant ALHF has an open reading frame that encodes an entire P450 protein. These results suggest that functional CYP4D35 in ALHF may play an important role in insecticide resistance.

7.3.3 Developmental expression of CYP4D35 and CYP4D36 in house flies

Diversity in the developmental expression and regulation of insect P450s is well established. It was thus of interest to determine the expression patterns of *CYP4D35* and *CYP4D36* during a house fly's life cycle. Northern blot analysis was performed to compare expression levels of these 2 P450 genes for four developmental stages (1^{st}+2^{nd} instar, 3^{rd} instar, pupa, and adult) of both the CS and ALHF strains. The expression of *CYP4D36* was not detected in the 1^{st}+2^{nd} instars, was detected in the 3^{rd} instar, not detected in the pupa, and then rose to a maximum in the adult of CS and ALHF (Fig. 7.4A, B). There is no significant difference in the expression of *CYP4D36* detected between CS and ALHF. *CYP4D35* was expressed at very low levels in the 1^{st}+2^{nd} instars, increased in the 3^{rd} instar, declined dramatically in the pupa stage, and then reached its

highest level in both CS and ALHF adults with a significant overexpression in ALHF flies (2.8-fold) compared to the CS susceptible flies (Fig. 7.4A, C).

7.3.4 Tissue specific expression of CYP4D35 between ALHF and CS house flies

Insect P450s may also vary as to the tissues where they are expressed in response to physiological and environmental stimulators. In insects, the midgut and fat body tissue have generally been considered to be the primary detoxification organs (Hodgson, 1985) where most insect detoxification P450s are expressed (Scott *et al*., 1998). To investigate whether *CYP4D35* expression was tissue specific, RNAs from the head+thorax and abdomen of both CS and ALHF adults were subjected to Northern blots. The Northern blot analyses revealed that there was no significant difference in the expression of *CYP4D35* in the head+thorax and abdomen tissues of the CS strain (Fig. 7.5A, B). However, in the ALHF strain, although the level of *CYP4D35* RNA in the head+thorax was similar to that in the CS strain, it increased 3.7-fold in the abdomen compared with CS (Fig. 7.5A, B), suggesting the importance of *CYP4D35* in the detoxication of insecticides in resistant ALHF house flies.

7.4 Discussion

In the current study, the detoxification-related tissue specific constitutive overexpression of *CYP4D35* is only detected in the resistant ALHF strain, suggesting the functional importance of this P450 gene in the detoxification of insecticides in ALHF. My previous studies have demonstrated that several other P450 genes (Chapter 4, 5, and 6) are overexpressed and/or induced in the resistant ALHF house fly strain. My current

study further indicated that *CYP4D35* was overexpressed in resistant house flies. Taken together, the studies have strongly indicated that the constitutive overexpression and/or induction of multiple P450s are important factors conferring the high level of resistance in ALHF house flies. These factors have been thought to be selected by natural selection over hundred of millions of years and selected by insecticide in the last 60 years (Cohen and Feyereisen, 1995). However, in the susceptible house fly strains, CS and aabys, without selection force (diversity of natural products and insecticides) in the evolution, all of these factors cannot be detected.

In my study, an important and interesting finding is that the P450 gene *CYP4D35* in CS and aabys has become a pseudogene with many mutations, deletions, and insertions in the sequence that cause no ORF to encode an entire functional protein. Pseudogenes are not uncommon in the vertebrates and even in insects. Nevertheless, my study, for the first time, identified pseudogene that was present in the susceptible house flies but not in resistant house flies, suggesting the functional *CYP4D35* in the resistant house flies plays very important role in detoxification of insecticides and, in turn, in the development of insecticide resistance in resistant house flies. I hypothesize that the presence of non-functional pseudogenes in susceptible house fly strains is the consequence of the lack of selection force because the function of these gene products have become unnecessary in these strains. This phenomenon also occurs in other genes or gene families. For example, one of the largest known gene families in mammals is the olfactory receptor (OR) gene family with more than 1,000 members (Glusman et al., 2001). ORs are the cell-surface proteins that confer the sense of smell. Researchers found that humans lost a large number of functional olfactory receptor genes during evolution. There are fewer than 500

functional olfactory receptor genes in humans' genome. More than 300 human olfactory receptor pseudogenes still have functions in the genome of rats and mice (Gilad et al., 2003; Gerstein and Zheng, 2006). Studies indicated that the accumulation of OR pseudogenes was consistent with a reduction of the sense of smell in humans (Sharon et al., 1999; Rouquier et al., 2000). Another example is the GLO gene (L-gulono-gamma-lactone oxidase) required for synthesizing ascorbic acid. It has been found that guinea pigs and primates lack a functional GLO gene since they could acquire ascorbic acid from their diet and this gene became unnecessary for them (Nishikimi et al., 1994), while most other species have it. Both examples strongly support my hypothesis that susceptible strains may not need too many P450s to metabolize insecticides, to which they are never exposed.

