REGULAR ARTICLE

Profiling of abundant proteins associated with dichlorodiphenyltrichloroethane resistance in *Drosophila melanogaster*

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Dichlorodiphenyltrichloroethane (DDT) metabolism-based resistance in *Drosophila melanogaster* is a complex metabolic system associated with the transcription of detoxification related genes, ion transport, lipid and sugar metabolism pathways. However, little is known about the differences regarding the proteome of field- and laboratory-selected resistant *Drosophila* genotypes. We investigated the impact of DDT resistance in the abundant proteome of field- and laboratory-selected resistant *Drosophila* using a two-dimensional gel electrophoresis DDT reference map. Proteomic profiling was performed in two DDT susceptible genotypes (Canton-S and 91-C) and three DDT resistant lines (*Rst(2)DDT*$^{91\text{-}R}$, *Rst(2)DDT*^{Wisconsin} and *Rst(2)DDT*$^{Hikone\text{-}R}$). Protein spots were stained with Coomassie blue and compared using PDQuest software. Selected protein spots were cut out and analyzed using matrix assisted laser desorption-time of flight mass spectrometry. Querying the NCBInr. 10.21.2003 database with mass spectrometric data yielded the identity of 21 differentially translated proteins in *Rst(2)DDT*$^{91\text{-}R}$, *Rst(2)DDT*^{Wisconsin} and Canton-S representing proteins putatively involved in biochemical pathways such as glycolysis and gluconeogenesis, the pentose phosphate pathway, the Krebs cycle and fatty acid oxidation. We hypothesize that both strategies are aimed to use of the pentose phosphate pathway to increase glucose utilization while *Rst(2)DDT*$^{91\text{-}R}$ relies primarily on glycolysis to produce reduced NADP and increase DDT detoxification. DDT exposure in Canton-S induced six proteins, while four proteins were repressed in *Rst(2)DDT*$^{Hikone\text{-}R}$. Our data suggest that insecticide resistance appears to impact different metabolic pathways in *Drosophila* genotypes selected with the same pesticide (DDT).

Keywords:
Dichlorodiphenytrichloroethane / *Drosophila* / Profiling / Two-dimensional gel

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Abbreviation: DDT, dichlorodiphenytrichloroethane

1 Introduction

Dichlorodiphenyltrichloroethane (DDT) has been extensively used for over fifty years as an insecticide for the protection of crops and for control of vector borne diseases like typhus and malaria [1–2]; and it is still used in Southeastern Asia and African countries due to (i) its low cost, (ii) broad spectrum of insecticidal activity and (iii) ease of application [3]. Nevertheless, DDT is highly durable in the environment, bioaccumulates and biomagnifies through the food chain. These combined factors have resulted in DDT being banned for widespread use in the developed world [4]. Although DDT is thought to affect primarily sodium channels, there is overwhelming evidence that this pesticide also affects several metabolic pathways. Previous findings have shown that DDT increases glucose utilization in insects and other organisms [2, 5–10]. It has been hypothesized that increased metabolism of glucose provides greater levels of reduced NADP, which is a limiting cofactor for reductive dechlorination of DDT [2, 5–10]. DDT has also been the primary cause of active transport inhibition of *D*-glucose and *L*-tyrosine in the brush border of rat intestine [11–14] and rats exposed to DDT show considerable increases of total lipids, phospholipids and tryglyceride contents in their microvilli membranes [14]. Additionally, DDT increases glucose metabolism in marine microorganisms, [6] and it has been shown to influence the Krebs cycle, the glyoxilate pathway and glucose metabolism in *Pseudomonas aeruginosa* [7]. Dietary DDT in rats has been shown to increase (i) serum lipids and thiobarbituric acid reactive substances in the liver, (ii) lipid peroxidation and (iii) hepatic activities of lipogenic enzymes. These lipogenic enzymes include the malic enzyme, glucose-6-phosphate dehydrogenase and the fatty acid synthethase. Furthermore, DDT has been shown to induce hepatic cytochrome P450 content and the activities of drug metabolizing enzymes such as (i) aminopyrine *N*-demethylase, (ii) glutathione-*S*-transferase and (iii) 4-nitrophenol-UDP glucuronosyltransferase [5, 15].

The most common types of resistance found in insects are increased enzymatic detoxification and target insensitivity [16–19]. Monooxygenase mediated-resistance is probably the most frequent type of metabolism-based insecticide resistance [18–19] although esterases are also important in some species [20]. Monooxygenase-mediated metabolism was first demonstrated in *Musca domestica* and provided the first evidence that resistant insects could have elevated monooxygenase-mediated metabolism of xenobiotics [21]. DDT-metabolism based resistance in *Drosophila* was initially mapped to multiple locations on chromosomes 2 and 3 [22–27]. Subsequently, low-level DDT resistance was recombinatorially mapped to 64.5 $\pm$ 2 cM on the second chromosome [28]; a locus (loci) known as *Rst(2)DDT*. Daborn *et al.* [29] and Le Goff *et al.* [30] suggested that resistance to DDT in the field is monogenic and is due to the over-expression of a single P450 gene, *Cyp6g1*. They also hypothesized that resistance in field isolates of both *Drosophila melanogaster* and *Drosophila simulans* is associated with over-transcription of *Cyp6g1*, while prolonged laboratory selection with DDT apparently coselects additional genes such as *Cyp12d1* [31] and *Cyp6a8* [32]. On the other hand, Brandt *et al.* [31] have shown that over-expression of at least two cytochrome P450 genes, *Cyp6g1* and *Cyp12d1*, are associated with DDT resistance. The fact that multiple cytochrome P450 genes may be associated with the *Rst(2)DDT* resistance locus (loci) [31] and the evidence that DDT-metabolism based resistance may be polygenic [22–27] made clear that a genome-wide transcription profile would be necessary to characterize genes involved in DDT resistance.

The premise that the same chemical could affect different metabolic pathways and the possibility that cytochrome P450 monooxygenases have some degree of plasticity in response to selection [19, 33] made crucial whole transcriptome studies in *Drosophila*. Recently, Pedra *et al.* [34] have demonstrated that DDT-metabolic resistance in *Drosophila* is associated with over- expression of detoxification related genes but also over-expression of ion transport, signal transduction, RNA transcription, and lipid and sugar metabolism pathways. We observed 158 genes putatively associated with DDT resistance. However, the statistical approach used has a false discovery rate of 0.56. In other words, one would expect to find approximately 88 genes being truly differentially transcribed in $Rst(2)DDT^{91\text{-}R}$ or $Rst(2)DDT^{Wisconsin}$ when compared to the wild-type Canton-S genotype. Nevertheless, as previously pointed out, elevated messenger RNA could initially be used to satisfy the criteria of pesticide resistance, however, use of protein information is clearly the most relevant measurement (*i.e.* the protein carries out detoxification, not the mRNA) [19]. In addition, RNA and protein expression do not necessarily agree for some genes and it is clear that RNA expression data is only the first step in understanding molecular processes involved in metabolic pesticide resistance. Also, in eukaryotic cells, there are on average 6–8 proteins produced per gene [35], and this number may actually be greater when splice variants and post-translational modifications are taken into consideration [36–38].

