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RNAi Validation of Resistance Genes and Their Interactions in the Highly DDT-Resistant 91-R Strain of Drosophila Melanogaster

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RNAI VALIDATION OF RESISTANCE GENES AND THEIR INTERACTIONS IN THE HIGHLY DDT-RESISTANT *91-R* STRAIN OF DROSOPHILA MELANOGASTER

A Thesis Presented

By

KYLE GELLATLY

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ABSTRACT

RNAI VALIDATION OF RESISTANCE GENES AND THEIR INTERACTIONS IN THE HIGHLY DDT-RESISTANT *91-R* STRAIN OF DROSOPHILA MELANOGASTER

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4,4'-dichlorodiphenyltrichloroethane (DDT) has been re-recommended by the World Health Organization for malaria mosquito control. Previous DDT use has resulted in resistance, and with continued use resistance will increase in terms of level and extent. *Drosophila melanogaster* is a model dipteran that has many available genetic tools, numerous studies done on insecticide resistance mechanisms, and is related to malaria mosquitoes allowing for extrapolation. The *91-R* strain of *D. melanogaster* is highly resistant to DDT (>1500-fold), however, there is no mechanistic scheme that accounts for this level of resistance. Recently, reduced penetration, increased detoxification, and direct excretion have been identified as resistance mechanisms in the *91-R* strain. Their interactions, however, remain unclear. Use of UAS-RNAi transgenic lines of *D. melanogaster* allowed for the targeted knockdown of genes putatively involved in DDT resistance and has validated the role of several cuticular proteins (*Cyp4g1* and *Lcp1*), cytochrome P450 monooxygenases (*Cyp6g1* and *Cyp12d1*), and ATP binding cassette transporters (*Mdr50*, *Mdr65*, and *Mrp1*) involved in DDT resistance.

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CHAPTER 1

INTRODUCTION

4,4'-dichlorodiphenyltrichloroethane (DDT) (Fig. 1), a neurotoxic organochlorine insecticide that was phased out of use in the 1980s due to environmental impacts, has since been re-recommended by the World Health Organization for use indoors to control mosquito populations in malaria prone areas [1, 2]. Malaria, an infectious disease spread through mosquito vectors, was responsible for nearly 655,000 deaths in 2010, the majority being less than 5 years of age [3]. Through both indoor residual spraying (IRS) and insecticide-treated nets (ITNs), DDT has been shown to be effective at reducing malaria transmission rates in disease prone areas. Its relatively low cost, long term effectiveness, and lack of environmental impact when applied properly, has led to fourteen African Sub-Saharan countries, and others around the globe such as India, to once again use DDT as a preventative measure against malaria.

This widespread use, however, has come at a cost; by 1984, a world survey showed that 233 species, mostly insects, were resistant to DDT [4]. Even more alarming is that DDT resistance has been reported in more than 50 species of anopheles mosquitoes, many of which are vectors of malaria [2]. The use of DDT for vector control continues to this day, and its use will increase as insect-borne diseases expand [5,6]. *Drosophila melanogaster (D. melanogaster)*, a genetic model organism for the last several decades with many genetic tools and a wealth of information about insecticide resistance mechanisms [7], has also been shown to be highly resistant to DDT [8-11]. Both the mosquito and *D. melanogaster* belong to order Diptera, and because they are highly related genetically, studies done on *D. melanogaster* can be extrapolated to the mosquito as well [12].

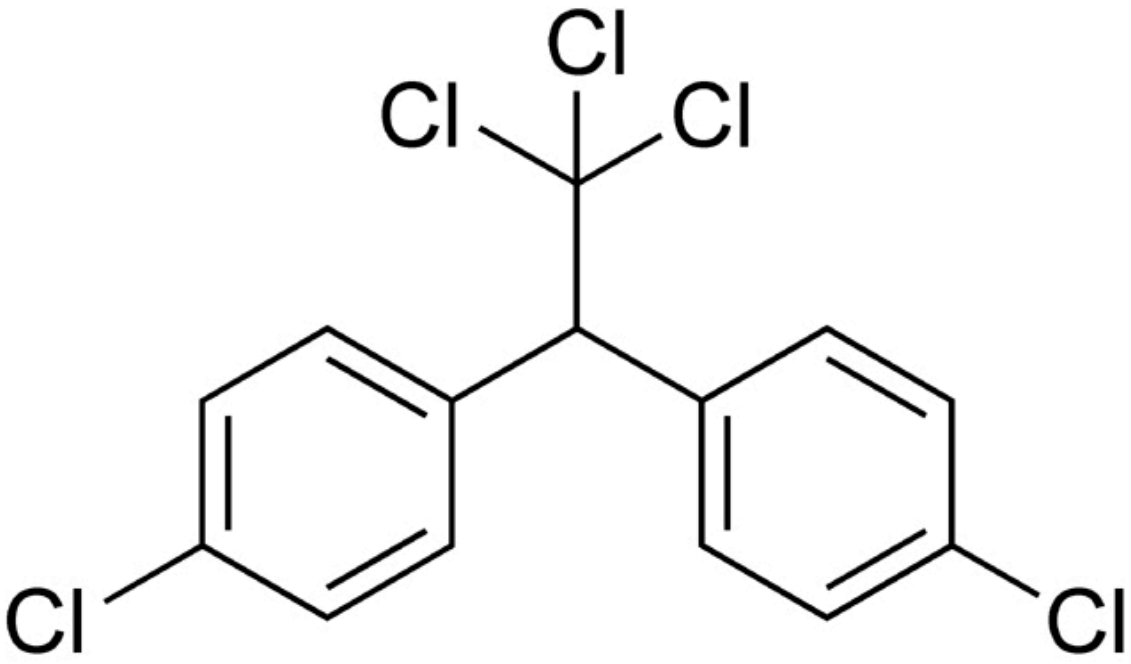


Figure 1. The chemical structure of 4,4'-dichlorodiphenyltrichloroethane (DDT).

Several strains of *D. melanogaster* have often been used in DDT resistance studies, such as the *Canton-S* (*CS*, DDT-susceptible), *91-C* (slightly DDT-resistant), and the *91-R* strain (highly DDT-resistant). The highly DDT-resistant *91-R* strain of *D. melanogaster* is over 1500-fold more resistant to DDT than *CS* [13]. As of yet, however, there is no definitive mechanistic model to accurately account for this level of resistance. Genome-wide transcription profiling, as well as protein mapping, has suggested that there are a number of factors involved in DDT resistance [14-16]. Both target-site insensitivity and enhanced xenobiotic metabolism have been shown in some cases to be responsible for DDT resistance [17-19]. It has been previously reported that the increased cellular excretion of DDT in the *91-R* strain is caused by increased expression of ATP-Binding Cassette Transporters (ABC-transporters) and the increased metabolism of DDT is caused in part by over expression of cytochrome P450 monooxygenase (P450s) [13]. With mortality bioassays, it was estimated that increased excretion and metabolism conferred 10- and 2.2-fold resistance levels, respectively. In the same investigation, a penetration factor was attributed to a 68-fold increase in resistance. Using gas-liquid chromatography with flame ionization detection (GC-FID), 5 cuticular hydrocarbons were identified to be significantly more abundant ($p<0.05$) in the *91-R* strain compared with *CS* flies. It is likely that the penetration factor associated with DDT resistance is due, in part, to the increase in cuticular hydrocarbons in the epicuticle of *91-R* flies. However, the molecular mechanism of this resistance factor had not yet been identified.

In 2007, a large step forward was taken towards the ultimate goal of identifying the role of nearly every gene in *D. melanogaster* by creating a transgenic RNAi library that contained transformants for 97% of all genes in *D. melanogaster* [20]. The RNAi library was created by

inserting gene activation control elements that are normally found in yeast into the *D. melanogaster* genome. This yeast system, called Gal4/UAS, works through the combination of two distinct parts: Gal4 and the upstream activation sequence (UAS). Gal4 is a modular protein in yeast that is responsible for DNA binding in order to activate gene expression. The UAS is a sequence before the coding region of the gene to which Gal4 binds to initiate transcription (Fig. 2) [21,22].

In *D. melanogaster*, lines were generated that expressed Gal4 in a variety of different ways: including constitutive, time specific, tissue specific, or inducible through chemical or environmental conditions such as a heat shock. Transformants were also created that contained an UAS preceding gene specific inverted repeats. When induced, these insertions express RNA that folds back onto itself to form a dsRNA hairpin loop, which is processed by RNAi machinery to lead to gene silencing [23]. By crossing a driver strain (which contains a Gal4 expression insertion) with a transformant (that contains an inverted repeat of a target gene under Gal4 promoter), RNAi can be induced in the resulting F1 progeny.

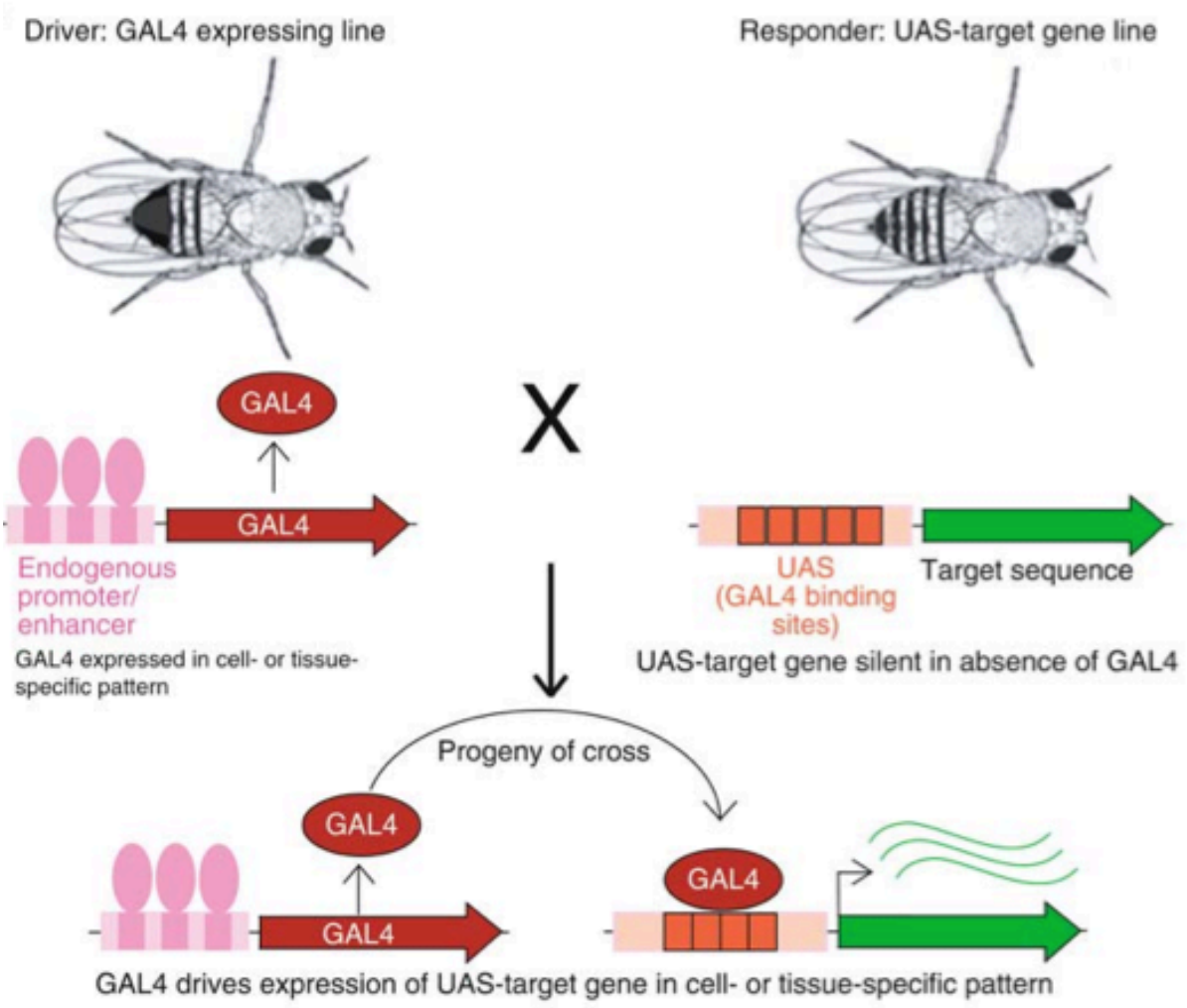


Figure 2. Through the cross of a Gal4 expressing driver and a transformant with an inverted repeat for a gene of interest, RNAi can be achieved. The expression of the dsRNA is processed by RNAi machinery to lead to gene silencing. [22]

Decreased penetration, increased xenobiotic metabolism, and increased xenobiotic excretion have all been shown to be at least partially responsible for overall DDT resistance levels in the *91-R* strain of *D. melanogaster* [13]. Before xenobiotic metabolism can take place, however, DDT must first penetrate into the target organism [24]. Decreased cuticular penetration therefore can impede the rate of distribution of DDT into the hemolymph [25] and ultimately the nervous system of the insect. In order for this to occur, DDT must pass through a variety of different layers of the insect cuticle that make up the protective barrier between the fly and its surrounding environment (Fig. 3). First, there is an epicuticle, which contains among other components, an external layer of waxy cuticular hydrocarbons that have evolved to prevent desiccation [26]. This external barrier is followed by the exocuticle and endocuticle, which consists of various cross-linked proteins and chitin. Lastly, there is a layer of epidermal cells that are responsible for the production and excretion of many of the proteins and compounds that are found within the cuticle itself [27]. A specialized cell type within the epidermis, the oenocyte, is responsible for the decarbonylation of long chain aldehydes, which are then transported to the outer surface by way of pore canals to form the waxy cuticular hydrocarbon layer [28].

Decreased penetration is an important factor for DDT resistance. Due to its lipophilicity, the ability of DDT to penetrate through the cuticular waxy hydrocarbon layer is largely dependent on the amount of hydrocarbon present. Changes in the protein content of the cuticle itself may also be responsible for the decreased rate of penetration, such as changes in the chitin content [29], laccase gene expression [30], or larval cuticle proteins such as *Lcp1* [31]. There is a strong correlation between the presence of this chitinous cuticle and sensitivity to DDT [32]. It has been hypothesized that an increase in cuticular hydrocarbons would decrease the rate by which DDT can penetrate into the target organism giving rise to resistance [13].

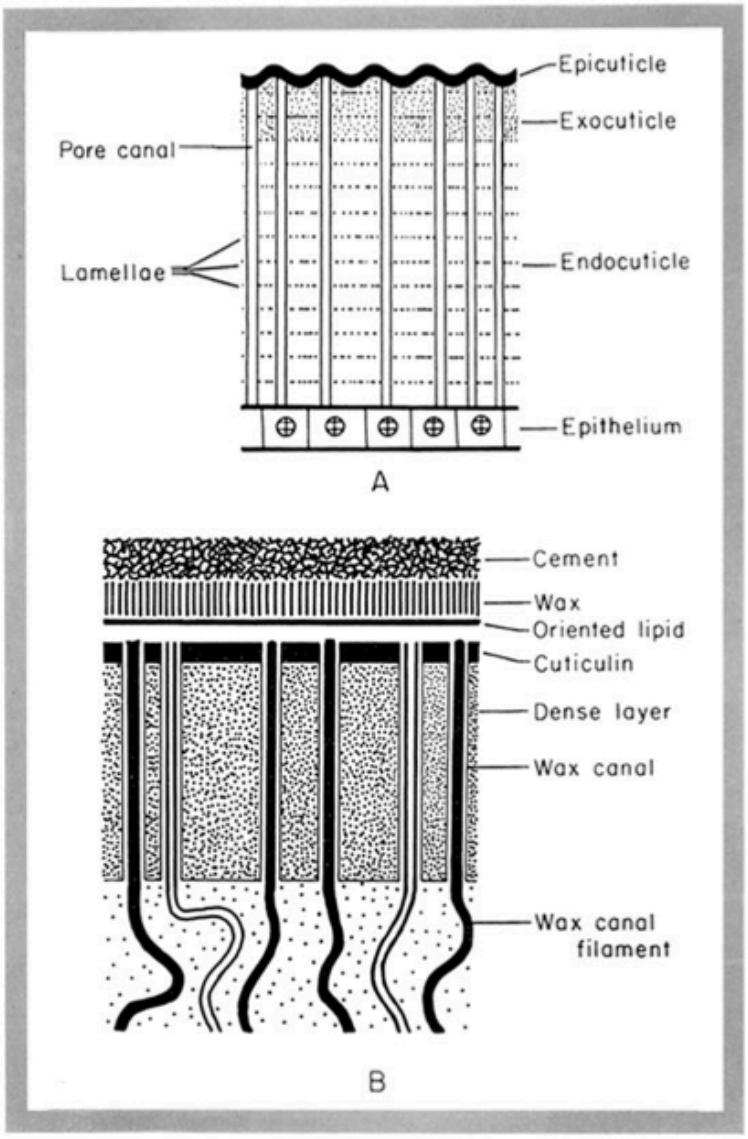


Figure 3. The protective barrier between the insect and its surrounding environment contains several distinct layers. Panel A shows the entire insect cuticle, while panel B is an inset of the epicuticle. The typical insect cuticle is composed of an outer epicuticle, which contains the waxy cuticular hydrocarbons to prevent desiccation, followed by the exo and endocuticle. These layers are produced by an underlying layer of epidermal cells. The oenocyte, a specialized epidermal cell, is responsible for the production of cuticular hydrocarbons. [33]

Reductive metabolism of DDT has been shown to occur in several P450 systems [34,35]. Increased metabolism, as a mechanism of DDT resistance in *D. melanogaster*, has been shown to be largely due to either increased expression or structural changes in P450s [14]. Several isoforms of P450s metabolize DDT into its primary and secondary metabolites. Specifically, *Cyp6g1, Cyp12d1, Cyp6a8,* and *Cyp6a2* have been shown to be significantly over expressed in several DDT-resistant strains [15, 36-39]. Microarray analysis showed that *Cyp6g1* was inducible by DDT [40]. DDT has also been shown to be metabolized by *Cyp6g1* expression in cell cultures of *Nicotiana tabacum* [41]. Further, when *Cyp6g1* expression was induced in *D. melanogaster* using a Gal4/UAS system, a greater level of DDT resistance was demonstrated [42,43]. Investigations into the reason behind the over expression of *Cyp6g1* have led to the identification of cis-acting elements [44]. Some suggest that it is due to the *Accord* retrotransposon, which results in tissue specific over expression [45-47]. While *Cyp6g1* may be considered by some to be the only P450 responsible for DDT metabolism in *D. melanogaster*, there are others who argue against this suggestion. One study found that *Cyp6g1* over expression alone does not confer DDT resistance in *D. melanogaster* [48], while another study reported that *Cyp6g1* knockout has no effect on DDT susceptibility [49].