ACKNOWLEDGEMENTS

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FIGURE LEGEND

Figure 7.1. The cDNA/deduced protein sequences of *CYP4D35* in ALHF, CS, and aabys. Invariant and highly conserved motifs in all P450 proteins are doubly underlined and conserved residues are underlined with stars. The primers used for generating the *CYP4D35* cDNA fragments are indicated by arrowheads.

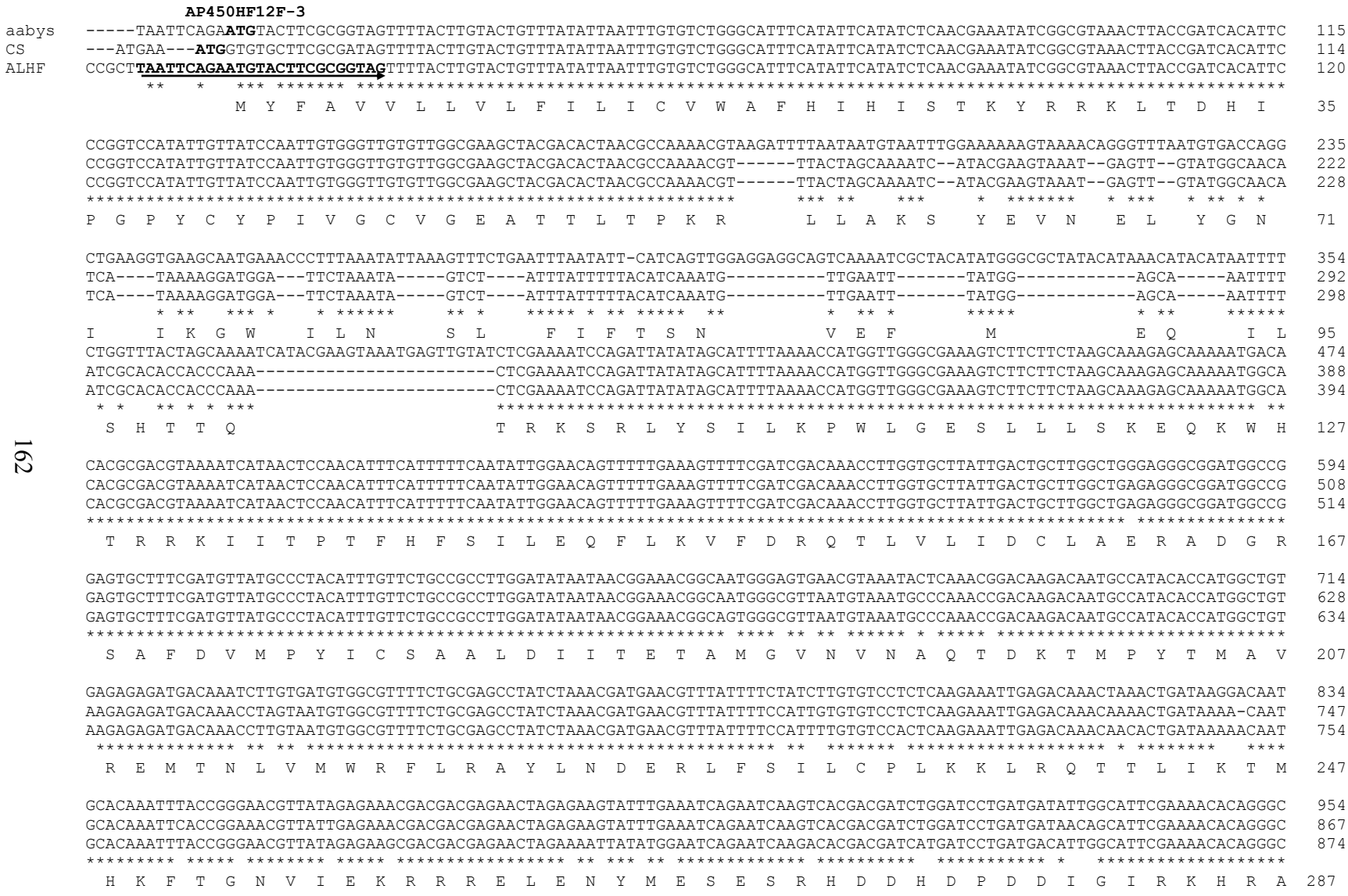
Figure 7.2. The cDNA/deduced protein sequences of *CYP4D36* in ALHF, CS, and aabys. Invariant and highly conserved motifs in all P450 proteins are doubly underlined and conserved residues are underlined with stars. The primers used for generating the *CYP4D36* cDNA fragments are indicated by arrowheads.

Figure 7.3. Alignment of the deduced amino acid sequences of CYP4D35, CYP4D36, and CYP4D8. Conserved amino acid residues among all three and two of the P450 proteins are indicated by boxes (gray) and asterisks, respectively. Invariant and highly conserved motifs in the P450 proteins are underlined.

