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Splice form variant and amino acid changes in MDR49 confers DDT resistance in transgenic *Drosophila*

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The ATP-binding cassette (ABC) transporters represent a superfamily of proteins that have important physiological roles in both prokaryotes and eukaryotes. In insects, ABC transporters have previously been implicated in insecticide resistance. The *91-R* strain of *Drosophila melanogaster* has been intensely selected with DDT over six decades. A recent selective sweeps analysis of *91-R* implicated the potential role of *MDR49*, an ABC transporter, in DDT resistance, however, to date the details of how *MDR49* may play a role in resistance have not been elucidated. In this study, we investigated the impact of structural changes and an alternative splicing event in *MDR49* on DDT-resistance in *91-R*, as compared to the DDT susceptible strain *91-C*. We observed three amino acid differences in *MDR49* when *91-R* was compared with *91-C*, and only one isoform (*MDR49B*) was implicated in DDT resistance. A transgenic *Drosophila* strain containing the *91-R-MDR49B* isoform had a significantly higher LD₅₀ value as compared to the *91-C-MDR49B* isoform at the early time points (6 h to 12 h) during DDT exposure. Our data support the hypothesis that the *MDR49B* isoform, with three amino acid mutations, plays a role in the early aspects of DDT resistance in *91-R*.

Post World War II, many agriculturally or medically important pests have been managed through the application of second-generation insecticides. This form of selection pressure has led to the evolution of pesticide resistance in many of the target insect populations¹. Insecticide resistance is a valuable model for studying molecular evolution, as it is a well-defined system of man-made selection using known insecticides^{2,3}. One such insecticide is the neurotoxic organochlorine pesticide 4,4'-dichlorodiphenyltrichloroethane (DDT), which affects the arthropod nervous system by interfering with normal nerve impulses. The insecticidal properties of DDT were discovered by Paul Müller in 1939 and due to its widespread use and effectiveness, DDT went from being a panacea for insect control to being banned for use in most countries in the 1970s. Nevertheless, the use of DDT for vector control continues and may increase as insect-borne diseases expand^{4,5}.

Resistance to DDT has been documented across many pest species along with the non-target species, such as *Drosophila melanogaster* (*Drosophila*)^{3,5}. DDT resistance in *Drosophila* has been used for the study of the evolution of insecticide resistance⁶. Historically, two major mechanisms of DDT resistance in *Drosophila* have been reported. First, resistance has been associated with amino acid changes in the voltage-gated sodium channel resulting in channel insensitivity to DDT⁷. Second, metabolic resistance to DDT has been observed in field populations of *Drosophila*⁸. Enhanced xenobiotic metabolism is an important form of resistance and is associated with alterations (or some cases structural changes) in the activities or levels of detoxification enzymes, such as cytochrome P450s, glutathione-S-transferases (GSTs), esterases or a combination of these activities⁸⁻¹¹. Furthermore, proteomics-based profiling identified abundant proteins associated with DDT resistance in field- and laboratory-selected resistant *Drosophila*¹².

The DDT resistance phenotype in *Drosophila* is not uniform, resulting in varying levels of resistance observed across different *Drosophila* strains, and resistance can be generally categorized into low, medium and high levels as measured by lethal concentration 50 (LC₅₀)^{8,10,11}. One pair of *Drosophila* strains, which are notably important for the study of the high level DDT resistance phenotype, are *91-R* and its DDT susceptible counterpart *91-C*. The two strains originated from a common population¹³⁻¹⁵. The laboratory selected DDT resistance strain *91-R* has been exposed to prolonged and periodic artificial selection with DDT for 60 years and has thus become highly resistant to DDT whereas *91-C* has not been exposed to DDT selection.

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In the *91-R* strain, constitutive over-expression of *Cyp12d1*, *Cyp6a2* and *Cyp6g1* have been observed^{8,16,17}, however, a recent selective sweeps analysis between *91-R* and *91-C* demonstrated that thirteen major and three minor effect chromosome intervals, with reduced nucleotide diversity, were identified only in the *91-R* strain¹⁸. Interestingly, of these thirteen major and three minor loci, the only cytochrome P450 observed was *Cyp4g1*, which is thought to be associated with the reduced curricular penetration phenotype¹⁹. Another gene, multidrug resistance 49 (*MDR49*) was located in one of the other major effect chromosome intervals in the selective sweeps analysis.

Multidrug resistance genes are known to code for ATP-binding Cassette (ABC) transporter proteins. ABC transporters are ATP-dependent efflux pumps belonging to extensive family of transmembrane proteins. The ABC protein family is present in the cellular membrane in all organisms and mediate the efflux of a wide variety of substrates, including sugars, amino acids, lipids, and xenobiotics to the outside of the cell and these transporters prevent the accumulation of harmful toxicants inside the cells^{20,21}. ABC transporters are structurally characterized by four functional units: two highly conserved nucleotide-binding domains (NBDs), which are responsible for ATP-binding and hydrolysis, providing the energy for active transporting substrates across cellular membrane; and, two highly hydrophobic transmembrane domains (TMDs), which are involved in physical pathway for substrate translocation. Unlike the NBDs, TMDs vary in sequence, length and helix number²².

Based on their sequence similarity, domain structures, and organization, ABC transporters can be subdivided into eight subfamilies, designated ABC-A to ABC-H. Insect ABC transporters have diverse functions that affect molting, metabolism, cuticle differentiation, and egg development^{23–25}. They are also thought to be associated with defense or resistance to plant defensive compounds and numerous insecticides by reducing toxic concentrations in tissues²⁶. Unlike the other subfamilies, the ABC-B, ABC-C, and ABC-G have been associated with drug resistance and detoxification in insect pests^{26,27}. For example, involvement of ABC transporters in pyrethroid resistance has been reported in *Helicoverpa armigera*²⁸, *Apis mellifera*²⁹ and *Culex pipiens*³⁰.

ABC transporters have also been implicated in DDT resistance in *Drosophila*. The ABC-B subfamily multiple drug resistance (*MDR*) genes, *MDR50*, *MDR65*, as well as the ABC-C subfamily multidrug resistance-associated protein gene, *MRP1*, are constitutively overexpressed in the DDT-resistant *91-R* strain when compared with the DDT-susceptible *Canton-S* strain. The ABC-B subfamily multiple drug resistance gene *MDR49*, however, was not overexpressed in DDT-resistant *91-R* strain^{19,31,32}. Additionally, RNAi knockdown of *MDR50*, *MDR65*, and *MRP1*, using transgenic GAL4/UAS-RNAi flies, in conjunction with DDT bioassays, confirmed the potential role of these genes in DDT resistance/susceptibility. Interestingly, *MDR49*, which was implicated as a putative resistance locus in the selective sweeps analysis, was not over transcribed in *91-R* versus the *Canton-S* strain^{31,32}. These combined observations lead us to test the hypothesis that *MDR49* may play a role in DDT resistance through structural changes in the gene (and the resultant proteins) as opposed to increased levels of expression.