The main objective of this study was to profile the proteome of laboratory selected $Rst(2)DDT^{91\text{-}R}$ and field collected $Rst(2)DDT^{Wisconsin}$ DDT resistant *Drosophila* genotypes. We also attempted to interrogate the proteomics response of the *Drosophila* genotypes Canton-S, 91-C, $Rst(2)DDT^{Hikone\text{-}R}$, $Rst(2)DDT^{Wisconsin}$, and $Rst(2)DDT^{91\text{-}R}$ to DDT exposure. Querying the NCBInr. 10.21.2003 database with mass spectrometric data yielded the identity of twenty-one differentially translated spots representing proteins putatively involved in carbohydrate and lipid metabolism. DDT exposure resulted in the up-regulation of six proteins in Canton-S, while four proteins were repressed in $Rst(2)DDT^{Hikone\text{-}R}$. Integration of functional genomics and proteomics allows for the testing of the hypothesis that only a single detoxification enzyme, *Cyp6g1*, is associated with DDT resistance [29–30].

2 Materials and methods

2.1 D. melanogaster

Drosophila lines were maintained in plastic bottles on Jazz Mix™ *Drosophila* Food (Applied Scientific, San Francisco, CA, USA). Flies were transferred to new bottles every three weeks. For all lines, Canton-S, 91-C, $Rst(2)DDT^{Hikone\text{-}R}$, $Rst(2)DDT^{Wisconsin}$ and $Rst(2)DDT^{91\text{-}R}$, the insects were reared at a constant room temperature of approximately 25°C with a photophase of 16 h and a scotophase of 8 h. The susceptible fly line, Canton-S, was obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN, USA). The resistant fly line $Rst(2)DDT^{Wisconsin}$ was collected from the field (Door County, Wisconsin, USA) and selected by initial exposure to 200 μg/vial of DDT (Sigma, St Louis, MO, USA) for 24 h [31]; the resultant males were crossed into a balancer stock, and a fly line isochromosomal for both the second and third chromosome was subsequently established. The DDT resistant line Hikone-R was originally collected in the field in Japan, and was obtained from the Bloomington *Drosophila* Stock Center. The DDT resistant line $Rst(2)DDT^{91\text{-}R}$ line is descended from a large collection of wild flies, and was kindly provided by Professor Dr. Ranjan Ganguly (University of Tennessee, Knoxville, USA) [39–44]. To select highly resistant flies in the $Rst(2)DDT^{91\text{-}R}$ strain, we performed bioassays with 4000 μg *per* vial; survivors were collected, and the population was reestablished. Overlapping *Drosophila* generations were used in our experiments.

2.2 Bioassays

The LC_{25} of each genotype was used for DDT exposed treatments (Canton-S, 0.15 μg/vial; 91-C 1.61 μg/vial; $Rst(2)DDT^{Hikone\text{-}R}$, 8.30 μg/vial; $Rst(2)DDT^{Wisconsin}$, 34.68 μg/vial; $Rst(2)DDT^{91\text{-}R}$, 142.18 μg/vial) (data not shown). Bioassays were performed according to Brandt *et al.* [31].

2.3 2-DE

One hundred *Drosophila* adults were grinded using the ReadyPrep™ Protein Extraction Kit (Bio-Rad, Hercules, CA, USA) and total proteins were extracted. Neither age nor sex discrimination was used for these experiments. Protein quantification was performed utilizing the Coomassie Plus Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA). Protein samples were extracted from each independent bioassay; one protein extraction was used for each 2-D gel replicate. Three 2-D gel replicates were used *per* treatment. The treatments are as follows: (i) Canton-S, (ii) Canton-S exposed to DDT, (iii) $Rst(2)DDT^{Wisconsin}$, (iv) $Rst(2)DDT^{Wisconsin}$ exposed to DDT, (v) $Rst(2)DDT^{91\text{-}R}$; (vi) $Rst(2)DDT^{91\text{-}R}$ exposed to DDT, (vii) 91-C, (viii) 91-C exposed to DDT, (ix) $Rst(2)DDT^{Hikone\text{-}R}$, and (x) $Rst(2)DDT^{Hikone\text{-}R}$ exposed to DDT. Three hundred micrograms of protein homogeneized in rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 0.8% ampholyte, 65 mM dTT, 0.02% bromophenolblue) were loaded onto an immobilized pH 3–10, NL 17 cm Readystrip IPG strip (Bio-Rad). Proteins were passively rehydrated for 12 h. *Drosophila* samples were then focused with a Protean IEF Cell (Bio-Rad) with a three-step program (250 V for 15 min, 1000 V for 3 h, and 3000 V for 70 000 Vh). A limiting current (50 μA *per* strip) was maintained. After IEF, the strips were stored at −80°C until they were analyzed with SDS-PAGE. The IEF strips were equilibrated for 10 min with 2% w/v DTT in 2.1 ml/strip of an equilibration solution (0.38M Tris base, pH 8.8, 6 mM urea, 2% w/v SDS and 20% v/v glycerol) at room temperature, and for 10 min with 2.5% w/v iodoacetamide with a trace of bromophenol blue in the above equilibration solution. Each strip was loaded onto a 12% acrylamide gel, and the proteins were electrophoresed (200 V for 420 min) with a Protein-Plus Dodeca Cell (Bio-Rad) in a buffer containing 25 mM Tris, 190 mM glycine, and 0.1% SDS. The 2-DE separated proteins were put in a 150 ml fixing solution (50% methanol and 7% acetic acid) and stained using Coomassie blue G250 (0.05% Coomassie blue, 25% methanol and 0.35% acetic acid) followed by a 2 h destaining (10% methanol, 1.4% acetic acid).

2.4 Protein spot identification

Coomassie blue stained gels were scanned (300 dpi resolution) using a Calibrating Densitometer Model GS-800 (Bio-Rad). The digitized gel images were analyzed using PDQuest 7.1.1 software (Bio-Rad) for spot detection, quantification, and comparative analysis. The PDQuest software models (i) protein spots mathematically using a 3-D Gaussian distribution and (ii) determines maximum absorption after raw image correction and background subtraction. Spot intensities were then obtained by integration of the Gaussian function with unit of intensity calculated as Intensity × Area as parts *per* million (INT × Area PPM). The intensity of each protein spot was normalized to the total intensity of the entire gel. We elected to consider a differential expression when a protein was absent in all three replicates of the DDT susceptible *Drosophila* but present in the DDT resistant mutant replicates or *vice-versa*. The probability of observing such a result by chance is 1 out of 64 or $p < 0.02$. Tables 1, 2 and 3 categorize the spot numbers according to the probabilistic event of $p < 0.02$. Manual selection identified 52 spots for subsequent analysis. Twenty-one differentially translated spots were identified based on genotype comparisons among the DDT susceptible Canton-S, the laboratory selected $Rst(2)DDT^{91\text{-}R}$ fly line and the field collected $Rst(2)DDT^{Wisconsin}$ strain. Six spots were differentially translated in the treatment comparison of Canton-S and Canton-S exposed to DDT. Four spots were differentially translated in the treatment comparison $Rst(2)DDT^{Hikone\text{-}R}$ and $Rst(2)DDT^{Hikone\text{-}R}$ exposed to DDT. The remaining proteins were not differentially translated but were also fingerprinted. Gel spots were excised using the Proteomics Plus Spot Cutter System (Bio-Rad) software and digestion was

Table 1. MALDI-TOF MS identification of differentially expressed proteins in Canton-S, *Rst(2)DDT*^{Wisconsin} and *Rst(2)DDT*$^{91\text{-}R}$ *Drosophila* genotypes.

