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Genome-wide analysis of genes associated with moderate and high DDT resistance in *Drosophila melanogaster*

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Abstract

BACKGROUND: Moderate to high DDT resistance in generally associated with overexpression of multiple genes and therefore has been considered to be polygenic. However, very little information is available about the molecular mechanisms that insect populations employ when evolving increased levels of resistance. The presence of common regulatory motifs among resistance-associated genes may help to explain how and why certain suites of genes are preferentially represented in genomic-scale analyses.

RESULTS: A set of commonly differentially expressed genes associated with DDT resistance in the fruit fly was identified on the basis of genome-wide microarray analysis followed by qRT-PCR verification. More genes were observed to be overtranscribed in the highly resistant strain (*91-R*) than in the moderately resistant strain (*Wisconsin*) and susceptible strain (*Canton-S*). Furthermore, possible transcription factor binding sites that occurred in coexpressed resistance-associated genes were discovered by computational motif discovery methods.

CONCLUSION: A glucocorticoid receptor (GR)-like putative transcription factor binding motif (TFBM) was observed to be associated with genes commonly differentially transcribed in both the *91-R* and *Wisconsin* lines of DDT-resistant *Drosophila*.
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Supporting information may be found in the online version of this article.

Keywords: dichlorodiphenyltrichloroethane (DDT); pesticide resistance; transcription factor binding motif; gene transcription

1 INTRODUCTION

Insecticide resistance is both an ongoing challenge for pest management and an important example of a man-made evolutionary force.[1,2] Resistance is an ideal system for studying the microevolution of environmental adaptation.[2,3] DDT (dichlorodiphenyltrichloroethane) resistance in the fruit fly, *Drosophila melanogaster*, has been used extensively for the study of the evolution of insecticide resistance. Two types of DDT resistance in *Drosophila* have been observed: target-site insensitivity and metabolism-based resistance. With respect to target-site insensitivity in *Drosophila*, DDT resistance has been associated with amino acid changes in the voltage-gated sodium channel encoded by the *para* gene.[4,5] Metabolic insecticide resistance is associated with overexpression of (or in some cases structural changes in) cytochrome P450s, glutathione-*S*-transferases (GSTs), esterases or a combination of these genes.[6–8]

Moderate to high-level metabolic DDT resistance in *Drosophila* is generally considered to be polygenic;[6,8–12] however, low-level DDT resistance may in some cases be monogenic.[13] Metabolic DDT resistance is not a single phenotype; the lethal concentration 50 (LC_{50}) for DDT varies considerably across pesticide-resistant *Drosophila* strains.[11] For example, the two metabolically pesticide-resistant fly strains *Wisconsin* and *91-R* show highly different LC_{50} values when bioassayed with DDT; *91-R* is far more resistant to DDT than *Wisconsin*. In some *Drosophila* strains with moderate to high levels of DDT resistance, considerable evidence supports the hypothesis that DDT resistance is polygenic and is at least partially associated with overexpression of cytochrome P450s.[6,8,11] Additionally, Amichot *et al*. have elucidated that, in some *Drosophila* strains, amino acid changes in CYP6A2 are associated with increased resistance to DDT.[7] To date, very little is known about the molecular 'steps' that insect populations use to evolve increasing levels of resistance. For example, do highly resistant pest populations (i) 'build upon' the resistance mechanisms of

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moderately resistant populations (do they produce more of the same gene products) or (ii) do they achieve resistance through different mechanisms (do they produce new gene products or different allelic forms of those gene products)?

The *Drosophila* genome, like other eukaryotic organisms, comprises both non-coding and protein-coding DNA. Non-coding DNA harbors a variety of regulatory elements containing binding sites for specific combinations of transcription factors (TFs) that interact in a coordinated fashion.[14] The discovery and characterization of such regulatory modules of coexpressed genes, based on gene expression profiling, are critical for understanding how resistance has evolved and for determining whether there is a master switch (or multiple regulatory factors) that may have changed in response to selection, and that could be exploited for resistance management purposes. To date, most studies have addressed the regulation of individual genes in conferring resistance to DDT.[15,16] Thus, there are no reports of multigene analyses of putative transcription factor binding sites for coexpressed genes associated with DDT resistance.