Figure 7.4. Expression analysis of *CYP4D35* and *CYP4D36* in different life stages of CS and ALHF house flies. A: Northern blot analysis of *CYP4D35* and *CYP4D36* in 1st and 2nd larval instars, 3rd instars, pupae, and adults of CS and ALHF. Blots were hybridized with the cDNA probes of *CYP4D35* and *CYP4D36* fragments. The ethidium bromide stain of 18S ribosomal RNA in agarose gel is shown at the bottom. B: Relative *CYP4D36* RNA levels for different life stages of house flies. C. Relative *CYP4D35* RNA levels for

different life stages of house flies. The blots from three independent experiments were scanned. The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of P450 expression among the samples with the same alphabetic letter (i.e., a, b, or c).

Figure 7.5. Expression of *CYP4D35* in head+thorax and abdomen tissue of adult house flies. A: Northern blot analysis of *CYP4D35* in head+thorax and abdomen tissue of CS and ALHF. Blots were hybridized with the cDNA probes of *CYP4D35* fragment. The ethidium bromide stain of 18S ribosomal RNA in agarose gel is shown at the bottom. B: Relative *CYP4D35* RNA levels in head+thorax and abdomen tissue. The blots from three independent experiments were scanned. The results are shown as the mean ± S.E. There was no significant difference ($P \leq 0.05$) in the levels of P450 expression among the samples with the same alphabetic letter (i.e., a, b, or c).