In the present study, we focused on the ABC-B subfamily genes, *MDR49*, *MDR50*, and *MDR65*. Using RNA-seq data, we compared open reading frames of all three genes and alternative splicing in *MDR49*. As *MDR49* was the only *MDR* to be in a major effect chromosome interval in the selective sweeps analysis¹⁸, we also created transgenic *Drosophila* containing both alternative splice forms of *MDR49*, with the respective amino acid differences between *91-R* and *91-C*, to determine if structural differences in *MDR49* proteins, between *91-R* and *91-C*, may play a role in DDT resistance.

Results

Sequence differences in *MDR49*, *MDR50*, and *MDR65* between the *91-R* and *91-C* strains.

We compared the sequences of the *MDR49*, *MDR50*, and *MDR65* genes between *91-R* and *91-C* to determine non-silent mutations within the ORFs that may lead to potential amino acid replacement. Using the GenBank database, two different transcript isoforms were observed from the *MDR49* gene, designated as *MDR49A* and *MDR49B*. Although the sequences of both transcript isoforms were completely identical, the first four exons were alternatively spliced out of *MDR49A*, giving rise to *MDR49B*. Several SNPs were detected when *91-R* was compared with *91-C*. There were 39 SNPs observed when the *MDR49A* isoform from *91-R* was compared with the *MDR49A* isoform from *91-C*. Similarly, there were 37 SNPs observed when the *MDR49B* isoform from *91-R* was compared with the *MDR49B* isoform from *91-C*. Most SNPs resulted in silent mutations, however, three SNPs resulted in non-silent mutations in both the *MDR49A* and *B* genes from *91-R* when compared with *91-C*.

Thr374, Met388, and Glu666 in *MDR49A* protein from *91-C* were replaced with Ile374, Leu388, and Asp666 from *91-R*; Thr173, Met187, and Glu465 in *MDR49B* protein from *91-C* was substitute with Ile173, Leu187, and Asp465 from *91-R* (Fig. 1). *MDR50* and *MDR65* were also determined to have SNPs (4 and 17, respectively) when *91-R* was compared with *91-C*. These SNPs resulted in three non-silent mutations: Thr393, Ser881, and Val1012 in *MDR50* protein from *91-C* was replaced with Ala393, Phe881, and Leu1012 in *91-R* (Supplementary Fig. S1); Lys277, Ile646, and Leu990 in *MDR65* protein from *91-C* was substituted with Arg277, Met646, and Pro990 in *91-R* (Supplementary Fig. S2). Interestingly, a total of three non-silent mutations were detected in each of the three *MDR* genes from the *91-R* strain and each mutation was novel, suggesting that these residue changes likely cause structural alternation in these three *MDR* proteins from the DDT-resistant strain.

Prediction of structure for three *MDR* proteins. Using the NCBI CDS-Conserved-Domains-prediction-server, the structural features of each of the aforementioned *MDR* proteins were predicted, including the alternative splice forms for *MDR49* (discussed further in the next section). The deduced amino acid sequences for each of the three *MDR* proteins had characteristic features of ABC transporters, including one cytoplasmic N-terminus nucleotide binding domain (NBD) and several conserved motifs (Walker A motif, Walker B motif, and C-motif) (Fig. 1 and Supplementary Fig. S1, S2). The two alternative splice forms of *MDR49* showed atypical variations in their transmembrane domains (TMDs), which were different from each other. For the *MDR49A* isoform, two TMDs at the C-terminus, both consisting of six transmembrane α -helix segments per TMD were predicted (Fig. 2A). For *MDR49B*, however, two TMDs at the C-terminus, one consisting of three transmembrane α -helix