Pedra *et al*. used oligoarray analysis to address whether moderate and high-level DDT resistance is associated solely with the overtranscription of *Cyp6g1* or also is complemented by overtranscription of other genes.[8] Using the *Drosophila* strains *Wisconsin* and *91-R*, they concluded that DDT resistance is associated with the overexpression of multiple genes. However, they did not define a core set of genes associated with moderate and high-level DDT resistance, nor did they differentiate between gene expression patterns within moderately and highly resistant strains. By doing this, it would be possible to begin to identify resistance-associated genes for further investigation of shared regulatory motifs. To address these issues, the present authors reanalyzed the data of Pedra *et al*.,[8] using the more robust significance analysis of microarrays (SAM) method, and verified by qRT-PCR analyses a subset of the identified over- and undertranscribed genes. In addition, an analysis was made of the regulatory motifs of genes coexpressed within the two DDT resistance phenotypes in order to identify candidate putative transcription factor binding motifs that may be associated with regulation of at least some resistance-associated genes.

2 MATERIALS AND METHODS

2.1 *Drosophila* strains

Three *Drosophila melanogaster* lines were used: *Canton-S* (susceptible to DDT), *Wisconsin* (moderately resistant to DDT) and *91-R* (highly resistant to DDT). Detailed descriptions of these fly strains, rearing conditions and sample preparation were given in Brandt *et al*.[17] and Pedra *et al*.[8] The LC_{50} values were 12.9, 447.0 and 8348.0 μg DDT in 100 μL of acetone, respectively, for *Canton-S*, *Wisconsin* and *91-R*.[12]

2.2 Microarray and statistical analysis

Microarray procedures and data collection were described in Pedra *et al*.[8] However, use was made of two-class significance analysis of microarrays (SAM) to analyze the data.[18] SAM is based on a permutation test and has recently been used widely in microarray data analysis.[19] The data were first normalized using the housekeeping gene *Actin-3*. The data were divided into three subsets: (i) *Canton-S* versus *91-R*; (ii) *Wisconsin* versus *91-R*; (iii) *Canton-S* versus *Wisconsin*. In each subset, those genes observed to have significant over- or undertranscription, were determined using a 5% false discovery rate (FDR).

2.3 Quantitative real-time PCR (qRT-PCR)

For each strain of fly, three RNA samples were prepared. Experimental adult flies were collected as virgins by using CO_2 anesthesia, and male and female flies were reared separately. Three-day-old flies were used for RNA extraction. RNA was extracted from 30 flies (1:1 male/female ratio) using the Qiagen RNeasy kit (Qiagen, Valencia, CA) with an 'on-column' DNase digestion procedure. The first-strand cDNA was synthesized by using 0.5 μg of total RNA with iScript cDNA kit from Bio-Rad (Hercules, CA) in a 20 μL reaction volume. Each first stranded cDNA was diluted tenfold for qRT-PCR, which was performed with iQ SYBR Green Supermix from Bio-Rad with SYBR Green dye on an iCycler thermal cycler. For each cDNA, three qRT-PCR reactions were performed. The threshold cycle (CT) was calculated by the iCycler IQ software. The relative expression levels were calculated as given in Pfaffl *et al*.[20] SAS software (SAS Institute, Cary, NC) was used to analyze the data. The *rp49* gene was used as a reference. All the primers for the assayed genes are listed in supporting information Table S1.

2.4 Analysis of putative transcription factor binding motifs of genes commonly differentially expressed with DDT resistance

Five different programs, AlignACE,[21] MEME,[22] MDscan,[23] BioProspector[24] and Weeder,[25] were used as motif discovery tools to analyze a set of coexpressed genes. For this set of genes, promoter sequences of 1000 bp were selected to contain 800 bp upstream and 200 bp downstream from the transcription start site (TSS). The results of each motif discovery program were represented in a format of position weight matrices (PWMs), the columns of which defined the probabilities of each nucleotide (A, C, G, T) at each position of the motif. Significant motifs identified from each of the five programs were pooled and clustered using either a k-medoids algorithm or a hierarchical clustering algorithm. The center of each cluster was selected as a potential transcription factor binding motif. The identified binding motifs were further verified by scanning the promoter sequences. Significant hits were evaluated by *P*-values, defined as the probability that a motif with a similar matching score could be obtained in scanning randomly selected sequences. A motif with a *P*-value of less than 0.05 was considered to be significant. The predicted motifs were compared with transcription factors in the TRANSFAC[26] database. Transcription factor names were assigned according to the closest known motifs in other organisms in the TRANSFAC[26] database. The closest match from TRANSFAC was used to annotate the identified motifs. The distance value (*d*-value) was defined as a dissimilarity measurement between any pair of PWMs. A distance of 0.07 or less suggested a relatively similar TF motif to that of a mammalian TF motif. The smaller the *d*-value, the closer the TF motif was to that found in mammals.

