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TOXICOLOGY AND CYTOGENETIC ANALYSIS OF A *Drosophila melanogaster* MUTANT RESISTANT TO IMIDACLOPRID AND DDT

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Resistance to all major insecticide classes has developed in numerous and diverse insect field populations. Imidacloprid, the worldwide most used neonicotinoid, has been extensively applied during the last decade for the control of different insect pests. Lately, cases of sporadic resistance also to neonicotinoids, including Imidacloprid, have been reported. *Drosophila melanogaster* is one of the most popular model organisms in biology and, although not a pest species, a promising model system for insecticide resistance research. In this study, we present a toxicological and karyotypic analysis of a *Drosophila* mutant (MiT[w^-]3R2) resistant to Imidacloprid and cross-resistant to DDT. Karyotype analysis of polytene chromosome of MiT[w^-]3R2 flies did not identify any apparent structural change of the polytene chromosome linked with the resistance phenotype.

Key words: *Drosophila melanogaster*, Imidacloprid, toxicology analysis, insecticide resistance

INTRODUCTION

The increase in productivity of the agricultural industry during the last century can, to a large extent, be attributed to an increased use of synthetic chemical insecticides. The neonicotinoids are one of the most effective insecticide classes. They act as agonists of the

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nicotinic acetylcholine receptor (nAChR), opening the channel and causing continuous depolarization and firing of postsynaptic neurons, resulting in paralysis and death (ZHANG *et al.*, 2000; NAUEN *et al.*, 2001). As a result of their specific mode of action (MoA), there is no cross-resistance to the long-established conventional insecticide classes (NAUEN and DENHOLM, 2005). Imidacloprid, the first commercially introduced neonicotinoid, became quickly the most successful and best-selling insecticide worldwide (MENCKE and JESCHKE, 2002). Imidacloprid is effective against a wide range of targeted insects, including sucking insects, beetles, lepidoptera, leafminers, some diptera, termites, locusts and fleas (CLOYD and BETHKE, 2011). Although Imidacloprid is still an invaluable agent for managing some of the world's most destructive crop pests, sporadic cases of resistance to neonicotinoids, including Imidacloprid, have been reported worldwide in the last 10 years (JESCHKE and NAUEN, 2008).

Insecticides are primarily used to target pest species, but in many cases non-targeted field populations, like *Drosophila*, are affected too. The application of comprehensive and refined methods for resistance mechanism analysis available for *Drosophila* is in most cases not possible in other non-targeted insects (WILSON, 2001).

In this study, a *Drosophila melanogaster* mutant (MiT[w^-]3R2) resistant to Imidacloprid and DDT, retrieved during a transposon *Minos* based genome-wide mutagenesis screen, was used for the analysis of insecticide resistance.

The generation of the resistant line MiT[w^-]3R2 is described in KALAJDZIC *et al*. (2012). Transcriptomic footprint analysis of the resistant mutant revealed expression patterns and gene groups that could be involved in the mechanism of insecticide resistance (KALAJDZIC *et al.,* 2012). Combined results of single nucleotide polymorphism analysis and P-element recombination mapping placed the resistance locus within a ~1Mb region in the vicinity of the *Cyp6g1* gene (KALAJDZIC *et al*., 2012).

We present here more information on the toxicology resistance profile and the resistance mechanism of line MiT[w^-]3R2 . Also, the karyotype and behavioural aspects of resistant flies were analyzed.

MATERIALS AND METHODS

Chemicals

Bioassays were carried out with active ingredients diluted in acetone (Merck). Imidacloprid (98.7 %) was kindly provided by Bayer CropScience GmbH-Germany, while DDT (4,4' – DDT PESTANAL®), paraquat (analytical standard) and orcein were purchased from SIGMA-ALDRICH Laborchemikalien GmbH, Germany.