While *Cyp6g1* appears to be important in DDT metabolism, there are several other P450 candidates that may lead to the extremely high level of resistance found in *91-R* flies. *Cyp12d1* is one such candidate. Several studies have shown that *Cyp12d1* is over expressed in resistant lines, and is inducible by DDT exposure [37,50]. Transgenic over expression of *Cyp12d1* resulted in increased survival times of flies exposed to DDT [43]. Other P450s of interested include *Cyp6a8* and *Cyp6a2*. *Cyp6a8* has been shown to be over expressed in DDT-resistant flies, likely due to

factors in the 5' region of the gene [51]. Both *Cyp6a8* and *Cyp6a2* are constitutively over expressed, as well as being inducible with phenobarbital [52, 53]. Another study suggests that the over expression of these genes is actually due to factors found on the third chromosome [38]. *Cyp6a2* has had several studies done on its ability to metabolize DDT. One study shows that while it is over expressed in resistant flies, its ability to be induced by insecticides is actually greater in susceptible flies [54]. Some studies looking to identify the reason for *Cyp6a2* over expression have suggested that it is due to either 5' transcriptional binding sites or changes in the 3' region of the gene [51, 55].

The ability to metabolize DDT using wild-type *Cyp6a2* seems to be negligible, while a R335S, L336V, V476L, (*Cyp6a2*SVL) mutant form has been reported to metabolize DDT when transformed into *Escherichi coli* [56]. Expression of the *Cyp6a2*SVL mutant was shown to increase the production of DDA, DDD, and dicofol, known DDT metabolites. The highly DDT-resistant *91-R* strain was shown to produce greater amounts of dicofol and DDD, as well as 2 unidentified metabolites (perhaps one of which may be DDA), compared to the DDT-susceptible *CS.* As *Cyp6a2* is the most highly over expressed P450 in the *91-R* strain, it would make sense to offset that energetic cost with the fitness advantage of direct DDT metabolism and detoxification. However, because wild type *Cyp6a2* does not metabolize DDT, there is a possibility that the *91-R* strain of *D. melanogaster* contains the SVL mutations. Sequencing *Cyp6a2* across the *CS, 91-C, 91-R,* UAS-Cyp6a2 (*Cyp6a2* RNAi line) strains and comparing them to the *Cyp6a2*SVL strain would show if these mutations are present in the DDT-resistant *91-R* strain. The possibility remains, nevertheless, that additional *Cyp6a2* mutations may exist in the *91-R* strain which may enable DDT metabolism even if the SVL mutations are not present.

With increased excretion driven by phase III metabolism, xenobiotics are effluxed out of the cell by ABC transporters, a class of proteins that span membranes and use ATP to drive this efflux [57]. ABC transporters have been shown to be an insecticide resistance mechanism in several insects [58-61]. Several ABC transporters have been implicated in DDT resistance in the *91-R* flies such as *Mdr49, Mdr50, Mdr65,* and *Mrp1* [62-65]. The multiple drug resistance (*Mdr*) genes are ABC-B type transporters, while the multidrug resistance-associated proteins (*Mrp*) are ABC-C type transporters. Over transcription of p-glycoproteins (*Mdr*, ABC-B type transporters) in epidermal cells has previously been shown to lead to insecticide resistance [66]. The first identification and characterization of *D. melanogaster* ABC-B transporters homologues occurred in 1991 and reported that they were similar to the mammalian *Mdr* cell lines [65]. *Mdr49* was shown to be expressed in all stages of the flies' life cycle, suggesting that it may be one of the transporters involved in the efflux of DDT [67]. Similarly, a study that compared the expression of *Mdr49* and *Mdr65* showed that they were inducible by stress, including pesticides that are well known inducers of stress [68]. The identification of another *Mdr* gene, *Mdr50* [64] showed that *D. melanogaster* had a variety of different ABC transporters in its genome, any of which may be responsible for effluxing DDT. *Mrp1* was identified as an orthologue of human MRP1, MRP2, MRP3, and MRP6, which were known to efflux a variety of therapeutic agents [69]. Further, *Mrp1* was shown to be inducible by DDT exposure [70].

Once DDT has penetrated the insect exoskeleton and is distributed within the hemolymph, both metabolism and excretion work cooperatively in an additive manner [71]. Penetration, however, has been described as a multiplicative resistance mechanism. Resistance to DDT in *91-R* flies is extremely high and is likely due to a combination of several of the

aforementioned mechanisms. Contact penetration of DDT was ~30% less with *91-R* flies, possibly due to having significantly more cuticular hydrocarbons and a thicker, more laminated cuticle compared to *CS* flies [13]. DDT was metabolized ~1.6-fold more extensively by *91-R* than *CS* flies, resulting in dicofol, dichlorodiphenyldichloroethane (DDD), two unidentified metabolites and a number of unresolved polar conjugates being formed in significantly greater amounts [13]. *91-R* flies also excreted ~4-fold more DDT and its metabolites than *CS* flies [13]. Verapamil pretreatment reduced the LD50 value for *91-R* flies topically dosed with DDT by a factor of 10-fold, indicating that the increased excretion may involve ABC B-type transporters [13]. Thus, DDT resistance in *91-R* flies is multifactorial and includes mechanisms involved in reduced penetration, increased detoxification and direct excretion, some of which may interact synergistically and result in high levels of DDT resistance.

Through crosses between a driver strain that expressed Gal4 under a heat shock promoter and UAS-RNAi lines for genes that were putatively determined to be involved in DDT resistance in the *91-R* strain, we identified several cuticular proteins, P450s and ABC transporters involved in DDT resistance across all three chromosomes. Our working hypothesis is that RNAi knockdown of the selected genes in the insecticide-susceptible transgenic flies, which results in increased sensitivity to DDT, are likely to function as resistance factors in *91-R* flies where these genes are over transcribed.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Drosophila melanogaster* strains

The DDT-resistant *91-R* and insecticide-susceptible *CS* strains were obtained from Dr. Barry Pittendrigh (University of Illinois, Urbana-Champaign). The *91-R* strain has been shown to be ~1500-fold more resistant to DDT than susceptible strains through the use of contact bioassays [13]. The *91-R* strain has been continually selected by maintaining the flies in a colony jar in the presence of a 150 mg DDT/filter paper disk. All strains were reared at room temperature on Jazz-Mix Drosophila Food® from Fisher Scientific (Waltham, MA) prepared according to the labeled instructions. Flies were kept in colony vials, which were changed every 2 weeks. Flies were anesthetized for transfers by placing dry ice in a Büchner flask with attached tubing connected to a needle that released CO_2.

For heat shock experiments, virgin females of Driver 1799 from the Bloomington *Drosophila* Stock Center (BDSC) at Indiana University, Bloomington, IN, were selected. This driver strain contains a Gal4 insertion under the Hsp70 promoter, allowing Gal4 expression when heat shocked. Driver 1799 females were crossed with males of the UAS-RNAi lines from the BDSC and the Vienna Drosophila RNAi Center (VDRC, Vienna, Austria) (Table 1). These lines contain an inverted gene repeat under control of the Gal4 promoter region, which when expressed results in the formation of dsRNA hairpin loop structures [20,21]. Thus, a 1799 X UAS-RNAi cross results in the generation of dsRNA for RNAi only after heat shock and allows for control groups (non-heat shocked, NHS) to be of the same genetic background as the RNAi groups (heat shocked, HS).

Table 1
Fly lines used for UAS-RNAi genetic crosses.

Gene Class	CG#	Synonym	Stock #	Line Type	Vendor
N/A	N/A	N/A	1799	Gal 4 Driver	BDSC
Cuticular Protein	11650	*Lcp1*	106030	UAS-RNAi	VDRC
	4784	*Cpr72Ec*	29452	UAS-RNAi	VDRC
P450	3972	*Cyp4g1*	102864	UAS-RNAi	VDRC
	8453	*Cyp6g1*	104171	UAS-RNAi	VDRC
	30489	*Cyp12d1*	109256	UAS-RNAi	VDRC
	10248	*Cyp6a8*	100459	UAS-RNAi	VDRC
	8859	*Cyp6g2*	105333	UAS-RNAi	VDRC
	9438	*Cyp6a2*	108776	UAS-RNAi	VDRC
ABC B-type	3879	*Mdr49*	108327	UAS-RNAi	VDRC
	8523	*Mdr50*	51166	UAS-RNAi	VDRC
	10181	*Mdr65*	35035	UAS-RNAi	BDSC
	11897	N/A	105174	UAS-RNAi	VDRC
	7806	N/A	2804	UAS-RNAi	VDRC
	5789	N/A	1204	UAS-RNAi	VDRC
ABC C-type	6214	*Mrp1*	105419	UAS-RNAi	VDRC
Glutathione *S*-Transferase	17530	*GstE5*	100632	UAS-RNAi	VDRC
	17527	*GstE6*	25270	UAS-RNAi	VDRC

For 1799 X UAS-RNAi line crosses, fly rearing vials were emptied of all adult flies and newly eclosed virgin females were selected within 6 h of adult emergence. Mating pairs of virgin females and males were transferred to new vials, and the mating pairs removed after 24 h. This process ensured that the resulting F1 progeny were within 24 h of each other in age. All references to age were based on the number of days since egg oviposition. For all heat shock experiments, 9 day old flies were placed into a 37°C incubator for 45 min and then allowed to continuously develop under the standard rearing conditions described above.

2.2 Selection of genes for UAS-RNAi and dsRNA injection-induced RNAi

Genes selected for RNAi knockdown (Table 2) were determined through a combination of literature searches, toxicokinetic analysis [13] and bioinformatic approaches (Table 3).

Table 2
Genes selected for RNAi knockdown through literature search, toxicokinetic data, and bioinformatics approaches.

Gene Class	CG#	Synonym	Transcript Ratio	P - value	Selection Criteria
Cuticular Protein	11650	*Lcp1*	2.52	.0067	[38]
	4784	*Cpr72Ec*	4.39	<0.0001	[38]
Cytochrome P450	3972	*Cyp4g1*	*	*	[25]
	8453	*Cyp6g1*	2.83	0.0136	[10, 16, 17, 20, 38]
	30489	*Cyp12d1*	0.1	<0.0001	[15, 17, 38]
	10248	*Cyp6a8*	*	*	[14, 15, 50, 51]
	8859	*Cyp6g2*	1.66	.205	[38, 51]
	9438	*Cyp6a2*	34.3	<0.0001	[12, 38, 49, 50, 51]
ABC-B Type	3879	*Mdr49*	*	*	[39, 40]
	8523	*Mdr50*	*	*	[39, 41]
	10181	*Mdr65*	*	*	[39, 42]
	11897	N/A	*	*	Bioinformatics
	7806	N/A	*	*	Bioinformatics
	5789	N/A	*	*	Bioinformatics
ABC-C Type	6214	*Mrp1*	*	*	[39, 43]
Glutathione S-Transferase	17530	*GstE5*	3.03	<0.0001	[38]
	17527	*GstE6*	2.52	0.0016	[38]

* No information on *91-R* transcript levels.

Lcp1, *Cpr72Ec*, *Cyp6g1*, *Cyp12d1*, *Cyp6a2*, *GstE6*, and *GstE5* have all been referenced in the literature as over transcribed in DDT-resistant strains [15]. *Lcp1* and *Cpr72Ec* are cuticular protein genes and increased expression of these genes in DDT-resistant strains was suggested to confer DDT resistance by altering the physical structure of the cuticle, making it more difficult for DDT to penetrate [13]. *Cyp4g1* was selected due to its recent identification as the P450 in oenocytes of the epidermis responsible for the decarbonylation of long chain aldehydes to form

cuticular hydrocarbons [28]. Because *91-R* flies have an increased amount of cuticular hydrocarbons, *Cyp4g1* was a logical choice as being responsible for the increase in hydrocarbon abundance [13]. *Cyp6g1*, *Cyp12d1*, and *Cyp6a2* were also shown to be over transcribed P450 genes in DDT-resistant strains, and these P450s could confer DDT resistance through direct metabolism and detoxification of DDT to dicofol or DDD [13]. Further, *Cyp6a2* has been reportedly able to metabolize DDT [56], although this was only in a *Cyp6a2*SVL mutant form of the gene. *Cyp6a2, Cyp6g2,* and *Cyp6a8* were also shown to be over expressed due to changes in the promoter and or 3' region of the genes [39]. The glutathione-S-transferase genes, *GstE5* and *GstE6,* were found to be over expressed in the *91-R* strain and are likely necessary to offset the increased oxidative stress produced through constitutive over transcription of the P450 detoxification genes [15].

The ABC B-type multiple drug resistance (*Mdr*) genes, *Mdr49*, *Mdr50*, *Mdr65* as well as the ABC C-type multidrug resistance-associated protein *Mrp1,* were selected for RNAi due to preliminary reverse transcription-quantitative real time PCR (RT-qPCR) results that showed their over transcription in the *91-R* strain [62] and their prior implications in insecticide phase III xenobiotic metabolism (efflux) and resistance [63-69]. Additional ABC B-type transporter genes, CG11897, CG7806, and CG5789, were selected through a bioinformatic approach using Flybase. Search parameters, such as xenobiotic transporting ATPase activity and expression in the Malpighian tubules (Table 3), were tabulated and the overall match scores determined by comparison with the known DDT interacting genes such as *Mdr50*. Any genes which had 4 or more positive parameters were deemed worthy of investigation.

Table 3

Search parameters used in Flybase for selection of candidate ABC transporter genes potentially involved in DDT efflux.

Family	Gene	Alias	Xenobiotic transporting ATPase activity	Drug transmembrane transporter activity	Resistance	Drug	Malphigian tubules	Fat body	**# Positive Parameters**
A	(10) Genes	-	No	No	No	No	-	-	0
B	CG10181	*Mdr65*	Yes	Yes	Yes	Yes	Yes		5
B	CG10226			Yes			Yes		2
B	CG10441								
B	CG17338		Yes						
B	CG1824								
B	CG3156		Yes	Yes					2
B	CG3879	*Mdr49*		Yes	Yes	Yes	Yes	Yes	5
B	CG4225								
B	CG7955								
B	CG8523	*Mdr50*		Yes	Yes	Yes			3
C	CG10505								
C	CG11897		Yes	Yes		Yes	Yes	Yes	5
C	CG11898		Yes	Yes		Yes			3
C	CG14709								
C	CG4562								
C	CG5772								
C	CG5789		Yes	Yes			Yes	Yes	4
C	CG6214	*Mrp1*	Yes	Yes	Yes		Yes	Yes	5
C	CG7627		Yes	Yes				Yes	3
C	CG7806		Yes	Yes			Yes	Yes	4
C	CG8799								
C	CG9270		Yes	Yes			Yes		3
D	(2) Genes	-	No	No	No	No	-	-	0
E	(4) Genes	-	No	No	No	No	-	-	0
G	(15) Genes	-	No	No	No	No	-	-	0
H	(3) Genes	-	No	No	No	No	-	-	0
	56 Total								

2.3 Total RNA extraction and reverse transcriptase-quantitative real-time PCR analysis (RT-qPCR)

For all analyses, 30 females (14 days post oviposition) were collected per biological replicate and 3 biological replicates were analyzed. This time point ensured that all heat shock-treated flies had fully emerged from their pupae. Flies were placed into 2 ml vials with five 0.25 g stainless steel beads, loaded into an aluminum casing and homogenized with the GenoGrinder 2010 (SPEX Sample Prep, Metuchen, NJ) for 1 min at 1000 strokes per min. After homogenization, the total RNA was extracted using the Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with an on column DNase digestion. Total RNA was eluted from the provided column in RNAse free (DEPC-treated), double-distilled H_2O (ddH_2O). The concentration of RNA was determined using the NanoDrop 8000 (NanoDrop Technologies, Wilmington, DE), and then adjusted to a final concentration of 100 ng RNA/μL.