AP450HF12F-3
aabys
CS
ALHF

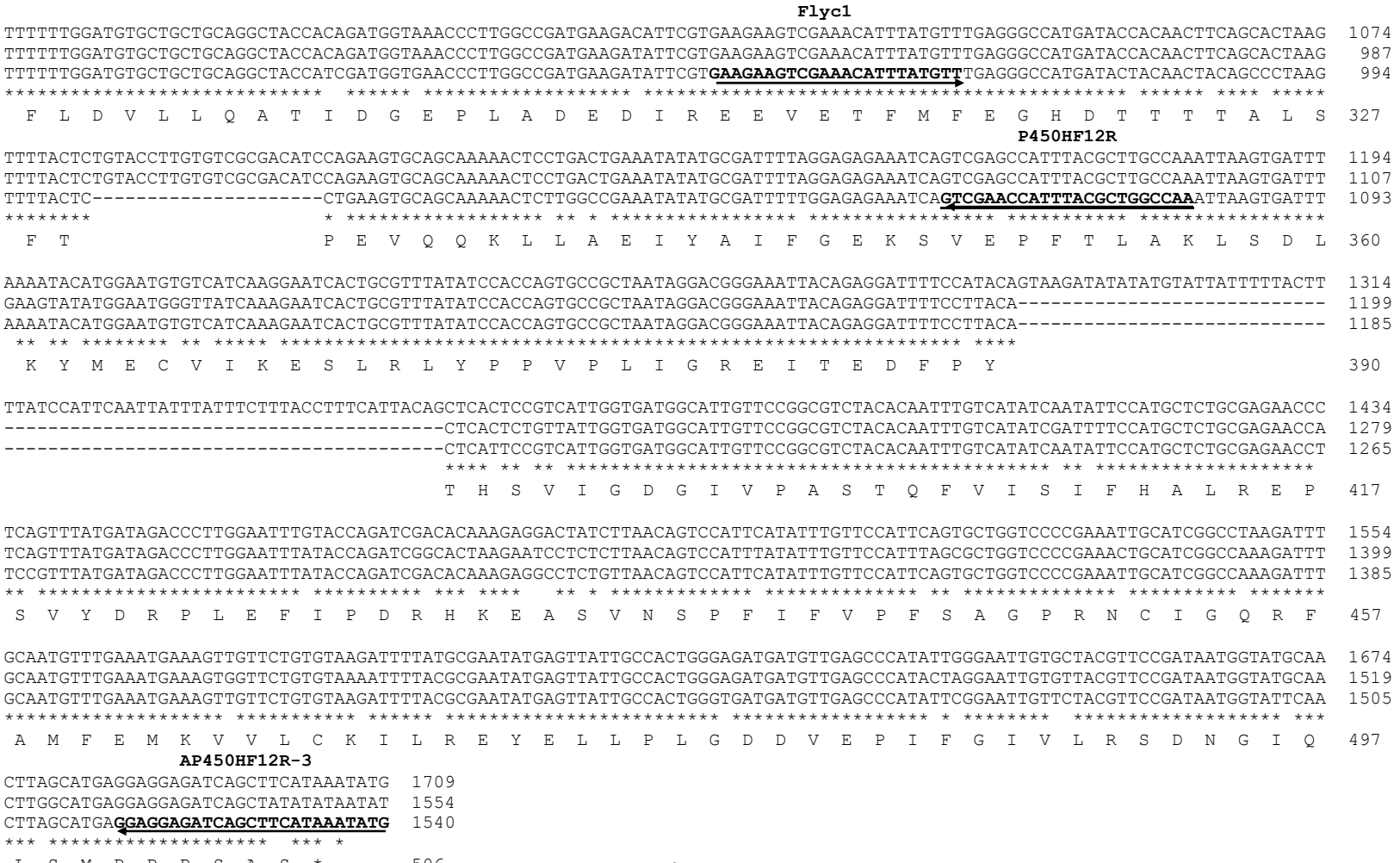

Flyc1
GAAGAAGTCGAAACATTTATGTT
P450HF12R
GTCGAACCATTTACGCTGGCCAA
AP450HF12R-3
GGAGGAGATCAGCTTCATAAATATG

Figure 7.1

```
                                                                                              ALHF    ATGAA   5
                                                                                              aabys   ATGAA   5
                                                                                              CS      ATGAA   5
       AP450HF12'F-1
ALHF   ATGGTGTGCTTCGCGATAGTTTTACTTGTGCTTTTCATAGCGATTTGTGTATGGGCATTACAAATTCACATCTCTACAAAATATCGCCGCAAACTTACCGATCGTATTCCCGGTCCATTT  125
aabys  ATGGTGTGCTTCGCGATAGTTTTACTTGTGCTTTTCATAGCGATTTGTGTATGGGCACTACAAATTCACATCTCTACAAAATATCGCCGCAAACTTACCGATCATATTCCCGGTCCATTT  125
CS     ATGGTGTGCTTCGCGATAGTTTTACTTGTGCTTTTCATAGCGATTTGTGTATGGGCATTACAAATTCACATCTCTACAAAATATCGCCGCAAACTTACCGATCGTATTCCCGGTCCATTT  125
       ********************************************************* ********************************************* ****************
        M  V  C  F  A  I  V  L  L  V  L  F  I  A  I  C  V  W  A  L  Q  I  H  I  S  T  K  Y  R  R  K  L  T  D R/H I  P  G  P  F

       TGTTATCCCATATTGGGTTGTGTGGGTGAAGCTACTACTCTAACGCCCAAACGTTTAATATCAAAATCTTTCGAGATGTATGAATTGTACGGCCACACCATAAAAGTATGGATATTAGAC  245
       TGTTATCCCATATTGGGTTGTGTGGGTGAAGCTACAACTCTAACGCCCAAACGTTTAATATCAAAATCTTTCGAGATGTATGAATTGTACGGCCACACCATAAAAGTATGGATATTAGAC  245
       TGTTATCCCATATTGGGTTGTGTGGGTGAAGCTACTACTCTAACGCCCAAACGTTTAATATCAAAATCTTTCGAGATGTATGAATTGTACGGCCACACCATAAAAGTATGGATATTAGAC  245
       *********************************** ************************************************************************************
        C  Y  P  I  L  G  C  V  G  E  A  T  T  L  T  P  K  R  L  I  S  K  S  F  E  M  Y  E  L  Y  G  H  T  I  K  V  W  I  L  D

       AAGCTGTTTATATTAACATCAAATGCCGATTTTATGGAGCAAATCTTATCGCATCCCACACAAACACGAAAAATTAGATTATATAACATCATAAAACCATGGGTGGGCGAGGGTCTTCTA  365
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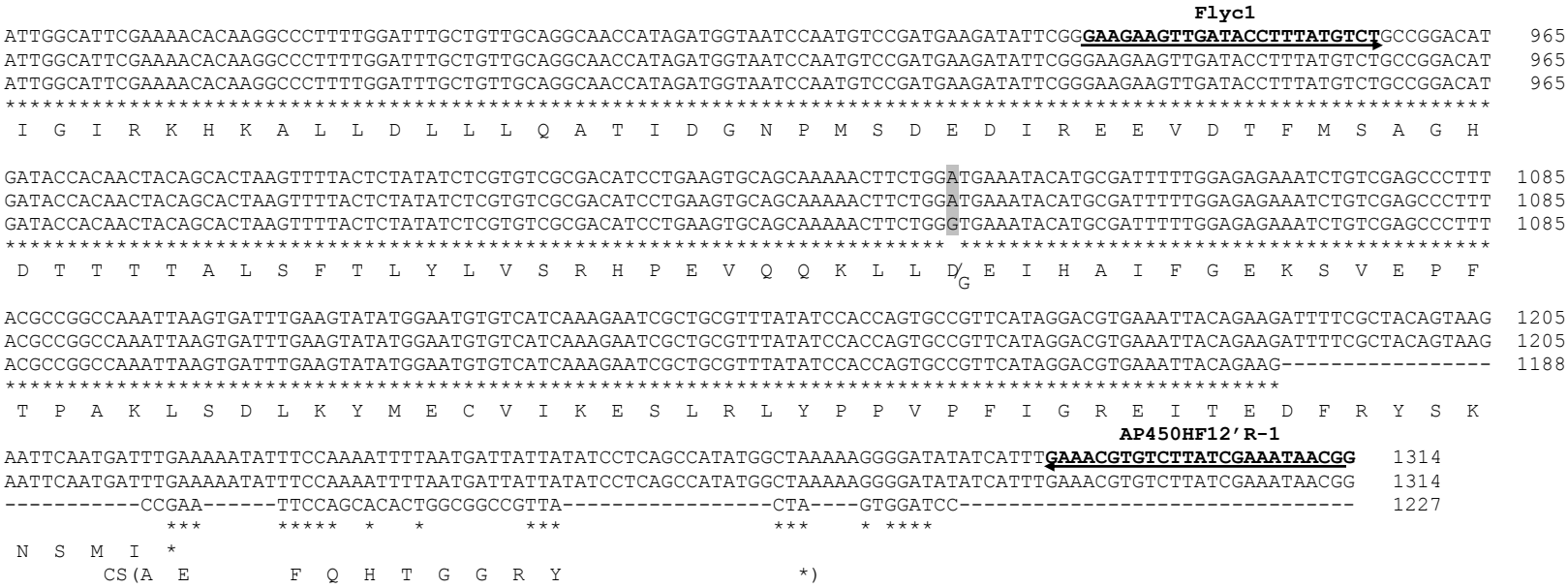

Figure 7.2