Spot number	Gene Symbol	% Coverage	MOWSE score	*Mr*/p*I*	Cytogenetic map	Molecular function
1	CG11620⇓⇓	12	1.2 e + 03	73071 5.9	86D6	Cell communication[a)]
2	CG7254⇓⇓	24	7.3 e + 06	96997 6.1	22C3-D1	Glycogen phosphorylase activity
3	CG7254⇔⇔	55	2.9 e + 14	96997 6.1	22C3-D1	Glycogen phosphorylase activity
4	CG3127⇓⇔	35	1.6 e + 06	43862 7.0	23A3	Phosphoglycerate kinase activity
5	Phosphoglucomutase⇓⇓	25	4.5 e + 12	60725 6.4	72D8	Phosphoglucomutase activity
6	CG17654⇔⇔	29	2.6 e + 06	46663 6.1	22B1	Phosphopyruvate hydratase activity
7	CG6058⇓⇓	23	6.8 e + 06	39048 7.0	97A6	Fructose-bisphosphate aldolase activity
8	CG6058⇓⇔	40	1.5 e + 06	39048 7.0	97A6	Fructose-bisphosphate aldolase activity
9	CG7010⇓⇔	34	2.4 e + 08	48877 8.4	4C14	Pyruvate dehydrogenase (lipoamide) activity
10	CG5028⇑⇔	46	1.8 e + 09	44432 6.3	96E9	Isocitrate (NAD) dehydrogenase activity
11	CG17246⇓⇓	53	4.3 e + 12	72344 6.6	56D3	Succinate dehydrogenase (ubiquinone) activity
12	CG17246⇔⇔	54	1.3 e + 11	72344 6.6	56D3	Succinate dehydrogenase (ubiquinone) activity
13	CG17246⇔⇔	41	1.2 e + 12	72344 6.6	56D3	Succinate dehydrogenase (ubiquinone) activity
14	CG7430⇔⇔	33	3.5 e + 07	53085 6.4	75A6	Dihydrolipoamide dehydrogenase activity
15	CG2286⇔⇔	16	5.1 e + 03	78631 6.4	7E1	NADH dehydrogenase activity
16	CG9042⇑⇓	36	1.9 e + 05	39343 6.3	26A3	Glycerol-3-phosphate dehydrogenase (NAD) activity
17	CG11154⇔⇔	24	2.1 e + 03	54108 5.1	102D1	Hydrogen-exporting ATPase activity, phosphorylative mechanism
18	CG11154⇓⇓	32	2.6 e + 10	54108 5.1	102D1	Hydrogen-exporting ATPase activity, phosphorylative mechanism
19	CG3612⇔⇔	34	3.4 e + 13	59422 9.1	59B1-2	Hydrogen-exporting ATPase activity, phosphorylative mechanism
20	CG3612⇔⇔	34	9.7 e + 04	59422 9.1	59B1-2	Hydrogen-exporting ATPase activity, phosphorylative mechanism
21	CG3680⇔⇔	24	1.0 e + 04	99995 6.4	77E8	Lipid metabolism[a)]
22	CG12262⇑⇓	23	4.2 e + 10	45872 8.1	66A10	Acyl-CoA dehydrogenase activity
23	CG32954⇔⇓	26	1.8 e + 05	27761 7.7	35B3	Oxidoreductase activity
24	CG7470⇑⇓	36	1.1 e + 10	84093 6.3	79A6	Glutamate 5-kinase activity
25	CG11241⇓⇓	12	1.8 e + 05	55847 8.6	80A1	Alanine-glyoxylate transaminase activity
26	CG4347⇔⇓	35	9.8 e + 08	57825 6.9	67A9-B1	UTP-glucose-1-phosphate uridylyltransferase activity
27	CG2979⇓⇓	23	3.6 e + 06	49661 7.7	9A5	Structural molecule activity
28	CG4843⇔⇓	27	6.3 e + 05	32807 4.7	88E13	Actin binding

Table 1. Continued

Spot number	Gene Symbol	% Coverage	MOWSE score	*M*r/p*I*	Cytogenetic map	Molecular function
29	CG32031⇔⇔	27	1.4 e + 07	47890 5.7	66F2-4	Arginine kinase activity
30	CG4432⇓⇓	19	2.1 e + 05	80759 6.6	67A9	Peptidoglycan recognition activity
31	CG5596⇓⇓	15	5.0 e + 04	17533 4.4	98A14-15	Myosin ATPase activity
32	LD13416p⇔⇔	18	5.6 e + 03	95558 6.4	61C1	Protein tyrosine phosphatase
33	CG7301⇔⇔	6	1.5 e + 03	13558 8.5	90C10	unknown
34	CG13363⇔⇓	10	2.1 e + 03	20805 7.7	1B13-14	unknown

Spot number, gene symbol, sequence coverage (% coverage), MOWSE score, and theoretical M_r and p*I* are indicated. Molecular function represents the molecular function category according to the Gene Ontology annotation. Cytogenetic map represents the gene location in the *Drosophila* cytological map (http://flybase.bio.indiana.edu). Symbols ⇔ (not differentially translated) ⇓ (down-regulated) ⇑ (up-regulated) represent the expression pattern of the respective protein or isoform when compared to the DDT susceptible Canton-S genotype (p– < 0.02). The symbol in the left represents the comparison *Rst(2)DDT*^{Wisconsin} *vs.* Canton-S and the symbol in the right represents the comparison *Rst(2)DDT*$^{91\text{-}R}$ *vs.* Canton-S. The $p < 0.02$ identifies differentially translated putative proteins that were absent in all three replicates in the DDT susceptible *Drosophila* but present in all three replicates of respective DDT resistant lines or *vice-versa*.
a) The biological process associated with the described protein according to gene antology annotation (http://geneontology.org)

Table 2. MALDI-TOF MS identification of differentially expressed proteins in Canton-S and Canton- S exposed to DDT LC_{25}

Spot number	Gene symbol	% Coverage	MOWSE score	M_r/p*I*	Cytogenetic Map	Molecular Function
1	CG6058⇑	17	6.3 e + 05	39048 7.0	97A6	Fructose-biphosphate aldolase activity
2	CG7010⇑	18	4.9 e + 09	43892 7.6	4C14	Pyruvate dehydrogenase (lipoamide) activity
3	CG5028⇔	25	9.9 e + 06	44432 6.3	96E9	Isocitrate dehydrogenase activity
4	CG5028⇑	46	1.8 e + 09	44432 6.3	96E9	Isocitrate dehydrogenase activity
5	CG6708⇔	15	8.5 e + 03	89235 5.6	96B10	Sterol binding
6	CG6030⇑	15	2.4 e + 05	20201 6.1	91F1	Hydrogen exporting ATPase activity/ phosphorylation mechanism
7	RH63796p⇔	9	5.1 e + 02	49199 6.4	25F3	Extracellular matrix[a)]
8	CG9031⇑	12	9.1 e + 02	30718 6.5	34C6	Small GTPase interacting/regulatory protein activity
9	CG6617⇑	8	3.1 e + 03	25997 4.9	17C7	Intracellular protein transport/ protein targeting[b)]