Drosophila lines

D. melanogaster stocks were maintained on standard cornmeal-agar-yeast medium at 24 °C with a 12-hour light/12-hour dark cycle. We analyzed *Drosophila melanogaster* lines MiT[w^-]3R2 and MiT[w^-]3R2/CyO, resistant to Imidacloprid (neonicotinoid), retrieved during a *Minos*-transposon based insertional mutagenesis screen. Mapping of the resistance to the second chromosome and generation of lines heterozygous (MiT[w^-]3R2/CyO) and homozygous (MiT[w^-]3R2) for the second resistant chromosome have been described (KALAJDZIC *et al.*, 2012). The isogenic line iso31 (w^{1118}iso; 2_{iso}; 3_{iso}) (RYDER *et al.*, 2004) was used as susceptible (wild-type) line.

Toxicology bioassays

Resistance was quantified by determining LC50 values, corresponding to insecticide concentrations that kill 50 % of treated individuals. Iso31 flies, in parallel with MIT[w^-]3R2 and MiT[w^-]3R2/CyO resistant flies, were tested for Imidacloprid and DDT LC50's. LC50 values of lines MiT[w^-]3R2 and iso31 have already been published in KALAJDZIC *et al*. (2012). The lethality of different concentrations of Imidacloprid was tested by analyzing egg-to-adult viability of the flies. Flies were mass-crossed and placed into fly cages, allowing females to lay eggs on cherry juice medium. Eggs were collected within 24 hours and placed into vials (50 eggs per vial), containing medium with different imidacloprid concentrations. For each concentration of Imidacloprid, eight replicas were set up; hence the total number of eggs was 400 per concentration. The number of emerged flies was determined for each concentration of Imidacloprid. For DDT susceptibility analysis, 3 days post-eclosion males and females were tested in a contact assay. The inside of 35 ml glass vials was coated with DDT by applying 200 µl of acetone (99.8 %, MERCK), containing different concentrations of DDT and rolling the vials horizontally, until the acetone was evaporated. Vials were plugged with cotton wool soaked in 5 % sucrose. Into each vial, 25 flies (both males and females) were placed, and mortality was scored after 24 hours. For this assay, four replicas per concentration were set up, with 100 flies per concentration in total. For both, Imidacloprid and DDT assays, the control mortality in the absence of insecticide was determined.

LC50 calculation and construction of dose-response curves

For both insecticides (Imidacloprid and DDT), flies were tested on at least 4 different concentrations plus control. The LC50 values were calculated with the computer program SPSS 16.0 (SPSS BASE 10.0 FOR WINDOWS SPSS INC., CHICAGO IL., 1999), using the probit regression model (FINNEY, 1971). Dose-response curves were derived using Sigma Plot 10.0 (SYSTAT SOFTWARE INC., 2007). Each dose-response curve was constructed from at least four concentrations.

Paraquat assay

Two to three days old MiT[w^-]3R2 and iso31 flies were collected. Ten males and ten females from each line were placed into vials with different concentrations of paraquat, in addition to a negative control lacking paraquat. Five replicas for each concentration plus control were set up. Paraquat was applied to paper filter discs mixed with a 1 % sucrose solution, which were placed in plastic vials. To each paper filter disc (1.5 cm diameter), 1 ml of paraquat in 1 % sucrose was applied. In the control, 1 ml of 1 % sucrose without additive was used. Three different concentrations of paraquat, 5 %, 10 % and 12.8 %, were tested. The mortality was scored after 24 hours.

Karyotype analysis of polytene chromosomes

Polytene chromosomes were prepared using an orcein polytene chromosome staining protocol (ASHBURNER, 1989). Six individual crosses between resistant line MiT[w^-]3R2 and line iso31 were set up. Individual larvae produced in these crosses were microscopically analyzed for the presence of aberrations on all 5 polytene chromosomes (X, 2L, 2R, 3L and 3R).

RESULTS

Resistance to Imidacloprid and DDT

Resistant lines, homozygous (MiT[W$^-$]3R2) or heterozygous (MiT[W$^-$]3R2/CyO) for the resistance locus on chromosome 2, as well as the susceptible line iso31, were tested for levels of resistance to Imidacloprid and DDT. For all lines, the levels of resistance were determined by analyzing egg to adult viability as LC50s (insecticide concentration causing 50 % lethality). Dose response curves were constructed from at least six concentrations.