500 ng of total RNA served as template for the first strand cDNA synthesis reaction using the iScript cDNA kit from Bio-Rad (Hercules, CA) in a 20 μL reaction volume following the manufacturer's instructions. The resulting cDNA was used as a template in the RT-qPCR reaction. RT-qPCR analyses were performed using the Power Cyber Green PCR Master Mix (Life Technologies, Carlsbad, CA) on a Step One Plus Real-Time PCR System (Life Technologies, Carlsbad, CA). The thermal cycle parameters were an initial hold at 95°C for 10 min, 40 cycles of 15 sec at 95°C (denature) and 60 sec at 60°C (anneal and extend). All samples were run in triplicate with 1.0 μL of a 1:10 dilution of cDNA template in a 20 μL reaction volume. Primer concentrations were 900 nM for all gene targets. The reference gene used for normalization of expression values was *Rp49*, a ribosomal protein gene from *D. melanogaster*.

Statistical analysis was performed on the Cycle Threshold (Ct) values that were calculated by the Step One Plus v2.2 software, and relative transcript levels were determined using the ΔΔCt method [72]. The specific primers for RT-qPCR (Table 4) were selected using the Drosophila RNAi Screening Center at Harvard Medical School's FlyPrimerBank (Boston, MA).

Table 4

Primer sequences used for RT-qPCR experiments.

Gene Class	CG #	Synonym	Primer Sequence (5'-3')
Ribosomal Protein	7939	*Rp49*	F: CGGTTACGGATCGAACAAGCG R: TTGGCGCGCTCGACAATCT
Cuticular Protein	11650	*Lcp1*	F: CACACCTCCAACGGAATCGA R: ACTTAACCTCGACGTGCTCG
	4784	*Cpr72Ec*	F: CACTGCTTCACCTGGACGATT R: CTCCTGGTAACCACTGCTCG
P450	3972	*Cyp4g1*	F: CCCCACCTTCCACCAGAGCAT R: AAGTTCTTATTACGATCCTTGACCACC
	8453	*Cyp6g1*	F: GGTTCCACAACCGATACGC R: TCCCTGACGAAGAACAGGTTAT
	30489	*Cyp12d1*	F: TATCTGGCCACGTCGTGATG R: GCCCCTGGGTTGCATAAAGA
	10248	*Cyp6a8*	F: CGTTGCACCGCATCTCATC R: GCCTTGCTTTCCCTGAATTTGTT
	8859	*Cyp6g2*	F: GCCTTGCTTTCCCTGAATTTGTT R: CTGATTTCGCGTACTCCCATT
	9438	*Cyp6a2*	F: CGGGTGATGCACGACTTCTT R: GTAAAAGCCCACGAAGGGAAA
ABC B-type	3879	*Mdr49*	F: TCCCGCAGAGAATACGGGT R: GAGCCATGCACAGGTAGATGA
	8523	*Mdr50*	F: AGAAGTCCAAGCATGACGAGT R: CGCTCGATCCTTTTTGGTGG
	10181	*Mdr65*	F: AGAGCCTATTGCATTCCTGAAAC R: GCAGCACATTATGAAGCCGAA
	11897	N/A	F: GACGAGGAGCTGTATCAACATAG R: CCTCCCACAGTTCCGCAAAT
	7806	N/A	F: GCCATATCCGCCTACAATTTCG R: AGATCCGCCGCAAACAGTT
	5789	N/A	F: AATACGGATGACCTGACACAATG R: CGTTTCTGAGATGCGGCTTCT
ABC C-type	6214	*Mrp1*	F: AATCGAAAGTATGGCGTGCAG R: GGGGAATCGACAGCACAGT
Glutathione *S*-Transferase	17530	*GstE6*	F: TACGGTTTGGACCCCAGTC R: ATATTCCGGTGAAAGTTGGGC
	17527	*GstE5*	F: CGTGCCGTCAAACTCACTCT R: GCTGCTCCTGACCCGAAAT

2.4 dsRNA synthesis for RNAi by injection

Total RNA was extracted from *91-R* flies and cDNA prepared as described above (section 2.3). For the *in vitro* transcription reactions yielding dsRNA, a T7-PCR protocol was utilized with Advantage HD Polymerase (Clonetech, Mountain View, CA) following the manufacturer's instructions. Gene specific primers containing the T7-Promoter on the 5' end (X=TAATACGACTCACTATAGGG) were F-XCATCTGGTATGGTGTGACGC; R-XTCCGTATCGGATATTCTCGC for *Mdr49*, F-XTAATTTCCGGAATCTGCTCG; R-XGCCGAAAAAGTGGTCATGTT for *Mdr50*, F-XCGAACTTCTTCCGCACTTTC; R-XCATAAAAGGGCCAACTTCCA for *Mdr65*, and F-XTGGAACAAGCTGAACGTGAG; R-XTCAGGTCCCACAGATCCTTC) for *Mrp1*, respectively. The PCR product was gel-extracted and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and used as a template in the dsRNA synthesis reaction using the MEGAscript T7 transcription kit (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. The resulting dsRNA was purified with a lithium-chloride precipitation step and re-suspended in 30 µL of *Drosophila* injection buffer (0.1 mM sodium phosphate, pH 6.8; 5 mM KCl). To ensure proper dsRNA formation and elimination of persistent secondary structure, the resulting dsRNA was diluted to 1 µg/µL and an annealing cycle was run on the thermal cycler by first incubating the sample at 95°C for 3 min, followed by sequential 5 min cooling steps at 85°C, 80°C, 75°C, 70°C, 65°C, 60°C, 50°C, 40°C, 30°C, before cooling to 4°C.

The purified and annealed dsRNA was electrophoresed on a 0.7% agarose gel with 1.0 µL of a 10 mg/mL ethidium bromide solution to determine its purity and confirm the expected product size. 200 nL of the dsRNA was injected using the Nanoliter 2000 injector (World

Precision Instruments, Sarasota, FL) into the abdomen of an anesthetized 3 day old adult female fly, which was approximately the same age as 14 days post oviposition.

2.5 Mortality bioassays

Ten female flies (14 days post oviposition) per biological replicate (3 total) were placed into a 20 mL glass scintillation vial that was pre-coated with 12.5 μg of DDT and capped with cotton moistened with 1 mL of a 5% (w/v) sucrose solution. Females were observed for 24 h and were considered dead when all movement and leg twitching had ceased. Log time versus logit percent mortality regression lines were generated in order to determine the median lethal time (LT_{50}) for the treated flies using the statistical software PoloPC (LeOra, Petaluma, CA). The maximum-log likelihood test was used to determine whether the resulting mortality curves (slope and Y-intercepts) from differently treated fly groups were statistically different ($p<0.05$).

2.6 Cuticular hydrocarbon analysis

Groups of 15 females from either the day 9 NHS or day 9 HS F1 progeny were surface-extracted with n-hexane and the cuticular hydrocarbon differences between the two treatments determined for three replicate experiments using the gas-liquid chromatography (GLC) method described by Strycharz et al. [13]. Structural identification by gas chromatography-mass spectrometry (GC/MS) was carried out on the five cuticular hydrocarbon peaks that were previously determined to be significantly more abundant in the *91-R* versus the *CS* flies [13].

2.7 Sequencing of *Cyp6a2*

Genomic DNA (gDNA) was extracted from groups of 15 females from either the *CS* (DDT-susceptible), *91-C* (slightly DDT-resistant), *91-R* (highly DDT-resistant), or UAS-Cyp6a2 (the inducible *Cyp6a2* RNAi) lines using the DNeasy Blood and Tissue Kit from Qiagen (Valencia, CA). Briefly, females were placed into 2 mL microcentrifuge tubes containing 180 µL Buffer ATL, 0.9 µL reagent DX and five stainless steel grinding balls (0.25 g, SPEX Sample Prep, Metuchen, NJ) and homogenized in an automated homogenizer (GenoGrinder 2010, SPEX Sample Prep, Metuchen, NJ) at 1250 strokes per min for 1 min. Proteinase K (20 µL) was added to the tube and incubated for 3-24 h at 56°C. Following incubation, the lysate was transferred to a 1.5 mL tube with 100% ethanol (200 µL) and Buffer AL (200 µL) and vortexed. The sample was transferred to a DNeasy Mini spin column (Qiagen), and centrifuged for 30 s at 6000g. The flow through was discarded and the column placed into a new collection tube before washing with 500 µL buffer AW1, followed by 500 µL buffer AW2 with centrifuging for 30 s at 20,000g at each wash. The column was dried by centrifugation for 3 min at 20,000g before DNA elution. The column was placed into a new collection tube and incubated for 1 min with 50 µL buffer AE and centrifuged for 1 min at 6,000g to elute the DNA. This step was repeated to maximize total DNA yield.

DNA was quantified by applying 1 µl of extracted gDNA from a single sample to a NanoDrop ND 8000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE), absorbance at 260 nm determined and used to calculate the DNA concentration in ng of DNA μL^{-1} using a modified Beer-Lambert equation. DNA purity was also determined by the ratio

between absorbencies at 260 and 280 nm, with ratios between 1.5 and 1.8 indicative of pure samples. Samples were diluted to a concentration of 10 ng DNA/μL and stored at -20°C.

The *Cyp6a2* sequence was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/nuccore/U78088.1) and was used as the template for primer design (Fig. 4). 15 μL of a master mix (12 μL ddH_2O, 5 μL 5X Advantage HD Buffer, 1.75 μL 2.857 mM dNTP mixture, 0.25 μL Advantage HD Polymerase and 1 μL of a 10 mM solution containing the forward and reverse primers (F-GCCTGCATATTATCGCCGAG, and R-ACTAGTCAGGTGGCGATTCG) were mixed with 5 μL of template DNA (10 ng/μL) and placed onto an Eppendorf Mastercycler Pro (Hamburg, Germany) thermal cycler operated using the following parameters: 1 cycle at 95°C for 1 min; 30 cycles of 98°C for 10 sec, 60°C for 10 sec, 72°C for 2 min; and 2 min at 72°C. Gel electrophoresis (0.9% agarose) was used to verify quality and quantity of PCR DNA fragment amplification using ethidium bromide (1 μL of 10 mg/mL stock) staining to visualize PCR products under UV light.

The QIAquick PCR Purification Kit (Qiagen) was used for PCR product purification following the manufacturer's instructions. Briefly, 5 volumes of buffer PB were added to 1 volume PCR product, mixed with 10 μL of 3M sodium acetate buffer, transferred to a QIAquick spin column placed in a 2 mL collection tube and centrifuged for 30 s at 20,000g. The column was first washed with 750 μL guanidine HCl (30 s) and then with 750 μL buffer PE (30 s). After drying by centrifugation, DNA was eluted into a 1.5 mL tube by applying 50 μL and then 30 μL of Buffer EB and centrifuging at 20,000g for 1 min. Gel electrophoresis (0.9% agarose) was used to verify quantity and quality of products as above.

Triplicates for each sample containing 5 µL of a 5 µM sequencing primer solution (F1-GCGTCGCAGGGGAATCTTAT, F2-AGCACCTGTTCAACCTGGAC, F3-ACGACTTCATGAACCTGCTG, F4-ACAGGTCATAATCCCCGCTT,R1-TGAACCTGCAAATCTGATGGG, R2-TGGGATCTCTGTCGTATCGC, R3-GTTCCTCCAGCACCGTTTG, R4-TCAGCTCCTTGATCTCGAGC,R5-CCGACCATGTTGCCATACAG) were mixed with 10 µL of purified PCR product at a concentration of (2 ng/µL), loaded into a 96 well plate and sent to GeneWiz (South Plainfield, NJ) for sequencing. Sequence chromatograms were analyzed using Chromas lite software (Technelysium Pty Ltd., Tewantin, Australia) to determine sequences, which were then loaded into CLC Sequence viewer (Boston, MA) for sequence alignment and protein translation.

GCCTGCATATTATCGCCGAGTCTGTAATCATGACAACAACTTAAAAGTAGTAGTCA
TGGTGATAGAAATATTTAGCTAGCTAGCTCACATGCTGTCATGCCTGTGCGTCGCAG
GGGAATCTTATAAAAAGTGTGCGAACATATTGTGGTGATCAGTAATTCGTCGTAGGT
CGAGCACGACGATTGCGAAAAGGGAGCAGCTACGCAAAATGTTTGTTCTAATATAC
CTGTTGATCGCGATCTCCTCGCTTTTGGCCTACTTGTACCACCGCAACTTCAACTACT
GGAATCGCCGCGGCGTGCCACACGATGCTCCTCACCCACTGTATGGCAACATGGTC
GGGTTCCGGAAGAACCGGGTGATGCACGACTTCTTCTACGACTACTACAACAAGTA
CCGGAAGAGCGGCTTTCCCTTCGTGGGCTTTTACTTTCTGCACAAGCCGGCCGCCTT
CATCGTGGACACCCAGCTGGCCAAGAACATCCTGATCAAGGATTTCTCGAACTTTG
CCGATCGTGGCCAGTTTCACAACGGGCGCGACGACCCGCTCACGCAGCACCTGTTC
AACCTGGACGGAAAGAAGTGGAAGGACATGCGCCAGAGGCTGACGCCGACTTTCA
CCTCGGGCAAGATGAAGTTCATGTTCCCGACGGTGATCAAGGTGTCTGAGGAGTTC
GTCAAGGTGATCACGGAGCAGGTGCCCGCCGCCCAGAACGGCGCTGTGCTCGAGA
TCAAGGAGCTGATGGCCAGGTTCACCACCGATGTGATTGGCACCTGTCGCTTCGGC
ATTGAGTGTAACACGCTGCGCACCCCTGTCAGTGATTTCCGCACCATGGGACAGAA
GGTGTTCACCGATATGCGCCACGGGAAACTGCTGACCATGTTCGTGTTCAGCTTTCC
CAAGCTGGCCAGCAGGTTGAGAATGCGCATGATGCCCGAGGACGTCCACCAGTTCT
TCATGCGCCTGGTCAACGACACGATTGCCCTCAGGGAGCGGGAGAACTTCAAGAG
GAACGACTTCATGAACCTGCTGATTGAACTGAAGCAGAAGGGGCGCGTCACCCTG
GACAACGGAGAGGTGATCGAGGGCATGGACATCGGCGAACTGGCCGCCCAGGTGT
TCGTCTTTTATGTGGCCGGATTTGAGACCTCCTCCTCGACAATGAGTTACTGCCTGTA
TGAGTTGGCTCAGAATCAGGACATTCAGGACAGGCTGCGCAACGAGATCCAAACG
GTGCTGGAGGAACAGGAGGGGCAGCTAACGTACGAATCCATCAAAGCCATGACCTA
CTTGAACCAGGTCATCTCAGG*TAGGTGTTCTGTCTGAAGCACAGCCCCAAGAGGGATT*
*GTAACTGGTTTTACCCAACATTGCTCTTCAG*AAACCCTGAGGCTCTACACACTGGTGCC
CCACCTCGAACGGAAGGCCCTCAACGACTACGTGGTGCCGGGCCATGAAAAGCTT
GTGATTGAGAAGGGCACACAGGTCATAATCCCCGCTTGCGCCTACCACCGCGACGA
GGATCTTTATCCGAATCCGGAGACCTTTGATCCGGAGCGCTTCTCGCCGGAGAAAGT
GGCCGCCCGGGAGTCCGTGGAGTGGCTGCCCTTCGGCGACGGGCCGCGGAACTGC
ATCGGGATGCGGTTTGGACAAATGCAGGCTCGCATCGGTTTGGCTCAGATCATCAGC
CGGTTCAGGGTATCCGTCTGCGATACGACAGAGATCCCACTGAAGTATAGTCCCATG
TCCATAGTTTTGGGCACCGTTGGGGGCATCTACTTGCGAGTGGAACGCATCTAACCT
CCATATTCGTTGCTCCCATGTATATAGCTTAGGATCCAAAGCTAAAGTGATGTACATTT
TAGACTGTTCAATTATTAAATAACCTTAACCTAAACAGCCATATTAACTTATTGGCCT
GTGATAAATCACTTATGTTCACAGTTTAGAAGATCTAATTGTCTACCAGTTAGATGCA
TTCAGGCAAATGTTGTTCCCATCAGATTTGCAGGTTCATAAATTGCATTCGGCTTAAG
TTGCAACATGCCACG**CGAATCGCCACCTGACTAGT**

Figure 4. The primer design for *Cyp6a2* sequencing. Blue text represents forward primers, while red text represents reverse primers. The bold primers are those used for the PCR amplification, while non-bolded primers were used for the sequencing reaction. The start and stop codons are underlined, while the only intron has been italicized.

CHAPTER 3

ROLE OF UAS-RNAi VALIDATED GENES IN DDT SENSITIVITY AND RESISTANCE

3.1 Role of cuticular genes in DDT sensitivity and resistance

In order to establish that the *Cyp4g1* gene was over transcribed by *91-R* versus *CS* flies, RT-qPCR analyses were carried out (Fig. 5). The expression level of *Cyp4g1* was significantly increased 1.4-fold in *91-R* flies compared with *CS* flies. This finding correlates to the increased abundance of cuticular hydrocarbons associated with the epicuticle layer in *91-R* flies and may be responsible, in part, for the decreased rate of DDT penetration when topically applied [13]. Three candidate cuticular genes, *Cyp4g1*, *Lcp1*, and *Cpr72Ec*, were then selected for UAS-RNAi testing using the criteria described in Table 2.