```
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CYP4D36    MVCFAIVLLV LLIAICVWAL QIHISTKYRR KLTDHIPGPF CYPILGCVGE  50
CYP4D8     .......... .......... .......... .......... .......MQL   3
              ** **** * * *****   ********* *********  **** *****

CYP4D35    ATTLTPKRLL AKSYEVNELY GNIIKGWILN SLFIFTSNVE FMEQILSHTT  99
CYP4D36    ATTLTPKRLI SKSFEMYELY GHTIKVWILD KLFIFTLNAD FMEQILAHPT  100
CYP4D8     MLRLNPKTFI KVGREYVLKF GHLQRVWIFN RLLIMSGDAE LNEQLLSSQE  53
           *** *  ***  **    ***  * ***  **   * ** *** **  * ** *

CYP4D35    QTRKSRLYSI LKPWLGESLL LSKEQKWHTR RKIITPTFHF SILEQFLKVF  149
CYP4D36    QTRKIRLYNI IKPWLGEGLL LSGEQKWYTR RKIITPAFHF SILEKFLEVF  150
CYP4D8     HLVKHPVYKV LGQWLGNGLL LSDGKVWHQR RKIITPTFHF SILEQFVEVF  103
           ***  **  * ***   **      *** **        *        * **

CYP4D35    DRQTLVLIDC LAERADGRSA FDVMPYICSA ALDIITETAM GVNVNAQTDK  199
CYP4D36    DRQTSVLIDC LAERADGKTA FDVMPYICSA ALDIITETAM GVNVNAQTDK  200
CYP4D8     DQQSNICVQR LAQKANG.NT FDVYRSICAA ALDIIAETAM GTKIYAQANE  152
            * * *****   ** *   *    ***  *       *      ****  ***

CYP4D35    TMPYTMAVRE MTNLVMWRFL RAYLNDERLF SILCPLKKLR QTTLIKTMHK  249
CYP4D36    TMPYTMAVRE MSSLITWRLV RAYLHDEWLF SMLYPLKKLR QTKLITTMHK  250
CYP4D8     STPYAEAVNE CTALLSWRFM SVYLQVELLF TLTHPHLKWR QTQLIRTMQE  202
           **  **  *  **      *  **   *     * *  * *          **

CYP4D35    FTGNVIEKRR RELENYMESE SRHDDHDPDD IGIRKHRAFL DVLLQATIDG  299
CYP4D36    FTRNVIEKRR RDLEKYIKSD INMENHDPDN IGIRKHRALL DLLLQATIDG  300
CYP4D8     FTIKVIEKRR QALE...DQQ SKLMDTADED VGSKRRMALL DVLLMSTVDG  249
              *       *       *  *   ****** * ***** *   *  ** *

CYP4D35    EPLADEDIRE EVETFMFEGH DTTTTALSFT P.......EV QQKLLAEIYA  342
CYP4D36    NPMSDEDIRE EVDTFMSAGH DTTTTALSFT LYLVSRHPEV QQKPLAEIYA  350
CYP4D8     RPLTNDEIRE EVDTFMFEGH DTTTSALSFC LHELSRHPEV QAKMLEEIVQ  299
             * ***      *   **       *    * *   ****    *   *  **

CYP4D35    IFGEKSVEPF TLAKLSDLKY MECVIKESLR LYPPVPLIGR EITEDFPYTH  392
CYP4D36    IFGEKSVEPF TLAKLSDLKY MECVIKESLR LYPPVPFIGR EITEDFRYSK  400
CYP4D8     VLGTDRSRPV SIRDLGELKY MECVIKESLR MYPPVPIVGR KLQTDFKYTH  349
           ** ***** * **** **               *      *   ****    **

CYP4D35    SVIGDGIVPA STQFVISIFH ALREPSVYDR PLEFIPDRHK EASVNSPFIF  442
CYP4D36    NSMI...... .......... .......... .......... ..........  404
CYP4D8     SVHGDGVIPA GSEIIIGIFG VHRQPETFPN PDEFIPERHE NGSRVAPFKM  399
           ** ***  **      * **    * *      * **** **    *   **

CYP4D35    VPFSAGPRNC IGQRFAMFEM KVVLCKILRE YELLPLGDDV EPIFGIVLRS  492
CYP4D36    .......... .......... .......... .......... ..........
CYP4D8     IPFSAGPRNC IGQKFAQLEM KMMLAKIVRE YELLPMGQRV ECIVNIVLRS  449
            ********* *** **  ** *  * ** ** ***** *  * * *  *****