The LC50 values for iso31 (susceptible line) and the resistant line MIT[w$^-$]3R2 are presented in table 1. LC50 values of lines MiT[*w*$^-$]3R2 and iso31 were already reported in KALAJDZIC *et al.* (2012). The susceptible line has a significantly lower LC50 of 0.18 µg/ml (with 95 % confidence limits of 0.15 µg/ml - 0.21 µg/ml) compared to resistant flies homozygous or heterozygous for the second "resistance" chromosome. Line MiT[W$^-$]3R2/CyO, heterozygous for the second chromosome carrying the resistance locus, has an LC50 of 2.15 µg/ml (with 95 % confidence limits of 1.59 µg/ml – 2.61 µg/ml). Analysis of the line MiT[W$^-$]3R2, homozygous for the second "resistance" chromosome, shows an LC50 that is ~18-fold higher than that of wild-type line iso31 (the LC50 for MiT[w$^-$]3R2 was 3.30 µg/ml, with 95 % confidence limits of 1.90 µg/ml - 4.10 µg/ml) (KALAJDZIC *et al.*, 2012). Importantly, line MiT[W$^-$]3R2 shows a higher LC50 compared to line MiT[W$^-$]3R2/CyO heterozygous for the second chromosome carrying the resistance locus (table 1).

Resistant lines MiT[W$^-$]3R2 and MiT[W$^-$]3R2/CyO were further tested for cross-resistance to DDT. The lines (resistant and susceptible) were tested for LC50 by analyzing adult mortality in a 24 hour DDT contact assay. LC50 values for the susceptible and the resistant line are given in table 1. The susceptible line iso31 has a significantly lower LC50 value than the resistant lines MiT[*w*$^-$]3R2 and MiT[W$^-$]3R2/CyO (iso31 was 0.37 µg/ml, with 95 % confidence limits of 0.15 µg/ml – 0.65 µg/ml). As in the case of Imidacloprid, there is an increase in the LC50 value in the presence of a second copy of the "resistance" chromosome. Resistance to DDT in the line homozygous for the second "resistance" chromosome is ~100 fold higher than in the wild-type line iso31 (the LC50 for iso31 was 0.37 µg/ml, with 95 % confidence limits of 0.15 µg/ml – 0.65 µg/ml; and for MiT[*w*$^-$]3R2 37.50 µg/ml (32.20 µg/ml - 41.90 µg/ml) (table 1).

Table 1. LC50s for Imidacloprid and DDT for susceptible and resistant flies (heterozygous or homozygous for the second chromosome)

	IMIDACLOPRID		DDT	
	LC50 µg/ml (95% confidence limits)	RR (resistance ratio)	LC50 µg/vial (95% confidence limits)	RR (resistance ratio)
iso31	0.18 (0.15 – 0.21)#	1.0	0.37 (0.15 – 0.65)#	1.0
MiT[w$^-$]3R2/CyO (heterozygous)	2.15 (1.59 -2.61)	11.9	5.50 (0.10 – 18.20)	14.9
MiT[w$^-$]3R2 (homozygous)	3.30 (1.90 – 4.10)#	18.3	37.50 (32.20 – 41.90)#	101.4

RR (resistance ratio) – LC50 value of the MiT[*w*$^-$]3R2 line/LC50 value of the iso31 line

- values reported in KALAJDZIC et al. (2012)

Karyotype analysis of the polytene chromosomes

Chromosomal inversion polymorphisms have been linked to DDT and dieldrin resistance in a laboratory strain of *Anopheles gambiae* (BROOKE *et al.*, 2002) and to DDT resistance in three populations of *Anopheles arabiensis* from Ethiopia (NIGATU *et al.*, 1995). This prompted us to analyze the resistant *Drosophila* line for the presence of putatively mutagenic inversions. The karyotype of the salivary glands of larvae from a cross between resistant line MiT[w^-]3R2 and susceptible line iso31 was microscopically analyzed for the presence of inversions on all five polytene chromosome. No rearrangements could be identified: all five polytene chromosomes (X, 2L, 2R, 3L, 3R) show the standard banding patterns (figure 1).