91-C-MDR49A	MVKKEESRLPQAGDFQLKEGSSVDATRKYSYDFDLFRYSTRCERFLIVVSLVATAASAFI	60
91-R-MDR49A	MVKKEESRLPQAGDFQLKEGSSVDATRKYSYDFDLFRYSTRCERFLIVVSLVATAASAFI	60
91-C-MDR49B	-----	0
91-R-MDR49B	-----	0
91-C-MDR49A	PFYFMIYGEFTSLVDRVTGVTGSSPAPALPMFGGGQQLTNSAKENQAIIDDATAFGI	120
91-R-MDR49A	PFYFMIYGEFTSLVDRVTGVTGSSPAPALPMFGGGQQLTNSAKENQAIIDDATAFGI	120
91-C-MDR49B	-----	0
91-R-MDR49B	-----	0
91-C-MDR49A	GSLVGSVMFLLITLAIIDLNRRIALNQIDRIKFLLEAMLQDIWYDTSGSSNFASKMT	180
91-R-MDR49A	GSLVGSVMFLLITLAIIDLNRRIALNQIDRIKFLLEAMLQDIWYDTSGSSNFASKMT	180
91-C-MDR49B	-----	0
91-R-MDR49B	-----	0
91-C-MDR49A	EDDLKKEGIGERKIVIVVELIMTFVIGIVSAFVYGNKLTLLVLSVCPFFIIAATSUVARLQ	240
91-R-MDR49A	EDDLKKEGIGERKIVIVVELIMTFVIGIVSAFVYGNKLTLLVLSVCPFFIIAATSUVARLQ	240
91-C-MDR49B	-----MTFVIGIVSAFVYGNKLTLLVLSVCPFFIIAATSUVARLQ	39
91-R-MDR49B	-----MTFVIGIVSAFVYGNKLTLLVLSVCPFFIIAATSUVARLQ	39
91-C-MDR49A	GSLAEKELKYSDAANVVEEVSFGIRTVFAFSGQEKERKFGKLLIPAENTGRKKGLYSG	300
91-R-MDR49A	GSLAEKELKYSDAANVVEEVSFGIRTVFAFSGQEKERKFGKLLIPAENTGRKKGLYSG	300
91-C-MDR49B	GSLAEKELKYSDAANVVEEVSFGIRTVFAFSGQEKERKFGKLLIPAENTGRKKGLYSG	99
91-R-MDR49B	GSLAEKELKYSDAANVVEEVSFGIRTVFAFSGQEKERKFGKLLIPAENTGRKKGLYSG	99
91-C-MDR49A	MGNALSWLIYLCLMALIWIYGVTLILDERLDPRVYTPAVIVIVLFAVIMGAQNLGFASP	360
91-R-MDR49A	MGNALSWLIYLCLMALIWIYGVTLILDERLDPRVYTPAVIVIVLFAVIMGAQNLGFASP	360
91-C-MDR49B	MGNALSWLIYLCLMALIWIYGVTLILDERLDPRVYTPAVIVIVLFAVIMGAQNLGFASP	159
91-R-MDR49B	MGNALSWLIYLCLMALIWIYGVTLILDERLDPRVYTPAVIVIVLFAVIMGAQNLGFASP	159
	Aromatic	
91-C-MDR49A	HVEAIVATAAAGQLFNIIDRPSQVDPDEKGNRPENTAGHIREFEGIRFRFYPARPDVEIL	420
91-R-MDR49A	HVEAIVATAAAGQLFNIIDRPSQVDPDEKGNRPENTAGHIREFEGIRFRFYPARPDVEIL	420
91-C-MDR49B	HVEAIVATAAAGQLFNIIDRPSQVDPDEKGNRPENTAGHIREFEGIRFRFYPARPDVEIL	219
91-R-MDR49B	HVEAIVATAAAGQLFNIIDRPSQVDPDEKGNRPENTAGHIREFEGIRFRFYPARPDVEIL	219
	Walker A	
91-C-MDR49A	KGLTVDVLPQGVAFVVASGCGKSTLIQLMQRFYDPEAGSVKLDGRDLRLTLNVGWLRSQI	480
91-R-MDR49A	KGLTVDVLPQGVAFVVASGCGKSTLIQLMQRFYDPEAGSVKLDGRDLRLTLNVGWLRSQI	480
91-C-MDR49B	KGLTVDVLPQGVAFVVASGCGKSTLIQLMQRFYDPEAGSVKLDGRDLRLTLNVGWLRSQI	279
91-R-MDR49B	KGLTVDVLPQGVAFVVASGCGKSTLIQLMQRFYDPEAGSVKLDGRDLRLTLNVGWLRSQI	279
	Q-loop	
91-C-MDR49A	GVVQGFVLFATTIGENIRYGRPSATQADIEKAARAANCHDFITRLPKGYDTQVGEKGAQ	540
91-R-MDR49A	GVVQGFVLFATTIGENIRYGRPSATQADIEKAARAANCHDFITRLPKGYDTQVGEKGAQ	540
91-C-MDR49B	GVVQGFVLFATTIGENIRYGRPSATQADIEKAARAANCHDFITRLPKGYDTQVGEKGAQ	339
91-R-MDR49B	GVVQGFVLFATTIGENIRYGRPSATQADIEKAARAANCHDFITRLPKGYDTQVGEKGAQ	339
	ABC Signature Walker B D-loop H-loop	
91-C-MDR49A	ISGGQKQRIARALVRPQVLLDEATSAIDPTESEKRVQSALDELASGPTTLVAHRLS	600
91-R-MDR49A	ISGGQKQRIARALVRPQVLLDEATSAIDPTESEKRVQSALDELASGPTTLVAHRLS	600
91-C-MDR49B	ISGGQKQRIARALVRPQVLLDEATSAIDPTESEKRVQSALDELASGPTTLVAHRLS	399
91-R-MDR49B	ISGGQKQRIARALVRPQVLLDEATSAIDPTESEKRVQSALDELASGPTTLVAHRLS	399
91-C-MDR49A	TITNADKIVFLKDGVAEGQTHEELMERRGLYCELVSIQRKEATEADGAVAGRPLQKS	660
91-R-MDR49A	TITNADKIVFLKDGVAEGQTHEELMERRGLYCELVSIQRKEATEADGAVAGRPLQKS	660
91-C-MDR49B	TITNADKIVFLKDGVAEGQTHEELMERRGLYCELVSIQRKEATEADGAVAGRPLQKS	459
91-R-MDR49B	TITNADKIVFLKDGVAEGQTHEELMERRGLYCELVSIQRKEATEADGAVAGRPLQKS	459
91-C-MDR49A	QNLSDEETDDDEDEEPELQTSGSSRDSGFRASRTRKRRSQRKKKDKKVVSKVS	720
91-R-MDR49A	QNLSDEETDDDEDEEPELQTSGSSRDSGFRASRTRKRRSQRKKKDKKVVSKVS	720
91-C-MDR49B	QNLSDEETDDDEDEEPELQTSGSSRDSGFRASRTRKRRSQRKKKDKKVVSKVS	519
91-R-MDR49B	QNLSDEETDDDEDEEPELQTSGSSRDSGFRASRTRKRRSQRKKKDKKVVSKVS	519
91-C-MDR49A	FTQLMKLNSPEWRFIVVGGIASVMHGATFLWGLFFGDFGILSDGDDVVRRAVLKISM	780
91-R-MDR49A	FTQLMKLNSPEWRFIVVGGIASVMHGATFLWGLFFGDFGILSDGDDVVRRAVLKISM	780
91-C-MDR49B	FTQLMKLNSPEWRFIVVGGIASVMHGATFLWGLFFGDFGILSDGDDVVRRAVLKISM	579
91-R-MDR49B	FTQLMKLNSPEWRFIVVGGIASVMHGATFLWGLFFGDFGILSDGDDVVRRAVLKISM	579
91-C-MDR49A	IFVGI L M A G L N M L Q T Y M F T T A G V K M T T R L R K R A F G T I I G Q D I A Y F D D E R N S V G A L C S R	840
91-R-MDR49A	IFVGI L M A G L N M L Q T Y M F T T A G V K M T T R L R K R A F G T I I G Q D I A Y F D D E R N S V G A L C S R	840
91-C-MDR49B	IFVGI L M A G L N M L Q T Y M F T T A G V K M T T R L R K R A F G T I I G Q D I A Y F D D E R N S V G A L C S R	639
91-R-MDR49B	IFVGI L M A G L N M L Q T Y M F T T A G V K M T T R L R K R A F G T I I G Q D I A Y F D D E R N S V G A L C S R	639
91-C-MDR49A	LASDCSNVQATGARVGTMLQAVATLVVGMVGVFVFSWQQLTLLVTLPLVCLSVYLEGR	900
91-R-MDR49A	LASDCSNVQATGARVGTMLQAVATLVVGMVGVFVFSWQQLTLLVTLPLVCLSVYLEGR	900
91-C-MDR49B	LASDCSNVQATGARVGTMLQAVATLVVGMVGVFVFSWQQLTLLVTLPLVCLSVYLEGR	699
91-R-MDR49B	LASDCSNVQATGARVGTMLQAVATLVVGMVGVFVFSWQQLTLLVTLPLVCLSVYLEGR	699
91-C-MDR49A	FIMKSAQKAKASIEEASQVAEAITNIRTVNGLCLERQVLDQYVQIDRVDAVCRKRVF	960
91-R-MDR49A	FIMKSAQKAKASIEEASQVAEAITNIRTVNGLCLERQVLDQYVQIDRVDAVCRKRVF	960
91-C-MDR49B	FIMKSAQKAKASIEEASQVAEAITNIRTVNGLCLERQVLDQYVQIDRVDAVCRKRVF	759
91-R-MDR49B	FIMKSAQKAKASIEEASQVAEAITNIRTVNGLCLERQVLDQYVQIDRVDAVCRKRVF	759
91-C-MDR49A	RGLV F A L G Q A A P F L A Y G I S M Y G G I L V A E E R M N Y E D I K V A E A L I F G S W M L Q Q A L Y A P N	1020
91-R-MDR49A	RGLV F A L G Q A A P F L A Y G I S M Y G G I L V A E E R M N Y E D I K V A E A L I F G S W M L Q Q A L Y A P N	1020
91-C-MDR49B	RGLV F A L G Q A A P F L A Y G I S M Y G G I L V A E E R M N Y E D I K V A E A L I F G S W M L Q Q A L Y A P N	819
91-R-MDR49B	RGLV F A L G Q A A P F L A Y G I S M Y G G I L V A E E R M N Y E D I K V A E A L I F G S W M L Q Q A L Y A P N	819
91-C-MDR49A	VNDAILSAGRIMDLFKRTSTQPNPPQSPYNTVEKSEGDIVYENGVFEYPTFRKGTPIIQGL	1080
91-R-MDR49A	VNDAILSAGRIMDLFKRTSTQPNPPQSPYNTVEKSEGDIVYENGVFEYPTFRKGTPIIQGL	1080
91-C-MDR49B	VNDAILSAGRIMDLFKRTSTQPNPPQSPYNTVEKSEGDIVYENGVFEYPTFRKGTPIIQGL	879
91-R-MDR49B	VNDAILSAGRIMDLFKRTSTQPNPPQSPYNTVEKSEGDIVYENGVFEYPTFRKGTPIIQGL	879
91-C-MDR49A	NLTIKKSTTVALVPGSGGKSTCVQLLLRYDVPVSGVNLGVPSTEFPLDTRLSKGLV	1140
91-R-MDR49A	NLTIKKSTTVALVPGSGGKSTCVQLLLRYDVPVSGVNLGVPSTEFPLDTRLSKGLV	1140
91-C-MDR49B	NLTIKKSTTVALVPGSGGKSTCVQLLLRYDVPVSGVNLGVPSTEFPLDTRLSKGLV	939
91-R-MDR49B	NLTIKKSTTVALVPGSGGKSTCVQLLLRYDVPVSGVNLGVPSTEFPLDTRLSKGLV	939
91-C-MDR49A	SQEPVLFDRITAEIAYGNFRDDVSMQEIIEAAKSNIHNFISALPQGYDTRLGKTSQL	1200
91-R-MDR49A	SQEPVLFDRITAEIAYGNFRDDVSMQEIIEAAKSNIHNFISALPQGYDTRLGKTSQL	1200
91-C-MDR49B	SQEPVLFDRITAEIAYGNFRDDVSMQEIIEAAKSNIHNFISALPQGYDTRLGKTSQL	999
91-R-MDR49B	SQEPVLFDRITAEIAYGNFRDDVSMQEIIEAAKSNIHNFISALPQGYDTRLGKTSQL	999
91-C-MDR49A	SGGQKQRIARALVRNPKLILDEATSAIDLESEKVVQALDEARSGRITCTIAHRLTT	1260
91-R-MDR49A	SGGQKQRIARALVRNPKLILDEATSAIDLESEKVVQALDEARSGRITCTIAHRLTT	1260
91-C-MDR49B	SGGQKQRIARALVRNPKLILDEATSAIDLESEKVVQALDEARSGRITCTIAHRLTT	1059
91-R-MDR49B	SGGQKQRIARALVRNPKLILDEATSAIDLESEKVVQALDEARSGRITCTIAHRLTT	1059
91-C-MDR49A	VRNADLICVLRKGVVEHGTDEIMALNKIYANLYLMQQVSG	1302
91-R-MDR49A	VRNADLICVLRKGVVEHGTDEIMALNKIYANLYLMQQVSG	1302
91-C-MDR49B	VRNADLICVLRKGVVEHGTDEIMALNKIYANLYLMQQVSG	1101
91-R-MDR49B	VRNADLICVLRKGVVEHGTDEIMALNKIYANLYLMQQVSG	1101

