Research Article

Received: 19 May 2013 Revised: 1 August 2013 Accepted article published: 4 September 2013 Published online in Wiley Online Library: 8 October 2013

(wileyonlinelibrary.com) DOI 10.1002/ps.3645

Nrf2/Maf-binding-site-containing functional *Cyp6a2* allele is associated with DDT resistance in *Drosophila melanogaster*

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Abstract

BACKGROUND: Increased insecticide detoxification mediated by cytochrome P450s is a common mechanism of insecticide resistance. Although *Cyp6a2* has been observed to be overexpressed in many 4,4′-dichlorodiphenyltrichloroethane (DDT)-resistant strains of *Drosophila melanogaster*, how *Cyp6a2* is regulated and whether its overproduction confers DDT resistance remain elusive.

RESULTS: Molecular analysis identified five *Cyp6a2* alleles ($Cyp6a2^{Canton-S-1}$, $Cyp6a2^{Canton-S-2}$, $Cyp6a2^{91-C}$, $Cyp6a2^{91-R}$ and $Cyp6a2^{Wisconsin-WD}$) from four *D. melanogaster* strains, notably differing in the presence or absence of an intact Nrf2/Maf (a transcription factor) binding site in the 5′-promoter core region, a 'G1410' frameshift deletion mutation in the heme-binding region and a long terminal repeat (LTR) of transposable element *17.6* in the 3′-untranslated region (UTR). Linkage analysis confirmed that DDT resistance was genetically linked to a Nrf2/Maf-binding-site-containing, LTR-lacking functional allele of *Cyp6a2* ($Cyp6a2^{91-R}$). The qRT-PCR results showed that overexpression of functional *Cyp6a2* was consistently associated with DDT resistance. Luciferase reporter gene assays revealed that an intact Nrf2/Maf binding site in the 5′-promoter core region enhanced the constitutive transcription of *Cyp6a2*.

CONCLUSION: The results suggest that the Nrf2/Maf binding-site-containing functional *Cyp6a2* allele is associated with DDT resistance in the *D. melanogaster* strains under study.

Supporting information may be found in the online version of this article.

Keywords: *Cyp6a2*; DDT resistance; frameshift mutation; long terminal repeat; Nrf2/Maf binding site; overexpression

1 INTRODUCTION

Cytochrome P450 monooxygenases (CYPs, P450) are a highly diverse family of heme-containing proteins found in bacteria, fungi, plants and animals.[1] They are characterized by a conserved heme-binding signature motif (FxxGxxxCxG) towards the C-terminus. Many studies have demonstrated that CYP-mediated insecticide detoxification is the common and an important mechanism of insecticide resistance. Insect CYPs have been implicated in conferring resistance to insecticides by increased enzyme production (via overexpression or gene duplication),[2] structural changes that may alter the catalytic properties of the enzymes (caused by point mutations),[3] or gene recombination of two closely linked genes by an unequal crossing-over event that creates a chimeric enzyme with a novel ability.[4]

4,4′-Dichlorodiphenyltrichloroethane (DDT, an organochlorine insecticide) had been widely and extensively used to control plant insect pests and insects that vector human diseases. The resurgence in the use of DDT (WHO, http://www.who.int/malaria/publications/atoz/htm_mal_2006_1112/eb/index.html) represents further DDT selection in the field insects. This situation points to the need for further research into other genes associated with DDT resistance. To date, in addition to the observations that overexpression of *Cyp6g1* is causally related to DDT resistance in numerous *Drosophila melanogaster* strains, CYP6Z1 and CYP6M2 have been characterized as being able to metabolize DDT and increased expression of CYP6Z1 or CYP6M2 confers DDT resistance in *Anopheles gambiae*.[5,6]

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P450-mediated metabolic resistance displays evolutionary plasticity.[7] In *D. melanogaster*, several CYPs such as *Cyp6a2*, *Cyp6a8*, *Cyp6g1* and *Cyp12d1* have been reported to be over-transcribed in various DDT-resistant fruit fly populations.[8–11] Although *Cyp6a8* is overexpressed in DDT-resistant strains, it is not able to metabolize DDT under aerobic conditions when heterologously expressed in yeast.[12] A constitutive high level of *Cyp12d1* transcript has been observed in one DDT-resistant strain.[10] In addition, *Cyp12d1* is differentially expressed in response to many xenobiotics including atrazine and caffeine, and under environmentally stressful conditions.[13–15] Higher inducibility of *Cyp12d1* has been detected in DDT-resistant 91-R and Wisconsin strains in response to DDT as compared with a susceptible Canton-S strain.[10,16] In addition, transgenic flies had increased survival on DDT when overexpressing *Cyp12d1* using the GAL4/UAS system.[17]

Several lines of evidence indicate that *Cyp6g1* is at least one gene involved in DDT resistance in some strains of insects. Genetic mapping of DDT resistance and microarray analysis of CYPs in a field-derived resistant strain, Hikone-R, support the hypothesis that *Cyp6g1* is one factor in the DDT resistance phenotype.[18] A more global examination of CYP levels has shown that the *Cyp6g1* is overexpressed in many field-evolved DDT-resistant strains of diverse origins.[19] In addition, transgenic overexpression has shown that *Cyp6g1* is important for resistance.[17,19] Homology modeling has also displayed that the active site cavity of CYP6G1 is both chemically and conformationally well suited to accommodate DDT.[20] A recent study has demonstrated that *Escherichia coli*-produced recombinant CYP6G1 can bind a number of pesticides, including DDT.[21] Heterologous expression of this gene using cultured cells of *Nicotiana tabacum* L. has confirmed that CYP6G1 is able to convert DDT to the reduced metabolite DDD under anaerobic conditions.[22] However, several other studies suggest that *Cyp6g1* is only one factor in moderate- to high-level DDT resistance. For example, some natural populations or laboratory strains with overexpression of *Cyp6g1* are DDT-susceptible.[16,23,24] In the strains used by Li *et al*,[24] *Cyp6g1* is genetically linked to nicotine resistance rather than DDT resistance. RNAi of *Cyp6g1* does not significantly increase fruit fly mortality to DDT in a susceptible strain.[25]

Another CYP suggested to be involved in DDT resistance in *D. melanogaster* is *Cyp6a2*. *Cyp6a2* is constitutively highly expressed in both laboratory-selected (e.g. 91-R and RDDT^{R})[8,26] and field-collected DDT-resistant strains (e.g. Wisconsin).[27] Following induction by DDT and other xenobiotics, *Cyp6a2* is highly expressed in both larvae and adults, in the midgut, Malpighian tubules and fat body, which are important organs for detoxification.[26,28] Although baculovirus-expressed CYP6A2 does not metabolize DDT under aerobic conditions,[29] *E. coli*-produced CYP6A2 enzyme with amino acid substitutions (R335S, L336V and V476L) displays elevated DDT metabolism under aerobic conditions.[3] However, transgenic overexpression of *Cyp6a2wt* does not increase survival on DDT in the susceptible genetic background.[17] These reports suggest that the role of *Cyp6a2* in DDT resistance remains 'conjectural'.[1]

Current understanding of the regulatory mechanisms of genes involved in insecticide resistance is very limited. *Cis*-acting factors have been suggested to be involved in the regulation of the constitutive expression of resistance-conferring genes. The insertion of a terminal direct repeat of the transposable element *Accord* was found in the 5′-untranslated region (UTR) of *Cyp6g1* gene of 20 different resistant strains from across the globe.[19] A correlation between the presence of the *Accord* insertion and resistance was established in a survey on samples of diverse origins.[30] Different alleles with duplicated *Accord* insertion, or with additional insertions of P element or HMS Beagle element within the original *Accord* insertion at the *Cyp6g1* locus were identified; and higher resistance and *Cyp6g1* transcription were found for the most complex allele (Cyp6g1-[BP]).[31] These findings imply that *cis*-acting factor contributes to the upregulation of *Cyp6g1*. A key sequence difference between the 5′-UTR of the *CYP6D1* allele of a resistant housefly strain (LPR) and of susceptible strains is the presence of a 15-bp insert that interrupts a binding site of the transcriptional repressor mdGfi-1, which reduces the binding of mdGfi-1 to the *CYP6D1* promoter in electrophoretic mobility shift assays.[32] This 15-bp insert is also found in some other pyrethroid-resistant strains from China and Turkey.[33–35]