To determine the most appropriate time for heat shock-induced RNAi experiments, a time course experiment was carried out where F1 progeny from crosses of the Driver 1799 and UAS-RNAi line 102864 (*Cyp4g1*) were either non-heat shocked (NHS, control) or heat shocked (HS) at 3, 6, or 9 days post oviposition. The same 5 hydrocarbon peaks that were significantly more abundant in the *91-R* strain compared with *CS* strain (peak 1, 9-triacosene; peak 2, triacosane; peak 3, pentacosane; peak 4, heptocosadiene; peak 5, heptocosane) [13], were analyzed for abundance changes in the HS versus the NHS groups ($p<0.05$) (Fig. 6). No significant differences in the five hydrocarbon peaks were found between the NHS and HS flies at either 3 or 6 days post oviposition. Following HS at 9 day post oviposition, hydrocarbon peaks 1, 3, 4, and 5 were all significantly reduced compared to the corresponding NHS flies ($p<0.05$). Peak 1 was reduced by 94%, peak 3 by 83%, peak 4 by 88%, and peak 5 by 86% (Fig. 6). Peak 2 was also reduced but not significantly.

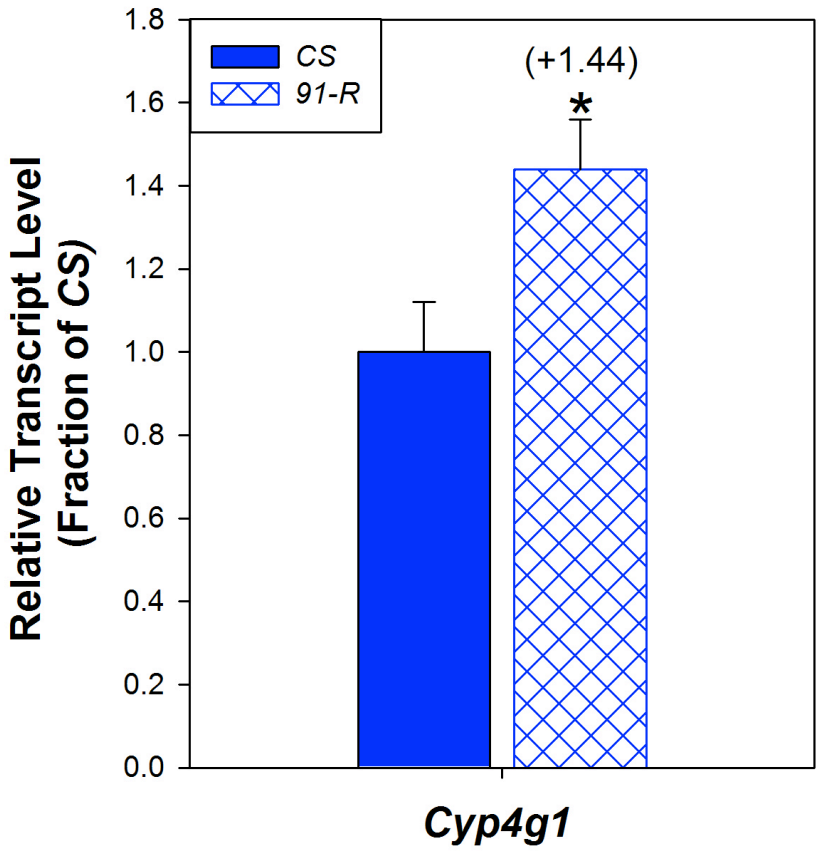


Figure 5. *Cyp4g1* was over transcribed in the DDT-resistant *91-R* strain compared to the insecticide-susceptible *CS* strain. Relative transcript levels were measure by RT-qPCR and analyzed using the ΔΔCT method with mean values ± S.D. of 3 biological replicates reported. The value in parentheses indicated the fold transcript increase over the *CS* strain. A single asterisks (*) indicates a statistically significant increase in the transcript level using Student's *t*-test ($p<0.05$).

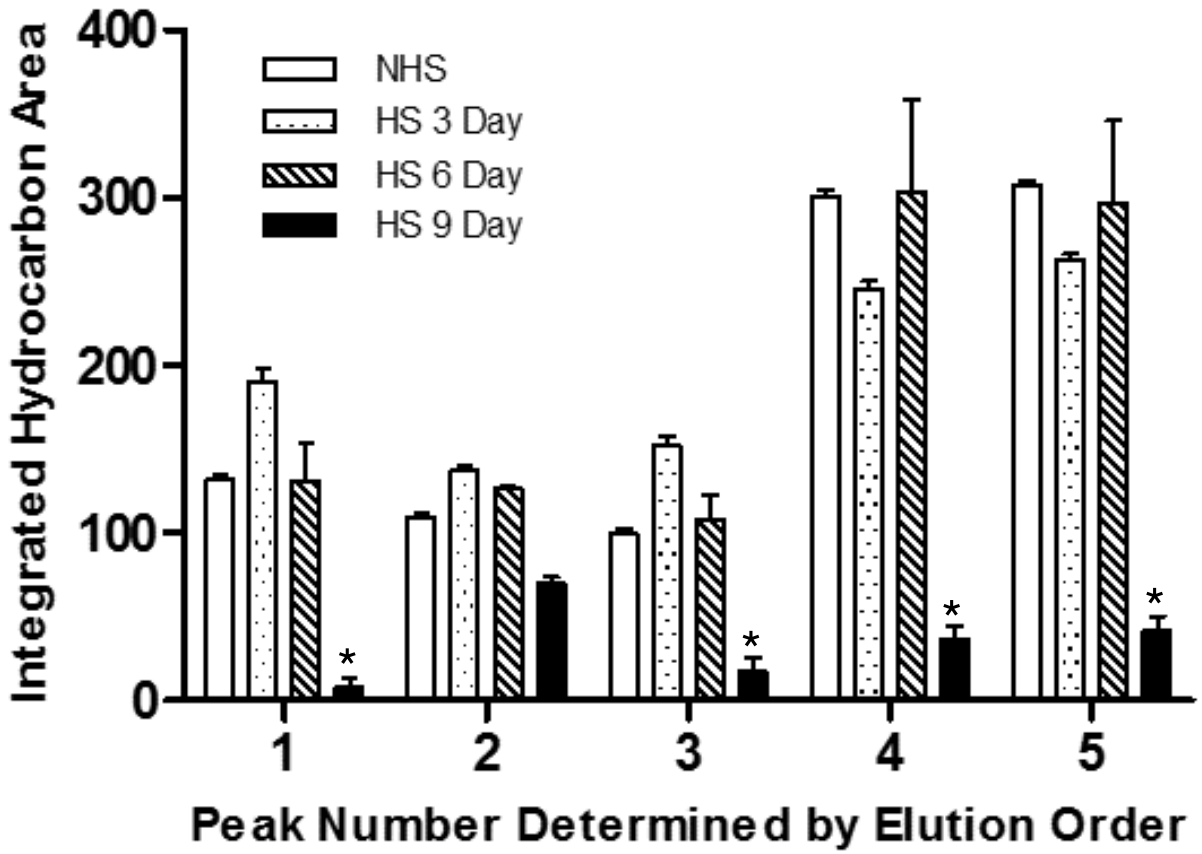


Figure 6. Cuticular hydrocarbon analyses by GC-FID of 3 biological replicates (15 females each) of crosses between Driver 1799 and UAS-RNAi line 102864 (*Cyp4g1*). Samples were either non-heat shocked (NHS), or heat shocked (HS) at 3, 6, or 9 days post oviposition. A grouped, two-way ANOVA analysis was performed to determine the significance of hydrocarbon peak reduction seen between HS versus NHS F1 females using S.E.Ms. A single asterisk (*) indicates statistically significant reduction in hydrocarbon abundances after HS induced RNAi compared with the NHS flies ($p<0.05$).

Following the establishment of a heat shock protocol (9 days post oviposition), crosses between the heat shock-inducible Gal4 Driver 1799 and the UAS-RNAi lines containing the inverted gene repeats for the 3 genes putatively involved in reduced cuticular penetration of DDT (*Cyp4g1, Lcp1*, and *Cpr72Ec*) were performed as described above. The F1 progeny were tested by RT-qPCR analysis to confirm gene knockdown and DDT sensitivity was determined by mortality bioassays. For *Cyp4g1, Lcp1*, and *Cpr72EC,* the relative transcript levels as determined by RT-qPCR were significant reduced ($p<0.05$) by 50% (+/- 6%), 57% (+/- 9%), and 48% (+/- 4%), respectively, and indicated that the UAS RNAi knockdown strategy was functional (Fig. 7A). In the DDT mortality bioassays, however, increases in susceptibility to DDT following RNAi knockdown were only significant in the *Cyp4g1* and *Lcp1* knockdown flies as determined by their respective LT_{50} values (Fig. 7B). *Cyp4g1* RNAi resulted in a 25% reduction in the LT_{50} value ($\chi 2$; d.f.; Tail Probability, 56.96; 2; <0.001), and *Lcp1* RNAi resulted in a 14% reduction ($\chi 2$; d.f.; Tail Probability, 16.04; 2; <0.001). These findings indicate that both *Cyp4g1* and *Lcp1* are involved in DDT sensitivity and may play a role in decreasing the rate of DDT penetration in resistant flies, perhaps in a synergistic fashion. It is also of interest to note that *91-R* flies had a significantly thicker cuticle than the *CS* flies and that the endocuticle of *91-R* flies was noticeably more laminated [13].

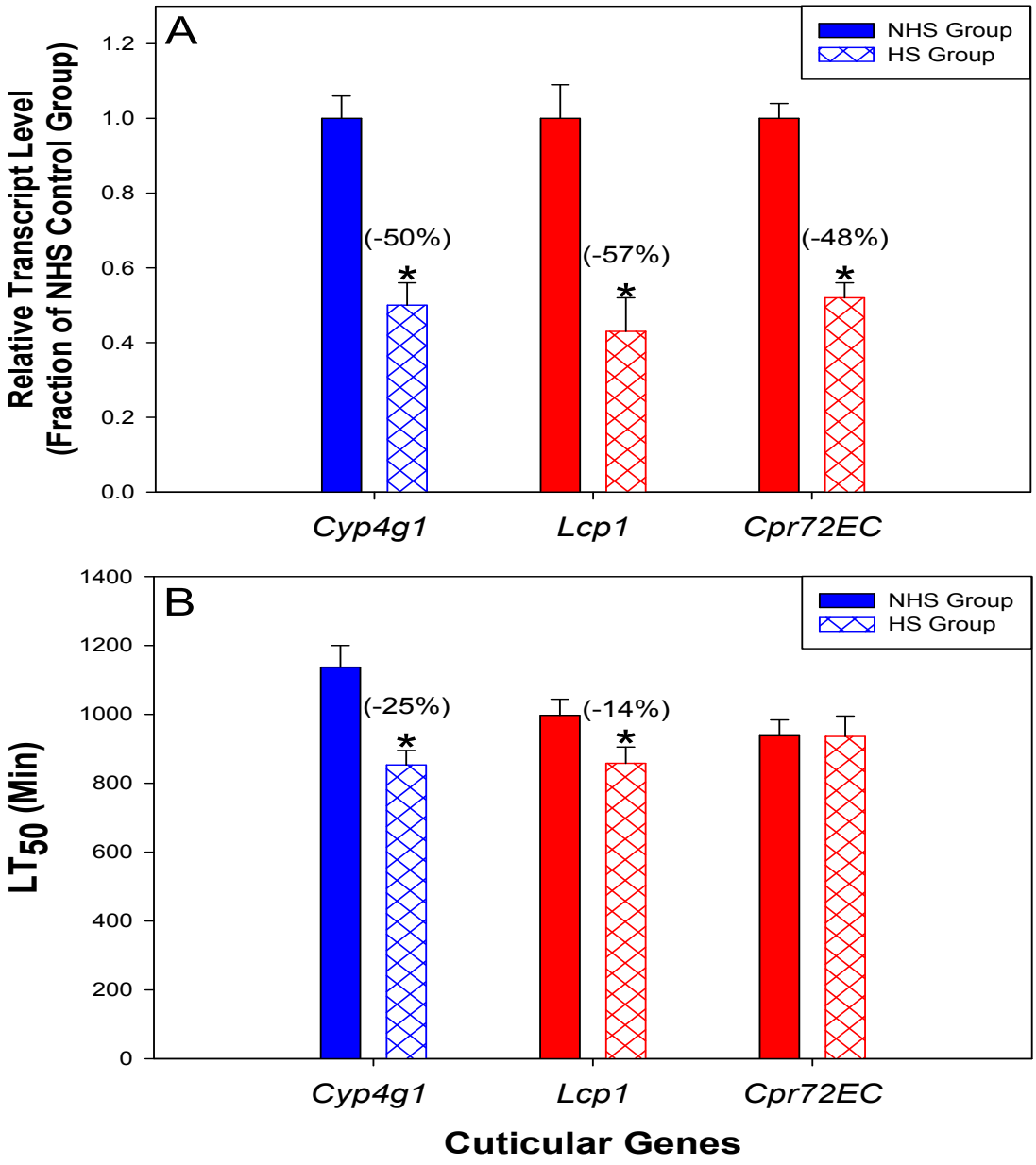


Figure 7. UAS-RNAi qPCR and mortality bioassay validation of genes putatively involved in the penetration of DDT. Following crosses of the Driver 1799 and appropriate UAS-RNAi lines, control NHS F1 females were compared to the day 9 HS RNAi groups. Three biological replicates of 30 F1 females each at 14 days post oviposition used for RT-qPCR analysis (Panel A). For mortality bioassays, three groups of 10 F1 females each were used and DDT mortality observations were taken every hour for 24 h (Panel B). Values in parentheses represent percent reductions in either transcript levels or LT_{50} values of HS versus NHS groups, respectively. A single asterisk (*) indicates statistically significant reductions in either transcript levels using Students *t*-test in Panel A or in LT_{50} values using the maximum-log likelihood ratio test in Panel B (both at $p<0.05$).

3.2 Role of phase I or II metabolism genes in DDT sensitivity and resistance

Seven candidate genes putatively involved in phase I (*Cyp6g1, Cyp12d1, Cyp6a8, Cyp6g2, Cyp6a2*) or phase II (*GstE5, GstE6*) metabolism of DDT were selected for UAS-RNAi testing using the criteria described in Table 2. Crosses between the heat shock-inducible Gal4 Driver 1799 and the UAS-RNAi lines containing the gene knockdown sequences of these genes were performed as described above. The relative transcript levels were significantly reduced ($p<0.05$) by 68% (+/- 4%), 59% (+/- 7%), 57% (+/- 6%), 50% (+/- 5%), 63% (+/- 3%) 51% (+/- 5%) and 55% (+/- 3%) for *Cyp6g1*, *Cyp12d1*, *Cyp6a8*, *Cyp6g2*, *Cyp6a2*, *GstE5* and *GstE6*, respectively, indicating successful gene knockdown (Fig. 8A). In the DDT mortality bioassays, however, increases in susceptibility to DDT following RNAi knockdown were only significant in the *Cyp6g1* and *Cyp12d1* knockdown flies as determined by their respective LT_{50} values (Fig. 8B). *Cyp6g1* RNAi resulted in a 16% reduction in the LT_{50} value ($\chi 2$; d.f.; Tail Probability, 22.00; 2; <0.001), and *Cyp12d1* RNAi resulted in a 10% reduction ($\chi 2$; d.f.; Tail Probability, 9.54; 2; 0.008). These findings indicate that both *Cyp6g1* and *Cyp12d1* are involved in DDT sensitivity in the transgenic flies and may play a role in the metabolism of DDT and perhaps in resistance. Of note is the finding that one of the major metabolites formed from DDT by *91-R* flies was dicofol, its hydrolytic breakdown product, dichlorobenzophenone, and their respective water-soluble conjugates [13]. Interestingly, *Cyp12d1* in *91-R* flies has a premature stop codon and therefore is not involved in the oxidative metabolism of DDT [15] and suggests a major role of *Cyp6g1* in the oxidative metabolism and detoxification of DDT in the *91-R* strain.