Spot number, gene symbol, sequence coverage (% coverage), MOWSE score, and theoretical M_r and p*I* are indicated. Molecular function represents the molecular function category according to the Gene Ontology annotation. Cytogenetic map represents the gene location in the *Drosophila* cytological map (http://flybase.bio.indiana.edu). Symbols ⇔ (not differentially translated) and ⇑ (up-regulated) represent the expression pattern of the respective protein when the comparison of Canton-S exposed to DDT *vs.* Canton-S is made ($p < 0.02$). The $p < 0.02$ identifies differentially translated putative proteins that were absent in all three replicates in the DDT susceptible *Drosophila* not exposed to DDT.
a) Cellular component category annotated according to Gene Ontology (http://geneontology.org)
b) Biological process category annotated according to Gene Ontology (http://geneontology.org)

Table 3. MALDI-TOF MS identification of differentially expressed proteins in *Rst(2)DDT*$^{Hikone\text{-}R}$ and *Rst(2)DDT*$^{Hikone\text{-}R}$ exposed to DDT LC_{25}

Spot number	Gene symbol	% Coverage	MOWSE score	M_r/p*I*	Cytogenetic Map	Molecular funtion
1	Tropomyosin 1⇔	25	2.1 e + 04	54560 4.4	88E12-13	Actin binding
2	Heat Shock Protein 83⇔	36	7.1 e + 09	81866 4.9	63B11	Chaperonin ATPase activity
3	CG3731⇔	17	1.8 e + 05	51875 5.7	88D6	Mitochondrial processing peptidase activity
4	CG5028⇔	19	1.4 e + 06	44432 6.3	96E9	Isocitrate dehydrogenase (NAD) activity
5	CG2331⇓	40	8.8 e + 10	88860 5.2	46D1	ATPase activity
6	CG5178⇓	31	1.3 e + 05	41700 5.3	88F5	Structural constituent of the cytoskeleton
7	CG30296⇓	7	1.5 e + 03	52812 6.3	57B9-12	unknown
8	CG11695⇓	13	1.1 e + 02	62490 8.0	10C7	unknown

Spot number, gene symbol, sequence coverage (% coverage), MOWSE score, and theoretical M_r and p*I* are indicated. Molecular function represents the molecular function category according to Gene Ontology annotation. Cytogenetic map represents the gene location in the *Drosophila* cytological map (http://flybase.bio.indiana.edu). Symbols ⇔ (not differentially translated) and ⇓ (down-regulated) represent the expression pattern of the respective protein when the comparison *Rst(2)DDT*$^{Hikone\text{-}R}$ exposed to DDT *vs. Rst(2)DDT*$^{Hikone\text{-}R}$ is made ($p < 0.02$). The $p < 0.02$ identifies differentially translated putative proteins that were present in all three replicates in the DDT resistant *Drosophila* not exposed to DDT.

performed according to the recommendation of the ProPrep Investigator software manual (Genomic Solutions, Ann Arbor, MI, USA). Each differentially expressed digested protein was desalted with Zip tips (Millipore, Billerica MA, USA) and the eluant was spotted onto a MALDI-TOF target plate. Analysis was performed on a Voyager-DE PRO MALDI MS (Applied Biosystems, Foster City, CA, USA) using delayed ion extraction in the positive ion reflector mode with α-cyano-4-hydroxy-trans-cinnamic acid used as the matrix. An accelerating voltage of 20 kV, 72% grid voltage and an acquisition mass range of 500–5000 Da were employed. All samples were mass calibrated using angiotensin and fragments of the adrenocorticotropic hormone. All spectra were internally mass calibrated with the protonated molecule of the selected trypsin auto digested peptide 842.92211 in the Data Explorer MS Data Processing software 4.0 (Applied Biosystems). Furthermore, peak selection was carefully analyzed using Peakfinder 1.2.12 (Efeckta Technologies, Steamboat Springs, CO, USA) and the masses due to the matrix, trypsin, and contaminant peaks were removed. The signal-to-noise ratio used was 6%. The selected tryptic peptides masses $(M+H)^+$ (mass range of 500–5000 Da) were used to search the *D. melanogaster* NCBInr. 10.21.2003 database monoisotopic module of the Protein Prospector program (http://prospector.ucsf.edu/). Proteins were confirmed as positive identifications if a minimum of four peptides with 200 ppm mass accuracy and one missed cleavage were matched with the *D. melanogaster* NCBInr. 10.21.2003 database.

3 Results and discussion

3.1 Protein profile comparison among *Rst(2)DDT*$^{91\text{-}R}$ *Rst(2)DDT*^{Wisconsin} and *Canton-S* genotypes

To test the hypothesis that only one cytochrome P450 enzyme, *Cyp6g1*, is associated with resistance in DDT resistant *Drosophila* strains, we attempted to profile the proteome of three *Drosophila* genotypes: *Rst(2)DDT*$^{91\text{-}R}$ (DDT-laboratory resistant), *Rst(2)DDT*^{Wisconsin} (DDT-field resistant) and Canton-S (wild-type) genotypes. All three *Drosophila* genotypes were not exposed to DDT and there was neither sex nor gender discrimination for these comparisons. 2-D gels were compared using PDQuest software and thirty-five spots were selected to be analyzed using MALDI-TOF MS. Twenty-five proteins were shown to have a theoretical p*I* occurring between 4 and 7 (Table 1). There was one spot that did not have any similarity with any other protein described in the *D. melanogaster* mono-isotopic peptide mass MS-fit module of the Protein NCBInr. 10.21.2003 program database (http://prospector.ucsf.edu). It is not shown in Fig. 1. This protein might have had a positive identification of less than four peptides or more than one trypsin cleavage was missed. Two spots were identified as protein coding genes but the molecular functions are unknown (CG7301, spot 33; CG13363, spot 34) (www.geneontology.org) (Table 1). Thirty-two excised spots (94%) were identified (Table 1). Thirteen identified proteins were not differentially translated but were