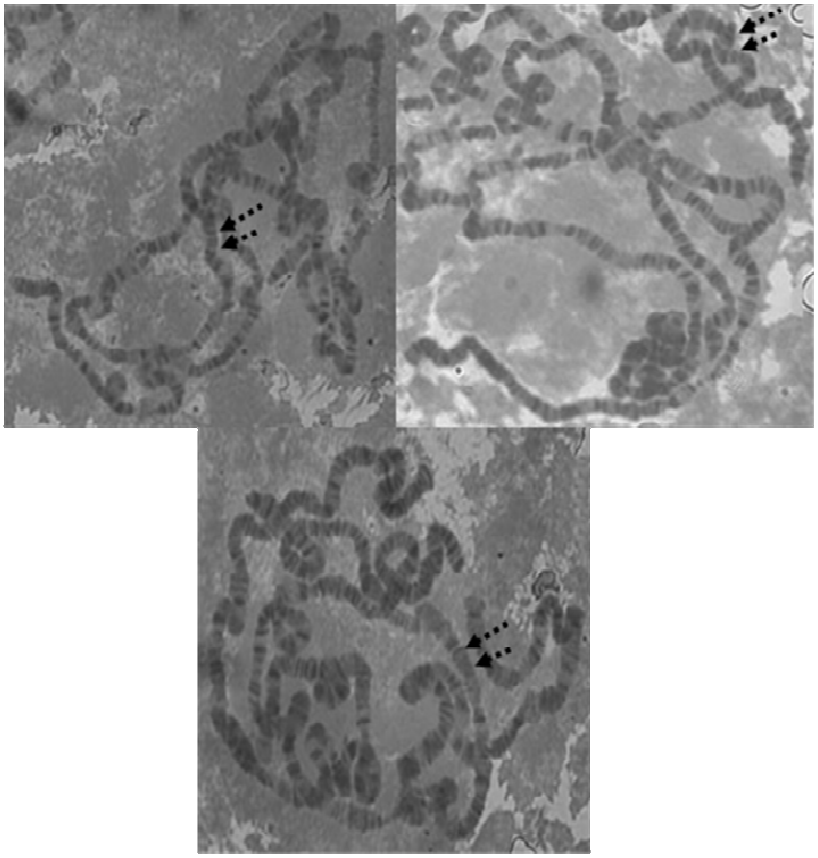

Figure 1. Salivary gland polytene chromosomes of larvae progeny from the cross between resistant and susceptible line, prepared with a squash technique (dashed arrows indicate the region where the resistance locus is mapped).

Paraquat assay

Unusual behavior, which manifested itself in upright wing posture and seizure-like episodes, was observed in resistant adults. Oxidative stress-mediated toxicity can cause such behaviour. Flies were analyzed for their resistance to paraquat in order to test if there is a decrease in antioxidant defense. The analysis did not yield any significant difference in survival between the resistant MiT[W$^-$]3R2 line and the susceptible iso31 line, as shown in table 2.

Table 2. Mortality (%) of the susceptible and resistant lines treated with different concentrations of paraquat

Concentrations	Mortality (%) iso31	Mortality (%) MiT[w$^-$]3R2
0 %	20	20
5 %	55	55
10 %	55	55
12.8%	80	75

DISCUSSION

The transcriptional profile of mutant MiT[w$^-$]3R2, as well as mapping of the mutation linked to Imidacloprid and DDT resistance in this line is described in KALAJDZIC *et al* (2012). The mutation linked to the resistance is located on the right arm of the second chromosome in the vicinity of the P450 gene *Cyp6g1* (KALAJDZIC *et al.*, 2012). The present study presents more information on the toxicology of the resistance mutation, and an analysis of possible karyotype alterations that may be linked to the resistance. Additionally, unusual behaviour of the resistant flies was tested for linkage with oxidative stress.