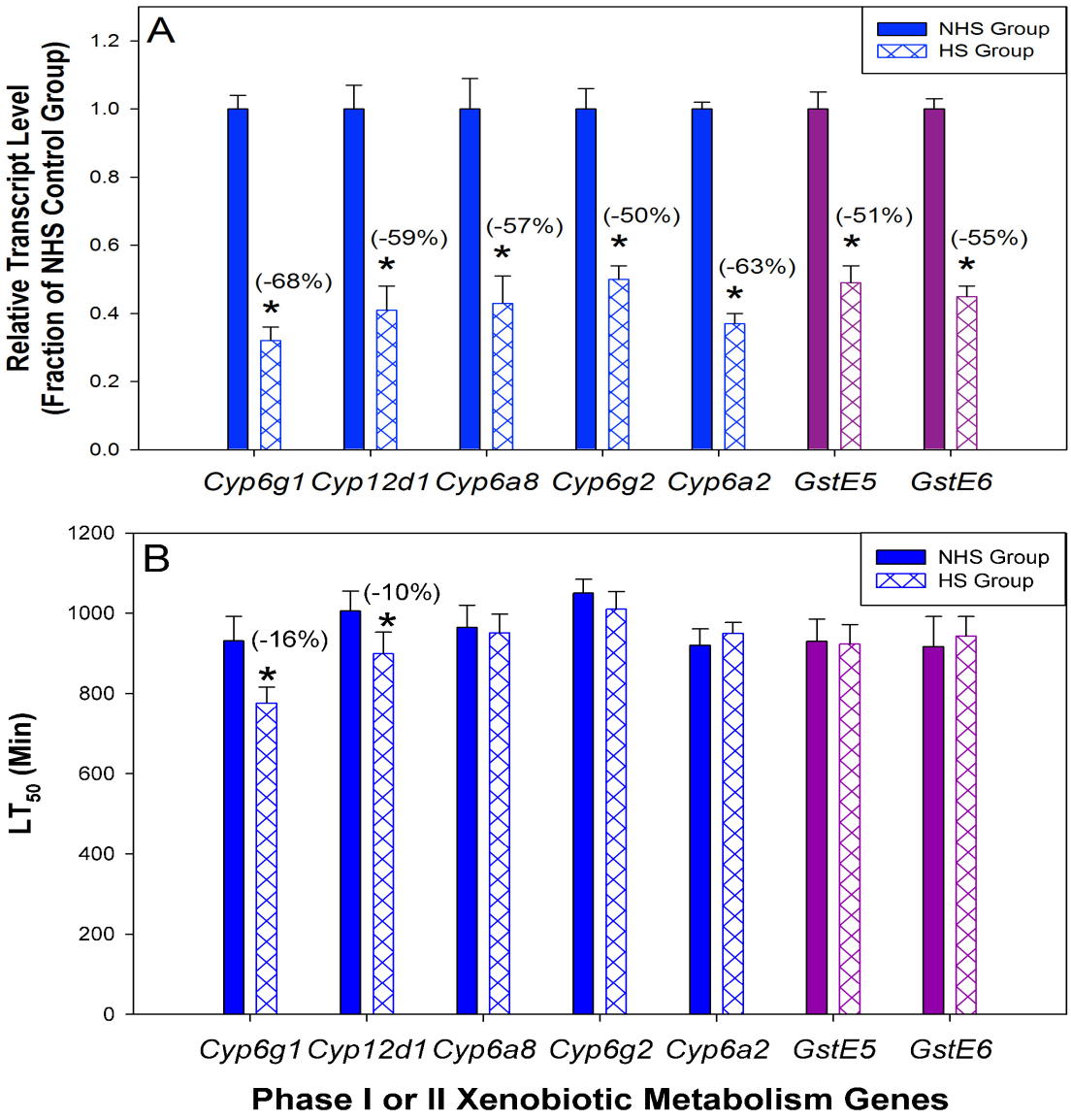


Figure 8. UAS-RNAi qPCR and mortality bioassay validation of genes involved in the phase I or II xenobiotic metabolism of DDT. Following crosses of the Driver 1799 and appropriate UAS-RNAi lines, control NHS F1 females were compared to the day 9 HS RNAi F1 females. Three biological replicates of 30 F1 females each at 14 days post oviposition were used for RT-qPCR analysis (Panel A). For mortality bioassays, three groups of 10 female flies each were used and DDT mortality observations were taken every hour for 24 h (Panel B). Values in parentheses represent percent reductions in transcript levels or LT_{50} values of HS versus NHS groups, respectively. A single asterisk (*) indicates statistically significant reductions in either the transcript levels using Students *t*-test in Panel A or in LT_{50} values as determined by the maximum-log likelihood ratio test in Panel B (both at $p<0.05$).

GstE5 and *GstE6* were also determined to be over transcribed in *91-R* flies and are hypothesized to provide indirect protection by scavenging reactive oxygen species (ROS) produced during the stress caused by DDT exposure and metabolism [15]. The lack of DDT effect in mortality bioassays using the respective heat shocked transgenic flies may be that these genes are simply not expressed or expressed only at low levels. An alternative explanation is that *GstE5* and *GstE6* are simply not needed in the insecticide-susceptible UAS-RNAi flies because they are not metabolizing DDT to the same extent as seen in the *91-R* flies. Nevertheless, they still may be necessary to offset the ROS generation through constitutive over expression of P450s seen in the *91-R* strain. It is possible therefore that RNAi of these genes in the DDT-resistant *91-R* strain may result in increases in the susceptibility to DDT due to impairment of the GSTs ability to offset ROS production, even though increases in susceptibility were not seen in the insecticide-susceptible UAS-RNAi flies.

3.3 Role of phase III metabolism genes in DDT sensitivity and resistance

Seven candidate genes involved in phase III metabolism, *Mdr49*, *Mdr50*, *Mdr65*, *Mrp1*, *CG11817*, *CG5789*, and *CG7806*, were selected for UAS-RNAi testing using the criteria described in Tables 2. Crosses were performed and tested as before. Relative transcript levels were significantly reduced ($p<0.05$) by 58% (+/- 5%), 49% (+/- 3%), 61% (+/- 4%), 54% (+/- 6%), 59% (+/- 5%), 52% (+/- 7%), and 56% (+/- 4%) for *Mdr50*, *Mdr65*, *Mdr49*, *Mrp1*, *CG11817*, *CG5789*, and *CG7806*, respectively, indicating successful gene knockdown (Fig. 9A). In the DDT mortality bioassays, however, increases in susceptibility to DDT following RNAi knockdown were only significantly increased in the *Mdr50*, *Mdr65*, and *Mrp1* knockdown flies as determined by their respective LT_{50} values (Fig. 9B). *Mdr50* RNAi resulted in a 13% reduction in the LT_{50} value ($\chi 2$; d.f.; Tail Probability, 16.56; 2; <0.001), *Mdr65* RNAi resulted in a 12% reduction ($\chi 2$; d.f.; Tail Probability, 11.94; 2; 0.002), and *Mrp1* RNAi resulted in a 15% reduction ($\chi 2$; d.f.; Tail Probability, 23.70; 2; <0.001). These findings indicate that both ABC B- and C-types of ABC transporters are involved in DDT sensitivity in the transgenic flies and may play a role in the ATP-driven efflux of DDT and perhaps in resistance. As shown previously, verapamil, a well-established substrate for ABC B-Type transporters, pretreatment significantly synergized the toxic action of DDT on *91-R* flies but had only limited effect on *CS* flies [13]. Furthermore, *91-R* flies effluxed 3.3-fold more unmetabolized DDT than *CS* flies.

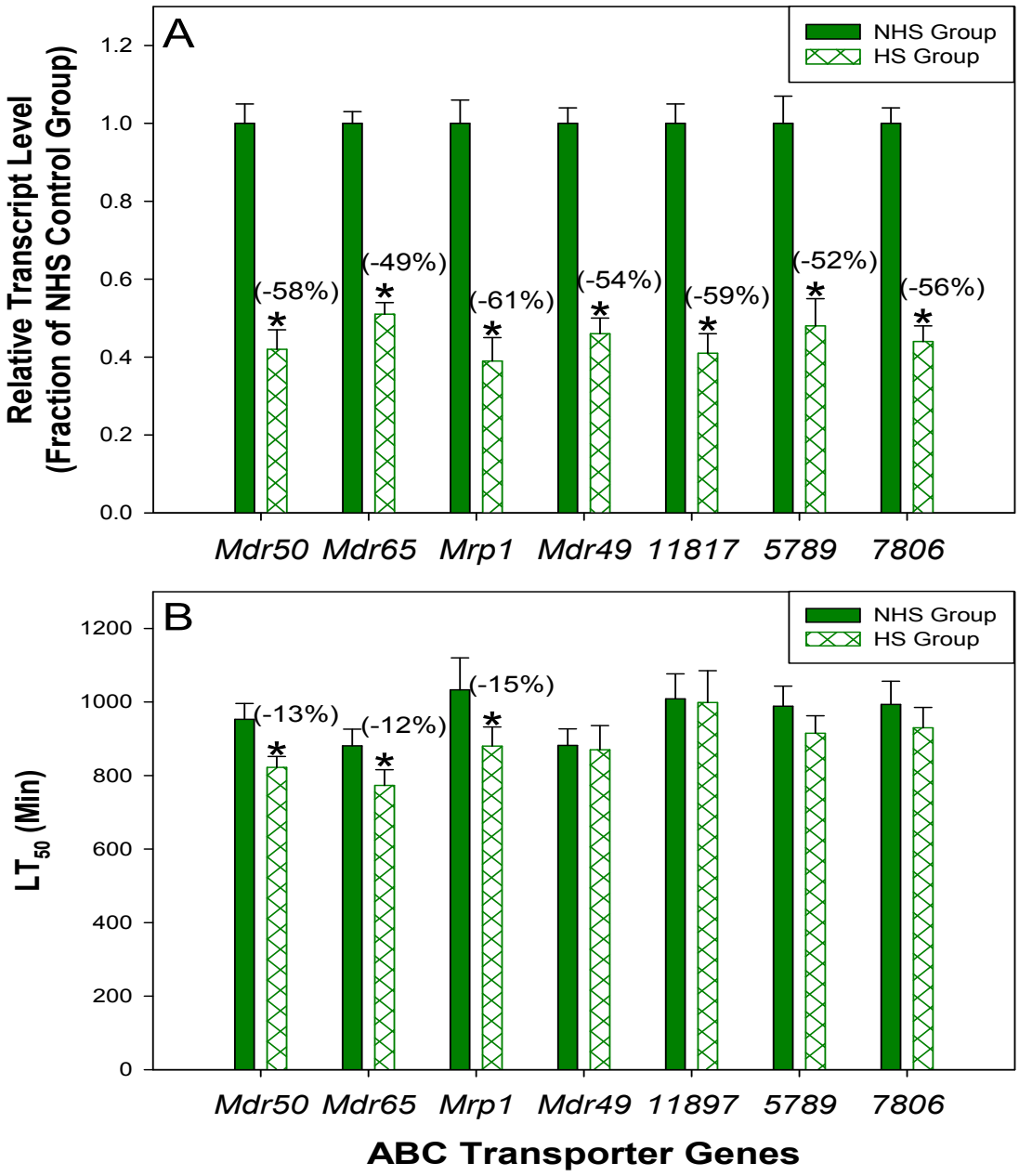


Figure 9. UAS-RNAi qPCR and mortality bioassay validation of genes involved in the phase III metabolism of DDT. Following crosses of the Driver 1799 and appropriate UAS-RNAi lines, control NHS F1 females were compared to the day 9 HS RNAi F1 females. Three biological replicates of 30 F1 females each at 14 days post oviposition were used for RT-qPCR analysis (Panel A). For mortality bioassays, three groups of 10 female flies each were used and mortality observations were taken every hour for 24 h (Panel B). Values in parentheses represent either percent reductions in transcript levels or LT_{50} values of HS versus NHS groups, respectively. A single asterisk (*) indicates statistically significant reductions in either the transcript levels using Students *t*-test in Panel A or in LT_{50} values as determined by the maximum-log likelihood ratio test in Panel B (both at $p<0.05$).

In an attempt to knock down the ABC transporters believed to efflux DDT in the *91-R* strain, intra-abdominal dsRNA injections were performed in anesthetized *91-R* female flies [73, 74]. First, the resulting T7-PCR product was visualized by (0.9%) agarose gel with ethidium bromide staining (Fig. 10A). Because there were multiple bands present after the T7-PCR, a gel purification protocol was utilized to extract the band of interest to use as a template in the dsRNA synthesis reaction. After gel extraction purification, the dsRNA was synthesized, purified, annealed, and electrophoresed on a (0.7%) formaldehyde agarose gel utilizing ethidium bromide staining for visualization (Fig. 10B). After the RNA synthesis, only a single band was present of the expected size (~500 bp).

After dsRNA injection, however, no decrease in the transcript levels of the ABC transporters were found in the 91-R line. There are several possible explanations for this, such as inefficient cellular uptake of the dsRNA, or perhaps inefficient dsRNA constructs against the genes of interest. In the future, it would make sense to design a dsRNA that is the same as that expressed by the UAS-RNAi line, in order to determine the reason why the particular dsRNAs that were injected did not have an effect on the transcript level.

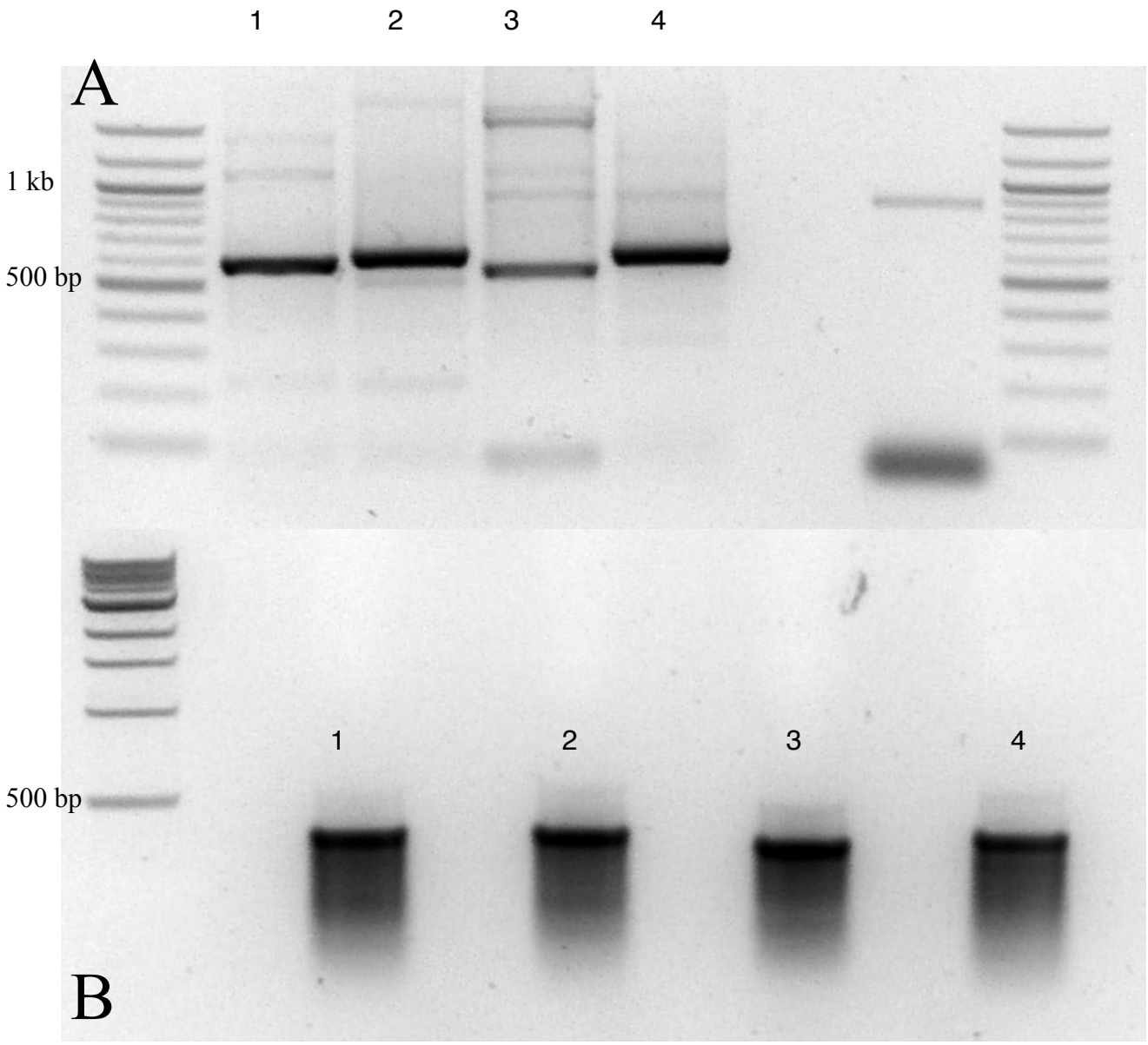


Figure 10. The synthesis of gene specific dsRNAs for intra-abdominal injection induced RNAi against the ABC-Transporters *Mdr49* (Lane 1, 553 bp), *Mdr50* (Lane 2, 509 bp), *Mdr65* (Lane 3, 508 bp), and *Mrp1* (Lane 4, 577 bp) consisted of two steps. Panel A shows the products after PCR amplification using cDNA as template. Due to the presence of multiple bands, the PCR products were gel purified before running the T7 MEGAscript RNA reaction. Panel B shows the resulting dsRNA that was annealed and electrophoresed on a formaldehyde denaturing agarose gel to confirm bands of the expected size were present.

3.4 Discussion

Of the 17 genes tested by UAS-RNAi, 7 were found to have a significant effect on susceptibility to DDT as determined by mortality bioassay (Fig. 11). Knockdown of *Cyp4g1* was found to increase susceptibility to DDT by 25% and this gene is located on the X chromosome (chromosome 1); the only sex-linked resistance factor that has been determined to date. *Lcp1*, *Cyp6g1*, *Cyp12d1*, and *Mdr50* are located on chromosome 2R and *Mrp1* is located on the left arm (2L) of chromosome 2. UAS-RNAi resulted in a 14%, 17%, 11%, 13% and 15% increase in susceptibility to DDT, respectively, making this chromosome prominent in DDT resistance. *Mdr65* is located on chromosome 3L and its knockdown by UAS-RNAi resulted in a 12% increase in susceptibility to DDT. Thus, multiple genes across all three chromosomes appear to be involved in DDT resistance in the highly resistant *91-R* strain. These results are consistent with the original suggestion that DDT resistance in the *91-R* strain is multifactorial and that factors on all three chromosomes are involved in DDT resistance [8-11]. Factors on chromosome 2 were suggested to play a major role, followed by factor(s) on chromosome 3, with the factor(s) on chromosome 1 showing a much smaller but statistically significant effect [19]. It was also suggested that the factors on chromosomes 2 and 3 interacted in a more than additive manner. Our results therefore suggest that the *Cyp4g1* gene on chromosome 1 is a likely candidate as the resistance factor associated with this sex chromosome. Also *Mdr65* on chromosome 3 is likely a resistance factor and may interact with either the P450 genes or the other ABC transporter genes on chromosome 2 in a synergistic fashion, resulting in extremely high levels of resistance. This conjecture, however, will need future experimental proof.