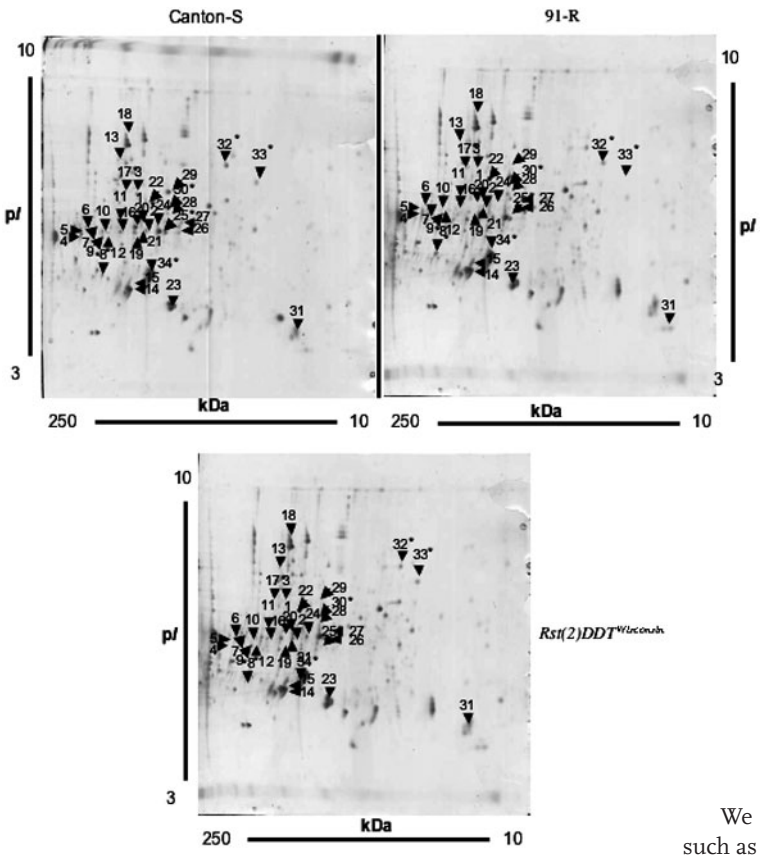

Figure 1. Representative example of a Coomassie blue stained 2-D gel of three *Drosophila* genotypes (Canton-S, *Rst(2)DDT*^{Wisconsin} and *Rst(2)DDT*$^{91\text{-}R}$). The pattern is oriented with acidic proteins at the bottom and basic proteins at the top, and with high M_r proteins towards the left and low M_r proteins towards the right. After in-gel digestion with trypsin, thirty-four spots were selected for comparison among the wild-type (Canton-S), the field collected genotype (*Rst(2)DDT*^{Wisconsin}) and the laboratory selected line (*Rst(2)DDT*$^{91\text{-}R}$) using MALDI-TOF MS. Each spot was assigned to a unique sample number, and is listed in Table 1. Asterisks indicate protein spots that did not match either the M_r or p*I*.

further fingerprinted for comparison purposes: glycogen phosphorylase (spot 3), phosphopyruvate hydratase (spot 6), succinate dehydrogenase (spots 12 and 13), dihydrolipoamide dehydrogenase (spot 14), NADH dehydrogenase (spot 15), ATPase (spots 17, 19, 20), CG3680 (spot 21), arginine kinase (spot 29), tyrosine phosphatase (spot 32), CG7301 (spot 33) (Table 1). Nevertheless, as listed in Table 1, putative isoforms of glycogen phosphorylase (spot 2), succinate dehydrogenase (spot 11) and ATPase (spot 18) were differentially translated. We suggest that different protein isoforms may be regulated in different manners.

The identified faint spots represent, almost exclusively, nonstructural proteins such as phosphoglycerate kinase (spot 4), phosphoglucomutase (spot 5), fructose bisphosphate aldolase (spot 7), and Acyl-CoA dehydrogenase (spot 22) among others (Fig. 2). Twenty-two out of the thirty-two (69%) identified protein spots with known or predicted functions in cellular processes belong to the functional protein classes involved in glycolysis, gluconeogenesis, the Krebs cycle, electron chain transport, fatty acid oxidation, and protein metabolism; these processes all account for the so-called house keeping pathway supporting cellular activity. Glycogen phosphorylase (spot 2), phosphoglucomutase (spot 5), succinate dehydrogenase (spot 11), ATPase (spot 18), alanine- glyoxylate transaminase (spot 25), CG2979 (spot 27), CG4432 (spot 30) and myosin (spot 31) appear to be repressed in both DDT resistant *Drosophila* strains. At the same time, the expression of fructose biphosphate aldolase (spots 7 and 8) and pyruvate dehydrogenase (spot 9) were down-regulated only in *Rst(2)DDT*^{Wisconsin}. Isocitrate dehydrogenase (spot 10) and glycerol-3-phosphate dehydrogenase (spot 16) were highly expressed in *Rst(2)DDT*^{Wisconsin} and the expression of Acyl-CoA dehydrogenase (spot 22), CG32954 (spot 23), glutamate 5-kinase (spot 24), UTP-glucose-1-phosphate uridilyltransferase (spot 26) and CG4843 (spot 28) were decreased only in *Rst(2)DDT*$^{91\text{-}R}$ (Table 1).

We observed a down-regulation of glycolytic enzymes such as aldolase (spots 7 and 8) and pyruvate dehydrogenase (spot 9) in *Rst(2)DDT*^{Wisconsin} (Fig. 3, Table 1). We hypothesize that because *Rst(2)DDT*^{Wisconsin} has lower levels of these two enzymes glucose 6-phosphate would enter the pentose phosphate pathway to produce reduced NADP and increase DDT detoxification (Fig. 3). On the other hand, we observed similar levels of aldolase expression (spots 7 and 8) and pyruvate dehydrogenase (spot 9) in *Rst(2)DDT*$^{91\text{-}R}$ when compared to Canton-S (Table 1, Fig. 3). We hypothesize that *Rst(2)DDT*$^{91\text{-}R}$ may rely primarily on glycolysis to metabolize glucose and therefore could produce more NADH than *Rst(2)DDT*^{Wisconsin}. The results confirm and extend previous findings in which DDT was shown to regulate glucose utilization [8–9]. Also, our data suggest that changes in glucose metabolism may be a factor in insect resistance to chlorinated insecticides. DDT has also been shown to affect β-oxidation of fatty acids [45–46]. In our study, an up-regulation of acyl-CoA dehydrogenase (spot 22) and isocitrate dehydrogenase (spot 10) in *Rst(2)DDT*^{Wisconsin} suggest that lipid metabolism and the Krebs cycle may both be associated with the DDT resistant phenotype. Maltseva and Golotseva [7] have shown that the activity of isocitrate and glucose-6-phosphate dehydrogenases were altered in *P. aeruginosa* strains capable of metabolizing DDT. It has been previously demonstrated that DDT-treated houseflies have decreased ATP and increased inorganic phosphate [47–48]. ATPase proteins were down-regulated in both resistant genotypes *Rst(2)DDT*^{Wisconsin} and 91-R (spots 18 and 31); (Table 1). Substrate-level phosphorylation may compete more effectively