Drosophila melanogaster is widely used in studies of chemical mutagenesis and selection for resistance to different insecticides (KIKKAWA, 1964; DABORN *et al.*, 2001). The present study is on the first resistant *Drosophila* line from an insertional mutagenesis screen using a transposon element and with selection on Imidacloprid. The susceptibility to Imidacloprid and DDT of resistant MiT[w$^-$]3R individuals, homozygous and heterozygous for the second chromosome, was analyzed. Daborn and colleagues (2001) generated *Drosophila* mutants with ethyl methanesulfonate (EMS) and selected for Imidacloprid resistance. During the screen, two resistant mutants were retrieved. Both resistant mutants, when homozygous for the resistance loci (also on the second chromosome) had LC50s of about 0.7 µg/ml (DABORN *et al.*, 2001). The MiT[w$^-$]3R2/CyO flies heterozygous for the second resistant chromosome show a more than 3-fold higher resistance (2.1 µg/ml) compared to these EMS mutants. The resistance increases in individuals homozygous for the second "resistance" chromosome to about 5-fold higher (3.3 µg/ml) compared to the EMS mutants.

Resistant heterozygous flies had an about ~12 fold higher LC50 compared to the susceptible line iso31, when analyzed for Imidacloprid resistance. Flies homozygous for the resistance locus increased their resistance to ~18 fold compared to the susceptible line (KALAJDZIC *et al.*, 2012). Cases of resistance to Imidacloprid showing cross-resistance to DDT in

Drosophila populations have been described (DABORN *et al.*, 2001; DABORN *et al.*, 2002; LE GOFF *et al.*, 2003). As for Imidacloprid, resistant flies, both homozygous and heterozygous for the second "resistance" chromosome, show an increased resistance to DDT. Flies heterozygous for the MiT[w$^-$]3R2 chromosome were ~15-fold more resistant compared to iso31 flies. This factor increases to ~100 fold in flies homozygous for the resistance locus (KALAJDZIC *et al.*, 2012). MiT[w$^-$]3R2 flies also show higher resistance to DDT than the mentioned EMS mutants (DABORN *et al.*, 2001). Transcription profiling results show that three genes (*Cyp4p2*, *Cyp6a2* and *Cyp6g1*) are highly overexpressed, with more than 15 fold expression difference in resistant MiT[w$^-$]3R2 line compared to susceptible line (KALAJDZIC *et al.*, 2012). Detoxification function of *Cyp6a2* and *Cyp6g1* by metabolizing DDT is documented in *Drosophila* (DUNKOV *et al.*, 1997; SANER *et al.*, 1996; JOUSSEN *et al.*, 2008). Also, homology modeling suggests that active sites of *Cyp6a2* and *Cyp6g1* genes are well suited to accommodate DDT (JONES *et al.*, 2010).

Transcriptional profiling data and biochemical assays suggest P450-metabolism as the main, or at least a major mechanism of resistance to Imidacloprid (neonicotinoid) in mutant line MiT[w$^-$]3R2 (KALAJDZIC *et al.*, 2012). These results strongly suggest one member of the Cyp family (*Cyp6g1*) as the main candidate gene responsible for the Imidacloprid and DDT resistance in the mutant. Genetic mapping placed the resistance locus on the right arm of the second chromosome, within a ~1 Mb region, in which the highly up-regulated *Cyp6g1* gene is located (KALAJDZIC *et al.*, 2012).