What is not yet known is how these resistance mechanisms interact with each other. Will

it be in a less than additive, additive, or greater than additive manner? By injecting dsRNA into *91-R* flies for each of the identified 7 genes individually, and then in combination, we should be able to determine how these multiple resistance mechanisms, found across all three chromosomes, interact.

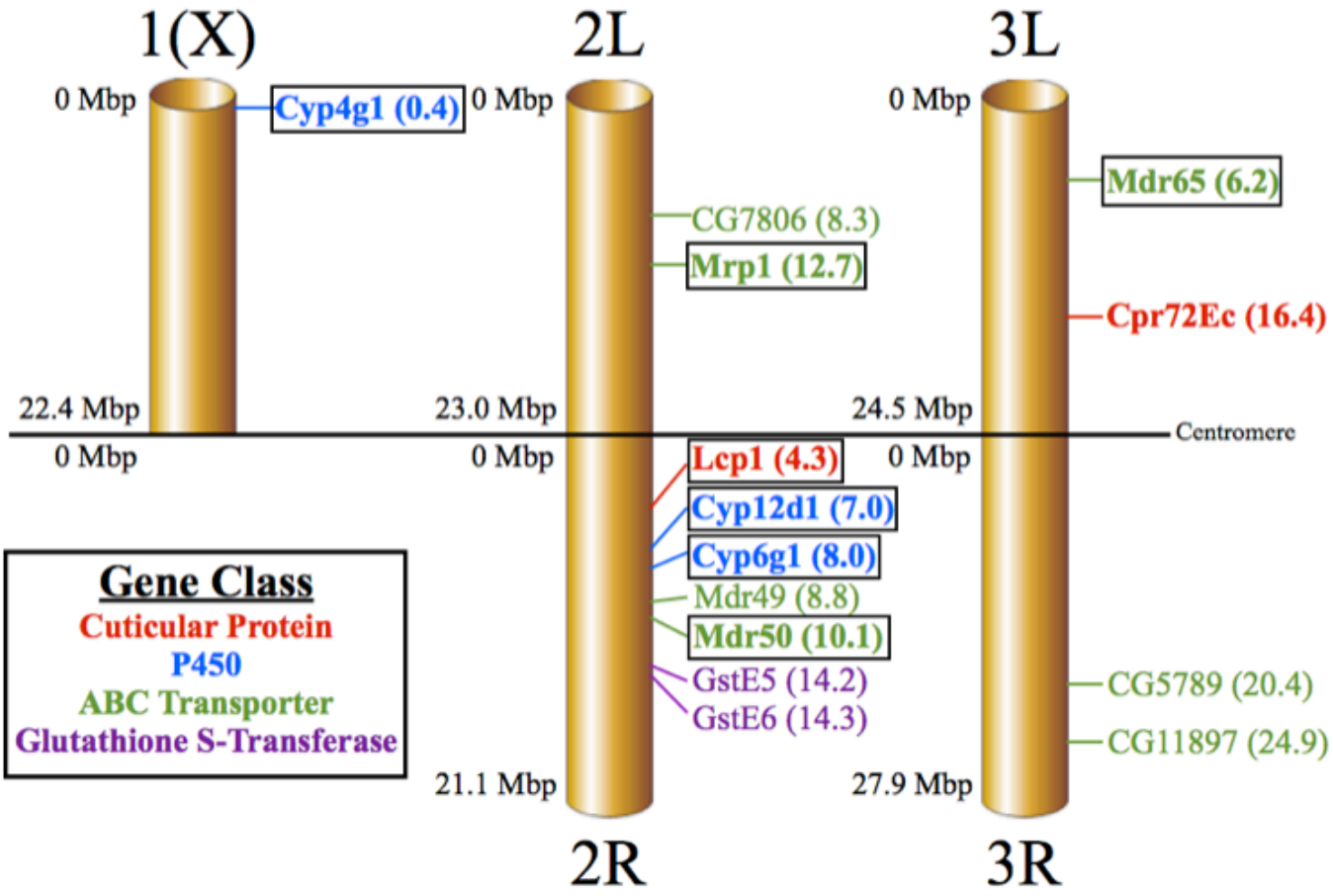


Figure 11. Genes putatively involved in DDT resistance through literature and bioinformatics review validated through use of the UAS-RNAi system (number in parentheses are approximate chromosome locations in mega-basepairs as determined by NCBI Map Viewer). The right arm of chromosome 2 seems to be highly involved in DDT resistance, with 4 of the 7 resistance factors present.

CHAPTER 4

SEQUENCING OF *CYP6A2*

4.1 Nucleotide sequence analysis

In order to determine if the mutations that enabled DDT metabolism found in the *Cyp6a2*SVL line were present in the highly DDT-resistant *91-R* strain, the *Cyp6a2* gene was sequenced across several strains of *D. melanogaster*. These strains included the DDT-susceptible *CS,* DDT-susceptible UAS-Cyp6a2 line (*Cyp6a2* RNAi*),* slightly DDT-resistant *91-C,* highly DDT-resistant *91-R,* and the *Cyp6a2*SVL mutant, which can metabolize DDT [56]. In order to assemble the full length sequence, the five forward and reverse sequencing reactions that spanned the gene were assembled as contigs. In all cases, the individual sequencing reactions overlapped by a minimum of 100 bp. The ends were trimmed to include only the highest quality nucleotide signals, and assembled end to end. The end result was a ~ 2100 bp continuous read length sequence that could be compared between all sequenced lines.

After PCR amplification, the resulting products were run on an agarose (0.9%) gel to confirm the bands were of the expected size. While bands were expected to be 2.0 kb in length based on the NCBI template, the *CS*, *91-C*, and UAS-Cyp6a2 lines all contained PCR products that were about 2.5 kb in length (Fig. 12). This discrepancy in band size was not due to changes in the coding region of the gene, instead it was due to the insertion of a ~500 bp fragment on the 3' region of the gene in the *CS*, *91-C*, and UAS-RNAi lines.

In the coding region, there were 21 Single Nucleotide Polymorphisms (SNP's) found (Fig. 13). While many of the SNP's found were present in only 1 of the 5 lines sequences, there were 5 mutations that were found across multiple sequenced lines, implying that these particular

mutations had been genetically selected for and were not purely random occurrences. At nucleotide position 408, both the *91-R* and *Cyp6a2*SVL strains had T, while the other 3 lines had G. At nucleotide position 679, the *CS* and *91-C* strains had A while the other lines had G. At nucleotide position 916, the *91-R* and *Cyp6a2*SVL strains had G while the other lines had A. At nucleotide position 933 the *91-R* and *Cyp6a2*SVL had A while the others had C. At nucleotide position 1301, the *91-R* and *Cyp6a2*SVL had C while the other lines had A. Of particular note, the *91-C* line has a base pair deletion at nucleotide position 1341. This deletion leads to a frame shift mutation, likely leading to a non-functional *Cyp6a2* gene. The *Cyp6a2*SVL line had been previously reported to metabolize DDT, however the highly DDT-resistant *91-R* line did not possess any of the mutations resulting in the *Cyp6a2*SVL amino acid changes associated with the *Cyp6a2*SVL line. Instead, our analysis showed additional mutations present that both the highly DDT-resistant *91-R* line and *Cyp6a2*SVL line shared (nucleotide positions 408, 916, 933, and 1301).

Further sequence analysis showed that the *CS*, *91-C*, and UAS-Cyp6a2 lines all contained a 500 bp insertion in the 3' region of *Cyp6a2,* while the *91-R* strain did not contain this insertion (Fig. 14) It is unclear what the exact consequence of this insert may be, although it is likely influential on transcript stability. The *91-R* line has *Cyp6a2* transcript levels much higher than the susceptible lines, and it is possible that this is due in part to the transposon insert.

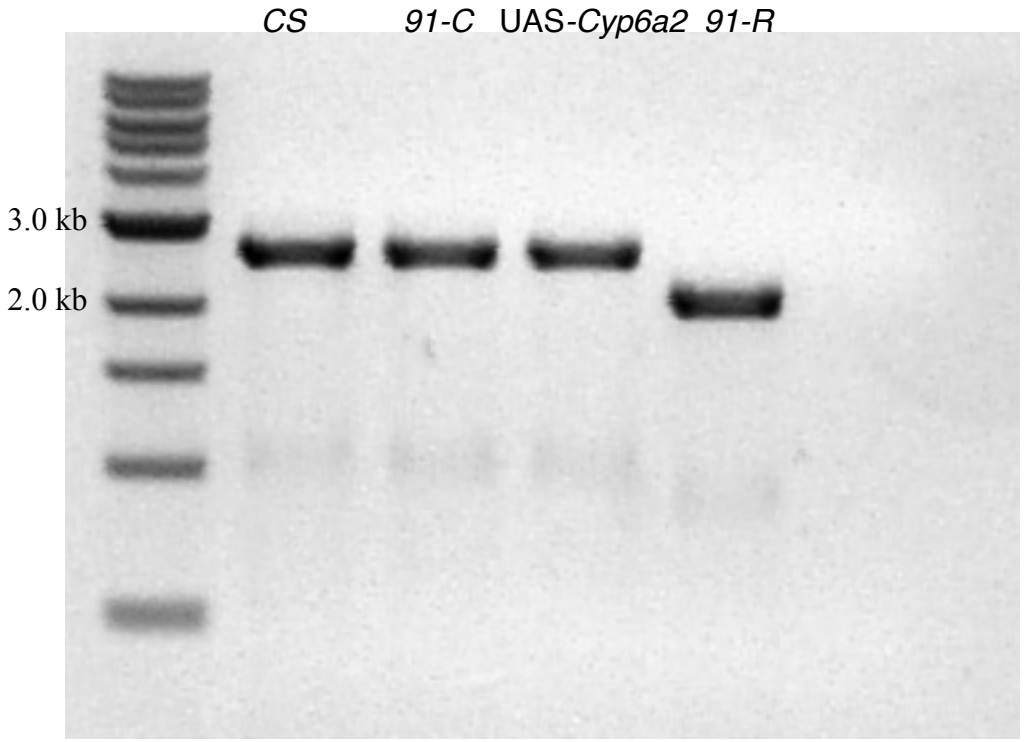
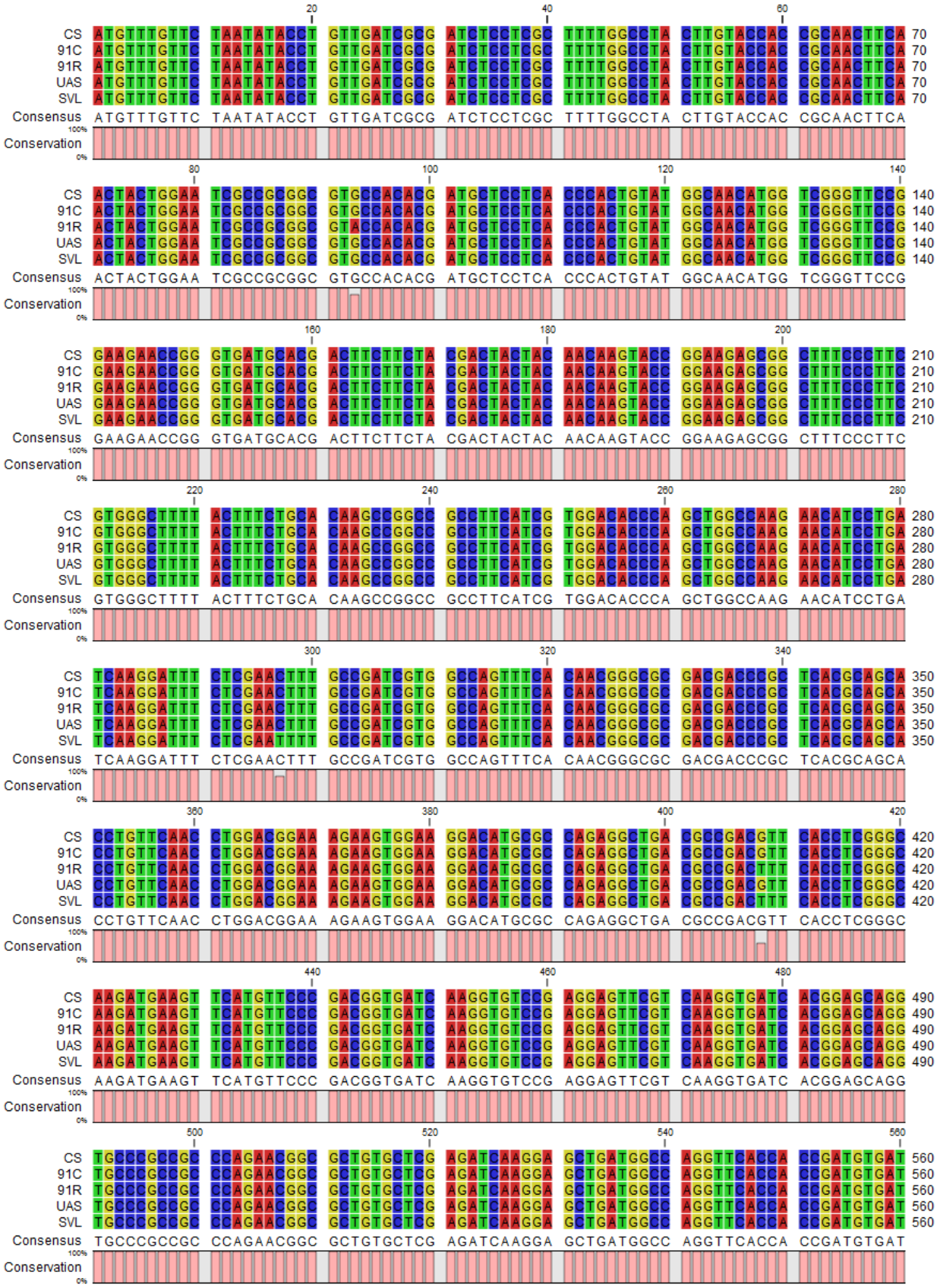