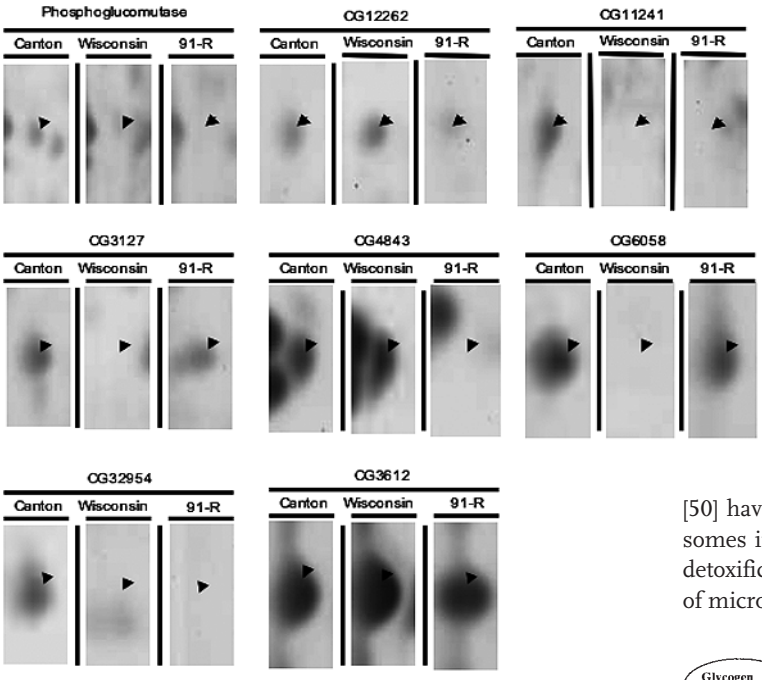

Figure 2. The 2-D electrophoresis pattern of the wild-type Canton-S (left side), *Rst(2)DDT*^{Wisconsin}(center) and *Rst(2)DDT*$^{91\text{-}R}$ (right side) genotypes were processed, matched and analyzed by PDQUEST software. The figure shows zoom-boxes of 2-D maps containing spots differentially translated (phosphoglucomutase, CG12262, CG11241, CG3127, CG4843, CG6058 and CG32954). The protein spot CG3612 was considered equally expressed.

for the available ADP. This may stimulate the pentose phosphate pathway and the Krebs cycle in *Rst(2)DDT*^{Wisconsin}.

Several factors can influence the interpretation of proteomic datasets, including variability in spot volumes, the influence of pipetting error, sensitivity, gel focusing, staining, scanning, and the software used. Thus, we elected to discuss only qualitative changes for spot identification. These differences represent proteins that were absent in all three replicates in the DDT susceptible *Drosophila* but present in all three replicates in the respective DDT resistant genotypes or *vice-versa*. The probability of observing such a result by chance is 1 out of 64 or $p < 0.02$. However, the rapid advance of proteomics tools should provide an effective system to detect quantitative proteome differences among *Drosophila* genotypes in the near future. Meanwhile, qualitative changes should serve as a DDT reference map for differentially translated proteins.

Pedra *et al.* [34] have shown in a genome-wide transcription profile that 158 probe sets (genes) are differentially transcribed in *Rst(2)DDT*$^{91\text{-}R}$ and *Rst(2)DDT*^{Wisconsin} as compared to Canton-S including cytochrome P450 enzymes, glutathione and glucuronosyl transferases. Our proteomics approach did not detect any detoxification enzymes being differentially translated except for a low UDP-glucuronosyltransferase (*Ugt86Dh*) MOWSE identity score with the spot identified as CG11620 (spot 1). We suggest that the absence of detoxification enzyme detection may be explained by (i) the methodology used to extract proteins (crude *vs.* microsomal extracts), (ii) instability of certain detoxification enzymes, (iii) the small number of spots identified, (iv) the use of the qualitative approach described, (v) the IEF range, (vi) there are so few changes in detoxification enzymes at the protein level that they are not detectable, or (vii) any combination of i–vi. The most likely explanation is centered in the methodology used. Crude protein extracts represent the most abundant polypeptides and microsomal proteins such as cytochrome P450s are not likely to be represented. In fact, Waters and Nix [49] and Scott [50] have described a successful strategy to separate microsomes in insects and they mention that the study of insect detoxification enzymes has been hampered by the instability of microsomal preparations. Nevertheless, we decided to use

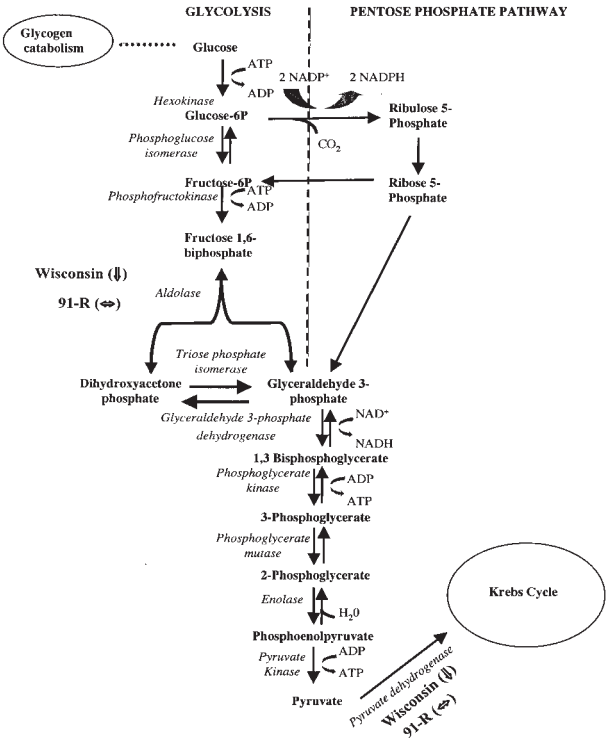

Figure 3. Partial diagram of glycolysis and the pentose phosphate pathway. The figure shows metabolic reactions occurring during glycolysis, and in the pentose phosphate pathway and the connections between these two pathways. The enzymes aldolase and pyruvate dehydrogenase were down- regulated (⇓) in *Rst(2)DDT*^{Wisconsin} and had similar levels of expression in *Rst(2)DDT*$^{91\text{-}R}$ (⇔) when compared to Canton-S. All enzymes are italicized and the substrates are bolded. Arrows indicate the direction of the chemical reaction. Dotted lines (.....) represent enzymatic reactions not shown. Dashed lines (——) separate chemical reactions occurring in either glycolysis or the pentose phosphate pathway.

the approach of crude extract proteins since total protein extracts give a better representation of the whole proteome of *Drosophila*.

3.2 Proteomics response of *Drosophila* genotypes to DDT exposure

We decided to use DDT LC_{25} to compare global proteome effects of DDT exposure in five *D. melanogaster* strains: 91-C, Canton-S, $Rst(2)DDT^{Wisconsin}$, $Rst(2)DDT^{91\text{-}R}$ and $Rst(2)DDT^{Hikone\text{-}R}$. Exposure of the following genotypes did not result in any qualitative differences, as compared to nonexposed gels: 91-C (wild-type), $Rst(2)DDT^{Wisconsin}$ (field collected line) and $Rst(2)DDT^{91\text{-}R}$ (laboratory selected genotype). However, we detected differentially translated proteins in Canton-S and the field collected line $Rst(2)DDT^{Hikone\text{-}R}$ both in the presence and absence of DDT exposure (Tables 2 and 3). The following proteins did not match either their theoretical p*I* or M_r: fructose-biphosphate aldolase (spot 1), isocitrate dehydrogenase (spot 4) and ATPase (spot 6) in Canton-S and ATPase (spot 5) and CG5178 (spot 6) in $Rst(2)DDT^{Hikone\text{-}R}$ (Fig. 4 and 5). The protein spots isocitrate dehydrogenase (spot 3), sterol binding protein (spot 5) and RH63796 (spot 7) were not differentially translated but were further fingerprinted in Canton-S (Table 2, Figure 6). Nevertheless, as listed in Table 2, a putative isoform of isocitrate dehydrogenase (spot 4) and fructose-biphosphate aldolase (spot 1), pyruvate dehydrogenase (spot 2), ATPase (spot 6), GTPase (spot 8) and a intracellular protein (spot 9) appear to be induced in Canton-S (Fig. 6, Table 2). Four identified proteins were not differentially translated in $Rst(2)DDT^{Hikone\text{-}R}$: tropomyosin 1 (spot 1), heat shock protein 83 (spot 2), a mitochondrial peptidase protein (spot 3) and a variant form of isocitrate dehydrogenase (spot 4) (Fig. 7, Table 3). However, the proteins listed as ATPase (spot 5), CG5178 (spot 6), CG30296 (spot 7), CG11695 (spot 8) were repressed in Hikone-R (Fig. 7, Table 3).