Figure 1. Sequence alignment of putative amino acids deduced from the MDR49 proteins associated with the 91-C and 91-R strains. Three mutations that resulted in amino acid replacements, T374I, M388L, and E666D for MDR49A; T173I, M187L, and E465D for MDR49B were observed in 91-R when compared with 91-C. The NBDs were homologous in both strains and each NBD had seven highly conserved motifs (aromatic, Walker A, Q-loop, ABC signature, Walker B, D-loop, and H-loop), which appear in boxes.

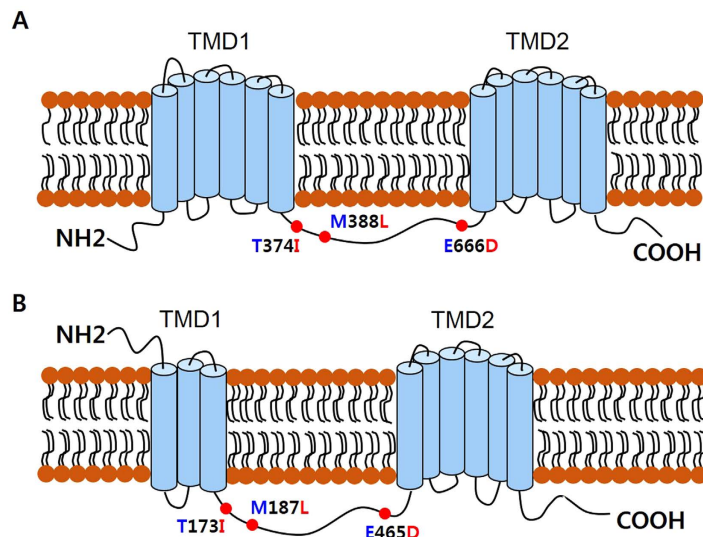


Figure 2. Schematic diagrams of the predicted structures of the MDR49 proteins, encompassing the T374I, M388L, and E666D for MDR49A; T173I, M187L, and E465D for MDR49B amino acid replacement sites. (A) Twelve transmembrane segments in two transmembrane domains (TMD 1 and TMD 2) were predicted in the MDR49A isoform. (B) Nine transmembrane segments were predicted in two TMDs in the MDR49B isoform. Amino acid replacements that may result in structural alterations are indicated by red dots. Blue letters represent 9I-C; red letters represent 9I-R.