3 RESULTS AND DISCUSSION

3.1 Differentially expressed genes among three *Drosophila* phenotypes based on microarray data

The results of gene expression profile analysis were summarized in a Venn diagram (Fig. 1). Differential transcription was found in 310 probe sets between *Wisconsin* and *91-R* strains, and a total of 246 genes displayed significantly different levels of expression between *Canton-S* and *91-R* strains, while transcription of 16 genes was significantly different between *Canton-S* and *Wisconsin*. The differences in gene expression among the strains may be

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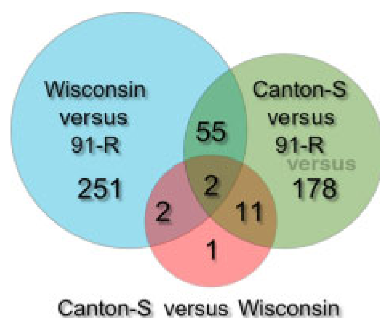

Figure 1. A Venn diagram representing the number of differentially expressed genes when comparing the three strains: (i) *Canton-S* versus *Wisconsin*; (ii) *91-R* versus *Canton-S*; (iii) *91-R* versus *Wisconsin*.

associated with a direct or indirect role in DDT resistance, with genetic background among various strains, with other biological or technical variations or with a combination of these factors. A relatively small number of genes were differentially transcribed in the *Wisconsin* line compared with the *Canton-S* strain, possibly reflecting the more natural conditions of selection for DDT resistance in *Wisconsin*. In contrast, a substantially larger number of genes corresponding to 310 and 246 probe sets, compared with *Wisconsin* and *Canton-S* line respectively, were differentially transcribed in the highly resistant *91-R* strain.

To identify a general list of overtranscribed genes that are associated with DDT resistance, the authors focused on shared subsets of genes that were observed to be differentially transcribed in at least two pairwise comparisons (Fig. 1). This method was intended to minimize the variation due to differences in genetic background among strains. For example, of the differentially expressed genes between the *Canton-S* and *91-R* strains, 13 genes were also differentially expressed between the *Canton-S* and *Wisconsin* strains, and therefore these 13 genes represent a set of genes that may confer (or at least be associated with) moderate levels of DDT resistance. Fifty-seven transcripts, including two transcripts (corresponding to *Cyp6a2* and *Cyp12d1*) in the above-mentioned 13 genes, were shared between the comparisons of *Wisconsin* versus *91-R* and *Canton-S* versus *91-R*. Thus, these 57 genes could be involved in conferring high levels of DDT resistance to *91-R* strain. These expression patterns are in keeping with the concept that high levels of DDT resistance may include both a core set of overexpressed genes shared with *Wisconsin* (13 genes in this study) and other genes (55 genes) that are not shared with *Wisconsin*.

3.2 Over- and undertranscribed genes among moderately and highly DDT-resistant *Drosophila* phenotypes based on qRT-PCR data

qRT-PCR analysis was used to confirm the differential expression of genes indicated by microarray results to be associated with moderate and high resistance. All of the 13 genes associated with moderate levels of DDT resistance were included. From the 55 genes proposed to be involved in conferring high levels of DDT resistance, 24 genes for which annotation information was available were then chosen. In addition, *Cyp6g1* and *Acp-1* (adult cuticle protein-1) were included for the qRT-PCR comparison, given that *Cyp6g1* has been implicated in playing an important role in DDT resistance in *Drosophila*,[13] and the homolog of *Acp-1* (*CPLC8*) is overtranscribed in a highly insecticide-resistant *Anopheles stephensi* DUB-R strain.[27]

The qRT-PCR results, along with the primers used for each gene, are given in Table 1 and S1. Transcripts were considered to be significantly over- or undertranscribed if they had *P*-values of <0.05 (Table 1). Of 38 genes assayed, nine were overtranscribed in both *Wisconsin* and *91-R* versus *Canton-S*, and an additional 12 genes were overtranscribed in *91-R* versus *Canton-S* (Table 1). Although qRT-PCR detected fewer over- and undertranscribed genes than did the microarray analysis, overall results from qRT-PCR showed that *91-R* overtranscribed more genes than *Wisconsin*.