Trans-acting factors were also found to affect the expression of resistance-related CYPs. For example, CYP6A1 is constitutively overproduced in several resistant strains of house flies including the Rutgers strain.[36] *CYP6A1* gene maps to chromosome 5;[37] however, its constitutive overexpression is linked to a semi-dominant factor on chromosome 2.[38] These results imply that the existence of a chromosome 2 *trans*-acting factor(s) regulates CYP6A1 expression.[38] Similarly, genetic crosses and chromosome substitution experiments conclusively showed that the expression of both *Cyp6a2* and *Cyp6a8* is repressed by factors on the third chromosome of the insecticides susceptible 91-C and rosy506 strains, while enhanced by the third chromosome of the resistant MHIII-D23 and 91-R strains,[39] indicating that loss-of-function mutations in gene(s) encoding negative regulators of P450 gene expression is on chromosome 3. Luciferase reporter analysis identified a −11/−761-bp region in the core promoter of the *Cyp6a8* gene of the 91-R strain that is sufficient to respond to the negative regulation by the rosy506 (wild-type) *trans*-acting factor.[40] *Drosophila* Jun protein (D-jun) encoded by a gene on chromosome 2R was identified to act as a repressor for *Cyp6a2* and *Cyp6a8* genes.[41]

Taken together, *D. melanogaster* has been adopted extensively as a model for the study of the molecular mechanism of DDT resistance. As of today, many critical questions on CYP-mediated resistance remain, such as: (1) how many CYPs participate in DDT resistance in a given strain; (2) which CYP allele confers resistance in specific strains; and (3) what are the regulatory mechanisms involved in constitutive over-transcription of resistance-conferring genes? The observation that *Cyp6a2* is overexpressed in several DDT-resistant strains led us to investigate the possible involvement of *Cyp6a2* in DDT resistance.[8,26,27] Specifically, we examined the sequence and expression level of *Cyp6a2* alleles in DDT-susceptible and -resistant strains and their genetic linkage with DDT resistance. We also investigated the possible *cis*-elements responsible for the constitutive overexpression of *Cyp6a2* gene.

2 MATERIALS AND METHODS

2.1 *Drosophila* strains

Two DDT-susceptible (Canton-S and 91-C) and two DDT-resistant strains (Wisconsin and 91-R) were used in this study. Detailed backgrounds of these strains were described elsewhere.[8,15,27]

2.2 DDT susceptibility bioassay

DDT susceptibilities were bioassayed according to the published method.[23] At least five concentrations of three to six replicates were used for each strain or single-pair family line. Bioassay data were analyzed using probit analysis in SPSS.

2.3 Cloning and sequencing of *Cyp6a2* alleles

The 5′- (∼ 1355 bp), 3′-flanking (622 or 105 bp) and coding regions (1521 bp) of *Cyp6a2* from the Canton-S, Wisconsin, 91-C and 91-R strains were cloned and sequenced. Thirty flies from each strain were used for DNA extraction, and DNA samples were digested with RNaseA for 5 min at 37 °C to avoid RNA contamination.[42] Total RNA was extracted from 30 three-day-old adult flies using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. RNA was treated with DNase I (Takara, Shiga, Japan) in order to eliminate the genomic DNA contamination. cDNA was synthesized using PrimeScript™ reverse transcriptase (Takara) and oligo(dT)$_{18}$ as the primer. The PCR primers used in this study are listed in Table S1. PCR conditions were set up as 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 2 min, then 72 °C for 10 min. All PCR products were sequenced by Invitrogen (Beijing Service Centre, Beijing, China).

2.4 Development of *Cyp6a2* allele genotyping methods

Allele-specific PCR methods were developed to genotype *Cyp6a2*$^{Canton\text{-}S\text{-}1}$, *Cyp6a2*$^{Canton\text{-}S\text{-}2}$ and *Cyp6a2*$^{91\text{-}R}$ alleles (Fig. 1). Three allele-specific reverse primers (CantonS-1-15 bp-R, CantonS-2-15 bp-R and 91-R-15 bp-R in Table S1) were designed based on the presence or absence of a 15-bp sequence and its immediate flanking sequence in the promoter of *Cyp6a2* alleles. An allele-specific PCR product of ∼ 1200 bp was generated using the common forward primer 6A2-pF (Table S1) and the corresponding reverse primer. The annealing temperature for the three allele-specific PCR was 62 °C.

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) was developed to detect the frameshift mutation of *Cyp6a2*. This frameshift mutation disrupts a *Sau*96I digestion site. Briefly, partial sequence (∼ 323 bp) of *Cyp6a2* covering the mutation site was amplified with the primers 6A2-1205 F and 6A2-1527R (Table S1) using DNA samples extracted from wings or the whole body.[43,44] The PCR products were digested with *Sau*96I restriction enzyme (New England Biolabs, Ipswich, MA, USA), run on a 2% agarose gel, and visualized with ethidium bromide staining. Homozygous *Cyp6a2*$^{91\text{-}C}$ allele exhibited a single band of 323 bp, heterozygous *Cyp6a2*$^{91\text{-}C}$ flies exhibited three bands of 323, 137 and 186 bp, and flies without *Cyp6a2*$^{91\text{-}C}$ exhibited two bands of 137 and 186 bp.

2.5 Establishment of homozygous single-pair family lines from the field collected heterozygous Wisconsin strain

A number of single-pair family lines were established using virgin males and females carrying different *Cyp6a2* alleles (*Cyp6a2*$^{Canton\text{-}S\text{-}2}$, *Cyp6a2*$^{91\text{-}C}$ and *Cyp6a2*$^{Wisconsin\text{-}WD}$) isolated from the Wisconsin strain. Virgin flies were genotyped for *Cyp6a2* allele using allele-specific PCR and PCR-RFLP. Then a male and a female homozygous for each *Cyp6a2* allele were mated to produce a homozygous single-pair family. In this way, a total of 12 single-pair family lines were established, i.e. four lines carrying the *Cyp6a2*$^{91\text{-}C}$ allele (named Wisconsin-MT-1, Wisconsin-MT-2, Wisconsin-MT-3 and Wisconsin-MT-4), four lines harboring the *Cyp6a2*$^{Canton\text{-}S\text{-}2}$ allele (named Wisconsin-2 bp-1, Wisconsin-2 bp-2, Wisconsin-2 bp-3 and Wisconsin-2 bp-4) and four lines possessing the *Cyp6a2*$^{Wisconsin\text{-}WD}$ allele (named Wisconsin-WD-1, Wisconsin-WD-2, Wisconsin-WD-3 and Wisconsin-WD-4).