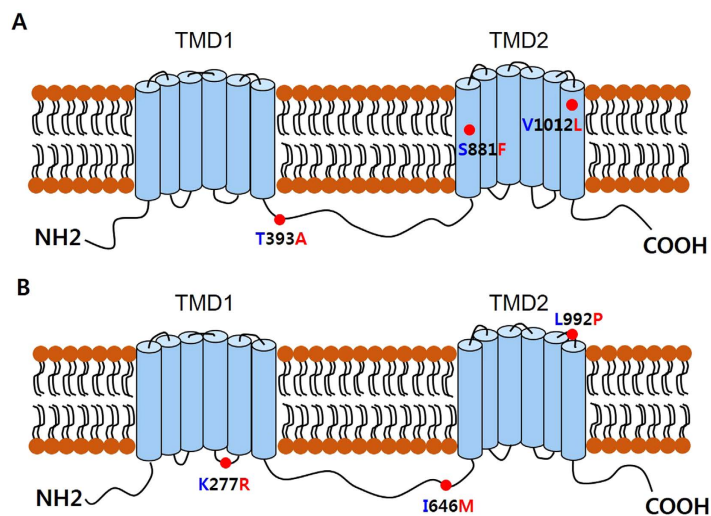


Figure 3. Schematic diagrams of the predicted structures of the MDR50 and MDR65 proteins, each encompassing twelve transmembrane segments in two TMDs. (A) The MDR50 protein had the T393A, S881F, and V1012L amino acid replacement sites. (B) The MDR65 protein had the K277R, I646M, and L992P sites. Amino acid replacements that may result in structural changes are indicated by red dots. Blue letters represent 9I-C; red letters represent 9I-R.

segments and the other consisting of six transmembrane α -helix segments were predicted (Fig. 2B). Typically, ABC transporters have six predicted membrane-spanning α -helix segments per TMD but the number of segments may vary in each TMD³³. The predicted proteins for both MDR50 and MDR65 displayed the more typical features of TMD structure, which consisted of six transmembrane α -helix segments per TMD, respectively (Fig. 3A,B).

The location of the three amino acid replacements in each of the three MDR proteins due to non-silent mutations found in 9I-R are given in Figs 2 and 3. T374I, M388L, and E666D for MDR49A; T173I, M187L, and E465D for MDR49B were predicted to be located on the intracellular loop between TMD 1 and TMD 2 for both MDR49A and MDR49B (Fig. 2A,B). For MDR50, T393A was located in the intracellular loop between TMD 1 and TMD 2 and S881F and V1012L were predicted to be in transmembrane segments 7 and 12, respectively (Fig. 3A). For MDR65, K277R was predicted to be in the intracellular loop between transmembrane segment 4 and 5 in TMD 1, I646M in the intracellular loop between TMD 1 and TMD 2, and L992P in the extracellular loop between transmembrane segment 11 and 12 in TMD 2 (Fig. 3B).

Strain	LD ₅₀ ^a (95% C.L.) ^b				χ ² (df)				Slope ± SD				RR ^c
	3 h	6 h	9 h	12 h	3 h	6 h	9 h	12 h	3 h	6 h	9 h	12 h	
91-R-MDR49A	3,920.3 (2,868.2–5,562)	833.9 (423.9–1,616.4)	241.6 (80.7–719.3)	103.8 (68–154.1)	2.79 (2)	18.43 (2)	17.14 (2)	0.03 (2)	1.6 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	1.0 ^d 0.9 ^e 0.5 ^f 1.0 ^g
91-C-MDR49A	3,893.2 (1,963.2–8862)	971.2 (509.9–1,777.9)	489.1 (164.6–2,251.5)	105.9 (58.6–180.3)					1.9 ± 0.2	2.0 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	
91-R-MDR49B	>50,000	11,834.8 [*] (9,603–16,025)	2,986.9 [*] (1,188–5968.5)	600.7 [*] (408–996.6)	–	131.1 (2)	101.1 (2)	36.4 (2)	–	2.1 ± 0.3	2.8 ± 0.2	2.0 ± 0.2	3.2 ^e 3.0 ^f 2.1 ^g
91-C-MDR49B	>50,000	3,284.4 [*] (1,481–6,398.7)	1,002.3 [*] (438.7–2,192.1)	299.9 [*] (185.6–501.8)					–	2.6 ± 0.2	2.7 ± 0.2	3.0 ± 0.3	
Strain	LD ₅₀ ^a (95% C.L.) ^b				χ ² (df)				Slope ± SD				RR ^c
	15 h	18 h	21 h	24 h	15 h	18 h	21 h	24 h	15 h	18 h	21 h	24 h	
91-R-MDR49A	39.8 (31.4–50.2)	17.1 (10.9–28.9)	12.2 (7.5–22.7)	7.8 (4.9–14.7)	1.86 (2)	5.1 (2)	3.84 (2)	9.54 (2)	1.7 ± 0.1	2.4 ± 0.2	2.7 ± 0.2	2.6 ± 0.2	1.2 ^h 1.1 ⁱ 1.0 ^j 1.4 ^k
91-C-MDR49A	32.3 (12.1–81.7)	15.1 (11.8–19.6)	11.9 (8.4–17.9)	5.7 (5.0–6.6)					1.8 ± 0.1	3.0 ± 0.2	3.3 ± 0.3	2.8 ± 0.2	
91R-MDR49B	68.1 (40.5–115.1)	46.2 (40.1–53.2)	35.9 (21.1–58.6)	18.6 (11.9–29.8)	3.34 (2)	5.19 (2)	1.66 (2)	5.18 (2)	2.8 ± 0.2	3.2 ± 0.3	3.1 ± 0.3	3.7 ± 0.4	1.2 ^h 1.3 ⁱ 1.1 ^j 1.2 ^k
91-C-MDR49B	56.8 (37.2–86.2)	36.7 (32.1–42.1)	29.9 (18.4–48.4)	17.9 (11.4–28.7)					2.4 ± 0.2	3.4 ± 0.3	2.9 ± 0.2	3.4 ± 0.3	

Table 1. Evaluation of DDT toxicity in two DDT-susceptible transgenic lines (91-C-MDR49A and 91-C-MDR49B) and two DDT-resistant transgenic lines (91-R-MDR49A and 91-R-MDR49B) of *Drosophila* using a contact exposure to DDT (μg/vial). ^aLethal Dose (ug/vial) that killed 50% of the flies. ^b95% Confidence limit. ^cResistance ratio = 91-R LD₅₀/91-C LD₅₀. ^dResistance ratio at 3 h DDT treatment. ^eResistance ratio at 6 h DDT treatment. ^fResistance ratio at 9 h DDT treatment. ^gResistance ratio at 12 h DDT treatment. ^hResistance ratio at 15 h DDT treatment. ⁱResistance ratio at 18 h DDT treatment. ^jResistance ratio at 21 h DDT treatment. ^kResistance ratio at 24 h DDT treatment. ^{*}Log DDT dose versus mortality regressions of 91-R-MDR49A versus 91-C-MDR49A and 91-R-MDR49B versus 91-C-MDR49B flies were significantly different using the maximum log-likelihood ratio test, respectively ($p < 0.05$).

Effects of three mutations in MDR49A and B from 91-R on DDT resistance. As *MDR49* was the only gene, of all the *MDR* genes in *Drosophila*, found to be associated with a major effect chromosome interval in the selective sweeps analysis¹⁸, four transgenic fly strains containing ORFs as follows: (1) *MDR49A* from 91-R (91-R-MDR49A); (2) *MDR49B* from 91-R (91-R-MDR49B); (3) *MDR49A* from 91-C (91-C-MDR49A); and (4) *MDR49B* from 91-C (91-C-MDR49B), were generated to investigate this finding in more detail.