3.3 Transcripts coding for detoxification enzymes

Transcriptional overexpression of genes, especially those coding for detoxification enzymes, has been suggested as a common and key mechanism in the development of resistance in insects.[28,29] Three P450 genes, *Cyp12d1*, *Cyp6g1* and *Cyp6a2*, which have been previously associated with DDT resistance,[6,7,17] were differentially expressed in *Wisconsin* and/or *91-R* when compared with *Canton-S*. In both strains, *Cyp6g1* was significantly overtranscribed at 2–3-fold higher than *Canton-S*, but, for *Cyp6a2*, *91-R* showed 30-fold higher expression compared with fivefold higher expression in *Wisconsin*. In addition, a transcript for a cytochrome P450 gene (*Cyp9c1*) not previously associated with DDT resistance was overexpressed in *91-R* but not *Wisconsin*. Thus, in general, the higher levels of DDT resistance found in *91-R* are associated with higher P450 expression levels of more P450 genes.

One exception to this observed effect was the mitochondrial P450 *Cyp12d1*, which was overtranscribed in *Wisconsin* but undertranscribed in *91-R* (Table 1) when compared with *Canton-S*. Recently, the *Cyp12d1* gene locus was shown to vary in copy number between *D. melanogaster* strains, with all three strains tested here showing a single copy of the gene.[30] In addition, sequencing of the *Cyp12d1* genomic region and mRNA transcript for the strain *91-R* revealed a splice site mutation that results in retention of the third intron, which contains a premature stop codon.[30] If the strain *91-R* produces a truncated CYP12D1 protein, it would be selectively advantageous to undertranscribe this gene, as observed in comparison with *Canton-S*. In the *Wisconsin* strain, overtranscription of *Cyp12d1* is associated with DDT resistance, which is consistent with evidence showing that using the GAL4/UAS system *Cyp12d1* overexpression fly lines confers resistance to DDT and dicyclanil.[30]

Two GSTs (GSTE5 and GSTE6) were overexpressed at similar levels in both resistant strains. In *Drosophila melanogaster*, GSTs constitute a gene superfamily and have important functions, such as to detoxify insecticides or other xenobiotic compounds by catalyzing the conjugation of reduced glutathione to lipophilic compounds,[31] thereby increasing their solubility and facilitating their excretion from the cell.[32] Insecticide resistance in *Anopheles gambiae* has been attributed to increased GST activity and DDT dehydrochlorinase activity; a GST also confers DDT resistance in the housefly.[32–36]

However, differential expression of P450s and GSTs in resistant strains does not necessarily mean that these genes are playing a direct role in pesticide resistance. Certain P450s may be directly involved in metabolizing DDT,[7] but many of these P450 genes probably have endogenous functions in cellular processes[37] that have changed in response to the differential expression of resistance genes. For instance, as P450 reactions are known for the production of reactive oxygen species (ROS), the high levels of expression of *Cyp6a2* observed in *91-R* may have necessitated the underexpression of another P450 (*Cyp12d1*), along with the

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Table 1. Over- and undertranscription of *Drosophila* genes in *Wisconsin* and *91-R*, as compared with *Canton-S*, based on qRT-PCR