2.6 Linkage analysis

Two genetic analyses were conducted to determine the linkage relationship between *Cyp6a2* alleles and DDT resistance. In Linkage 1 (Fig. 2A), a female adult from the DDT-resistant strain 91-R (homozygous for the *Cyp6a2*$^{91\text{-}R}$ allele, genotype: RR) was

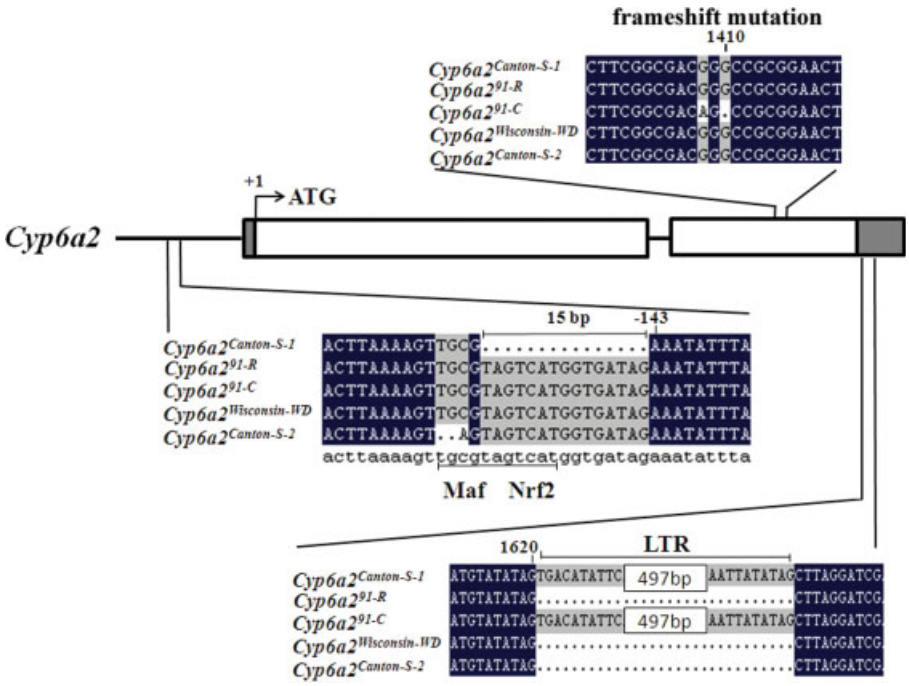

Figure 1. *Cyp6a2* alleles recovered from the four *D. melanogaster* strains. *Cyp6a2* is drawn to scale with transcript depicted as boxes, the flanking sequences and intron as black lines and translation initiation site as a vertical arrow with +1. Three major differences among the five *Cyp6a2* alleles: the presence or absence of an intact Nrf2/Maf binding site in the promoter, the presence or absence of a frameshift deletion mutation (G1410) in the coding region and the presence or absence of a terminal repeat (LTR) of transposable element *17.6* in the 3′-UTR were characterized in detail. Dots represented nucleotides deletions in the sequence.

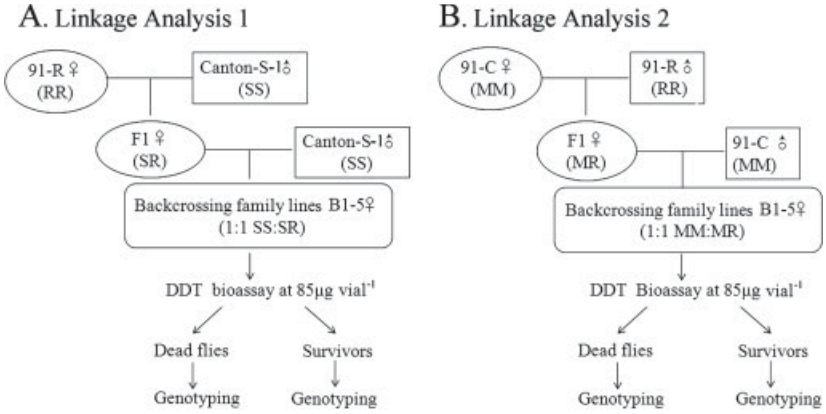

Figure 2. Crossing schemes for Linkage Analysis trial 1 (A) and trial 2 (B).

crossed with a male fly from the DDT-susceptible strain Canton-S (individuals homozygous for the *Cyp6a2*$^{Canton\text{-}S\text{-}1}$ allele, genotype: SS). Then five single-pair backcross families were produced by backcrossing the F_1 females (created by a 91-R × Canton-S cross) with the DDT-susceptible strain Canton-S (SS). In Linkage-2 (Fig. 2B), a female adult from the DDT-susceptible 91-C strain (homozygous for the *Cyp6a2*$^{91\text{-}C}$ allele: genotype: MM) was crossed with a male fly from the DDT-resistant strain 91-R (homozygous for the *Cyp6a2*$^{91\text{-}R}$ allele, RR). Likewise, five single-pair backcross families were generated by backcrossing the F_1 females (created by a 91-C × 91-R cross) with the DDT-susceptible strain 91-C (MM).

Three to five-day-old female adults of each backcross family lines were exposed to 85 μg vial^{-1} of DDT. This discriminating dose killed > 95% of susceptible (SS and MM) and < 5% of resistant (RR) individuals. Dead flies were collected at a 6 h interval up to 48 h and frozen at −80 °C for subsequent genotyping. The flies survived after 48-h exposure to DDT were also collected for *Cyp6a2* genotyping. The *Cyp6a2* genotype of each female was determined using the allele-specific PCR or PCR-RFLP. A chi-square test for each observation time point was performed to determine the significance of the difference.

2.7 Real-time PCR

The expression levels of the *Cyp6a2* gene and four other CYP6 genes (*Cyp6a8*, *Cyp6g1*, *Cyp6g2*, *Cyp6w1*) were determined by quantitative RT-PCR (qRT-PCR) using an Mx3005P qPCR System (Stratagene, La Jolla, CA, USA) and RealMasterMix SYBR Green PCR kit (Takara). The ribosomal protein gene *Rp49* was used as a reference gene to normalize the expression levels of these genes.[31] qRT-PCR was run in triplicate for each RNA sample in a 20-μL reaction containing 10 μL 2× RealMasterMix, 0.4 μL reference dye Rox II, 0.4 μL each of the corresponding forward and reverse primers (10 μM; Table S1), 3.8 μL ddH_2O and 5 μL cDNA.[45] The qRT-PCR cycling parameters were: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The specificity of the PCR amplification was checked by a melt curve analysis (MxPro 4.0 program, Stratagene) and by sequencing the PCR products. For each gene, a serial dilution from 10- to 1000-fold of each cDNA template was performed to assess their PCR amplification efficiency. The relative expression level of *Cyp6a2* was calculated by the comparative CT method.[46] Results were expressed as mean expression ratio (± SE) of three biological replicates. Statistical analysis was determined by one-way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple comparisons.

2.8 Construction of *Cyp6a2* promoter– and/or 3′-UTR–pGL3 reporter constructs

To investigate the effects of the 5′-core promoter and 3′-UTR of *Cyp6a2* on the expression of *Cyp6a2*, a ~ 217 bp DNA fragment covering the core of the 5′-promoter was PCR-amplified from the 5′-flanking region of *Cyp6a2*$^{Canton\text{-}S\text{-}1}$, *Cyp6a2*$^{Canton\text{-}S\text{-}2}$ and *Cyp6a2*$^{91\text{-}R}$, and subcloned into the *Kpn*I/*Xho*I sites of pGL3-Basic firefly luciferase reporter vector (Promega, Madison, WI, USA) using the primers 6A2-p217-F (*Kpn*I) and 6A2-pR (*Xho*I), respectively (Table S1). The resulting promoter–pGL3 constructs were called pGL3–Canton-S-1, pGL3–Canton-S-2 and pGL3–p217-promoter, respectively. To investigate the effect of the LTR of transposable element *17.6* (LTR *17.6* for short) in the 3′-UTR of the *Cyp6a2* alleles on the basal transcription of *Cyp6a2*, the 3′-UTR sequences of *Cyp6a2*$^{91\text{-}C}$ (containing LTR *17.6*) and *Cyp6a2*$^{91\text{-}R}$ (lacking LTR *17.6*) were PCR-amplified from the corresponding genomic DNA samples and subcloned into the *Xba*I site of the pGL3–p217-promoter construct using the primers 6A2-3UTR-F (*Xba*I) and 6A2-3UTR-R (*Xba*I), respectively (Table S1). In this manner, the 3′-UTR of the two *Cyp6a2* alleles was inserted immediately upstream of the SV40 late poly(A) signal of the vector sequence. Sequencing was performed to ensure the proper orientation.