The susceptibility of these transgenic strains following exposure to DDT is presented in Table 1. When mortality was evaluated at eight different time points (3, 6, 9, 12, 15, 18, 21, and 24 h post-treatment), the test evaluating the hypotheses of equality between the 91-R-MDR49A and 91-C-MDR49A determined that the regression lines were equal (Fig. 5). However, the test evaluating the hypotheses of equality between the 91-R-MDR49B and 91-C-MDR49B determined that regression lines were not equal (Fig. 6). Thus, the LD₅₀ values for 91-R-MDR49B were significantly larger than that from the 91-C-MDR49B after 6, 9, and 12 h following DDT treatment and yielded RR of 3.2, 3.0, and 2.1, respectively (Table 1). There were no significant differences in LD₅₀ values between two transgenic strains, however, after 12 h of DDT treatment and the test evaluating the hypotheses of equality between 91-R-MDR49B and 91-C-MDR49B from 15 to 24 h of DDT treatment determined that the regression lines were equal (Fig. 6). For 3 h post-treatment, precise estimation of the LD₅₀ values for the both 91-R-MDR49B and 91-C-MDR49B were not feasible because its mortality, even at the highest dose examined (50 mg/vial), was still >50%.

Our results support the hypotheses that the *MDR49A* isoform may not be involved in the DDT resistance phenotype, however, the *MDR49B* isoform may play a temporal role in DDT resistance at the early time points of DDT exposure. Furthermore, this finding implies that amino acid alternations in the protein coded for by the 91-R allele of the *MDR49* gene may be associated with potential resistance mechanisms, including regulating substrate binding affinity at the TMDs or with ATPase activity of the NBDs.

Discussion

To the authors' knowledge, the present study represents the first paper demonstrating the combination of amino acid replacements and splice form variants of a *Drosophila* ABC transporter in DDT resistance. Our data demonstrate that one of the two alternative splice forms, *MDR49B*, contributes to DDT resistance, however, the other isoform *MDR49A* does not, in spite of the fact that the resulting respective proteins both contain the same amino acid replacements. Transgenic over-transcription of either *MDR49A* or *MDR49B* obtained from 91-C did not result in changes in resistance levels to DDT, suggesting that over-transcription of *MDR49* does not represent a mechanism by which *MDR49* plays a role in resistance. These results are in line with previous work by Gellatly and coworkers, which demonstrated a direct relationship between expression levels of *MDR50*, *MDR65* and *MRP1* and DDT resistance whereas *MDR49* was not over-transcribed in 91-R³¹.

An additional observation from our RNA-seq data was the occurrence of a third alternative splice variant of *MDR49* from 91-R. Comparing the additional transcript sequence of *MDR49* with the *Drosophila* genome

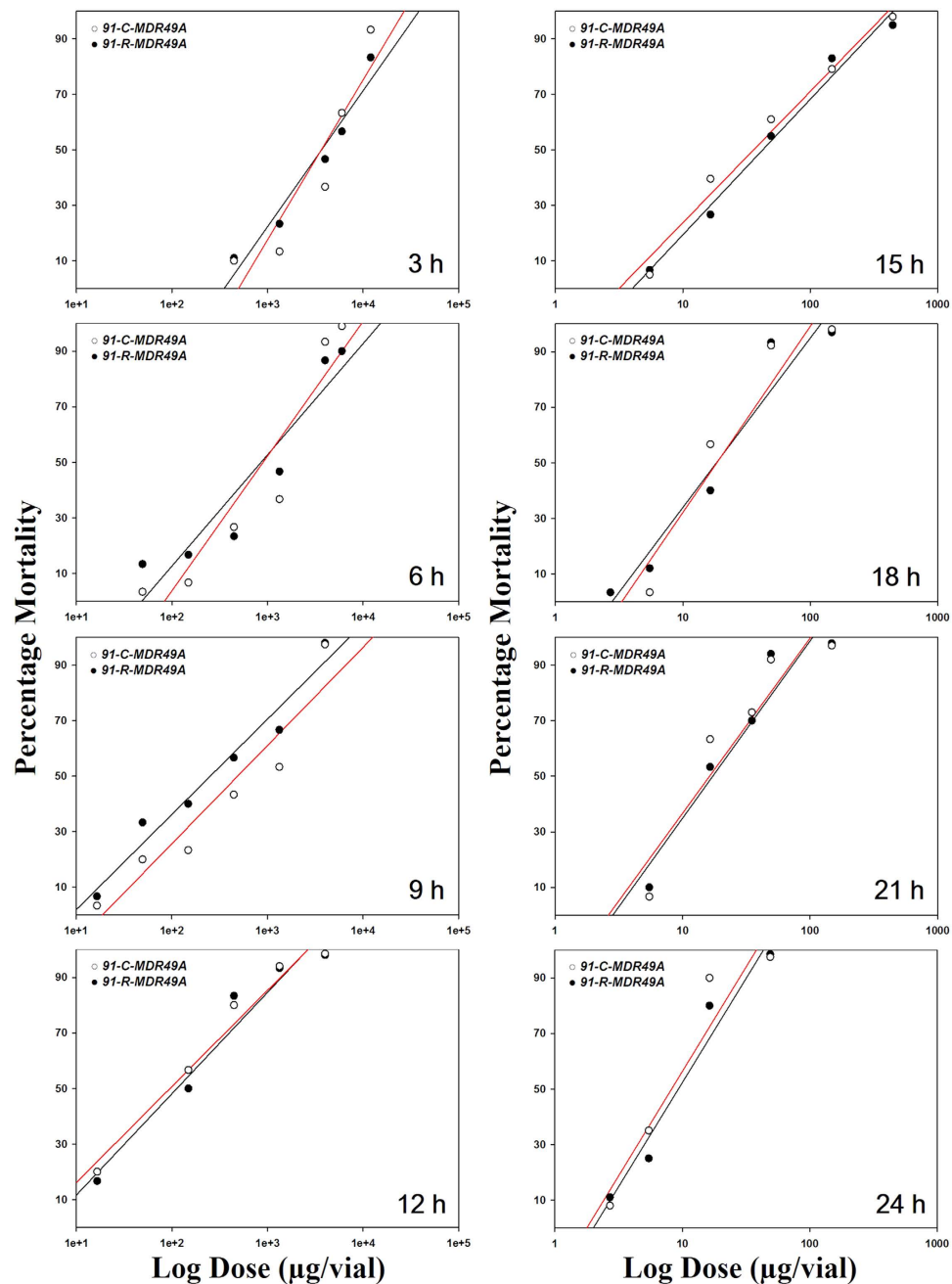


Figure 5. Dose-response curves for the transgenic strains, *91-C-MDR49A* and *91-R-MDR49A*. Log dose versus percent mortality response curves were determined at different time interval from 3 to 24 h following contact exposure to DDT ($\mu\text{g}/\text{vial}$), respectively. No significant differences between the two transgenic strains were observed at any time interval after DDT treatment.

revealed that the non-coding RNA region generated an optional exon region as a result of alternative splicing with premature stop codon. Thus, this third alternative splice form is unlikely to play any role in the resistance phenotype. Nevertheless, it would be interesting to determine the potential impact of such intense selection pressure by an insecticide upon the prevalence of such “extra” alternative splice forms across the insect genome. One could speculate that the *91-R* strain, under intense DDT selection pressure, acquired an additional transcript, including this optional exon from a non-coding RNA region. Additional research is necessary, however, to confirm this hypothesis. However, the phenomenon of an extra alternative splice form originating from intronic sequence is not without precedent, as previous studies have demonstrated that alternative exons with high homology probably originated from exons that were previously constitutively spliced in order to maintain the ancestral transcript as a major form, whereas alternative exons with low homology probably originated from exonization of intronic sequences³⁴.