CYP4D35    DNGIQLSMRR RSAS  506
CYP4D36    .......... ....
CYP4D8     ETGFQLGMRK RKHN  463
             * ** **  *
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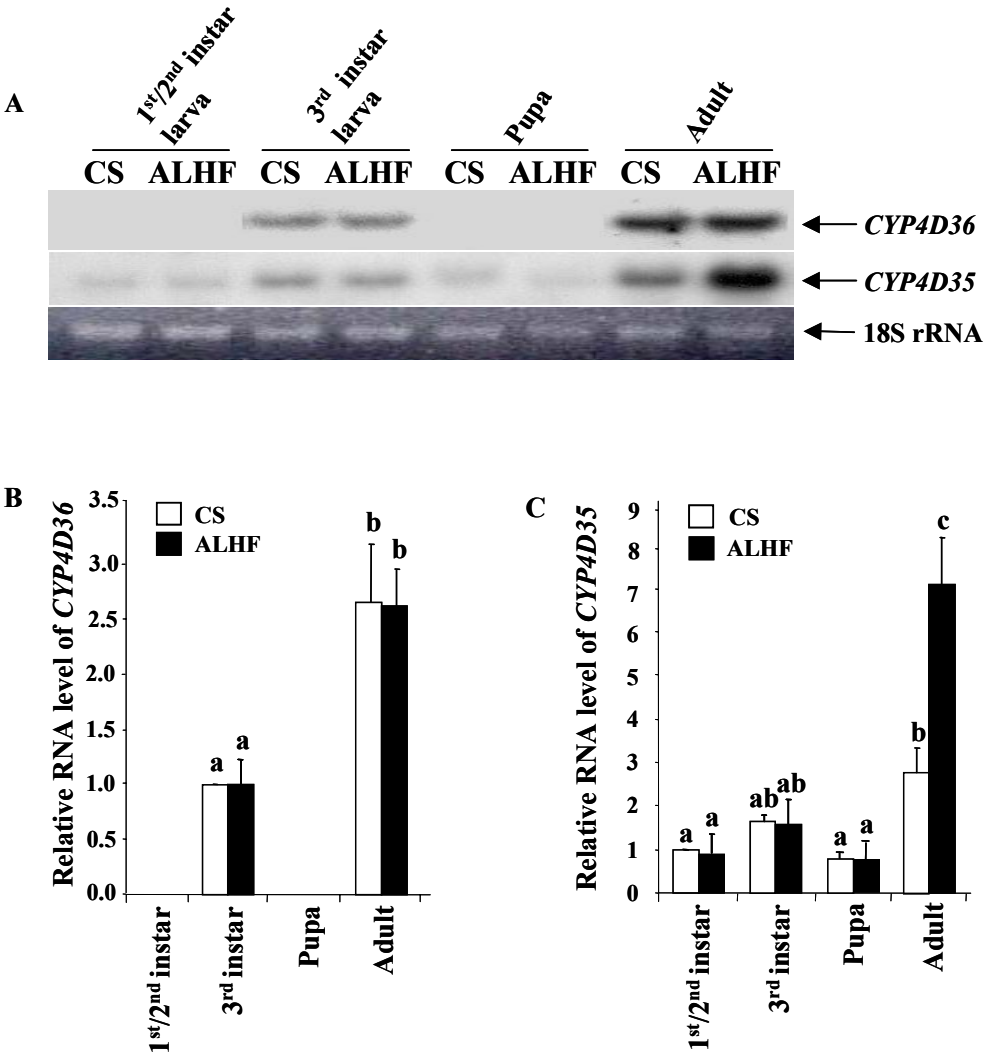
Figure 7.3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1st/2nd instar larva
3rd instar larva
Pupa
Adult
CS ALHF CS ALHF CS ALHF CS ALHF
CYP4D36
CYP4D35
18S rRNA
B
Relative RNA level of CYP4D36
CS
ALHF
1st/2nd instar
3rd instar
Pupa
Adult
C
Relative RNA level of CYP4D35
CS
ALHF
1st/2nd instar
3rd instar
Pupa
Adult