2.9 Reporter gene assays

Drosophila S2 cells were routinely maintained at 26 °C in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Transfection experiments were performed in 24-well cell culture plates. Briefly, recipient S2 cells were seeded at a density of 1 × 10^6 cell mL^{-1}. After removal of culture medium and single wash using fresh medium without FBS, the cells were cotransfected with 0.5 μg of each of the pGL3–*Cyp6a2* promoter/3′-UTR constructs and 0.05 μg of pRL-TK vector (an internal control for normalization transfection efficiency) per well using 2 μL Cellfectin Transfection Reagent (Invitrogen) in 100 μL medium without FBS. After incubation for 6 h, the transfection reagent mix was replaced with 0.5 mL of Schneider's *Drosophila* medium containing 10% FBS. Cells were harvested at 48 h post transfection. Firefly and Renilla luciferase activities were measured by the Dual-Glo luciferase assay system according to the manufacturer's instructions. Three independent transfections of three replicates for each construct were conducted. Differences in promoter activity among the pGL3–*Cyp6a2* promoter/3′-UTR constructs were tested by one-way ANOVA, followed by Tukey's HSD test for multiple comparisons.

Table 1. Dose–response data for *Drosophila melanogaster* exposed to DDT and the relative expression of *Cyp6a2*

Strain/lines	*Cyp6a2 allele*	*N*	Slope (± SE)	LC_{50}(95% CI)	RR	RE
	80% $Cyp6a2^{Canton\text{-}S\text{-}1}$					
Canton-S	20% $Cyp6a2^{Canton\text{-}S\text{-}2}$	746	1.4 (0.1)	2.64 (2.16–3.14)	1.0	1
91-C	$Cyp6a2^{91\text{-}C}$	891	2.7 (0.2)	4.67 (4.28–5.10)	1.8	48 (5.2)
Wisconsin-MT-3	$Cyp6a2^{91\text{-}C}$	540	1.9 (0.1)	10.0 (8.59–11.7)	3.8	74 (11)
Wisconsin-MT-2	$Cyp6a2^{91\text{-}C}$	540	1.2 (0.1)	11.0 (8.62–14.9)	4.2	74 (5.9)
Wisconsin-MT-4	$Cyp6a2^{91\text{-}C}$	630	1.5 (0.1)	11.3 (9.45–13.6)	4.3	63 (5.2)
Wisconsin-MT-1	$Cyp6a2^{91\text{-}C}$	540	1.1 (0.1)	17.5 (13.6–23.9)	6.6	73 (7.1)
Wisconsin-2 bp-3	$Cyp6a2^{Canton\text{-}S\text{-}2}$	540	1.2 (0.1)	18.4 (14.5–22.0)	7.0	24 (0.9)
Wisconsin-2 bp-1	$Cyp6a2^{Canton\text{-}S\text{-}2}$	540	1.2 (0.1)	23.6 (18.7–29.5)	8.9	30 (1.4)
Wisconsin-2 bp-2	$Cyp6a2^{Canton\text{-}S\text{-}2}$	540	1.3 (0.1)	24.3 (19.6–29.9)	9.2	31 (4.5)
Wisconsin-2 bp-4	$Cyp6a2^{Canton\text{-}S\text{-}2}$	540	1.3 (0.1)	26.0 (20.7–32.4)	9.9	29 (1.3)
Wisconsin (parent)	32% $Cyp6a2^{Canton\text{-}S\text{-}2}$	541	1.3 (0.1)	58.7 (45.7–79.9)	22.2	57 (6.7)
	23% $Cyp6a2^{Wisconsin\text{-}WD}$					
	45% $Cyp6a2^{91\text{-}C}$					
Wisconsin-WD-2	$Cyp6a2^{Wisconsin\text{-}WD}$	540	2.4 (0.2)	234 (203–264)	89	46 (7.4)
Wisconsin-WD-3	$Cyp6a2^{Wisconsin\text{-}WD}$	540	2.5 (0.2)	286 (252–320)	109	62 (6.2)
Wisconsin-WD-4	$Cyp6a2^{Wisconsin\text{-}WD}$	540	2.3 (0.2)	324 (286–363)	123	89 (10)
Wisconsin-WD-1	$Cyp6a2^{Wisconsin\text{-}WD}$	450	2.6 (0.3)	369 (329–414)	140	108 (19)
91-R	$Cyp6a2^{91\text{-}R}$	713	1.2 (0.1)	2057 (1660–2638)	779	336 (60)

RR, LC_{50} of a given strain or family/LC_{50} of the most susceptible strain (Canton-S). RE, Relative expression—level of *Cyp6a2* relative to that of Canton-S strain.

3 RESULTS

3.1 Sequence differences at the *Cyp6a2* locus in different DDT resistance phenotypes of *D. melanogaster*

To examine if any DDT genetic mutations occur at the *Cyp6a2* locus, we cloned and sequenced the genomic DNA and cDNA of *Cyp6a2* gene from multiple individuals of the two DDT susceptible (Canton-S and 91-C) and two DDT-resistant strains (Wisconsin and 91-R). Sequencing results showed that the 91-C and 91-R strains were homozygous at the *Cyp6a2* locus, whereas the wild type Canton-S strain and the field-collected Wisconsin strain were both heterozygous (Table 1). *Cyp6a2* alleles were further confirmed by sequencing of PCR products from at least 10 individual DNA samples from each of the four strains. The allele frequency was evaluated by genotyping at least 60 individuals using the methods established in this study (Fig. S1).

A single *Cyp6a2* allele was recovered from the homozygous 91-C [$Cyp6a2^{91\text{-}C}$ (GenBank ID: KC521475) and 91-R strain ($Cyp6a2^{91\text{-}R}$, KC521476], respectively. The wild-type Canton-S strain was composed of two *Cyp6a2* alleles named $Cyp6a2^{Canton\text{-}S\text{-}1}$ (at a frequency of 0.8; KC455540) and $Cyp6a2^{Canton\text{-}S\text{-}2}$ (0.2; KC521474; Table 1). Three *Cyp6a2* alleles were recovered from the field-collected Wisconsin strain and two of the three alleles were also identical to the allele found in the strain Canton-S ($Cyp6a2^{Canton\text{-}S\text{-}2}$) or 91-C ($Cyp6a2^{91\text{-}C}$). The frequency of the three alleles in the Wisconsin strain was 0.32 for $Cyp6a2^{Canton\text{-}S\text{-}2}$, 0.45 for $Cyp6a2^{91\text{-}C}$ and 0.23 for $Cyp6a2^{Wisconsin\text{-}WD}$ (KC521477).

Genomic sequence alignment revealed three major differences among the five *Cyp6a2* alleles: the presence or absence of an intact Nrf2/Maf binding site in the promoter,[47] the presence or absence of a frameshift deletion mutation in the coding region, and the presence or absence of an LTR of transposable element *17.6* in the 3′-UTR (Figs 1 and S2).[8]

Compared with the 5′-core promoter region of the $Cyp6a2^{91\text{-}R}$ allele, allele $Cyp6a2^{Canton\text{-}S\text{-}1}$ lacked a 15-bp fragment (TAGTCATG-GTGATAG), whereas another allele $Cyp6a2^{Canton\text{-}S\text{-}2}$ substituted the 'TGCG' quadrinucleotide immediately upstream of the 15-bp fragment with 'AG' (Figs 1 and S1). MatInspector scanning showed that the 15-bp fragment and its immediate upstream quadrinucleotide form a canonical binding site for the Nrf2/Maf heterodimer.[47] $Cyp6a2^{91\text{-}C}$ and $Cyp6a2^{Wisconsin\text{-}WD}$ alleles also contained the intact Nrf2/Maf binding site (Fig. 1).