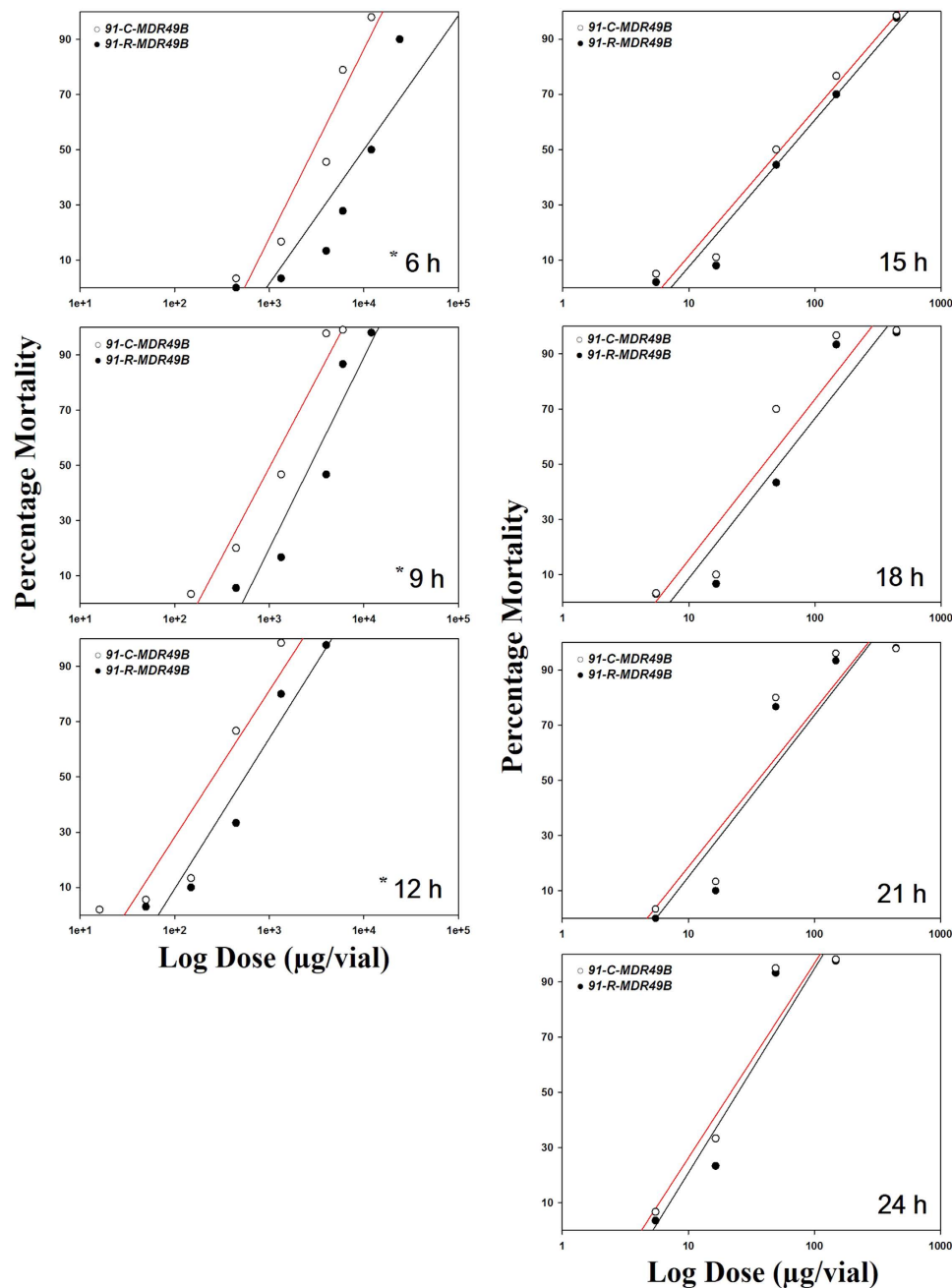


Figure 6. Dose-response curves for the transgenic strains, *91-C-MDR49B* and *91-R-MDR49B*. Log dose versus percent mortality response curves were determined at different time interval from 6 to 24 h following contact exposure to DDT ($\mu\text{g}/\text{vial}$), respectively. Asterisks indicate statistically significant differences in the dose responses when the two transgenic strains were compared at 6, 9, and 12 h after DDT treatment, as determined by the maximum-log likelihood ratio test ($p < 0.05$).

Nonetheless, previous studies characterized how alternative splicing of a voltage-gated sodium channel in mosquito contributed to insensitivity to pyrethroids, and feasibly indicated a role of splice variants in the pyrethroid resistance phenotype³⁵. The current results from the present study imply that *MDR49* may be associated with DDT resistance through a mechanism involving both alternative splicing and amino acid replacements in the protein.

As *MDR49* was the only *MDR* gene previously shown be associated with a major effect chromosome interval in a selective sweep analysis¹⁸, we tested the hypothesis that this gene was playing a direct role in DDT resistance through the use of DDT bioassays of transgenic *Drosophila* expressing the *MDR49* isoforms from *91-R* and *91-C*. Our results suggest that only the *MDR49B* transcript from *91-R* is involved in DDT resistance and importantly, significant mortality differences were observed only after DDT treatment for 6 h to 12 h intervals while no significant difference was observed after 15–24 h DDT exposure. Thus, the data from this study revealed that structural

alteration of the *MDR49* gene provides partial protection from DDT toxicity and suggests that *MDR49B* could play a role during the initial phase of the detoxification process after DDT exposure. Interestingly, MDR proteins are known to translocate exogenous substrates to the outside of the cell using energy obtained from ATP hydrolysis by the ATPase activity associated with the NBDs³⁶. In human, two mutations in NBDs disrupt the catalytic activity, causing reduced ATP binding and hydrolysis³⁷. Thus, amino acid replacements near NBDs of *MDR49B* in *91-R* could be directly involved in interrupting the transportation of exogenous substrates like DDT. Furthermore, the role of TMD is to recognize and mediate the passage of substrates across cell membrane. The TMD conformational alteration within *MDR49B* in *91-R* could enhance the large diversity of substrate specificity and mediate substrate transport.