Figure 12. Agarose gel showing the PCR product of *Cyp6a2* for the *CS*, *91-C*, UAS-*Cyp6a2*, and *91-R* strains. The *91-R* band was about 2.0 kb while the other strains PCR products were about 2.5 kb.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91C ATGTTTGTTC TAATATACCT GTTGATCGCG ATCTCCTCGC TTTTGGCCTA CTTGTACCAC CGCAACTTCA 70
91R ATGTTTGTTC TAATATACCT GTTGATCGCG ATCTCCTCGC TTTTGGCCTA CTTGTACCAC CGCAACTTCA 70
UAS ATGTTTGTTC TAATATACCT GTTGATCGCG ATCTCCTCGC TTTTGGCCTA CTTGTACCAC CGCAACTTCA 70
SVL ATGTTTGTTC TAATATACCT GTTGATCGCG ATCTCCTCGC TTTTGGCCTA CTTGTACCAC CGCAACTTCA 70
Consensus ATGTTTGTTC TAATATACCT GTTGATCGCG ATCTCCTCGC TTTTGGCCTA CTTGTACCAC CGCAACTTCA
Conservation
CS ACTACTGGAA TCGCCGCGGC GTGCCACACG ATGCTCCTCA CCCACTGTAT GGCAACATGG TCGGGTTCCG 140
91C ACTACTGGAA TCGCCGCGGC GTGCCACACG ATGCTCCTCA CCCACTGTAT GGCAACATGG TCGGGTTCCG 140
91R ACTACTGGAA TCGCCGCGGC GTACCACACG ATGCTCCTCA CCCACTGTAT GGCAACATGG TCGGGTTCCG 140
UAS ACTACTGGAA TCGCCGCGGC GTGCCACACG ATGCTCCTCA CCCACTGTAT GGCAACATGG TCGGGTTCCG 140
SVL ACTACTGGAA TCGCCGCGGC GTGCCACACG ATGCTCCTCA CCCACTGTAT GGCAACATGG TCGGGTTCCG 140
Consensus ACTACTGGAA TCGCCGCGGC GTGCCACACG ATGCTCCTCA CCCACTGTAT GGCAACATGG TCGGGTTCCG
Conservation
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91C GAAGAACCGG GTGATGCACG ACTTCTTCTA CGACTACTAC AACAAGTACC GGAAGAGCGG CTTTCCCTTC 210
91R GAAGAACCGG GTGATGCACG ACTTCTTCTA CGACTACTAC AACAAGTACC GGAAGAGCGG CTTTCCCTTC 210
UAS GAAGAACCGG GTGATGCACG ACTTCTTCTA CGACTACTAC AACAAGTACC GGAAGAGCGG CTTTCCCTTC 210
SVL GAAGAACCGG GTGATGCACG ACTTCTTCTA CGACTACTAC AACAAGTACC GGAAGAGCGG CTTTCCCTTC 210
Consensus GAAGAACCGG GTGATGCACG ACTTCTTCTA CGACTACTAC AACAAGTACC GGAAGAGCGG CTTTCCCTTC
Conservation
CS GTGGGCTTTT ACTTTCTGCA CAAGCCGGCC GCCTTCATCG TGGACACCCA GCTGGCCAAG AACATCCTGA 280
91C GTGGGCTTTT ACTTTCTGCA CAAGCCGGCC GCCTTCATCG TGGACACCCA GCTGGCCAAG AACATCCTGA 280
91R GTGGGCTTTT ACTTTCTGCA CAAGCCGGCC GCCTTCATCG TGGACACCCA GCTGGCCAAG AACATCCTGA 280
UAS GTGGGCTTTT ACTTTCTGCA CAAGCCGGCC GCCTTCATCG TGGACACCCA GCTGGCCAAG AACATCCTGA 280
SVL GTGGGCTTTT ACTTTCTGCA CAAGCCGGCC GCCTTCATCG TGGACACCCA GCTGGCCAAG AACATCCTGA 280
Consensus GTGGGCTTTT ACTTTCTGCA CAAGCCGGCC GCCTTCATCG TGGACACCCA GCTGGCCAAG AACATCCTGA
Conservation
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91C TCAAGGATTT CTCGAACTTT GCCGATCGTG GCCAGTTTCA CAACGGGCGC GACGACCCGC TCACGCAGCA 350
91R TCAAGGATTT CTCGAACTTT GCCGATCGTG GCCAGTTTCA CAACGGGCGC GACGACCCGC TCACGCAGCA 350
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Consensus TCAAGGATTT CTCGAACTTT GCCGATCGTG GCCAGTTTCA CAACGGGCGC GACGACCCGC TCACGCAGCA
Conservation
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91R CCTGTTCAAC CTGGACGGAA AGAAGTGGAA GGACATGCGC CAGAGGCTGA CGCCGACTTT CACCTCGGGC 420
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Conservation
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91R AAGATGAAGT TCATGTTCCC GACGGTGATC AAGGTGTCCG AGGAGTTCGT CAAGGTGATC ACGGAGCAGG 490
UAS AAGATGAAGT TCATGTTCCC GACGGTGATC AAGGTGTCCG AGGAGTTCGT CAAGGTGATC ACGGAGCAGG 490
SVL AAGATGAAGT TCATGTTCCC GACGGTGATC AAGGTGTCCG AGGAGTTCGT CAAGGTGATC ACGGAGCAGG 490
Consensus AAGATGAAGT TCATGTTCCC GACGGTGATC AAGGTGTCCG AGGAGTTCGT CAAGGTGATC ACGGAGCAGG
Conservation
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91C TGCCCGCCGC CCAGAACGGC GCTGTGCTCG AGATCAAGGA GCTGATGGCC AGGTTCACCA CCGATGTGAT 560
91R TGCCCGCCGC CCAGAACGGC GCTGTGCTCG AGATCAAGGA GCTGATGGCC AGGTTCACCA CCGATGTGAT 560
UAS TGCCCGCCGC CCAGAACGGC GCTGTGCTCG AGATCAAGGA GCTGATGGCC AGGTTCACCA CCGATGTGAT 560
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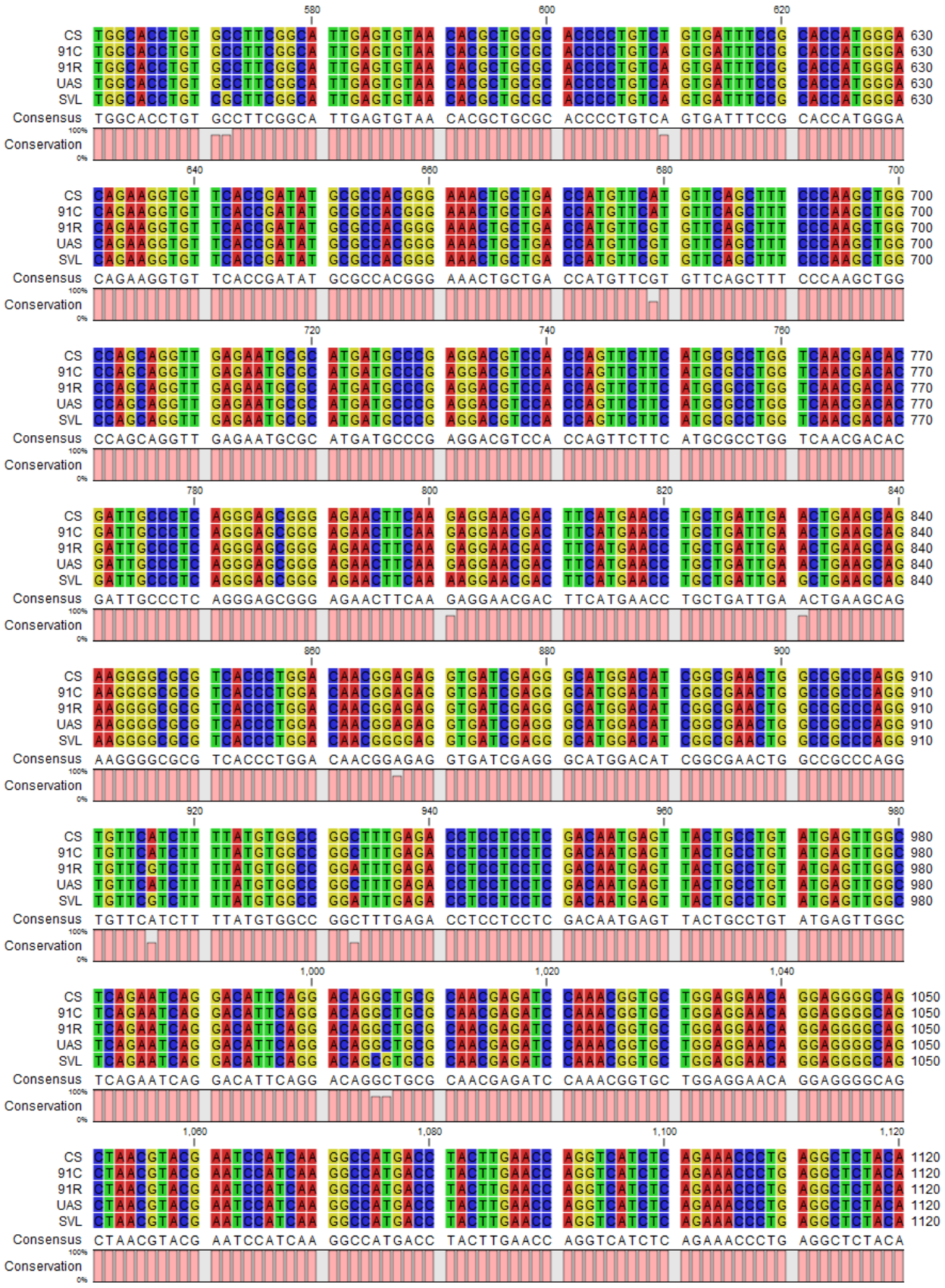
Conservation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600
620
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91R TGGCACCTGT GCCTTCGGCA TTGAGTGTAA CACGCTGCGC ACCCCTGTCA GTGATTTCCG CACCATGGGA 630
UAS TGGCACCTGT GCCTTCGGCA TTGAGTGTAA CACGCTGCGC ACCCCTGTCA GTGATTTCCG CACCATGGGA 630
SVL TGGCACCTGT GCCTTCGGCA TTGAGTGTAA CACGCTGCGC ACCCCTGTCA GTGATTTCCG CACCATGGGA 630
Consensus TGGCACCTGT GCCTTCGGCA TTGAGTGTAA CACGCTGCGC ACCCCTGTCA GTGATTTCCG CACCATGGGA
100%
Conservation
0%
640
660
680
700
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91C CAGAAGGTGT TCACCGATAT GCGCCACGGG AAACTGCTGA CCATGTTCAT GTTCAGCTTT CCCAAGCTGG 700
91R CAGAAGGTGT TCACCGATAT GCGCCACGGG AAACTGCTGA CCATGTTCGT GTTCAGCTTT CCCAAGCTGG 700
UAS CAGAAGGTGT TCACCGATAT GCGCCACGGG AAACTGCTGA CCATGTTCGT GTTCAGCTTT CCCAAGCTGG 700
SVL CAGAAGGTGT TCACCGATAT GCGCCACGGG AAACTGCTGA CCATGTTCGT GTTCAGCTTT CCCAAGCTGG 700
Consensus CAGAAGGTGT TCACCGATAT GCGCCACGGG AAACTGCTGA CCATGTTCGT GTTCAGCTTT CCCAAGCTGG
100%
Conservation
0%
720
740
760
CS CCAGCAGGTT GAGAATGCGC ATGATGCCCG AGGACGTCCA CCAGTTCTTC ATGCGCCTGG TCAACGACAC 770
91C CCAGCAGGTT GAGAATGCGC ATGATGCCCG AGGACGTCCA CCAGTTCTTC ATGCGCCTGG TCAACGACAC 770
91R CCAGCAGGTT GAGAATGCGC ATGATGCCCG AGGACGTCCA CCAGTTCTTC ATGCGCCTGG TCAACGACAC 770
UAS CCAGCAGGTT GAGAATGCGC ATGATGCCCG AGGACGTCCA CCAGTTCTTC ATGCGCCTGG TCAACGACAC 770
SVL CCAGCAGGTT GAGAATGCGC ATGATGCCCG AGGACGTCCA CCAGTTCTTC ATGCGCCTGG TCAACGACAC 770
Consensus CCAGCAGGTT GAGAATGCGC ATGATGCCCG AGGACGTCCA CCAGTTCTTC ATGCGCCTGG TCAACGACAC
100%
Conservation
0%
780
800
820
840
CS GATTGCCCTC AGGGAGCGGG AGAACTTCAA GAGGAACGAC TTCATGAACC TGCTGATTGA ACTGAAGCAG 840
91C GATTGCCCTC AGGGAGCGGG AGAACTTCAA GAGGAACGAC TTCATGAACC TGCTGATTGA ACTGAAGCAG 840
91R GATTGCCCTC AGGGAGCGGG AGAACTTCAA GAGGAACGAC TTCATGAACC TGCTGATTGA ACTGAAGCAG 840
UAS GATTGCCCTC AGGGAGCGGG AGAACTTCAA GAGGAACGAC TTCATGAACC TGCTGATTGA ACTGAAGCAG 840
SVL GATTGCCCTC AGGGAGCGGG AGAACTTCAA AAGGAACGAC TTCATGAACC TGCTGATTGA GCTGAAGCAG 840
Consensus GATTGCCCTC AGGGAGCGGG AGAACTTCAA GAGGAACGAC TTCATGAACC TGCTGATTGA ACTGAAGCAG
100%
Conservation
0%
860
880
900
CS AAGGGGCGCG TCACCCTGGA CAACGGAGAG GTGATCGAGG GCATGGACAT CGGCGAACTG GCCGCCCAGG 910
91C AAGGGGCGCG TCACCCTGGA CAACGGAGAG GTGATCGAGG GCATGGACAT CGGCGAACTG GCCGCCCAGG 910
91R AAGGGGCGCG TCACCCTGGA CAACGGAGAG GTGATCGAGG GCATGGACAT CGGCGAACTG GCCGCCCAGG 910
UAS AAGGGGCGCG TCACCCTGGA CAACGGAGAG GTGATCGAGG GCATGGACAT CGGCGAACTG GCCGCCCAGG 910
SVL AAGGGGCGCG TCACCCTGGA CAACGGGGAG GTGATCGAGG GCATGGACAT CGGCGAACTG GCCGCCCAGG 910
Consensus AAGGGGCGCG TCACCCTGGA CAACGGAGAG GTGATCGAGG GCATGGACAT CGGCGAACTG GCCGCCCAGG
100%
Conservation
0%
920
940
960
980
CS TGTTCATCTT TTATGTGGCC GGCTTTGAGA CCTCCTCCTC GACAATGAGT TACTGCCTGT ATGAGTTGGC 980
91C TGTTCATCTT TTATGTGGCC GGCTTTGAGA CCTCCTCCTC GACAATGAGT TACTGCCTGT ATGAGTTGGC 980
91R TGTTCGTCTT TTATGTGGCC GGATTTGAGA CCTCCTCCTC GACAATGAGT TACTGCCTGT ATGAGTTGGC 980
UAS TGTTCATCTT TTATGTGGCC GGCTTTGAGA CCTCCTCCTC GACAATGAGT TACTGCCTGT ATGAGTTGGC 980
SVL TGTTCGTCTT TTATGTGGCC GGATTTGAGA CCTCCTCCTC GACAATGAGT TACTGCCTGT ATGAGTTGGC 980
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100%
Conservation
0%
1,000
1,020
1,040
CS TCAGAATCAG GACATTCAGG ACAGGCTGCG CAACGAGATC CAAACGGTGC TGGAGGAACA GGAGGGGCAG 1050
91C TCAGAATCAG GACATTCAGG ACAGGCTGCG CAACGAGATC CAAACGGTGC TGGAGGAACA GGAGGGGCAG 1050
91R TCAGAATCAG GACATTCAGG ACAGGCTGCG CAACGAGATC CAAACGGTGC TGGAGGAACA GGAGGGGCAG 1050
UAS TCAGAATCAG GACATTCAGG ACAGGCTGCG CAACGAGATC CAAACGGTGC TGGAGGAACA GGAGGGGCAG 1050
SVL TCAGAATCAG GACATTCAGG ACAGGCTGCG CAACGAGATC CAAACGGTGC TGGAGGAACA GGAGGGGCAG 1050
Consensus TCAGAATCAG GACATTCAGG ACAGGCTGCG CAACGAGATC CAAACGGTGC TGGAGGAACA GGAGGGGCAG
100%
Conservation
0%
1,060
1,080
1,100
1,120
CS CTAACGTACG AATCCATCAA GGCCATGACC TACTTGAACC AGGTCATCTC AGAAACCCTG AGGCTCTACA 1120
91C CTAACGTACG AATCCATCAA GGCCATGACC TACTTGAACC AGGTCATCTC AGAAACCCTG AGGCTCTACA 1120
91R CTAACGTACG AATCCATCAA GGCCATGACC TACTTGAACC AGGTCATCTC AGAAACCCTG AGGCTCTACA 1120
UAS CTAACGTACG AATCCATCAA GGCCATGACC TACTTGAACC AGGTCATCTC AGAAACCCTG AGGCTCTACA 1120
SVL CTAACGTACG AATCCATCAA GGCCATGACC TACTTGAACC AGGTCATCTC AGAAACCCTG AGGCTCTACA 1120
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100%
Conservation
0%

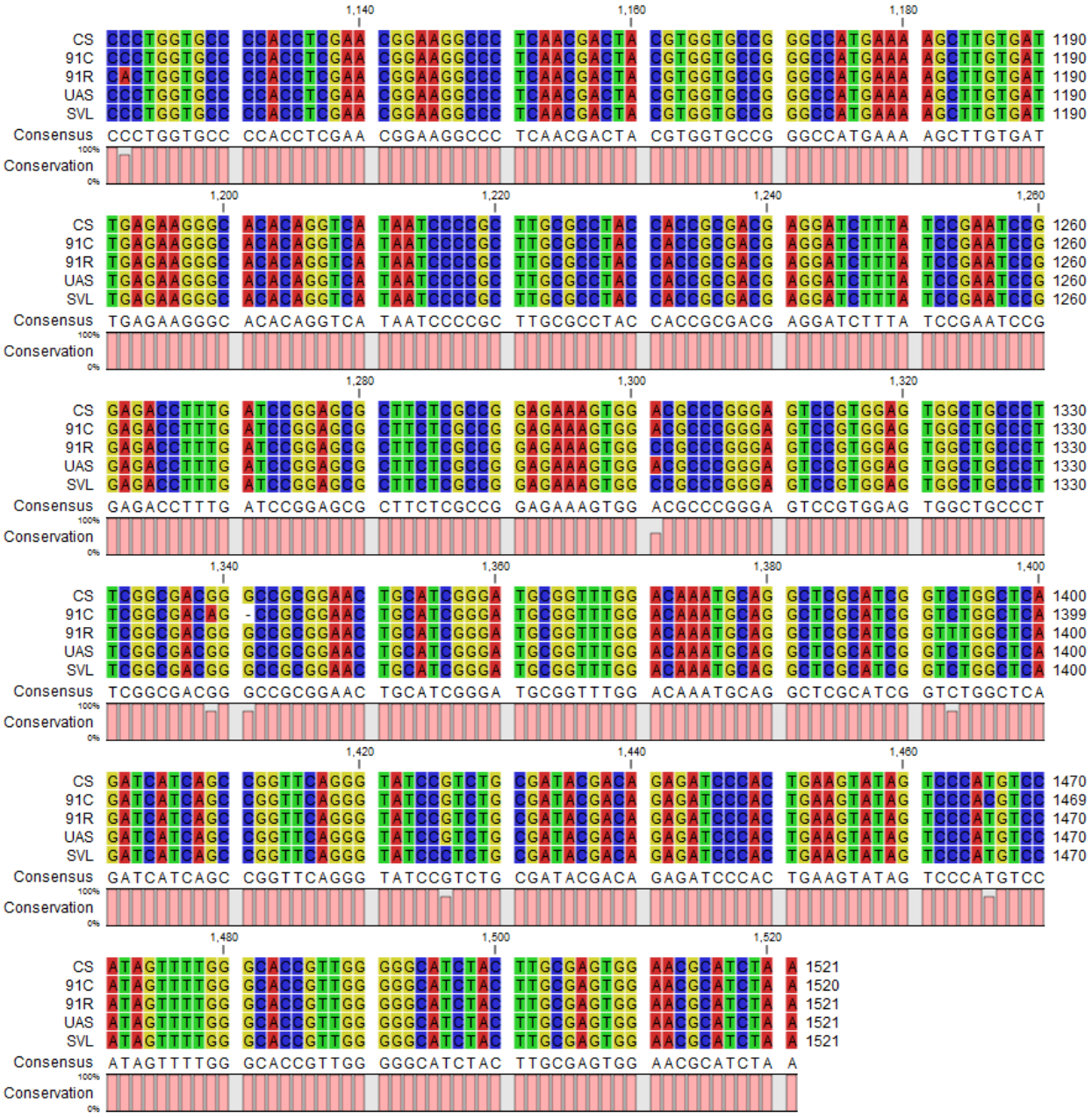

Figure 13. The CLC DNA sequence alignments between the *CS, 91-C, 91-R,* UAS-*Cyp6a2,* and *Cyp6a2*SVL strains showed 21 SNP's were present in the coding region of *Cyp6a2*. The overall consensus is signified by a pink bar underneath the sequences. The highly DDT-resistant *91-R* line and the *Cyp6a2*SVL line have mutations in common at nucleotide positions 408, 916, 933, and 1301. The *91-C* strain contained a single basepair deletion at position nucleotide 1341.