In the coding region, a single nucleotide of 'G' at position 1410 was deleted in the susceptible $Cyp6a2^{91\text{-}C}$ allele, in comparison with the resistant $Cyp6a2^{91\text{-}R}$ allele (Figs 1 and S1). The 'G1410' deletion shifted the downstream reading frame and created a premature stop codon. This mutant would putatively produce a C-truncated protein of 483 amino acids. This putative truncated protein would lack the Cys residue which is the absolutely conserved amino acid functioning as the fifth ligand to the heme.[1] Like $Cyp6a2^{91\text{-}R}$, $Cyp6a2^{Wisconsin\text{-}WD}$, $Cyp6a2^{Canton\text{-}S\text{-}1}$ and $Cyp6a2^{Canton\text{-}S\text{-}2}$ also did not have the 'G1410' frameshift deletion (Figs 1 and S1).

In the 3′-UTR, $Cyp6a2^{Canton\text{-}S\text{-}1}$ and $Cyp6a2^{91\text{-}C}$ alleles shared 513-bp insertion of the LTR transposable element *17.6*, whereas $Cyp6a2^{Wisconsin\text{-}WD}$, $Cyp6a2^{Canton\text{-}S\text{-}2}$ and $Cyp6a2^{91\text{-}R}$ did not contain this insertion (Figs 1 and S1).

While $Cyp6a2^{Wisconsin\text{-}WD}$ and $Cyp6a2^{91\text{-}R}$ were identical to each other in terms of the three major sequence differences, they did have four nucleotide substitutions (T906C, G739A, T688A and G260C) in the promoter region, five nucleotide substitutions (A93G, G675T, A831G, A867G and T1462C) in the coding region conferring an amino acid substitution at (M225I) and two nucleotide substitutions (C1128G and A2494T) in the intron (Fig. S2).

In addition, other 29 SNPs were observed among the 5 *Cyp6a2* alleles, i.e. 11 in the 5′-flanking region, 15 in the coding region resulting in seven amino acid substitutions (V186M, M225I, M227V, I306V, D434A, V476L, T489M) and 3 in the intron (Fig. S2).

3.2 Association between *Cyp6a2* alleles and DDT resistance

To explore the relationship between *Cyp6a2* genotype and DDT resistance, we established 12 homozygous single-pair family lines from the heterozygous resistant strain Wisconsin: four for $Cyp6a2^{Canton\text{-}S\text{-}2}$ (Wisconsin-2 bp-1–4), four for

Table 2. The death time course and final survival number of each five replicate backcross families upon DDT exposure

	12 h[a]		18 h[a]		24 h[a]		30 h[a]		36 h[a]		42 h[a]		48 h[a]		Alive[a,b]	
Trial 1	SS	SR	SS	SR	SS	SR	SS	SR	SS	SR	SS	SR	SS	SR	SS	SR
Family 1 ($n = 56$)	19	0	29	0	30	2	30	4	30	5	30	9	30	10	0	16
Family 2 ($n = 54$)	12	0	24	0	28	1	28	5	28	8	28	12	28	12	0	14
Family 3 ($n = 52$)	26	0	33	0	33	0	33	4	33	9	33	13	33	15	0	4
Family 4 ($n = 54$)	19	0	21	0	21	2	21	5	21	8	21	10	21	14	0	19
Family 5 ($n = 54$)	12	0	26	0	27	1	27	3	27	5	27	5	27	8	0	19
Total	88	0	133	0	139	6	139	21	139	35	139	49	139	59	0	72
Percentage (%)	100	0	100	0	95.9	4.1	86.9	13.1	79.9	20.1	73.9	26.1	70.2	29.8	0	100
	12 h[a]		18 h[a]		24 h[a]		30 h[a]		36 h[a]		42 h[a]		48 h[a]		Alive[a,b]	
Trial 2	MM	MR	MM	MR	MM	MR	MM	MR	MM	MR	MM	MR	MM	MR	MM	MR
Family 1 ($n = 74$)	11	0	28	0	33	1	33	3	33	7	33	11	33	13	0	28
Family 2 ($n = 74$)	22	0	38	1	39	1	39	7	39	11	39	14	39	14	0	21
Family 3 ($n = 74$)	23	0	44	0	45	1	45	4	45	6	45	7	45	8	0	21
Family 4 ($n = 74$)	22	0	36	0	38	3	38	7	38	8	38	9	38	12	0	24
Family 5 ($n = 69$)	7	0	24	0	24	3	24	5	24	7	24	13	24	16	0	29
Total	85	0	170	1	179	9	179	26	179	39	179	54	179	63	0	123
Percentage (%)	100	0	99.4	0.6	95.2	4.8	87.3	12.7	82.1	17.9	76.8	23.2	74.0	26.0	0	100

S, individual carrying $Cyp6a2^{Canton\text{-}S\text{-}1}$; R, individuals carrying $Cyp6a2^{91\text{-}R}$; M, individuals carrying $Cyp6a2^{91\text{-}C}$. Dead individuals were collected and genotyped at 6-h intervals. Numbers listed in the table are the accumulative numbers of dead individuals at each time point. Percentage, the number of individuals of one genotype dead at a given time point/the total number of individuals dead at the same time point. The value at the 6-h time point was not included because no fly was dead at that time. [a] The total accumulative death/survival distribution at the corresponding time point is significantly dependent on the $Cyp6a2^{91\text{-}R}$ genotype at $p < 0.05$ (Chi-square independent tests).
[b]Number surviving at 48 h.

$Cyp6a2^{91\text{-}C}$ (Wisconsin-MT-1–4) and four for $Cyp6a2^{Wisconsin\text{-}WD}$ (Wisconsin-WD-1–4). Bioassays of DDT showed the 91-R strain homozygous for $Cyp6a2^{91\text{-}R}$ allele had the highest LC_{50} (2057 μg vial^{-1}), followed by the four homozygous lines carrying $Cyp6a2^{Wisconsin\text{-}WD}$ (235–370 μg vial^{-1}), then the for homozygous lines carrying $Cyp6a2^{Canton\text{-}S\text{-}2}$ (18–26 μg vial^{-1}), the four homozygous lines carrying $Cyp6a2^{91\text{-}C}$ (10–18 μg vial^{-1}), the 91-C strain homozygous for $Cyp6a2^{91\text{-}C}$ (4.7 μg vial^{-1}), and finally the Canton-S strain heterozygous for $Cyp6a2^{Canton\text{-}S\text{-}1}$ and $Cyp6a2^{Canton\text{-}S\text{-}2}$ (2.6 μg vial^{-1}; Table 1). These data suggest that functional alleles with the intact Nrf2/Maf binding site ($Cyp6a2^{91\text{-}R}$ and $Cyp6a2^{Wisconsin\text{-}WD}$) are associated with higher DDT resistance (> 90-fold resistance relative to Canton-S), whereas nonfunctional alleles ($Cyp6a2^{91\text{-}C}$) or functional alleles without the intact Nrf2/Maf binding site ($Cyp6a2^{Canton\text{-}S\text{-}1}$ and $Cyp6a2^{Canton\text{-}S\text{-}2}$) are associated with much lower DDT resistance (Table 1).