Previously, Pedra *et al.* (2004) reported that the relative transcript expression of ABC transporter-like gene (CG9892) in the DDT-resistant *91-R* strain was greater than in the DDT-susceptible *Canton-S* strain using whole genome transcript profiles, suggesting a possible association between DDT resistance and ABC transporters⁸. Recently, Gellatly *et al.* (2015) also showed that the ABC-B subgroup (*MDR50*, *MDR65*) and the ABC-C subgroup (*MRP1*) were over-transcribed in *91-R* when compared to *Canton-S* and using a UAS/RNAi approach showed that knockdown of these genes increased DDT susceptibility in the transgenic flies³¹. Furthermore, the association of over transcription of ABC transporters with insecticide resistance has been confirmed in *Lygus hesperus*³⁸, *Cimex lectularius*³⁹, and *Myzus persicae*⁴⁰. Nevertheless, our current findings would suggest that *91-R* strains may use additional mechanisms allowing for DDT resistance besides over expression of ABC transporters. Indeed, the over expression of ABC transporters associated with resistance to insecticides at different time points has been previously reported in *Anopheles stephensi*⁴¹. The relative expression of an ABC-B subfamily gene was highly upregulated at early time points following permethrin treatment and ABC-G4 was over transcribed at later time points. Also, Atsumi *et al.* (2012) demonstrated that a single amino acid replacement in the second extracellular loop in an ABC transporter gene causes resistance to the Bt toxin *Cry1Ab* in the *Bombyx mori*⁴². To date, however, ABC transporter mutations associated with resistance to insecticides have not been reported in *Drosophila*.

In order to elucidate the actual effects of individual amino acid replacements on ABC transporter activity, a logical next step will be to focus on the heterologous expression of *MDR49* using cRNAs, containing these three mutation alone and in all combinations, injected into *Xenopus laevis* oocytes to improve our mechanistic understanding of this novel phase III xenobiotic metabolism reaction involved in DDT resistance in *Drosophila*.

Methods

***Drosophila melanogaster* strains.** DDT-resistant *91-R* and DDT-susceptible *91-C* strains were obtained from Dr. Ranjan Ganguly (University of Tennessee-Knoxville). Strains were reared on brown diet (Jazz-Mix *Drosophila* Food, Fischer Scientific, Cat. No. AS153) at 25 °C with 8:16 L:D in plastic bottles and transferred to new bottles about every three weeks. Populations of the *91-R* and *91-C* strains have been maintained in the Pittendrigh laboratory for over a dozen years and *91-R* strain has been under continuous selection at DDT concentrations of 100 mg/ml. For the transgenic lines *w*¹¹¹⁸ was used.

Structure analysis of MDR proteins. Sequence similarity and analysis of protein-specific motifs were performed using BLAST programs on NCBI. Sequence alignments were performed with Clustal Omega (EMBL-European Bioinformatics Institute, Cambridge, UK). The transmembrane domain and membrane topology was predicted with TOPCONS online software (<http://topcons.cbr.su.se/>)⁴³.

Identification of alternatively spliced transcripts. All RNA-seq databases from *91-R* and *91-C* were imported to the CLC genomic workbench 8.5 software according to the manufacturer's manual (Qiagen, Valencia, CA, USA). The "Transcript Discovery" plug-in was used to predict alternative splicing transcripts and genes. Our trimmed reads of each sample with three replications were mapped against *MDR49*, *MDR50*, and *MDR65* *Drosophila* genome sequences extracted from *Drosophila* genome assembly release 6.07 (file *dmel-all-chromosome-r6.07.fasta* downloaded from Flybase.org) including intergenic regions. The BAM files with mapped reads were deposited to NCBI Short Read Archive (SRA) with the accession number SRP068789. The matched reads for each transcript were visualized in the CLC interface. Each generated transcript was manually examined by comparing RNA-seq reads with the *Drosophila* genomic sequence to identify alternatively spliced variants.

Cloning of *MDR49* from *91-R* and *91-C* strains for transgenic expression. Two *MDR49* splice isoforms were cloned and sequenced using total RNA from each of the two strains (*91-R* and *91-C*). First-strand cDNA was synthesized by using Superscript™ III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) primed with oligo-d(T). Internal cDNA fragments of the *MDR49A* and *MDR49B* genes were amplified from the first-strand cDNA with a set of gene-specific primers for each strain. The amplified cDNA-specific products were purified using a PCR clean-up kit (Qiagen, CA) and directly sequenced using the gene-specific primers for both ends to cover the full length. All sequences were assembled and compared by using Vector NTI (Invitrogen, CA).

Transgenic expression of the two *MDR49* splice isoforms in *Drosophila*. The full-length *MDR49A* and *MDR49B* splice isoforms were amplified from the cDNA of both *91-R* and *91-C* strains using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). The sequence-specific primer pairs were used (Table S1). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, CA) and cloned into the pCR2.1 TOPO vector (Invitrogen, CA). Individual clone from both strains were purified with a QIAprep Miniprep kit (Qiagen, CA) and sequenced to locate the open reading frame and validate the correct amino acid sequences for *MDR49A* and *MDR49B*. After sequence analysis, the selected clones for both *MDR49* transcripts were sub cloned into the pUAST vector. Transgenic flies were generated by the BestGene Inc (Chino Hills, CA) using the *w*¹¹¹⁸ strain. For the expression of the transgene *MDR49A* and *B*, ubiquitous driver strain

($P\{w[+mC] = GAL4-elav.L\}2/CyO$) was obtained from Bloomington *Drosophila* Stock Center (Bloomington, IL). This female ubiquitous driver strain was crossed with our male transgenic strains and selected F1 progeny that showed both GAL4-MDR49A and B by examining wing shape and eye color for mortality bioassay.

Mortality bioassays. We used a previously described bioassay approach with some modifications³¹. To determine diagnostic doses of DDT, flies were exposed to vials coated a series of concentration of DDT, and mortality was determined after the flies were exposed for 3, 6, 9, 12, 15, 18, 21, and 24 h in the vials. Flies were considered dead when all movement and leg twitching had ceased. The median lethal dose (LD_{50}) values and their 95% confidential limits (CLs) were determined by Probit analysis (POLOPC, LeOra Software, Berkeley, CA). Test for the hypotheses of equality (slopes and intercepts are not significantly different) was performed as described by Robertson *et al.* (2007)⁴⁴. The maximum-log likelihood test was used to determine whether the resulting mortality curves from differently treated fly groups were statistically different ($p < 0.05$).

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Author Contributions

K.M.S. designed the experiments, analyzed the data and wrote the manuscript. W.S. performed experiments and data analysis. B.R.P. supervised the research, participated in the design of the experiments, interpretation of the results, and in the writing and editing of the manuscript. J.M.C. revised and discussed the results and commented on the manuscript. All authors contributed to the discussion and reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

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