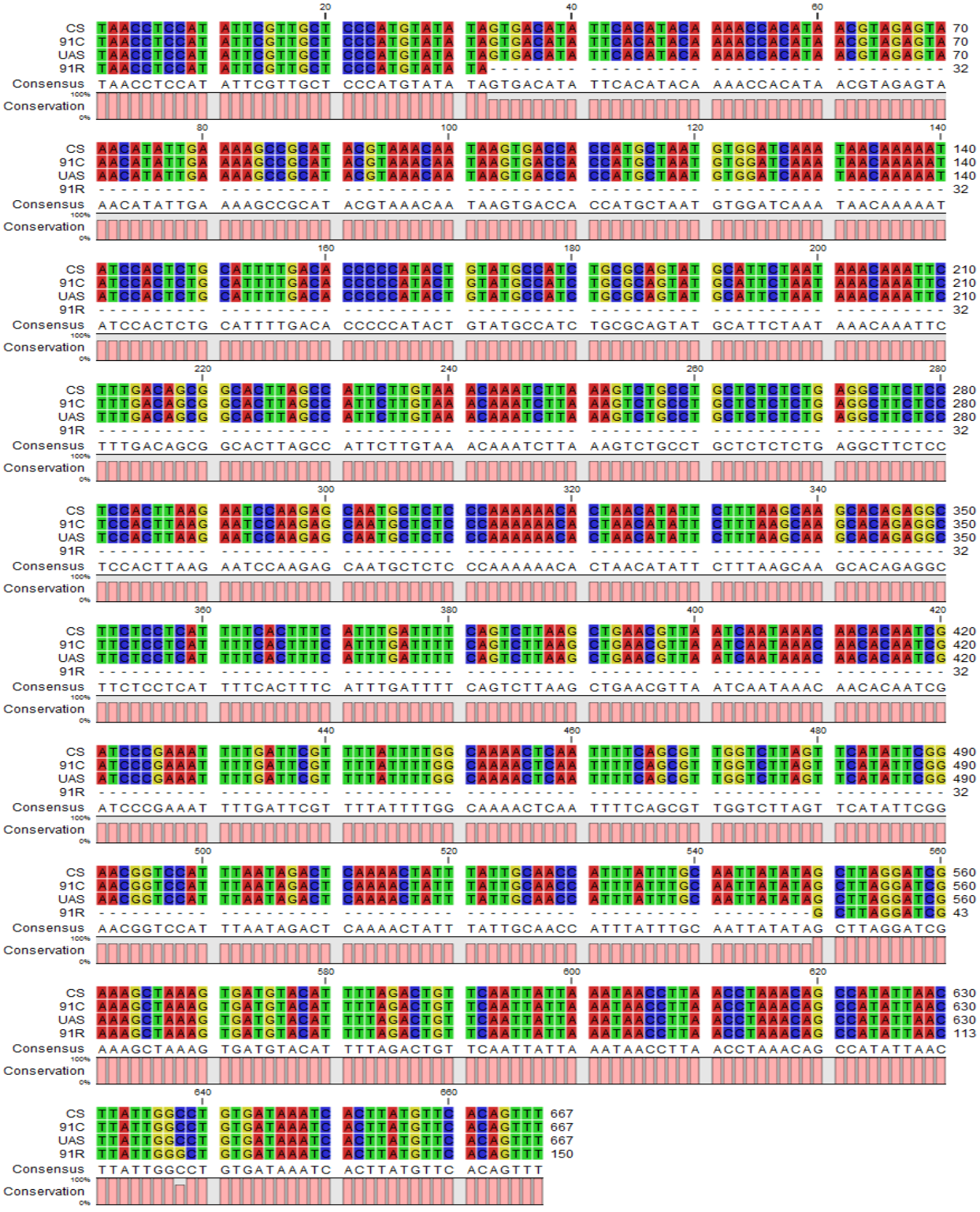

Figure 14. The CLC DNA sequence alignments between the *CS, 91-C,* UAS-*Cyp6a2*, and *91-R* strains showed a ~500 bp insert in the 3' region of the gene that was not present in the DDT-resistant 91-R strain. The overall consensus is signified by a pink bar underneath the sequences, starting with TTA, the stop codon.

4.2 Protein sequence analysis

While 21 SNP's were detected, only 9 of these point mutations led to amino acid changes (Table 5). The *Cyp6a2*SVL mutant, which is able to metabolize DDT, is named as such for the R335S, L336V, and V476L mutations [56]. These mutations were not found in the highly resistant *91-R* line. However, there are several additional mutations of interest present (Fig. 15). At amino acid position 191, the *Cyp6a2*SVL had an A to R substitution. Amino acid positions 204, 227, 306, 336, and 476 all resulted in amino acid substitutions, however, the character of the resulting amino acid substitution was the same (i.e., polar to polar, non-polar to non-polar). At amino acid position 434, both the *91-R* and *Cyp6a2*SVL had a D to A substitution, which may result in a significant change in the local protein structure and function. Similarly, at amino acid position 489, there was a M to T mutation in the *91-C* line. The *91-C* sequence becomes degenerate from the rest at amino acid position 447 due to the base pair deletion, which led to a frame shift mutation.

Table 5
Sequence analysis of *Cyp6a2* in DDT susceptible (*CS*, *91-C*, UAS-Cyp6a2) and resistant (*91-R*, *Cyp6a2*SVL) strains led to the identification of several mutations in addition to SVL.

Strain	191	204	227	306	**335***	**336***	434	**476***	489
CS	A	C	M	I	R	L	D	V	M
91-C	A	S	M	I	R	L	D	V	T
91-R	A	S	V	V	R	L	A	V	M
UAS-*Cyp6a2*	A	S	V	I	R	L	D	V	M
*Cyp6a2*SVL	R	S	V	V	**S***	**V***	A	**L***	M

Orange = Non-polar, Blue = Polar, Red = Polar Charged (+), Black = Polar Charged (-)
* Indicates the amino acid positions of the *Cyp6a2*SVL mutations

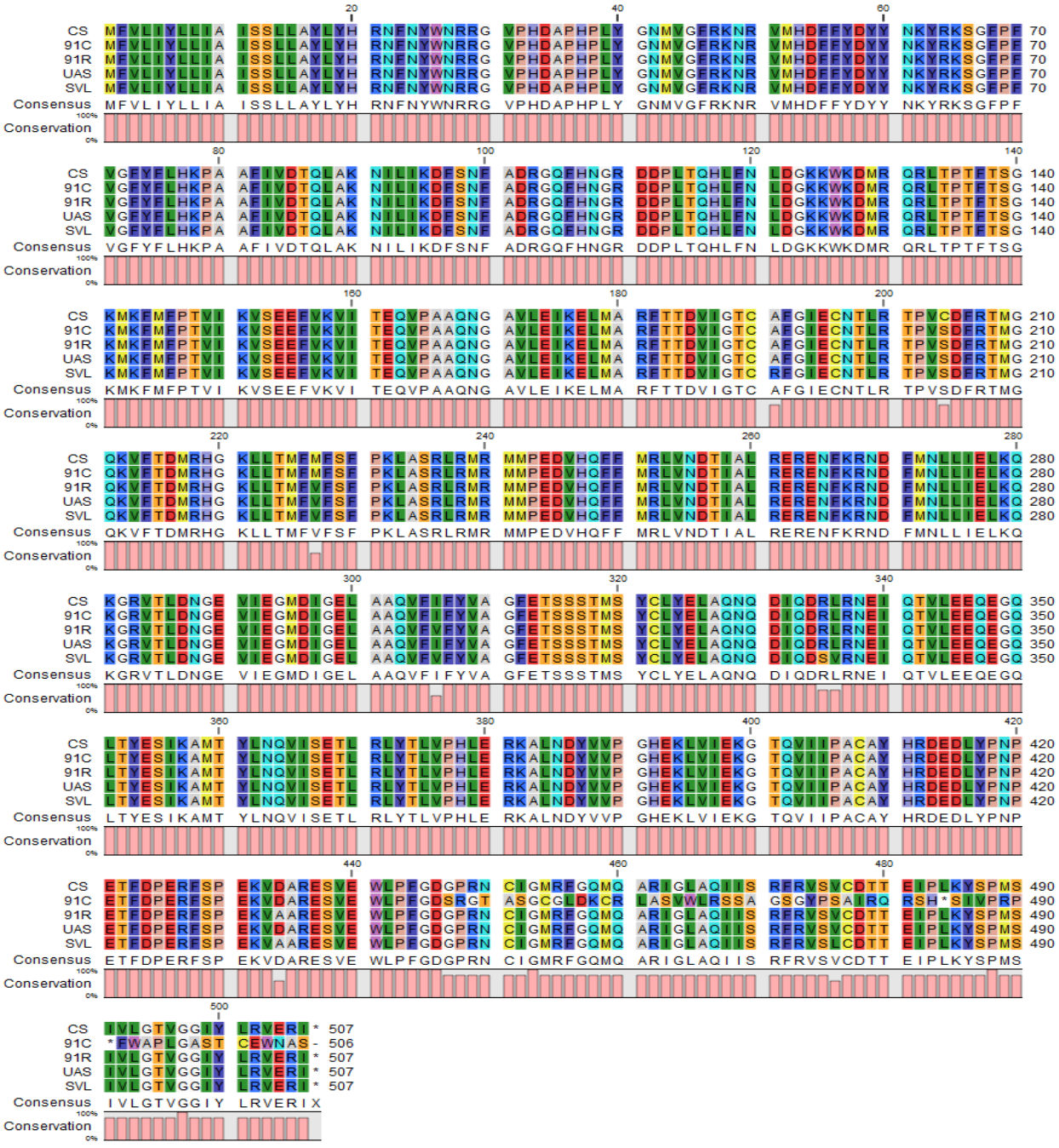

Figure 15. The CLC Protein sequence alignments between the *CS, 91-C, 91-R,* UAS-*Cyp6a2*, and *Cyp6a2*SVL strains showed 9 amino acid substitution in total. The overall consensus is signified by a pink bar underneath the sequences. The SVL amino acid substitutions (R335S / L336V / V476L) were only present in the *Cyp6a2*SVL line, however, the DDT-resistant *91*-R and the *Cyp6a2*SVL strains shared two mutations in common (I306C /D434A). At this time it is unclear which of these mutations may enable *Cyp6a2* to metabolize DDT. The *91-C* sequence is degenerate from 447 onward due to a base pair deletion leading to a frame shift mutation.

4.3 Discussion

In total, 21 SNP's were detected across the sequenced strains. Only 5 of these SNP's were found across multiple sequenced lines. These 21 SNP's led to a total of 9 amino acid substitutions. Of these 9 amino acid substitutions, 4 led to changes in the amino acid's character (i.e., polar to non-polar). The *Cyp6a2*SVL mutant, which can metabolize DDT, did not share the SVL mutations in common with any of the other sequenced lines, even the highly DDT-resistant *91-R*. Interestingly, 2 novel mutations were found in common between the highly DDT-resistant *91-R* line and the *Cyp6a2*SVL line. These 2 mutations resulted in amino acid substitutions at position I306V and position D434A. While the *Cyp6a2*SVL mutations were only found in the *Cyp6a2*SVL line, it is possible that the other mutations in common between the *91-R* and *Cyp6a2*SVL may enable this particular *Cyp6a2* variant to metabolize DDT directly. Further analysis is needed to determine the effect of the 3' UTR transposon insert as well as the effect of the amino acid substitutions. It is likely that the lack of the 3' transposon in the *91-R* line at least partially explains the higher transcript level found in the *91-R*. Further testing would need to be done to show prove this, however.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Of the 17 genes tested, 7 were found to have a significant effect on susceptibility to DDT as determined by UAS-RNAi (Fig. 7). Knockdown of *Cyp4g1* was found to increase susceptibility to DDT by 25% and this gene is located on the X chromosome (chromosome 1); the only sex-linked resistance factor that has been determined to date. *Lcp1*, *Cyp6g1*, *Cyp12d1*, and *Mdr50* are located on chromosome 2R and *Mrp1* is located on the left arm (2L) of chromosome 2. UAS-RNAi resulted in a 14%, 17%, 11%, 13% and 15% increase in susceptibility to DDT, respectively, making this chromosome prominent in DDT resistance. *Mdr65* is located on chromosome 3L and its knockdown by UAS-RNAi resulted in a 12% increase in susceptibility to DDT. While 10 of the genes tested by UAS-RNAi resulted in no increase in susceptibility to DDT, that does not mean that these genes play no role in DDT resistance. Instead, it may be an artifact of inefficient RNAi with the experimental conditions used (heat shock-inducible RNAi). To test this hypothesis, a constitutive Gal4 driver could be used in order to maximize chances of dsRNA induced RNAi. Additionally, constitutive over expression of all the genes tested should be tried, in order to further correlate the expression of these genes with DDT tolerance and resistance.

Multiple genes on all three chromosomes appear to be involved in DDT resistance in the highly resistant *91-R* strain. These results are consistent with the original suggestion that DDT resistance in the *91-R* strain is multifactorial and that factors on all three chromosomes are involved in DDT resistance [8-11]. Factors on chromosome 2 were suggested to play a major role, followed by factor(s) on chromosome 3, with the factor(s) on chromosome 1 showing a

much smaller but statistically significant effect [19]. It was also suggested that the factors on chromosomes 2 and 3 interacted in a more than additive manner. Our results therefore suggest that the *Cyp4g1* gene on chromosome 1 is a likely candidate as the resistance factor associated with this sex chromosome. Also *Mdr65* on chromosome 3 is likely a resistance factor and may interact with either the P450 genes or the other ABC transporter genes on chromosome 2 in a synergistic fashion, resulting in extremely high levels of resistance. This conjecture, however, will need future experimental proof.

What is not yet known is how these resistance mechanisms interact with each other. Will it be in a less than additive, additive, or greater than additive manner? By injecting dsRNA into *91-R* flies for each of the identified 7 genes individually, and then in combination, we will be able to determine how these multiple resistance mechanisms, found across all three chromosomes, interact.

While *Cyp6a2* RNAi in the UAS-RNAi cross did not result in an increased susceptibility to DDT, the possibility still remains that *Cyp6a2* in the *91-R* line may be able to process DDT. As *Cyp6a2* is one of the highest over transcribed (over 30-fold) P450s in the DDT-resistant *91-R* strain [38], the energetic cost of such expression should be balanced with a gain in fitness upon exposure to DDT. One possible explanation for this over transcription may be due to the changes in the 3' UTR of *Cyp6a2* in the *91-R* line. While all DDT-susceptible (*CS*, *91-C*, UAS-Cyp6a2) lines had a ~500 bp insertion in the 3' region of the *Cyp6a2* gene, the DDT-resistant *91-R* did not possess this insertion. Future studies should be done to evaluate the effect of this insertion, or lack thereof, on gene expression levels.

The reported *Cyp6a2*SVL mutant was able to metabolize DDT when expressed in *E. coli* [56]. While the *Cyp6a2*SVL amino acid substitutions were not found to be present in the *91-R*, there were still 2 amino acid substitutions shared between the DDT-resistant *91-R* and the *Cyp6a2*SVL strains. The I306V and D434A mutations were both found in the *91-R* and *Cyp6a2*SVL strains. These mutations, however, were not present in the susceptible strains (*CS*, *91-C*, UAS-Cyp6a2). The I306V amino acid substitution, while found in both the *91-R* and *Cyp6a2*SVL strains, results in a non-polar to non-polar amino acid substitution, which may not drastically change the structure of the resulting protein. Of more interest, the D434A mutation results in polar charged (aspartic acid) to non-polar hydrophobic (alanine) substitution. It is possible that this change may change the local structure of *Cyp6a2* allowing it to more efficiently bind DDT for phase I xenobiotic metabolism. Further, site directed mutagenesis studies should be run to evaluate the effect of these mutations in the ability of *Cyp6a2* to metabolize DDT.

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