Figure 7.4

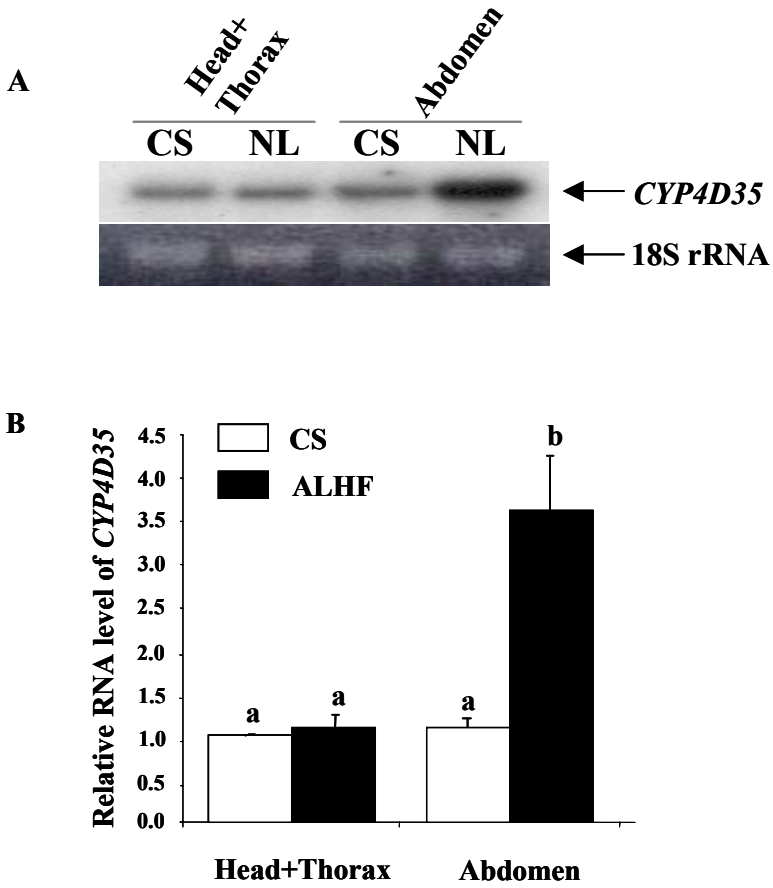

A
Head+ Thorax
Abdomen
CS
NL
CS
NL
CYP4D35
18S rRNA
B
Relative RNA level of CYP4D35
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CS
ALHF
a
a
a
b
Head+Thorax
Abdomen

Figure 7.5

CHAPTER 8: RESEARCH SUMMARY AND FUTURE STUDIES

8.1 Research Summary

My research project was focused on the characterization of cytochrome P450 genes that were involved in the development of insecticide resistance and their expressional regulation in response to insecticide stimulation in the house fly, *Musca domestica*. I cloned 19 new P450 genes from a resistant house fly strain, ALHF, and characterized the spatial and developmental regulation of these genes. I found that 6 of 19 P450 genes were constitutively overexpressed in ALHF compared with CS and 3 of them were induced by permethrin treatment in ALHF house flies. All these constitutively expressed and permethrin induced P450 genes were predominantly expressed in the abdomen tissue of ALHF, in which the primary detoxification organs of insects are located. I also conducted genetic linkage analyses to determine the causal link between these constitutively expressed and permethrin induced P450 genes and insecticide resistance. Five of 6 constitutively overexpressed and/or induced P450 genes were mapped on autosome 5, which is correlated with the linkage of resistance in ALHF. This proposed research is the first comprehensive effort to decipher P450 genes and understand their functions in insecticide resistance of house flies. My study strongly indicates that multiple P450 genes, whose expression is constitutively up-regulated and/or induced by insecticides, are involved in insecticide resistance in ALHF house flies. In addition, my study has

provided a solid framework for future studies on characterization P450 gene regulation and function in insecticide resistance, including the analysis of promoter sequences that are involved in the regulation of overexpression and induction of P450 genes and the functional study of these genes in insecticide resistance.

8.2 Future Studies

8.2.1 Characterization of the molecular mechanisms of P450 gene expression regulation in ALHF

To characterize the molecular mechanisms of P450 gene expression regulation in insecticide resistance, the promoter elements that are involved in the overexpression of P450 genes in resistant ALHF house flies will be examined by gel-shift assay (Fig. 8.1). The promoter sequences of P450 genes will be compared between resistant and susceptible house flies. The promoter sequences that are different between resistant and susceptible strains will be chosen for the further characterization of their importance in the interaction with transcriptional factors and the regulation of P450 gene expression. In the gel shift assay, the oligonucleotides (elements) will be labeled at both ends with [γ-^{32}P] ATP and will be incubated with nuclear extract proteins. The DNA–protein complexes will be resolved by electrophoresis on a non-denaturing polyacrylamide gel and visualized by autoradiography by exposure to X-ray film with intensifying screens (Fig. 8.1). The interaction between the elements and the transcriptional factors will be identified and compared according to the mobility of the molecules.