Resistance in this mutant could be based on modification of gene expression altered by *cis*- or *trans*-acting control, by duplication or amplification, or by post translation modification (TAYLOR and FEYEREISEN, 1996). Since a single mutation event appears to be responsible for resistance in MiT[w$^-$]3R2 mutant (KALAJDZIC *et al.*, 2012), the molecular mechanism that gives rise to resistance is most likely modification of gene expression. Results of this study show that the resistance is manifested in individuals heterozygous and homozygous for the resistance locus, suggesting that the resistance is dominantly inherited. Higher resistance was observed for both insecticides in homozygous individuals compared to heterozygous second chromosome resistant flies, indicating dosage dependence of dominant mutant allele.

Drosophila natural populations have been widely used for studying chromosomal inversion polymorphism (ANDJELKOVIC *et al.*, 2003; KALAJDZIC *et al.* 2006; JELIC *et al.*, 2012). Chromosomal inversion polymorphisms have been associated with DDT and dieldrin resistance in *Anopheles gambiae* (BROOKE *et al.*, 2002), as well as with DDT resistance in *Anopheles arabiensis* (NIGATU *et al.*, 1995). Karyotype analysis of the larvae from the cross between resistant line MiT[w$^-$]3R and the susceptible line iso31 did not show the presence of any discernible chromosomal aberrations of any of the five polytene chromosome arms (X, 2L, 2R, 3L, 3R). Specifically, there were no aberrations or cytological changes on the right arm of the second chromosome within the region of 1 Mb in the vicinity of *Cyp6g1* gene that is directly linked to the resistance in laboratory line (figure 1).

It has been suggested that oxidoreductase enzymes, including the P450 cytochromes, could be involved in the detoxifying processes that follow oxidative stress in *Drosophila* (GIRARDOT *et al.*, 2004). Oxidative stress is strongly correlated with neurodegenerative diseases in humans, and *Drosophila* is one of the model organism in which this phenomenon is increasingly studied (ANDERSEN, 2004; BOTELLA *et al.*, 2009; SYKIOTIS and BOHMANN, 2010). Resistant MiT[w$^-$]3R2 male and female adults display an unusual behaviour: the wings are held in an upright posture, and seizures were observed. In order to test for a correlation between this

behaviour and oxidative stress, resistant flies were analyzed for resistance to paraquat. Paraquat is used as an inducer of oxidative stress by catalyzing the formation of reactive oxygen species (ROS) (BUS and GIBSON, 1984). If there is a pre-existing oxidative stress in the analyzed individuals, treatment with paraquat should cause an increased lethality. The analysis shows, however, that there is no significantly higher lethality in the treated resistant flies compared to susceptible iso31 flies (table 2). Thus, there is no evidence for a decrease in the antioxidant defense of the resistant line.

In conclusion, toxicological and karyotype analyses provided additional data regarding the nature of resistance in line MiT[w^-]3R2. No changes on the chromosomal level were detected, supporting the hypothesis that the resistance mutation occurred on the nucleotide level.

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TOKSIKOLOŠKA I CITOGENETIČKA ANALIZA *Drosophila melanogaster* MUTANTA REZISTENTNOG NA IMIDAKLOPRID I DDT

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Izvod

Otpornost na sve važnije klase insekticida razvila se kod prirodnih populacija mnogobrojnih insekata koji pripadaju različitim vrstama. Imidakloprid, najkorišćeniji neonikotinoid na svetu, ekstenzivno se primenjuje tokom poslednje decenije u kontroli različitih vrsta insekata štetočina. U istom periodu detektovani su sporadični slučajevi rezistentnosti na neonikotinoide, uključujući i Imidakloprid. *Drosophila melanogaster* je jedan od najkorišćenijih model organizama u biološkim istraživanjima. Iako nije insekt štetočina postaje interesantan model organizam u istraživanjima rezistentnosti na insecticide. U ovoj studiji smo predstavili toksikološku i kariotipsku analizu mutantne *Drosophila* linije (MiT[w^-]3R2) rezistentne na Imidakloprid i kros rezistentne na DDT. Analiza politenih hromozoma MiT[w^-]3R2 mušica nije pokazala prisustvo vidljive strukturne promene na hromozomima koja bi mogla da se poveze sa rezistentnim fenotipom.

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