3.3 Genetic linkage between *Cyp6a2* alleles and DDT resistance

To further confirm the association of *Cyp6a2* genotypes and DDT resistance, two genetic linkage analyses were conducted. Results in Linkage trial 1 showed that SS individuals (homozygous for the $Cyp6a2^{Canton\text{-}S\text{-}1}$ allele) had significantly higher mortality than RS individuals (heterozygous for the $Cyp6a2^{91\text{-}R}$ and $Cyp6a2^{Canton\text{-}S\text{-}1}$ alleles) at all the seven time points (Table 2). Most SS individuals (96%) died within 18 h and all the SS individuals had died at the 24 h time point. By contrast, none of RS individuals died within 18 h and all the survivors at 48 h were RS individuals (Table 2). These data suggested that higher DDT resistance was genetically linked to the Nrf2/Maf binding-site-containing, LTR *17.6*-lacking functional allele of *Cyp6a2* (e.g. $Cyp6a2^{91\text{-}R}$). Results in the Linkage trial 2 showed that the MM individuals died significantly faster than the MR individuals (Table 2). All the MM individuals (homozygous for the $Cyp6a2^{91\text{-}C}$ allele) had died at the 24 h time point and all the surviving individuals at 48 h were MR (heterozygous for the $Cyp6a2^{91\text{-}C}$ and $Cyp6a2^{91\text{-}R}$ alleles). These data also indicated that higher DDT resistance was genetically linked to the LTR 17.6-lacking functional *Cyp6a2* allele (e.g. $Cyp6a2^{91\text{-}R}$).

3.4 The expression levels of *Cyp6a2* and in different DDT resistance phenotypes

To ascertain whether DDT resistance is correlated with the transcription levels of *Cyp6a2*, real-time qPCR was employed to determine the expression levels of *Cyp6a2* and four other P450 genes (*Cyp6a8, Cyp6g1, Cyp6g2* and *Cyp6w1*). The five P450 genes were chosen largely because these P450s were previously reported to be involved in insecticide resistance, and all of them are located on the right arm of chromosome 2.[10,12,17,19,27,48]

The expression of *Cyp6a2* mRNA in descending order was 91-R strain (allele $Cyp6a2^{91\text{-}R}$) > > Wisconsin-WD-1–4 ($Cyp6a2^{Wisconsin\text{-}WD}$) ≥ Wisconsin-MT-1–4 ($Cyp6a2^{91\text{-}C}$) ≥ 91-C ($Cyp6a2^{91\text{-}C}$) > Wisconsin-2 bp-1–4 ($Cyp6a2^{Canton\ S\text{-}2}$) > Canton-S (Fig. 3), indicating that DDT resistance was positively correlated with the expression level of functional *Cyp6a2* alleles (Table 1). Notably, the *Cyp6a2* alleles in strains or lines with more abundant *Cyp6a2* transcripts (e.g. 91-R and Wisconsin-WD-1–4) contained the Nrf2/Maf binding site but lacking the LTR *17.6*, whereas those in strains with lower *Cyp6a2* transcripts (e.g. Canton-S and Wisconsin-2 bp-1–4) lacked the intact Nrf2/Maf binding site. This suggests that the Nrf2/Maf binding site is potentially a positive *cis*-element regulating the constitutive transcription of *Cyp6a2* in these strains or lines.

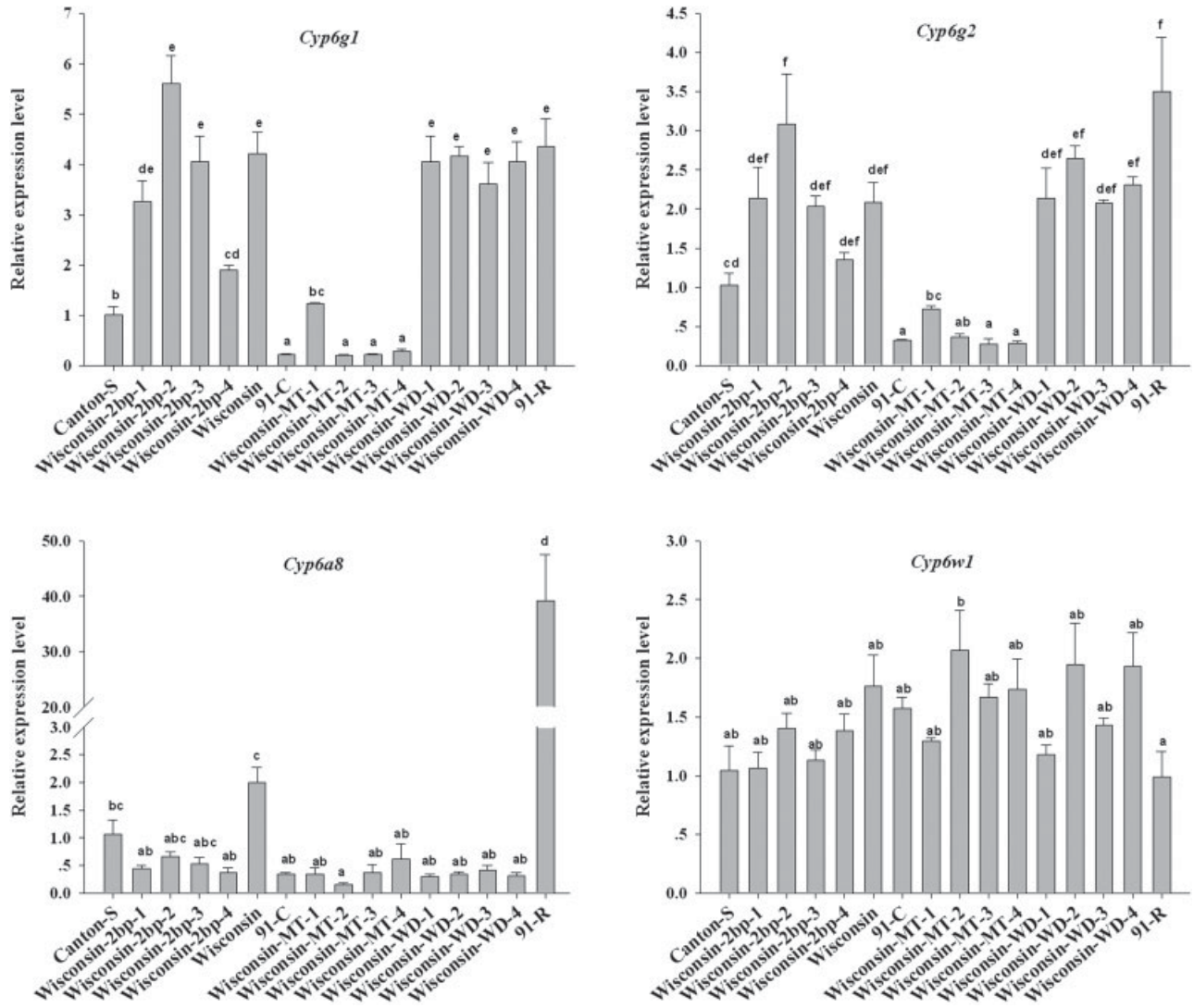

Figure 3. Relative expression levels of CYPs.

DDT resistance (RR in Table 1) in 91-R (779-fold) was much greater than Wisconsin-WD family lines (89–140-fold), and than Wisconsin-2 bp family lines (7.0–9.9-fold), but no significance difference in *Cyp6g1* expression was found among these strains/lines. Although the LC_{50} value of Canton-S was significantly lower than those of 91-C and Wisconsin-MT lines (Table 1), the expression level of *Cyp6g1* in Canton-S was significantly higher than that of 91-C strain and the Wisconsin-MT lines. The expression profile of *Cyp6g2*, a tandem duplication of *Cyp6g1*, was similar to that of *Cyp6g1*, and thus also not associated with DDT resistance in the 16 strains/lines (Fig. 3).

Although the *Cyp6a8* was significantly overproduced in both the high resistant strain 91-R (RR = 779-fold) and the moderate resistant Wisconsin-WD lines (RR = 89–140-fold), its expression levels in the moderate resistant lines showed no significant difference with those of the susceptible or low resistance strain (Canton-S, 91-C, Wisconsin-MT and Wisconsin-2 bp; Fig. 3).

The expression level *Cyp6w1*, the P450 gene near to *Cyp6a2* on the chromosome, was no significant difference between the highly resistant 91-R strain and most other strains or family lines (Fig. 3). The expression data for the six P450 genes implied that *Cyp6a2* in these strains was independently upregulated.