Gene category/name	*Wisconsin* versus *Canton-S*			*91-R* versus *Canton-S*		
	Ratio	ΔCT (SE)	*P*-value	Ratio	ΔCT (SE)	*P*-value
Monooxygenase						
Cyp6a2	5.79	−2.53 (0.31)	0.0002	34.30	−5.10 (0.31)	<0.0001
Cyp6g1	2.14	−1.10 (0.44)	0.0446	2.83	−1.50 (0.44)	0.0136
Cyp6g2	1.20	−0.27 (0.52)	0.624	1.66	−0.73 (0.52)	0.205
Cyp9c1	1.09	−0.013 (0.28)	0.653	2.96	−1.57 (0.28)	0.0014
Cyp12d1	2.46	−1.30 (0.22)	0.001	0.10	3.27 (0.22)	<0.0001
Glutathione transferase						
GstE5	2.09	−1.07 (0.16)	0.0005	3.03	−1.60 (0.16)	<0.0001
GstE6	2.05	−1.03 (0.24)	0.006	2.52	−1.33 (0.24)	0.0016
Serine-type endopeptidase						
CG1304	0.51	0.97 (0.74)	0.242	10.31	3.37 (0.74)	0.004
CG9377	0.79	0.33 (0.38)	0.410	8.19	−3.03 (0.48)	0.0002
Ser6	1.66	−0.73 (0.25)	0.027	0.26	1.97 (0.25)	0.0002
Spn	1.66	−0.73 (0.36)	0.090	11.31	−3.50 (0.36)	<0.0001
Triacylglycerol lipase						
CG3635	1.95	−0.97 (0.09)	0.0001	3.17	−1.67 (0.09)	<0.0001
Structural constituent of cuticle						
CG4784	1.59	−0.067 (0.14)	0.003	4.39	−2.13 (0.14)	<0.0001
Lcp1	1.26	−0.33 (0.33)	0.3499	2.52	−1.33 (0.33)	0.0067
Lysozyme						
Lysp	0.91	0.13 (0.20)	0.526	9.62	−3.26 (0.20)	<0.0001
Epoxide hydrolase						
Jheh1	1.02	−0.03 (0.19)	0.867	2.35	−1.23 (0.19)	0.0006
Alcohol dehydrogenase binding						
Adh	1.95	−0.70 (0.37)	0.105	1.62	−0.97 (0.37)	0.039
Sulfotransferase binding						
Pip	1.02	−0.03 (0.21)	0.879	12.41	−3.63 (0.21)	<0.0001
Others						
AC1	1.18	−0.23 (0.23)	0.354	1.70	−0.77 (0.20)	0.013
Acp1	1.02	−0.03 (0.34)	0.925	0.83	0.27 (0.34)	0.460
ATtrans	0.95	0.07 (0.41)	0.876	0.93	0.10 (0.41)	0.814
Boss	0.93	0.10 (0.29)	0.738	1.20	−0.27 (0.29)	0.386
CG11893	1.62	−0.70 (0.26)	0.038	1.66	−0.73 (0.26)	0.032
CG14715	1.62	−0.70 (0.28)	0.044	1.18	−0.23 (0.28)	0.430
CG7708	0.95	−0.07 (0.41)	0.876	1.30	−0.37 (0.41)	0.406
CG7955	1.02	−0.03 (0.28)	0.908	1.52	−0.60 (0.28)	0.073
Cpn	0.68	0.57 (0.29)	0.101	1.12	−0.17 (0.29)	0.590
Dbi	1.70	−0.77 (0.14)	0.0013	1.05	−0.07 (0.14)	0.642
Delpha	0.95	0.07 (0.25)	0.802	1.45	−0.53 (0.25)	0.080
Gad	2.14	−1.10 (0.51)	0.074	2.30	−1.20 (0.51)	0.057
Hrb87F	1.38	−0.47 (0.32)	0.193	1.48	−0.56 (0.32)	0.126
map205	1.07	−0.10 (0.28)	0.733	1.05	−0.07 (0.28)	0.812
Nhe2	0.85	0.23 (0.46)	0.627	0.87	0.20 (0.46)	0.677
mthl8	1.48	−0.57 (0.24)	0.053	1.62	−0.70 (0.24)	0.025
Sbb	1.30	−0.37 (0.23)	0.16	1.26	−0.33 (0.26)	0.196
Pdh	0.98	0.03 (0.22)	0.884	1.26	−0.33 (0.22)	0.180
Pgrp	0.93	0.10 (0.26)	0.716	1.87	−0.90 (0.26)	0.014
Sbb	1.30	−0.37 (0.23)	0.16	1.26	−0.33 (0.26)	0.196

overtranscription of ROS-conjugating GSTs, in order to maintain an equilibrium. Given that the enzymatic functions of most P450s in *D. melanogaster* are unknown, their role in DDT resistance can only be speculated, but having a global view of their expression patterns allows for hypothesis-driven testing of their functions.

3.4 Transcripts coding for lipid metabolism

Lipid metabolism and mobilization could also be altered as a result of the physiological changes occurring in response to insecticide resistance. Araujo *et al*. have documented that lipases are higher in resistant populations of the maize weevil, *Sitophilus zeamais*.[38] Both *Drosophila* resistant strains show increased

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expression of *CG3635*, a gene with high amino acid identity with the lipase 3 precursor of *Drosophila* and several mammalian gastric triacylglycerol lipase precursors, and predicted to have triacylglycerol lipase activity. The few insect lipases that have been characterized play roles in the digestion of dietary fats, in the hydrolysis of lipids[39,40] and in lipid mobilization from the insect fat body.[41] Higher efficiency in lipid hydrolysis, resulting from higher lipase activities, may increase energy mobilization in resistant insects, which is required to maintain their resistance mechanisms.[38] Although these data suggest a possible association between insecticide resistance and lipid metabolism, the direct or indirect role of lipases in resistance of *Drosophila* to insecticide remains to be determined.

3.5 Transcripts coding for protein metabolism

Four genes related to protein metabolism were differentially expressed in *91-R*, including a serine proteinase (*Ser 6*) that was undertranscribed, two serine-type endopeptidases (*CG1304, CG9377*) and a serine-type endopeptidase inhibitor that was overtranscribed. None of these genes was differentially transcribed in *Wisconsin* as compared with *Canton-S* (Table 1).