4 Concluding remarks

The publication of the *D. melanogaster* genome sequence provided an excellent foundation for novel insecticide resistance studies. Possible interrelationships between chlorinated insecticides and monooxygenase mediated-resistance in insects have been previously investigated, often with contradictory results. A possible reason for this was the use of the single gene approach to detect global effects of DDT resistance in *Drosophila*. However, as the diversity of cytochrome P450 genes and other detoxification enzymes become more and more obvious with the use of modern genomics and proteomics tools, it appears unlikely that only one gene is solely responsible for metabolism-based pesticide resistance. Another possibility for confusing results is that there is strong evidence that there is evolutionary plasticity of monooxygenase-mediated resistance [33]; *i.e.* different pesticide resistant strains over-express different P450 genes. Resistance may evolve using different detoxification enzymes and possibly different regulatory signals controlling gene expression. For example, some *D. simulans* [51] and

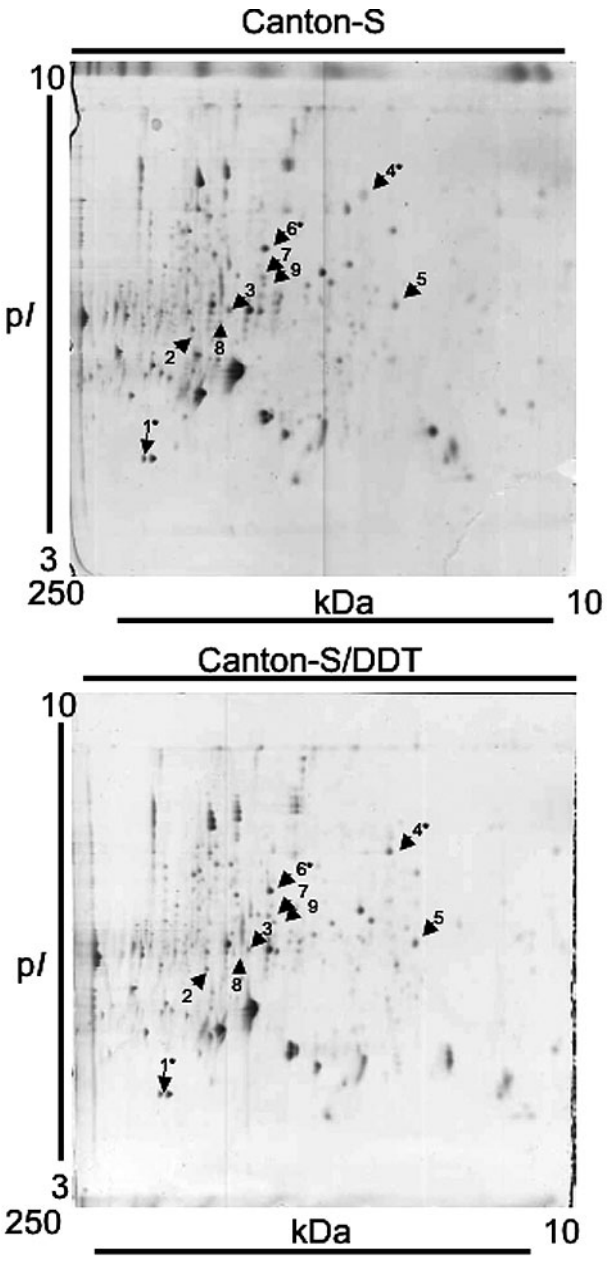

Figure 4. Representative example of a Coomassie blue stained 2-D gel of Canton-S and Canton- S/DDT (exposed to DDT at the LC_{25} level). The pattern is oriented with acidic proteins at the bottom and basic proteins at the top, and with high M_r proteins towards the left and low M_r proteins toward the right. After in-gel digestion with trypsin, nine spots were selected for comparison between the Canton-S genotype and the wild-type genotype exposed to DDT using MALDI-TOF. Each spot was assigned a unique sample number, and is listed in Table 2. Asterisks indicate protein spots that did not match either the M_r or p*I*.

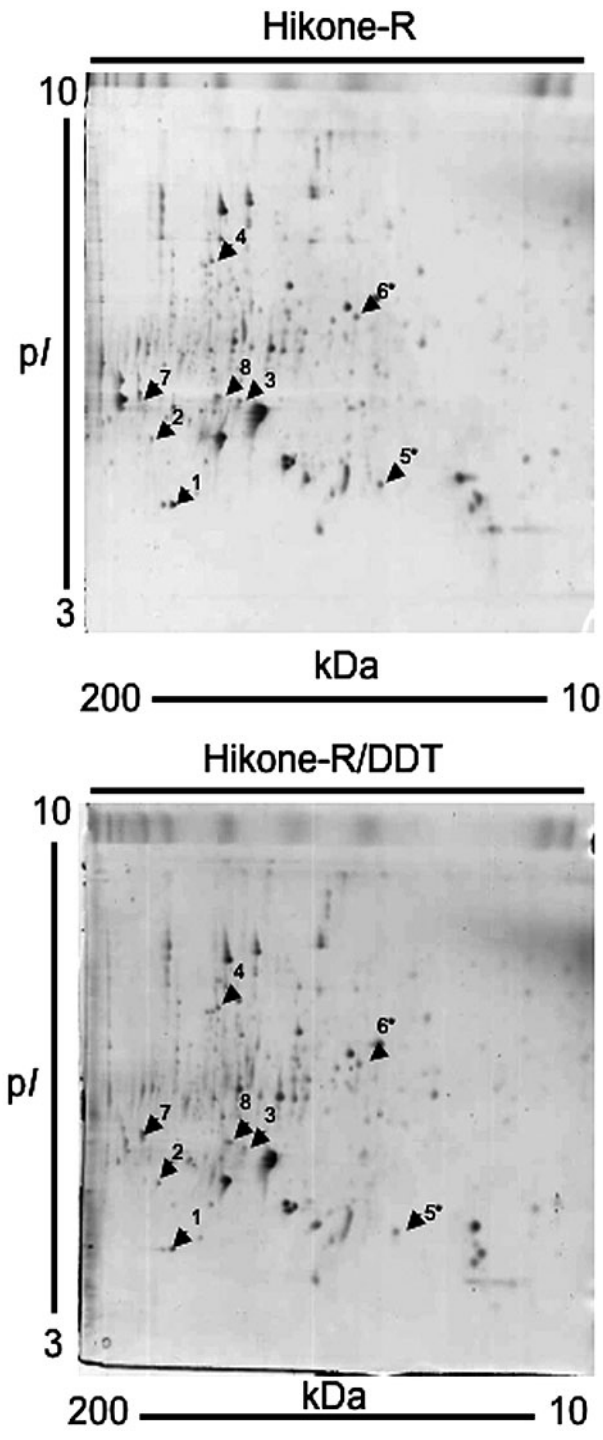

Figure 5. Representative example of a Coomassie blue stained 2-D gel of *Rst(2)DDT*$^{Hikone\text{-}R}$ and *Rst(2)DDT*$^{Hikone\text{-}R}$/DDT (exposed to DDT at the LC_{25} level). The pattern is oriented with acidic proteins at the bottom and basic proteins at the top, and with high M_r proteins towards the left and low M_r proteins toward the right. After in-gel digestion with trypsin, eight spots were selected for comparison between the *Rst(2)DDT*$^{Hikone\text{-}R}$ and *Rst(2)DDT*$^{Hikone\text{-}R}$ genotypes exposed to DDT using MALDI-TOF. Each spot was assigned a unique sample number, and is listed in Table 3. Asterisks indicate protein spots that did not match either the M_r or p*I*.