3.5 Effects of the Nrf2/Maf binding site and LTR of transposable element *17.6* on the constitutive expression of *Cyp6a2* alleles

To confirm the role of the Nrf2/Maf binding site in regulation of constitutive expression of *Cyp6a2*, we subcloned the proximal 217-bp 5′-flanking sequences (−1 to −217, immediately upstream of the translation initiation site) of the $Cyp6a2^{Canton\text{-}S\text{-}1}$, $Cyp6a2^{Canton\text{-}S\text{-}2}$ and $Cyp6a2^{91\text{-}R}$ alleles into the pGL3–Basic plasmid, respectively (Fig. 4A). Luciferase analysis showed that S2 cells transfected with the promoter sequence of the $Cyp6a2^{91\text{-}R}$ allele that contains the intact Nrf2/Maf binding site had significantly higher promoter activity than S2 cells transfected with the promoter sequence of the $Cyp6a2^{Canton\text{-}S\text{-}1}$ or $Cyp6a2^{Canton\text{-}S\text{-}2}$ allele (Fig. 4A). Both $Cyp6a2^{Canton\text{-}S\text{-}1}$ and $Cyp6a2^{Canton\text{-}S\text{-}2}$ alleles lacked the intact Nrf2/Maf binding site due to loss of the 15-bp fragment ($Cyp6a2^{Canton\text{-}S\text{-}1}$) or the upstream 'TGCG' quadrinucleotide ($Cyp6a2^{Canton\text{-}S\text{-}2}$) (Fig. 1). These data demonstrated that the intact Nrf2/Maf binding site could enhance the promoter activity of *Cyp6a2*.

To test whether the LTR of the transposable element *17.6* (LTR *17.6*) in the 3′-UTR of *Cyp6a2* reduces the basal expression of *Cyp6a2*, we subcloned the 3′-UTR sequences of the $Cyp6a2^{91\text{-}R}$

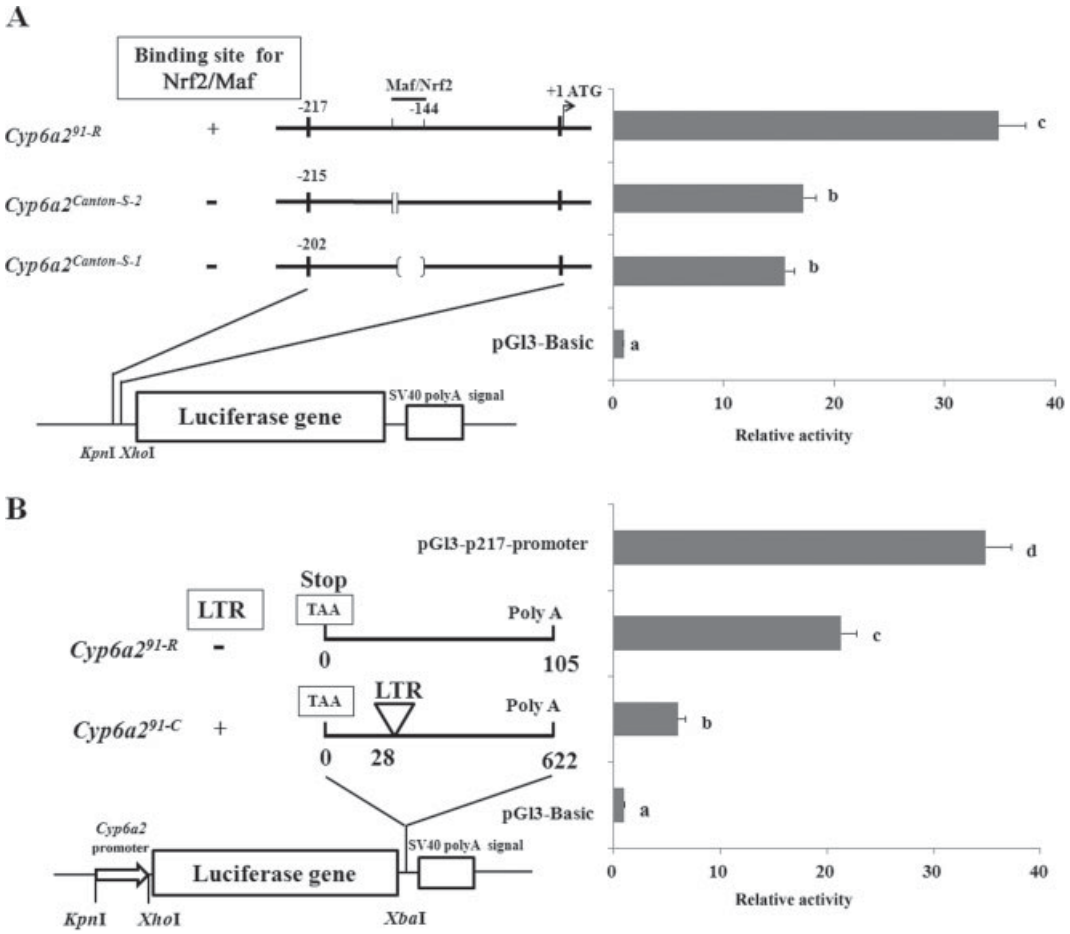

Figure 4. Expression of three *Cyp6a2* promoter–pGL3 constructs (A) and two *Cyp6a2* 3′-UTR–pGL3 constructs (B). The average relative luciferase activity and standard errors of three independent transfections are presented. Bars marked with different letters are significantly different at $p < 0.05$.

(105 bp, without the LTR *17.6*; Fig. 1) and *Cyp6a2*$^{91\text{-}C}$ (622 bp containing the LTR *17.6*; Fig. 1) alleles into the pGL3–p217-promoter plasmid (Fig. 4B). Luciferase analyses showed that S2 cells transfected with the promoter only sequence of the *Cyp6a2*$^{91\text{-}R}$ allele had the highest basal level of *Cyp6a2* expression, followed by S2 cells transfected with the promoter plus the 3′-UTR of the *Cyp6a2*$^{91\text{-}R}$ allele, and then S2 cells transfected with the promoter plus the 3′-UTR of the *Cyp6a2*$^{91\text{-}C}$ allele (Fig. 4B). This result indicated that the LTR transposable element *17.6* might contribute to a decreased constitutive expression level of *Cyp6a2*.

4 DISCUSSION

The focus of this study was to address the potential role of *Cyp6a2* in mediating DDT resistance and the factors likely to regulate *Cyp6a2* transcription. Two susceptible (Canton-S and 91-C) strains, a DDT-resistant field strain (Wisconsin) and a laboratory selected high DDT resistance strain (91-R) were investigated. A total of four functional (*Cyp6a2*$^{91\text{-}R}$, *Cyp6a2*$^{Wisconsin\text{-}WD}$, *Cyp6a2*$^{Canton\text{-}S\text{-}1}$ and *Cyp6a2*$^{Canton\text{-}S\text{-}2}$) and one nonfunctional (*Cyp6a2*$^{91\text{-}C}$) *Cyp6a2* alleles were recovered (Fig. 1).

Our genetic and molecular analyses support the hypothesis that *Cyp6a2* is associated with DDT resistance in the resistant strains/lines under this study. First, functional alleles with the intact Nrf2/Maf binding site but without the LTR *17.6* (*Cyp6a2*$^{91\text{-}R}$ and *Cyp6a2*$^{Wisconsin\text{-}WD}$) were associated with higher LC_{50} values, whereas nonfunctional alleles (*Cyp6a2*$^{91\text{-}C}$) or functional alleles without the intact Nrf2/Maf binding site but with (*Cyp6a2*$^{Canton\text{-}S\text{-}1}$) or without (*Cyp6a2*$^{Canton\text{-}S\text{-}2}$) the LTR *17.6* associated with lower LC_{50} values (Table 1). Second, the Nrf2/Maf binding-site-containing and LTR *17.6*-lacking functional allele (*Cyp6a2*$^{91\text{-}R}$) was genetically linked to a higher LC_{50} value (Table 2). Third, DDT resistance was positively correlated with the expression level of *Cyp6a2* in all the tested strain/lines carrying functional alleles (Table 1).