These results hold out the possibility of a relationship between high levels of DDT resistance, as seen in *91-R* but not *Wisconsin*, and serine proteinase activity, which supports similar observations in the housefly,[42] *Anopheles gambiae*,[43] *Culex pipiens pallens*[44] and *Bemisia tabaci*.[45] Ahmed *et al*. documented that proteinases have higher enzymatic activities in DDT-resistant houseflies than in susceptible ones.[42]

Several putative peptidases, including serine protease, showed remarkable constitutive overtranscription in DDT-resistant strains of *A. gambiae*.[43] Gong *et al*. suggested that overtranscription of trypsin and chymotrypsin in *Cx. pipiens pallens* is associated with insecticide resistance.[44] One hypothesis is that the increased proteolytic activity in resistant insects may help the insects meet energy demands,[8] thus balancing protein degradation and synthesis during stress.[44] In addition, serine proteases regulate several invertebrate defense responses, including hemolymph coagulation, antimicrobial peptide synthesis and melanization of pathogen surfaces.[46] An alternative hypothesis is that the activation of immune pathways may enhance the capacity of insect to adapt to the insecticide pressure.

3.6 Differentially expressed cuticle-associated genes

Interestingly, two transcripts for genes (*CG4784* and *Lcp-1*) that encode for structural molecules of cuticle were overtranscribed in *91-R*. Only one of them, *CG4784*, was overtranscribed in *Wisconsin* compared with *Canton-S*. The cuticle is known to be a major route by which insecticides penetrate insects. Insects can reduce penetration of insecticides into their bodies by a thickening or a change in the chemical composition of the cuticle.[47,48] Reduced insecticide penetration has been proposed to be an important mechanism in insecticide resistance. For example, penetration-based resistance has been observed in organophosphate-resistant strains of *Culex quinquefasciatus*[47] and *Culex tarsalis*.[48] Therefore, the high level of DDT resistance in *91-R* may be partially explained by reduced penetration, which may be associated with the change in expression of both *CG4784* and *Lcp-1*. The moderate DDT resistance in *Wisconsin* could be partially explained by expression of *CG4784* without *Lcp-1*.

The present authors also measured the expression of *CG7216* (*Acp-1*), a gene encoding for cuticle strengthening/thickening protein in *Drosophila*, because its ortholog is overtranscribed in a highly insecticide-resistant *Anopheles stephensi* DUB-R strain.[27] However, no differences in its transcription were observed among the three phenotypes studied (Table 1), which indicates that insects may utilize different genes to acquire penetration-based resistance. Another example, reported by Djouaka *et al*., found that the overexpression of two cuticular precursors was associated with pyrethroid resistance in *Anopheles gambiae* s.s. from Southern Benin and Nigeria.[49] Compared with target insensitivity and metabolic resistance, the mechanism of cuticular resistance is less understood and clearly warrants further investigation.

3.7 Additional genes associated with high DDT resistance

In the artificially selected DDT resistant strain, *91-R*, certain overtranscribed genes encode for proteins involved in the defense response (*Jheh1*), immune response (*Lysp*) and regulation (*Adh* and *pip*). Epoxide hydrolases, including *Jheh1*, have been implicated in juvenile hormone degradation in insects and have been shown to be inducible by a diversity of xenobiotics.[50,51] Although these researchers hypothesized that these enzymes play a role in xenobiotic metabolism, the roles of these genes and gene products in insecticide resistance have not been investigated.

3.8 Common regulatory factor binding motifs of genes associated with DDT resistance

Using the identified set of coexpressed genes associated with both moderate and high levels of DDT resistance, the authors investigated whether these nine genes shared regulatory motifs. If these genes changed in concert, in response to selection for DDT resistance, then a single shared regulatory mechanism by which they could all be partially or fully controlled would be expected. The presence of a 'regulatory switch' would suggest that a single transcription factor, upstream of the coregulated genes, had changed. On the other hand, if the expression of these genes changed in a stepwise pattern over time, then more variation in the predicted binding sites would be expected, and perhaps single nucleotide polymorphisms in the binding sites. These predictions are not mutually exclusive, as it is possible to imagine a scenario in which a single transcription factor upstream of these genes mutates, which then leads to differential regulation of the genes but also changes their evolutionary trajectories, allowing them to mutate and undergo additional transcriptional changes. Computational motif discovery methods were used to identify putative transcription factor (TF) binding sites associated with the aforementioned coexpressed genes. The predicted TF binding sites that occurred in more than half of the nine genes include sites for glucocorticoid receptor (GR)-like, androgen receptor (AR)-like, D-site binding protein (DBP)-like, and CCAAT/enhancer binding protein (C/EBP) gamma-like (Fig. 2). These results demonstrate that the coexpressed genes share one predicted TF binding site, a GR-like motif, supporting the prediction for a common coregulation of at least some of these genes, but beyond that, the genes do not necessarily share other TF binding sites, suggesting that additional pathways may also contribute to individual gene transcription. Interestingly, the DBP has been associated with circadian expression of at least one P450 in the liver of rats.[52] In *Drosophila*, P450 expression and DDT resistance are both influenced by circadian rhythms.[53] Additionally, CCAAT/enhancer-binding proteins have also been shown to be involved in P450 expression in human hepatocytes[54] and in a furanocoumarin-inducible lepidopteran P450.[55]