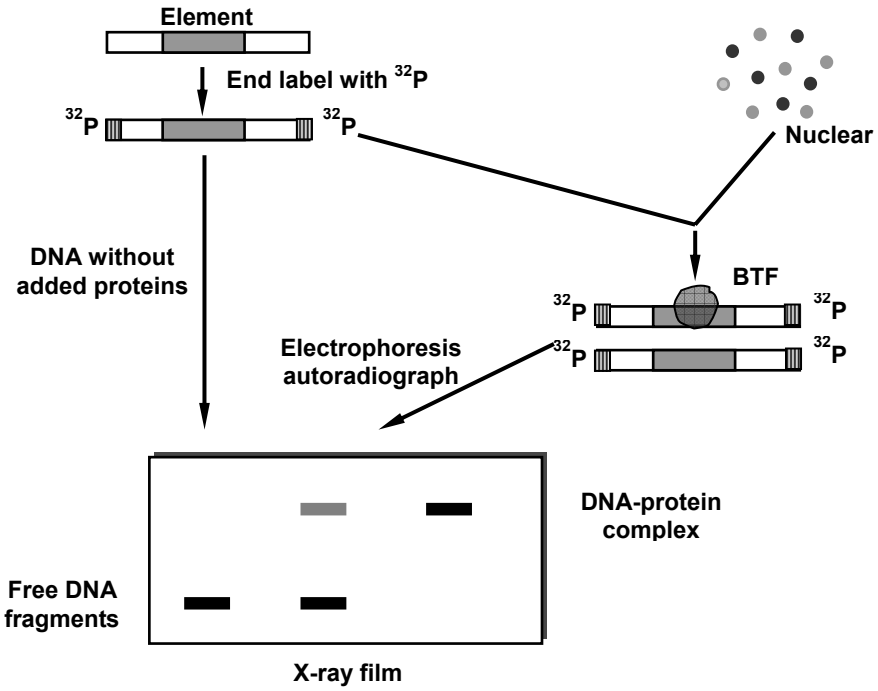
8.2.2. Functional study of overexpressed and permethrin induced P450 genes in insecticide resistance by RNA interference (RNAi) technique

To further investigate the functions of the P450 genes that are constitutively overexpressed and/or up-regulated by the insecticide stimulation in the permethrin resistant ALHF house flies, the RNAi technique (Hannon, 2002; Fig. 8.2) will be carried out. A two-step process is involved in the principle of RNAi as shown in Fig. 8.2. In the first step, the dsRNA will be digested into siRNA with 21 to 25 nucleotides in length by the ribonuclease-III-like enzyme, called Dicer. The second step involves incorporation of the siRNA into a RNA-induced silencing complex (RISC) and unwound by RISC. The siRNA will subsequently serve as a guide for RISC to search for the homologous single-stranded target mRNA and the target mRNA will then be degraded by RISC. To carry out RNAi experiments and to knockdown the target P450 genes in ALHF house flies, P450 specific fragments will be generated using the primers designed according to the sequences of P450 genes. Subsequently, the dsRNA will be synthesized using the PCR product as template. The dsRNA will then be injected into the adult ALHF house flies. The expression of the target P450 genes in dsRNA injected and control (un-injected or buffer injected) house flies will be examined by quantitative real-time PCR and compared. At the same time, the permethrin resistance levels of injected and control house flies will be compared. Eventually, the function of the target P450 genes in insecticide resistance will be analyzed.

FIGURE LEGEND

Figure 8.1. The scheme of gel shift assay.

Figure 8.2. The scheme of RNA interference.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End label with ^{32}P
^{32}P
^{32}P
Nuclear
DNA without
added proteins
BTF
^{32}P
^{32}P
^{32}P
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Electrophoresis
autoradiograph
DNA-protein
complex
Free DNA
fragments
X-ray film

Figure 8.1

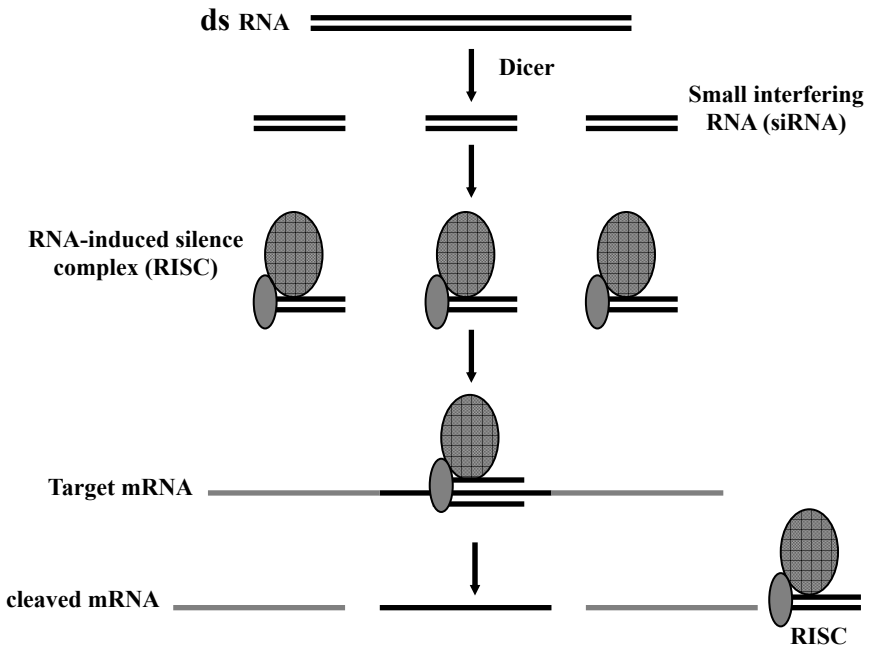
ds RNA
Dicer
Small interfering
RNA (siRNA)
RNA-induced silence
complex (RISC)
Target mRNA
cleaved mRNA
RISC

Figure 8.2

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