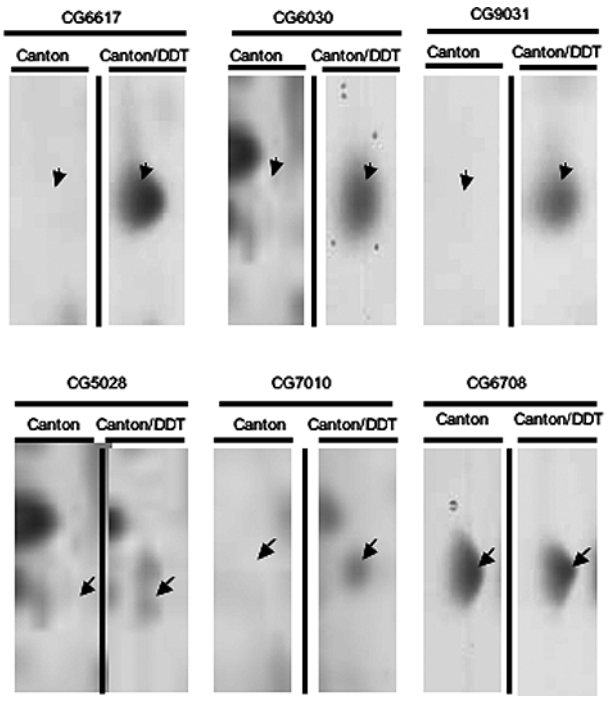

Figure 6. 2-D electrophoresis pattern of wild-type Canton-S (left side) and Canton-S exposed to DDT (LC_{25}) (right side). Gels were processed, matched and analyzed by PDQUEST software. The figure shows zoom-boxes of 2-D maps containing spots differentially translated (CG6617, CG6030, CG9031, CG5028 and CG7010). The protein CG6708 was considered equally expressed.

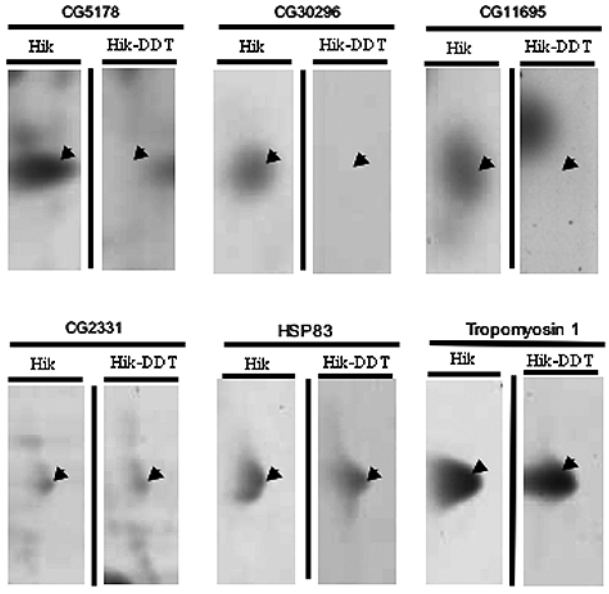

Figure 7. 2-D electrophoresis pattern of the field collected DDT resistant *Drosophila* genotype *Rst(2)DDT*$^{Hikone\text{-}R}$ (left) and *Rst(2)DDT*$^{Hikone\text{-}R}$ (right side) exposed to DDT (LC_{25}). Gels were processed, matched and analyzed by PDQUEST software. The figure shows zoom-boxes of 2-D maps containing spots differentially translated (CG5178, CG30296, CG11695 and CG2331). The proteins HSP83 and Tropomyosin 1 were considered equally expressed.

D. melanogaster [29] populations have up-regulation of *Cyp6g1* due to a transposon insertion, which seems to be associated with DDT resistance. *D. melanogaster* lines harboring the *Accord* transposon insertion upstream in *Cyp6g1* had a significant lower mortality in the presence of DDT than lines without the *Accord* transposon insertion in *Cyp6g1* [29, 51]. However, abundant variation in DDT resistance was found within each class of *D. melanogaster* harboring *Cyp6g1* alleles. In addition, Californian *D. simulans* lines harboring the *Doc* transposon insertion associated with *Cyp6g1* and African *Drosophila simulans* lines not carrying the *Doc* transposon insertion in *Cyp6g1* showed the same levels of DDT resistance. In fact, some populations carrying *Cyp6g1* alleles with the *Doc* transposon insertion faired worse in the presence of DDT than lines lacking the *Doc* transposon [51]. Thus, CYP6G1 high-expression and DDT resistance may be population-specific and not a universal phenomenon as previously described [29].

It is important to understand intricate molecular changes in the whole proteome of *Drosophila* because protein information is clearly the most relevant measurement. Proteomics studies should help to design target-specific chemicals to be deployed in the environment. To our knowledge, we report the first proteomics study to detect differentially translated proteins in the laboratory selected DDT resistant genotype $Rst(2)DDT^{91\text{-}R}$, the DDT resistant field collected fly line $Rst(2)DDT^{Wisconsin}$ and the wild-type Canton-S. By combining 2-DE with an extended pH range (3–10) and high sensitivity protein identification by MALDI-TOF MS, identification was obtained for 34 out of 35 excised and analyzed spots. Most of the proteins identified were apparently involved in biochemical pathways such as glycolysis and gluconeogenesis, the pentose phosphate pathway, the Krebs cycle and fatty acid oxidation. The $Rst(2)DDT^{Wisconsin}$ genotype seems to make use of the pentose phosphate pathway to increase glucose utilization while $Rst(2)DDT^{91\text{-}R}$ relies primarily on glycolysis to metabolize glucose. We hypothesize that both evolutionary strategies resulted in an increase in the production of reduced NAD or NADP. Cytochrome P450 enzymes use reduced NADPH to metabolize DDT. Moreover, we were able to detect the induction of six protein spots in Canton-S after exposure at the DDT LC_{25} level. This included enzymes involved in glycolysis and the Krebs cycles. On the other hand, the DDT LC_{25} exposure in $Rst(2)DDT^{Hikone\text{-}R}$ seems to have a repressive effect on ATPases. We suggest that protein induction in Canton-S and translation repression in $Rst(2)DDT^{Hikone\text{-}R}$ were responses to DDT according to the organism's genetic background.

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