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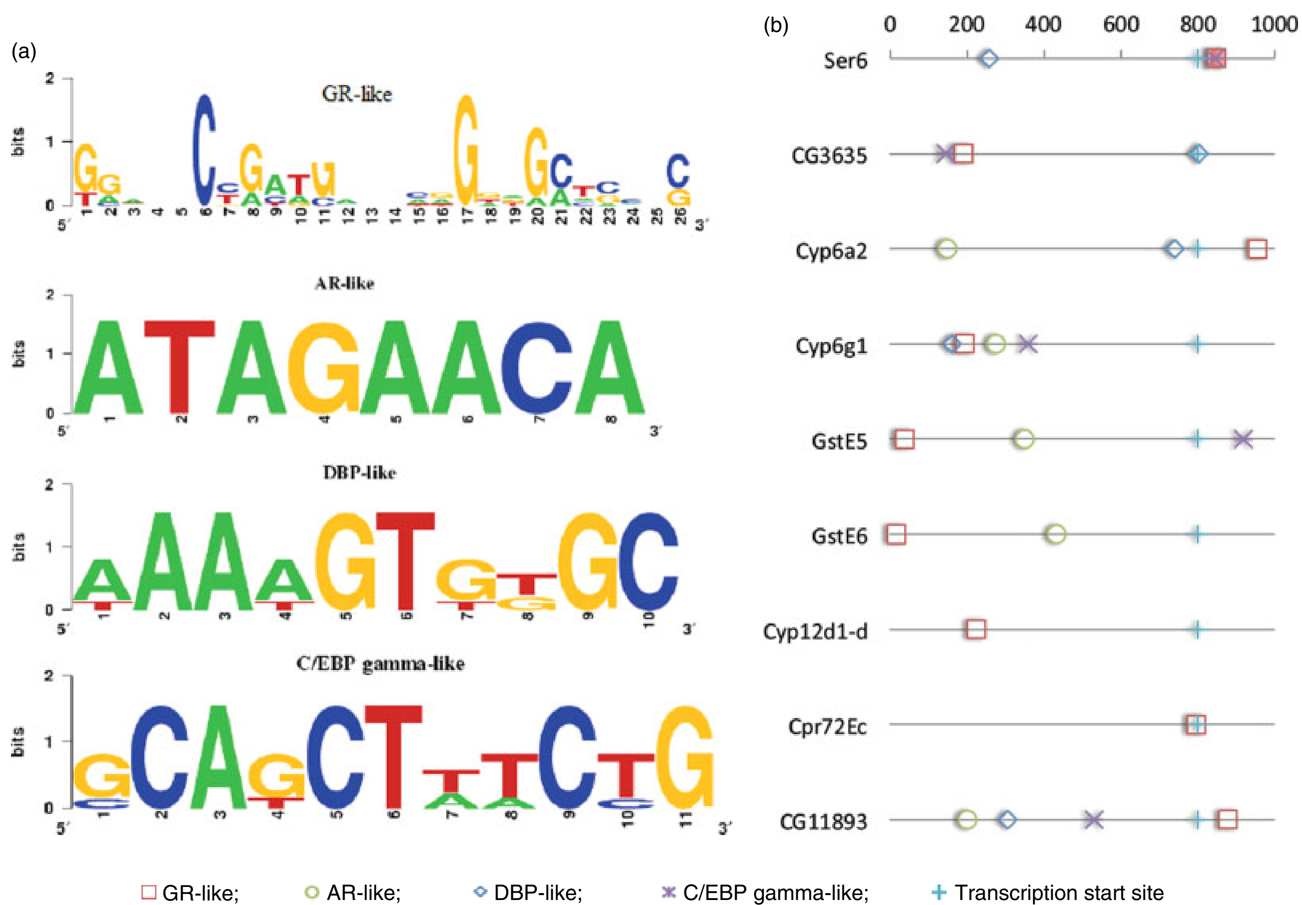