DDT resistance was found to be positively correlated with the expression level of functional *Cyp6a2* alleles in all the tested strain/lines carrying functional alleles, while lack of correlation was observed for *Cyp6a8*, *Cyp6g1*, *Cyp6g2* and *Cyp6w1* (Fig. 3). It has been widely recognized that increased expression of insecticide detoxification CYP enzyme is a major mechanism of P450-mediated resistance, although a recent study provided a case showing that pyrethroid resistance is associated with a novel P450 CYP337B3.[4] The hypothesis that overexpression of CYP6A2 confers DDT resistance have been challenged largely due to the conflicting results of DDT metabolism studies. Dunkov *et al.* reported that baculovirus-expressed CYP6A2 showed no detectable activity to metabolize DDT under aerobic conditions.[29] By contrast, Amichot *et al.* presented that *E. coli* produced CYP6A2wt (from *y; cn bw sp* strain) enzyme could metabolize DDT to dicofol, DDD and DDA, and much greater capacity of DDT metabolism was observed in the CYP6A2vSVL (present in the DDT-resistant RDDT^{R} strain with amino acid substitutions R335S,

L336V and V476L).[3] In addition, Daborn *et al.* did not observe any increased survival in a toxicity assay at two doses (1 and 5 μg vial^{-1}) using the GAL4/UAS system where *Cyp6a2wt* was overexpressed in a susceptible genetic background.[17] The discrepancy between the above two metabolism studies may be because the two groups used different approaches to heterologous P450 expression and insecticide metabolism. No increased survival in the overexpressed transgenic lines may be because the expression level of the low-capacity *Cyp6a2* allele is not high enough to cause any phenotypic difference that can be distinguished by the two-dose assay. Based on these understandings, we argue that *Cyp6a2* is associated with DDT resistance in the Wisconsin and 91-R strains, although the contribution of CYP6G1 may not be denied. More precise and comprehensive metabolism studies are required to clarify the potential of different *Cyp6a2* allele and other candidate CYPs in DDT resistance in these strains under current investigation.

Constitutive overexpression of P450s can be caused by *trans*- or *cis*-acting factors, and in a few cases by the duplication or amplification of the P450 genes.[31,38,49–51] A preliminary study by real-time qPCR showed that no duplication of *Cyp6a2* gene occurred in Canton-S, 91-C and 91-R strains (data not shown). *Cis*-acting regulation of insecticide resistance-conferring P450s (e.g. *Cyp6g1* and *CYP6D1*) is often achieved through indels or mutations in their promoter region. The observation that the *Cyp6a2* alleles with the intact Nrf2/Maf binding site (*Cyp6a2*$^{91\text{-}R}$, *Cyp6a2*$^{91\text{-}C}$ and *Cyp6a2*$^{Wisconsin\text{-}WD}$) had higher basal expression of *Cyp6a2* than alleles without the intact binding site (*Cyp6a2*$^{Canton\text{-}S\text{-}1}$ and *Cyp6a2*$^{Canton\text{-}S\text{-}2}$; Table 1) suggests that gain of the Nrf2/Maf binding site is a significant change in *cis*-acting elements that are responsible for overexpression of *Cyp6a2*. Our luciferase reporter gene assays further confirmed this suggestion, because containing the intact Nrf2/Maf binding site significantly increased the luciferase activity (Fig. 4A).

The negative effect of the LTR *17.6* in the 3′-UTR on *Cyp6a2* transcription was inferred from the expression data and confirmed by our luciferase analysis (Table 1, Fig. 4B). A likely explanation is that the LTR *17.6* insertion reduces the stability of the CYP6A2 mRNA, probably via introduction of target sequences for micro RNAs or RNA-binding proteins.[8,52,53] The fact that the expression of *Cyp6a2*$^{91\text{-}C}$ in Wisconsin MT lines (average RE = 71, Table 1) (carrying an allele with both the Nrf2/Maf binding site and the LTR *17.6*) was only marginally decreased compared with that (average RE = 76, Table 1) of *Cyp6a2*$^{Wisconsin\text{-}WD}$(an allele with the Nrf2/Maf binding site but without the LTR *17.6*; Fig. 4), indicates that the LTR *17.6* insertion can only result in a limited impact on the expression of *Cyp6a2*. The minor influence of the LTR *17.6* can be easily masked by other alternations. This may partially explain why previous studies failed to show a correlation between the LTR *17.6* insertion and the expression of *Cyp6a2* or DDT susceptibility.[54,55]

Our finding of the Nrf2/Maf binding site as an enhancer for constitutive overexpression of *Cyp6a2* is consistent with previous results, in which deletion of the 15-bp fragment which encompasses an Nrf2/Maf binding site is responsible for a low basal level expression of *Cyp6a2* in *Met*[1] mutants.[47] Interestingly, a 15-bp element identified in the promoter of resistant allele of *CYP6D1* increases the expression of *CYP6D1* via disrupting a mdGfi-1 binding site. However, the Nrf2/Maf binding site alone cannot fully explain the differences in *Cyp6a2* mRNA levels among strains. For example, *Cyp6a2*$^{91\text{-}R}$ and *Cyp6a2*$^{Wisconsin\text{-}WD}$ are identical to each other in terms of the three major sequence differences (Fig. 1), but the 91-R had significantly much more CYP6A2 transcript (3-7fold) than Wisconsin-WD(−1∼−4) (Table 1). Although the enhancement effect of Nrf2/Maf binding site in regulation of basal transcription (according to our luciferase assay in S2 cells) is limited (around twofold), this site may play a more ecologically and evolutionarily important role in the induction of *Cyp6a2* gene, based on the observation that this Nrf2/Maf binding site in the *Cyp6a2* promoter is both necessary and sufficient to mediate xenobiotic inducible transcription.[47] The induction of *Cyp6a2* expression will provide flies a more efficient protection, given the broad substrate specificity of CYP6A2.[29]

The differences of *Cyp6a2* mRNA level among various strains (alleles) strongly suggest that other unknown *cis*-element(s) or/and *trans*-acting factor(s) may also contribute to the differences in *Cyp6a2* expression. This proposal is supported by other previously reported evidences. For example, sequence in −983/−522 region of *Cyp6a2* promoter has the boost basal transcription activity;[41] one or more *trans*-acting factors on the third chromosome are required for the elevated expression of *Cyp6a2* in DDT-resistant lines.[39] A more extensive study is needed to reveal the factors that define the constitutive overexpression of *Cyp6a2* gene in resistant strains.

In addition, upregulation of *Cyp6a2* alone is not necessary to ensure DDT resistance at high levels in the strains we investigated. Our findings in this study do not rule out other mechanisms involved in DDT resistance. Multifactorial resistance to DDT has been reported in the 91-R strain.[27] A recent study showed that decreased penetration, increased metabolism and direct excretion play a role in resistance of 91-R strain.[56] These results suggest that the mechanisms underlying DDT resistance are more complicated than previously suggested.

ACKNOWLEDGEMENTS

We are grateful to Dr. Qilian Qin (Institute of Zoology, CAS) for the *Drosophila* S2 cells culture, and to Dr Yi-ming Mu (Department of Endocrinology, Chinese PLA General Hospital) for providing pGL3-basic and pRL-TK plasmids. We thank the reviewers for their constructive comments. This work was supported by grants from National Basic Research Program of China (973 program, No. 2012CB114103) and National Natural Science Foundation of China (No. 30870342).

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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