Figure 2. Predicted transcription factor binding sites identified within nine genes associated with DDT resistance. GR-like (high affinity binding site for glucocorticoid receptor, $d = 0.0989$); AR-like (androgen receptor, $d = 0.0730791$), DBP-like (D-site binding protein-like, $d = 0.156497$); C/EBP gamma-like (CCAAT/enhancer-binding gamma-like proteins, $d = 0.156066$). TF names were assigned according to the closest known motifs in other organisms in TRANSFAC database.[26] The distance was defined as a dissimilarity measurement between any pair of PWMs. A distance d of 0.07 or less suggested a relatively similar TF motif to that of mammalian. The smaller the value of d, the closer the TF motif was to that found in mammals. (A) The logo height of the letter indicates the probability of appearing at the position in the motif. (B) The position of putative transcription factor binding sites within 800 nucleotides (nt) and 200 nt downstream of the transcription start site.

Notably, the GR-like motif was present in all nine genes and also with a small d-value (0.0989). GR is a classical steroid receptor, belonging to the superfamily of nuclear receptors that function as ligand-dependent transcription factors, which regulates the expression of target genes negatively or positively.[56] Transcriptional regulatory mechanisms through GR have been extensively studied in mammals, and it has been demonstrated that GR can either up- or downregulate target gene expression through direct binding to specific DNA sequences [glucocorticoid response elements (GREs)] or through interaction with transcription factors such as activator protein-1, nuclear factor-kB, signal transducers and activators of transcription (STAT).[57] Through these versatile regulatory mechanisms, GR participates in the regulation of genes involved with resistance to stress, regulation of intermediary metabolism and immunosuppressive and anti-inflammatory effects.[58,59] Specifically, GR has been implicated in regulating expression of several drug-metabolizing cytochrome P450s (CYP3A4, CYP2C9, CYP2C19) in mammalian livers.[54] The presence of GR binding motifs within the nine identified coregulated genes associated with DDT resistance implicates a GR-like homolog in *D. melanogaster* as a regulatory factor that may have changed in response to selection (insects are not known to have glucocorticoids per se). Additional studies are now needed to test the role of GR-like in the regulation of these genes and to identify whether its regulatory function has changed in the *Wisconsin* and *91-R* strains.

Although several potential TF binding sites were found by referring to the TRANSFAC database, the existence and/or functions of these putative TFs in *Drosophila* still need to be investigated using biological tools. Present understanding of the regulatory mechanisms of genes involved in insecticide resistance in insects is very limited, even at the level of identifying which transcription factors are involved. Yet this study demonstrates how the identification of coregulated genes and predictive bioinformatics can offer better insight into the possible regulatory mechanisms involved in the development and evolution of insecticide resistance.

4 CONCLUSION

A set of coexpressed genes associated with DDT resistance in the fruit fly has been identified on the basis of genome-wide microarray analysis followed by qRT-PCR verification. Observations indicate that, in an artificially selected and highly resistant strain (*91-R*), more genes were differentially expressed than in a naturally resistant and moderately resistant strain (*Wisconsin*) compared

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with a susceptible strain (*Canton-S*), which suggests that *91-R* achieves high levels of resistance via unique mechanisms. Yet, for those differentially expressed genes shared by *91-R* and *Wisconsin*, overwhelmingly they were more highly expressed in *91-R* than in *Wisconsin*, suggesting that highly resistant pest populations also build upon common resistance mechanisms, such as detoxification, energy metabolism and cuticle penetration, for additional levels of resistance. Furthermore, putative transcription factor (TF) binding sites that occur in promoters of coexpressed genes from both strains have been discovered by computational motif discovery methods. From this analysis, a GR-like homolog is postulated to play an important role in regulating some resistance-associated genes in *Drosophila*.

However, it cannot be claimed at this time that any of these genes, other than those that have been previously tested in transgenics or in other assay systems,[30] are directly involved in resistance. Inferences from this study are limited to the three fly strains that were used. As techniques improve and it becomes cheaper to identify point mutations across the genome and expression level differences of transcripts between different organisms, future researchers may be able to conduct allele-specific expression analyses that will ascertain whether variation is due to polymorphisms at the gene locus or in other genes that influence its expression. Ultimately, understanding those resistance-associated traits that are common across insect strains may enable researchers (i) to understand how resistance evolves and (ii) to use pesticide resistance as a mechanism to gain a better understanding of gene regulation associated with resistance.

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SUPPORTING INFORMATION

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

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